Characterisation and propagation of *Hibiscus coddii* subsp. *barnardii*, an indigenous plant with ornamental potential from Sekhukhuneland, Limpopo Province, South Africa

by

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2020

DECLARATION

“I declare that the thesis hereby submitted to the University of Limpopo for the degree of Doctor of Philosophy in Botany has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged”.

....28/02/2020...

Du Plessis, HJ **Date**

DEDICATION

This thesis is dedicated to Stephan, Nicola and Johan

"You are the best things in my life"

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ABSTRACT

South Africa is known globally for its rich floral biodiversity. A number of the country's indigenous plants are commercialised and grown worldwide. An increased interest in growing drought-tolerant plants due to limited water resources, created a need for introducing additional novel wild species onto the horticultural market. One such plant occurring in the drought-prone areas of the Sekhukhuneland Centre of Plant Endemism is *Hibiscus coddii* Exell subsp. *barnardii* (Exell) Leistner & P.J.D. Winter. In nature, the plant is an upright, branched perennial herb that produces bright red flowers for about four months during the summer season. Its characteristics makes it suitable for growing as a small shrub, especially in sunny rockeries, and as a pot plant. Effective propagation is, however, imperative for any commercial production of plants. This study, therefore, addresses the lack of reliable propagation protocols for *ex situ* conservation purposes and for large-scale production of this valuable ornamental plant with commercial potential.

The study showed that plants could be propagated by both conventional (*in vivo*) methods, using seeds and stem cuttings, and *in vitro* culture methods under controlled conditions. The effect of seed scarification with various concentrations of sulfuric acid (25%, 50%, and 98%) for various durations (5–40 minutes) and of temperature (15°C, 20°C, 25°C, 30°C, and 35°C) on seed germination were studied. The best response for both seed germination and seedling development was respectively obtained with 98% sulfuric acid for 30 minutes at 25°C in moist vermiculite. This protocol for seed germination can be used for the production of seedlings and of plants that could serve as stock for further propagation. Seedlings and mature plants grown in pots require adequate nutrients supplied once per week to ensure optimum performance.

Mature plants grown under a controlled environment exhibited strong apical dominance. Removal of the apex from the main stem of four to five-month-old plants promoted outgrowth of axillary shoots suitable for use as cuttings for vegetative propagation. The effect of cutting type, exogenous application of commercial rooting hormone powder (Dynaroot^{TM} No.1 and No.2) containing 0.1% and 0.3% Indole-3-butyric acid (IBA) respectively, and culture media on rooting of stem cuttings was

investigated. Both types (apical and basal) of axillary shoot cuttings showed high rooting percentages (89–91%) and formation of numerous roots per cutting when treated with both strengths of rooting hormone, which was more pronounced with Dynaroot No.2. The best rooting responses were observed in vermiculite medium alone, and in combination with coco peat, followed by a coco peat and sand mixture. High rooting percentages (>80%) were observed in these media.

For establishment of aseptic *in vitro* cultures, seeds and nodal explants from wild and *in vivo* grown plants were surface disinfected with 70% ethanol and 25% and 50% commercial bleach (Jik®) solution. Nodal explants from both sources did not survive the treatment and proved unsuitable for *in vitro* shoot culture. Disinfection with 70% ethanol (90 seconds) and 50% commercial bleach solution (25 minutes) proved the best for establishment of aseptic seed cultures. Germination of scarified seeds and seedling development under controlled environmental conditions (24±2°C with a 16-hour photoperiod at 55–60 µmol m^{-2} s^{-1}) was the best on full strength Murashige and Skoog medium (MS). *In vitro* grown seedlings proved suitable as an aseptic explant source for *in vitro* shoot culture. The aseptic excision of the apex from five-week-old seedlings directly in the culture vessel, without the need for transplanting to a new culture medium, resulted in the proliferation of axillary shoots (up to 8) over a short period. Nodal explants obtained from these shoots, as well as directly from *in vitro* grown seedlings, were used for *in vitro* shoot multiplication on MS medium with and without plant growth regulators (PGRs). Basal shoot explants (3–4 nodes) obtained directly from *in vitro* grown seedlings and cultured on PGR-free MS medium in a vertical position was the most suitable for generating axillary shoots and for plant regeneration. Low shoot regeneration efficiency in all types of explants was observed in the presence of various concentrations of 6-Benzylaminopurine (BAP) (0.25–4 mg L^{-1}) alone and in combination with 0.5 mg L^{-1} IBA and 1-Naphtalene-acetic acid (NAA). Microcuttings derived from axillary shoots of seedlings with a removed apex were used for *in vitro* and *ex vitro* rooting. Poor *in vitro* rooting responses were observed in both PGR-free (15% rooting) and MS media supplemented with 0.5 and 1 mg L^{-1} IBA and NAA (5–20% rooting). In contrast, high rooting percentages (80–100%) were attained when microcuttings treated with Dynaroot™ No.1 commercial rooting powder (0.1% IBA) were rooted *ex vitro* in moist vermiculite. *In vitro* seedlings and plantlets were successfully

acclimatised (>90%) for 2–3 weeks under controlled environmental conditions (24°C±2°C and a 16-hour photoperiod at 150–200 µmol m^{-2} s^{-1}). Further hardening-off in a greenhouse (uncontrolled environment) resulted in well-established mature plants, which proved suitable for transplanting to an open environment.

This study revealed that *H. coddii* subsp. *barnardii* can be propagated both by *in vivo* and *in vitro* cultures using seeds and cuttings. However, *in vitro* culture could be more suitable for large-scale plant production under controlled environmental conditions, since it is unaffected by seasonal variations and requires limited space and low maintenance of the plant material. This approach also resulted in a shorter time for seedling establishment (7–8 weeks) in an artificial nutrient medium as compared to conventional *in vivo* propagation in soil (12 weeks), where cultures require constant care (water and nutrients) for optimum growth. Plant regeneration by microcuttings derived from *in vitro* grown seedlings was also shorter (up to 4 months) than the regeneration by stem cuttings (6 months) derived from *in vivo* grown plants. This study could form the basis for domestication of *H. coddii* subsp. *barnardii* and its introduction as ornamental xeriscaping plant, especially in dry, rocky places, or for cultivation as pot plants. This would contribute to the popularisation of indigenous plants for gardening purposes. The study also supports *ex situ* conservation of the species threatened by the mining industry, expansion of human settlements and destruction of its natural habitat.

Keywords: *Hibiscus coddii* subsp. *barnardii*, ornamental plant, Sekhukhuneland, seed propagation, chemical scarification, vegetative propagation, stem cuttings, *in vitro* shoot culture, *ex vitro* rooting.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
ERI	Emergence rate index
FEP	Final emergence percentage
FGP	Final germination percentage
GRI	Germination rate index
IBA	Indole-3-butyric acid
LSD	Least significant difference
MET	Mean emergence time
MGT	Mean germination time
MS	Murashige and Skoog's culture medium
NAA	1-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
PGRs	Plant growth regulators
T_{50}	Time taken to reach 50% seed germination/seedling emergence based on the total seed population

Chapter 1

CHAPTER 1
Introduction

1.1 Background information

Ornamental plants have one or more aesthetic feature such as attractive and colourful flowers, an aromatic scent or an overall eye-catching foliage texture (Davidson and Miller, 1990). These plants are widely used for decorative purposes in gardens and landscape design projects, as houseplants, for cut flowers and specimen display. They also contribute to humanity's health, well-being and creativity (Davidson and Miller, 1990; Schmidt, 2009). Gardening is becoming a popular hobby of many people who also require new plant varieties to suit their changing lifestyles, such as smaller living places and the fast paced standard of living (Kleynhans and Spies, 2011; Middleton and Vosloo, 2011). The creation of jobs in the horticultural sector and the growing awareness of environmental issues are other driving forces for expanding the ornamental plant market (Kamenetsky and Shlomi, 2012).

In South Africa ornamental plants that have either attractive foliage, or flowers, or both, constitute a significant part of the horticultural industry. Consumers and growers are constantly on the lookout for new suitable plants that could be introduced to the market (Bester *et al.*, 2009). Middleton (2015) conducted extensive research amongst consumers in South Africa on their attitude towards indigenous ornamental plants and reported that they are knowledgeable about the benefits of such plants. There is also increasing pressure to provide such indigenous plants, but to satisfy this market demand it must be possible to produce these plants on a large scale. One such potential indigenous ornamental is *Hibiscus coddii* Exell subsp. *barnardii* (Exell) Leistner & P.J.D. Winter (family Malvaceae), an endemic subspecies that is mainly found in the Northern Dry Mixed Bushveld vegetation type of Sekhukhuneland (Siebert, 2001; Van Wyk and Smith, 2001; Craib, 2003). This perennial plant, that is restricted to rocky outcrops, bears attractive bright orange-red flowers mostly during the summer season from November to March (Retief and Herman, 1997; Craib, 2003; Leistner *et al.*, 2005). According to Craib (2003) the

plant has horticultural potential and it will be suitable as a small shrub, especially in sunny rockeries, and for growing as a pot plant.

1.2 Motivation for study

South Africa has a rich floral biodiversity with several species that have the potential to become popular garden or indoor plants (Bester *et al*., 2009). Despite the fact that research in various fields of ornamental plants and their production has already been conducted in South Africa, the selection of new species, especially those that are indigenous, lags behind (Bester *et al*., 2009; Van Staden *et al*., 2008).

Middleton and Vosloo (2011) pointed out that both consumers and growers in South Africa prefer indigenous plants as these are already adapted to local conditions. The Sekhukhuneland Centre of Plant Endemism (SCPE), located mainly in the Limpopo province, has enormous potential concerning ornamental plants. There are around 2200 plant species, including 30 endemic and 50 near-endemic plant species and infraspecific taxa occurring in the area (Siebert and Van Wyk, 2001; Siebert *et al*., 2001). The attractive red flowers and its ability to grow and survive in drought-prone areas, makes *H. coddii* subsp. *barnardii* ideal as a possible new ornamental plant for South African gardens (Siebert and Van Wyk, 2001; Craib, 2003). Successful introduction of a potential ornamental plant to the market necessitates research on suitable and reliable propagation methods. To the best of my knowledge, no propagation protocols are known for this species and these are an essential requirement for establishing this species in the horticultural milieu. Reliable *in vivo* and *in vitro* propagation protocols, as well as the development of appropriate growth conditions, are required for the plant. In addition, knowledge of the natural habitat of the species in Sekhukhuneland is also important to understand its possible effect on plant propagation.

1.3 Research hypotheses

The research hypotheses for the study are as follows:

I. Assessment of the natural habitat of *H. coddii* subsp. *barnardii* (Chapter 3)

The mineral composition of the soil determines the growth location of *H. coddii* subsp. *barnardii* plants.

II. *In vivo* propagation

By seeds (Chapter 4)

i. Scarification with sulfuric acid improves the germination performance (final germination percentage, mean germination time, germination rate index) of *H. coddii* subsp. *barnardii* seeds.

ii. Temperatures of 25–35°C are the most suitable for germination of *H. coddii* subsp. *barnardii* seeds.

iii. Removal of the apical bud of *H. coddii* subsp. *barnardii* plants stimulates outgrowth of axillary shoots.

By stem cuttings (Chapter 5)

i. Cutting type, Indole-3-butyric acid (IBA) application and root culture medium have a significant effect on rooting performance of *H. coddii* subsp. *barnardii* stem cuttings.

III. *In vitro* propagation (Chapter 6)

i. Various strengths of Murashige and Skoog (MS) medium have a significant effect on *in vitro* seed germination and seedling performance of *H. coddii* subsp. *barnardii*.

ii. Explant source, explant type, cytokinin [6–Benzylaminopurine (BAP)] and auxin [Indole-3-butyric acid (IBA) and 1-Naphtaleneacetic acid (NAA)] affects *in vitro* axillary shoot and multiplication.

iii. Axillary shoots produced *in vitro* successfully root in *in vitro* and *ex vitro* cultures with the application of IBA and NAA.

1.4 Purpose of the study

1.4.1 Aim

The aim of the research was to develop efficient *in vivo* and *in vitro* regeneration protocols for mass propagation of this potential ornamental species.

1.4.2 Objectives

The objectives of the study were to:

I. Assess the natural habitat of *H. coddii* subsp. *barnardii* plants in Sekhukhuneland by describing selected sites of occurrence, collecting and analysing of soil samples, and listing habitat-associated plant species.

II. Evaluate the suitability of various *in vivo* and *in vitro* techniques for plant propagation:

In vivo

i. Seed propagation

Evaluate the effect of various factors on seed germination (seed coat and temperature) as well as on seedling and plant development (culture medium, nutrients, apex removal).

ii. Vegetative propagation

Test the effect of cutting type, exogenous application of Indole-3-butyric acid (IBA) and various culture media on rooting of stem cuttings obtained from *in vivo* grown plants.

In vitro

i. Seed culture

Establish a protocol for *in vitro* germination and development of seedlings that can serve as an aseptic explant source for shoot multiplication.

ii. Shoot culture

Evaluate the effect of various explant sources, types of explants and a range of concentrations and combinations of cytokinins [6–Benzylaminopurine (BAP)] and auxins [IBA and 1-Naphtaleneacetic acid (NAA)] on *in vitro* shoot induction and multiplication.

iii. Root culture

Evaluate the effect of different concentrations and combinations of auxins (IBA and NAA) on the *in vitro* and *ex vitro* rooting of microcuttings.

iv. Acclimatisation

Establish a procedure for successful acclimatisation of *in vitro* produced seedlings and plantlets under controlled environmental conditions.

v. Harden-off acclimatised plants in a greenhouse with uncontrolled environmental conditions.

1.5 Significance of the study

The research is expected to contribute to the popularisation of indigenous and drought-resistant plants such as *H. coddii* subsp. *barnardii* suitable for growing in areas with limited water availability. The propagation protocols developed in this study could be used for *ex situ* conservation purposes and be communicated to plant growers and nurseries for possible commercialisation. Introducing new indigenous South African plants to the market could also contribute to an increase in revenue for plant growers and local nurseries. The research is also in line with the Bio-economic Strategy announced by the Minister of Science and Technology [CSIR International Convention Centre, Pretoria (14/01/2014)] that will promote research on the conservation and commercialisation of South Africa's rich plant biodiversity. As development in Sekhukhuneland expands, the mining industry and human settlements threaten the natural habitat of the plant and there is a need to grow plants *ex situ* for conservation. This work showcases effective propagation methods for the cultivation of a potential ornamental plant, which will lead to its conservation and supply for commercial use.

1.6 Ethical considerations

The research did not involve animals or humans; therefore, ethical clearance was not required. Permits (0090-CPM402-00010 and ZA/LP/73663) for collection of seeds and a limited number of plants from nature were obtained from the Limpopo Department of Economic Development, Environment and Tourism (LEDET).

1.7 Layout of thesis

The thesis consists of seven chapters and five appendices that is presented as follows:

CHAPTER 1

This chapter gives background information on the study topic and a motivation for undertaking the study as well as the aim and objectives of the study. It also mentions the scope and limitations of the study.

CHAPTER 2

A general literature review is presented in this chapter. This includes information on ornamental plants and *H. coddii* subsp. *barnardii* and its natural habitat in Sekhukhuneland. It describes the impact of factors such as apical dominance and mineral nutrition on plant architecture and plant growth. Detailed information on various *in vivo* (seeds and stem cuttings) and *in vitro* (seed, shoot and root culture) propagation methods is provided.

CHAPTER 3

This chapter focuses on *H. coddii* subsp. *barnardii* in its natural habitat and includes a condensed description of plant characteristics. Plant species occurring in the same habitat are listed. Soil analyses from the plant's habitat provide for the screening of mineral elements present.

CHAPTER 4

This chapter deals with *in vivo* seed propagation and the effect of chemical scarification and temperature on seed germination. It also provides information on factors (culture medium, nutrients, apex removal) affecting seedling and plant growth and development. A graphic overview of plant development from a seed to a mature plant is also provided.

CHAPTER 5

In vivo propagation by means of axillary stem cuttings (vegetative) is outlined in this chapter. It presents the effect of cutting type, auxin treatment and various culture media on adventitious root formation and growth parameters of cuttings as well as a graphic overview of vegetative propagation *via* stem cuttings.

CHAPTER 6

This chapter deals with *in vitro* seed germination and seedling development. It also investigates the suitability of such seedlings as aseptic explant source for *in vitro* shoot culture. The effect of plant growth regulators such as BAP, IBA and NAA on shoot induction of various types of nodal explants is included. Finally, *in vitro* rooting and *ex vitro* rooting responses of *in vitro* produced microcuttings are given and the chapter concludes with a graphic overview of *in vitro* culture.

CHAPTER 7

In this last chapter, general conclusions and recommendations for future studies are given.

APPENDICES

Appendices related to Chapters 3–6 are presented at the end of the thesis.

1.8 Scope and limitations of the study

The focus of the study is on various *in vivo* and *in vitro* methods suitable for propagation of *H. coddii* subsp. *barnardii* plants. It will not address the marketability of the plant as a new ornamental. The effect of mineral nutrients on plant growth and development is limited to morphological observations of deficiency symptoms.

The study also gives information on characterisation of *H. coddii* subsp. *barnardii* in its natural habitat, although the plant features are not described in detail. Further, the assessment is limited to basic soil analyses for screening the presence of mineral elements in the soils and does not offer extensive details or an explanation for the plant's occurrence in these specific soils. Material from wild plants was not analysed for mineral element content and will be a subject for further studies. Plant species occurring near *H. coddii* subsp. *barnardii* plants were merely listed and by no means does it offer a complete floristic survey or an ecological study of the plant's habitat.

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Chapter 2

CHAPTER 2
Literature review

The literature review deals with concepts of ornamental plants and the potential of South African indigenous plants to be introduced as new ornamentals. *Hibiscus coddii* Exell subsp. *barnardii* (Exell) Leistner & P.J.D. Winter is such a candidate and is described with its natural habitat in Sekhukhuneland, an important centre of plant endemism. The importance of mineral nutrition to plant growth is highlighted and apical dominance and its impact on plant architecture and flowering is mentioned. Detailed information on various *in vivo* (seeds and stem cuttings) and *in vitro* (seed, shoot and root culture) propagation methods is provided.

2.1 Ornamental plants

Ornamental plants are grown for decorative purposes in gardens and for landscape projects, as indoor plants, for cut flowers and for specimen display. It is also used to enclose areas to provide privacy, cover the ground, prevent erosion and give shade (Simpson and Ogorzaly, 1996; Armstrong, 2000). Ornamentals have one or more aesthetic attributes such as attractive and colourful flowers, eye-catching leaves, an aromatic scent or overall striking foliage texture (Davidson and Miller, 1990; Rout *et al.*, 2006). These plants form an important part of human life as they improve the environment when planted outdoors, while indoor use contribute to people's health, well-being and creativity (Armstrong, 2000; Schmidt, 2009). In a South African context, plants are also cultivated in gardens for cultural reasons such as honouring the ancestors, protection from lightning and in addition, grown for their medicinal value (Constant and Tshisikhawe, 2018).

Plants in an urban environment are often people's only link to the natural growing plant world. It reduces the impact of tarred roads and concrete buildings and improves the quality of life of its residents (Relf and Lohr, 2003; Yournis *et al.*, 2010; Wilson *et al.*, 2016). Gardens and the active involvement when taking care of the plants are used positively in the therapy of handicapped and disabled people (Kwack, 2007). Indoor plants contribute to healthier air in offices and may help to reduce sick-building syndrome (Relf and Lohr, 2003). It is reported that plants have

the ability to remove indoor air pollutants such as benzene, formaldehyde, ozone and toluene. When plants are present in the working environment, it could lead to reduced stress, increased pain tolerance and improved productivity in humans (Lohr, 2010; Thomsen *et al*., 2011; Husti *et al*., 2015).

2.1.1 Selection criteria for ornamental plants

As the cost of creating and maintaining public spaces, corporate and private gardens increase, people select the most suitable plants to meet their needs (Simpson and Ogorzaly, 1996; Noordegraaf, 2000). The preferences of growers and consumers should be kept in mind when there is an intention to introduce new ornamental plants to the market. Therefore, there should be collaboration between researchers, growers, breeders and consumers. Such collaboration can be achieved by doing market-orientated research amongst all interested parties (Kamenetsky and Shlomi, 2012; Middleton, 2015a).

Noordegraaf (2000) stated that new ornamental plants should be chosen keeping its purpose and use in mind. The plant should have attractive flowers or colourful foliage and not be dangerous or toxic to humans. Consumers in Slovenia prefer flowering shrubs with red flowers followed by yellow, pink and white flowers. Individual flowers are also preferred above small flowers borne in large inflorescences (Kravanja, 1995). Yournis *et al*. (2010) stated that people in Pakistan prefer plants with colourful and fragrant flowers and with attractive foliage. They also prefer flowering plants that will attract wildlife (birds, bees and butterflies) and more than 80% of the respondents indicated that they would grow plants from the wild if those were available. Middleton (2015b) reported related results by growers and consumers specifically for South African indigenous ornamental plants. The general appearance (colourful flowers or foliage) and final size and shape of the plant are important attributes. Plants should also complement people's modern lifestyle including smaller living spaces. Furthermore, South African consumers also want plants with a sweet scent and aroma that would attract wildlife such as birds and butterflies. Sought-after plants should perform well in gardens, be drought-tolerant and resistant to pests and diseases (Middleton, 2015b). However, a study by Davoren *et al*. (2016) revealed that people with a lower socio-economic status prefer plants that also have use-value, such as medicinal and food plants. People of higher socio-economic status

are more interested in the aesthetic value of plants and prefer ornamental species in their gardens. There is thus a need to identify unknown plants with ornamental potential that can satisfy the ongoing demands of growers and consumers for new ornamental plants.

2.1.2 Sources of new ornamental plants

Consumers worldwide and in South Africa have a large selection of ornamental plants from which they can choose when buying. Despite the many varieties that are available, the dynamic floriculture industry is continually looking for new products (Noordegraaf, 2000; Reddy *et al*., 2012). According to Kamenetsky and Shlomi (2012), the demand for new ornamentals in the market can be attributed to the increasing popularity of gardening as a hobby. The creation of jobs in the horticultural sector and the growing awareness of environmental issues are other driving forces. People's lifestyles also change and as such, they require new products to fit in with this (Kleynhans and Spies, 2011).

Middleton and Vosloo (2011) reported that in South Africa new ornamental plants are sourced from botanical gardens, plant collectors, specialist nurseries, and heritage plants from old gardens. Plants that are becoming fashionable again or known plants used in a different way, as well as, new cultivars of existing ornamental plants are also considered. However, most plant growers (92%) in South Africa regard indigenous plants as the most important source of new ornamental plants. This preference is based on the adaptation of indigenous plants to specific climatic conditions and soil types. South African consumers also show a positive attitude towards indigenous plants and approximately 50% of them will ask nurseries for such plants (Middleton, 2012). They are aware of the various benefits of planting indigenous plants such as drought-resistance, attraction of wildlife and their contribution to conserving the plants when they grow it in their gardens (Middleton, 2012).

The southern African region (south of the Cunene, Okavango and Zambezi rivers) has an extremely rich plant diversity of more than 30 000 species (Van Wyk and Smith, 2001; Germishuizen and Meyer, 2003). What makes this more unique is that more than 10% of the world's vascular plants are located on less than 2.5% of the

earth's land surface area, and that more than 60% of these species are endemic to the region (Van Wyk and Smith, 2001; Moyo *et al*., 2011). As such, various centres of plant endemism have been identified in southern Africa of which the Cape Floristic Region with its 8500 species is probably the most well known (Van Wyk and Smith, 2001). Despite this rich floristic diversity, referred to as "green gold", South Africa has contributed relatively little to the number of commercial crops in the international market (Van Staden *et al*., 2008). According to Middleton (2015b), this could be due to several inhibitory or restraining factors including competition from exotic plants, the unmet needs of consumers and growers and established shopping habits of customers that are difficult to change. Although the South African public seems to appreciate indigenous plants and are aware that they are contributing to the well-being of nature as a whole when they buy such plants, most of them lack specialised knowledge of indigenous plants. There is thus a need to educate the public more on the specific species, their characteristics and growing conditions (Middleton, 2015b). It seems that wholesale retailers and landscapers are more knowledgeable in the field of indigenous plants; therefore, growers of these plants rely on these people to promote new plants on the market. On the other hand, plant growers and people that do research on indigenous plants need to supply the wholesale retailers and landscapers with detailed knowledge on available indigenous plants. In general, the communication flow between all role players needs to be improved to promote the use of indigenous plants (Middleton and Vosloo, 2011).

Notwithstanding the various types of research that are performed on aspects of ornamental plants in South Africa, it lags behind in the field of the selection of new ornamental plants and then specifically indigenous plants with ornamental potential. There is potential to commercialise some of South Africa's indigenous plants, but the ornamental plant market has specific requirements in terms of production, the global trade and consumer preferences (Bester *et al*., 2009). The question is what can be done to rectify the situation and to convince researchers, growers and consumers of the existence of numerous plants that can be successfully introduced onto the market. Middleton (2015a) indicated that fulfilment of the preferences and needs of consumers and growers of plants, appropriate marketing strategies and affordable pricing could contribute to the overall usage of indigenous ornamental plants in South Africa. The attractive features of *H. coddii* subsp. *barnardii* and its limited

occurrence in drought-prone areas of the Sekhukhuneland Centre of Plant Endemism suggests it ornamental potential and possible application in the horticultural industry.

2.2 Sekhukhuneland

2.2.1 Boundaries

The greater Sekhukhune district, commonly known as Sekhukhuneland, is an area of approximately 13 300 km^2 situated in the Limpopo province of South Africa (Victor *et al.*, 2005; Greater Sekhukhune District Municipality, 2011). Although it is important to know the political boundaries of the region, this study does not concern the political or historical background of the region, but rather focuses on the Sekhukhuneland Centre of Plant Endemism (SCPE). The SCPE covers an area of approximately 4000 km^2 and is located in both the Limpopo and Mpumalanga provinces west of the north-eastern Drakensberg escarpment (Siebert *et al.*, 2002). The Strydpoort Mountains to the north, the Highveld escarpment to the south, Steenkampsberg and Drakensberg on the eastern side and the Springbok Flats in the west, delimits the centre (Van Wyk and Smith, 2001).

2.2.2 Topography and climate of the SCPE

The heterogeneous topography of the SCPE is characterised by parallel belts of mountains and rocky ridges that are combined with flat to undulating valleys (Siebert *et al.*, 2002). The Leolo mountain (1932 m above sea level) and the Steelpoort River valley (700 m above sea level) are the most prominent topographical features (Van Wyk and Smith, 2001; Siebert *et al.*, 2002; Victor *et al.*, 2005).

Sekhukhuneland has warm, moist summers and cool, dry winters (Victor *et al.*, 2005). The drier northern and western parts have warmer temperatures with a maximum daily average of 28.3°C and a minimum of 7.2°C, whereas temperate average daily temperatures exist in the southern and eastern regions (Siebert *et al.*, 2002). The mean annual temperature is 20°C, but can reach a maximum of 38°C in summer and a minimum of -4.5°C in winter in the high-lying areas (Siebert *et al.*, 2003b). The valleys have a subtropical climate with little or no frost in winter (Van Wyk and Smith, 2001). The SCPE lies in the summer rainfall area and receives

nearly half of its rain between December and February, while spring rains contribute 28% of the total rainfall (Siebert *et al.*, 2002). The semi-arid savannah found in the northern part lies in the rain shadow of the Drakensberg escarpment and are drier than the southern parts (Van Wyk and Smith, 2001; Siebert *et al.*, 2003b). The average rainfall for the SCPE is 600 mm, but can be as low as 400 mm in the drier areas and valleys and up to 700 mm on the Leolo Mountains and the southern parts (Van Wyk and Smith, 2001; Siebert *et al.*, 2002).

2.2.3 Geology and soils of the SCPE

The rocks and soils in the SCPE is part of the Bushveld Igneous Complex (BIC) which is the world's largest layered rock intrusion covering an area of about 66 000 km^2 (Scoon and Mitchell, 2009; Roelofse and Ashwal, 2012). The BIC is made up by rocks of the Rooiberg Felsic Group, the Lebowa Granite Suite and the Rustenburg Layered Suite (RLS) and is underlain by rocks from the Transvaal Supergroup and overlain by Karoo sediments (Von Gruenewaldt *et al.*, 1985; Scoon and Mitchell, 2009). The Rustenburg Layered Suite is the outermost of these suites and contains mainly ultramafic and mafic rocks that are well exposed (Haldar, 2011; Roelofse and Ashwal, 2012). It is divided into a prominent western and eastern limb or lobe with further northern and southern extensions. The SCPE is located within the eastern lobe of the RLS (Van Wyk and Smith, 2001; Haldar, 2017). The RLS is subdivided vertically, from bottom to top, into several zones namely Marginal, Lower, Critical, Main and Upper Zone. The Critical Zone is sometimes divided into a Lower, Middle and Upper Critical Zone (Mungall *et al.*, 2016). The main rock types and minerals in each zone are shown in Table 2.1.

Ultramafic and mafic rocks and the soils that derive from them are low in SiO_2 (40–50%) and contain high concentrations of Mg, Fe, Ca, Na, K, and Al compared to other rocks. The magnetite in the Upper Zone is rich in V. The Critical Zone, and more specifically the Upper part, contains large deposits of platinum group elements (PGE) and chromium in the UG-2 chromitite layer and the Merensky Reef. Notable amounts of Cu, Ni, Co and Au are found as by-products. These Pt and Cr deposits in the Critical Zone are of the richest in the world and led to the establishment of many mining activities in Sekhukhuneland (Von Gruenewaldt *et al.*, 1985; Kinnaird, 2005; Cawthorn *et al.*, 2006).

Table 2.1. The main rock types and minerals contained in the various zones of the Rustenburg Layered Suite (Kinnaird, 2005; Scoon and Mitchell, 2009; Mungall *et al.*, 2016).

Zone	Rock types	Minerals
Upper	Gabbro, Anorthosite, Magnetite	Olivine, Clinopyroxene, Plagioclase, Magnetite, Apatite
Main	Pyroxenite, Anorthosite, Gabbronorite	Orthopyroxene, Clinopyroxene, Plagioclase
Upper Critical	Pyroxenite, Anorthosite, Norite, Chromitite	Orthopyroxene, Plagioclase, Chromite
Lower Critical	Pyroxenite, Chromitite	Orthopyroxene, Chromite
Lower	Dunite, Pyroxenite, Harzburgite	Olivine, Orthopyroxene
Marginal	Norite, Pyroxenite	Plagioclase, Clinopyroxene, Hornblende

Soils in the SCPE tend to be rich in clay. The rocks give rise to mainly red or black heavy metal-containing or ultramafic montmorillonite clay soils that are rich in smectite clay minerals and Ca, K, Na and Mg ions (Siebert, 2001; Siebert *et al.*, 2001). The most common soil types are red apedal soils, loamy Valsrivier soils on the plains and shallow Glenrosa soils on the low-lying rocky hills (Mucina and Rutherford, 2006).

2.2.4 Vegetation types and flora of the SCPE

The most part of the SCPE fits into the Sekhukhune Plains Bushveld (SVcb 27) vegetation type that is part of the Savannah Biome (Mucina and Rutherford, 2006). The vegetation is broadly defined as mountain bushveld that forms a mosaic with moist grasslands in the south and semi-arid bushveld in the north (Siebert *et al.*, 2003a). Small patches of Afromontane forest and fynbos-type vegetation occur on the summit of the Leolo Mountains (Van Wyk and Smith, 2001). Siebert (2001) and Siebert *et al.* (2002) recognised three major floristic regions in the SCPE namely Arid Bushveld, Mountain Bushveld and Grassland, which are further characterised by six major vegetation types. These include the Northern Dry Mixed Bushveld, Closed Mountain Bushveld, Open Mountain Bushveld, Rock Outcrop vegetation, Cool Moist Grasslands and Wetland Vegetation (Siebert *et al.*, 2002). According to Siebert and Van Wyk (2001) these vegetation types are the result of the topography, soils and

rocks, varying regional climate and changes in altitude from 700 m to 1500 m over short distances in which the plants of the region have developed.

The SCPE is home to about 2200 plant species with an estimated 4.5% endemic or near-endemic species (Van Wyk and Smith, 2001). Most of these species are known only from specific localities in Sekhukhuneland and are considered rare. Siebert *et al.* (2001) recognised 30 endemic and 50 near-endemic plant species and infraspecific taxa in the SCPE. The endemic plant taxa belong to 20 plant families of which the Euphorbiaceae, Vitaceae and Araceae has three or more endemic plant species. Some of these include *Euphorbia barnardii*, *E. sekukuniensis*, *Rhoicissus sekhukhuniensis* and *Zantedeschia jucunda* and *Z. pentlandii* that have very attractive yellow flowers making them sought-after garden plants (Siebert *et al.*, 2001). The superficial tubers of *Z. jucunda* and *Z. pentlandii* plants are easily removed which led to a marked decline in wild populations (Van Wyk and Smith, 2001). Other noteworthy endemic plant species that could be researched for ornamental potential include *Searsia batophylla, S. sekhukhuniensis* (Anacardiaceae), *Plectranthus venteri* (Lamiaceae) *Jamesbrittenia macrantha* (Scrophulariaceae), *Triaspis glaucophylla* (near-endemic, Malpighiaceae), the highly threatened *Prototulbaghia siebertii* (Alliaceae) and *H. coddii subsp. barnardii* (Malvaceae) which will be the topic of research for this study (Siebert and Van Wyk, 2001; Van Wyk and Smith, 2001; Vosa, 2007).

The Sekhukhune Plains Bushveld vegetation type has been extensively transformed and is classified as vulnerable mainly due to residential developments, mining activities, severe overgrazing, and both man-made and natural erosion dongas (Rouget *et al.* 2004; Victor *et al.*, 2005; Mucina and Rutherford, 2006). Propagation of endemic species that are found here would contribute to their *ex situ* conservation and could add to the pool of indigenous species suitable for horticultural purposes.

2.3 *Hibiscus coddii* subsp. *barnardii*

The plant species belongs to the family Malvaceae that worldwide contains 90 genera with 2000 species. Members are mostly herbs and shrubs that occur in tropical and subtropical areas extending into temperate regions of the world. The family is widespread in southern Africa (South Africa, Namibia, Botswana, Swaziland, and Lesotho) with 21 genera and 165 species, but the highest diversity is found in the northern areas of South Africa (Koekemoer *et al*., 2013). Well known genera include *Abutilon*, *Anisodontea*, *Gossypium*, *Hibiscus*, *Pavonia* and *Sida*. Plants are mainly found in savannah, scrub and forest edge habitats (Koekemoer *et al*., 2013).

Hibiscus coddii and *Hibiscus barnardii* were previously described by Exell as different species, but Leistner *et al*. (2005) reviewed this and divided *H. coddii* into two subspecies namely *H. coddii* subsp. *coddii* and *H. coddii* subsp. *barnardii* mainly on its clear geographic separation and also morphological appearance. The latter subspecies is endemic to Sekhukhuneland and is mainly found in the Northern Dry Mixed Bushveld vegetation type as described by Siebert (2001). Plants often grow amongst dark-coloured rocks where they prefer intense north or west sunlight and are exposed to extremely hot conditions (Van Wyk and Smith, 2001).

The *H. coddii* subsp. *barnardii* plant is described as an upright, slender perennial herb with a height of 600–1500 mm and bears single, suborbicular-cordate hairy leaves arranged in an alternate fashion on the stems. The leaf margin is coarsely serrated and filiform stipules are borne at the base of the petiole (Leistner *et al*., 2005). Bright red to orange-red flowers appear during the summer season from November to March (Retief and Herman, 1997; Craib, 2003; Leistner *et al*., 2005). Flowers are borne singly, but sometimes also in terminal racemes or corymbs due to reduction of the upper leaves. A calyx and epicalyx is present and the five petals are adnate to a staminal tube at the base. The style branches into five capitate stigmas. Ripe fruit capsules open widely during dry weather to release the seeds that are covered with white cottony floss (Craib, 2003; Leistner *et al*., 2005). Labels on specimens located in the National Herbarium in Pretoria indicate that the plant is used medicinally, but does not specify the exact use.

Van Wyk and Smith (2001) and Craib (2003) indicated that the plant has horticultural potential. It forms abundant seeds, but might require hand pollination. The plant will perform best where it receives at least four hours of direct sunlight per day, which makes them ideal as rockery plants. The small size of the plant and its attractive red flowers also makes it suitable as a pot plant (Craib, 2003). These characteristics and possible use of *H. coddii* subsp. *barnardii* as a drought-tolerant plant merited research, since no substantial information on propagation protocols for this plant is available.

2.4 Propagation of plants

In the horticulture industry, propagation of ornamental plants is essential to ensure large numbers of plants, while at the same time preserving important plant characteristics (Ibironke, 2017). Plants are propagated and cultivated in special growth rooms under controlled temperatures and relative humidity with artificial light supplied by special lamps and tubes. Outdoor structures such as greenhouses, tunnels and shade nets are used to grow plants under both controlled and uncontrolled environmental conditions. Plants can also be grown in the field where environmental conditions cannot be controlled, but mineral nutrition can be manipulated (Sadhu, 1989; Hartmann *et al*., 2011).

Various factors such as water, light, temperature and mineral nutrition influence the growth and development of plants. Insufficient or lack of any of these factors will cause stress in plants that will lead to stunted growth, reduced flowering and seed formation and even death of the plant. Insufficient water supply can cause drought stress and affect plant performance and survival. Light is needed for photosynthesis, seed germination in some species and induction of flowering. Plant growth is favoured at warmer temperatures and slows down during cooler periods or plants even become dormant during winter (Sadhu, 1989; Hartmann *et al*., 2011). Sufficient mineral elements are also required to ensure optimum plant growth and healthy plants that will flower and produce fruits and seeds.

2.4.1 Plant mineral nutrition

Plants require 17 essential elements for their normal growth, development and reproduction. These include C, H, and O, so called macronutrients that plants can obtain from water and air. The other 14 elements are divided into soil-derived macronutrients (N, P, K, S, Ca, Mg) and micronutrients (B, Cl, Cu, Fe, Mn, Mo, Ni, and Zn). The level of macronutrients in plants is normally more than 0.1% of the plant's total dry weight, whereas micronutrients are present at lower concentrations, a few parts per million (ppm) of the plant's total dry weight (Uchida, 2000; Mahler, 2004). The presence of mineral elements in the soil does not necessarily imply that they are available for uptake by plant roots since absorption is determined by the surface charge, pH, structure and texture of the soil. Mineral cations such as K^+ and $NH4^+$ are adsorbed by the negatively charged surface of organic and inorganic soil particles, which can be replaced by other cations during cation exchange. Mineral elements with a negative charge (anions) such as NO^{3-} and PO_4^{3-} remain dissolved in the soil solution and easily leach from the soil. Most nutrients become available to plants when the pH ranges between 5.5 and 7. Outside this range most nutrients are not available for uptake, which could lead to deficiency symptoms in plants (Pandey, 2015). Once absorbed, some mineral elements (N, P, K, Mg, Cl, Na, Zn and Mo) can move from one part in the plant to another part (mobile elements), whereas others (Ca, S, Fe, B, Cu) are immobile. Lack of or insufficient amounts of mobile elements will first cause visible deficiency symptoms in the older leaves since these elements are transported to younger plant parts to support growth. Insufficient amounts of immobile elements will first cause visible deficiency symptoms in the younger leaves (Mahler, 2004; Taiz and Zeiger, 2010).

Essential elements have different biochemical functions in plants. Nitrogen and S form part of C-containing compounds such as amino acids, nucleic acids, proteins, and vitamins. Plants absorb nitrogen in NO_3^- or NH_4^+ form and lack of sufficient N causes stunted growth and so-called 'V'-shaped chlorosis that starts from the leaf tip of older leaves. The entire plant becomes pale green and the leaf petiole and veins turn purple due to synthesis of anthocyanins (Uchida, 2000; Pandey, 2015). Sulfur is taken up by plants mostly as sulfate ions (SO_4^{2-}) and observable deficiency symptoms are similar to that of N. The chlorosis symptoms however, first appear in young leaves rather than old leaves (Mahler, 2004; Taiz and Zeiger, 2010). Plant

roots take up P as $H_2PO_4^-$, HPO_4^{2-} or PO_4^{3-} depending on the soil pH and it is a component of nucleic acids, sugar phosphates and coenzymes. Insufficient amounts of P cause stunted growth and dark-green to purple leaves often with small necrotic spots (Uchida, 2000; Taiz and Zeiger, 2010). The other essential macronutrients, K, Ca and Mg, remain in ionic form in the plant. Potassium, taken up as K^+, is a cofactor for more than 40 enzymes and maintains turgor pressure. Chlorosis in older leaves near the base of the stem is the first noticeable symptom of K deficiency, which later turns into necrosis at the leaf tip, and along the leaf margin. Leaves may also crinkle and curl and the size and quantity of fruits and seeds are reduced (Uchida, 2000; Taiz and Zeiger, 2010; Pandey, 2015). Calcium, taken up as Ca^{2+}, is an integral part of the middle lamella of cell walls and acts as second messenger in metabolic regulation. It is immobile in plants and deficiency symptoms such as necrosis and deformed young leaves are first visible at the growing tips of roots and shoots followed by severe stunted growth (Mahler, 2004; Taiz and Zeiger, 2010). Magnesium (Mg^{2+}) is part of the porphyrin structure of the chlorophyll molecule and is important for photosynthesis. Interveinal chlorosis in the older leaves is an evident sign of Mg deficiency which can culminate into yellow or white leaves and premature leaf abscission (Uchida, 2000; Taiz and Zeiger, 2010; Pandey, 2015).

Boron, taken up as H_3BO_3, $H_2BO_3^-$ or HBO_3^{2-}, promotes translocation of sugars and cell elongation and when deficient the growing stem tips are often malformed (Mahler, 2004). The elements Cl, Mn and Na remain in ionic form in plants. Chlorine (Cl^-) is required for the water-splitting reaction of photosynthesis and for cell division in leaves and roots. Wilting of leaf tips, and general leaf chlorosis and necrosis are typical deficiency symptoms (Taiz and Zeiger, 2010). Plant species that use the C_4 and CAM pathways of photosynthesis need Na^+ ions. If not available, these plants will show chlorosis and necrosis of leaves and no flower formation (Taiz and Zeiger, 2010). Manganese (Mn^{2+}) is important in the Hill reaction of photosystem II and it activates decarboxylases and dehydrogenases enzymes in the citric acid (Krebs) cycle (Mahler, 2004; Taiz and Zeiger, 2010). Noticeable deficiency symptoms include small yellow spots and interveinal chlorosis of younger leaves (Pandey, 2015).

The other micronutrients, Fe, Cu, Zn, Mo and Ni, are involved in redox reactions in plants. Iron (Fe^{2+}) plays a role in photosynthesis, mitochondrial respiration, scavenging of reactive oxygen species and synthesis of chlorophyll and certain plant growth regulators (PGRs). Copper (Cu^{2+}) has an essential role in photosynthesis, carbon and nitrogen metabolism, cell wall synthesis and oxidative stress protection. Insufficient Fe results in interveinal leaf chlorosis, whereas lack of enough Cu leads to short internodes, stunted growth and dark green leaves with necrotic spots in the younger part of the plants (Taiz and Zeiger, 2010; Pandey, 2015). Plants take up Zn^{2+} ions, which are required for breakdown of proteins and activation of enzymes such as carbonic anhydrase. Visible symptoms of Zn deficiency include short internodes that causes a rosette growth form, small leaves and interveinal chlorosis with white necrotic spots in older leaves (Mahler, 2004; Taiz and Zeiger, 2010; Pandey, 2015). Molybdenum ions (Mo^{4+}) are components of several enzymes such as nitrate reductase and nitrogenase, and lack of or insufficient quantities cause chlorosis and necrosis in older leaves. Other symptoms include suppression of flower formation and premature flower-fall (Taiz and Zeiger, 2010). Nickel is part of the urease enzyme in plants and deficiencies are seldom reported. However, Ni can become toxic to plants in high concentrations and causes chlorosis and necrosis in leaves (Taiz and Zeiger, 2010; Pandey, 2015). Sufficient minerals are thus required for optimum growth and healthy plants that will flower and produce fruits and seeds. This is especially important for pot plants that do not have access to natural soils with mineral elements and, therefore, these plants need to be supplied with nutrients.

2.4.2 Plant architecture and flowering

Plant growth is characterised by an increase in the size, weight and volume of the plant and is affected by various internal and external factors. Plant development refers to the change in the life stages of the plant from seed germination to the mature flowering plant. Development causes different growth forms or shapes (architecture) depending on the plant species (Rowe and Speck, 2005; Taiz *et al.*, 2015).

Plant architecture

Flowering plants have different growth forms because of different branching patterns, leaf size and position of leaves, flowers and fruits. These are also referred to as plant

architecture (Teichmann and Muhr, 2015). Plant species can be identified by their unique plant architecture since it is mostly genetically determined, although environmental factors such as light, temperature, moisture, mineral nutrition and plant density can influence it to some extent (Napoli *et al*., 1999; Reinhardt and Kuhlemeier, 2002; Barthélémy and Caraglio, 2007; Wang and Li, 2008).

As the apical shoot meristem of an herbaceous plant develops, axillary buds arise exogenously from superficial cell layers in the axils of leaf primordia. The formation of an axillary shoot involves first the initiation of the axillary bud followed by the outgrowth of the bud into a shoot or branch. Once initiated an axillary meristem (bud) may remain dormant or it can develop into an axillary shoot (Teichmann and Muhr, 2015; Tian and Jiao, 2015). The architecture or form of a mature flowering plant is determined by the outgrowth of axillary shoots that form laterally at some distance away from the apical meristem of the shoot. In many plant species, the axillary buds become dormant because the primary shoot apex inhibits the outgrowth of these buds through the suppressing action of auxin produced in the apical buds. Therefore, branching of axillary shoots is delayed (Hartmann *et al*., 2011). This phenomenon is known as apical dominance since the main shoot is active for its entire life span (indeterminate growth) and will first produce leaves and later flowers (Cline 1997; Reinhardt and Kuhlemeier, 2002; Barthélémy and Caraglio, 2007; Wang and Li, 2008). Different types of apical dominance in plants are recognised. It is negligible or even non-existent when there is no rest or dormancy period between axillary bud formation and outgrowth of axillary shoots. In intermediate apical dominance, the axillary buds are partially inhibited since there is a certain level of shoot outgrowth even without removing the apical bud. Strong apical dominance is characterised by complete inhibition of the axillary buds and no bud will grow out unless the apical bud is removed (Cline 1997; Napoli *et al*., 1999). In apical shoots with indeterminate growth, monopodial branching occurs since the apical bud continues with growth and lateral shoots only grow out lower down the stem where the hormonal effect of the apical bud has diminished. In sympodial branching the apical bud dies or is transformed into a flower or tendril where after axillary shoots grow out (Reinhardt and Kuhlemeier, 2002; Barthélémy and Caraglio, 2007).

When the apical bud of a stem is removed by human interference or by natural causes, so-called de-topping, it induces the sprouting of axillary buds lower down the stem. The outgrowth of axillary shoots will also eventually increase the number of flowers, fruits and seeds that are produced per plant (Cline 1997; Mollah *et al.*, 2017). Huhta *et al.* (2000) removed the apical bud of *Erysimum strictum* and *Rhinanthus minor* plants where after they produced the highest number of lateral branches and nodes, while the control plants with an intact apical bud failed to form lateral shoots. The plant growth form or architecture can thus be manipulated by pruning and removal of the stem apex. This will lead to more flower and fruit formation, while also inducing outgrowth of axillary stems that could be used for vegetative propagation by cuttings (Cline 1997; Mollah *et al.*, 2017).

Flowering

Various developmental phases are recognised in the plant life cycle. Juvenile plants in the vegetative phase grow at the shoot regions where apical meristems produce leaves and cannot flower even if environmental cues for flowering are present. A plant in the adult vegetative phase is competent to flower and it ends with the reproductive phase when the meristems produce flowers (Evert and Eichhorn, 2013; Taiz *et al.*, 2015). Flowering affects the growth form or architecture of plants. In some plants, the apical meristem of the main stem shows indeterminate growth and is active for the entire life span of the plant. It will first form leaves and later flowers. In other plants, the shoot apical meristem will terminate into a single flower and development continues from the lateral meristems, a so-called determinate apical meristem (Barthélémy and Caraglio, 2007).

For flowering to occur in plants, certain events have to take place in the apical meristem that commits the meristem to produce flower buds and floral leaves instead of normal leaves. This process of floral evocation can be brought about by both internal and external factors. The ability of a plant to flower is linked to the size and age of the plant. In some plants certain environmental signals such as daylength and temperature are required, although some plants also flower independently from environmental cues (Evert and Eichhorn, 2013; Taiz *et al.*, 2015).

2.4.3 *In vivo* propagation of plants

Different methods such as, division, grafting, budding and layering, are employed during *in vivo* propagation, although the most conventional practices are to grow plants by seeds and vegetatively (clonally) by cuttings (Sadhu, 1989; Hartmann *et al.*, 2011).

2.4.3.1 Seed propagation

In nature, seeds are the beginning of the next generation of sexual reproduction in plants. Commercial propagation of plants for agricultural and horticultural purposes is commonly done by seeds (Hartmann *et al.*, 2011). Seed germination is an important stage in plant growth and refers to the physiological and developmental processes that take place in seeds when they are exposed to appropriate environmental conditions for germination (McNair *et al.*, 2012).

2.4.3.1.1 Factors affecting seed germination

Various internal and external factors influence seed germination. External environmental factors such as water, temperature, gas exchange and light play a role in the success of seed germination (Hartmann *et al.*, 2011; Baskin and Baskin, 2014). Seeds are considered to be dormant when no germination occurs despite the presence of favourable environmental conditions. Dormancy is not just the absence of germination; it is also a seed characteristic unique to each plant species that determines conditions for germination (Finch-Savage and Leubner-Metzger, 2006).

External and internal factors associated with the seed coat and embryo itself can cause primary dormancy. Various types of primary dormancy are recognised depending on plant species and seed properties. Endogenous dormancies include physiological (most common), morphological and morpho-physiological types that are associated with the embryo itself and is considered to be controlled by physiological inhibiting mechanisms. Exogenous physical dormancy exists outside of the embryo and is caused by an impermeable seed coat (hard seeds) and or chemical inhibitors present in the seed coverings (Hartmann *et al.*, 2011; Baskin and Baskin, 2014). A seed without primary dormancy will germinate readily when exposed to optimal environmental conditions (Baskin, 2003; Finch-Savage and Leubner-Metzger, 2006). Some seeds have one or other form of primary dormancy

that prevents germination even when favourable environmental conditions exist (Hartmann *et al*., 2011). Since seeds of plants that belong to the Malvaceae family, including *Hibiscus* species, are known for their hardiness or physical dormancy (Serrato-Valentini *et al*., 1992; Van Assche and Vandelook, 2006), only this type of dormancy will further be mentioned.

a. Hard seed coat

Hard seeds with physical dormancy are a way of survival, but can pose a problem in plant cultivation practices as it might prevent simultaneous germination and uniform seedlings (Serrato-Valentini *et al*., 1992). Such seeds are unable to take up water even when environmental conditions are favourable for seed germination. The water impermeability starts during the later stages of seed maturation when the seed loses water. The seed coat dries out and the integument develops layer(s) of palisade cells with thick lignified secondary walls (macrosclereids) resulting in a hard seed (Erickson *et al*., 2016). Natural openings on the seed (hilum, micropyle and chalazal area) also become impermeable to water. Several water-repelling substances such as cutin and waxes can also be deposited onto the seed coat. (Poljakoff-Mayber *et al*., 1992; Baskin *et al*., 2000; Gama-Arachchige *et al*., 2013; Geneve *et al*., 2018). Before imbibition of water and subsequent germination phases can occur, the hard seed coat must become permeable to water. The water gap, a small, specialised opening on the seed coat, needs to be disrupted or removed to allow for water absorption. These water gaps differ between plant families and even between genera within the same family and type I, II and III water gap complexes are recognised (Gama-Arachchige *et al*., 2013; Geneve *et al*., 2018). Several genera in the Malvaceae family have a type III water complex which can be a chalazal oculus, a chalazal blister gap or a chalazal slit (Hartmann *et al*., 2011; Gama-Arachchige *et al*., 2013; Geneve *et al*., 2018). According to Egley and Paul (1981), the water gap structure in *Sida spinosa* is a chalazal blister, while a chalazal slit exists in other members of the Malvaceae family such as *Kosteletzkya virginica* and *Berrya cordifolia* (Poljakoff-Mayber *et al*., 1994; Gama-Arachchige *et al*., 2013; Geneve *et al*., 2018). The cap-like structure associated with the chalazal region in *Sphaeralcea munroana* seeds detached when seeds were placed in boiling water allowing inflow of water into the seeds (Kildisheva *et al*., 2011).

A hard seed coat prevents the sufficient uptake of water and emergence of the radicle and needs to be softened before imbibition of water can take place. The exact mechanism for uplifting physical dormancy under natural conditions is not known for plant species with physical dormancy (Van Assche and Vandelook, 2006). Factors such as fire, drying, freezing alternated with thawing, passage through the gut of animals, as well as high and widely fluctuating temperatures might be involved (Baskin *et al*, 2000; Baskin and Baskin, 2014). Various studies showed that temperature is the primary factor that regulates the opening of the water gap and it is reported that the water gap structure in the seeds might be able to detect high or fluctuating temperatures that will support seed germination and subsequent seedling development. It can also act as a rain gauge that will prevent germination until the soil is moist enough (Chawan, 1971; Baskin, 2003; Kildisheva *et al*., 2011; Baskin and Baskin, 2014; Erickson *et al*., 2016).

Uplifting the physical dormancy for commercial or research purposes is normally done through mechanical scarification, wet and dry heat treatments as well as softening the seed coat with acids such as sulfuric acid (H_2SO_4) which will allow for the uptake of water (Hartmann *et al*., 2011; Van Assche and Vandelook, 2006; Baskin and Baskin, 2014). An increase in germination percentage and improvement of other germination parameters after scarification of seeds are widely documented in literature. Germination of *Sphaeralcea coccinea* seeds increased ten times from 4.7% in untreated seeds to 51.3% in seeds mechanically scarified with sandpaper (Dunn, 2011). Chauhan *et al*. (2006) reported 87% germination of *Malva parviflora* seeds nicked with a scalpel, while the lowest germination (12%) was found in non-scarified seeds. Although mechanical scarification is effective in uplifting dormancy it is tedious and time consuming and other more practical methods are also employed (Hartmann *et al*., 2011; Kildisheva *et al*., 2011).

The best treatment for hard seeds of *Sphaeralcea munroana* that resulted in 49% germination was scarification in boiling water (100°C for 10 seconds) (Kildisheva *et al*., 2011). Al-Sherif (2007) reported 100% germination in seeds of *Prosopis farcta* when scarified in 98% H_2SO_4 for 15–30 minutes compared to no germination in non-scarified seeds. Seeds of *Sida rhombifolia* needed an extended time (120 minutes) of scarification in concentrated sulfuric acid to reach the highest germination

percentage (65%). Scarification in H_2SO_4 for shorter periods (5, 10, and 30 minutes) resulted in less than 20% germination, while only 5% of non-scarified seeds germinated (Chauhan and Johnson, 2008).

b. Water

Seeds start to dry out when reaching maturity and can lose up to 90% or more water leaving the seed extremely dehydrated and sufficient uptake of water is, therefore, required to hydrate the embryo and endosperm. Seed germination starts with rapid uptake of water by the dry seed (imbibition) followed by a lag phase with little water uptake but active metabolism and embryo expansion, and following additional water uptake, culminates in the appearance of the radicle through the seed coverings (Finch-Savage and Leubner-Metzger, 2006; Hartmann *et al.*, 2011). The stored food in the endosperm is hydrolysed by enzymes to release the energy required by the embryo. These biochemical reactions require water in order to activate germination. After germination, the seedling needs to photosynthesise and a continuous supply of water is necessary for this (Evert and Eichhorn, 2013).

c. Temperature

Temperature affects the percentage and rate of seed germination and the further development of seedlings (Hartmann *et al.*, 2011; Baskin and Baskin, 2014; Taghvaei and Nasrolahizadehi, 2016). The optimum temperature for seed germination results in the highest percentage of germination in the shortest time, while the minimum and maximum temperatures are respectively the lowest and highest temperatures at which seeds will germinate (Taghvaei and Ghaedi, 2010; Hartmann *et al.*, 2011). The optimal temperature for most seeds that are not dormant is 25 to 30°C, but it is dependent on the time of the year when seeds are formed and also on the geographic origin of the species (Serrano-Bernardo *et al.*, 2007). In general, seeds of cool-season plants such as *Primula* and *Freesia* species germinate best at temperatures below 25°C, whereas seeds of warm-season plants such as *Gossypium hirsitum* and *Glycine max* need higher temperatures (25–30°C) and fail to germinate below 10°C or 15°C. Seeds of several other species germinate over a wide range of temperatures, while some seeds germinate better when there is a daily temperature fluctuation of 10°C between the maximum and minimum temperature (Hartmann *et al.*, 2011; Evert and Eichhorn, 2013).

Serrano-Bernardo *et al.* (2007) reported 60% germination when seeds of *Hormathophylla spinosa* were kept at 15°C, which significantly reduced to 26% when seeds were exposed to a higher temperature (25°C). Temperatures of 25°C and 30°C resulted in 70% germination in seeds of *Arthrocereus glaziovii*, but lower (15°C) and higher (35°C) temperatures only achieved 10% germination (Cheib and Garcia, 2011). The optimum constant temperature for germination of *K. virginica* seeds was 28°C to 30°C (Poljakoff-Mayber *et al.*, 1994). Seeds of *Sida cordifolia* exposed to alternating temperatures (12 hours at 5–7°C, 12 hours at 100°C) germinated better (69%) than seeds kept at constant temperatures of 25°C (10%) and 60°C (no germination) (Mishra, 2016). Chachalis *et al.* (2008) also found less than 40% germination when *H. trionum* seeds were kept at constant temperatures of 15°C to 40°C (5°C intervals) and no germination at 10°C and 45°C. Alternating day/night temperatures of 30°C/20°C resulted in the highest germination percentage (60%).

d. Light

The intensity, wavelength and duration (photoperiod) of light have an effect on seed germination. Light sensitivity in seeds is regulated by the phytochrome pigment which exists in two forms, the inactive phytochrome red (P_r) and the biologically active phytochrome far-red (P_{fr}). Small seeds that have limited nutrient reserves mostly need light to germinate and will germinate better when placed on or just below the soil surface (Chachalis *et al.*, 2008). Most seeds are not affected by light, but many seeds, including forest species or weeds, will not germinate until an opening in the canopy allows sufficient light for growth of the seedling (Evert and Eichhorn, 2013).

Germination responses may vary depending on plant species and light conditions. Small seeds of *Arthrocereus melanurus* subsp. *odorus* achieved 75% germination when germinated in the light at 25°C, but no seeds germinated in the dark. Small seeds have limited nutrient supplies and when buried too deep in the soil the seedling will not be able to penetrate above the soil surface due to insufficient food reserves (Cheib and Garcia, 2011). Constant darkness favoured germination of *Thymus serpylloides* seeds (78%), but was detrimental to germination (28%) in *Genista versicolor* seeds. When exposed to alternating light:dark (12:12 hours)

conditions the germination in *T. serpylloides* reduced to 62%, but improved to 42% *in G. versicolor* seeds (Serrano-Bernardo *et al*., 2007). Seeds of *Lavatera agrigentina* have no light preference and showed similar germination responses when kept in constant darkness (60%) and in light:dark (12:12 hours) conditions (63%) (Santo *et al*., 2015).

e. <u>Gas exchange</u>

Germinating seeds respire at a high rate and an adequate supply of oxygen is necessary for germination and seedling growth. Oxygen supply and diffusion is limited in hard soils and poorly aerated or water-flooded germination media. Carbon dioxide can accumulate around the seed under poor aeration conditions and in deep soils, which might inhibit germination. In some seeds, dormancy can be uplifted by high carbon dioxide levels (Sadhu, 1989; Hartmann *et al*., 2011).

Seed germination patterns differ between plant families, genera and even between species and, therefore, should be studied for each species to establish the most suitable protocol.

2.4.3.1.2 Germination parameters

The completion of germination is considered as the emergence of the radicle from the seed coat or the appearance of cotyledons above soil level (Hartmann *et al*., 2011; McNair *et al*., 2012). Plant biologists are interested in the measurement of germination since it differs between plant species, populations, seed lots and seed pre-treatments (Thomson and El-Kassaby, 1993; Soltani *et al*., 2015). Researchers developed numerous formulas or measurements of seed germination, which includes the final germination percentage and an indication of the speed and distribution of germination (Al-Mudaris, 1998). The most common parameter used to quantify seed germination is the final germination percentage (FGP) which is the number of seeds in a seed population that produces a seedling expressed as a percentage. A higher FGP indicates a better germination performance of a seed population. Although the FGP of different seed lots might be the same, the germination patterns can be very different, therefore, other germination parameters are also considered (Hartmann *et al*., 2011; McNair *et al*., 2012). Amongst these are the germination rate index (GRI) and the mean germination time (MGT) (Cheib and

Garcia, 2011; Rehman *et al.*, 2014). The germination rate or speed (GRI) measures how fast a seed lot germinates. A higher GRI suggests a higher percentage and rate of germination (Al-Mudaris, 1998; Hartmann *et al.*, 2011). The mean germination time (MGT) is the mean time a seed lot needs to initiate and end germination. A lower MGT shows a faster germination by the seed population. According to Soltani *et al.* (2015), the MGT does not give the time from the start of imbibition to a specific germination percentage. It is not the real time to mean germination, but just an index of germination speed. In certain cases it is therefore also important to determine the time required for a seed lot to reach a predetermined percentage based on the FGP, for example the time to reach 50% germination (T_{50}). In cases of a lower FGP the time required for a seed lot to reach 20% germination, the T_{20} value, is determined (Hartmann *et al.*, 2011). Some other parameters not dealt with in this study include the coefficient of velocity of germination (CVG), the time spread of germination (TSG), Kotowski's coefficient of velocity, Czabator's germination value, Maguire's speed of germination and Timson's cumulative germination (Ranal and De Santana, 2006).

The development of a suitable protocol for seed germination is useful for commercial production, which requires seedlings at the same developmental stage. Mature plants developed from seedlings can also serve as a source for conventional propagation by cuttings and *in vitro* multiplication. Such plants are also useful for *ex situ* conservation purposes and for re-introduction of plants into nature (Papafotiou and Martini, 2016).

2.4.3.2 Vegetative propagation by stem cuttings

During vegetative propagation, a piece (cutting) of a plant is used to develop a new plant without fertilisation (Megersa, 2017). Cuttings obtained from the stem, root or leaves of plants are used for multiplication of plants (Relf and Ball, 2009; Abdullateef and Osman, 2012). Stem cuttings is the most common way of vegetative or clonal propagation in many plants, including ornamental species (Thakur *et al.*, 2018). This ensures that the unique characteristics of the original mother plant are preserved in the newly formed plants (Relf and Ball, 2009; Hartmann *et al.*, 2011; Megersa, 2017). It is also a fast and cost-effective way of multiplying plants, especially in

hybrids that do not form seeds and in plants that are difficult to propagate by seeds (Manokari *et al*., 2016; Aslam *et al*., 2017).

Successful propagation by stem cuttings relies on the formation of adventitious roots at the base of the cutting since terminal and axillary buds that can continue with stem growth are already present on the cutting (Geiss *et al*., 2009; Hartmann *et al*., 2011; Pop *et al.,* 2011). Bertram (1992) mentioned that fast adventitious root formation on cuttings promotes increased axillary bud sprouting, proliferation of longer axillary shoots and a larger leaf area, which in turn leads to a better quality plant. Adventitious roots usually derive from stem or leaf cells, but not from the radicle of the embryo (Haissig, 1974; Blakesley *et al*., 1991; Geiss *et al*., 2009). Steffens and Rasmussen (2016) distinguished between adventitious roots that are formed during normal development such as the nodal roots on stolons of strawberry plants, those formed in response to stress for example in flooded plants, and adventitious roots that are formed in response to wounding when making stem cuttings.

Wound response signalling pathways are initiated at the base of the cutting after its excision from the main stem. Auxin and a sufficient supply of carbohydrates in the root regeneration zone is needed for root initiation and development (De Klerk *et al*., 1999; Geiss *et al*., 2009; Hartmann *et al*., 2011; Pop *et al*., 2011). Furthermore, levels of compounds that promote adventitious root formation such as jasmonic acid, hydrogen peroxide, hydrogen sulfide and polyphenols also increase after wounding. Polyphenols play a role in reducing auxin degradation, while high hydrogen peroxide levels improve auxin signalling (Kurepin *et al*., 2011; Steffens and Rasmussen, 2016).

Adventitious root primordia that originate in response to wounding develop adjacent to and outside the central core of the vascular tissue and involves a four stage process namely dedifferentiation, induction, initiation (outgrowth in stem) and expression (outgrowth from stem) each with its own physiological requirements. During the dedifferentiation stage specific existing cells in the stem tissues are transformed into meristematic cells (root initials or meristemoids) under the influence of wound related compounds and auxin (De Klerk *et al*., 1999; Geiss *et al*., 2009). The location of the meristemoids in stem tissues differs between plant species.

Adventitious roots originate from living parenchyma cells in the young secondary phloem, from vascular rays, phloem, cambium, callus, or lenticels in woody stem cuttings, and in herbaceous stem cuttings generally develop from root initials just outside and between the vascular bundles (Haissig, 1974; Geiss *et al.*, 2009; Hartmann *et al.*, 2011). In the induction phase the meristemoids undergo cell divisions to form new meristematic growing points under the influence of auxin transported to the base of the cutting (De Klerk *et al.*, 1999; Hartmann *et al.*, 2011). The initiation phase is characterised by the outgrowth of dome-shaped primordia in the stem tissue and it culminates in the outgrowth of the root primordia through the cortex and emerging from the stem epidermis. Vascular tissue develops in the new root primordium and becomes connected to the nearest vascular bundle (Haissig, 1974; Geiss *et al.*, 2009; Hartmann *et al.*, 2011).

Various factors affect adventitious root formation on stem cuttings such as the type of cutting, the time when cuttings are collected, the application of exogenous auxin, the rooting medium and the temperature and light conditions at which the cuttings are kept (Leakey, 2004; Geiss *et al.*, 2009; Hartmann *et al.*, 2011; Hassanein, 2013).

2.4.3.2.1 Type of stem cuttings

Stem cuttings are generally divided into hardwood, semi-hardwood, softwood and herbaceous cuttings. Hardwood cuttings on deciduous woody plant species derive from the mature and lignified stems of the previous season's growth. Leafless cuttings are taken in the dormant season. Semi-hardwood and softwood cuttings also originate from woody species, but some leaves are retained on the cuttings. Semi-hardwood cuttings contain the partially matured wood of the current season's growth and are usually taken in summer, while softwood cuttings are taken from the soft new growth in spring (Leakey, 2004; Hartmann *et al.*, 2011). Herbaceous cuttings are the soft cuttings of non-woody plants and are divided into apical (with or without intact apex) and basal cuttings depending on the position on the stem from where they are taken (Hartmann *et al.*, 2011).

Varying rooting responses were reported depending on the cutting type and plant species. During the rooting process, hardwood cuttings without leaves depend on carbohydrates stored within the stem tissues, whereas softwood leafy cuttings would

make use of the photosynthetic products produced while they are in the rooting medium (Leakey, 2004; Hartmann *et al*., 2011). Rooted hardwood cuttings of *Pogostemon heyneanus* formed the highest mean number (4.6) of axillary branches, although it did not differ significantly from the mean number of branches on softwood (4.3) and semi-hardwood (7.7) cuttings (Rathnayake *et al*., 2015). In *Pterocarpus santalinoides*, semi-hardwood cuttings showed the best rooting response with the highest percentage (31.7%) of rooting and more roots (8.2) per cutting, while significantly less (9.2%) of the softwood cuttings rooted with fewer (1.5) roots per cutting (Ky-Dembele *et al*., 2016). The best cutting type for vegetative propagation of *Duranta repens* was also hardwood and semi-hardwood cuttings that formed the highest number of roots per cutting, 11 and 8 respectively, while only 3 roots per cutting formed on softwood cuttings (Ibironke, 2013).

Apical cuttings of *Berberis aristata* showed a higher (24.2%) rooting percentage and dry root mass (0.6 g) than basal cuttings with 22.6% rooting and 0.2 g dry root mass (Ali *et al*., 2008). In *Hedera helix* and *Schefflera arboricola* basal cuttings performed better than apical cuttings with more roots that formed on the basal cuttings (Hartmann *et al*., 2011). Abdullateef and Osman (2012) also reported a higher number of roots (4) on basal cuttings of *Stevia rebaudiana* than on apical cuttings (1.8 roots). Kako Al-Zebari and Al-Brifkany (2015) found no significant difference in the rooting percentage of untreated apical (70%) and basal (73.3%) cuttings of *Citrus medica*.

It is reported that cuttings with leaves root more easily than those where all the leaves are removed. Auxin synthesised in young leaves and buds on the cuttings is transported to the base of the cutting where it initiates root formation (Rathnayake *et al*., 2015; Yeshiwas *et al*., 2015). Stem cuttings of *Hibiscus* plants with intact leaves showed improved rooting responses (Hartmann *et al*., 2011; Aslam *et al*., 2017). Leafless cuttings of *Garcinia lucida* failed to root, whereas those with leaves (50 cm^2) showed 23% rooting (Takoutsing *et al*., 2014). Tchoundjeu *et al*. (2002) also reported no rooting in leafless cuttings of *Prunus africana*. However, the rooting percentage increased with an increase in the leaf area, from 68% in cuttings with a leaf area of 10 cm^2 to 80% in cuttings with a leaf area of 20cm^2. On the other hand, leaves on cuttings can increase transpiration that could lead to water loss and drying

out of cuttings. It is, therefore, important to maintain high humidity around the cuttings initially (Ibironke, 2017).

Flower buds or flowers on a cutting can act as a competing sink for metabolites, which can be disadvantageous for the rooting of cuttings (DeVier and Geneve, 1997; Hartmann *et al*., 2011). Recent research suggested that this decline in adventitious root formation is linked to signals that control the switch from the vegetative phase to the reproductive phase and not to a stimulus or competition for resources (Rasmussen *et al*., 2015). In commercially grown plants, the flower buds are usually removed to ensure fast root development and better vegetative growth (DeVier and Geneve, 1997; Hartmann *et al*., 2011).

2.4.3.2.2 Exogenous application of auxins

Many stem cuttings only need the endogenous source of natural auxin, mostly Indole-3-acetic acid (IAA), produced in the growing tips and young leaves of plants to induce adventitious root formation (Pop *et al*., 2011). Removal of the stem cutting from the stock plant would lead to accumulation of IAA at the bottom of the cutting in order to restore the wound by inducing dedifferentiation and induction stages as described above (Blakesley *et al*., 1991; De Klerk *et al*., 1999). Exogenous auxin is applied to cuttings to enhance adventitious root formation since it is reported that it can increase hydrogen peroxide and amino acid production levels and respiration at the base of the cutting which promotes root formation (Izadi and Zarei, 2014; Steffens and Rasmussen, 2016). Auxins such as IAA, Indole-3-butyric-acid (IBA) and 1-Naphtaleneacetic acid (NAA) are most often used and applied in the form of talc powder and solutions that contain a specific concentration of the auxin (Geneve, 2000; Hartmann *et al*., 2011).

The basal end of the cutting is dipped for a few (1–5) seconds into solvent solutions that contain varying [500 ppm (0.05%) to 10 000 ppm (1%)] auxin concentrations (quick dip). The solvent, such as ethanol used in the quick dip method seems to facilitate auxin movement through the cut end and the epidermis of the cutting (Geneve, 2000). Cuttings can also be soaked for longer periods (up to 48 hours) in an aqueous auxin solution. The wounded end of the cutting is also inserted into a talc powder, which contains various strengths of auxin, and that acts as a carrier for

the auxin (Geneve, 2000; Pop *et al.*, 2011). The applied auxin enters the stem cutting via the cut surface. From here it is taken up in the cells by influx carriers and pH trapping and further up the stem by translocation via the transpiration stream (De Klerk *et al.*, 1999; Geneve, 2000). This activates starch hydrolysis and movement of sugars and nutrients to the base of the cutting that provides the energy for cell division and differentiation in the rooting zone of cuttings (Leakey, 2004; Husen and Pal, 2007; Gehlot *et al.*, 2014).

The use of exogenously applied auxins (IBA, IAA and NAA) to enhance rooting responses is widely documented in literature. The rooting response of cuttings however, varies greatly amongst different species and even cultivars of the same species. It is also dependent on the physiological state of each cutting and the environmental conditions (Carpenter and Cornell, 1992; Hartmann *et al.*, 2011). Abu-Zahra *et al.* (2013) also illustrated such differential responses. In cuttings of *Syngonium podophyllym*, *Rosmarinus officinalis* and *Gardenia jasminoides* the rooting percentage was improved with the application of NAA as compared to the control, although the optimum concentration differed between the species. The application of 1000 mg L^{-1} NAA resulted in 77.5% rooting in *S. podophyllym*, while *R. officinalis* cuttings gave the best (58.8%) rooting response with 3000 mg L^{-1} and *G. jasminoides* cuttings (57.5%) with 4000 mg L^{-1} NAA. None of the cuttings rooted when treated with 5000 mg L^{-1} NAA except for 11% rooting in *G. jasminoides* cuttings. Anaz *et al.* (2017) reported that the rooting percentage in semi-hardwood cuttings of *Salacia gambleana* increased with the increase in the IBA concentration, from 55% with 4000 ppm IBA to 98% with 8000 ppm IBA. However, in *Salacia brunoniana* the rooting percentage decreased from 35% with 4000 ppm IBA to only 15% with 8000 ppm IBA.

In general, the application of auxin to cuttings results in an improved rooting percentage, formation of more roots per cutting and a higher dry root mass (Leakey, 2004). The application of 4000 ppm IBA solution to stem cuttings of *Tectona grandis* increased the rooting percentage from 35% in the control to 65%, and the average number of roots from 2.2 to 3.3 roots per cutting (Husen and Pal, 2007). Aslam *et al.* (2017) also reported a significant improvement in the rooting response of *Taxus wallichiana* cuttings with IBA treatment. The rooting percentage (8%) and number of

roots (3.6) per cutting in the control (no IBA) increased to 76.7% with 12.3 roots per cutting with application of 500 ppm IBA. Cuttings of *Citrus medica* showed a significant increase in the rooting percentage (90–93.3%) when treated with 500, 1000 and 2000 ppm IBA as compared to 71.1% in untreated cuttings (Kako Al-Zebari and Al-Brifkany, 2015). It is preferable that the optimum auxin concentration, which varies between plant species and cutting types, should be used for adventitious root induction. Application of excessive amounts of exogenous auxin can sometimes be inhibitory to root induction since the formation and the number of root primordia is reduced (Carpenter and Cornell, 1992; Kanmegne *et al*., 2017). In softwood cuttings of *Bauhinia purpurea*, the rooting percentage reduced from 65% in cuttings treated with 2000 ppm IBA to 50% with a higher concentration of IBA (6000 ppm), while the highest rooting (87.5%) was obtained in untreated cuttings (Razvi *et al*., 2014). The rooting percentage in untreated cuttings (50%) of *Ricinodendron heudelotti* did not significantly improve (56%) with the application of 0.1 mg L^{-1} IBA (Tchinda *et al*., 2013). Therefore, the synergistic effect of endogenous and exogenous auxin should be kept in mind when designing experiments (Geiss *et al*., 2009; Pop *et al*., 2011).

2.4.3.2.3 Rooting media

The medium used for rooting of cuttings also determines the success of vegetative propagation. The rooting medium provides support for the cuttings and should be porous for water drainage and aeration, but also have good water and nutrient retention (Relf and Ball, 2009; Szajdak *et al*., 2015). Secondary requirements include cost-effectiveness, availability and easy of manageability (Barrett *et al*., 2016).

The composition of the rooting medium varies depending on plant species and cutting types. A medium with high porosity and low bulk density would allow for sufficient exchange of gases between the root zone and the medium, since oxygen has a positive effect on rooting. On the other hand, the medium should be able to hold sufficient water, but not become waterlogged which would lead to rotting of cuttings (Agbo and Omaliko, 2006; Szajdak *et al*., 2015). The pH of media also differs, but should be between 5.5 and 7 since most nutrients in the medium will then be available to plants, (Szajdak *et al*., 2015). The medium can contain an organic component or a mineral component, or a combination of both. The most commonly used organic substrates include peat moss, coco peat and bark, whereas sand,

perlite and vermiculite are commonly used mineral substrates (Schmilewski, 2008; Hartmann *et al*., 2011; Barrett *et al*., 2016). The use of other media such as sawdust, wood fibres, compost, rice husks, clay, rockwool, pumice, sewage sludge and mineral soil are also reported (Wilson, 1983; Hartmann *et al*., 2011; Barrett *et al*., 2016).

Peat moss (*Sphagnum* sp.) harvested from peat bogs is a lightweight medium with a low bulk density. The pH is highly acidic (3.2–4.5) and is increased with the supplementation of lime. Peat moss is porous for good air exchange, has a very high water holding capacity (10–20 times its weight in water), but it is not easy to re-wet once it dried out (Schmilewski, 2008; Hartmann *et al*., 2011; Barrett *et al*., 2016). Coco peat also known as coir dust, coir pith, and coir meal is a light weight waste product of the coconut (*Cocos nucifera*) industry and consists of the dust and short fibres derived from the mesocarp of the fruit when chopped in smaller pieces (Abad *et al*., 2002; Barrett *et al*., 2016). Since coco peat is a renewable, biodegradable resource, it is used as a substitute for peat moss (Abad *et al*., 2002; Schell, 2013). It has a low bulk density with a neutral to alkaline pH. Coco peat also has a high water holding capacity that could result in poor aeration, which improves when mixing it with a coarse substrate such as sand (Hume, 1949; Hartmann *et al*., 2011). Coco peat decomposes slowly because of its high cellulose and lignin content and thus used as mulching substrate (Abad *et al*., 2002). The properties of coco peat can vary as it is a waste product not intended specifically for horticultural use and if originating from a coastal region, it could be saline with high levels of Na and K (Barrett *et al*., 2016). The most commonly used bark medium that is milled into smaller pieces and often composted derives from pine trees. It is highly porous and used as a filler component in culture medium mixes (Hartmann *et al*., 2011; Barrett *et al*., 2016).

Vermiculite derives from the mineral mica that produces a lightweight, loose product with low bulk density when heated. The pH varies from 7 to 9.5 depending on its origin. It has good water holding capacity and aeration, but may dry out quickly (Hartmann *et al*., 2011; Farhan *et al*., 2018). Perlite is a siliceous material derived from crushed volcanic rocks that expands into tiny white particles when heated. It is lightweight, has a low bulk density and drains well (Farhan *et al*., 2018). Sand is weathered rock and consists of small (0–2 mm in diameter) particles. It is heavy, has

a high bulk density and is often mixed with organic substrates to improve the drainage. It has almost no mineral nutrients (Hartmann *et al.*, 2011). A mix with equal amounts of peat moss, vermiculite and perlite is a good rooting medium (Hartmann *et al.*, 2011; Relf and Ball, 2009). The rooting medium that results in the optimum rooting response should be researched for each plant species.

Hassanein (2013) used sand, peat moss and perlite alone or as a mixture [1:1:1 (v/v/v)] to root woody cuttings of *Ficus hawaii* and herbaceous cuttings of *Chrysanthemum morifolium*. The woody cuttings had the highest survival and rooting percentage in the sand and peat moss alone, while perlite alone and the mixture gave the best results in the *Chrysanthemum* cuttings. Stem cuttings of *Simmondsia chinensis* rooted in sand and peat moss [1:1 (v/v)] achieved the highest rooting response (37.3%) compared to 31.7% rooting in soil medium (Eed and Burgoyne, 2014). On the other hand, *Mentha piperata* cuttings took the longest time (25 days) to root in a sand and peat moss mixture, while the time to rooting was the shortest in perlite (15 days) followed by 20 days in a peat and perlite mixture (Buta *et al.*, 2014). The best rooting medium for cuttings of *Milicia excelsa* was sawdust alone where 80% of the cuttings rooted followed by 70% in a mixture of sawdust and sand [1:1 (v/v)], while the lowest rooting percentage (30%) was found in fine sand (Ofori *et al.*, 1996). Sawdust was also the best rooting medium for *Gongronema latifolia* with formation of 7.5 roots per cutting compared to rice hull and soil with 4.9 and 6.3 roots per cutting respectively (Agbo and Omaliko, 2006). In contrast, Takoutsing *et al.* (2014) found that sawdust alone and mixed with sand [1:1 (v/v)] resulted in less than 10% rooting in cuttings of *Garcinica lucida*. Cuttings of *R. heudelotti* rooted in sand had a significantly higher rooting percentage (53.6%) than cuttings rooted in sawdust alone and in combination with sawdust [1:1 (v/v)], 42.5% and 51.2% respectively (Tchinda *et al.*, 2013). The higher rooting of stem cuttings (77.9%) of coffee hybrid varieties in red soil compared to 19.6% rooting in peat moss was ascribed to the higher micronutrient content of the red soil medium (Magesa *et al.*, 2018).

From the above, it is evident that the vegetative propagation of plants by stem cuttings highly depends on the plant species, the cutting type, auxin treatment and the rooting medium used. The optimum protocol for *H. coddii* subsp. *barnardii* is not

known and should be studied to ensure a fast and cost-effective way of vegetative propagation.

2.4.4 *In vitro* propagation of plants

Plant tissue culture is a generic term for several techniques used to propagate plants by culturing small, living plant parts ("explants") on artificial nutrient media under axenic conditions. Other commonly used terms include *in vitro* ("in glass") propagation, clonal propagation and micropropagation which refers to the small size of the explant and the small amount of space required for maintaining and multiplying the plants (Thiart, 2003; Iliev *et al*., 2010; Hartmann *et al*., 2011). These techniques may include organ, cell, meristem, callus, protoplast, anther and embryo cultures (Iliev *et al*., 2010; Sidhu, 2010; Yildiz, 2012). It relies on the totipotency characteristic of cells that implies that a single cell has the genetic program to develop into an entire plant (Sidhu, 2010; Kumar and Reddy, 2011; Hussain *et al*., 2012). Clonal micropropagation in a controlled environment is an alternative to conventional vegetative clonal propagation, which is time consuming, has lower multiplication rates and may be dependent on seasonal variations (Rout *et al*., 2006; Sidhu, 2010; Solanki and Siwach, 2012).

2.4.4.1 Stages of micropropagation

Micropropagation starts with the selection of an explant from a healthy, vigorous growing donor plant. The process is generally divided into five distinct stages namely Stage 0 to Stage IV. In Stage 0, the donor plants are selected from their natural environment or from plants grown in a garden. Plants are also grown in a clean controlled environment such as a greenhouse and can be pre-conditioned before excision of explants. Pre-treatment includes spraying with fungicide and cytokinin or gibberellic acid solutions. Lateral growth of shoots can also be stimulated by trimming the plants (Thiart, 2003; Kane, 2005; Hartmann *et al*., 2011). During Stage I, an aseptic culture is established when surface disinfected explants are inoculated on sterile culture media to initiate shoot production. Cytokinins or auxins can be added to enhance explant survival and shoot development (Kane, 2005; Hussain *et al*., 2012). This stage usually takes from 4–6 weeks, but might be as long as 12 months in woody plants (Iliev *et al*., 2010; Kumar and Reddy, 2011; Sharma *et al*., 2015). The purpose of Stage II is to maintain the culture in a stabilised state and to

increase the number of proliferated shoots. Axillary shoots are produced from shoot tips and single or multiple node cultures, whereas adventitious shoots are formed directly on explants or indirectly on callus tissue (Thiart, 2003). Plant growth regulators (PGRs) are added to enhance shoot formation. Higher cytokinin concentrations improve shoot proliferation, but results in the formation of shorter shoots. This can be counteracted by addition of auxins to the medium, although the likelihood for callus formation is also increased (Kane, 2005). Multiple shoots are separated and subcultured frequently (every 2–8 weeks) to fresh medium (Iliev *et al*., 2010; Hartmann *et al*., 2011; Hussain *et al*., 2012). Shoots formed during the multiplication stage (Stage II) are elongated and rooted in Stage III with or without auxins. Alternately, shoots are only elongated during this stage and then rooted *ex vitro* in a soil medium (Kane, 2005; Iliev *et al*., 2010; Hussain *et al*., 2012). After root formation the regenerated plants are transferred to *ex vitro* conditions and acclimatised (Stage IV) in preparation for growth under uncontrolled environmental conditions. Plants are gradually exposed to low humidity and high light intensity, transferred to a solid substrate, such as soil or sand, and hardened-off in a greenhouse (Rout *et al*., 2006; Iliev *et al*., 2010; Hartmann *et al*., 2011).

2.4.4.2 Factors affecting micropropagation

Various factors affect the successful formation of organs such as shoots and roots in *in vitro* cultures. These include the source and type of explant, the disinfection procedure, the type of culture medium, and PGRs. Temperature, the quality of light and the photoperiod at which cultures are kept also affect organogenesis (Da Silva *et al*., 2017).

2.4.4.2.1 Plant material and explants

The physiological stage and age of the donor plant, and the position on the donor plant from where explants are taken, determine the success of *in vitro* cultures. This might be due to different levels of endogenous PGRs in the plant parts (Sidhu, 2010; Yildiz, 2012). According to Yildiz (2012), plants grown in a greenhouse give better results than field-grown plants, but the best results are obtained when explants are taken from *in vitro* grown seedlings. Vegetative plant parts also regenerate easier *in vitro* than generative ones. The size of the explant is also important. Yildiz (2012) stated that larger explants have higher amounts of plant hormones and nutrient

reserves and will perform better in culture than small explants. The most commonly used explants are leaves, petioles, cotyledonary leaf, hypocotyl, epicotyl, internodes, shoot tips, nodal buds and roots (Sidhu, 2010; Kumar and Reddy, 2011).

In vitro culture of *Hibiscus* species made use of plants collected from an outside garden and plants grown *in vivo* in a greenhouse, as well as *in vitro* grown seedlings. Airò *et al*. (2009) and Dar *et al*. (2012) collected nodal explants from *H. rosa-sinensis* plants grown in an outside garden, while Christensen *et al*. (2008) and Bhalla *et al*. (2009) obtained nodal explants from cultivated pot plants in a greenhouse. The shoot apex with two leaf primordia and nodal segments obtained from ten-day-old seedlings of *H. sabdariffa* were used as explants for multiple shoot induction (Gómez-Leyva *et al*., 2008). Sultana *et al*. (2016) used the root tip, cotyledons and hypocotyl of *in vitro* grown *H. cannabinus* seedlings as explants, whereas Sakhanokho (2008) used shoot apices of *H. acetosella* seedlings as explants.

2.4.4.2.2 Aseptic conditions

Explants obtained from donor plants in nature or from *in vivo* grown plants, as well as seeds used for *in vitro* germination, must be surface disinfected before inoculation on the culture medium. This is necessary to remove microbial contaminants from the surface of the explants and seeds without damaging the plant material (Iliev *et al*., 2010; Hartmann *et al*., 2011). Bacterial and fungal microbes compete with the growing explant for nutrients and might release chemical compounds that could alter the culture medium. Several aseptic procedures are employed during preparation and inoculation of shoot and seed cultures to avoid microbial combination (Sidhu, 2010).

Plant material is removed from the donor plant with clean secateurs or a knife and is first washed with soapy water and rinsed thoroughly with clean water. This is followed by surface disinfection with a sterilant for a specified period. The concentration, application period and temperature of the disinfectant have an effect on the regeneration ability of the explant (Yildiz, 2012). Generally, a shorter disinfection period is needed with a high sterilant concentration, whereas longer exposure is necessary with a lower concentration of sterilant. The best practise is to use the lowest concentration of disinfectant for the shortest time (Yildiz, 2012). The

most common surface disinfectants include ethyl alcohol (50–95%), 5–50% sodium hypochlorite (NaOCl) and calcium hypochlorite [$Ca(ClO)_2$], mercuric chloride ($HgCl_2$, 0.01–0.1%), hydrogen peroxide (H_2O_2) and silver nitrate ($AgNO_3$) solution (Rout *et al.*, 2000; Gaba, 2005). Commercial bleach such as Jik® and Clorox® contain varying concentrations of NaOCl (3.5–5.25%) and are used in aqueous solutions for surface disinfection. $HgCl_2$ is a highly effective surface disinfectant, but is toxic and needs to be disposed of safely. It is used as a 0.1% (w/v) aqueous solution for 1–3 minutes for soft tissue, 8–10 minutes for woody explants and 10–20 minutes for seeds (Iliev *et al.*, 2010). Two to three drops of Tween 20 (polyoxyethylene sorbitan monolaurate), a non-ionic detergent and wetting agent is added to the sterilant solution before disinfecting the explants. Plant material is rinsed several times with sterile distilled water after each disinfection step to remove the sterilant (Oseni *et al.*, 2018). The optimum surface disinfection for internodal explants of *H. rosa-sinensis* was 15 minutes in 10% commercial Clorox [5.25% (w/v) NaOCl] solution (Dar *et al.*, 2012). Sami *et al.* (2016) surface disinfected nodal explants of *H. syriacus* in 70% ethanol (v/v) for 60 seconds, then 10 minutes in 20% commercial NaOCl solution followed by 7 minutes in 0.1 g L^{-1} $HgCl_2$. Nodal explants of *H. moscheutos* were successfully surface disinfected by West and Preece (2004) with a low dilution of 40% commercial bleach solution (2% NaOCl) for 10 minutes.

Establishment of aseptic seed cultures also requires effective disinfection of the seed material before inoculation on the germination medium. Surface disinfection of *Hibiscus* seeds were successfully performed with ethanol, sodium hypochlorite, and $HgCl_2$. Seeds of *H. cannabinus* were surface disinfected with 50% (v/v) Clorox (5.25% NaOCl) solution for 40 minutes (Srivatanakul *et al.*, 2000), whereas *H. sabdariffa* seeds were disinfected with 70% ethanol for 3 minutes followed by 50% (v/v) NaOCl solution for 20 minutes (Gómez-Leyva *et al.*, 2008). Chen *et al.* (2010) successfully surface disinfected seeds of *H. cannabinus* in 70% (v/v) ethanol for 30 seconds followed by 14 minutes in $HgCl_2$.

The culture medium, culture vessels, all instruments such as forceps, scalpel holders, scissors, and glassware are sterilised in an autoclave (100 kPa) at 121°C for 15–20 minutes. Culture media used for *Hibiscus* cultures were also sterilised in an autoclave at 121°C (100 kPa) for 15–20 minutes (Manokari *et al.*, 2016; Sami *et*

al., 2016). Heat-labile compounds are filter-sterilised and added to media that have cooled-down to less than 60°C (Sidhu, 2010; Beyl, 2005). Furthermore, surface disinfection and inoculation procedures are performed in a laminar flow hood to eliminate contamination. The working area and the hands are regularly sprayed with 70% ethanol. Instruments are flamed between inoculation steps, but sterilisation in a glass bead steriliser is also common (Collin and Edwards, 1998; Beyl, 2005).

2.4.4.2.3 Culture media

The culture medium on which the surface-disinfected explant is inoculated can either be liquid or solid. The medium supports the explant and also provides the necessary nutrients for plant regeneration. The medium consists of 95% water and contains some or all of the following components: macronutrients (N, P, K, Ca, Mg, S) micronutrients (Fe, Mn, Zn, B, Cu, Cl, Mo, Ni), vitamins (thiamine, nicotinic acid, pyridoxine), amino acids or nitrogen supplements and PGRs (Rout *et al*., 2000; Beyl, 2005; Saad and Elshahed, 2012). Stock solutions of the different components are prepared and used to make the desired medium. In certain instances, the level of salts is reduced or increased to different medium strengths, depending on the requirements of the plant material. The pH of the medium is adjusted to 5.4–5.8 before sterilisation (Beyl, 2005; Iliev *et al*., 2010; Hussain *et al*., 2012; Lima *et al*., 2012). Various plant tissue culture media are used depending on the goal of the research, the plant species and the age of the explant. Murashige and Skoog (MS) medium has a high N and K salt content and was originally developed for tobacco culture. Linsmaier and Skoog (LS) medium is similar to MS medium, but with fewer organic compounds whereas White's medium was developed for tissue culture of tomato roots. Schenk and Hildebrand developed SH medium for callus culture of mono- and dicotyledonous plants, while Gamborg's B-5 medium was developed for soybean callus culture particularly in suspension culture. Lloyd and McCown developed the woody plant medium (WPM) to compensate for the salt sensitivity of some woody plant species, while the Nitsch and Nitsch (NN) medium with salt concentrations between that of MS medium and White's medium, was devised for anther cultures (Beyl, 2005; Hartmann *et al*., 2011; Saad and Elshahed, 2012). The most commonly used medium for plant regeneration from plant tissues and callus is the high salt MS medium (Beyl, 2005; Sidhu, 2010; Oseni *et al*., 2018).

Further, a carbon source [2–6% (w/v)] is added to the culture medium, which can serve as an energy source for the explants since they have no or reduced photosynthetic capacity. Sucrose is commonly used because it is cheap and readily available, but lactose, galactose, maltose and starch are also used (Kumar and Reddy, 2011; Saad and Elshahed, 2012). Gelling agents are used to create a semi-solid gel-like surface on or in which explants can be placed (Beyl, 2005; Saad and Elshahed, 2012; Sharma *et al*., 2015). Agar, agarose and gellan gums (Gelrite^{TM} and Phytagel^{TM}) are most commonly used for this purpose. Agar is a polysaccharide extracted from seaweeds (red algae) and its concentration in the medium ranges from 0.5 to 1% (w/v). It contains organic and inorganic contaminants, such as organic acids, phenolic compounds and long chain fatty acids, which might interfere with successful shoot and seed cultures (Beyl, 2005). Agarose is a purified extract of agar and is used for protoplast and single cell cultures (Sharma *et al*., 2015). Gellan gums are made from a polysaccharide produced by the *Pseudomonas elodea* bacterium. It is cheaper than agar and produces a clear medium that facilitates observation of cultures and it is used at concentrations ranging from 0.1–0.3% (w/v) (Sharma *et al*., 2015). Mechanical support for explants such as filter paper bridges does not need a gelling agent and can be used with liquid media (Beyl, 2005).

Research on *in vitro* propagation of *Hibiscus* species mostly made used of MS medium. Jeon *et al*. (2009) cultured explants of *H. syriacus* on MS medium supplemented with 3% sucrose and solidified with 0.3% Gelrite. Explants of *H. sabdariffa* were cultured on MS medium with 3% sucrose and that was solidified with 0.35% Phytagel (Gómez-Leyva *et al*., 2008). Sami *et al*. (2016) solidified MS medium containing 3% sucrose with 0.8% agar for cultivation of *H. syriacus* explants. On the other hand, nodal explants of *H. moscheutos* gave better results when cultured on Driver and Kuniyuki Walnut (DKW) medium than on MS medium (West and Preece, 2004).

2.4.4.2.4 Growth conditions

In vitro cultures are kept in culture rooms where the temperature and light conditions are controlled. The temperature usually ranges between 20°C and 27°C, but may vary depending on the plant species (Rout *et al*., 2000; Beyl, 2005; Hartmann *et al*., 2011). High temperatures should be avoided since it can reduce cytokinin-induced

shoot formation (Hartmann *et al.*, 2011). The light irradiance, light quality, and photoperiod can have an impact on the *in vitro* cultures (Rout *et al.*, 2000; Kumar and Reddy, 2011). The light irradiance in the culture room varies between 40–80 µmol m^{-2} s^{-1}, but might be much lower in the culture vessel. Since sucrose is provided in the culture medium, the heterotrophic culture does not need high light irradiance. Cool, white fluorescent lamps are generally used in tissue culture growth rooms, although red light lamps and light emitting diodes (LEDs) are also common (Hartmann *et al.*, 2011). The type of culture vessel used for cultures affects the light quality. Polycarbonate vessels transmit light wavelengths longer than 390 nm, whereas glass vessels do not transmit wavelengths shorter than 290 nm (Hartmann *et al.*, 2011; Kumar and Reddy, 2011). Since light inhibits root growth in general, activated charcoal is sometimes added to the *in vitro* rooting medium to provide dark conditions for root initiation (Beyl, 2005; Hartmann *et al.*, 2011). The photoperiod usually varies between 12 and 16 hours (Beyl, 2005; Hartmann *et al.*, 2011; Yildiz, 2012).

Similar growth conditions are reported for *in vitro* cultures of *Hibiscus* species. Shoot cultures of *H. rosa-sinensis* were kept in a growth room at 24°C with a 16 hour photoperiod at 45 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Christensen *et al.*, 2008). Gómez-Leyva *et al.* (2008) kept cultures of *H. sabdariffa* at higher temperatures (28°C±2°C) and a 16-hour photoperiod at lower light intensity (25 µmol m^{-2} s^{-1}). Manokari *et al.* (2016) kept cultures of the same species at 25°C±2°C with a 12 hour photoperiod at higher (40–50 µmol m^{-2} s^{-1}) light intensity provided by cool, white fluorescent tubes. All these growth conditions were conducive for culture growth.

2.4.4.2.5 Plant growth regulators

Plant hormones, also called plant growth regulators (PGRs), control various physiological and morphological processes in living, intact plants. Although they are used in low concentrations (0.1–100 µM) in *in vitro* cultures, they play an important role in determining the developmental pathway of plant cells and tissues into shoots or roots. This effect also depends on the plant species and explant type used (Beyl, 2005; Oseni *et al.*, 2018).

Auxins, cytokinins and to a lesser extent gibberellins are considered to be the most important PGRs for regulating growth and development in micropropagation. Auxins are used in cultures to stimulate cell growth and callus production and to initiate shoots, roots and somatic embryogenesis (Gaba, 2005). In general, lower auxin concentrations initiate root formation, while higher concentrations will cause callus formation. Auxins are also used to generate adventitious roots on axillary and adventitious shoots formed during the multiplication stage (Beyl, 2005; Saad and Elshahed, 2012). In micropropagation, the most widely used auxins are the naturally occurring Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA), and the synthetic 1-Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxy-acetic acid (2,4-D) as well as 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) (Beyl, 2005; Saad and Elshahed, 2012). The weakest auxin, IAA is used at concentrations between 0.01–10 mg L^{-1}, whereas the more active auxins, IBA, NAA, 2,4-D and picloram are used at concentrations ranging from 0.001–10 mg L^{-1}. Picloram and 2.4-D are used to induce and regulate somatic embryogenesis (Beyl, 2005; Kumar and Reddy, 2011).

Cytokinins stimulate cell division in cultures, induce shoot formation and axillary shoot proliferation, release dormancy of axillary buds and can also induce formation of adventitious buds (Gaspar *et al*., 1996; Saad and Elshahed, 2012). When the cytokinin concentration is too high, it leads to the formation of numerous small shoots that fail to develop. It also blocks adventitious root development (Gaba, 2005). The most commonly used cytokinins in micropropagation are zeatin, 2-iP, kinetin (Kin), thidiazuron (TDZ) and 6-Benzyl-aminopurine (BAP) which is considered to be the most effective in shoot induction (Sharma *et al*., 2015). Gibberellins comprise more than 20 compounds of which gibberellic acid (GA_3) is the most commonly used. It enhances callus growth and is used for shoot elongation (Kumar and Reddy, 2011; Saad and Elshahed, 2012), however, it was not much used in the *in vitro* culture of *Hibiscus* species.

Combinations of auxins and cytokinins are also used in *in vitro* cultures. Generally, a high ratio of auxin to cytokinin induces root formation, whereas a low ratio of auxin to cytokinin induces axillary and adventitious shoot production in shoot cultures. Equal concentrations of auxins and cytokinins initiate callus formation (Gaba, 2005; Kumar and Reddy, 2011; Oseni *et al*., 2018). In the multiplication stage (Stage II), the

cytokinin levels should be higher than the auxin levels, whereas higher levels of auxin can be used in the rooting stage (Stage III). Cytokinins (BAP, Kin, and TDZ) and auxins (IBA, IAA, and NAA) were used extensively in the micropropagation of *Hibiscus* species (Sakhanokho, 2008). Their use in different types of cultures are more detailed below.

2.4.4.3 Types of *in vitro* cultures

2.4.4.3.1 Seed culture

In contrast to *in vivo* seed germination, the *in vitro* germination of seeds ensures a constant moisture level without the need for watering seeds and it reduces the time required to obtain viable seedlings (Roni *et al*., 2018). The use of *in vitro* seed cultures for the asymbiotic germination of orchid seeds is well known and reported, and used almost exclusively for orchid propagation (Hartmann *et al*., 2011; Pant *et al*., 2011; Jualang *et al*., 2014; Bae *et al*., 2015). However, seeds of other plant species are also germinated *in vitro* and the resulting seedlings then served as an explant source for further multiplication of plants. Since these seedlings are aseptic, surface disinfection of explants is not necessary (Hartmann *et al*., 2011).

The *in vitro* seed germination of several *Hibiscus* species and subsequent use of different parts of uniform seedling as explants were widely reported (Srivatanakul *et al*., 2000; Khatun *et al*., 2003; Herath *et al*., 2004; Ayadi *et al*., 2011; Sultana *et al*., 2016). Surface disinfected seeds are placed on a culture medium with or without PGRs for germination. Ayadi *et al*. (2011) and Samanthi *et al*. (2013) reported high germination percentages (90–100%) within two to three days when scarified seeds of *H. cannabinus* were germinated on full strength MS medium with no added PGRs. Scarified and disinfected seeds of *H. moscheutos* germinated on MS medium and the 3–5 day old seedlings were used as an explant source (Sakhanokho and Kelley, 2009). Seeds of *H. sabdariffa* placed on MS medium supplemented with 1 mg L^{-1} BAP germinated and gave rise to seedlings with a short and wide apical bud, which facilitated excision of the apex explants (Gómez-Leyva *et al*., 2008). Daffalla *et al*. (2016) germinated scarified seeds of *Grewia tenax* (Malvaceae) on two strengths of MS medium (½ and full) and achieved 100% seed germination on both strengths within fourteen days. In general, germination of *Hibiscus* seeds *in vitro* resulted in

uniform seedlings that could be used as aseptic starting material for *in vitro* shoot culture establishment.

2.4.4.3.2 Shoot culture

Micropropagation makes use of various techniques to regenerate plants of which shoot culture is considered the most common way. It aims to produce multiple shoots on one explant (Stage II) which can be separated and used for further shoot induction, or the shoots are placed on suitable media for rooting purposes where after the regenerated plantlets are acclimatised (Kane, 2005; Sakhanokho, 2008; Hartmann *et al.*, 2011).

Axillary shoots are initiated from pre-existing meristems (axillary buds) on single-or multiple node explants usually in the presence of cytokinins. It is also referred to as nodal culture (Kane, 2005; Iliev *et al.*, 2010). Relatively large (up to 20 mm) explants can be used for culture establishment (Stage I) and for subsequent shoot culture (Stage II) which ensures better survival and more rapid growth responses. It can however, be more difficult to surface disinfect larger explants (Kane, 2005). The stem segments with one or more nodes can be inoculated in a vertical or horizontal (stool shoots) position on the culture medium (Iliev *et al.*, 2010; Hartmann *et al.*, 2011). Axillary shoots provide genetic stability and are most reliable to produce true-to-type plants. It is also attainable for many plant species (Kane, 2005; Iliev *et al.*, 2010; Sharma *et al.*, 2015). Adventitious shoots develop *de novo* from non-meristematic plant tissues such as leaf pieces, petioles, bulb scales, stem internodes and roots (Schwarz *et al.*, 2005; Hartmann *et al.*, 2011). These shoots can arise directly from the explant (direct organogenesis) or from callus that develops on the wound edges of the explant (indirect organogenesis). Callus is an unorganised mass of dedifferentiated parenchyma cells that could indirectly give rise to adventitious shoots in the presence of PGRs such as BAP, NAA, IAA, IBA, and 2,4-D, although this might increase the chance for genetic variability (Thiart, 2003; Samanthi *et al.*, 2013).

Shoot culture (axillary and adventitious shoots) has been used extensively in micropropagation of several *Hibiscus* species. Various types of explants, such as shoot tips, cotyledonary petioles, hypocotyl and nodal stem segments, have been

used for shoot induction in the presence or absence of PGRs (Khatun *et al.*, 2003; Herath *et al.*, 2004; Airò *et al.*, 2009; Bhalla *et al.*, 2009; Ayadi *et al.*, 2011; Sultana *et al.*, 2016). Nodal explants of *H. sabdariffa* cultured on MS medium supplemented with 1 mg L^{-1} BAP gave the best shoot induction response (96%) with the most (4.2) shoots formed per explant (Manokari *et al.*, 2016). In contrast, Govinden-Soulange *et al.* (2009) reported only 39.6% shoot induction response with 0.6 shoots per nodal explant of *H. sabdariffa* with 1 mg L^{-1} BAP, whereas a lower BAP concentration (0.1 mg L^{-1}) resulted in 73% shoot formation with 1.4 shoots per explant. Application of 15 µM BAP to MS medium increased shoot induction in nodal explants (1.94 shoots per explant) of *H. rosa-sinensis* compared to 1.14 shoots per explant in the BAP-free medium (Bhalla *et al.*, 2009). Sakhanokho (2008) found the best shoot induction response on shoot apex explants of *H. acetosella* with a combination of cytokinins. The most number of shoots (3.7) was produced with a combination of 8.9 µM BAP and 0.6 µM TDZ compared to lesser shoots when the PGRs were used alone, 1.1 and 3 shoots respectively. Agrawal *et al.* (1997) reported 100% shoot induction response in cotyledonary nodal explants obtained from seedlings of *Gossypium hirsitum* in the presence of 2.50 mg L^{-1} BAP and Kin each.

Combinations of cytokinins and auxins also resulted in shoot induction responses. A combination of 1 µM TDZ and 0.50 µM 2,4-D improved shoot formation in shoot apex explants of *H. cannabinus* cv. Everglades 71 to 80% as compared to 60% shoot formation with 20 µM TDZ alone (Srivatanakul *et al.*, 2000). On the other hand, PGRs also suppressed shoot induction in *Hibiscus* species. The highest shoot induction (91%) on nodal explants of *H. cannabinus* was obtained on PGR-free medium while 1 mg L^{-1} BAP significantly reduced shoot induction to 69%. The lowest response (32%) was reported with the combination of 0.5 mg L^{-1} BAP and 1 mg L^{-1} NAA (Ayadi *et al.*, 2011). Govinden-Soulange *et al.* (2009) also reported the best shoot induction response (81%) on nodal explants of *H. sabdariffa* in the absence of PGRs.

Yang *et al.* (1995) reported direct adventitious shoot formation from leaf and petiole explants of *H. syriacus* on MS medium supplemented with 0.1 mg L^{-1} NAA in combination with 2 mg L^{-1} 2-ip and BAP, respectively. The highest adventitious shoot regeneration (60%) from cotyledonary petioles of *H. cannabinus* was obtained with

5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA in combination, whereas lower BAP (1 mg L^{-1}) combined with 0.5 mg L^{-1} IAA resulted in no shoot regeneration (Khatun *et al.*, 2003). Adventitious shoots also formed indirectly from callus initiated on internodal stem segments and cotyledonary leaf explants of *H. cannabinus* with 0.3 mg L^{-1} each of 2,4-D and Kin, and in the presence of 1.5 mg L^{-1} BAP & 0.5 mg L^{-1} IBA (McLean *et al.*, 1992; Samanthi *et al.*, 2013).

2.4.4.3.3 Root culture

Adventitious roots must be induced on shoots formed during micropropagation before the regenerated plantlets can be transplanted to solid medium and acclimatised. *In vitro* rooting is used for this purpose when the cytokinin level in the medium is reduced and the auxin level is increased. Sometimes root formation on explants can occur simultaneously with multiple shoot induction on the same medium and there is no need for an extra *in vitro* rooting step (Rout *et al.*, 2000; Govinden-Soulange *et al.*, 2009; Hartmann *et al.*, 2011). The successful rooting of shoots formed during micropropagation of *Hibiscus* species have been reported by various researchers. In some cases, shoots rooted without the addition of auxins, but in others, the medium was supplemented with various combinations and concentrations of IAA, IBA and NAA to induce root formation (Sakhanokho, 2008; Bhalla *et al.*, 2009; Ayadi *et al.*, 2011). Chen *et al.* (2010) found that 2 mg L^{-1} IAA and NAA were the best for root induction (100% and 93.8% respectively) on *in vitro* produced shoots of *H. cannabinus*. This was contradicted by Ayadi *et al.* (2011) who reported the best rooting performance (91%) in *H. cannabinus* on PGR-free medium. Shoots of *H. sabdariffa* were successfully rooted (98%) on half-strength MS medium supplemented with 2 mg L^{-1} IBA (Manokari *et al.*, 2016).

Due to the fact that *in vitro* induction of a viable adventitious root system that is able to withstand transplanting to a soil medium poses a challenge, the *ex vitro* rooting of microshoots or microcuttings has also been the focus of research. In general, the adventitious roots that are formed *ex vitro* are longer, thicker and more flexible than those formed with *in vitro* techniques. In addition, the plant survival rate is improved by using *ex vitro* techniques (Ranaweera *et al.*, 2013; Shiji and Siril, 2018). Many commercial growers prefer to root microcuttings *ex vitro* since it decreases the cost, resources and labour involved with *in vitro* rooting. It also reduces the time for

rooting and acclimatisation of rooted plantlets (Hartmann *et al.*, 2011; Ranaweera *et al.*, 2013; Shiji and Siril, 2018). Ranaweera *et al.* (2013) reported that *ex vitro* rooting of microshoots of *Camellia sinensis* reduced the cost of micropropagated plants by 71% when compared with the standard micropropagation method.

The basal end of microcuttings (microshoots) are treated with varying concentrations of auxin solution for different time periods, or dipped in auxin-containing rooting powder before planting it in a porous medium such as mixtures of vermiculite, peat, sand or perlite (Thiart, 2003; Dev and Kaur, 2017). Reports on *ex vitro* rooting in microcuttings of *Hibiscus* species are not widely available in literature, although Christensen *et al.* (2008) recommended *ex vitro* rooting after attaining poor *in vitro* rooting response (<50%) in microshoots of *H. rosa-sinensis*. Microshoots of another Malvaceae member, *Gossypium hirsitum*, successfully rooted *ex vitro* (89%) after treatment with Rootone® hormone rooting powder [0.2% Nicotinamide adenine dinucleotide (NAD)] (Hemphill *et al.*, 1998). *Ex vitro* rooting of microshoots of other plant species was also reported. *In vitro* produced microshoots of *Piper longum* were successfully rooted (96%) after dipping for five minutes in 300 mg L^{-1} IBA solution and planting in Soilrite®, a mixture of perlite, peat moss and vermiculite (Ravindran *et al.*, 2016). Microshoots of *Pistacia vera* rooted successfully (78.6%) when dipped in Rhizopon® AA rooting powder (2% IBA) and planted in a peat-perlite-vermiculite (80-15-5%) mixture (Benmahioul *et al.*, 2012).

2.4.4.3.4 Other *in vitro* cultures

Meristem, callus cultures, and somatic embryo formation are other techniques practiced in plant tissue culture. Meristem culture, mostly employed for virus elimination, makes use of the meristematic region or apical dome (less than 1 mm) of the stem tip to allow outgrowth of the apex directly into a shoot (Iliev *et al.*, 2010; Hartmann *et al.*, 2011). Callus, an undifferentiated mass of cells, forms in plants in response to stress such as wounding or a pathogen infection. It can be induced *in vitro* in the presence of an intermediate ratio of auxins and cytokinins. Cultures are started from any vegetative tissue and are used for breeding and genetic transformation studies. Enzymes, medicines, natural flavours and colours can also be obtained from callus cultures (Sidhu, 2010; Ikeuchi *et al.*, 2013). Sié *et al.* (2010)

reported large callus (7.9 mm diameter) on 100% of cotyledon explants of *H. sabdariffa* on MS medium supplemented with 0.1 mg L^{-1} BAP and NAA each.

Somatic embryogenesis gives rise to a non-zygotic embryo from plant tissues or cells. It may take place directly from explants or indirectly through an intermediate callus stage in the presence of the auxins 2,4-D and NAA (Iliev *et al*., 2010; Kumar and Reddy, 2011). These somatic embryos can develop into whole plants without undergoing sexual fertilisation (Hussain *et al*., 2012). Somatic embryos were initiated from callus formed on hypocotyl and cotyledon explants of *H. sabdariffa* cultured on DKW medium in the presence of 2,4-D and TDZ (Sié *et al*., 2010).

2.4.4.4 *Ex vitro* establishment of regenerated plants

Ex vitro establishment is important for the success of *in vitro* plant propagation. This stage involves acclimatisation of plants under controlled environmental conditions and further hardening-off in a greenhouse under uncontrolled conditions (Thiart, 2003; Rout *et al*., 2006).

a. Acclimatisation

In vitro cultured plants are grown in closed culture vessels under high humidity that causes structural changes such as a thin cuticle, poorly differentiated mesophyll and improperly functioning or absent stomata. These plants are also not entirely autotrophic since they are grown in a carbon source under low light conditions (Kane, 2005; Hazarika, 2006). Plantlets regenerated *in vitro* are transplanted to *ex vitro* conditions into a solid medium, such as vermiculite, and covered with perforated plastic bags to ensure gradual exposure to lower humidity (Thiart, 2003; Rout *et al*., 2006; Hartmann *et al*., 2011). This allows for acclimatisation under controlled environmental conditions with a higher light intensity than in the *in vitro* culture (Hazarika, 2003; Hartmann *et al*., 2011; Kumar and Reddy, 2011).

b. Hardening-off

During hardening-off, successfully acclimatisated plants are gradually acclimated to an environment with lower relative humidity and increasing temperature and light irradiance. It enables plants to absorb nutrients and water through the newly formed root system, and develop new stems and leaves that can withstand the changing

environment. After transferring plants to a potting soil mixture of varying composition, they are hardened-off for 2–3 weeks under uncontrolled environmental conditions in a greenhouse or under shade netting that allow for different light irradiance conditions. Plants are then ready to be moved to outdoor or field conditions (Hazarika, 2003; Hartmann *et al.*, 2011; Hussain *et al.*, 2012).

Various authors reported on the successful acclimatisation and hardening-off of regenerated *Hibiscus* plantlets (Khatun *et al.*, 2003; Herath *et al.*, 2004; Sakhanokho, 2008). Sakhanokho and Kelley (2009) successfully acclimatised plantlets of *H. moscheutos* in a peat medium kept in a growth room at 22°C at a 16-hour photoperiod. Plantlets of *H. rosa-sinensis* were successfully acclimatised in peat medium under clear plastic at 20°C and a 16-hour photoperiod and hardened-off in a greenhouse at day/night temperatures of 22°C/20°C (Christensen *et al.*, 2008). Rooted plantlets of *H. sabdariffa* were acclimatised in Soilrite®, a mixture of perlite, peat moss and vermiculite, for four weeks and then successfully hardened-off in a greenhouse (26–28°C) for two weeks in pots containing a mixture of Soilrite®, manure and soil (1:1:1) (Manokari *et al.*, 2016).

2.4.4.5 Advantages and disadvantages of micropropagation

The main advantage is that a large number of clonal plants can be obtained in a short period of time, independent of seasonal changes, especially slow-growing plants or ones that are difficult to propagate (Thiart, 2003; Sharma *et al.*, 2015; Oseni *et al.*, 2018). As it is performed *in vitro* (in vessels), the culture environment, levels of nutrients and plant growth regulators (PGRs) necessary for growth can be manipulated to achieve the best results (Christensen *et al.*, 2008; Sakhanokho, 2008; Hartmann *et al.*, 2011). Furthermore, virus-free plants can be produced with meristem techniques and endangered and rare plant species can be conserved by micropropagation, whereas somaclonal variations could lead to the production of improved crop plants. More advantages include rejuvenation of plant material and long-term storage of vegetatively produced material (Thiart, 2003; Hussain *et al.*, 2012; Oseni *et al.*, 2018). On the other hand, micropropagation of plants can be expensive and requires a specialised production facility and advanced skills for its operation (Thiart, 2003; Kane, 2005). Labour costs can account for 50–70% of the production costs (Sharma *et al.*, 2015). Plantlets generated *in vitro* must also

undergo a transitional phase to adapt to the outside environment with low humidity and high light intensity. This critical phase can reduce the survival percentage of plants substantially (Thiart, 2003; Sharma *et al*., 2015).

In vitro culture responses and successful acclimatisation of regenerated plantlets vary in *Hibiscus* species and should, therefore, be researched for each species. An extensive literature review indicated that to date no research on the *in vitro* culture of *H. coddii* subsp. *barnardii* was published before. Hence, research on its *in vitro* culture propagation is needed to optimise the procedure for mass multiplication of this potential ornamental plant.

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Chapter 3

CHAPTER 3

Characterisation of *Hibiscus coddii* subsp. *barnardii*

3.1 Introduction

South Africa is renowned for its rich plant biodiversity and various floristic regions and centres of plant endemism of which the Cape Floristic Region with its fynbos is probably the best known (Van Wyk and Smith, 2001). This rich floral heritage holds several plants that have the potential to become popular garden or indoor plants (Bester *et al*., 2009). Middleton (2015) pointed out that both consumers and growers in South Africa prefer indigenous plants as these are already adapted to local growing conditions. The striking foliage and flowers of many of these plants, together with the fact that they often do not require excessive water makes them an attractive option for South African gardeners (Middleton and Vosloo, 2011; Middleton, 2015).

One of the lesser known, but equally important centres of plant endemism, is the Sekhukhuneland Centre of Plant Endemism (SCPE) located in the Limpopo and Mpumalanga provinces. It is a unique floristic region located on the ultramafic and mafic rocks of the Rustenburg Layered Suite of the Bushveld Igneous Complex (Siebert *et al*., 2002). A vast number (2 200) of plant species grow in the unique soils of this complex (Van Wyk and Smith, 2001). Of these at least 50 species are endemic or near-endemic, with *Hibiscus coddii* subsp. *barnardii* being one of these species (Siebert and Van Wyk, 2001; Siebert *et al*., 2001). Craib (2003) suggested the suitability of this plant species as a new ornamental because of its striking red flowers and water-wise properties. He also mentioned that it would be suitable for growing as a pot plant or a small shrub, especially in sunny rockeries. These characteristics warranted research into the natural growing conditions of the plant species in order to establish protocols for the propagation and cultivation of the plant.

This chapter therefore provides information on the growing conditions of *H. coddii* subsp. *barnardii* plants in their natural habitat and documents accompanying plant species.

3.2 Materials and Methods

3.2.1 Study sites

Three sites in the SCPE where *H. coddii* subsp. *barnardii* plants occur were chosen at random to study plants in their natural habitat.

3.2.2 Plant species

Plant species occurring in the same habitat as *H. coddii* subsp. *barnardii*, were documented by means of surveys on three consecutive days in January 2017. At each site, the area where the *H. coddii* subsp. *barnardii* plants were growing was divided into quadrats and the plant species diversity was recorded using Whittaker's plant diversity sampling method as described by Shmida (1984). Plant species within the quadrats were identified at the Larry Leach Herbarium, University of Limpopo (UL). In addition, the number of individual *H. coddii* subsp. *barnardii* plants per site was recorded.

3.2.3 Plant characterisation in nature

Measurements of *H. coddii* subsp. *barnardii* plants in their natural environment were taken according to Pérez-Harguindeguy *et al*. (2013). In February 2017, 25 plants from Sites 2 and 3 in the Potlake Nature Reserve (in full sun) were randomly selected. The number of branches at soil level was counted per plant and the length of the longest stem (axillary branch) was measured using a measuring tape. This was taken as the height of the plant (mm). The width and length of 30 leaves (randomly selected from the middle of the axillary branches from different plants) were measured with a ruler. A voucher plant specimen of *H. coddii* subsp. *barnardii* was collected and deposited at the Larry Leach herbarium at the University of Limpopo (Voucher number UNIN121948).

3.2.4 Soil properties

Soil samples (four replicates) were collected during 2016 (February, June, September and November) from the three sites. At each site, six collection points (Appendix A: Table 2) were chosen at random around the rocky outcrop where the plants are found. At each point, a sample pair was collected. Each pair consisted of soil collected next to a growing plant (+Plant) and soil collected at its nearest

proximity (5–10 m) where no plants were growing (–Plant). The top 300 mm of soil (without obvious organic material) was collected using a soil auger and small spade and placed in a plastic bag.

Two methods were followed during analysis of soil mineral composition. This allowed for triangulation of results and although not directly comparable, showed overall trends in soil components. Soil samples were analysed at the Limpopo Agro-Food Technology Station situated on the University of Limpopo campus. Levels of macro-nutrients (Ca, K, Mg, P, and S) and micro-nutrients (B, Cu, Fe, Mn, Mo, Na, and Zn) and also Cr, which is a heavy metal and non-essential element, were determined. Samples were dried in a Memmert UF30 (Lasec) oven at 60°C for one hour, ground to a fine powder with a mortar and pestle and then digested with a PerkinElmer® Titan MPS microwave sample preparation system before mineral analyses. Soil (3.5–4 g) from each sample was placed in a digestion vessel and 10 ml of 70% nitric acid (HNO_3) was added. The mixtures were stirred, left for 10 minutes and then heated in a microwave at the temperature and time recommended by the manufacturer (PerkinElmer, 2014). The digested soil mixtures were transferred to centrifuge tubes and filled to 50 ml with de-ionised water. The samples were analysed with a Shimadzu ICPE-9000 (Inductively Coupled Plasma Atomic Emission Spectrometry) instrument (Shimadzu Corporation, 2012). The concentration of elements in the soil sample solutions was determined in mg L^{-1}.

The total organic carbon (C) in the soil samples was determined based on the Walkley-Black chromic acid wet oxidation method. Oxidisable matter in the soil was oxidised with a 1N potassium dichromate solution. Two volumes of sulfuric acid were mixed with one volume of the dichromate solution and the remaining dichromate was titrated with ferrous sulphate. The titre was inversely related to the amount (%) of C present in the soil sample (Soil Organic Carbon Test, Sa). The pH values of the different soil samples were determined with the standard pH-KCl method (Burt, 2014).

The data were analysed with the GenStat64-bit Release 18.2 (PC/Windows 8) statistical package (VSN International, 2016). Analysis of variance (ANOVA) was

used to determine whether there was a difference in soil chemical composition between the various sites (Site 1, 2, and 3) and between soils collected at the plants (+Plant) and away from the plants (–Plant). Fisher's protected least significant difference (LSD) test at the 5% level of significance was performed to distinguish between different means (Williams and Abdi, 2010).

In addition, the chemical composition of soil samples was analysed with the non-destructive X-Ray Fluorescence Spectroscopy (XRF) method (La Tour, 1989) in the Department of Geology and Mining at the University of Limpopo. A soil sample was first milled to a fine powder (<60 μm), then a 30 g aliquot was mixed with a cellulose binder (9 g). The mixture was placed in an aluminium cup and pressed into a pellet with a manual press machine at 20 tons (Takahashi, 2015). Pellets thus produced were loaded in an Epsilon 3^{XLE} benchtop Energy Dispersive X-Ray Fluorescence spectrometer (EDXRF) which operates within a He atmosphere (Malvern Panalytical, Sa) for analysis. As per convention (Potts, 1987; Eales, 2001), the XRF results are reported as major oxides and trace elements. Values for the major oxides (SiO_2, TiO_2, Al_2O_3, Fe_2O_3, MnO, MgO, CaO, Na_2O, K_2O, and P_2O_5) are given as weight % and those for trace elements (Cr, Cu, Ni, Rb, Sr, V, Y, Zr, and Zn) as ppm.

The soil texture was determined by hand texturing according to the method described by Ritchey *et al.* (2015).

3.3 Results and Discussion

3.3.1 Study sites in Sekhukhuneland

The SCPE spreads over both the Limpopo and Mpumalanga provinces with the largest part occurring in the Limpopo Province (Figure 3.1). According to Siebert *et al.* (2002), *H. coddii* subsp. *barnardii* occurs in the drier northern bushveld part of the SCPE (Figure 3.2). This corresponds to the Sekhukhune Plains Bushveld vegetation type (SVcb27) as mapped by Mucina and Rutherford (2006). Figure 3.2 indicates the chosen sites where the *H. coddii* subsp. *barnardii* plants are growing.

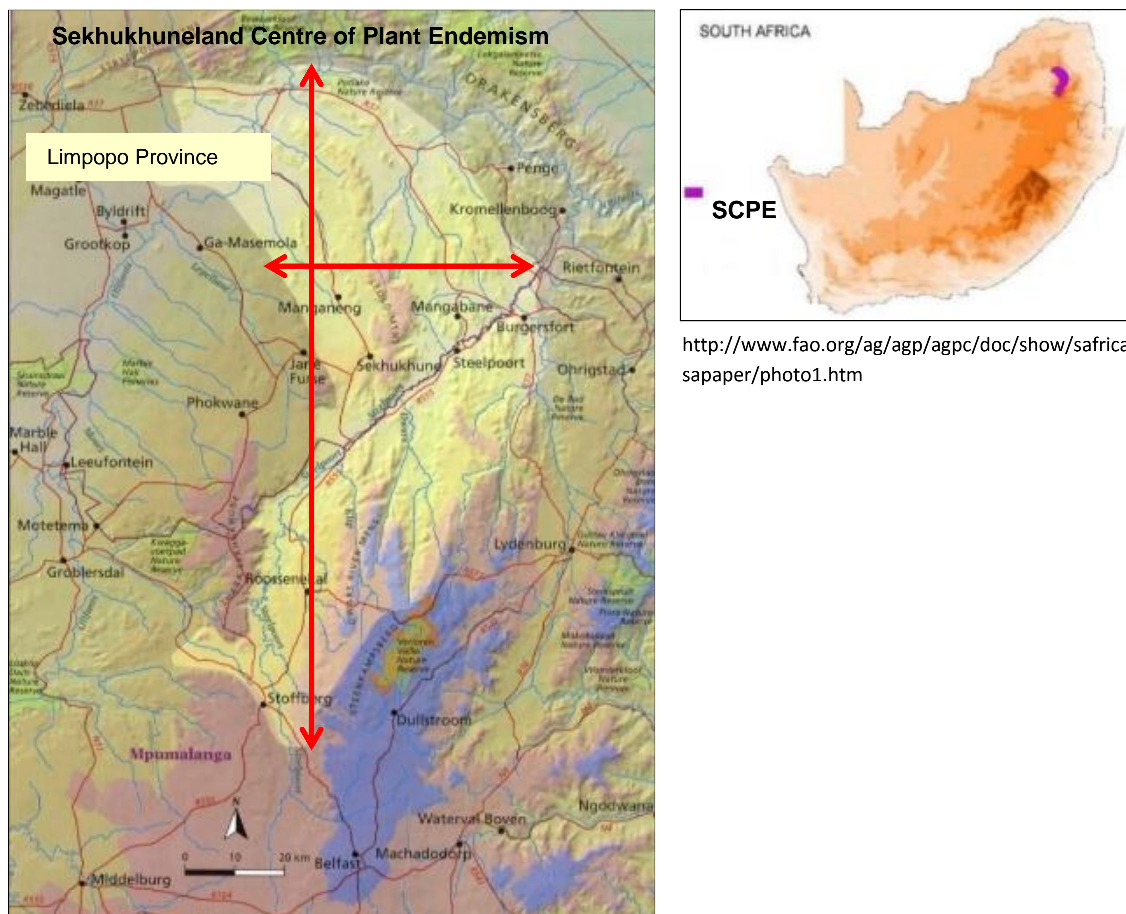

Figure 3.1. Map showing the location of the Sekhukhuneland Centre of Plant Endemism (SCPE) (light-yellow area on the map indicated by red arrows). (Map adapted with permission from Van Wyk and Smith, 2001).

The first site was next to the D4190 (Apèl) gravel road approximately 5 km from the turnoff at the R37 (Polokwane-Burgersfort) road. The area is situated at 748–756 m above sea level with GPS co-ordinates 24°17'34.5"S; 29°47'31.1"E. This site is located in an unprotected area and is subjected to grazing by local livestock (goats and cattle) as well as human activities such as firewood and medicinal plant collection.

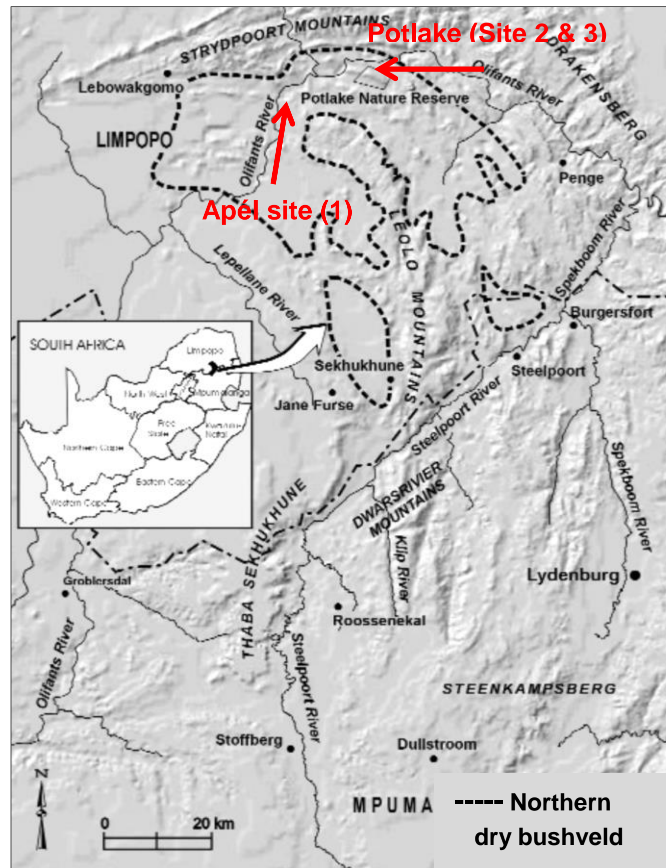

Figure 3.2. Map showing the location of the different sites in Sekhukhuneland. (Modified from Siebert *et al.*, 2003). Red arrows indicate study sites.

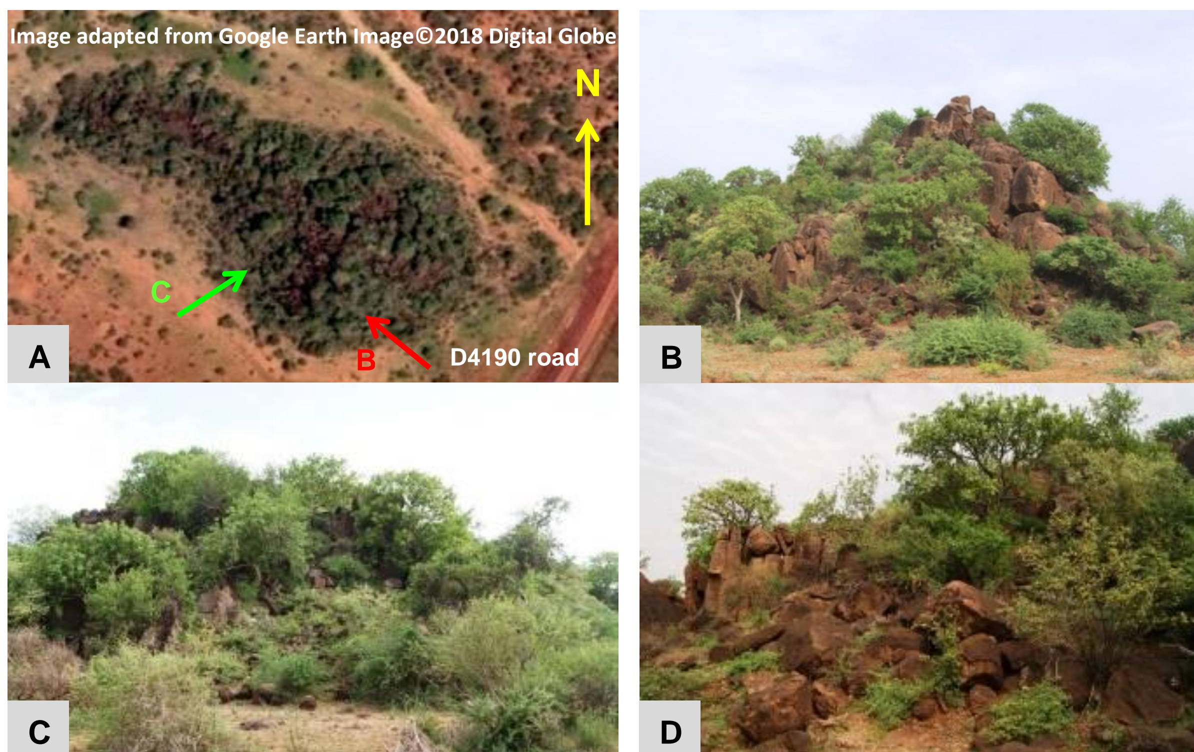

Figure 3.3. Site 1 next to the D4190 Apél road. (A) Aerial image; (B) Southern view, next to the road (red arrow); (C) Western side (green arrow); (D) Close-up showing the rocks.

The site is characterised by an almost bean-shaped rocky outcrop made up of large dark-coloured boulders (Figure 3.3A & D). The *H. coddii* subsp. *barnardii* plants are restricted to the rocks and the foot of the outcrop. The rest of the vegetation is characterised by forbs and shrubs with a few small to medium-sized trees (Figure 3.3B & C). The other two sites were inside the Potlake Nature Reserve which is situated next to the R37 (Polokwane-Burgersfort) road approximately 80 km from Polokwane and 37 km from Lebowakgomo. The nature reserve covers a total area of 2 784 ha (NCC Environmental Services, Sa). These two sites were chosen since large concentrations of *H. coddii* subsp. *barnardii* plants are found here. Site 2 is located at the co-ordinates 24°15' 16.9"S; 29°54'44.0"E with the height above sea level varying from 824 to 830 m. Two rocky outcrops with a longitudinal rocky ridge on their northern side occur at this site (Figure 3.4A, C & D) where the plants are found amongst and in between the rocks (Figure 3.4B).

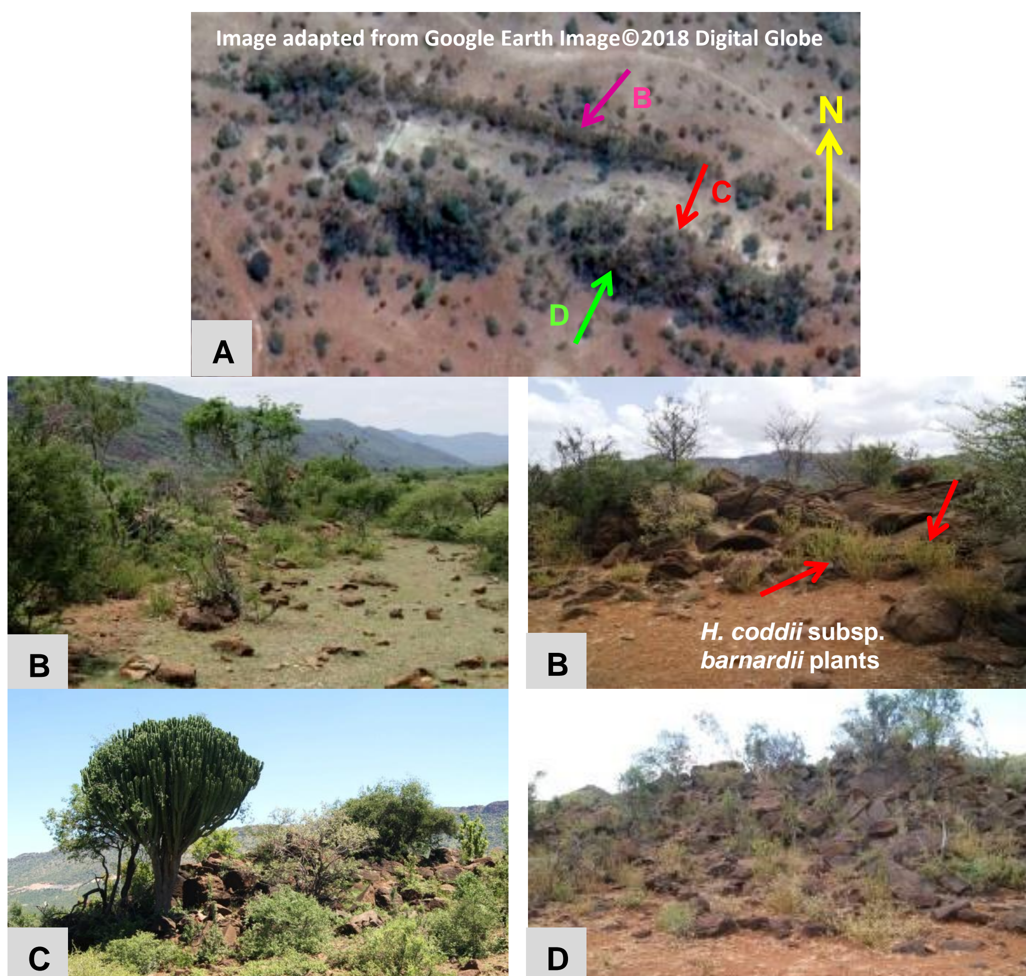

Figure 3.4. Site 2 in Potlake Nature Reserve. (A) Aerial view; (B) Rocky ridge on northern side (pink arrow); Rocky outcrop: (C) Northern side (red arrow); (D) Southern view (green arrow).

The third site is situated approximately 1 km from Site 2 and consists of one large rocky outcrop and a smaller outcrop on the southern side (Figure 3.5A & C). It is located at 24°15' 18.0"S; 29°54'22.8"E and at 830–840 m above sea level. Forbs, shrubs and small trees with sparse grass cover characterise the vegetation at both sites (Figure 3.4C & D; Figure 3.5B, C & D).

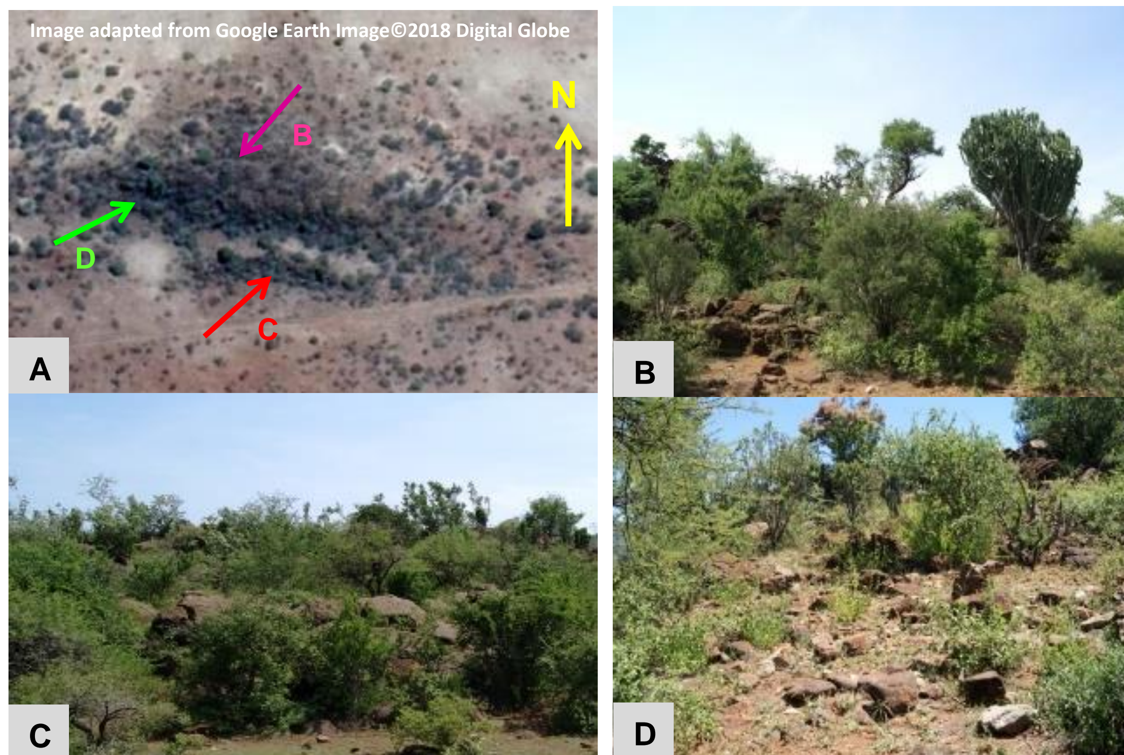

Figure 3.5. Site 3 in Potlake Nature Reserve. (A) Aerial view; (B) Northern side of rocky outcrop (pink arrow); (C) South side of smaller rocky ridge (red arrow); (D) Southern view of rocky outcrop (green arrow).

3.3.2 Topography

The east-flowing Olifants River is the driver behind the heterogeneous topography of the SCPE. Over millions of years, the river has eroded the landscape into characteristic parallel belts of rocky ridges and mountains interspersed with heavily eroded valleys (Van Wyk and Smith, 2001; Siebert *et al*., 2002). In the study area, hills and small mountains form chains that run parallel to the escarpment creating open valleys interspersed with semi-arid to arid plains. In some areas, heavy degradation of the landscape due to cultivation, mining and urbanisation occurs (Mucina and Rutherford, 2006).

3.3.3 Climate

Sekhukuneland lies in the summer rainfall area and receives nearly half of its rain (48%) between December and February, while spring rains contribute 28% of the total rainfall in a single year (Siebert *et al.*, 2002). Winters are typically very dry with infrequent frost (Mucina and Rutherford, 2006). The semi-arid savannah characteristic of the larger northern and north-central parts of Sekhukhuneland lies in the rain shadow of the Drakensberg escarpment and rainfall can be as low as 400 mm per year (Van Wyk and Smith, 2001; Siebert *et al.*, 2003). The mean annual temperature in Sekhukhuneland is 20°C with daily temperatures ranging from a minimum of 8°C in winter to a maximum of 38°C in summer (Siebert *et al.*, 2003). The drier northern and western parts have warmer temperatures with a maximum daily average of 28.3°C and a minimum of 7.2°C (Siebert *et al.*, 2002). The valleys where *H. coddii* subsp. *barnardii* plants occur have a subtropical climate with little or no frost in winter (Van Wyk and Smith, 2001; Mucina and Rutherford, 2006).

The temperature and rainfall data for 2015, 2016 and 2017 recorded in the Potlake Nature Reserve are shown in Appendix A: Table 1. The average minimum and maximum temperature over the three years in winter months (June, July, and August) were 10.5°C and 25°C respectively. The lowest minimum temperature (4°C) was recorded in June 2017 and the highest maximum temperature (32°C) in August 2015. In the summer months (December, January, and February), the average minimum and maximum temperatures were 14.5°C and 32.8°C respectively. The lowest minimum temperature for this period was 13°C and the highest maximum was 38°C. Overall, 2017 was a cooler year than 2015 and 2016 with 12°C the average minimum temperature and 27°C the average maximum temperature compared to 14°C and 30°C for the other two years. During 2015, the average rainfall (278 mm) was well below the minimum. This, and the high temperatures during the summer months, caused very low production of *H. coddii* subsp. *barnardii* seeds in 2016. The rainfall in 2016 and 2017 was above average for this area, being 583 and 494 mm respectively (Appendix A: Table 1).

3.3.4 Vegetation

Hibiscus coddii subsp. *barnardii* is a component of the Sekhukhune Plains Bushveld vegetation type (SVcb27) that extends into the northern region of the SCPE. This vegetation type is characterised by a pre-dominance of succulents, such as *Aloe cryptopoda* and *A. castanea*, within open to closed thornveld. *Senegalia mellifera* subsp. *detinen*s, *Vachellia nilotica*, and *V. tortilis* subsp. *heteracantha* are some of the prominent thorn trees found in this vegetation type. Bush encroachment by both indigenous and alien plant species is common (Mucina and Rutherford, 2006). Based on extensive floristic surveys in the SCPE, Siebert *et al*. (2002) divided the SCPE into six main vegetation types that belong to three major floristic regions. According to them, *H. coddii* subsp. *barnardii* plants occur in the drier northern part of the SCPE (Figure 3.2) in the *Acacia tortilis*-*Dichrostachys cinerea* Arid Northern Bushveld vegetation type. The Sekhukhune Plains Bushveld vegetation type is vulnerable, as 25% of the area has been transformed and eroded as well as targeted by chromium and platinum mining (Mucina and Rutherford, 2006). Other threats include urban development, overgrazing, pollution and transformation by alien plants (Siebert and Van Wyk, 2001).

3.3.4.1 Plant species

The plant survey showed that more individuals of *H. coddii* subsp. *barnardii* plants were recorded at Site 2 (765) and 3 (425) than at Site 1 (205). The higher numbers might be due to the location of Sites 2 and 3 within a conservation area (Potlake Nature Reserve). According to the Red List of South African Plants, *H. coddii* subsp. *barnardii* has a status of Least Concern (Raimondo *et al.*, 2009); however, mining activities in the area are expanding and threatening the plant's habitat. Since the conclusion of the fieldwork for this study, the area wherein Site 1 is located has been transformed by mining.

Grasses, forbs, climbers, shrubs, geophytes and trees were encountered during the plant surveys (Appendix A: Table 9). Site 1 had slightly more species (112) than sites 2 and 3 with 107 and 106 species respectively (Table 3.1).

Table 3.1. Various plant growth forms found at selected sites in Sekhukhuneland.

	Site 1 Apèl Road	Site 2 Potlake	Site 3 Potlake
Total number of species	112	107	106
	Site 1 Apèl Road	**Site 2 Potlake**	**Site 3 Potlake**
Growth form			
Climber or Creeper	5	6	5
Fern	2	1	2
Forb	51	43	41
Geophyte	2	1	1
Grass	10	10	8
Shrub	20	19	18
Succulent	3	2	3
Tree	19	25	28

There was not a great variation in species found between sites, however, some species found at Site 1 were not found at Sites 2 and 3 and *vice versa*. For example, *Blepharis elegans* and *Ruellia patula* were found only at Site 1, whereas *Turraea obtusifolia* and *Pristimera longipetiolata* were not at Site 1, only at Sites 2 and 3 (Appendix A: Table 9). Site 1, which is not in a protected area, contained more alien plant species than the other sites. These included amongst others, *Chenopodium album*, *Flaveria bidentis*, *Opuntia ficus-indica*, and *Schkuhria pinnata*. The most common plant growth form recorded, were forbs (Table 3.1) for example *Aptosimum lineare* (Figure 3.6A), *Gloriosa superba* (Figure 3.6B), *Aneilema longirrhizum* (Figure 3.6D) and *Barleria crossandriformis* (Figure 3.6F). Trees and shrubs included *Karomia speciosa* (Figure 3.6C), *Turraea obtusifolia* (Figure 3.6E), *Triaspis glaucophylla* (Figure 3.6G) and *Adenia fruticosa* subsp. *fruticosa* (Figure 3.6H).

Plants of *H. coddii* subsp. *barnardii* are restricted mainly to rocky outcrops and grass species were less plentiful amongst these rocks, thus explaining the small number of grass species recorded (Table 3.1). Graminoid species found at all three sites included *Aristida congesta*, *Heteropogon contortus*, *Melinis repens*, *Panicum maximum* and *Tragus berteronianus* (Appendix A: Table 9). There is a lack of tall trees (>10 m) in all sites, probably due to the shallow soils between the rocks. Noteworthy trees include *Balanites maughami*, an important medicinal plant in the

Figure 3.6. Examples of plant species found in the same habitat as *H. coddii* subsp. *barnardii.* (A) *Aptosimum lineare* [1,2,3]; (B) *Gloriosa superba* [3]; (C) *Karomia speciosa* [2,3]; (D) *Aneilema longirrhizum* [2,3]; (E) *Turraea obtusifolia* [2,3]; (F) *Barleria crossandriformis* [2,3]; (G) *Triaspis glaucophylla* [1]; (H) *Adenia fruticosa* subsp.*fruticosa* [1].
The number in square brackets indicates the site where the plants occurred.

area of which the population is decreasing due to overharvesting and habitat destruction (Raimondo *et al*., 2009). Three *Commiphora* tree species were recorded of which *C. marlothii* is most well known. The peeling bark of the tree was previously used to make paper and the root is chewed for its sweet juices (Van Wyk and Van Wyk, 1997).

Some of the species encountered in this study are South African endemics, which occur in the Limpopo (LP) and Mpumalanga (MP) provinces. These include *Adenia fruticosa* subsp. *fruticosa* (LP & MP) (Figure 3.6H), *Aneilema longirrhizum* (LP) (Figure 3.6D), *Searsia englerii* (LP & MP) and *Triaspis glaucophylla* (LP & MP, and the North West Province) (Figure 3.6G). *Cyphostemma sulcatum* is also endemic to Limpopo, Gauteng and North West provinces (Raimondo *et al*., 2009). Wild populations of *Adenia fruticosa* subsp. *fruticosa* and *Aneilema longirrhizum* are reported to be decreasing due to habitat transformation caused by mining activities, human settlements and severe overgrazing (Raimondo *et al*., 2009).

Siebert *et al*. (2002) reported the diagnostic tree, forb and grass species for the entire Northern Dry Mixed Bushveld vegetation type, which is a sparse thornveld with an open grassy layer. The most prominent and dominant tree species are *Vachellia tortilis*, *Boscia foetida* subsp. *rehmanniana*, and *Dichrostachys cinerea*, whereas *Becium filamentosum*, *Felicia clavipilosa*, *Gisekia africana*, *Hermannia odorata*, and *Melhania rehmannii* are the most frequently occurring forb species. Prominent grass species include *Aristida congesta*, *Enneapogon cenchroides*, *E. scoparius* and *Urochloa mossambicensis*. Some of these species were also found in the surveys at the different sites (Appendix A: Table 9).

Craib (2003) mentioned that *H. coddii* subsp. *barnardii* plants would be suitable for planting in sunny rockeries. Some of the species found in this survey, such as *Heteropyxis natalenis*, *Karomia speciosa*, *Mundulea sericea* subsp. *sericea*, *Aloe* spp., and *Scadoxus puniceus*, would be excellent companion species in such a rockery. Most of these plants are already available on the ornamental plant market. Van Wyk and Smith (2001) reported that *Triaspis glaucophylla* has ornamental potential due to its unique flowers, which could be further researched.

3.3.5 Plant characterisation in nature

Hibiscus coddii subsp. *barnardii* plants are sun-loving and grow amongst or near dark-coloured rocks mostly in full sun (Figure 3.7A & B). The open valleys between rocky outcrops are devoid of the plants (Figure 3.7A) with seedlings usually germinating in close vicinity to sheltering rocks (Figure 3.7C). Plants are frequently observed lodged between two rocks in a protected environment conducive to growth (Figure 3.7D & E).

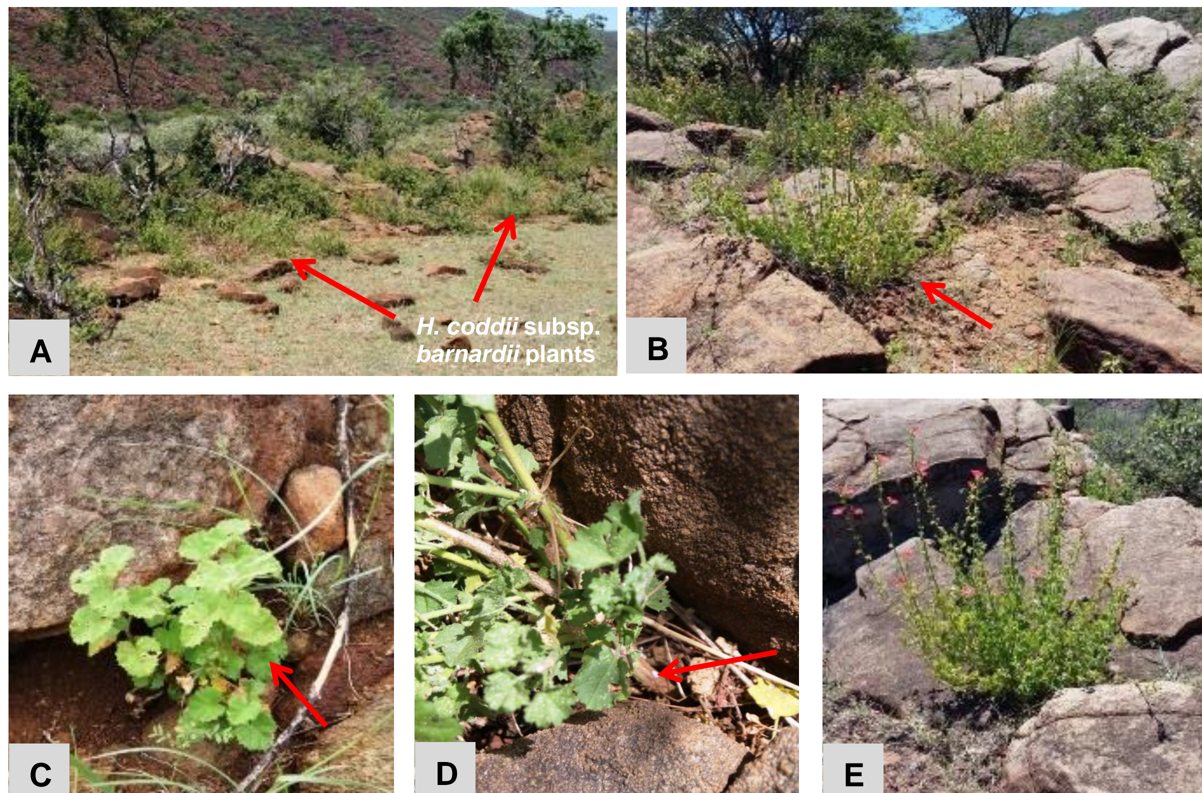

Figure 3.7. *H. coddii* subsp. *barnardii* plants in nature. (A & B) Plants grow amongst and between dark-coloured rocks; (C) Seedling germinated next to a rock; (D) Stem of the plant tightly lodged between two rocks; (E) Multi-stemmed plant with flowers growing between rocks.

Leistner *et al.* (2005) described the plant as an upright, long and slender perennial herb with a height of 600–1500 mm. Plants growing at Site 2 (Figure 3.7E) averaged 744±184.48 mm in height with 6.88±3.02 branches sprouting from the base. The plants at Site 3 were slightly shorter with an average height of 605.20±137.54 mm and 6.56±2.38 branches per plant, thus falling within Leistner's *et al.* (2005) height parameters. Leaves are arranged in an alternate fashion on cylindrical stems. They are almost circular in shape, have a deep notch at the leaf base and a serrated

margin. The width and length of leaves taken from plants in nature were 31.03±6.04 mmx29.23±5.36 mm and 34.47±4.95 x32.70±4.56 mm for Sites 2 and 3 respectively. (Appendix A: Table 3). These measurements correspond with those of Leistner *et al.* (2005) who reported that leaves are 10–35 mm in diameter. Densely stellate trichomes (hairs) cover the entire plant. Trichomes act as structural defence mechanism against herbivores and fungal infections and protect plants from abiotic stressors such as drought and heat. It also reflects excess sunlight and in this way protects the underlying tissues. Accumulation of ultraviolet-absorbing compounds such as flavonols in trichomes can also protect the underlying photosynthetic tissues from harmful UV-A and UV-B radiations (Hauser, 2014). The trichomes of *H. coddii* subsp. *barnardii* might protect the plants from the harsh sunlight and extreme heat associated with their habitat, particularly in the close vicinity of the dark-coloured rocks of the area.

The plant is predominantly summer flowering with most flowers appearing from mid-February (Craib, 2003; Leistner *et al*., 2005). The bright orange-red to red bisexual flowers (Figure 3.8A) are usually borne singly in the axils of the leaves but can also occur in terminal racemes or corymbs due to the reduction of upper leaves (Leistner *et al*., 2005). Field visits revealed that flowering starts during late November after good rains, albeit sporadically at first. The greatest density of flowers was observed from middle January until the end of March, although a few odd flowers could still be seen in May. During the extremely dry 2015/2016 season, flowering was adversely affected with very few flowers formed during January and February 2016.

Fruit capsules with seeds were observed on plants at all three sites confirming that pollination of the flowers and fertilisation took place (Figure 3.8B). The pollination agent, if any, is not known. However, insects crawling around on the plants and flowers were observed (Figure 3.8C) and could play a role. Pollination by agents such as insects and birds was reported for other members of the Malvaceae family, for example, flowers of *Sida rhombifolia* are pollinated by *Perditomorpha brunerii* bees (Gaglianone, 2000). McDade and Davidar (1984) reported pollination of flowers of *Pavonia dasypetala* plants by the hummingbird, *Phaethornis supercilliosus*. Self-pollination is an adaptation to a scarcity in pollinators and was reported by Klips and Snow (1997) for *Hibiscus laevis* flowers. They observed backwards curvature of non-

pollinated stigmas on older flowers that enabled contact with the pollen on stamens resulting in self-pollination, followed by fruit set. This backward curvature of stigmas was not observed in *H. coddii* subsp. *barnardii* flowers. Pollination of the flowers in nature could be further investigated.

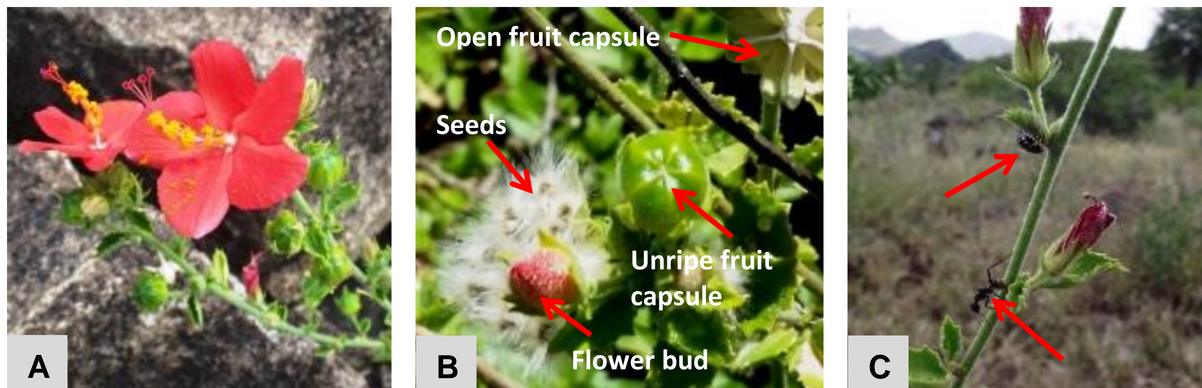

Figure 3.8. *H. coddii* subsp. *barnardii* plants in nature. (A) Flowers appear in warmer summer months; (B) Flower bud, fruit capsule (open and unripe), and seeds; (C) Ant and other insects (arrows) crawling around on plant.

In its natural habitat, apical dominance is seldom observed in the plants. The tips of the shoots are either eaten off by animals (Figure 3.9B), or might be broken off by passing animals or the wind. This leads to a more rounded and bushy appearance. Craib (2003) pointed out that the plants tend to become dormant and die back (Figure 3.9C) during the winter months and sprout again from the base (Figure 3.9D) in spring.

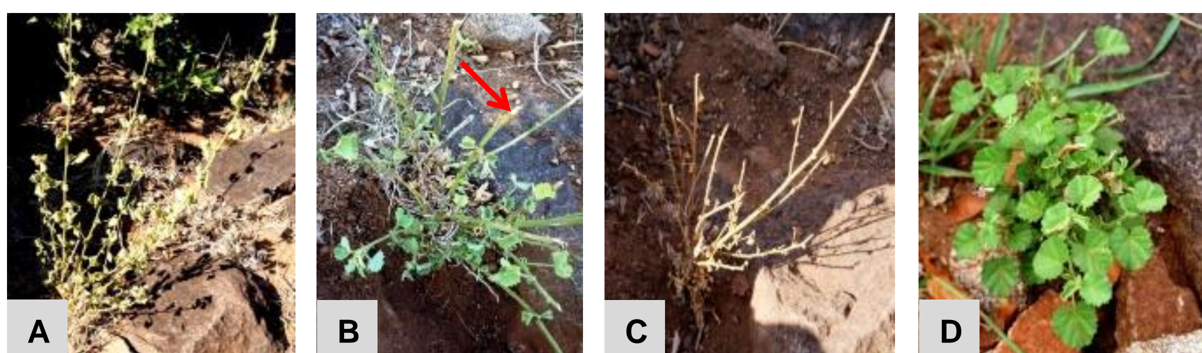

Figure 3.9. *H. coddii* subsp. *barnardii* plant in nature (Site 1) grazed (red arrow) by animals and its recovery after rain during 2016. (A) February; (B) June; (C) September; (D) November.

During field visits, it was noticed that the plants are extremely hardy. To illustrate this, the same plant from Site 3 in the Potlake Nature Reserve was photographed in February, June, September and November 2016. Rainfall in the Potlake Nature

Reserve was well below average in 2015 with only 278 mm of rainfall for the year. This was coupled with high maximum temperatures (21°C in July and 38°C in January) recorded for 2016 (Appendix A: Table 1). When photographing this plant (and others) for the first time in February 2016, it appeared lifeless and dry. Very little or no flowers were observed (Figure 3.10A). This drought period led to unavailability of seeds in nature. After receiving some rain in March and April 2016 (212 mm and 61 mm respectively) the same plant recovered by the end of June 2016. The leaves were green and some flowers were observed on the plant (Figure 3.10B). During the dry winter months that followed most of the leaves fell from the plant and by the end of September 2016 the plant had an unattractive and dry appearance (Figure 3.10C). In November 2016, 108 mm of rain was recorded in the nature reserve and this completely changed the appearance of the plant. By the end of November, it sprouted fresh leaves and it had numerous flowers (Figure 3.10D). It seems that the plant can withstand periods of drought and high temperatures, making it a suitable candidate for water-wise gardening in South Africa.

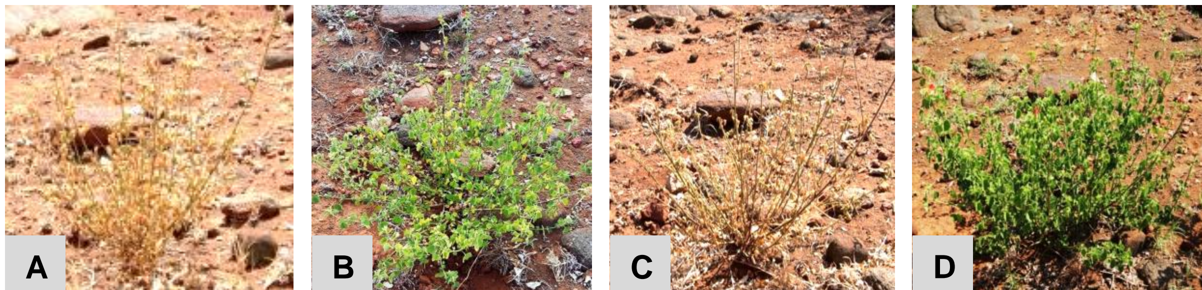

Figure 3.10. The same *H. coddii* subsp. *barnardii* plant at Site 3 photographed throughout 2016. (A) Dry appearance in February after a very dry summer; (B) Green leaves and flowers in June; (C) Dry plant in September (after winter); (D) Revived, green plant with flowers in November.

3.3.6 Geology and soils

Soils derive from weathered rock immediately below them or from alluvial material deposited from running water and are dominated by the silicate group of minerals. Silicate clays consist of O_2, Si, Al, and Fe and combine with Mg, Ca, Na and K in various ratios to form several minerals. More than 80 other elements also occur in soils depending on the rocks (parent material) from which the soil is formed (Kabata-Pendias and Pendias, 2001). The parent material from where soils in Sekhukhuneland are formed varies greatly in type and abundance of elements.

The three chosen sites in Sekhukhuneland are part of the eastern lobe of the Rustenburg Layered Suite (RLS) of the Bushveld Igneous Complex (BIC). The RLS consists mainly of ultramafic and mafic rocks that are well exposed in contrast to other flat areas of the BIC that are covered by deep clay soils (Siebert *et al*., 2001; Cawthorn *et al*., 2006). The layers of the RLS are subdivided vertically, on the basis of regional-scale variations in lithology, into five major stratigraphic units. These are, from bottom to top, the Marginal Zone, Lower Zone, Critical Zone (upper and lower), Main Zone, and Upper Zone (Molyneux and Klinkert, 1978; Bamisaiye *et al*., 2016; Mungall *et al*., 2016). The two sites in the Potlake Nature Reserve (Sites 2 and 3) are in the Lower Zone, while Site 1 is located in the transition area from the Lower Zone to the Upper Critical Zone (Eales, 2001). The Lower Zone is characterised by ultramafic dunite and mafic pyroxenite and harzburgite rocks containing olivine and orthopyroxene cumulus minerals (Haldar, 2017). The ultramafic to mafic Upper Critical Zone shows layering of ortopyroxenite, anorthosite, norite, harzburgite and chromitite rocks, which contain olivine, orthopyroxene, plagioclase feldspar (anorthite) and chromite cumulus minerals (Cameron, 1978; Cawthorn *et al*., 2006; Yudovskaya *et al*., 2013). In general, the soils derived from these parent rocks are rich in Mg and Fe with moderate amounts of Ca and low amounts of K, P and Na. It can hold high percentages of additional metals such as Al, Cr, Mn, Na, and Ti Anorthite, a plagioclase feldspar contains Ca and Al and is low in Na and K (Eales, 2001; Kabata-Pendias and Pendias, 2001; Reeves and Adigüzel, 2008).

3.3.6.1 Soil properties

Plants of *H. coddii* subsp. *barnardii* are mostly associated with dark-coloured harzburgite and pyroxenite rocks from rocky outcrops in the study area where they grow in the shallow soils between rocks. The reason for this is not known, therefore, soil analyses were performed to screen for the presence of mineral elements in the soils where plants are growing.

3.3.6.1.1 Collection sites

i. <u>ICPE method</u>

The ANOVA procedure indicated significant differences between the sites (Site 1, 2, and 3) for the macro-elements Ca, K, and Mg, and for the micro-elements Mn and

Mo. There were also differences between sites for Cr, % C, and pH (Appendix A: Table 4). The highest concentration of macro-elements was recorded for Mg with 61.63 and 38.72 mg L^{-1} at Sites 2 and 3 respectively. The concentration of Ca at Site 1 (38.27 mg L^{-}1) was approximately four times higher than at Site 2 (8.17 mg L^{-1}) and Site 3 (12.59 mg L^{-1}). The P and S concentrations were both lower than 10 mg L^{-1} at all three sites. The lowest macro-element was K, with less than 5 mg L^{-1} at Sites 1, 2 and 3 (Figure 3.11A, Appendix A: Table 5).

The highest concentration of micro-elements at all three sites was recorded for Fe (23.30–33 mg L^{-1}) followed by Mn and Na with concentrations of less than 5 mg L^{-1}, except for Na at Site 1 which was 6.71 mg L^{-1}. The other micro-elements (B, Cu, Mo, and Zn) had concentrations of less than 1 mg L^{-1} at all three sites (Figure 3.11B, Appendix A: Table 5). The level of Cr was the highest at Sites 2 and 3 (10.44 and 9.35 mg L^{-1} respectively) with significantly less (7.87 mg L^{-1}) Cr at Site 1 (Figure 3.11B).

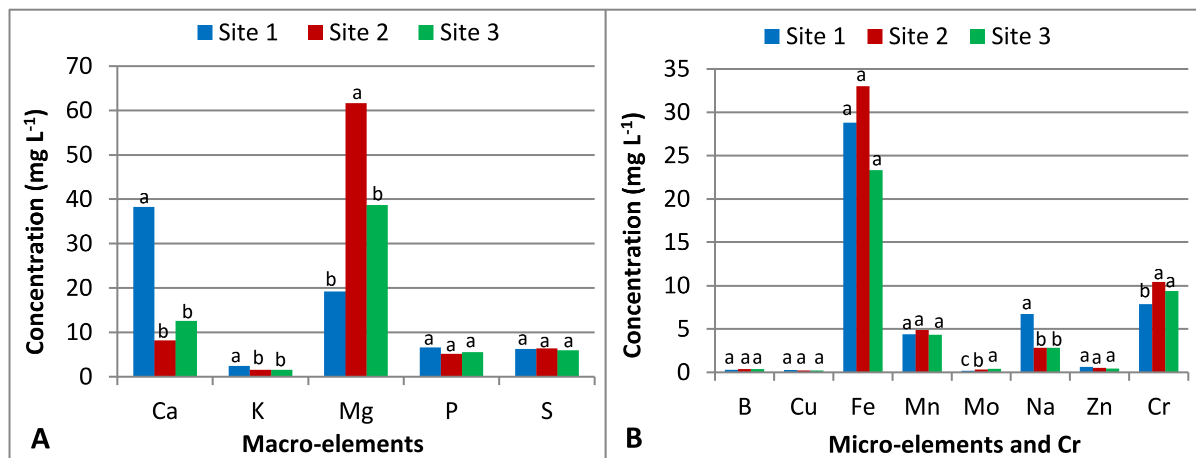

Figure 3.11. Concentration of (A) Macro-elements; (B) Micro-elements and Cr of soil samples collected from three sites in Sekhukhuneland and analysed with the ICPE method. For each element, means (bars) with the same letters indicate no significant differences at the 5% level of significance.

ii. XRF method

Soil analyses revealed concentrations of elements typical of what could be expected from soils occurring in the Lower and Upper Critical Zones of the RLS. Major and trace elements were found in the same proportions and ranges as reported in the literature for similar soils (Cameron, 1978; Eales, 2001; Kabata-Pendias and Pendias, 2001; Cawthorn *et al.*, 2006).

The largest portion (40–50% of the mass) of the soil samples at all three sites was made up by SiO_2 (Figure 3.12A). The MgO in samples from Site 2 (17.57%) and 3 (16.92%) was 6 times higher than at Site 1 (2.86%), whereas Fe_2O_3 at Site 1 (5.24%) was half that of Site 2 (9.97%) and 3 (11.27) (Figure 3.12A). The highest Al_2O_3 content (16.86%) was at Site 1, and the value decreased by two-thirds to 5.46% at Site 2, and by three-quarters to 3.46% at Site 3. The same trend was observed for the CaO values. The other major oxides (TiO_2, MnO, Na_2O, K_2O, P_2O_5) each contributed less than 1% to the total mass (Figure 3.12A, Appendix A: Table 7).

For trace elements, the highest Cr level was recorded at Sites 2 (5429 ppm) and 3 (5870 ppm). Although the level of Cr was substantially less at Site 1 (339 ppm) it is still very high in comparison with the normal range (70 ppm) in most soils (Kabata-Pendias and Pendias, 2001; Hajar *et al.*, 2014). Relatively high levels of Ni were recorded at Sites 2 (2486 ppm) and 3 (1104 ppm) with 10–20 times less Ni at Site 1 (109 ppm) (Figure 3.12B). The element with the next highest level was Sr from soils collected at Site 1, with a value of 281 ppm (Figure 3.12 B). The concentrations of the other elements (Cu, Zn, V, Rb, Y, Zr) were very low, less than 120 ppm (Figure 3.12B, Appendix A: Table 8).

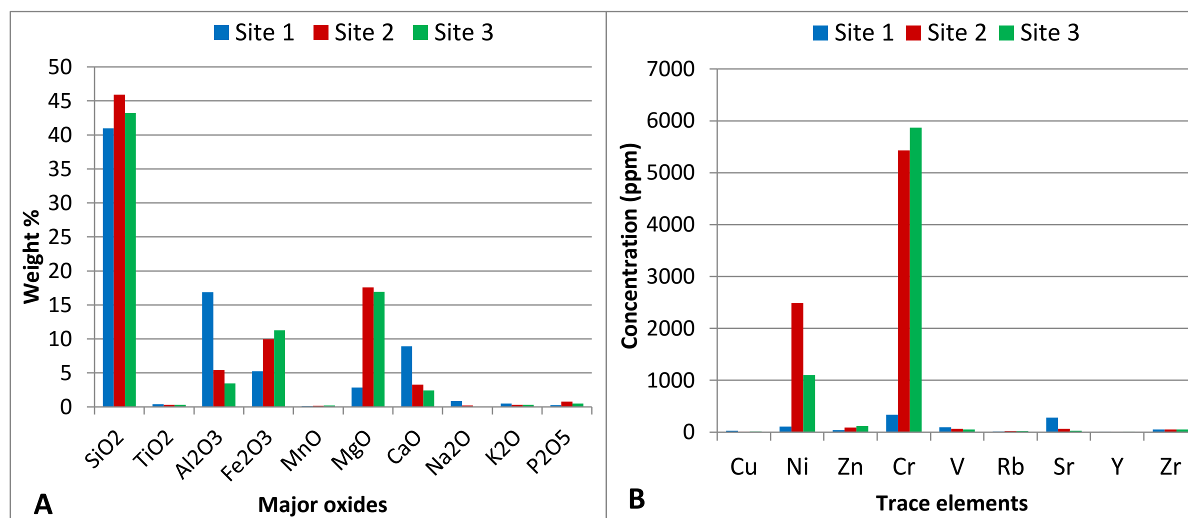

Figure 3.12. (A) Major oxides and (B) Trace elements of soil samples collected from three sites in Sekhukhuneland and analysed with X-Ray Fluorescence Spectroscopy (XRF). Values are the mean of twelve replicates.

Results from the ICPE and XRF methods cannot be directly compared since the first one measured the elements from soils in solution and the XRF method measured the elements in the soil directly. However, a similar trend in terms of the proportions of the elements K, P, Ca, Mg, Cu, Fe, Mn, Na, Zn, and Cr was observed in results from both methods for the sites and pair samples (+Plant and –Plant). The values from only the XRF method are therefore used in discussions that follow.

The level of SiO_2 of soils collected at the three sites in Sekhukhuneland is between 40% and 50% of the total mass that is consistent with soils derived from ultramafic and mafic rocks (Eales, 2001; Cawthorn *et al.,* 2006). The three sites in Sekhukhuneland where soils were collected are located in the Lower and Upper Critical Zone of the RLS of which the rocks mainly contain olivine, orthopyroxene, plagioclase feldspar (anorthite) and chromite cumulus minerals (Cameron, 1978; Cawthorn *et al*., 2006; Yudovskaya *et al*., 2013). Olivines have the overall formula of [Mg_2SiO_4, Fe_2SiO_4 or $(Mg,Fe)_2$ SiO4], while for orthopyroxenes it is either $MgSiO_3$ or $(Mg,Fe)SiO_3$ when it contains Fe as well. The composition of these minerals might explain the high levels of Mg and Fe found in soils at selected sites in Sekhukhuneland (Figure 3.11, Figure 3.12). The Fe content in soils collected from the three sites varied from 5.24–11.27%. According to Kabata-Pendias and Pendias (2001), this is within the range for the Fe concentration in ultramafic and mafic rocks (5.6–10%) Soils derived from such rocks carry high levels of other elements such as Ni and Cr (Reeves and Adigüzel, 2008) as found in this survey (Figure 3.11, Figure 3.12). These rocks can also hold small percentages of additional metals such as Mn, Na, and Ti (Eales, 2001) that might explain the lower levels of these elements found at the selected sites. Anorthite ($CaAl_2Si_2O_8$), a plagioclase feldspar contains Ca and Al, and is low in Na and K. The higher levels of Ca and Al at Site 1 might be due to the presence of anorthite, which is reported to be a mineral of the Upper Critical Zone (Cawthorn *et al*., 2006; Yudovskaya *et al*., 2013). Strontium (Sr) is often associated with Ca which might explain the higher level of Sr at Site 1, which also had the highest Ca level (Figure 3.11, Figure 3.12) (Kabata-Pendias and Pendias, 2001). Chromite is an iron chromium oxide (FeO,Cr_2O_3 or $FeCr_2O_4$±Mg) (Haldar, 2017). The chromitite rocks in the eastern lobe of the RLS contain 60–90% chromite with up to 43.5% Cr_2O_3 (Kinnaird, 2005). The average Cr content for ultramafic and mafic soils is 1600–3400 ppm (Kabata-Pendias and Pendias, 2001), although in this

survey higher Cr concentrations were found at Sites 2 (5429 ppm) and 3 (5870 ppm). The average P content in soils is 0.6% but can be as low as 0.03–0.13% as also found in soils at these sites (0.10%) (Figure 3.12). Levels of other elements in the collected soils at the three sites are in accordance with levels given by Kabata-Pendias and Pendias (2001) for these elements that derive from ultramafic and mafic rocks.

3.3.6.1.2 Pairs of soil samples

i. ICPE method

The ANOVA procedure showed significant differences between pairs of soil samples (+Plant and –Plant) for Ca, K, Mg, Cr, Mn, Zn, % C, and pH (Appendix A: Table 4).

The highest concentration for the macro-elements was recorded for Mg and Ca in soils collected away from the plants. The Mg concentration away from the plants (49.90 mg L^{-1}) was 1.7 times higher than that at the plants (29.80 mg L^{-1}). The same trend was observed for the Ca concentration (Figure 3.13). The lower levels of Mg and Ca at the plants (+Plant) might be due to the uptake of these macronutrients by the plants, but this needs to be confirmed with further mineral analysis of plant material. The P and S concentrations were both lower than 10 mg L^{-1} and were almost the same at the plants and away from the plants. The lowest macro-element was K with less than 3 mg L^{-1} at both the plants and away from the plants (Figure 3.13A, Appendix A: Table 6). Soils derived from ultramafic and mafic rocks are reported to be low in K, P and Na (micronutrient) as also seen in this study (Eales, 2001; Kabata-Pendias and Pendias, 2001; Reeves and Adigüzel, 2008).

The highest concentration of micro-elements, both at the plants and away from the plants, was recorded for Fe (28.40 and 25 mg L^{-1} respectively) followed by Mn and Na with concentrations of less than 5 mg L^{-1}. The other micro-elements (B, Cu, Mo, and Zn) had concentrations of less than 1 mg L^{-1} both at the plants and away from the plants (Figure 3.13B, Appendix A: Table 6). The level of Cr was significantly higher at the plants (10.13 mg L^{-1}) than away from the plants (8.31 mg L^{-1}) (Figure 3.13 B).

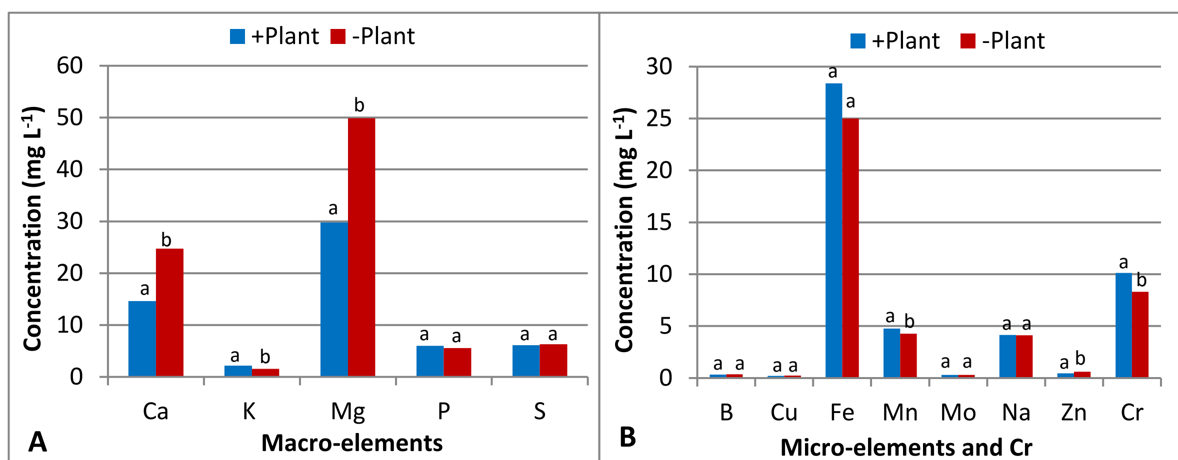

Figure 3.13. Concentration of (A) Macro-elements; (B) Micro-elements and Cr of soil samples collected at the plants (+Plant) and away from the plants (–Plant) in a pairwise design and analysed with the ICPE method.

For each element, means (bars) with the same letters indicate no significant difference at the 5% level of significance.

ii. XRF method

The largest portion (43% of the mass) of the soil samples was contributed by SiO_2, both at the plants and away from the plants (Figure 3.14A). The next highest mass for the major oxides was recorded for MgO, which was 12.18% at the plants and 12.73% away from the plants. The value of Fe_2O_3 in soils at the plants was slightly higher (9.37%) than in soils away from the plants (8.29%) similar to values for the ICPE method. The opposite was observed for CaO where soils away from the plants had a higher content (5.60%) than soils at the plants (4.12%) as also found for values from the ICPE method (Figure 3.13). Values for Al_2O_3 barely differed (8.72% and 8.46%) between soils at the plants and away from the plants. The other major oxides (TiO_2, MnO, Na_2O, K_2O, P_2O_5) each contributed less than 1% to the total mass both at the plants and away from the plants. The content of P_2O_5 was 2.5 times higher (0.74%) at the plants than away from the plants (0.30%) (Figure 3.14A, Appendix A: Table 7).

The highest levels of trace elements were observed for Cr and Ni. A higher average level of Cr was found at the plants (3956 ppm) than away from the plants (3803 ppm) (Figure 3.14) as also found with the ICPE method (Figure 3.13). These Cr levels are extremely high as compared to chromium levels (70 ppm) in other soils (Hajar *et al.*, 2014). The Ni levels were high, both at the plants (1247 ppm) and away from the

plants (1219 ppm) (Figure 3.14). The average level of Ni (1233ppm) where the plants occur was much higher than the grand mean (22 ppm) reported for world soils (Kabata-Pendias and Pendias, 2001). The value for Sr was slightly higher (134 ppm) in soils away from the plants than at the plants (118 ppm). Levels of Zn, V, and Zr were less than 100 ppm in soils both at the plants and away from the plants. The lowest levels (<25 ppm) was recorded for Cu, Rb and Y (Figure 3.14B, Appendix A: Table 8).

This study suggests that plants of *H. coddii* subsp. *barnardii* grow in soils with high levels of Ni (average of 1247 ppm) and Cr (average of 3956 ppm), but the results do not show if these metals are in bio-available form in the soils. Further research, including mineral analysis of plant material, will be required to investigate this. It is reported that plants growing in soils with heavy metals have developed various strategies for tolerance and detoxification in the presence of high levels of such elements (Viehweger, 2014; Singh *et al.*, 2015). Hyperaccumulator plants accumulate heavy metals in their organs at concentrations from 100 to 1000 times higher than found in non-hyperaccumulator species without showing any apparent phytotoxic effects (Muszyńska and Hanus-Fajerska, 2015).

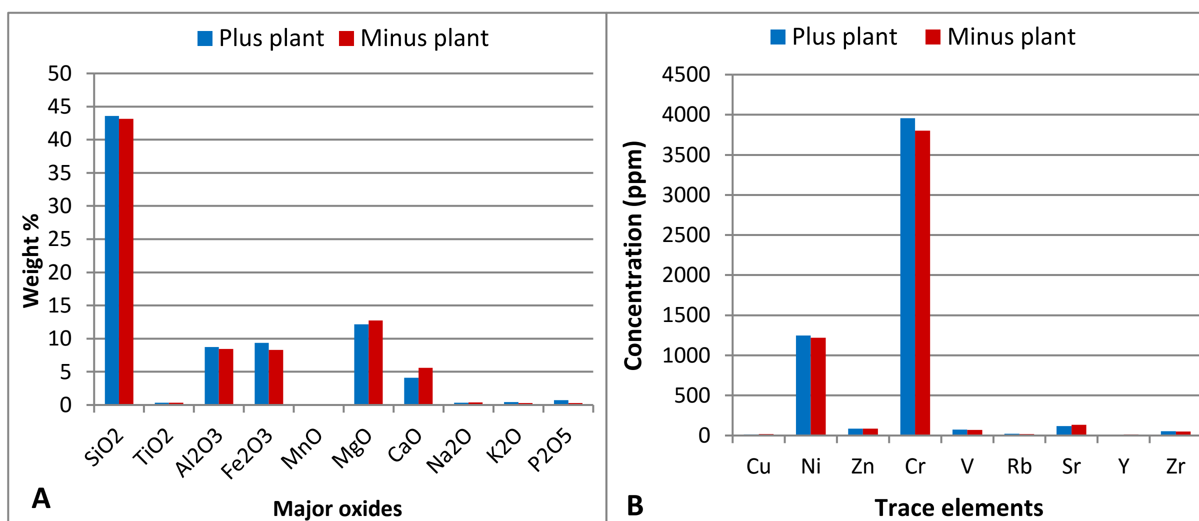

Figure 3.14. (A) Major oxides and (B) Trace elements of soil samples collected at the plants (+ Plant) and away from the plants (–Plant) in a pairwise design and analysed with X-Ray Fluorescence Spectroscopy (XRF). Values are the mean of 18 replicates.

Hypertolerator plants have developed various strategies to cope with high levels of heavy metals in the soil. In excluder plants, physical barriers, such as a thick cuticle, trichomes, the cell wall and sometimes, mycorrhizal associations, prevent the uptake of heavy metals from the soil or air (Emamverdian *et al.*, 2015; Muszyńska and Hanus-Fajerska, 2015). Hauser (2014) reported that trichomes could serve as heavy metal storage sites for detoxification purposes or could secrete secondary metabolites to counteract the harmful effect of heavy metals. Plants of *H. coddii* subsp. *barnardii* are densely covered by trichomes, which might play a role in metal tolerance. This, and the tolerance or exclusion mechanism to such high concentrations of heavy metals, if any, of *H. coddii* subsp. *barnardii* plants, is not known and could be further researched. Exclusion of heavy metals, especially Cr, Al and Fe, have been reported for *Euclea sekhukhuniensis* (Retief *et al.*, 2008) and *Polygala sekhukhuniensis* plants growing in calciferous, ultramafic soils (Siebert *et al.*, 2010). Both species are also Sekhukhuneland endemics.

Plants with the ability to hyperaccumulate high levels of heavy metals in their cells or tissues could be used for phytoremediation of contaminated sites or for phytomining purposes (Zhang *et al.*, 2007). Hyperaccumulation of Ni and Cr by plants has been widely reported in the literature. Plants require Ni in small amounts to activate the urease enzyme for nitrogen metabolism, but when in excess [10 to 100 ppm (dry weight)] it becomes phytotoxic. Normal soils contain about 7–50 ppm Ni, while soils formed from ultramafic and mafic rocks can contain from 700–5 000 ppm (Reeves and Adigüzel, 2008; Emamverdian *et al.*, 2015). The South African species, *Berkheya coddii* is reported to be a fast-growing, high biomass hyperaccumulator of Ni and the most Ni (4375 ppm) was found in the cuticle of the upper epidermis of the older leaves when grown hydroponically in a solution that contained 100 µM Ni (Robinson *et al.*, 2003).

Chromium is a non-essential element and not needed by plants for normal growth, instead it can be toxic to plants in high concentrations (75–100 ppm) causing chlorosis and necrosis (Amin *et al.*, 2013). In non-accumulating plants, most Cr accumulates in the root cells where it is immobilised in the vacuoles of the root cells that could be a protective mechanism against Cr toxicity (Kumar *et al.*, 2016). Zhang *et al.* (2007) identified *Leersia hexandra* as a hyperaccumulator of Cr which can

accumulate up to 1636 ppm Cr in its leaves under natural conditions and up to 5608 ppm Cr (III) and 2164 ppm Cr (VI) in the leaves in pot culture experiments. Other plants such as *Sutera fodina* (Baker and Brooks, 1989) and *Convolvulus arvensis* (Gardea-Torresdey *et al*., 2004) were also reported to accumulate high levels of Cr. Amin *et al*. (2013) reported Cr toxicity in *Hibiscus esculentus* seeds and seedlings exposed to high (50 and 100 mg kg $^{-1}$) concentrations of Cr. The seed germination percentage was significantly reduced by 42% and 49% in the presence of 50 and 100 mg kg^{-1} Cr respectively. Root and shoot elongation and dry root mass of seedlings were also adversely affected by 50 mg kg^{-1} Cr, while all seedlings exposed to 100 mg kg^{-1} Cr died. In contrast, *H. coddii* subsp. *barnardii* plants grow in soils with high levels of heavy metals such as Ni and Cr without obvious adverse effects. This study suggests that the Cr might not be in a bio-available form for plants and further studies are required to identify the mineral in which the Cr resides.

% C and pH

The percentage C was significantly higher at Sites 1 and 3 (4.39 and 4.21% respectively) than at Site 2 (3.40%) (Appendix A: Table 5). It was also significantly higher at the plants (4.85%) than away from the plants (3.14%) (Appendix A: Table 6) which could be due to the presence of more organic material where the plants occur. The pH values for all three sites were slightly alkaline. Sites 2 and 3 had a pH value of 7.7, which was significantly higher than at Site 1 (7.4) (Appendix A: Table 5. It was also significantly lower in soils collected at the plants (7.23) than in soils away from the plants (7.94), but was still alkaline (Appendix A: Table 6). Soils formed under low rainfall conditions as in Sekhukhuneland where these plants occur tend to be more alkaline with soil pH readings around 7.0 (Hamza, 2008).

The lower pH measured at soils collected at the plants (7.23) might be due to the presence of organic matter around the plants since it is reported that decomposition of organic matter lowers the soil pH (Taiz and Zeiger, 2010). Soil pH is important since it directly affects availability of mineral elements to the roots. Plant roots can only absorb nutrients after transformation into certain ionic forms and this can only happen within a suitable pH range. Most plants grow best in soils where the pH ranges from 5.5 to 7 (Hamza, 2008; Taiz and Zeiger, 2010). In general, the presence of mineral elements in the soil only gives an indication of what is potentially available

to plants for uptake by roots and not that the plants actually absorb the elements or nutrients (Taiz and Zeiger, 2010). Further studies on mineral analyses could be performed to identify levels of nutrients in *H. coddii* subsp. *barnardii* plants.

3.3.6.1.3 Soil texture

According to Siebert (2001) the deep and shallow soils from low altitudes in the SCPE, as in the study area, have a sandy-loam to clay texture and vary in colour from deep red to brown. The soil collected at the plants (for all three sites) was also found to have a sandy loam texture and the texture for the soils collected away from the plants also ranged from sandy loam to sandy clay loam with a few silty clay loam textured soils (Ritchey *et al*., 2015). Furthermore, the soil collected at the plants had a darker colour (dark-brown) compared to the light brown and red colour of soils collected away from the plants. From the results obtained it seems that the endemic *H. coddii* subsp. *barnardii* species prefers to grow in the ultramafic and mafic soils rich in Mg and Fe that are associated with the Lower Zone and Upper Critical Zone. Siebert *et al*. (2001) reported a highly significant positive correlation between the basaltic rocks (norite, pyroxenite, and anorthosite) and the endemic flora in Sekhukhuneland. They further speculated that the floristic-geological correlation could be due to the heavy metal rich montmorillonite clays derived from the basaltic rocks.

The results of this study do not conclusively show that the *H. coddii* subsp. *barnardii* plants prefer the rock habitat because of the presence or absence of certain mineral elements since there was no difference in the level of most elements at the plants and away from the plants. Physical factors such as wind might play a more important role. It is possible that the released seeds are blown about by the wind and when they land between or near rocks the seed is lodged into the soil and will germinate there as also reported by Craib (2003). A so-called “dead-wind” zone is created against the rock when the wind blows over it. The seeds would accumulate on the downwind side of the rocks and when it rains or dew collects, the runoff water from the rocks could assist in establishment and subsequent growth of the seedling. Navarro and Guitián (2003) indicated anemochory (wind dispersal) in seeds of *Petrocoptis grandiflora* and *P. viscosa*. They also stated that seeds, which land in rock crevices, have a better chance of germinating and seedlings are less exposed

to herbivores and competition from other plants. Smith and Capelle (1992) reported increased germination and establishment of seedling roots when seeds of *Cichorium intybus* germinated in gaps between rocks or soil clods creating sites with increased moisture retention. Craib (2003) also reported that *H. coddii* subsp. *barnardii* seeds that land between rocks are trampled into the soil by goats that walk around the rocks. He further reported that goats eat seedlings that germinated in open exposed soil patches between rocks, which might be another reason why *H. coddii* subsp. *barnardii* plants are only found between the rocks.

Baskin and Baskin (1988) reported that high light requirements, less competition from other plants, reduced predation from herbivores, and not edaphic factors, might explain the occurrence of endemic plant species of the eastern United States on rocky outcrops. These plant species grew well when cultivated in a potting mixture of topsoil and river sand. This also applies to *H. coddii* subsp. *barnardii* plants which could be grown successfully in pots in a mixture of potting soil, vermiculite and sand [5:3:1 (v/v/v)] indicating that the plant is not dependent on soils from its natural habitat. The plants in pots, however, showed deficiency symptoms when not supplied with a nutrient solution (Chapter 4). These interveinal chlorosis symptoms in older and younger leaves might be related to a deficit in Mg and Fe, which occur in high concentrations in the plant's natural habitat.

The *H. coddii* subsp. *barnardii* plants occur naturally in dry areas and can withstand periods of drought. These traits make it an ideal plant for the dry South African conditions where water-wise gardening is becoming more popular and necessary. Despite the fact that the plants are endemic to ultramafic soils in Sekhukhuneland with a high chromium and nickel concentration, they could be grown in pots and in an outside garden near Polokwane. Although plants prefer warmer winter temperatures with no frost, they will re-sprout and recover quickly when planted outdoors in colder areas (Chapter 4).

3.4 References

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Chapter 4

CHAPTER 4

In vivo propagation of *Hibiscus coddii* subsp. *barnardii* by seeds

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4.1 Introduction

Seeds are primarily used to propagate commercial crop and ornamental plants (Hartmann *et al.*, 2011). Seed propagation might lead to genetic diversity, which is desirable when native ornamentals are introduced as landscape plants or if the aim is *ex situ* conservation (Papafotiou and Martini, 2016). In its natural environment, the endemic *Hibiscus coddii* subsp. *barnardii* species produce seeds in the summer months, which are responsible for natural reproduction of the plant. Seeds released from the fruit capsules are dispersed by wind until they come to rest in the soil between rocks (Craib, 2003). The plant has horticultural potential as an ornamental plant because of its attractive red flowers, but before it can be introduced to the market, reliable propagation protocols need to be established.

The seeds produced by *H. coddii* subsp. *barnardii* plants are small (3–5 mm long), covered with white cotton-like hairs and have a hard seed coat which imposes physical dormancy. This adversely affects germination as also reported for other *Hibiscus* species and members of the Malvaceae family (Liu and Spira, 2001; Gama-Arachchige *et al.*, 2013; Geneve *et al.*, 2018). The exact mechanism for uplifting physical dormancy under natural conditions is not well known, although factors such as fire, drying, freezing alternated with thawing, passage through the gut of animals, as well as, high and widely fluctuating temperatures have been reported (Baskin *et al.*, 2000; Hartmann *et al.*, 2011; Baskin and Baskin, 2014; Erickson *et al.*, 2016).

Synchronised seed germination is an important requirement in the commercial production of uniform seedlings (Sakhanokho, 2009; Kak *et al.*, 2015). Artificial ways of uplifting physical seed dormancy, such as mechanical and chemical scarification and wet and dry heat treatments are used to improve seed germination (Hartmann *et al.*, 2011; Baskin and Baskin, 2014). Kak *et al.* (2015) researched the effect of hot water treatments (60–80°C) and chemical scarification with H_2SO_4 (50% and 100%) on breaking seed dormancy of seven *Hibiscus* species. Hot water treatment of 80°C for 10 min was efficient in only two of the *Hibiscus* species where more than 90%

germination was attained. Seed germination in the other five *Hibiscus* species was achieved only when scarified with 50% and 100% H_2SO_4, although the germination responses varied amongst the plant species, depending on the seed size, degree of hardseededness and H_2SO_4 concentration. Other researchers reported that the breaking of physical dormancy with concentrated H_2SO_4 in seeds of some *Hibiscus* species depended on the duration of scarification that could vary between species (Chachalis *et al*., 2008; Sakhanokho, 2009; Seo *et al*., 2012). A single scarification treatment (concentration and duration) can, therefore, not be recommended for all *Hibiscus* species (Kak *et al*., 2015).

Temperature is probably the most important environmental factor that affects seed germination responses and the subsequent growth and development of seedlings (Carberry and Abrecht, 1990; Taghvaei and Nasrolahizadehi, 2016). Some seeds require exposure to a constant temperature for effective germination, whereas other seeds prefer fluctuating day/night temperatures (Hartmann *et al*., 2011, Baskin and Baskin, 2014; Taghvaei and Nasrolahizadehi, 2016). The constant optimum temperature for germination of *Kosteletzkya virginica* seeds ranged from 28°C to 30°C (Poljakoff-Mayber *et al*., 1994). Chauhan and Johnson (2008) reported that alternating day/night temperatures of 30°C/20°C resulted in 59% germination in seeds of *Sida rhombifolia* in the dark, and 64% germination when seeds were exposed to a 12 h/12 h, light/dark period. Wild plants of *H. coddii* subsp. *barnardii* grow in dry bushveld conditions and in summer are exposed to a wide range of temperatures from 15°C to 35°C and sometimes higher (Mucina and Rutherford 2006). These high temperatures could have an effect on seed germination and should be investigated.

The germination medium also affects seed germination. It should be firm and dense to hold the seeds in place and retain moisture and permit gas exchange (Gairola *et al*., 2011). Common seedling mixes that are commercially available, or that can be mixed on site, include combinations of peat moss, coco peat, perlite, bark and vermiculite. Mineral nutrients or slow-release fertilisers may be added to seedling mixes. Smaller seeds require a finer and more compact germination medium than larger seeds (Hartmann *et al*., 2011). Papafotiou and Kalantzis (2009) reported the

highest germination (80–88%) when seeds of *Sideritis athoa* were planted in a peat-perlite mixture and 100% in perlite alone.

Various factors such as water, light, temperature and mineral nutrition affect further seedling development and plant growth and also determine the architecture or form of the mature plant (Taiz *et al.*, 2015). Branching of axillary shoots also governs the plant architecture. Indeterminate monopodial branching occurs when the apical bud continues with upward growth and axillary shoots only form lower down the stem where the suppressing effect of the apical bud is diminished. In sympodial branching the apical bud dies or is transformed into a flower or other structure resulting in outgrowth of axillary shoots (Reinhardt and Kuhlemeier, 2002; Barthélémy and Caraglio, 2007). The apical bud can be removed (de-topping) by natural causes or by humans to induce axillary branching resulting in a bushy plant (Cline 1997; Mollah *et al.*, 2017). Conditions such as water stress, mineral deficiency, low light and temperature would delay growth of the plant. The plant would remain in the juvenile phase without flowering. On the other hand, conditions that promote strong and fast growth in plants can accelerate the transition to the adult phase and the plant will start to flower if environmental prerequisites have been met (Evert and Eichhorn, 2013; Taiz *et al.*, 2015).

Collection of vegetative material from wild *H. coddii* subsp. *barnardii* plants for propagation purposes is restricted due to legislation. Thus, the development of a protocol for seed propagation was required to produce plants that can be used as stock for further propagation. Hence, the aim of this study was to research the effect of factors such as chemical scarification and temperature on seed germination in order to develop a reliable seed propagation protocol. Furthermore, the effect of the apical bud and different nutrient regimes on growth and flowering of mature plants under controlled and uncontrolled growth conditions were studied to recommend the most suitable practice for plant growth.

4.2 Materials and methods

4.2.1 Factors affecting seed germination and seedling emergence

4.2.1.1 Seed coat

4.2.1.1.1 Plant material

Fruit capsules with seeds were collected from *H. coddii* subsp. *barnardii* plants growing at two localities in the Potlake nature reserve (24°15' 16.9"S; 29°54'44.0" and 24°15' 18.0"S; 29°54'22.8"E), Limpopo Province. Three batches of fruit capsules were collected during the seed production cycle from February to March 2016. Seeds were removed from the capsules and each batch was separately stored in paper envelopes at ambient temperature. The three batches of seeds (±1000 seeds per batch) were used as replicates for the scarification experiments (a specific replicate only contained seeds of one of the collected batches).

4.2.1.1.2 Chemical scarification of seeds

Seeds were scarified by incubation in various concentrations of H_2SO_4 for different time periods at ambient temperature. To select the best acid concentration, 12 scarification treatments consisting of three concentrations (25%, 50%, and 98%) of H_2SO_4 and four scarification time periods (10, 20, 30, and 40 minutes), were investigated. To define the optimum duration for scarification, eight durations of scarification treatments (5, 10, 15, 20, 25, 30, 35, and 40 minutes) with the best H_2SO_4 concentration were then examined. After scarification, the seeds were rinsed under running tap water followed with distilled water to remove the H_2SO_4 and hair debris from the seeds. Non-scarified, dry seeds and seeds imbibed for 40 minutes in distilled water were used as two controls.

4.2.1.1.3 Seed germination

Filter paper and vermiculite cultures were used for all seed germination experiments. In the filter paper culture, seeds were aseptically inoculated on sterile, moist filter paper bridges placed in Magenta™B-cap (Sigma) glass bottles layered with sterile distilled water. In the vermiculite culture, seeds were sown (3–5 mm depth) in plastic trays filled with moist vermiculite. Trays were covered with a transparent plastic lid with small holes, and mist sprayed regularly to maintain adequate moisture until seed germination. The vermiculite cultures allowed for further observation of

seedling establishment and survival. After cotyledon emergence, vermiculite cultures were watered three times a week with tap water for four weeks for further seedling development.

4.2.1.1.4 Growth conditions

All seed cultures were kept in a growth room under controlled environmental conditions at 24°C±2°C with a 16 hour photoperiod at 150–200 µmol m^{-2} s^{-1} provided by a mixture of cool-white (Phillips and Osram) and Gro-lux (Sylvania) fluorescent tubes. The relative humidity varied from 50–80% in a 24 hour cycle.

4.2.1.1.5 Experimental design

In the first scarification experiment there were 14 treatments [three concentrations (98%, 50%, 25%) of H_2SO_4 and four scarification time periods (10, 20, 30, 40 minutes), dry seeds and seeds imbibed for 40 minutes in distilled water] with three replicates (the three seed batches).

The second scarification experiment consisted of two trials which will further be referred to as trial A and trial B. Both trials had ten treatments [scarification with 98% H_2SO_4 for 5, 10, 15, 20, 25, 30, 35, and 40 minutes as well as two controls (dry seeds and seeds imbibed in water)]. For trial A, there were three replicates using the three seed batches, whereas trial B had four replicates. Three of the replicates were set up as in trial A and the fourth replicate consisted of a mixture of seeds taken randomly in equal numbers from the three seed batches.

For both the first and second scarification experiments, the experimental unit was 10 seeds per bottle for filter paper bridge cultures and 10 seeds per tray for vermiculite cultures. Both types of seed cultures were arranged in a randomised complete block design (RCBD) in the growth room.

4.2.1.1.6 Data collection and analysis

Successful seed germination on filter paper bridge cultures was considered as protrusion of the radicle (1–2 mm) through the seed coat (Figure 4.1A). The emergence of the two cotyledons above the vermiculite (Figure 4.1B) was considered as germination for this culture and is further referred to as emergence

(Hartmann *et al.*, 2011). Germination of seeds from all scarification treatments in both cultures and seedling survival in vermiculite cultures were recorded daily for 28 days. Microbial contamination of seeds on filter paper cultures was noted. Seedling survival in vermiculite cultures was also recorded after 28 days.

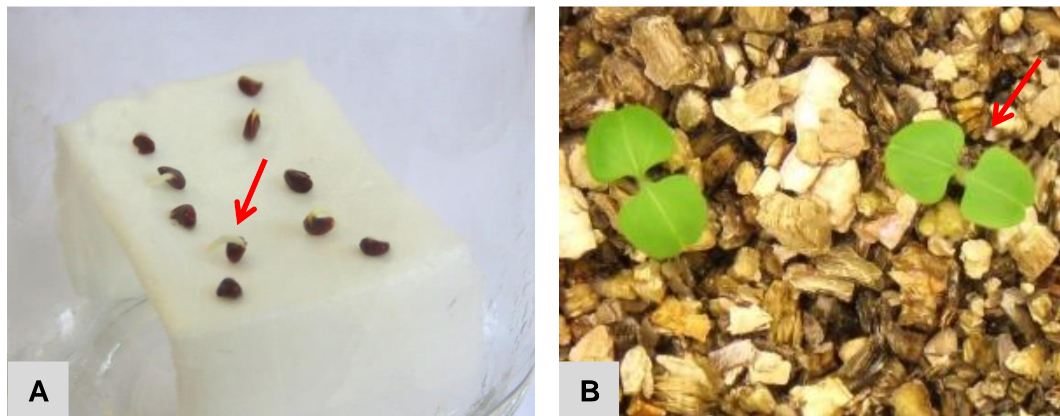

Figure 4.1. Seed germination in different types of seed cultures. (A) Radicle protrusion on filter paper bridges; (B) Emergence of cotyledons in moist vermiculite.

Germination responses of both seed cultures were evaluated by assessing the following germination parameters: final germination (%) (FGP) and final emergence (%) (FEP), mean germination time (MGT) and mean emergence time (MET) as well as germination rate index (GRI) and emergence rate index (ERI).

i. Final germination (%) (FGP) and Final emergence (%) (FEP) (Rehman *et al.*, 2014):

$$FGP\ or\ FEP = \frac{No\ of\ seeds\ that\ germinated/emerged}{Total\ no\ of\ seeds} x100$$

ii. Mean germination time (MGT) and Mean emergence time (MET):

$$MGT\ or\ MET = \frac{\sum Dn}{\sum n}$$

where n is the number of seeds with protruded radicle or that emerged on day D and day D is the number of days counted from the beginning of germination (Ellis and Roberts, 1981).

iii. Germination rate index (GRI) and Emergence rate index (ERI):

$$GRI\ or\ ERI = \frac{\#of\ seeds\ with\ radicle\ or\ emerged\ seedling}{Days\ of\ first\ count} \pm - - \mp \frac{\#of\ seeds\ with\ radicle\ or\ emerged\ seedling}{Days\ of\ final\ count}$$

(Al-Mudaris, 1998; Ranal and De Santana, 2006)

The T_{50} values [time in days taken to reach 50% seed germination (GT_{50}) and seedling emergence (ET_{50}) based on the total seed population] were also

determined. The SAS/NLIN (non-linear procedure) and the Gauss-Newton algorithm was used to fit the non-intercept sigmoid function, as described in TableCurve® 2D (2002), on the cumulative germination and emergence percentage to determine the time to 50% germination and emergence (T_{50}) (Jami Al-Ahmadi and Kafi, 2007):

$$y = \frac{a}{1 + e^{\left(-\frac{x-b}{c}\right)}}$$

where *a* is the maximum germination and emergence percentage, *b* is the turning point, *c* is slope of the line, *x* is the time, and *y* is the germination and emergence %. GT_{50} and ET_{50} means were calculated and subjected to an appropriate analysis of variance (ANOVA).

Data for the various H_2SO_4 treatments were analysed with the GenStat64-bit Release 17.1 (PC/Windows 7) statistical package (VSN International, 2014). An analysis of variance (ANOVA) was used to test for treatment effects. Fisher's protected least significant difference (LSD) test, at the 5% level of significance, was performed to distinguish between different means (Williams and Abdi, 2010). Data for the duration of scarification were analysed with the SAS® Version 9.3 statistical software (SAS Institute, 2011). The data was first tested for homogeneity of variances using Levene's test (Levene, 1960), where after it was subjected to analysis of variance (ANOVA) using the linear models procedure (PROC GLM). The means of the treatments were separated with the T test (LSD) at the 5% level of significance (Snedecor and Cochran, 1989). The Shapiro-Wilk's test was performed on the standardised residuals to test for deviations from normality (Shapiro and Wilk, 1965).

Based on the two scarification experiments, scarification with 98% H_2SO_4 for 30 minutes was selected for all further *in vivo* and *in vitro* seed germination experiments.

4.2.1.2 Temperature

4.2.1.2.1 Plant material

Seedlings from the scarification experiments grown in vermiculite were transplanted to pots containing a mixture of potting soil, vermiculite and sand [5:3:1(v/v/v)]. The plants were maintained in a greenhouse for seed production due to the limited

availability of seeds in nature. Flowers formed after five to six months and were hand-pollinated. Seeds were removed from the ripe fruit capsules and stored in paper envelopes at ambient temperature. A pilot study on germination of these seeds, after scarification with 98% H_2SO_4 for 30 minutes, showed the same germination responses as found when seeds from nature were used for germination. Therefore, the seeds from cultivated plants were used for all further *in vivo* and *in vitro* seed germination experiments.

4.2.1.2.2 Temperature treatments

Scarified seeds from cultivated plants were inoculated on filter paper bridges and moist vermiculite as described in 4.2.1.1.3. Both types of seed cultures were kept in Labcon growth chambers (in darkness) at varying temperatures from 15°C to 35°C with 5°C increments according to the experimental design. The vermiculite cultures were kept moist by mist spraying.

4.2.1.2.3 Experimental design

The experimental design was a 5 x 5 Latin square with five temperature treatments (15°C, 20°C, 25°C, 30°C, and 35°C) replicated five times. Replicates were thus conducted consecutively to allow for changing the temperature of a specific cabinet according to the Latin square design. The experimental unit was 30 seeds per treatment for both filter paper and vermiculite seed cultures.

4.2.1.2.4 Data collection and analysis

Successful seed germination was considered as radicle protrusion and cotyledon emergence as described in 4.2.1.1.6 and was recorded on a daily basis (every 6 hours) for 14 days. Different germination indices namely FGP and FEP, MGT and MET (in hours), GRI and ERI, and GT_{50} and ET_{50} values (in hours) for the different treatments were also determined as described in 4.2.1.1.6 above.

The data obtained were subjected to an analysis of variance (ANOVA) using the linear models (PROC GLM) procedure in the SAS® Version 9.3 software package (SAS Institute, 2011). The means of the treatments were separated with the T test (LSD) at the 5% level of significance (Snedecor and Cochran, 1989). The Shapiro-

Wilk's test was performed on the standardised residuals to test for deviations from normality (Shapiro and Wilk, 1965).

4.2.2 Factors affecting growth and development of seedlings and plants

4.2.2.1 Seedlings of *H. coddii* subsp. *barnardii*

Pilot trials were conducted to observe the effect of culture media, nutrients and growth conditions on seedling development over a period of 12 weeks.

4.2.2.1.1 Culture medium

a. Vermiculite

Scarified (98% H_2SO_4 for 30 minutes) seeds were germinated in plastic seedling trays with removable plugs filled with moist vermiculite which were covered with transparent plastic bags until seedling emergence. Vermiculite was mist-sprayed with water to maintain adequate moisture for seed germination. After five weeks the seedlings were transplanted to pots filled with a potting mix [potting soil, vermiculite and sand 5:3:1 (v/v/v)].

b. Commercial seedling mix

Plastic seedling trays (with six compartments) were filled with moist commercial (Culterra®) seedling mix that is made from well-composted selected raw materials including coco peat to improve water retention. It also contains essential elements such as K, Ca and Mg, which according to the provider's instructions, should provide sufficient nutrients to seedlings for up to 3 months. Scarified seeds were placed on the surface of the medium and covered with a 3–5 mm layer of moist vermiculite. The trays were covered with transparent plastic bags until seedling emergence and the medium was mist-sprayed to maintain adequate moisture for seed germination.

4.2.2.1.2 Growth conditions

The vermiculite seed cultures (50 seeds) were kept under controlled environmental conditions in a growth room as described in Section 4.2.1.1.4. The seedling mix cultures were placed in different environmental conditions. One group of cultures (36 seeds) was kept in a growth room under controlled environmental conditions (Section 4.2.1.1.4) and the other group (36 seeds) in a greenhouse (Section 4.2.2.2.1) under uncontrolled environmental conditions. The average minimum

temperature in the greenhouse ranged between 10°C–16°C (lowest 8°C; highest 20°C) and the average maximum temperature between 24°C–27.9°C (lowest 20°C; highest 33°C).

4.2.2.1.3 Nutrients

Seedlings from the vermiculite culture transferred to pots at five weeks were watered three times a week with tap water and once per week with nutrient solution (Culterra® Multisol 'N', Table 4.1). Emerged seedlings in the seedling mix medium were subjected to two nutrient regimes. Half (18) of the seedlings were only given tap water three to four times per week. The other half (18) were given tap water two to three times per week and a nutrient solution [Nulandis® Dr Fisher's Multifeed Classic 19:8:16 (43), Table 4.1] once per week. The amount of nutrient solution per seedling gradually increased [5 ml (0.0125 g)–50 ml (0.125 g)] over the period of 12 weeks.

4.2.2.1.4 Data collection

Visual observations and photographs of seedlings from all treatments were collected over a period of 12 weeks to evaluate seedling performance.

4.2.2.2 Plants of *H. coddii* subsp. *barnardii*

4.2.2.2.1 Effect of apex removal on biometric parameters and flowering

a. <u>Plant material and treatments</u>

Five month old plants (56) grown from seedlings and at the vegetative stage were randomly selected and further used to evaluate the effect of apex removal on axillary shoot induction (branching) and flowering. The effect of different nutrient regimes on plant growth was also evaluated.

Half of the plants (28) were kept in a growth room under controlled environmental conditions as described above (Section 4.2.1.1.4), while the other half (28) were kept in a greenhouse under uncontrolled environmental conditions. The greenhouse is an aluminium structure with glass panels on the side and roof louvres that could be opened or closed depending on the light and temperature requirements. The section on the northern side of the greenhouse was used for the experiment and the temperature and relative humidity were measured with a Huato S100 data logger for

the duration of the experiment (Appendix B: Table 4). The mean measurements were as follows: minimum temperature 17.5°C, maximum temperature 30°C, minimum relative humidity 39% and maximum relative humidity 84%. The light intensity in the greenhouse varied depending on the season and the time of day. The two places where the plants were grown are further referred to as location growth room and location greenhouse (Figure 4.2).

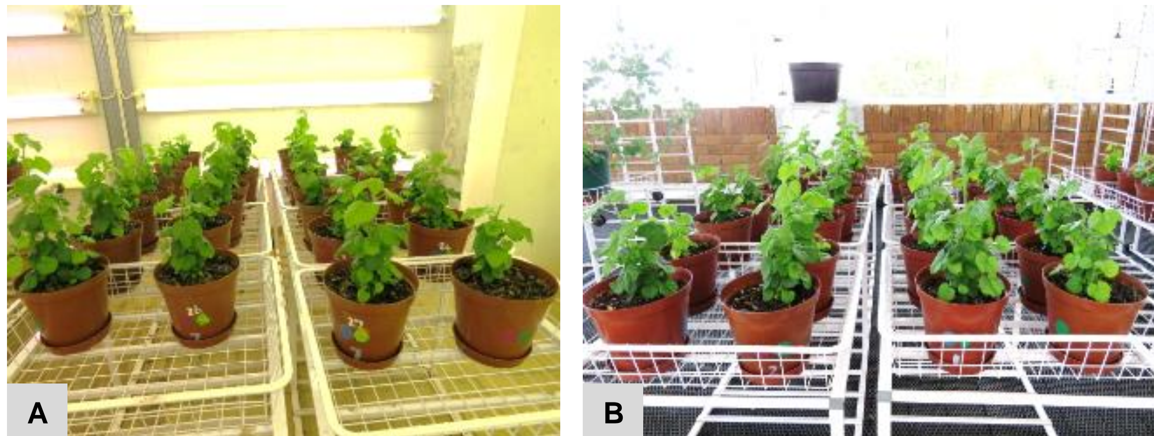

Figure 4.2. Examples of five-month-old *H. coddii* subsp. *barnardii* plants in different locations. (A) Growth room; (B) Greenhouse.

The apex, including the apical bud and 1–2 nodes below it, of half (14) of the plants in each location was removed [minus apex (-A)] with secateurs to induce axillary shoot proliferation. The other half (14) of the plants were left with the apex intact [with apex (+A)]. All the plants were watered three times a week with tap water (100 ml per pot). In addition, half of the plants with an apex (7) and minus an apex (7) were provided with water-soluble nutrients (Table 4.1) which were applied as a soil drench once per week. The two commercial nutrients, Nulandis® Dr Fisher's Multifeed Classic [19(N):8(P):16(K) (43), 2.5 g L^{-1} (w/v)] and Culterra Multisol 'N' [6(N):1(P):3(K) (44), 1 g L^{-1} (w/v)], were alternated weekly to balance the nutrients. Each plant received 100 ml of nutrient solution for the first four weeks of the experiment and 200 ml of solution for the last four weeks. The other half of the plants with an apex (7 plants) and minus an apex (7 plants) that did not receive the nutrient solution were watered with tap water at the same time and in equal amounts, as those that received the nutrient solution.

Table 4.1. Composition of two commercial nutrients used for the experiment.

Active ingredients	Nulandis® Dr Fisher's Multifeed Classic ($g\ kg^{-1}$)	Culterra Multisol 'N' ($g\ kg^{-1}$)
Nitrogen (N)	193	264
Phosphorus (P)	83	43
Potassium (K)	158	133
Sulphur (S)	6.1	–
Magnesium (Mg)	4.6	0.9
Zinc (Zn)	0.7	0.350
Boron (B)	1.054	1.005
Molybdenum (Mo)	0.063	–
Iron (Fe)	0.751	0.763
Manganese (Mn)	0.273	0.310
Copper (Cu)	0.075	0.077

b. Experimental design

The experiment was a RCBD with four treatments [(+A+N), (+A-N), (-A+N), and (-A-N)] (Table 4.3) replicated seven times. One plant in a pot formed the experimental unit. The whole experiment was repeated twice.

Table 4.2. Various treatments applied to five-month-old *H. coddii* subsp. *barnardii* plants under controlled and uncontrolled environmental conditions.

Treatment	Legend
+A+N	Plants with intact apex that received nutrients
+A-N	Plants with intact apex where nutrients were withheld
-A+N	Plants minus apex that received nutrients
-A-N	Plants minus apex where nutrients were withheld

c. Data collection and analysis

The following biometric parameters were recorded for all plants in both locations: plant height, number of nodes and basal diameter of the main shoot and the number of sprouted axillary shoots (10–80 mm and ≥90 mm in length). The plant height was taken from the cotyledon position to the top of the main shoot. In plants with a removed apex, the axillary shoot that sprouted closest to the end of the main shoot was stretched and the height ("stretched length") was measured from the cotyledon

position to the apex of this axillary shoot (Cornelissen *et al.*, 2003). The plant height (mm) was measured with a ruler. The number of nodes on the main shoot was counted from the cotyledon position to the end of the main shoot excluding the apical bud. The basal shoot diameter (mm) was measured at the cotyledon position or cotyledon scar with a digital vernier calliper (Mexal and Landis, 1990). All measurements were taken at the beginning of the experiment (week 0) and then again every second week until the end of the experiment (8 weeks). The presence of flower buds and flowers on all plants were also recorded according to the qualitative seven class descriptive ranking scale presented in table 4.3.

Table 4.3. Descriptive ranking scale used to indicate the presence or absence of flower buds and flowers on the apex of the main shoot and on axillary shoots.

Abbreviation	Legend
NFBF	No flower buds or flowers on the plant.
FBAp	Flower buds at the apex of the main shoot (in plants with intact apex).
FBFAp	Flower buds and flowers at the apex of the main shoot (in plants with intact apex).
FBFApFBAs	Flower buds and flowers at the apex of main shoot and flower buds and/or flowers on the lower axillary shoots (in plants with intact apex).
FBAs	Flower buds on the axillary shoots (in plants with removed apex).
FBFAs	Flower buds and flowers on the axillary shoots (in plants with removed apex).
FBFApNN	Flower buds and/or flowers at the apex of main shoot; no new buds are formed. (in plants with intact apex).

The collected data were analysed with the GenStat64-bit Release 18.2 (PC/Windows 8) statistical package (VSN International, 2016). An analysis of variance (ANOVA) was used to test for treatment and location effects on the variables. Fisher's unprotected least significant difference (LSD) test at the 5% level of significance was performed to distinguish between different means (Williams and Abdi, 2010). Flower data were analysed with the SAS® Version 9.3 statistical software (SAS Institute, 2011). The frequency procedure was used to test for associations (patterns) between different variables. The Pearson's chi-square test (Cochran, 1952) was performed to test the relationship between the categorical variables (location and treatment) and the distribution of flower frequencies. In case of significant evidence, graphs were constructed to demonstrate differences in patterns.

4.3 Results and Discussion

4.3.1 Effect of chemical scarification on seed germination

4.3.1.1 Effect of scarification with various sulfuric acid concentrations on seed germination and seedling emergence

Scarification of *H. coddii* subsp. *barnardii* seeds with various H_2SO_4 concentrations for different durations caused highly significant differences in the final germination (%) (FGP) and final emergence (%) (FEP) and the germination rate index (GRI) and emergence rate index (ERI). Mean germination time (MGT) and mean emergence time (MET) and contamination percentage (CP) were not affected by the scarification treatments. In the vermiculite cultures, the appearance of seedlings with first leaves and the survival of seedlings were significantly affected by the treatments (Appendix B: Table 1).

Scarification with 98% H_2SO_4 significantly enhanced seed germination (FGP of 73.3%–80%) and seedling emergence (FEP of 70–90%), although no significant differences were observed between the different scarification durations (10, 20, 30, and 40 minutes) of the 98% H_2SO_4 treatments. Scarification with lower concentrations of H_2SO_4 (50% and 25%) resulted in either poor (≤10%) or no germination in both seed cultures. Similar poor responses (≤4% germination) were observed in the non-scarified seeds (Table 4.4). Comparable germination responses were reported for various *Hibiscus* species. Kak *et al*. (2015) reported no germination in non-scarified seeds of five *Hibiscus* species (*H. calyphyllus*, *H. lobatus*, *H. radiatus*, *H. surattensis* and *H. vitifolius*). Furthermore, they found that scarification with 50% H_2SO_4 for 5 and 10 minutes was either not effective in uplifting seed dormancy or resulted in low (<30%) FGPs. In contrast, scarification with concentrated H_2SO_4 increased the FGP up to 90% in some of the *Hibiscus* species. Similar significant improvements of germination percentages of seeds scarified with 98% H_2SO_4 compared to non-scarified seeds were also reported for *H. acetosella* and *H. dasycalyx* (Sakhanokho, 2009) and *H. hamabo* (Seo *et al*., 2012).

Table 4.4 Seed germination and seedling emergence indices of *H. coddii* subsp. *barnardii* seeds scarified with various concentrations of sulfuric acid for various time durations after 28 days of incubation.

Treatment[1]	Germination on filter paper FGP (%)	MGT (days)	GRI (No. of seeds/day)	CP (%)	Emergence in vermiculite FEP (%)	MET (days)	ERI (No. of seedlings/day)	SS (%)
C98D10	80.00[a]	1.68	5.61[a]	3.33	70.00[a]	4.24	1.69[b]	96.67[a]
C98D20	76.67[a]	1.78	5.66[a]	0.00	83.33[a]	3.58	2.37[a]	96.33[a]
C98D30	76.67[a]	1.53	6.11[a]	3.33	76.67[a]	3.33	2.30[a]	92.67[a]
C98D40	73.33[a]	1.56	5.50[a]	10.00	90.00[a]	4.12	2.47[a]	93.00[a]
C50D10	3.33[b]	6.33	0.02[b]	0.00	3.33[b]	1.33	0.08[c]	33.33[bc]
C50D20	6.67[b]	6.67	0.03[b]	6.67	3.33[b]	1.33	0.08[c]	0.00[c]
C50D30	6.67[b]	8.50	0.03[b]	0.00	0.00[b]	0.00	0.00[c]	0.00[c]
C50D40	6.67[b]	18.67	0.03[b]	3.33	3.33[b]	7.00	0.02[c]	33.33[bc]
C25D10	0.00[b]	0.00	0.00[b]	3.33	6.67[b]	7.00	0.06[c]	66.67[ab]
C25D20	0.00[b]	0.00	0.00[b]	20.00	3.33[b]	8.00	0.01[c]	0.00[c]
C25D30	0.00[b]	0.00	0.00[b]	16.70	3.33[b]	1.33	0.08[c]	0.00[c]
C25D40	0.00[b]	0.00	0.00[b]	3.30	0.00[b]	0.00	0.00[c]	0.00[c]
Control Dry	0.00[b]	0.00	0.00[b]	0.00	3.33[b]	8.67	0.01[c]	33.33[bc]
Control Imb	3.33[b]	7.33	0.02[b]	0.00	0.00[b]	0.00	0.00[c]	0.00[c]
F pr.	<.001	0.235	<.001	0.418	<.001	0.806	<.001	<.001
LSD	23.17	–	2.31	–	20.28	–	0.51	54.01

[1]C: H_2SO_4 concentration, D: duration of treatment (minutes).
FGP: final germination (%), FEP: final emergence (%), MGT: mean germination time, MET: mean emergence time, GRI: germination rate index, ERI: emergence rate index, CP: contamination percentage, SS: seedling survival (%). Means with the same letter within a column are not significantly different at the 5% level of significance.

Other indices such as MGT and MET are used to measure speed and spread of germination (Hartmann *et al.* 2011). The MGT on filter paper for *H. coddii* subsp. *barnardii* seeds scarified with 98% H_2SO_4 was 1.53–1.78 days, whereas the MGT for seeds scarified with 50% H_2SO_4 was 3–9 times longer depending on the duration of the treatment. The MET in vermiculite of seeds scarified with 98% H_2SO_4 was 3.33–4.24 days. Although some of the treatments with lower concentrations of H_2SO_4 had three times faster METs, these treatments resulted in poor FEPs of 3.3%–6.7% (Table 4.4). According to Demir *et al.* (2008), the mean emergence time of seedlings will determine the size of seedlings and when germination is spread out over time (high MET) it will lead to smaller and non-uniform seedlings. Scarification of *H. coddii* subsp. *barnardii* seeds with 98% H_2SO_4 resulted in high FEPs and short METs and ensured uniform seedlings of the same size and age which is desirable in commercial production of seedlings (Demir *et al.*, 2008; Hartmann *et al.*, 2011).

Scarification of seeds with H_2SO_4 also improved the speed of germination. The highest GRI (5.50–6.11 seeds/day) and ERI (1.69–2.47 seedlings/day) were attained for seeds scarified with 98% H_2SO_4 that were significantly higher than these indices for 50% and 25% H_2SO_4. The duration of scarification with 98% H_2SO_4 did not affect the GRI on filter paper cultures, while in vermiculite cultures a significantly lower ERI was obtained with 10 minutes of scarification (Table 4.4).

Scarification of seeds of *H. coddii* subsp. *barnardii* with 98% H_2SO_4 significantly improved the germination in both cultures, irrespective of the duration of scarification. In contrast, lower concentrations of H_2SO_4 (50% and 25%) and lack of scarification, negatively affected the germination (Table 4.4). This suggests that concentrated H_2SO_4 can be used to soften the hard seed coat of *H. coddii* subsp. *barnardii* seeds and uplift the physical dormancy, and thus promote seed germination. It is generally proposed that chemical scarification improves the water permeability of hard seeds due to changes caused in the water gap structure or by removing the hilum cap depending on the plant species (Baskin *et al*., 2000; Alderete-Chávez *et al*., 2011; Seo *et al*., 2012). Scarification with 98% H_2SO_4 also contributed to the complete removal of the numerous soft cotton-like hairs (Figure 4.3C) that cover the hard seeds of *H. coddii* subsp. *barnardii*, while with lower concentrations of H_2SO_4 (Figure 4.3A & B), and in the controls (Figure 4.3D & E), only partial or no removal of the hairs was observed. Low (≤20%) and erratic fungal contamination was observed on seeds germinated on filter paper cultures (Table 4.4). The variation is most probably incidental and might be ascribed to the use of seeds collected from plants in nature. However, it is important to restrict contamination of cultures. Removal of the hairs from the *H. coddii* subsp. *barnardii* seeds with H_2SO_4 could be beneficial for prevention of contamination in seed cultures since the hairs might harbour micro-organisms. Seeds collected from cultivated plants showed no contamination of filter paper seed cultures.

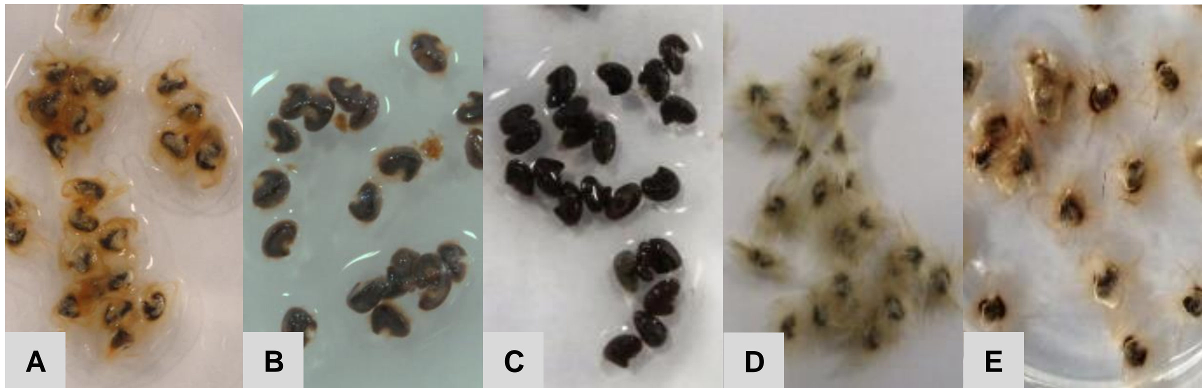

Figure 4.3. (A–C) Seeds scarified with different concentrations of sulfuric acid for 30 minutes before rinsing with water. (A) 25% H_2SO_4; (B) 50% H_2SO_4; (C) 98% H_2SO_4; (D) Dry seeds (control); (E) Seeds imbibed in distilled water for 40 minutes (control).

Seedlings that developed from the scarification treatments had two cotyledons and a normal appearance (Figure 4.4). In vermiculite cultures, seedlings derived from seeds scarified with 98% H_2SO_4 (all durations of scarification) had the highest survival percentage after four weeks in culture as compared to the other scarification treatments and the controls (Table 4.4). The first true leaf was visible on seedlings from all 98% H_2SO_4 scarification treatments 12–14 days after planting the seeds and on day 18 to 20 most of the seedlings (90–100%) from these treatments had at least one true leaf (Figure 4.4).

Figure 4.4. Appearance of first true leaf (arrow) on emerged seedling of *H. coddii* subsp. *barnardii* 12–14 days after seed planting.

Based on the above results, scarification with 98% H_2SO_4 was selected to define the optimum duration of scarification on filter paper and in vermiculite cultures.

4.3.1.2 Effect of various scarification durations with 98% sulfuric acid on seed germination and seedling emergence

Both seed germination (FGP, GRI, GT_{50}) and seedling emergence (FEP, ERI, ET_{50}) indices were significantly affected by the duration of chemical scarification, whereas no significant differences were observed for the MGT and the MET (Appendix B: Table 2). The two trials (repeat experiments) were also compared and the trial only affected the GT_{50} on filter paper at day 28. Significant interactions between the trials and treatments were only observed for the GT_{50} values on filter paper and the mean emergence time (MET) in vermiculite. (Appendix B: Table 2).

Scarification of seeds in 98% H_2SO_4 for 10 to 40 minutes resulted in significantly higher FGPs (77.1–88.6%) as compared to the 5 minute scarification duration (52.9%) and the non-scarified seeds (<5%). A similar trend was observed for the FEP (67.1–80%) of seeds in vermiculite cultures (Table 4.5). Likewise, scarification of *H. dasycalyx* seeds with 98% H_2SO_4 for 20–25 minutes improved germination up to 70% as compared to 19% in seeds scarified for 5 minutes, and 15% in non-scarified seeds (Sakhanokho, 2009). Chachalis *et al.* (2008) also reported 30 minutes as the optimum time for scarification with 97% H_2SO_4 (>90% FGP) for seeds of *H. trionum* without reduction in seed germination and viability. Scarification of seeds of several *Hibiscus* species with concentrated H_2SO_4 for 10–30 minutes was also effective in uplifting the physical dormancy and enhancing seed germination, although the optimum scarification duration differed between species (Kak *et al.*, 2015). Scarification of *H. tridactylites* seeds with 98% H_2SO_4 for 5–30 minutes increased the germination percentage compared to the control (13%). The highest germination (80%) was achieved with 20 minutes of scarification (Chauhan, 2016), whereas the highest FGP (88.6%) in *H. coddii* subsp. *barnardii* seeds was achieved with 30 minutes of scarification (Table 4.5).

Longer exposure (35 and 40 minutes) of *H. coddii* subsp. *barnardii* seeds to scarification resulted in the shortest MGT and MET, respectively (Table 4.5). Nonetheless, some of the seeds from these scarification treatments showed physical damage and disintegrated during the rinsing process. This shows that longer exposure to scarification could be detrimental for these seeds. Kak *et al.* (2015) also reported an increase in seed mortality for other *Hibiscus* species when the seeds

were scarified for periods longer than for which the maximum germination was achieved.

Table 4.5. Seed germination and seedling emergence indices of *H. coddii* subsp. *barnardii* seeds scarified with 98% sulfuric acid for various durations after 28 days of incubation.

Treatment	Germination on filter paper				Emergence in vermiculite			
	FGP (%)	MGT (days)	GRI (No. of seeds/day)	GT_{50} (days)	FEP (%)	MET (days)	ERI No. of seedlings/day	ET_{50} (days)
5 min	52.86[b]	5.41	1.60[c]	11.47[a]	48.57[b]	6.15	0.99[b]	5.39[a]
10 min	77.14[a]	3.18	3.45[b]	2.92[b]	67.14[ab]	4.43	1.97[a]	3.35[b]
15 min	81.43[a]	1.95	5.41[a]	1.51[bc]	68.57[a]	3.62	2.07[a]	2.99[b]
20 min	84.29[a]	2.07	5.82[a]	1.17[c]	80.00[a]	3.72	2.33[a]	3.20[b]
25 min	77.14[a]	1.66	5.64[a]	1.67[bc]	80.00[a]	3.60	2.50[a]	3.44[b]
30 min	88.57[a]	1.99	6.33[a]	1.77[bc]	80.00[a]	3.47	2.37[a]	3.03[b]
35 min	78.57[a]	1.46	6.17[a]	1.00[c]	78.57[a]	3.50	2.41[a]	3.19[b]
40 min	84.29[a]	3.01	5.68[a]	1.32[bc]	72.86[a]	3.37	2.24[a]	2.81[b]
Control Dry	2.86[c]	1.86	0.06[cd]	–	4.29[c]	2.14	0.06[c]	–
Control Imbibed	4.29[c]	5.00	0.03[d]	–	5.71[c]	9.43	0.06[c]	–
F pr.	<.0001	0.205	<.0001	<.0001	<.0001	0.054	<.0001	<.0001
LSD	15.12	–	1.54	1.64	19.34	–	0.66	0.78

FGP: final germination (%), MGT: mean germination time, GRI: germination rate index, GT_{50}: time to 50% germination, FEP: final emergence (%), MET: mean emergence time, ERI: emergence rate index, ET_{50}=time to 50% emergence. Means with the same letter within a column are not significantly different at the 5% level of significance.

Besides the MGT and MET indices, the time taken to reach 50% germination (T_{50}) is also used to indicate the speed of germination (Hartmann *et al.*, 2011, Nin *et al.*, 2017). The duration of scarification caused no significant difference in the MGT and MET in the seeds of *H. coddii* subsp. *barnardii*, but it did cause significant differences in the GT_{50} and ET_{50} (Table 4.5). For both cultures, a significantly longer time was required for seeds scarified for five minutes to reach 50% germination than the other scarification durations. With five minutes of scarification, the MGT was 5.41 days, although it took 11.47 days for the seeds to reach 50% germination (GT_{50}). This time was significantly longer than the time it took the seeds from all other scarification durations to reach 50% germination (Table 4.5). It is, therefore, also important to consider the T_{50} when analysing speed of germination. The number of seeds that germinated per day in both cultures (GRI and ERI) was also significantly higher when seeds were scarified for durations longer than five minutes (Table 4.5). Likewise, scarification of *Bauhinia divaricata* seeds with 98% H_2SO_4 for 20 minutes

resulted in a significantly lower T_{50} (6 days) than seeds scarified for 7 minutes (21 days). Unlike in *H. coddii* subsp. *barnardii*, 30 minutes of scarification significantly increased the T_{50} to 19 days (Alderete-Chávez *et al*., 2011). Based on the germination indices, scarification with 98% for 30 minutes was chosen as suitable treatment for uplifting physical dormancy in *H. coddii* subsp. *barnardii* seeds.

The cotyledons of *H. coddii* subsp. *barnardii* seeds from the scarification treatments started to appear above the vermiculite 3 to 4 days after planting, followed by normal seedling development. It took 12–14 days from planting the seed until the first true leaf of the seedling appeared regardless of the type of prior seed treatment. After 28 days, the different scarification treatments did not cause any significant differences between the percentages of seedlings with first real leaves, neither between the seedling survival percentages (>80%) (data not shown).

It is reported that the germination response may also depend on the type of seed culture. Scarified seeds (20 minutes in 97% H_2SO_4) of *H. hamabo* cultured on both filter paper and in a potting soil mix achieved almost similar germination percentages, 80% and 81% respectively (Seo *et al*., 2012). For *H. coddii* subsp. *barnardii*, a higher FGP (88%) and shorter MGT (1.99 days) were attained on filter paper cultures than in vermiculite cultures (80% FGP, 3.47 days MGT) (Table 4.5). Germination of seeds on filter paper bridge cultures in a closed system, as in this study, provided an aseptic environment and constant and even supply of moisture. Such cultures could be suitable for testing the germination capacity of seeds in a short time. Vermiculite cultures, on the other hand, proved to be suitable for further seedling establishment. In such cultures, appearance of the first true leaf of *H. coddii* subsp. *barnardii* seedlings was observed from the 12th day onwards and after 28 days the seedlings were well established with 4–5 leaves. Such seedlings were suitable for transplanting for further observation of plant development.

Comparison of the results of the two trials (A and B) showed only significant differences between the GT_{50} values. The other germination indices did not differ significantly between the trials (Appendix B: Table 2). Seeds from trial B took a significantly longer time (2.99 days) to reach 50% germination than the seeds from trial A (1.88 days) on filter paper, but not in vermiculite. The minimal significant

differences in germination parameters between the two trials could be indicative of good repeatability of results.

Table 4.6. Seed germination and seedling emergence indices for trial A and B.

Trial	Germination on filter paper				Emergence in vermiculite			
	FGP (%)	MGT (days)	GRI (#seeds/ day)	GT_{50} (days)	FEP (%)	MET (days)	GRI (#seedlings/ day)	ET_{50} (days)
A	64.00	2.30	3.94	1.88[b]	63.33	3.57	1.86	3.39
B	62.50	3.11	4.08	2.99[a]	55.00	4.92	1.58	3.25
F pr.	0.660	0.287	0.671	0.009	0.061	0.146	0.075	0.448
LSD	–	–	–	0.81	–	–	–	–

FGP: final germination (%), MGT: mean germination time, GRI: germination rate index, GT_{50}: time to 50% germination, FEP: final emergence (%), MET: mean emergence time, ERI: emergence rate index, ET_{50}: time to 50% emergence. Means with the same letter within a column are not significantly different at the 5% level of significance.

The only highly significant interaction between trial and treatment was observed for the GT_{50} values (Appendix B: Table 2). Scarification of seeds for 5 and 10 minutes caused significant differences in the time that it took seeds from trial A and trial B, respectively, to reach 50% germination (data not shown). Seeds from trial B scarified for 5 minutes took almost 7 times longer (20.25 days) to reach 50% germination than seeds from trial A (2.69 days). In contrast, seeds from trial A scarified for 10 minutes took almost double the time (4.20 days) to reach 50 % germination than the seeds of trial B (1.65 days). The discrepancy could possibly be explained by the short scarification time since it was not observed in seeds that were scarified for longer times (15–40 minutes). A significant interaction between trial and treatment was observed for the MET only (Appendix B: Table 2), but is not discussed here.

4.3.2 Effect of temperature on seed germination and seedling emergence

The breaking of seed dormancy must also coincide with favourable environmental conditions to ensure germination and subsequent survival of the seedling (Baskin *et al.*, 2000). Members of the Malvaceae family, including *Hibiscus* species, seem to favour higher temperatures for germination of seeds that are permeable to water (Carberry and Abrecht, 1990; Angelini *et al.*, 1998; Taghvaei and Nasrolahizadehi, 2016). The different temperatures (treatments) caused highly significant differences in all the seed germination and seedling emergence indices of *H. coddii* subsp. *barnardii*, except for the FGP (Appendix B: Table 3).

High FGPs (>92.67%) were obtained for *H. coddii* subsp. *barnardii* seeds germinated on filter paper cultures irrespective of the temperature. The highest FEP (94%) in vermiculite cultures was obtained for seeds germinated at 25°C which was significantly different from the FEP for all other temperatures, except 30°C (92%). However, no germination was observed in seeds in vermiculite subjected to the lower temperature of 15°C (Table 4.7). In corroboration, seeds of *H. sabdariffa* germinated at 30°C had the highest FGP (96%), but seeds kept at 10°C and 40°C reached only 50% and 69% germination respectively (Taghvaei and Nasrolahizadehi, 2016). Carberry and Abrecht (1990) reported no significant differences in final germination percentages for seeds of *H. cannabinus* cv. Guatemala-4 germinated at 15°C–35°C on filter paper and recorded a mean germination of 83% for these temperatures. Similarly, there were no significant differences in final germination percentages for *H. coddii* subsp. *barnardii* seeds germinated at 15°C–35°C where 95% mean FGP on filter paper cultures was attained for these temperatures (Table 4.7). In contrast, Chachalis *et al.* (2008) reported less than 35% germination in seeds of *H. trionum* on filter paper at 25–35°C and less than 20% germination at 15–20°C. Unlike these results, germination of *H. coddii* subsp. *barnardii* seeds at constant temperatures of 20°C–35°C resulted in high (>80%) FGPs and FEPs (Table 4.7).

The shortest MGT and MET, shortest GT_{50} and GE_{50} and highest GRI and ERI were attained when seeds were germinated at 25°C and 30°C which differed significantly from the indices for other tested temperatures (Table 4.7). Taghvaei and Nasrolahizadehi (2016) also reported the highest germinate rate (0.66 seeds/day) for *H. sabdariffa* seeds germinated at 30°C which was lower than the GRI (1.19 seeds/hour) for *H. coddii* subsp. *barnardii* seeds (Table 4.7) In contrast, Carberry and Abrecht (1990) found that the best germination response on filter paper for seeds of *H. cannabinus* cv. Guatemala-4 was attained at 35°C. Although seeds of *H. coddii* subsp. *barnardii* germinated at 15°C on filter paper cultures had a high FGP (96%), they had a longer MGT, GT_{50} and the lowest GRI (0.29 seeds per hour). Failure of the seedlings to emerge when seeds were kept at 15°C (Table 4.7) suggests that higher temperatures (>15°C) are required for germination of *H. coddii* subsp. *barnardii* seeds. Taghvaei and Nasrolahizadehi (2016) also reported significantly lower germination rates for *H. sabdariffa* seeds on filter paper when

germinated at 10°C (0.17 seeds/day) and 15°C (0.20 seeds/day). This was lower than the 0.29 seeds of *H. coddii* subsp. *barnardii* that germinated per hour at 15°C on filter paper (Table 4.7). In contrast, the best germination response (52.4%) and fastest ERI (6.1 seeds/day) for *Senecio douglasii* seeds in vermiculite was obtained at 20°C and a significantly lower response (34.8% and 2.5 seeds/day) was found at 30°C (Pivetta *et al*., 2013). Some members of the Malvaceae family also performed better at lower germination temperatures. The seed germination percentage was the highest (63.3%) when seeds of *Lavatera agrigentina* were incubated at 10°C, while higher temperatures (20°C and 25°C) resulted in significantly lower germination percentages, 35% and 5% respectively. This response could be due to the natural ecosystem conditions from where the seeds originated (Santo *et al*., 2015).

Table 4.7. Effect of temperature on seed germination and seedling emergence of *H. coddii* subsp. *barnardii*.

	Germination on filter paper				Emergence in vermiculite			
Treat-ment	**FGP (%)**	**MGT (hours)**	**GRI (# seeds /hour)**	**GT_{50} (hours)**	**FEP (%)**	**MET (hours)**	**ERI #seedlings /hour**	**ET_{50} (hours)**
15°C	96.00	108.11^{a}	0.29^{d}	103.79^{a}	0.00^{d}	0.00^{d}	0.00^{d}	–
20°C	96.00	39.91^{b}	0.80^{b}	34.51^{b}	88.00^{bc}	178.93^{a}	0.15^{c}	176.83^{a}
25°C	94.67	26.14^{b}	1.26^{a}	22.95^{b}	94.00^{a}	85.74^{bc}	0.35^{b}	82.31^{bc}
30°C	95.33	30.10^{b}	1.19^{a}	26.08^{b}	92.00^{ab}	64.90^{c}	0.46^{a}	60.92^{c}
35°C	92.67	84.86^{a}	0.51^{c}	82.43^{a}	83.33^{c}	99.97^{b}	0.33^{b}	103.48^{b}
F pr.	0.502	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
LSD	–	23.67	0.19	26.48	5.81	24.99	0.06	29.73

FGP: final germination (%), MGT: mean germination time, GRI: germination rate index, GT_{50}: time to 50% germination, FEP: final emergence (%), MET: mean emergence time, ERI: emergence rate index, ET_{50}: time to 50% emergence. Means with the same letter within a column are not significantly different at the 5% level of significance.

Results showed that the best germination indices for scarified seeds (98% H_2SO_4) on filter paper cultures were attained at 25°C (Table 4.7). In vermiculite cultures, seedling emergence indices (MET, ERI and ET_{50}) were the best at 30°C (Table 4.7), although this and higher temperatures (35°C) resulted in drying out of the hypocotyl and subsequent yellowing and death of the cotyledons (Figure 4.5B). In contrast, the cotyledons that emerged from seeds germinated at 25°C were green (Figure 4.5A). The results of this study showed that rapid and efficient germination of *H. coddii* subsp. *barnardii* seeds can be achieved by scarification with 98% H_2SO_4 for 30 minutes and germination at 25–30°C, although 25°C would be better for further seedling development.

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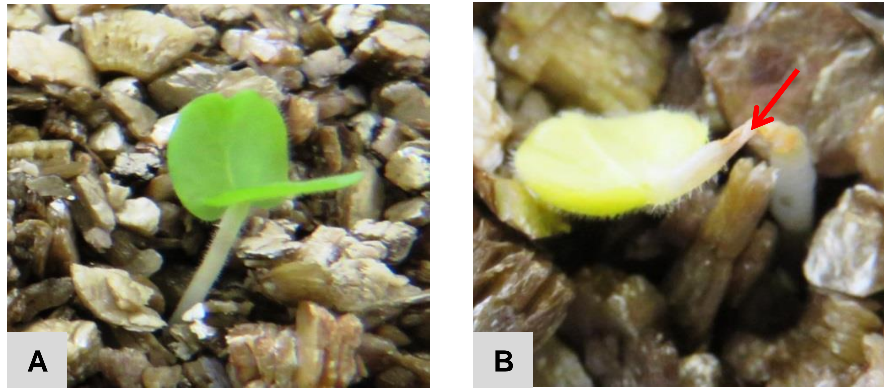

Figure 4.5. Effect of temperature on seedling emergence. (A) Normal healthy seedlings germinated at 25°C; (B) Hypocotyl of emerged seedlings dried out at 30°C and 35°C.

4.3.3 Factors affecting growth and development of seedlings and plants

4.3.3.1 Seedlings of *H. coddii* subsp. *barnardii*

The effect of culture medium, nutrients and growth conditions on seedling development was studied over a period of 12 weeks in order to define the best conditions for *in vivo* seedling production.

4.3.3.1.1 Effect of culture medium and nutrients

Chemically scarified seeds were used to grow seedlings on moist vermiculite and on Culterra® commercial seedling mix containing slow released nutrients under controlled (Section 4.2.1.1.4) and uncontrolled (Section 4.2.2.2.1) environmental conditions. Cotyledons in more than 80% of seedlings from both cultures started to emerge three days after sowing. Gairola *et al*. (2011) also reported a high percentage of seedling emergence of *Jatropha curcas* seeds in vermiculite (85%) and sand (83%) followed by perlite, coco-peat and garden soil ranging from 71% to 76%. Kaintura *et al*. (2016) reported that a mixture of sand, soil, coco peat and vermicompost [1:1:1:1 (v/v/v/v)] was the most suitable germination medium for *Angelia glauca* seeds.

The best growth performance for *H. coddii* subsp. *barnardii* seedlings was observed on Culterra® seedling mix supplemented with nutrients. Seedlings had 5–6 well-developed large green leaves (Figure 4.6A) and thicker roots (Figure 4.7A) after 4 weeks of culture. In contrast, seedlings grown on the same medium without additional nutrient supplementation had 3–4 smaller leaves with visible chlorosis symptoms (Figure 4.6B). This suggests that Culterra® seedling mix with slow

release nutrients (K, Ca and Mg) recommended for seedling growth up to 12 weeks was not sufficient to support healthy seedling growth of *H. coddii* subsp. *barnardii*. Additional nutrients were required for healthy growth. Seedlings grown on vermiculite (without nutrients) also showed similar symptoms (leaves with interveinal chlorosis and thin roots) (Fig 4.6C, Figure 4.7B) and died after 5–6 weeks. It is preferable that seedlings germinated in vermiculite should be transplanted to a potting mix at five weeks and fertilised with adequate nutrients to ensure further seedling and plant growth.

Both Culterra® seedling mix and vermiculite could be used for early stages of seedling growth if they are fertilised with sufficient mineral nutrients once per week after cotyledon emergence. The International *Hibiscus* Society (2004) also recommends a commercial seedling mix or sand, vermiculite, peat, and perlite or mixture of any of these for germination and seedling development of *H. rosa-sinensis*.

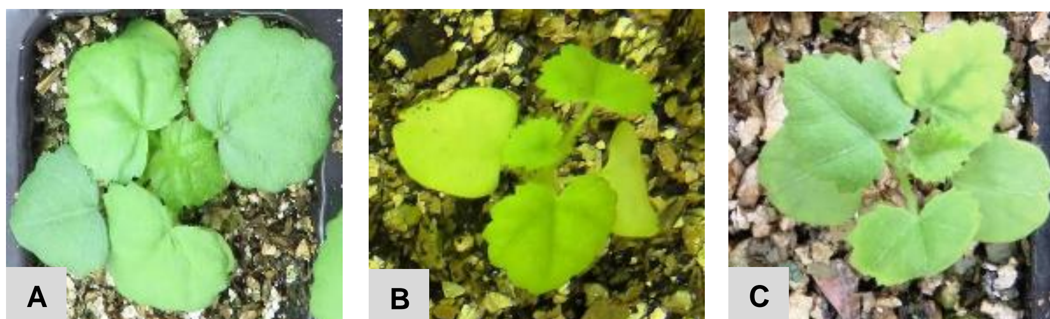

Figure 4.6. Examples of 4–5-week-old *H. coddii* subsp. *barnardii* seedlings grown in (A) Culterra® seedling mix with additional nutrients; (B) Culterra® seedling mix without additional nutrients; (C) Vermiculite.

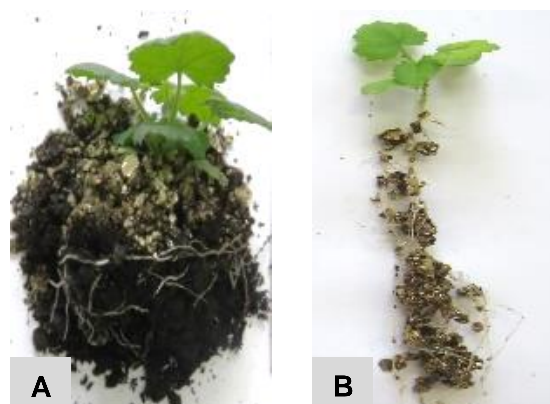

Figure 4.7. Examples of root formation on seedlings of *H. coddii* subsp. *barnardii* grown in (A) Culterra® seedling mix with additional nutrients; (B) Vermiculite.

4.3.3.1.2 Effect of growth conditions

The environmental growth conditions also had an effect on the development of seedlings grown in the seedling mix. At 8 weeks, seedlings grown under controlled conditions had thicker stems (average height 95 mm), larger leaves (Figure 4.8A) and a strong intact root system (Figure 4.8C). Thinner stems (average height 70 mm), smaller leaves and a weak root system (Figure 4.8E) were observed for seedlings in the greenhouse at this stage (Figure 4.8B). These differences could be due to daily and seasonal temperature and light variations in the greenhouse. After 12 weeks, well-developed seedlings with a strong intact root system were established under both controlled (Figure 4.8D) and uncontrolled (Figure 4.8F) environmental conditions. However, seedlings in the growth room (average height of 165 mm) had thicker stems, shorter internodes and a more compact appearance. Axillary shoots (5–6) proliferated at the base of seedlings (Figure 4.8D) in the growth room and a few seedlings (10%) had flower buds.

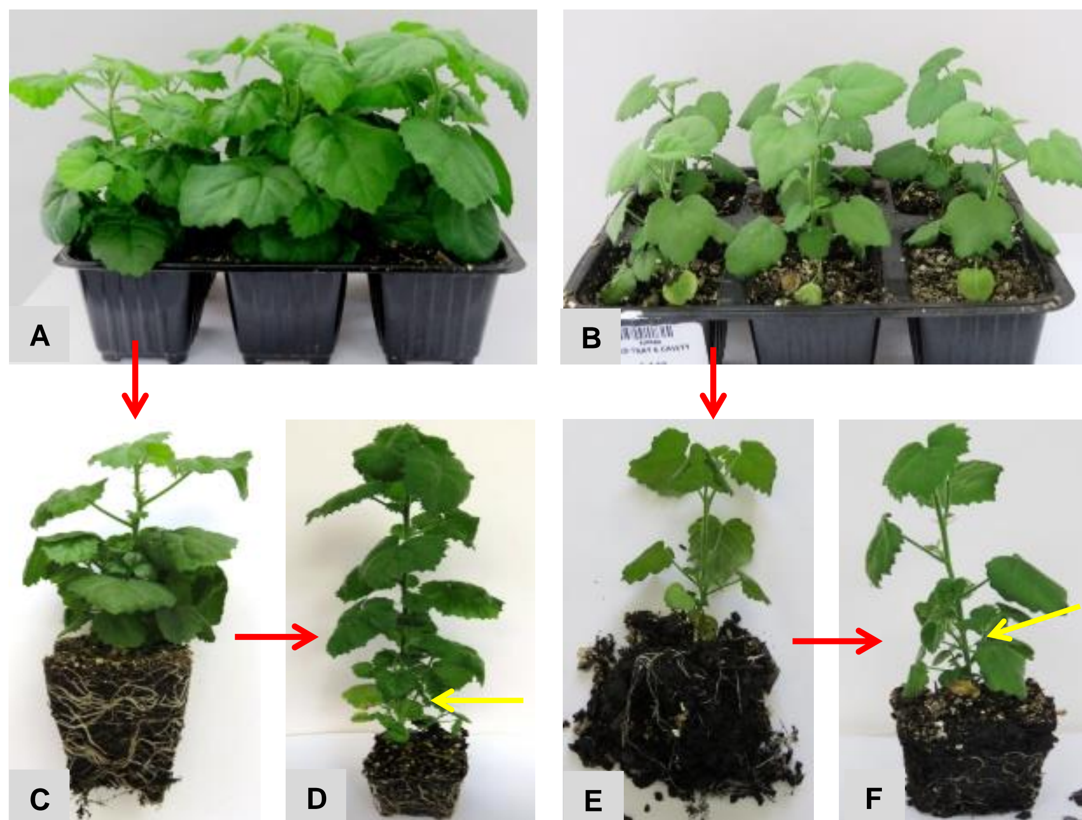

Figure 4.8. Seedlings of *H. coddii* subsp. *barnardii* developed under different growth conditions in Culterra® seedling mix with additional nutrients. Eight-week-old seedlings in (A) Growth room; (B) Greenhouse. Root system of eight-week-old seedlings in (C) Growth room; (E) Greenhouse. Twelve-week-old seedlings in (D) Growth room; (F) Greenhouse. Yellow arrows indicate proliferated axillary shoots.

Seedlings in the greenhouse had thinner stems (average height 110 mm), longer internodes and a more slender appearance with fewer (3–4) and shorter proliferated axillary shoots (Figure 4.8F). When seedlings are grown in an uncontrolled outside environment it should preferably be done during the warmer months of the year (October until March).

These results show that well-established seedlings of *H. coddii* subsp. *barnardii* could be produced in a seedling mix (with additional nutrients) under controlled and uncontrolled environmental conditions in 8–12 weeks. At this stage seedlings had a well-developed root system which will ease transplanting of seedlings to soil. Plants grown from these seedlings showed the same phenotypic characteristics as that of plants in nature.

4.3.3.2 Plants of *H. coddii* subsp. *barnardii*

4.3.3.2.1 Effect of apex removal and nutrients on biometric parameters

Cline (1997) described the ideal plant for studying the release of apical dominance as one that rapidly grows upward, has moderately strong apical dominance that will be released when the apical bud is removed, and internodes with axillary buds that are widely spaced from one another to make observations easier. Plants of *H. coddii* subsp. *barnardii* meet these requirements. Munir and Naz (2006) reported that removal of the apical bud will release the lower axillary buds from apical dominance and initiate outgrowth of axillary shoots.

In this study, five month old *H. coddii* subsp. *barnardii* plants at the vegetative stage were used to evaluate the removal of the apex and the effect of nutrients on axillary shoot induction (branching) and flowering. Reference to the different times (2, 4, 6 and 8 weeks) indicates the time after the plants were placed in the growth room and greenhouse respectively. The applied treatments (plus and minus apex and nutrients) caused significant differences in biometric parameters of plants grown in the different locations (Appendix B: Table 5 & 6).

Plant height

There was no significant difference in plant height, irrespective of the treatment and location when the experiment commenced. Thereafter, both plants with apex and

plants without apex (“stretched length”, Section 4.2.2.2.1) showed a gradual increase in height, although it was more pronounced in plants that received nutrients (+A+N). In both the growth room (Figure 4.9A) and the greenhouse (Figure 4.9B) the plants with apex and nutrients (+A+N) were significantly taller than plants from the other treatments after 8 weeks. Furthermore, such plants grown in the greenhouse were significantly taller (593 mm) than these plants (510.6 mm) in the growth room. This significant difference in height between plants in the growth room and in the greenhouse was also observed for the other three treatments (Figure 4.9A & B). In both locations, plants with apex and nutrients (+A+N) were also significantly taller than plants with apex and without nutrients (+A-N) (Figure 4.9A & B).

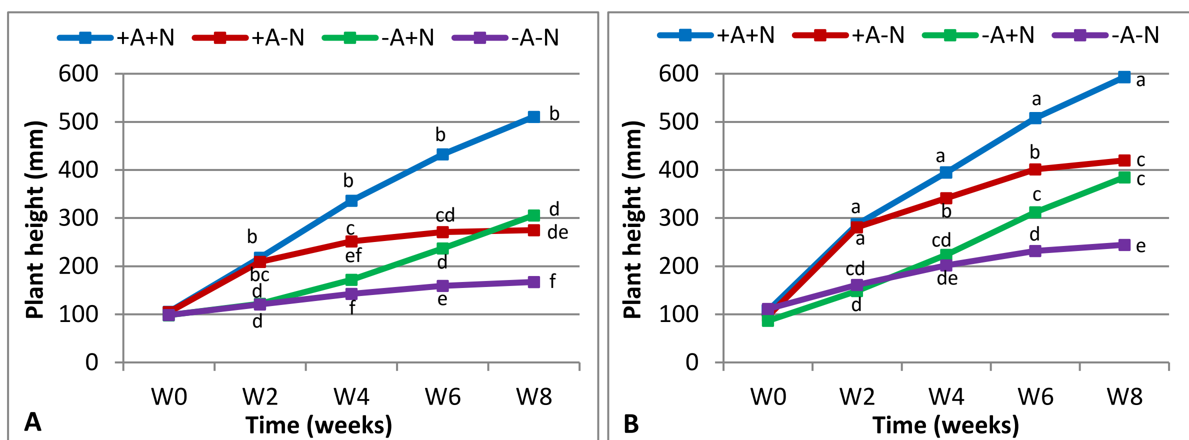

Figure 4.9. Height of *H. coddii* subsp. *barnardii* plants grown over a period of 8 weeks in (A) Growth room; (B) Greenhouse.

Values with the same letters within a graph (per week) and between the two graphs are not significantly different at the 5% level of significance. +A+N: Plants with intact apex that received nutrients; +A-N: Plants with intact apex where nutrients were withheld; -A+N: Plants minus apex that received nutrients; -A-N: Plants minus apex where nutrients were withheld.

The difference in height between locations could be due to daily and seasonal fluctuations in temperature and light in the greenhouse compared to the constant temperature and light intensity in the growth room. Mineral nutrients are essential for normal plant growth, such as increase in height, which might explain the highest increase in height in plants that received nutrients (Taiz *et al*., 2015).

Number of nodes

The significant difference in node number between plants with and without apex is probably due to the lack of further growth of the main stem after apex removal. Plants with an apex from both nutrient regimes showed a progressive increase in the

number of nodes, however, it was more distinct in plants that received nutrients. In both locations, plants with apex and nutrients (+A+N) had significantly more nodes than the plants with apex and without nutrients (+A-N) after 8 weeks (Figure 4.10A & B). Although plants in the growth room with nutrients (+A+N) were shorter in height (Figure 4.9A) than in the greenhouse they had significantly more nodes (Figure 4.10A) probably due to shorter internodes. In contrast, significantly more nodes were observed on plants without nutrients in the greenhouse (33.3 nodes) than on plants in the growth room (28.7 nodes) which could be due the longer height of plants in the greenhouse (Figure 4.9B).

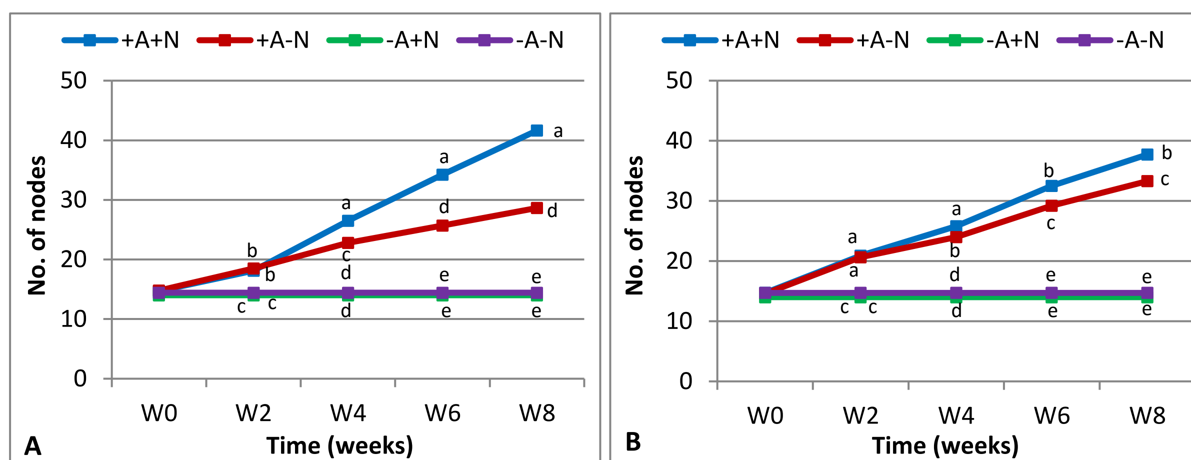

Figure 4.10. Number of nodes on main stem of *H. coddii* subsp. *barnardii* plants grown over a period of 8 weeks in (A) Growth room; (B) Greenhouse.

Values with the same letters within a graph (per week) and between the two graphs are not significantly different at the 5% level of significance. +A+N: Plants with intact apex that received nutrients; +A-N: Plants with intact apex where nutrients were withheld; -A+N: Plants minus apex that received nutrients; -A-N: Plants minus apex where nutrients were withheld.

Basal diameter

Plants from all four treatments showed a continuing increase in the basal diameter for the duration of the experiment in both the growth room and the greenhouse (Figure 4.11A & B). In both locations the increase was, however, significantly more in the plants that received nutrients than in plants without nutrients, irrespective of the status of the apex. Plants without an intact apex and with nutrients (-A+N) and grown in the growth room (Figure 4.11A) had significantly thicker stems (4.6 mm) than these plants in the greenhouse (4.2 mm) (Figure 4.11B). The thinner stems in plants that did not receive nutrients could be due to S deficiency (McCauley *et al*., 2011).

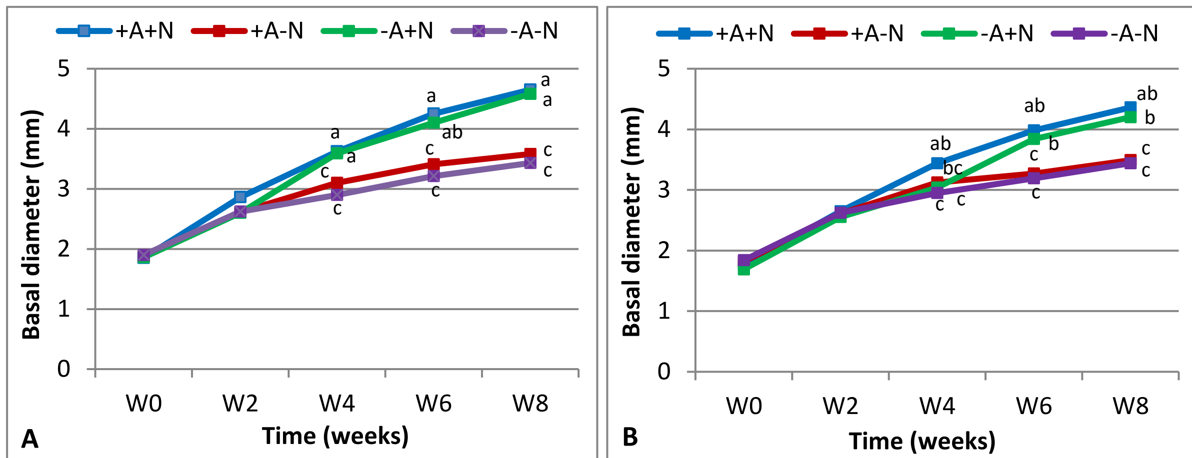

Figure 4.11. Basal diameter of the main stem of *H. coddii* subsp. *barnardii* plants grown over a period of 8 weeks in (A) Growth room; (B) Greenhouse.

Values with the same letters within a graph (per week) and between the two graphs are not significantly different at the 5% level of significance.+A+N: Plants with intact apex that received nutrients; +A-N: Plants with intact apex where nutrients were withheld; -A+N: Plants minus apex that received nutrients; -A-N: Plants minus apex where nutrients were withheld.

In both locations, the plants with an intact apex (+A+N and +A-N), irrespective of the nutrient regime, showed apical dominance and continued to grow in height while sprouting and subsequent outgrowth of the axillary shoots were delayed. Axillary buds would only sprout when there was a relative distance between the plant apex and the basal axillary buds, and or when flower buds (Figure 4.12B) started to appear on the apex. A cluster of short axillary shoots (Figure 4.12A) sprouted first at the base of these stems, while the apical bud continued with stem growth, so-called monopodial branching (Barthélémy and Caraglio, 2007). In plants with removed apex, the axillary buds started to sprout a few days after removal (Figure 4.12C) and two weeks after de-topping some of the proliferated shoots were already 90 mm or longer (Figure 4.12D).

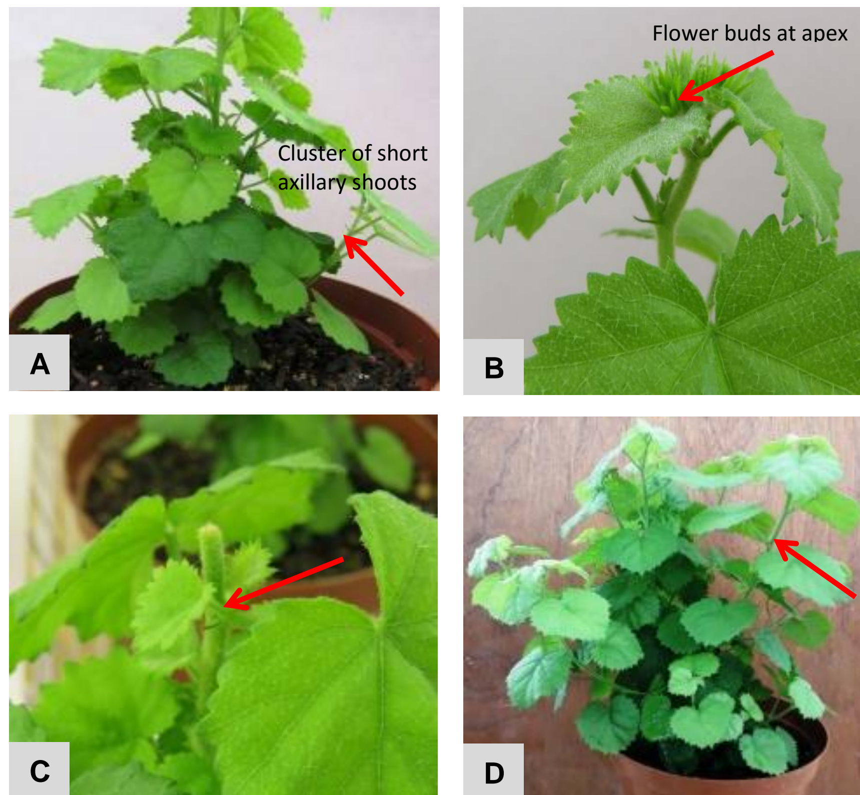

Figure 4.12. (A) Cluster of short axillary shoots (arrow) at the base of a plant with an intact apex (+A+N); (B) Flower buds (arrow) on a stem with an intact apex; (C) Axillary shoot sprouting (arrow) a few days after apex removal of the main stem (-A+N); (D) Numerous long axillary shoots (arrow) on a plant 4½ weeks after the apex was removed. All photographs from plants that received nutrients.

Auxin synthesised in the shoot apex, mainly by the young expanding leaves, is known to be transported basipetally where it supresses outgrowth of axillary buds. When the apical bud and thus the main site of auxin synthesis is removed, it is considered to facilitate axillary bud proliferation (Taiz *et al*., 2015; Hartmann *et al*., 2011; Teichmann and Muhr, 2015). It is also reported that proliferation of axillary shoots is not only affected by a decrease in the auxin concentration, but also by an increase in the cytokinin concentration. Cytokinin synthesised in the roots is transported upwards in the xylem and will transmit the reduced auxin concentration signal towards the dormant axillary buds facilitating bud outgrowth. Cytokinin thus acts antagonistically to auxin with respect to axillary bud outgrowth since it releases bud dormancy (Ross, 1986). Strigolactones, also considered long distance messengers, are likewise involved in relaying the decreased auxin concentration to the dormant axillary buds (Evert and Eichhorn, 2013; Teichmann and Muhr, 2015).

Axillary shoots (10–80 mm)

The most proliferated short axillary shoots (10–80 mm) were observed in plants with a removed apex after two weeks, irrespective of the nutrient regime (Figure 4.13A & B). However, plants in the growth room with apex and nutrients (+A+N) formed significantly more short shoots (16.6) than plants from all the other treatments in the growth room after 8 weeks (Figure 4.13A). The number of shoots from all the other treatments did not differ significantly from each other in both the growth room and the greenhouse (Figure 4.13A & B). Von Richter and Offord (2006) applied commercial fertiliser (Nutricote® Total: 18N:2.6P:6.6K) to *Actinotus helianthi* plants in pots and also reported a higher number of stems (9) on plants that received nutrients than the three stems formed on control plants with no nutrients. In both the growth room and greenhouse the least number of sprouted axillary shoots (10–80 mm) was observed in *H. coddii* subsp. *barnardii* plants with apex and without nutrients, 10.29 and 7.43 shoots respectively (Figure 4.13A & B). The combination of apical dominance and no nutrients could have been detrimental to axillary shoot development in these plants. It is reported that suboptimal or lack of nutrients in plants, especially N and P, causes decreased branching. This is probably due to resources being translocated to the roots rather than to the shoots in order to facilitate nutrient uptake from the soil (Teichmann and Muhr, 2015).

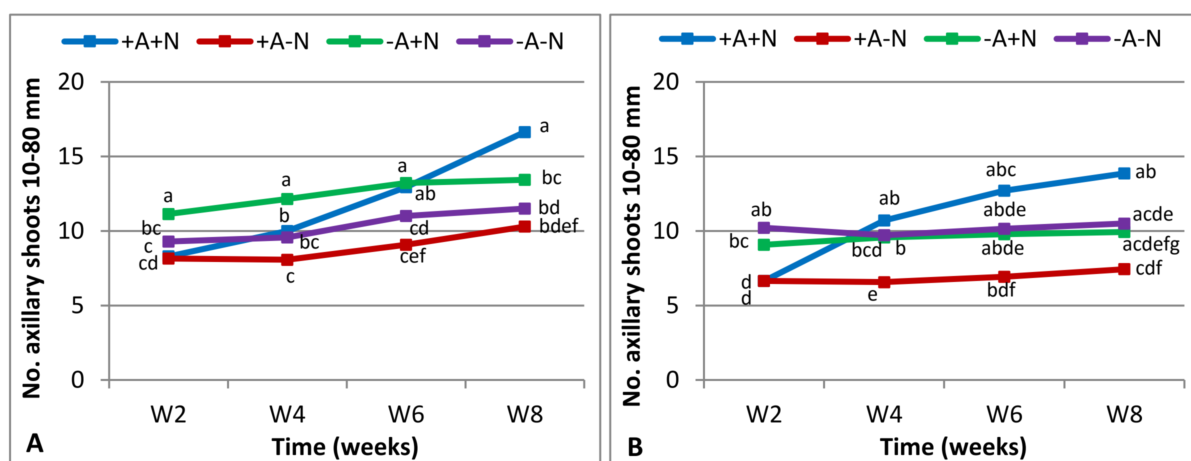

Figure 4.13. Number of proliferated axillary shoots (10–80 mm) on *H. coddii* subsp. *barnardii* plants grown over a period of 8 weeks in (A) Growth room; (B) Greenhouse.

Values with the same letters within a graph (per week) and between the two graphs are not significantly different at the 5% level of significance. +A+N: Plants with intact apex that received nutrients; +A-N: Plants with intact apex where nutrients were withheld; -A+N: Plants minus apex that received nutrients; -A-N: Plants minus apex where nutrients were withheld.

Axillary shoots ≥90 mm

After 8 weeks, the highest number of longer axillary shoots (≥90 mm) in both locations was formed on plants with nutrients irrespective of the apex status (+A+N and –A+N) (Figure 4.14A & B). However, in the growth room the plants without apex and with nutrients had significantly more shoots (13) than the plants with apex and nutrients (9.21 shoots), while there was no significant difference between these treatments in the greenhouse. The removal of the apex in *Antirrhinum majus* plants also resulted in outgrowth of more shoots (84) compared to 77 shoots in plants with an apex (Munir and Naz, 2006). In the growth room, *H. coddii* subsp. *barnardii* plants without nutrients, irrespective of the apex status, formed significantly less shoots (≥90 mm) than plants with nutrients (Figure 4.14A & Figure 4.14B).

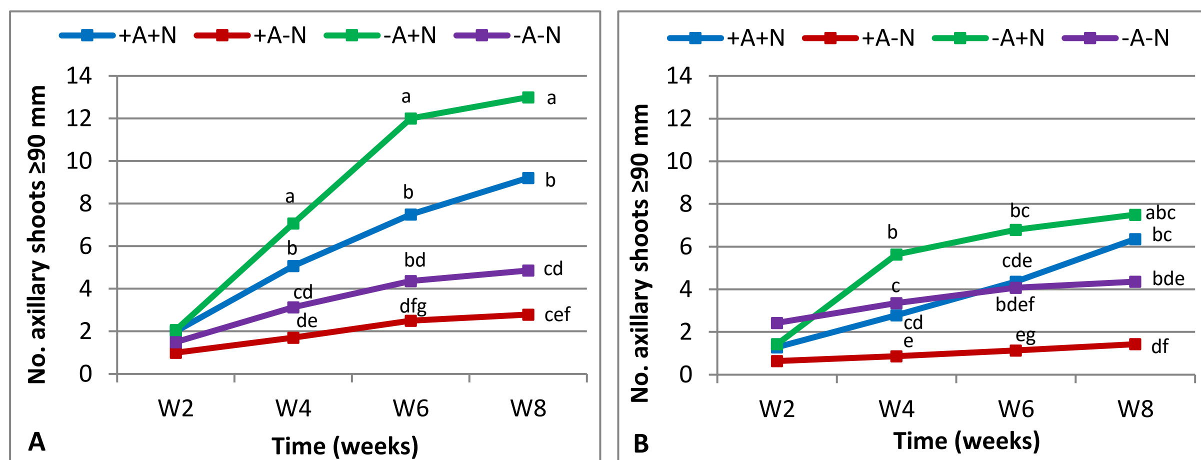

Figure 4.14. Number of proliferated axillary shoots (≥90 mm) on *H. coddii* subsp. *barnardii* plants grown over a period of 8 weeks in (A) Growth room; (B) Greenhouse.

Values with the same letters within a graph (per week) and between the two graphs are not significantly different at the 5% level of significance. +A+N: Plants with intact apex that received nutrients; +A-N: Plants with intact apex where nutrients were withheld; -A+N: Plants minus apex that received nutrients; -A-N: Plants minus apex where nutrients were withheld.

Plants of *H. coddii* subsp. *barnardii* have one leaf per node and one axillary bud in the leaf axil and therefore, gives rise to one axillary shoot per node. A high number of shoots (10–12 or more), depending on the remaining number of nodes on the plant, can be produced on one plant 6–8 weeks after apex removal. These axillary shoots can be used to propagate the plant vegetatively through cuttings (Chapter 5). The outgrowth of axillary shoots also gave rise to a more striking bushy plant. Flower buds and flowers were formed after 5–6 weeks on axillary shoots all over the plant increasing its attractiveness (Figure 4.15). Removal of the plant apex changes the

architecture of the plant making it more suitable for a specific purpose, such as branching and flowering in ornamental plants or formation of fruits and seeds in food plants (Vasudevan *et al*., 2008). Such plants will be more appealing to buyers of ornamental plants (Reinhardt and Kuhlemeier, 2002).

Figure 4.15. Example of a *H. coddii* subsp. *barnardii* plant (with nutrients) in the growth room 8 weeks after apex removal.

Plants require essential macro- and micronutrients for normal growth, functioning and reproduction and lack of or insufficient nutrients will cause deficiency symptoms in plants. Visible deficiency symptoms include stunted growth, complete and interveinal leaf chlorosis, purple or reddish leaf colour and necrosis (Taiz and Zeiger, 2010; McCauley *et al*., 2011). Plants of *H. coddii* subsp. *barnardii* where nutrients were withheld did not increase in height, axillary buds did not proliferate into shoots and old and young leaves were chlorotic (Figure 4.16). Lack of mobile elements (N, P, K, Mg, Cl, Na, Zn, Mo) would cause symptoms first in the older leaves as these elements move from older leaves to support younger plant parts. In contrast, symptoms will first appear in the younger leaves when there are insufficient immobile elements (Ca, S, Fe, B, Cu, Mn) (Uchida, 2000; Mahler, 2004; Taiz and Zeiger, 2010; McCauley *et al*., 2011).

The commercial nutrient fertilisers used in this study (Table 4.2) contain most of the essential nutrients required by plants for normal growth and development. The

H. coddii subsp. *barnardii* plants watered with these nutrient solutions had a healthy, green appearance and formed flower buds and flowers continuously in both the growth room (Figure 4.16A & C) and the greenhouse (Figure 4.16B & D). Reduced growth and visible deficiency symptoms were observed in plants where nutrients were withheld (Figure 4.16). The older leaves of plants without nutrients (+A-N and -A-N), irrespective of the apex status, showed symptoms of interveinal chlorosis and yellowing in both locations from the second week onwards. The lack of or insufficient N and Mg (a component of chlorophyll) could have caused the stunted growth and leaf chlorosis that were first visible in the older leaves (Mahler, 2004; McCauley *et al.*, 2011). The interveinal and complete chlorosis symptoms visible on the younger leaves of *H. coddii* subsp. *barnardii* plants from the fourth week onwards (Figure 4.16) could be due to deficiency of immobile elements such as Fe, Mn and S that are involved in chlorophyll synthesis and photosynthetic reactions (Taiz and Zeiger, 2010; McCauley *et al.*, 2011). Some plants also had leaves with marginal necrosis which could be due to K deficiency (Mahler, 2004; McCauley *et al.*, 2011). In addition, no new flower buds formed and existing flower buds did not open in plants without nutrients (Figure 4.16).

It was possible to grow *H. coddii* subsp. *barnardii* plants in the greenhouse under uncontrolled environmental conditions, at least during the warmer months of the year. It seems that the restricting factor for growth is the lack of essential nutrients since plants in the greenhouse with nutrients performed equally well as those in the growth room. The plants increased in height, axillary buds sprouted and elongated, and flowers were formed. However, differences in the height, length of axillary shoots and overall appearance of the plants were visible depending on where they were grown. Plants in the growth room had a shorter, stockier appearance (Figure 4.16A & C), whereas those in the greenhouse had a more lengthened and slender appearance (Figure 4.16B & D).

Differences in morphological appearance could be due to light and temperature differences between the two locations. It is reported that light quality (wavelength), quantity (intensity) and the photoperiod controls plant growth and development (Chen *et al.*, 2014). Sunlight provides the complete range of wavelengths, although plants use mostly blue and red light for vegetative growth and flowering (Taiz *et al.*,

2015). Plants in the greenhouse were only exposed to natural light that was transmitted through the glass panels and it varied depending on the time of the day and the season. Plants of *H. coddii* subsp. *barnardii* performed better under constant photoperiod and light intensity in the growth room.

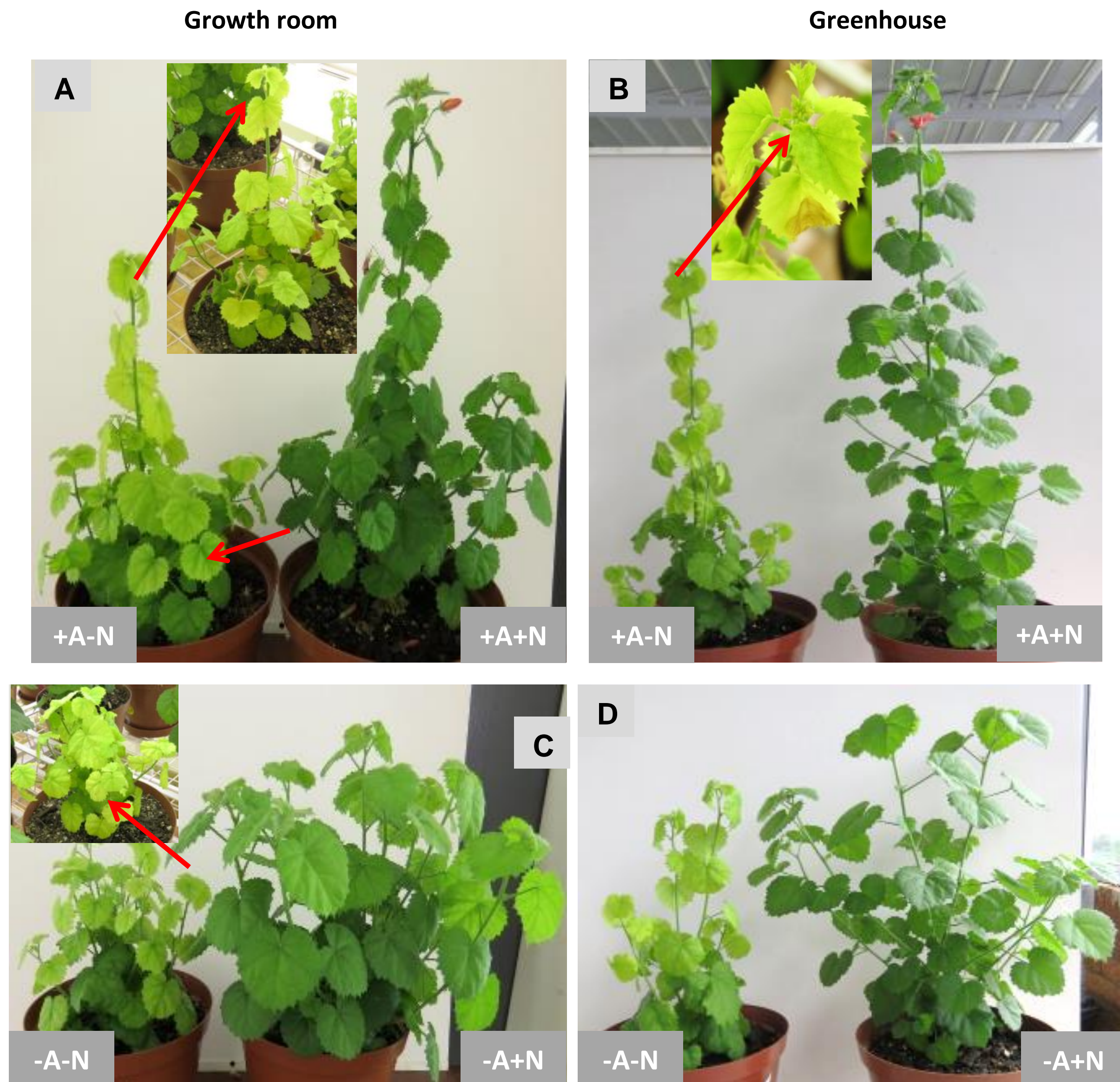

Figure 4.16. Examples of *H. coddii* subsp. *barnardii* plants in the growth room and greenhouse after 6–8 weeks. (A) Growth room with an intact apex: No nutrients (left), Plus nutrients (right); (B) Greenhouse with an intact apex: No nutrients (left), Plus nutrients (right); (C) Growth room with removed apex: No nutrients (left), Plus nutrients (right); (D) Greenhouse with removed apex: No nutrients (left), Plus nutrients (right).

4.3.3.2.2 Effect of apex removal and nutrients on flowering characteristics

The treatments (plus and minus apex and nutrients) had a significant effect on the flowering pattern (percentage of plants with formed flower buds and open flowers) from week 2 to week 8 (Appendix B: Table 7).

Flower buds (FB) and flowers (F) were visible on plants with an apex from about 5½ months (2 weeks after the start of the experiment) onwards. At this stage, most of the plants from both nutrient regimes only had flower buds at the apex (FBAp), namely 67.9% (+A+N) and 75% (+A-N) respectively. However, more of the plants that received nutrients (17.9%) also had open flowers at the apex (FBFAp) than the plants without nutrients (7.1%). Plants with removed apex from both nutrient regimes had no flower buds or flowers (NFBF), thus, the significant difference in frequency distributions (Figure 4.17).

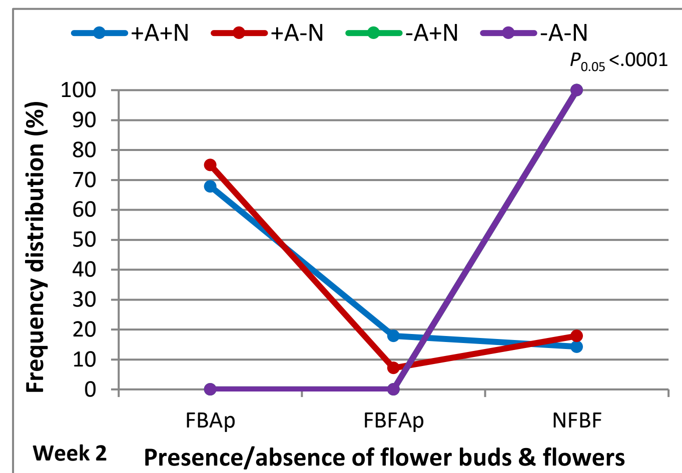

Figure 4.17. Effect of treatment on frequency distribution percentages of flower buds and flowers at Week 2. (Note: -A+N and -A-N are lying on top of each other).
FBAp: Flower buds at the apex of the main shoot, FBFAp: Flower buds and flowers at the apex of the main shoot, NFBF: No flower buds or flowers on the plant.

After four weeks, 60.7% of plants with apex, both with (+A+N) and without nutrients (+A-N), had flower buds and open flowers on the apex (FBFAp), while 35.7% and 28.6% respectively, had only flower buds at the apex (FBAp) at this stage (Figure 4.18). Most of the plants (96%–100%) with removed apex from both nutrient regimes (-A+N and -A-N) were still without flower buds or flowers (NFBF) (Figure 4.18). It is reported that removal of the apical bud leads to a more equal distribution of photosynthetic products to several growing points instead of only one. This also

causes a delay in flowering as the juvenile phase of the plant is maintained for a longer time and the proliferated axillary shoots is in a less advanced physiological state than stems with an intact apex (Rubinstein and Nagao, 1976; Munir and Naz, 2006). This delay in flowering was also seen in *H. coddii* subsp. *barnardii* plants where the apex was removed. Flower buds and flowers were visible on plants with an intact apex from the second week onwards and only after 5–6 weeks on the axillary shoots in plants with a removed apex.

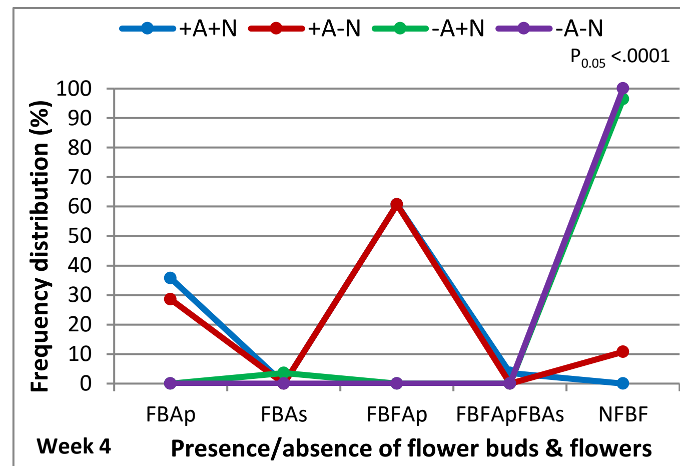

Figure 4.18. Effect of treatment on frequency distribution percentages of flower buds and flowers at Week 4. Note: (-A+N and -A-N are lying on top of each other).

FBAp: Flower buds at the apex of the main shoot, FBAs: Flower buds on the axillary shoots, FBFAp: Flower buds and flowers at the apex of the main shoot, FBFApFBAs: Flower buds and flowers at the apex of main shoot and flower buds and/or flowers on the lower axillary shoots, NFBF: No flower buds or flowers on the plant.

By the end of week 6 and 8, the significant effect of the nutrient treatment on formation of flower buds and flowers was more pronounced. Most (92.9%) of the plants with apex and nutrients (+A+N) had flower buds and open flowers at the apex (FBFAp) after six weeks, whereas a high percentage (64.3%) of plants with apex and without nutrients (+A-N) had no new flower buds (FBFApNN). The effect of nutrients was more evident in plants with removed apex where 60.7% of plants with nutrients (-A+N) had flower buds on the axillary shoots (FBAs) and plants without nutrients (-A-N) had no flower buds or flowers (NFBF) (Figure 4.19).

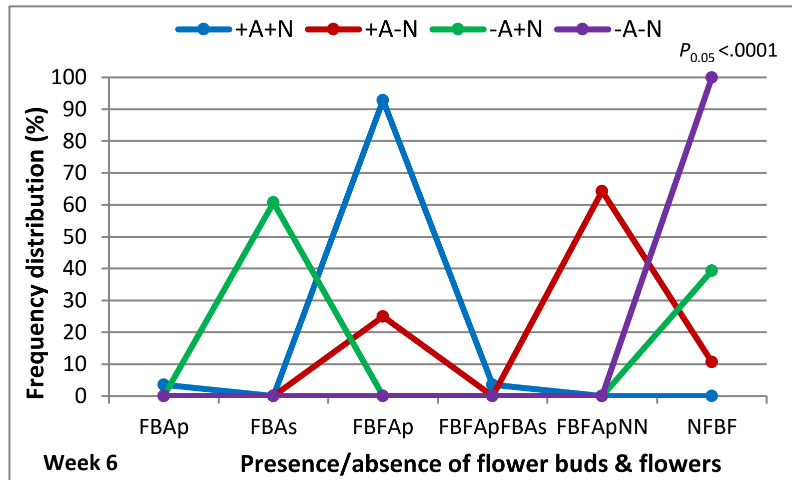

Figure 4.19. Effect of treatment on frequency distribution percentages of flower buds and flowers at Week 6.

FBAp: Flower buds at the apex of the main shoot, FBAs: Flower buds on the axillary shoots, FBFAp:Flower buds and flowers at the apex of the main shoot, FBFApFBAs: Flower buds and flowers at the apex of main shoot and flower buds and/or flowers on the lower axillary shoots, FBFApNN: Flower buds and/or flowers at the apex of main shoot; no new buds are formed, NFBF: No flower buds or flowers on the plant.

Two thirds (67.9%) of the plants with apex and nutrients (+A+N) had flower buds and flowers on the apex, whilst a third (32.1%) of these plants also had flower buds on the lower proliferated axillary shoots at the end of the experiment (week 8). On the other hand, most (78.6%) of the plants with intact apex and without nutrients (+A-N) had no new flower buds or flowers at this stage (Figure 4.20). In plants with removed apex, almost 60% of plants with nutrients (-A+N) already had flower buds and open flowers on the axillary shoots. Only flower buds were present on the axillary shoots in 40% of these plants at this stage. Plants without apex and nutrients (-A-N) had no flower buds or open flowers on the axillary shoots after 8 weeks (Figure 4.22). The immobile nutrient, B accumulates in reproductive tissues of plants and is necessary for flower bud formation (McCauley *et al.*, 2011). Lack of B could have been responsible for the poor flower performance of the plants that did not receive a nutrient solution.

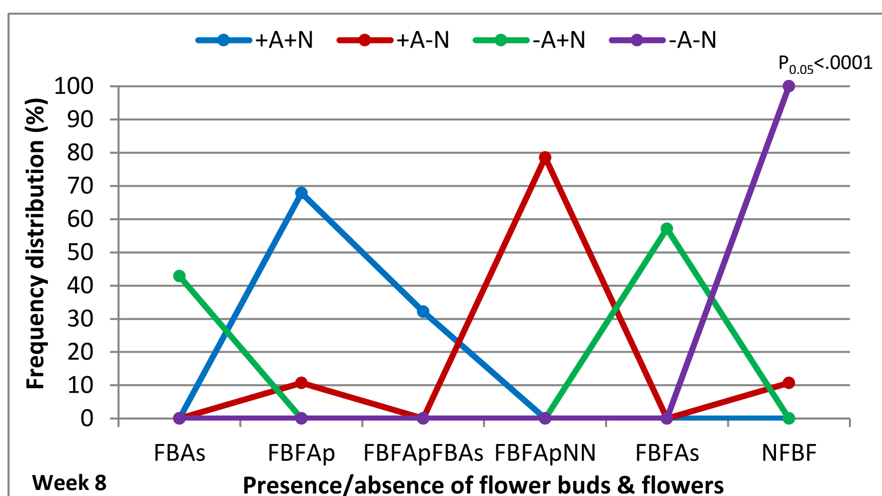

Figure 4.20. Effect of treatment on frequency distribution percentages of flower buds and flowers at Week 8.
FBAs: Flower buds on the axillary shoots, FBFAp: Flower buds and flowers at the apex of the main shoot, FBFApFBAs: Flower buds and flowers at the apex of main shoot and flower buds and/or flowers on the lower axillary shoots, FBFApNN: Flower buds and/or flowers at the apex of main shoot; no new buds are formed, FBFAs: Flower buds and flowers on the axillary shoots, NFBF: No flower buds or flowers on the plant.

The location caused a significant difference in the frequency distribution patterns of flower buds and flowers after 8 weeks (Appendix B: Table 7). The more controlled environment in the growth room seemed to have favoured the occurrence of flowers on the plants. Plants (16.1%) in the growth room, in addition to flower buds and open flowers on the apex (FBFAp), had flower buds on the axillary shoots as well (FBFApFBAs). None of the plants in the greenhouse had flower buds on the axillary shoots in addition to the apex at this stage (Figure 4.21). Furthermore, 14.3% of plants without an apex in the growth room had flower buds on the axillary shoots, while only half (7.1%) of these plants in the greenhouse had flower buds after 8 weeks. The location had no effect on the percentages of plants with no flower buds or open flowers (NFBF) (Figure 4.21).

The exact cue for flower formation in *H. coddii* subsp. *barnardii* was not studied, but it is thought that age and the vegetative mass of the plants could be the signal for flower induction since plants under controlled and uncontrolled environmental growth conditions formed flowers almost at the same time. It is reported that plants should be in the adult vegetative phase and be committed to flowering, while it should have enough leaves to perceive the signal for flowering and sufficient biomass to support the formation of the flowers (Taiz and Zeiger, 2010).

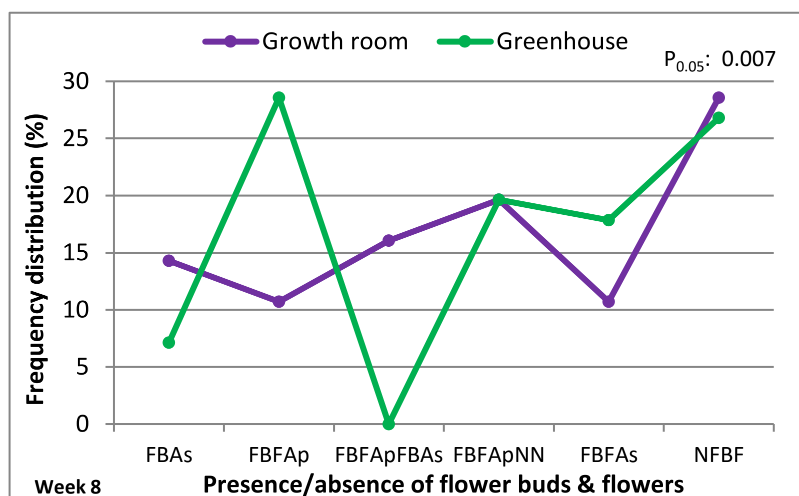

Figure 4.21. Effect of location on frequency distribution percentages of flower buds and flowers at Week 8.

NFBF: No flower buds or flowers on the plant, FBFAp: Flower buds and flowers at the apex of the main shoot, FBFApFBAs: Flower buds and flowers at the apex of main shoot and flower buds and/or flowers on the lower axillary shoots, FBAs: Flower buds on the axillary shoots, FBFAs: Flower buds and flowers on the axillary shoots, FBFApNN: Flower buds and/or flowers at the apex of main shoot; no new buds are formed.

The photoperiod determines the flowering time of plants and short-day, long-day and day-neutral plants are distinguished (Hartmann *et al*., 2011). In nature, plants of *H. coddii* subsp. *barnardii* flower in summer which suggests that they might be long-day plants, but this has to be further investigated. The photoperiod in the growth room was 16 hours, while the average photoperiod in the glasshouse was 13½ hours for the duration of the experiment. The minimum photoperiod was 12 hours, 26 minutes and the maximum 13 hours, 29 minutes which is 2½–3½ hours shorter than the photoperiod in the growth room. Despite the difference, this did not seem to have affected flowering since it started in both locations two weeks after the start of the experiment.

Temperature might also induce flowering as *H. coddii* subsp. *barnardii* plants in nature flower in the summer months (Chapter 3), but in previous observations plants kept in the growth room under controlled growth conditions (constant temperature 24°C±2°C) would flower throughout the year. The temperature in the greenhouse varied between 17.5°C and 30°C for the duration of the experiment that could have been warm enough for flower bud initiation. Plants could also flower independently from environmental conditions (Taiz *et al*., 2015) and this needs to be investigated further.

Flower buds first appeared on *H. coddii* subsp. *barnardii* plants with intact apex around 5 ½ months. The main stem continued to grow while flower buds were visible at the apex (Figure 4.22A), so-called indeterminate flowering (Reinhardt and Kuhlemeier, 2002). Flower buds borne in the axil of a leaf opened in succession from the base towards the apex resulting in one to three open flowers per plant per day (Figure 4.22B).

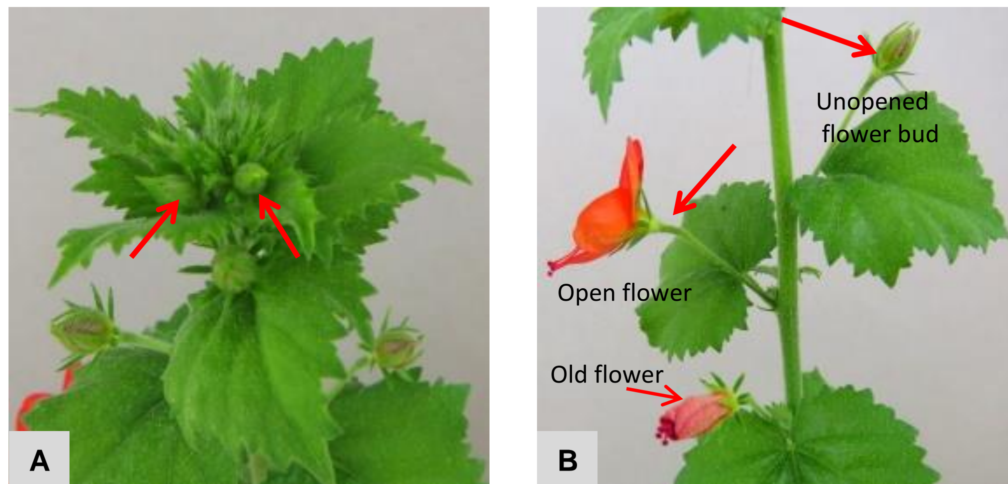

Figure 4.22. (A) A group of flower buds (arrows) at the apex of the plant; (B) Flower buds and flowers develop in the axils of leaves.

Two weeks elapsed between flower bud appearance (Figure 4.23A) and opening of the flower (Figure 4.23B). A flower was open for only one day and after closing the petals (Figure 4.23D) the next flower bud along the stem would open. In cultivated plants, the flowers should be hand-pollinated if seeds are desired. Pollen was brushed off the stamens and placed on the stigma of the flower (Figure 4.23C). Fruit capsules with seeds formed irrespective of the origin of pollen. Pollen from the same flower (self-pollination), pollen from another flower on the same plant, or even pollen from flowers on other plants could be used (cross-pollination). The fruit capsule was visible two weeks after pollination (Figure 4.23E). The ripe fruit capsule opened 3–3½ weeks later to release the seeds (Figure 4.23F). Plants grown from these seeds stayed true to type, at least phenotypically.

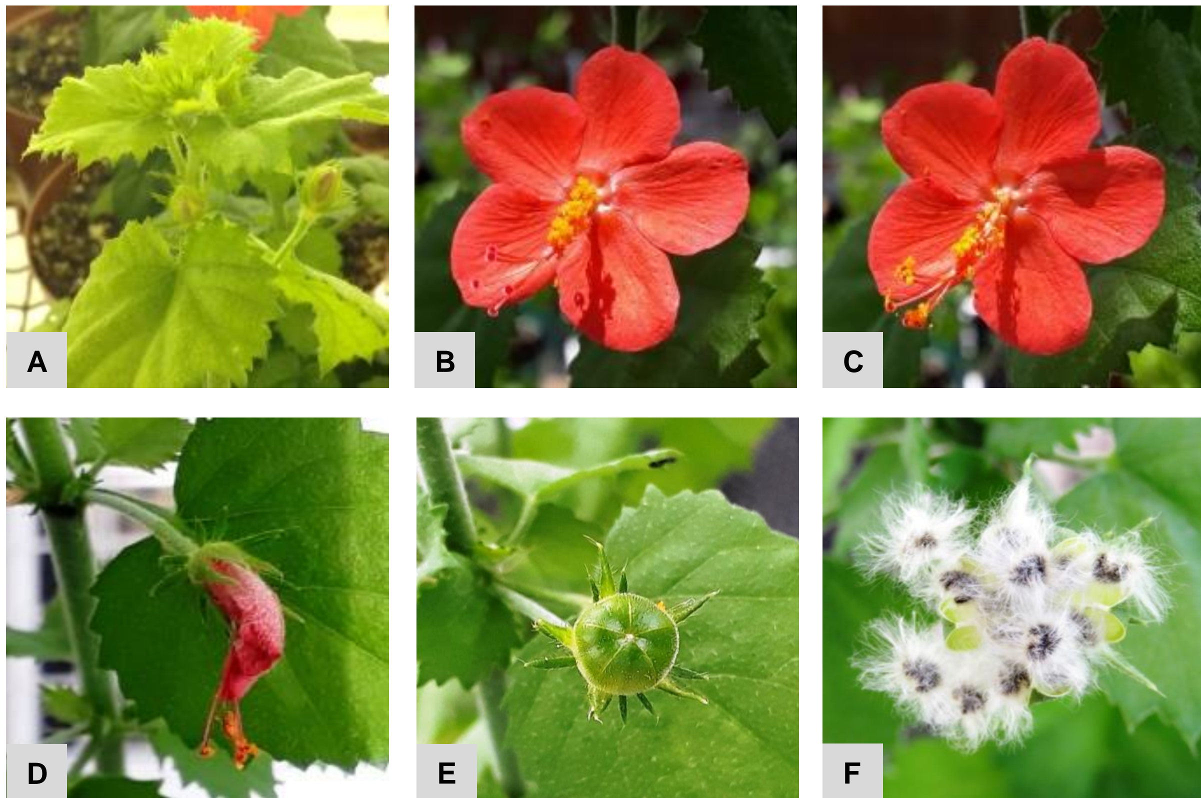

Figure 4.23. Flower and seed formation on a *Hibiscus coddii* subsp. *barnardii* plant grown in a greenhouse. (A) Flower bud; (B) Open flower after two weeks; (C) Hand-pollinated flower; (D) Dead flower one day after pollination; (E) Fruit capsule one week after pollination; (F) Open fruit capsule with seeds 3½ weeks after pollination.

4.4 Characterisation of *H. coddii* subsp. *barnardii* plants in cultivation

A basic description of the principal stages (Table 4.8) of potted plants grown under controlled conditions is given below. It is complemented with micrographs taken with a Zeiss Discovery V12 stereomicroscope (except Figure 4.25B).

Table 4.8. Principal growth stages* of *H. coddii* subsp. *barnardii* in cultivation.

Stage	Description	Time period
0	Seed germination	3–4 days after sowing
1	Seedling development	First leaf 10–12 days after germination
2	Plant development	
	2.1 Vegetative growth	4–5 months
	2.2 Maturation and reproduction	
	• Flower formation	4–5 months
	• Development of fruit capsule	10–14 days after pollination
	• Dehiscence of capsule and release of seeds	3–4 weeks after pollination
3	Senescence	Perennial plant. Older leaves on plant show senescence and fall off. Die back during dry winter months. Sprouting in warmer months.

*Stages modified from Javadzadeh (2018).

Stage 0: Seed germination

Seed germination starts with radicle protrusion (Figure 4.24A) followed by emergence of the cotyledons (Figure 4.24B) above the germination medium, known as epigeal germination (Hartmann *et al.*, 2011). The apical meristem from where shoot growth would commence can be observed between the cotyledons (Figure 4.24C & D).

Stage 1: Seedling development

The first true leaf (Figure 4.24D) appears 10–12 days after cotyledon emergence followed by stem elongation. Well-developed seedlings with a strong taproot system are established after 12 weeks.

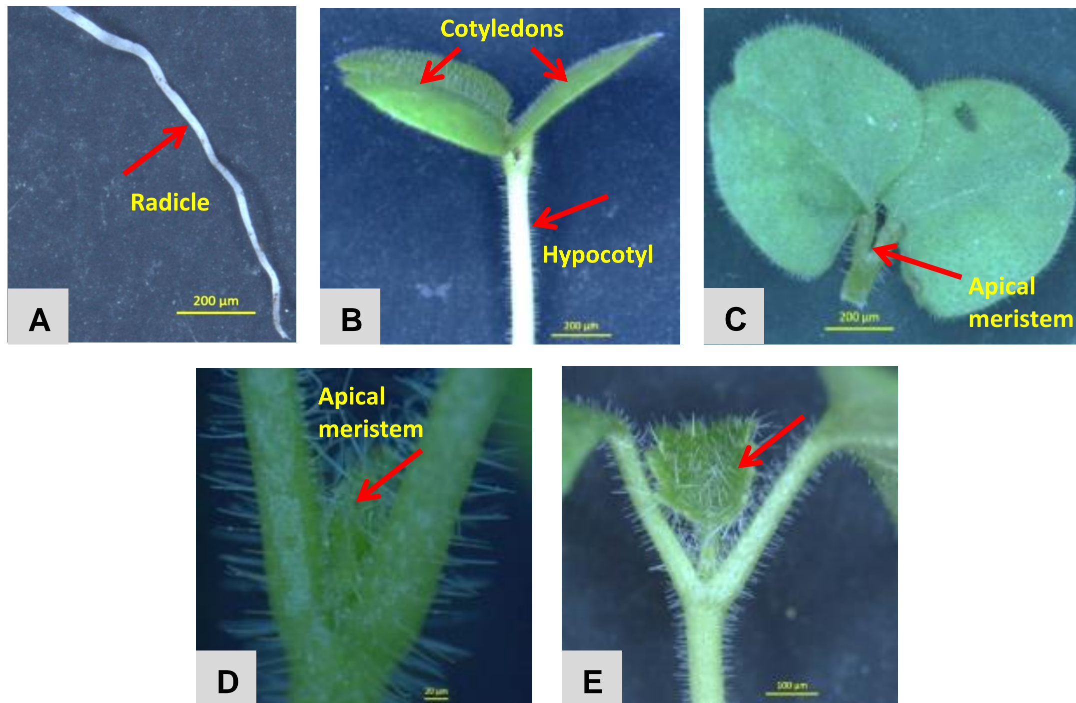

Figure 4.24. Micrographs showing morphological appearance of plant organs during Stages 0 and 1. (A) Radicle [5.04x]; (B) Cotyledons and hypocotyl [5.04x]; (C) Cotyledons with apical meristem [20.16x]; (D) Apical meristem (arrow) with leaf primordia [25.2x]; (E) First leaf (arrow) ten days after seedling emergence [10.08x].

Stage 2: Plant development

Stage 2.1 Vegetative growth

The plants are herbaceous with a single stem and show strong apical dominance, so-called monopodial branching (Reinhardt and Kuhlemeier, 2002). Axillary shoots begin to grow out from the base of the stem after 4–5 months. An average of 9.21±2.45 branches were recorded after seven months of cultivation. Plants (with an intact apex) had an average height of 510.57±85.38 mm after seven months of cultivation.

The simple, petiolate leaves are arranged alternately on the main stem and have a serrated margin (Figure 4.25A) and palmate netted venation. Leaves (49.43±6.47x42.98±6.42 mm) were found to be larger than those in nature (Chapter 3). A pair of slender stipules is borne at the base of the petiole (Figure 4.25B). The entire plant is covered in stellate trichomes (hairs) that are grouped together and radiate from the point of origin in a horizontal position (Figure 4.25C) (Koekemoer *et*

al., 2013). It is advisable to wear gloves and long sleeves when working with the plants since the trichomes could irritate the skin.

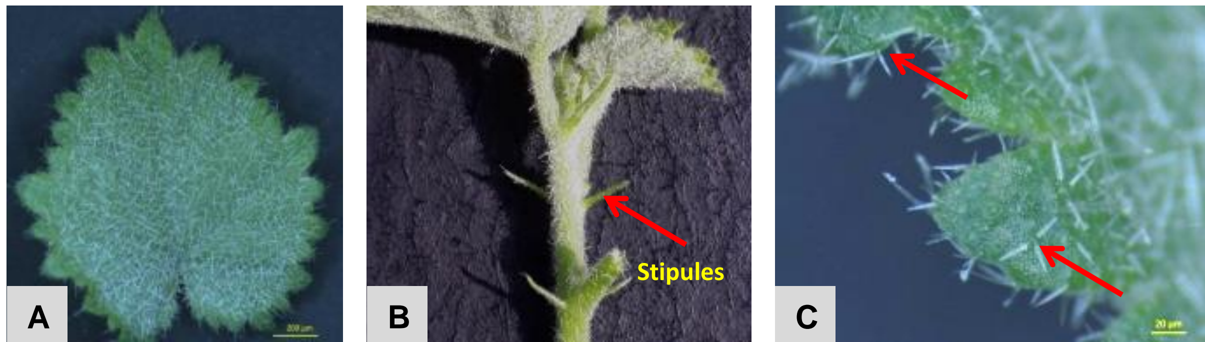

Figure 4.25. Micrographs showing morphological appearance of plant organs during Stages 2.1. (A) Mature leaf with serrated margin and deep notch at leaf base [5.04x]; (B) Filiform stipules [2x]; (C) Stellate hairs (arrows) on leaf [31.5x].

A liquid drop (Figure 4.26A) is visible on the underside of leaves usually in the mornings. It has a sweet taste and might contain a sugary substance that attract ants since they were seen walking around on the plants (Figure 4.26B) The chemical composition and reason for the formation of the drop is not known and could be further researched.

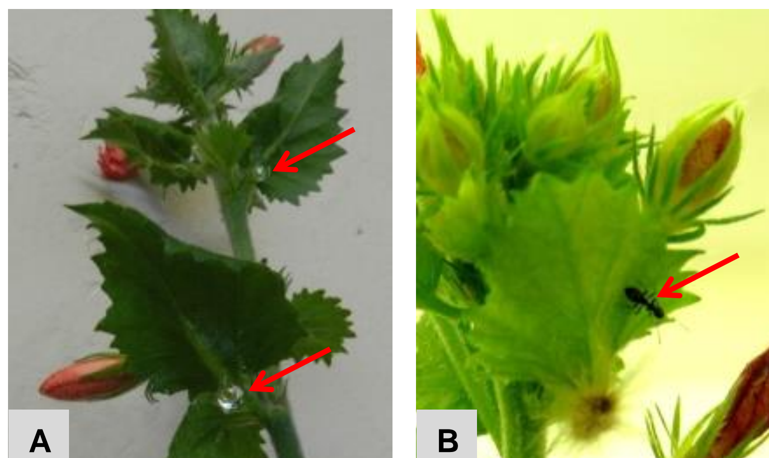

Figure 4.26. (A) Liquid drop (arrow) on the underside of leaves; (B) Ant (arrow) crawling around on plant.

Stage 2.2: Maturation and reproduction

<u>*Flower formation*</u>

Flower buds (Figure 4.28A & B) form on 4–5 month old plants which suppressed the dominance of the vegetative apex and promotes axillary shoot formation. A single flower with a pedicel is borne in the leaf axil. The flowers are regular (actinomorphic) and bisexual and varied in diameter from 25–35 mm. A variation in the shape and colour of the flowers was observed in cultivated plants grown from seeds (Figure 4.27). The reason for the difference might be genetic and could be further investigated. The flowers consist of a calyx with five free sepals with valvate aestivation and an epicalyx with 9–10 whorl members (Figure 4.28A). The corolla (Figure 4.27, Figure 4.28C) is bright red to orange-red in colour and consists of five free petals.

The androecium possesses numerous stamens that are monadelphous to form a staminal tube (Figure 4.28C & D) that unites with the base of the corolla and surrounds the style (Koekemoer *et al*., 2013). The stamens have short filaments (Figure 4.28D) and free reniform monothecous (one lobe with two pollen sacs) (Klips and Snow, 1997) anthers (Figure 4.28E). The anther opens by means of a longitudinal slit (Figure 4.28F) to release the spherical, spiny pollen grains (Figure 4.28 H). The gynoecium is composed of five fused carpels and the hypogynous flower has a superior ovary with five locules. The style ends in five short branches with five capitate stigmas; one for each carpel (Figure 4.28I & J). The stamens with pollen sacs open in the morning at approximately 10h00 after which the pollen can be transferred by hand to the five stigmas (Figure 4.29K). After anthesis, which lasts one day, the corolla, staminal tube and upper parts of the gynoecium are shed simultaneously from the plant.

Figure 4.27. Different shapes of flowers found on cultivated plants.

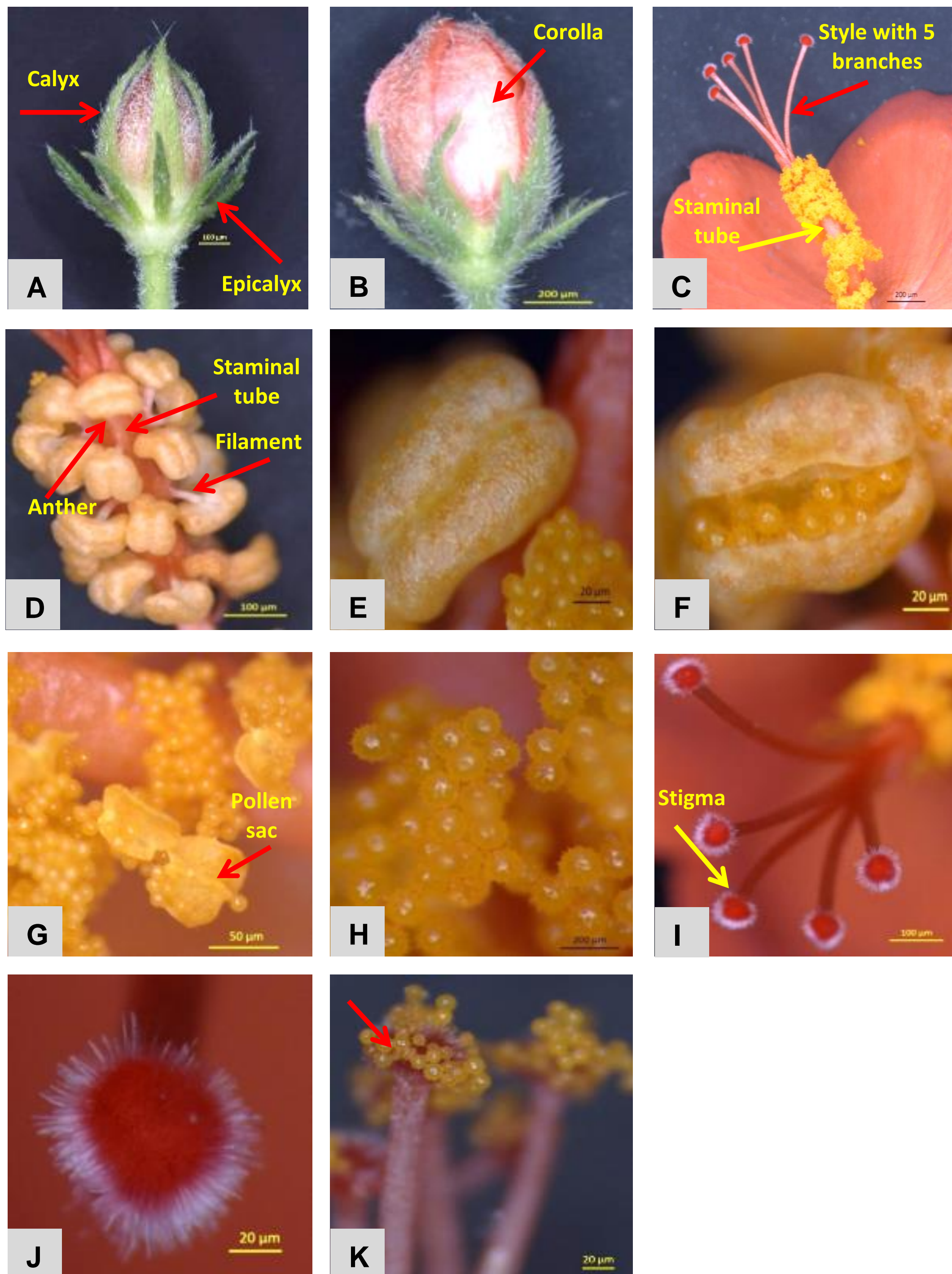

Figure 4.28. Flower of *H. coddii* subsp. *barnardii*. (A) Young flower bud [6.3x]; (B) Mature flower bud [5.04x]; (C) Part of an open flower with stamens and stigma [5.04x]; (D) Staminal tube with stamens [10.08x]. (E) Closed monothecous anther [31.5x]; (F) Anther dehiscence [31.5x]; (G) Open pollen sacs with released pollen [20.16x]; (H) Pollen structure [63x]; (I) Style divided into branches with five capitate stigmas [10.08x]; (J) Close-up of stigma [1.5x]; (K) Pollen on the stigma [25.2x].

Development of fruit capsule

The young fruit capsule (Figure 4.29A) was visible 10–14 days after pollination and has five locules (Figure 4.29B) with axile placentation.

Dehiscence of capsule and release of seeds

The fruit capsule ripened 3–4 weeks after pollination. It opens in five segments to release the seeds (Figure 4.29C & D). The number of seeds in the capsule varied from 8–10.

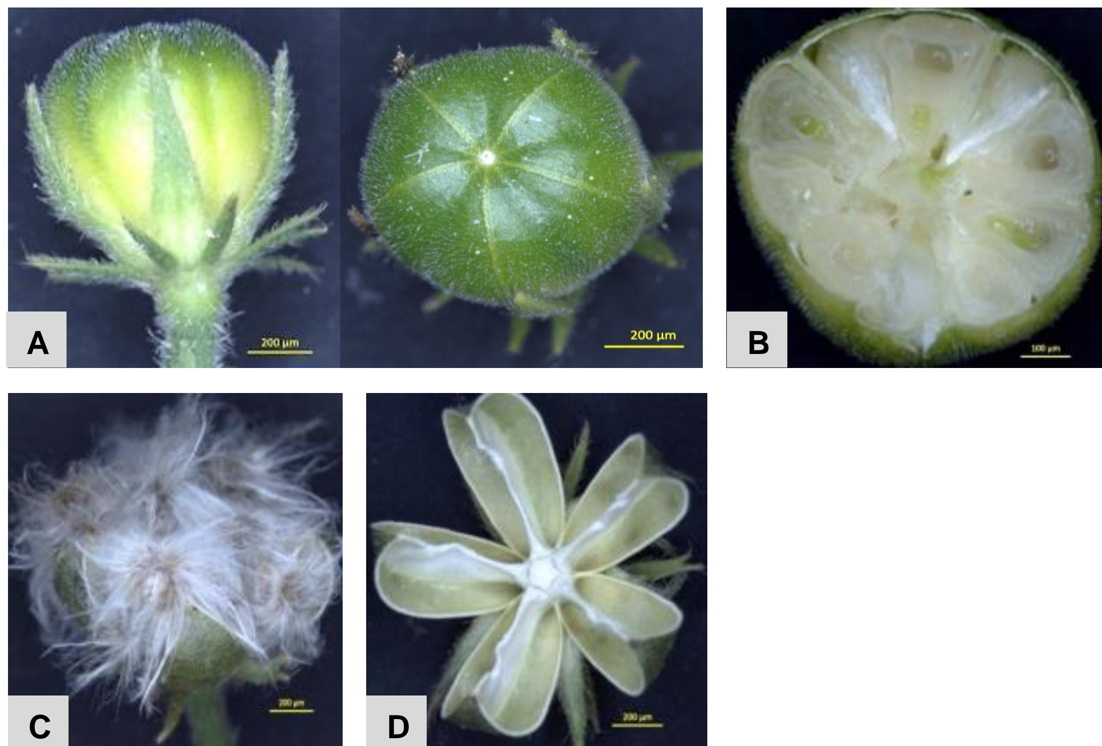

Figure 4.29. (A) Young fruit capsule [5.04x]; (B) Cross-section of unripe fruit capsule [10.08x]; (C) Open fruit capsule with seeds [5.04x]; (D) Empty fruit capsule [5.04x].

The seeds are kidney-shaped and measure about 3–5 mm in length and are covered with soft white (in fresh seeds) to beige coloured (in older seeds) hairs (Figure 4.30A). Seeds have a hard seed coat and must be scarified to uplift the physical dormancy (Section 4.3.1.2). After chemical scarification, the shape and markings on the seed are clear (Figure 4.30B & D). The water gap complex, possibly a chalazal slit, is visible on the one side of the seed (Figure 4.30C).

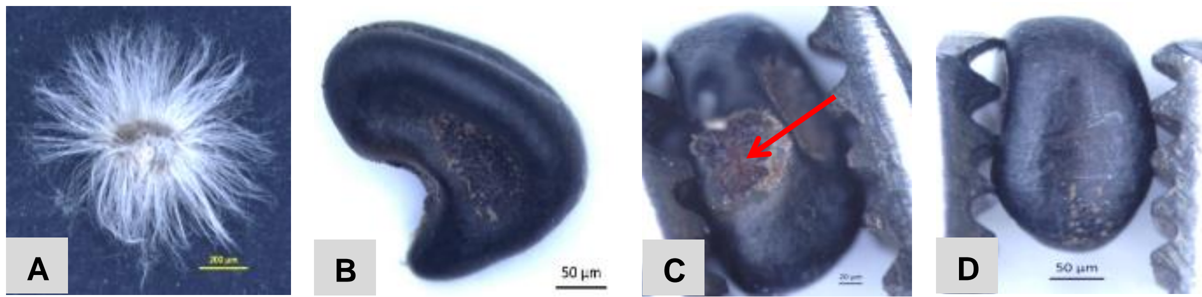

Figure 4.30. Seed of *H. coddii* subsp. *barnardii*. (A) Mature, dry seed with hairs [5.04x]; (B) Chemically scarified seed [12.6x]; (C) Chalazal/hilar region with chalazal slit (arrow) [25.2x]; (D) Seed coat, opposite side of chalazal region [12.6x].

Stage 3: Senescence

Cultivated plants, hardened-off in a greenhouse, were transferred to a local garden with sandy clay soil. The plants showed vigorous growth and flowering during the warmer summer months and grew to 500–750 mm in height (Figure 4.31A). The above ground parts of plants die back during winter, but plants sprout again in spring (Figure 4.31B). Plants were also used for a landscaping project at a local school in Polokwane where they grew well and formed flowers.

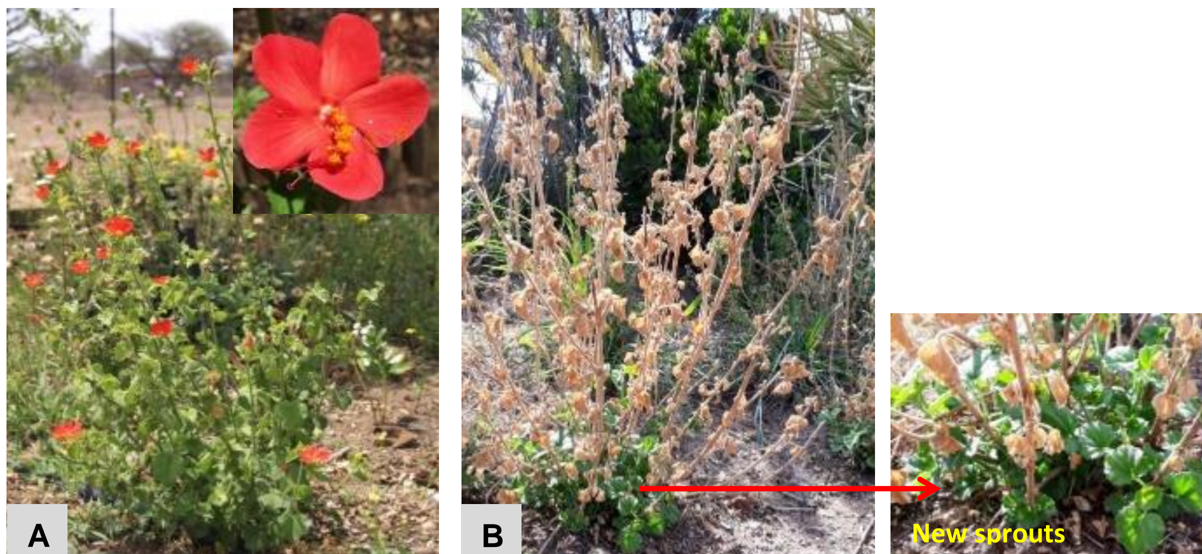

Figure 4.31. *H. coddii* subsp. *barnardii* plants transferred to a garden. (A) Vigorous growth and flowering in the summer months; (B) Die-back of branches in winter and new sprouting in spring.

Propagation of *H. coddii* subsp. *barnardii* from seeds collected in nature up to plant maturation and subsequent transfer of a hardened-off plant to outdoor garden conditions is presented in an overview below (Figure 4.32).

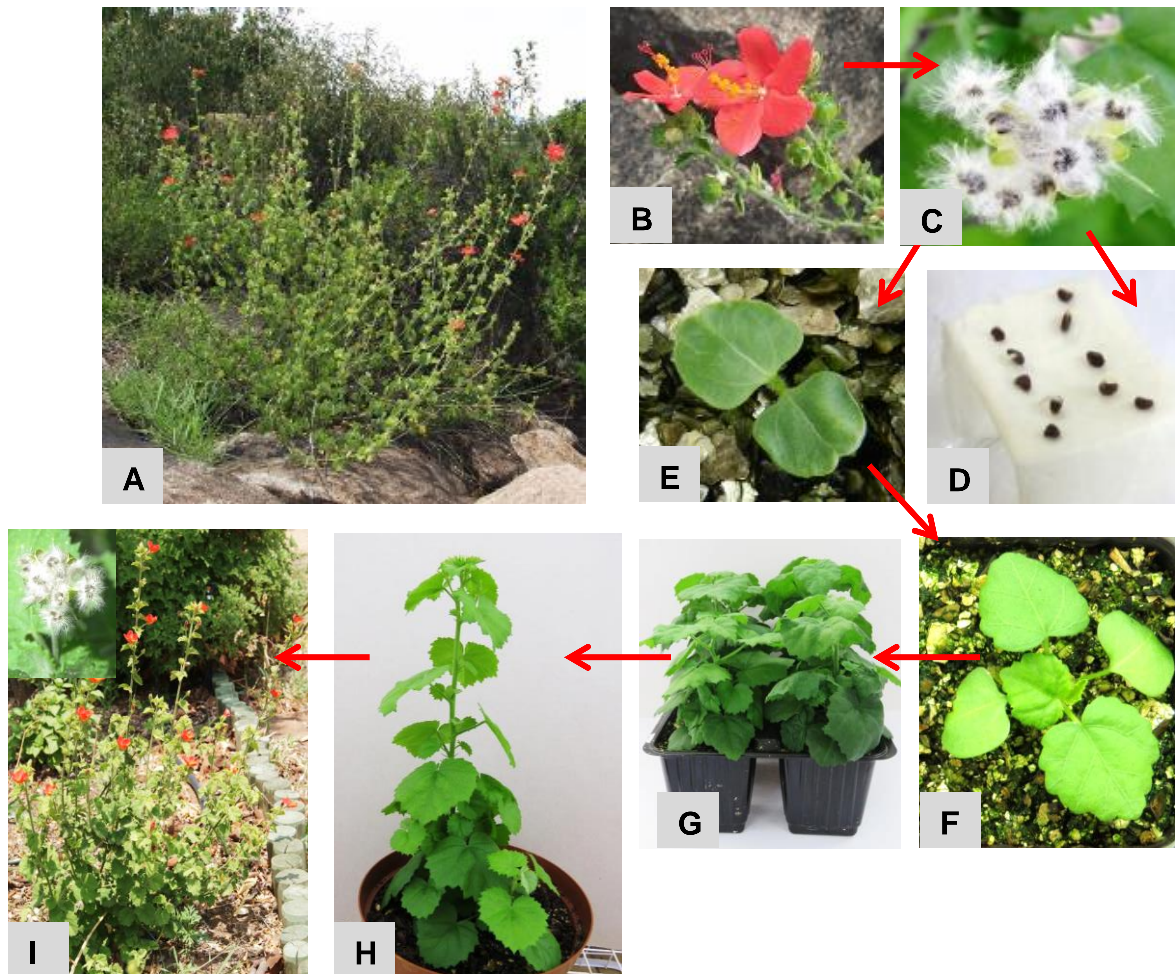

Figure 4.32. Overview of seed propagation of *H. coddii* subsp. *barnardii*. (A) Wild plant growing amongst rocks in nature; (B) Bright red flowers produced during summer months; (C) Hairy seeds on open fruit capsule; Germination of scarified seeds on (D) Filter paper bridges; (E) Vermiculite cultures showing emerged cotyledons; (F) Three week old seedling; (G) Cultivated seedlings (8 weeks old) developed from germinated seeds in vermiculite; (H) Cultivated young plant (4 months old); (I) Hardened-off plant transferred to outdoor garden showing flowers and seeds.

In conclusion, this complete procedure for propagation of plants by means of seeds would be useful for horticultural purposes and for the production of plants that can serve as stock plant source for vegetative propagation by stem cuttings. Propagated plants could also be useful for *ex situ* conservation purposes and for re-introduction of plants into nature.

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Chapter 5

CHAPTER 5
In vivo propagation of *Hibiscus coddii* subsp. *barnardii* by stem cuttings

5.1 Introduction

Ornamental plants are propagated using various methods including seed germination and tissue culture, but *in vivo* vegetative or clonal propagation by stem cuttings is probably the most common (Salaš *et al.*, 2012; Izadi and Zarei, 2014). It is possible to propagate *Hibiscus coddii* subsp. *barnardii* by means of seeds (Chapter 4), however, seed propagation can lead to genetic variation in the offspring and seeds are not always available as also reported by Mabizela *et al.* (2017). Vegetative propagation on the other hand, ensures that the unique characteristics of the mother plant are preserved in the newly formed plants (Relf and Ball, 2009; Baul *et al.*, 2011; Hartmann *et al.*, 2011). It is also a fast and cost-effective way of propagating plants, especially of hybrids that do not form seeds as in most ornamental varieties of *Hibiscus rosa-sinensis* (Baldotto *et al.*, 2012; Torkashvand and Shadparvar, 2012; Chowdhuri *et al.*, 2017).

The pre-requisite for successful propagation by stem cuttings is the formation of adventitious roots since the buds that initiate stem growth are already present (Geiss *et al.*, 2009; Hartmann *et al.*, 2011; Krajnc *et al.*, 2013). Adventitious roots usually derive from stem or leaf cells and it involves the induction of several new meristematic areas (Haissig, 1974; Geiss *et al.*, 2009; Yan *et al.*, 2014). The initiation phase of adventitious root development is characterised by outgrowth of dome-shaped primordia in the stem tissue and it ends with outgrowth of root primordia through the cortex and emergence of roots from the stem epidermis. Vascular tissue that develops in the new root primordium connects to the nearest vascular bundle (Geiss *et al.*, 2009; Hartmann *et al.*, 2011; Krajnc *et al.*, 2013).

Various factors such as cutting type, the time when the cutting is taken, application of exogenous auxin, the rooting medium as well as temperature and light conditions affect adventitious root formation on stem cuttings (Geiss *et al.*, 2009; Baul *et al.*, 2011; Singh, 2018).

The original position from where the cutting is taken from the stem of the stock plant may also influence its rooting capacity (Bona *et al.*, 2012). Hardwood (mature and dormant), semi-hardwood (partially matured, current season's growth) and softwood (new, soft growth) cuttings are distinguished in woody plants. Semi-hardwood cuttings of *H. sabdariffa* performed better than softwood cuttings in terms of rooting and cutting parameters, except for the increase in cutting diameter (Govinden-Soulange *et al.*, 2009). Cutting types distinguished in herbaceous species include apical (with or without intact apex), middle and basal cuttings (Hartmann *et al.*, 2011). In plant species where cuttings root easily, the cutting type is of less importance, although it is reported that basal cuttings could have a better rooting capacity than apical cuttings, but this should be investigated for each species (Hartmann *et al.*, 2011). Apical cuttings of *Cyclopia genistoides* showed a higher rooting percentage (74.8%) and higher number of roots (2.9) per cutting than the basal cuttings (40.8%) with 1.1 roots per cutting (Mbangcolo *et al.*, 2013). In contrast, basal cuttings of *Dalbergia melanoxylon* showed a higher rooting percentage (35.8%) and had more roots (7.3) per cutting than apical cuttings with 12.3% rooting and 1.8 roots per cutting (Amri *et al.*, 2010).

Auxin is one of the major plant growth regulators involved in adventitious root formation on cuttings (Krajnc *et al.*, 2013). Some stem cuttings have sufficient endogenous auxin for adventitious root induction, but the application of exogenous auxin could lead to faster root formation, increased rooting percentage and better root quality (Krajnc *et al.*, 2013; Ibironke, 2017). Auxins such as Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) are mostly used for adventitious root induction on stem cuttings. It can be applied as rooting hormone powder or by soaking cuttings in auxin solutions (Hartmann *et al.*, 2011). The most commonly used auxin for root induction is IBA since it has a high ability to promote root initiation in a wide range of plant species and is not easily oxidised by peroxidase enzymes and remains stable at the base of the cutting (Hartmann *et al.*, 2011; Kurepin *et al.*, 2011; Izadi and Zarei, 2014).

An increase in rooting percentage, number of roots per cutting and root length, and a reduction in time to rooting have been reported with exogenous application of auxin (Yan *et al.*, 2014). Cuttings of *Lippia javanica* treated with Seradix® No. 2 rooting

powder (0.3% IBA) had a significantly higher number of roots per cutting (22.5) than the untreated cuttings with 17.5 roots per cutting (Soundy *et al.*, 2008). Salaš *et al.* (2012) reported an improvement in the rooting percentage of *Cornus alba* cuttings from 48.9% in the control to 70.4% with the application of Rhizopon AA powder (1% IBA). The number of roots per cutting also increased from 2.5 roots on untreated to 3.1 roots on IBA treated cuttings. Shadparvar *et al.* (2011) also reported a higher rooting percentage (67.7%) after dipping *H. rosa-sinensis* cuttings in a solution containing 3000 mg L^{-1} IBA. Torkashvand and Shadparvar (2012) reported a significant reduction in time to adventitious root formation in *H. rosa-sinensis* cuttings, from 71.7 days in the control to 54.6 days in cuttings treated with 4000 mg L^{-1} IBA. It is noteworthy that poor rooting responses after application of exogenous auxins have also been reported. Untreated cuttings of *Lippia alba* had a high (83.3%) rooting percentage which reduced to 46.7% and 20% with the application of 2000 and 4000 mg L^{-1} IBA respectively (Herrera-Moreno *et al.*, 2013). The effect of auxin on other parameters was also reported. The percentage of *Toona ciliata* cuttings with sprouted buds increased from 7.8% in the control to 52.2% in cuttings treated with 8000 ppm IBA (Thakur *et al.*, 2018). On the other hand, Pholo *et al.* (2013) reported a reduction in fresh mass and stem diameter of *Pelargonium sidoides* cuttings when treated with Dynaroot No. 2 (0.3% IBA).

The rooting medium is one of the most important factors that influences the successful rooting of cuttings and since there is no universal medium, the most suitable one for each species should be investigated (Hartmann *et al.*, 2011; Sardoei, 2014; Szczepaniak *et al.*, 2016). The medium must provide adequate support for the cutting while at the same time have a high porosity that allows for sufficient gas exchange. It should also supply enough moisture to the cutting, but should have a low water holding capacity (Sardoei, 2014). Organic compounds such as peat moss, coco peat and bark, and mineral components such as vermiculite, coarse sand and perlite are mostly used as rooting media. Combinations of an organic and mineral component are also used where the mineral component improves porosity and drainage, for example in coco peat and vermiculite. The mineral component can also be used alone or in combination with other mineral components as in vermiculite and sand (Biondo and Noland, 2000; Hartmann *et al.*, 2011).

Torkashvand and Shadparvar (2012) used a number of media namely sand, coco peat, a mixture of sand and perlite and a mixture of coco peat and perlite for rooting stem cuttings of *H. rosa-sinensis* (Yellow double hybrid). The best results for cutting survival, rooting percentage and number of roots on cuttings were achieved with the coco peat and perlite mixture. Baldotto *et al*. (2015) successfully rooted apical stem cuttings of *H. rosa-sinensis* in carbonised rice husks, while Govinden-Soulange *et al*. (2009) used a mixture of soil, compost and sand [3:2:1 (v/v/v)] to root cuttings of *H. sabdariffa*. *Perovskia atriplicifolia* stem cuttings rooted better (68.8%) in perlite and peat [2:1 (v/v)] than in perlite and sand [2:1 (v/v)] medium (39.9%) (Dumitraşcu, 2008). A soil, sand and coirdust [1:1:1 (v/v/v)] mixture was more beneficial for bud sprouting of *Stevia rebaudiana* cuttings (81.2%) than a soil and sand [1:1:1 (v/v/v)] mixture where 73.8% of cuttings had bud sprouts (Smitha and Umesha, 2012). There is thus no ideal rooting medium for cuttings since it depends on the plant species and cutting type and hence, the most suitable medium should be researched for each species.

Stem cuttings offer a fast and cost-effective way to propagate clones of plants. A literature survey indicated that to date and to the best knowledge of the author, information on the use of stem cuttings for vegetative propagation of *H. coddii* subsp. *barnardii* is lacking. The objective of this chapter was, therefore, to study the effect of cutting type, IBA treatment and rooting medium on the rooting of axillary stem cuttings and to recommend the most suitable protocol for vegetative propagation.

5.2 Materials and Methods

5.2.1 Plant material

Scarified seeds were germinated in moist vermiculite and after five weeks, the seedlings were transplanted to pots filled with a potting mix [potting soil, vermiculite and sand [5:3:1(v/v/v)]. The plants were kept in a growth room for further development at 24°C±2°C and a 16 hour photoperiod at 150–200 µmol m^{-2} s^{-1} provided by a mixture of cool-white (Phillips and Osram) and Gro-lux (Sylvania) fluorescent tubes. The relative humidity varied from 50–80% in a 24-hour cycle. Plants received tap water three times a week and a nutrient solution (Culterra® Multisol 'N', Table 4.1) once per week. After four months the apex of the plants were removed to induce axillary bud sprouting and outgrowth of axillary shoots. These primary axillary shoots were used as cuttings for the rooting experiment.

5.2.2 Plant growth media

5.2.2.1 Composition

Growing media such as potting soil, coco peat, vermiculite and sand utilised commercially by plant nurseries in South Africa, were used in the following combinations, based on a literature survey, to test the rooting responses of axillary shoot cuttings:

- Potting soil and coco peat (PS & CP) [1:1 (v/v)];
- Potting soil, vermiculite and river sand (PS, V & S) [5:3:1 (v/v/v)];
- River sand and coco peat (S & CP) [1:1 (v/v)];
- Vermiculite and coco peat (V & CP) [1:1 (v/v)], and
- Vermiculite (V) only.

The vermiculite, river sand and coco peat were all Culterra® brand products. The coco peat (coir fibre) is made from the ground-up fibrous material between the outer seed husk and the coconut of the coconut palm tree and is compacted in brick form. The potting soil, primarily made from plant material with unknown components, was supplied by the Compost Guy, Garden Technologies cc, Polokwane. These media will further be referred to as the rooting media.

5.2.2.2 Preparation

The different media were thoroughly mixed manually in the specified volume per volume ratios. The compressed coco peat bricks were moistened in tap water and the loose granules dried in the sun before it was mixed with other components. Removable plugs of Unigro plastic trays (98 plugs per tray; supplier Plasgrow, White River, Mpumalanga) were used for the rooting experiment. The open end of the tapered plug measures 50 mm x 50 mm with a length of 100 mm. Plugs were filled with the respective rooting media and moistened with tap water prior to the insertion of the cuttings.

5.2.2.3 Analysis of media

5.2.2.3.1 Mineral nutrients

Mineral analysis (Ca, K, Mg, P) of each rooting medium was performed at the Limpopo Agro-Food Technology Station situated on the University of Limpopo campus. After drying the rooting media in a Memmert UF30 (Lasec) oven at 60°C for one hour, each medium was ground to a fine powder with a mortar and pestle. Thereafter, it was digested with a PerkinElmer® Titan MPS microwave sample preparation system before analysis. A sample (3.5–4 g) of each rooting medium was placed in a digestion vessel and 10 ml of 70% nitric acid (HNO_3) was added. The mixture was stirred carefully, left for 10 minutes, and then heated in the microwave at the temperature and time recommended by the manufacturer (PerkinElmer, 2014). The digested soil mixtures were transferred to centrifuge tubes and filled to 50 ml with de-ionised water. The samples were analysed with a Shimadzu ICPE-9000 (Inductively Coupled Plasma Atomic Emission Spectrometry) instrument (Shimadzu Corporation, 2012). The values for the mineral elements were recorded in mg L^{-1}. The total organic carbon (C) in the soil samples was determined based on the Walkley-Black chromic acid wet oxidation method. Oxidisable matter in the soil was oxidised with a 1N potassium dichromate solution. Two volumes of sulfuric acid were mixed with one volume of the dichromate solution and the remaining dichromate was titrated with ferrous sulphate. The titre was inversely related to the amount (%) of carbon present in the soil sample (Soil Organic Carbon Test, Sa).

5.2.2.3.2 Water holding capacity and pH

The water holding capacity (WHC) of each medium was determined according to the procedure described by Dianming (2012). The percentage (%) WHC was determined using the following formula:

$$\frac{\text{mass of the water (g) contained in the saturated medium}}{\text{mass of the saturated soil (g)}} \text{ X } 100$$

The pH value (in water) of each medium was determined according to the procedure described by the Geotechnical Test Method (2015).

5.2.3 Rooting of axillary shoot cuttings

The experiment was conducted to evaluate the effect of cutting type (original position on stem of stock plant), auxin treatment and rooting medium on the rooting performance. Axillary stem cuttings with an intact apex were taken randomly from the main shoot of *in vivo* grown plants (Figure 5.1A) using clean secateurs. Cuttings taken from the upper four nodes on the stem are further referred to as apical cuttings whilst the ones taken from the lower four nodes are referred to as basal cuttings. Most of the leaves were removed from the cuttings except for the upper two to three leaves (Figure 5.1B).

The basal cut end of the excised cutting was first dipped in water and then in rooting hormone powder (10 mm from the base upward, including the lowest node). Commercial rooting powders, Dynaroot™ No.1 and Dynaroot™ No.2 containing respectively 0.1% and 0.3% IBA as active ingredient, were tested. Excess rooting powder was tapped off before cuttings were planted in the different media into pre-made openings at a depth of 20 mm (Figure 5.1C). Cuttings without rooting powder served as the control. The trays with cuttings were covered with a perforated, transparent plastic bag and mist sprayed for the first one to two weeks to maintain the moist condition of the rooting media (Figure 5.1D) where after it was removed. During the second week each cutting received 5 ml of tap water twice per week and 10 ml of water was given in week 3 and 4. Cuttings were not provided with mineral nutrients for the duration of the experiment. The trays with cuttings were kept in a growth room under controlled environmental conditions as described in section 5.2.1 above.

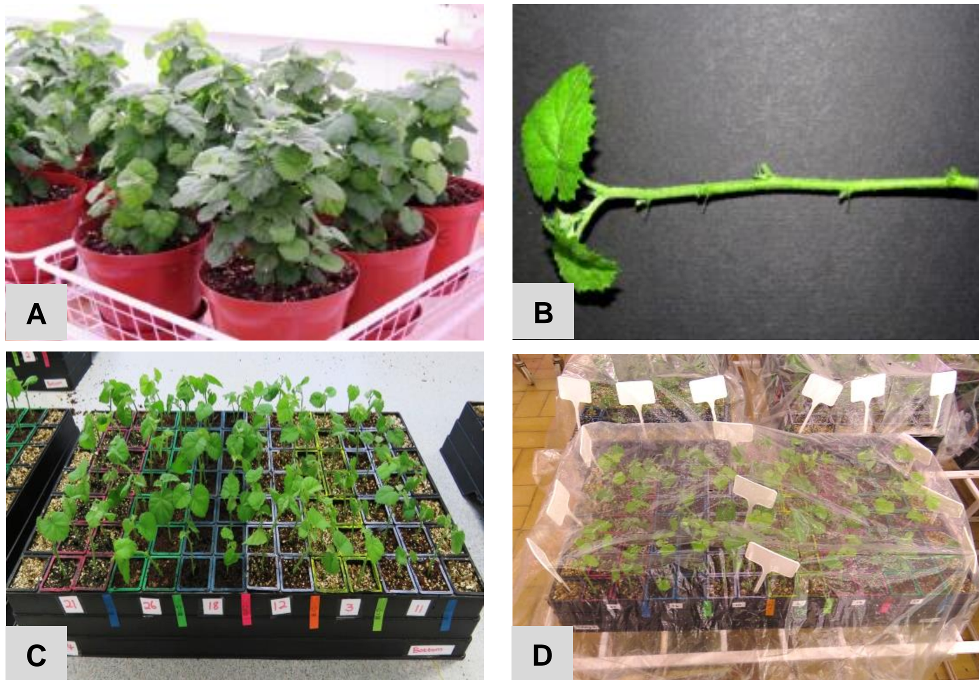


Figure 5.1. (A) *In vivo* grown donor plants of *H. coddii* subsp. *barnardii* used for stem cutting collection; (B) Cutting with lower leaves removed before planted into the rooting medium; (C) Rooting media with planted cuttings; (D) Tray with cuttings covered with plastic bag for 1–2 weeks.

At the end of the experiment (four weeks), the surviving cuttings (rooted and unrooted), were carefully removed from the plugs and the rooting medium was gently removed. Care was taken to remove all rooting medium particles using forceps and a small wet paintbrush to prevent breakage of the roots. The roots of each cutting were separated from the stem and the fresh and dry root biomass for each rooted cutting were measured with a Sartorius M-power AZ214 balance. The roots were dried in a Memmert Model100–800 oven at 70°C until a constant dry root mass was reached.

5.2.4 Experimental design

The design followed a 2 x 3 x 5 factorial design: two axillary stem cutting types (apical and basal), three IBA treatments [no IBA (Control), Dynaroot 1 (0.1% IBA), Dynaroot 2 (0.3% IBA)] and five rooting media [potting soil and coco peat (PS & CP), potting soil, vermiculite and sand (PS, V& S), sand and coco peat (S & CP), vermiculite (V) and vermiculite and coco peat (V & CP)]. There were 30 treatments and the experimental unit was 10 cuttings per treatment. Treatments were arranged in a randomised complete block design (RCBD) in the growth room and replicated three times.

5.2.5 Data collection and analysis

The following variables were gathered four weeks after the planting of the cuttings:

Survival

The survival of each cutting was evaluated based on the appearance (green and rigid) of the cutting at the end of the experiment and both rooted and unrooted surviving cuttings were used in data analysis. Cuttings were considered dead if wilted and necrotic stem or leaf tissue was visible.

Root formation

Due to the large number of roots formed on some of the cuttings a five-point score or point system (descriptive and quantitative) were used to evaluate the rooting response of each surviving cutting after four weeks (Salaš *et al*., 2012). The position of origin of adventitious roots was also evaluated with a three point scale. The fresh and dry root biomass of each rooted cutting were also determined.

Root score system

1 No roots (Figure 5.2A)
2 Visible root primordia (Figure 5.2B)
3 Single root (Figure 5.2C)
4 Two to ten roots (Figure 5.2D)
5 More than 10 roots (Figure 5.2E)

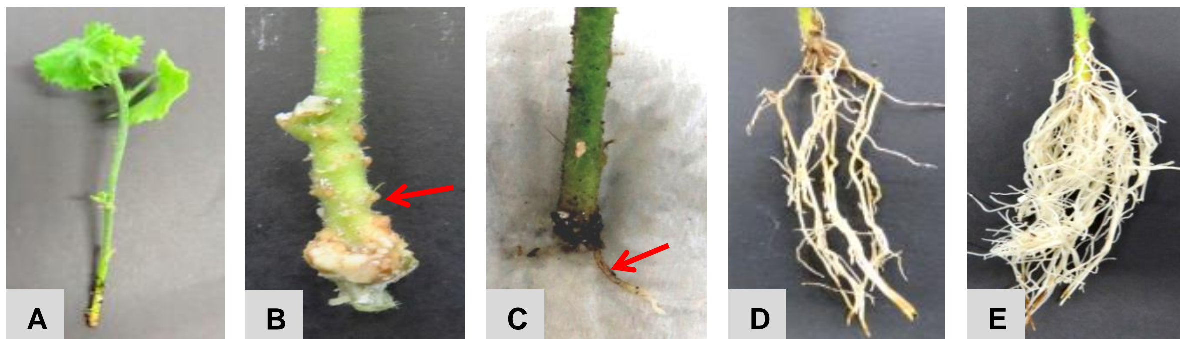


Figure 5.2. Examples of scores used for the evaluation of adventitious root formation on stem cuttings. (A) No roots [1]; (B) Visible root primordia [2]; (C) Single root [3]; (D) Two to ten roots [4]; (E) More than 10 roots [5].

Position of origin of adventitious roots on cutting

1 Roots at the wound edge (Figure 5.3A)
2 Roots at the wound edge and lowest node (Figure 5.3B)
3 Roots at the wound edge, at and above the lowest node, and sometimes at upper node(s) (Figure 5.3C)

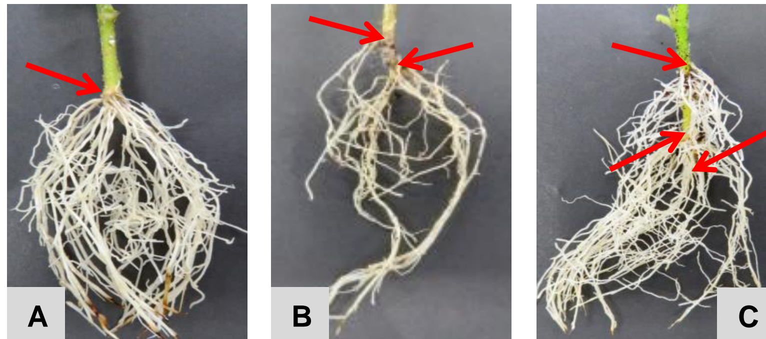


Figure 5.3. Examples of the position of origin of adventitious roots on stem cuttings (arrows indicate position). (A) Wound edge [1]; (B) Wound edge and lowest node [2]; (C) Wound edge, at and above lowest node, might include upper nodes [3].

Additional cuttings from each treatment were planted to observe the rooting response at 7, 14 and 21 days. These cuttings did not form part of the final evaluation and data analysis.

Stem cuttings

Different growth parameters (length, number of nodes, basal diameter) for each stem cutting were recorded at the beginning (initial measurements) and at the end (harvest measurements) of the rooting experiment. The length (mm) was measured with a ruler starting from the last node (at the base) up to the apical bud of the cutting. The number of nodes was counted from the bottom of the cutting until the last node at the apex, excluding the apical bud. The basal diameter just above the last node (at the base) was measured with a digital vernier calliper (Carpenter and Cornell, 1992). After the experiment, the difference between the initial and harvest measurements for length, number of nodes and basal diameter of each rooted cutting was determined. The presence or absence of a flower bud at the beginning of the experiment and the sprouting of axillary buds at the end of the experiment were also noted for each cutting.

Data analyses were performed with the SAS® Version 9.3 statistical software package (SAS Institute, 2011). Data were subjected to analysis of variance (ANOVA) using the linear models procedure (PROC GLM) to determine the effect of three factors (cutting type, IBA treatment and rooting medium) on rooting and cutting parameters (Cochran, 1952). The means of the treatments were separated with T tests (LSD) at the 5% level of significance (Snedecor and Cochran, 1989). The

Shapiro-Wilk's test was performed on the standardised residuals to test for deviations from normality (Shapiro and Wilk, 1965).

Pearson's correlation (for normally distributed data) and Spearman's correlation (for monotonic data) were run using XLSTAT (version 2015.04.36025) software to determine any correlation or relationship between initial cutting parameters and the harvest measurements and observations of the variables at the end of the experiment (Mukaka, 2012; McDonald, 2014). The correlation or relationship between the initial length, number of nodes and basal diameter and the harvest parameters, including the fresh and dry root biomass, were determined with Pearson's correlation (p=0.05). Spearman's ranked correlation (p=0.05) was used to determine the correlations or relationships between initial flower buds, survival and rooting of cuttings at week 4, the root score and root origin and the sprouting of axillary buds. The size of the correlation coefficient was interpreted according to Mukaka (2012) (Appendix C: Table 6).

5.3 Results and Discussion

5.3.1 Characteristics of rooting media

The media used for rooting of the cuttings differed in their mineral nutrient composition, pH values and water holding capacities (WHC) (Table 5.1).

Table 5.1. Mineral nutrients and other characteristics of media used to root stem cuttings of *H. coddii* subsp. *barnardii*.

Media	Ca (mg L^{-1})	K (mg L^{-1})	Mg (mg L^{-1})	P	%C	pH (H_2O)	WHC (%)
Potting soil & coco peat (PS & CP)	14.50	12.20	11.60	n.d.	3.25	6.71	75.54
Potting soil, vermiculite & sand (PS, V & S)	17.20	18.09	27.50	n.d.	3.17	7.54	25.80
Sand and coco peat (S & CP)	3.18	30.01	1.73	n.d.	3.25	7.46	36.35
Vermiculite (V)	39.90	64.70	70.00	n.d.	5.55	9.19	68.65
Vermiculite & coco peat (V & CP)	24.40	39.10	41.50	n.d.	3.20	6.37	79.53

n.d.: not detected, below instrument detection limit. WHC: water holding capacity.
Values are the average of three readings.

The results showed that vermiculite had the highest Ca, K and Mg content followed by the vermiculite and coco peat (V & CP) medium. Sand and coco peat (S & CP) was much lower in Ca and Mg content. The level of P in all media was below the

detection limit of the instrument. The percentage C was the highest in vermiculite (>5%) and up to 3.3% for the other media. The pH varied from slightly acidic (6.37) in vermiculite and coco peat (V & CP) to slightly alkaline (7.54) in potting soil, vermiculite and sand (PS, V & S), except for vermiculite where it was highly alkaline (9.19). Rooting media that contained coco peat (PS & CP and V & CP) had high water holding capacities (WHC) except in combination with sand, which had the second lowest WHC (36.4%). The medium with the lowest WHC (25.8%) was the combination of potting soil, vermiculite and sand (Table 5.1).

Potting soil is usually made up from a combination of components such as compost, perlite, vermiculite, coco peat, peat moss, topsoil and shredded pine bark which might lead to varying characteristics depending on its composition (Biondo and Noland, 2000; Hartmann *et al.*, 2011). Vermiculite is obtained from the mineral mica and might contain high levels of Ca, Mg and K (Biondo and Noland, 2000) as evident by the high concentration of these elements found in the vermiculite used in this study (Table 5.1). Vermiculite obtained from South African sources has a high average pH (8.5) (Brooking, 1976) as also found in this study (pH 9.19) (Table 5.1). It is a lightweight medium with a low bulk density and mixes easily with other mediums (Hartmann *et al.*, 2011). Coco peat has a slightly acidic pH (5.2–6.8) as also found in this study (Table 5.1) and depending on the source, it can be rich in minerals such as K, Fe, Mn, Cu and Zn. It holds water well and at the same time provides good drainage and aeration, especially when mixed with coarse sand (Herrera-Moreno *et al.*, 2013). Some of the coco peat-containing media used in this study (PS & CP and V & CP) also had high water holding capacities compared to the other media used (Table 5.1).

Sand is a heavy medium with a high bulk density and provides good support for cuttings. It has a low water holding capacity, but improves water drainage and aeration when mixed with other substrates. Hume (1949) reported that the water holding capacity of sand was improved from 24.3% to 33.2% when coco peat was added, similarly as reported here for the sand and coco peat (S & CP) medium. The most suitable rooting medium for cuttings must be porous with a pH of 5.5–6.5 and well-drained to ensure availability of oxygen and nutrients (Magesa *et al.*, 2018). Media used in this study mostly meet these requirements.

5.3.2 Rooting responses

Both cutting types (apical and basal) from the control and the IBA treatments had formed adventitious roots in all five tested rooting media after four weeks of culture, although the rooting performance and growth parameters of cuttings differed between treatments. The effect of cutting type, IBA treatment [Dynaroot 1 (0.1% IBA) and Dynaroot 2 (0.3% IBA)] and rooting medium [potting soil and coco peat (PS & CP), potting soil, vermiculite and sand (PS, V & S), sand and coco peat (S & CP), vermiculite (V) and vermiculite and coco peat (V & CP)] on survival and rooting of cuttings, and cutting growth parameters are presented below.

5.3.2.1 Survival of cuttings

5.3.2.1.1 Effect of cutting type

The cutting type had no significant effect on the survival of cuttings (Appendix C: Table 1.1). High survival percentages in both apical (89%) and basal (91%) cuttings of *H. coddii* subsp. *barnardii* were observed after four weeks. The survival percentage in other plant species was dependent on cutting type. Apical cuttings of *Momordica dioica* showed better survival (61.7%) than basal cuttings (41%) (Padekar *et al.*, 2018). However, De Souza *et al.* (2015) reported 100% survival in basal cuttings of *H. rosa-sinensis* cv. Snow Queen, while significantly fewer (30%) apical cuttings survived. Mahmood *et al.* (2017) also reported a higher survival (48.5%) in basal cuttings of *Paulownia tomentosa* compared to 31.2% survival of apical cuttings. The apical cuttings are less lignified, have less phenolic compounds and more meristematic activity, but are more susceptible to moisture loss that cause drying-out of cuttings which could lead to lower survival percentages (Bona *et al.*, 2012; Ibironke, 2013; De Souza *et al.*, 2015).

5.3.2.1.2 Effect of IBA treatment

The IBA treatment and the rooting medium significantly affected the survival of *H. coddii* subsp. *barnardii* cuttings (Appendix C: Table 1.1). The survival percentage significantly improved with the application of IBA-containing rooting hormone powder. Almost all (99%) of the cuttings that received an IBA treatment [Dynaroot 1 (0.1% IBA) and Dynaroot 2 (0.3% IBA)] survived probably due to root formation, whereas untreated cuttings (control) had a significantly lower survival percentage (71.3%) (Figure 5.4).

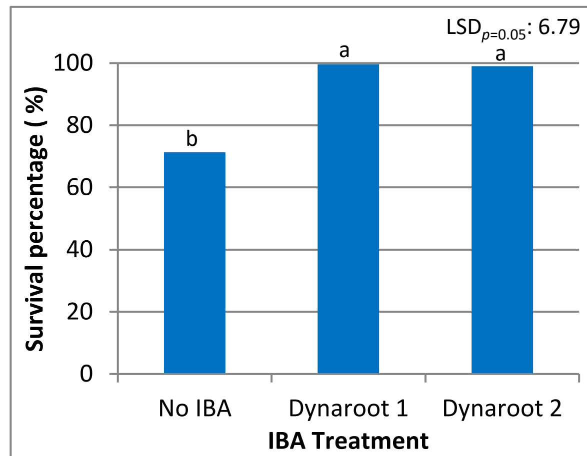


Figure 5.4. Effect of IBA treatment on survival of *H. coddii* subsp. *barnardii* cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA.

Swarts *et al.* (2018) also reported an improved survival percentage (72.7%) in *Lobostemon fruticosus* cuttings with the application of Seradix® B No.2 rooting powder (0.3% IBA) compared to 63.7% survival in control cuttings. This was lower than the survival for treated and untreated cuttings of *H. coddii* subsp. *barnardii*. Similarly, Torkashvand and Shadparvar (2012) reported a higher survival rate (68%) for *H. rosa-sinensis* (Yellow double hybrid) hardwood cuttings treated with 2000 and 4000 mg L^{-1} IBA, while only 23.3% of untreated cuttings survived. Untreated cuttings of *Camellia sinensis* also had a low survival (24.6%) compared to 57.6% survival of cuttings treated with 4000 ppm IBA solution (Hoque, 2016). In contrast, Sabatino *et al.* (2014) found no difference in the survival percentage (97–98%) of *Teucrium fruticans* control cuttings and cuttings treated with 0.5% IBA solution. Similar as in *H. coddii* subsp. *barnardii*, the IBA concentration (0.3% and 0.8%) caused no significant difference in the survival percentage, 69% and 68.3% respectively, of *Cyclopia subternata* cuttings treated with rooting powder (Mabizela *et al.*, 2017). Cuttings of *H. coddii* subsp. *barnardii* treated with Dynaroot 1 and 2 formed roots faster than untreated cuttings, which could have contributed to the higher survival percentage as also reported by Torkashvand and Shadparvar (2012).

5.3.2.1.3 Effect of rooting medium

Survival of *H. coddii* subsp. *barnardii* cuttings in the various rooting media ranged from 83.3% to 96.7%. Cuttings rooted in media with potting soil namely mixtures of potting soil and coco peat (PS & CP) and potting soil, vermiculite and sand (PS, V &

S) showed the highest survival, 96.7% and 95% respectively. A significantly lower survival rate for cuttings in vermiculite (V) and vermiculite and coco peat (V & CP), 85.6% and 83.3% respectively, was observed (Figure 5.5).

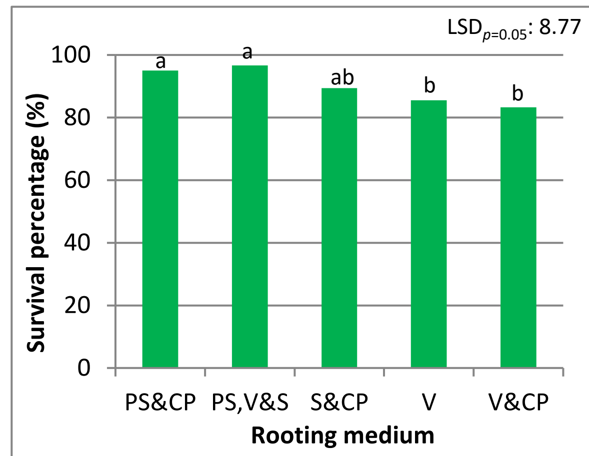


Figure 5.5. Effect of rooting medium on survival of *H. coddii* subsp. *barnardii* cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance. PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

Cuttings from other plant species showed varying survival percentages depending on the rooting medium used. Sardoei (2014) reported an average survival of 98% for *Simmondsia chinensis* cuttings rooted in four media namely a peat moss, vermiculite and perlite [(1:1:1 (v/v/v)] mixture, coco peat alone and mixed respectively with sand and perlite in a 1:1 (v/v) ratio with no significant differences between the media. Mabizela *et al.* (2017) found a significantly higher survival (90%) of *C. subternata* cuttings in a 3-mix medium (Canadian peat moss, sterilised river sand and polystyrene balls) and bark mix medium (fermented pine bark and sterilised river sand) than the 80% survival in a peat mix medium (Canadian peat moss and sterilised river sand). Hardwood cuttings of *H. rosa-sinensis* showed the highest cutting survival percentage (65%) in a coco peat and perlite mixture followed by 57% in coco peat and only 35% survival in sand (Torkashvand and Shadparvar, 2012). Mesén *et al.* (1997) reported rotting of *Cordia alliodora* cuttings rooted in sawdust, which was attributed to the dense medium. Such media with low porosity and limited space between particles could retain too much water and cause reduced oxygen availability at the base of cuttings. This would result in rotting of cuttings and a low survival percentage (Sardoei, 2014; Sedaghat *et al.*, 2017; Magesa *et al.*, 2018). Limited rotting of *H. coddii* subsp. *barnardii* cuttings was observed in more dense

media containing potting soil [(PS & CP) and (PS, V & S)] since high survival percentages were noticed on these media. On the other hand, loose media such as vermiculite (V) and vermiculite and coco peat (V & CP), if not watered regularly, can be prone to drying out (Farhan *et al*., 2018) which might have contributed to the lower cutting survival observed in these media.

5.3.2.2 Rooting percentage of cuttings

Adventitious roots formed in both types of *H. coddii* subsp. *barnardii* cuttings in all tested rooting media, but the response was strongly influenced by the IBA treatment. First roots were visible after seven days of culture in IBA treated cuttings only in the vermiculite (V) and the vermiculite and coco peat (V & CP) media (Figure 5.6A). Adventitious roots were visible in IBA treated cuttings in all the rooting media after 14 days (Figure 5.6B) and on cuttings from all treatments, including the control, after three weeks (Figure 5.6C). Bertram (1992) also observed formation of adventitious roots after 14 days on cuttings of three varieties of *H. rosa-sinensis* and stated that fast root formation results in better growth and higher quality of the newly generated plant. More roots per cutting would also lead to improved transplanting success and survival of the new plant, which are required in commercial production of plants (Baldotto *et al*., 2015; Kleynhans *et al*., 2017).

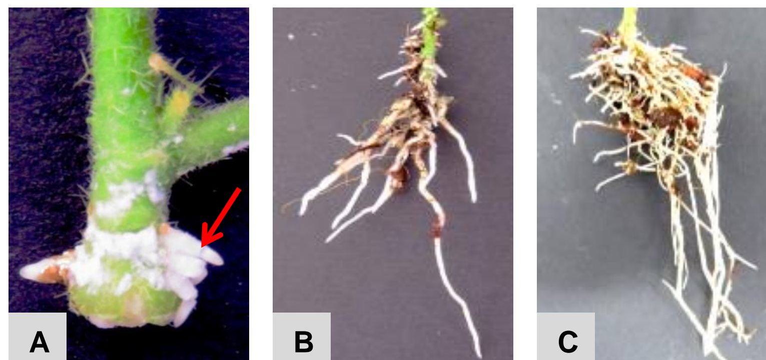


Figure 5.6. Examples of adventitious root formation on (A) Apical stem cuttings treated with Dynaroot 1 showing protrusion of short roots 7 days after planting in vermiculite. Basal stem cutting treated with Dynaroot 2 in vermiculite and coco peat medium [V & CP] with longer roots (B) after 14 days and (C) 21 days of culture.

Root formation on apical and basal cuttings of *H. coddii* subsp. *barnardii* from the control and IBA treatments in the five rooting media is shown in Figure 5.7. The length of the formed roots was not measured, but roots would mostly cover the full length (100 mm) of the seedling plug after four weeks of culture.

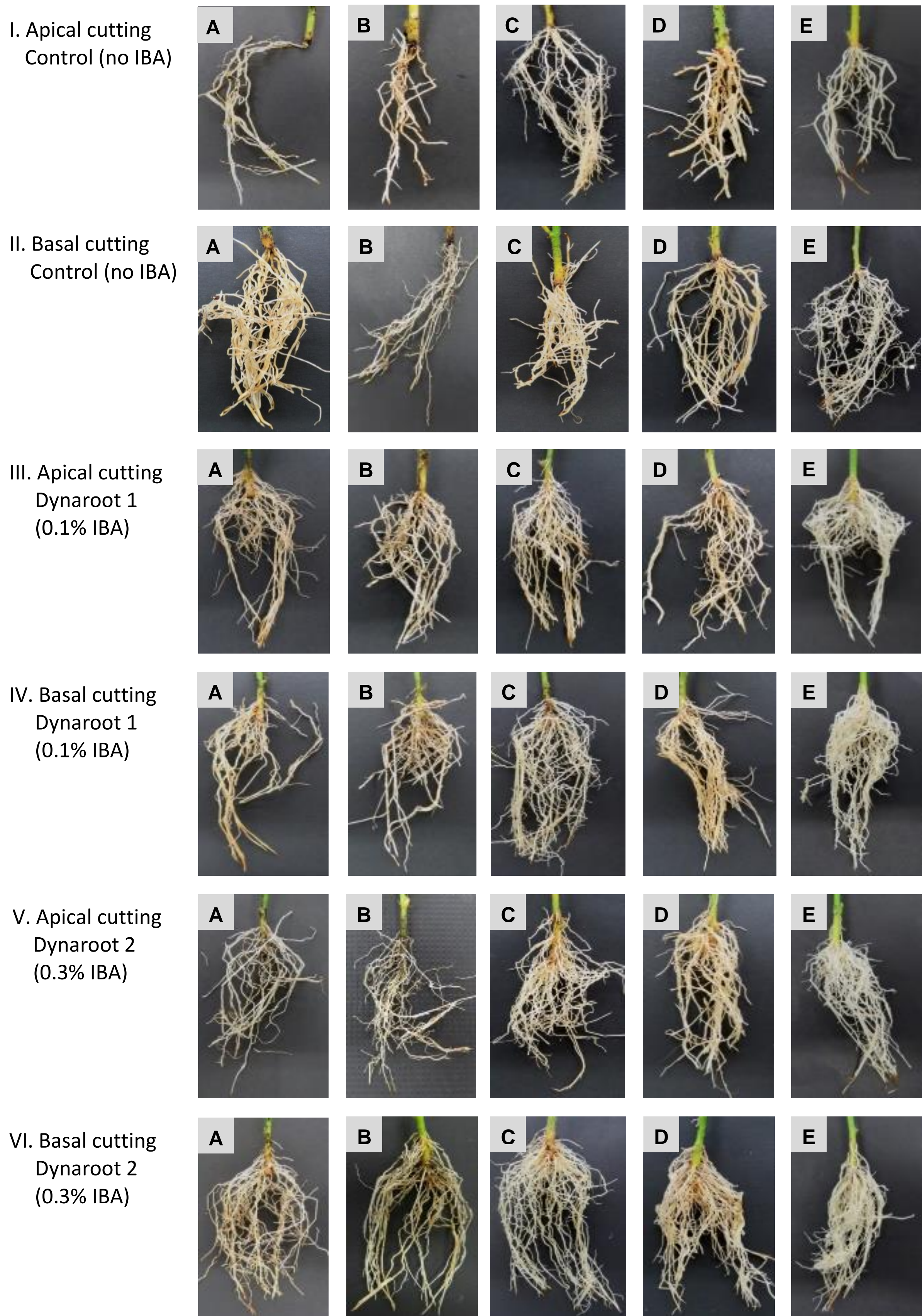


Figure 5.7. Examples of adventitious root formation on apical and basal cuttings from different IBA treatments in various rooting media. (A) Potting soil and coco peat [PS & CP]; (B) Potting soil, vermiculite and sand [PS, V & S]; (C) Sand and coco peat [S & CP]; (D) Vermiculite [V]; (E) Vermiculite and coco peat [V & CP].

Formation of vigorous (thick and strong) roots was visible in cuttings in sand and coco peat (S & CP), vermiculite (V) and in vermiculite and coco peat (V & CP) media, mainly in cuttings that received an IBA treatment (Figure 5.7 III–VI, C–E) that was more pronounced on basal cuttings treated with Dynaroot 2 as compared to other media. Roots would break off easily from cuttings rooted in media with potting soil, especially in apical cuttings not treated with rooting powder (Figure 5.7 I, A & B).

5.3.2.2.1 Effect of cutting type

The cutting type did not significantly affect the rooting percentage (Appendix C: Table 1.2). Apical cuttings rooted slightly better (78.2%) than the basal cuttings (77.1%), but there was no significant difference between rooting percentages. Bona *et al*. (2012) also reported no significant difference between rooting percentages of apical and basal *Lavandula dentata* cuttings, 97.9% and 93.7% respectively. However, the rooting percentage in apical cuttings (89.9%) of *Athrixia phylicoides* was significantly higher than the 44.7% rooting in the basal cuttings (Araya *et al*., 2007). This was ascribed to the presence of endogenous auxin that is concentrated in the apex of the plant and that promoted root formation (Bona *et al*., 2012). In contrast, De Souza *et al*. (2015) reported a significantly higher rooting percentage (75%) in basal cuttings of *H. rosa-sinenis* cv. Snow Queen than the 3.3% rooting found in apical cuttings. In corroboration, Ibrahim *et al*. (2015) reported better rooting (31%) in basal cuttings of *Lippia citriodora* than in apical cuttings (16.3%). Chowdhuri *et al*. (2017) reported a higher rooting percentage (51%) in semi-hardwood cuttings of *H. rosa-sinensis* in comparison with 34% rooting in softwood cuttings. Overall, the rooting response of cutting types varies between species, although Hartmann *et al*. (2011) reported that basal cuttings could have a better rooting capacity than apical cuttings. Hence, it should be investigated for each plant species.

5.3.2.2.2 Effect of IBA treatment

Treatment of *H. coddii* subsp. *barnardii* cuttings with IBA-containing rooting powder significantly affected the rooting percentage (Appendix C: Table 1.2). High rooting percentages were achieved, although there was no significant difference in the rooting percentage for Dynaroot 1 (88.7%) and Dynaroot 2 (90.7%). Both rooting powders differed from the control where the rooting percentage was significantly reduced to 53.7% (Figure 5.8). Untreated cuttings also developed fewer roots

(Figure 5.7 I & II) than cuttings treated with rooting powder. Despite the lower rooting response in untreated cuttings of *H. coddii* subsp. *barnardii*, roots were still formed which could be due to the presence of endogenous auxin in the apex and leaves of cuttings (Hartmann *et al*., 2011). It is reported that endogenous auxin is transported to the base of the cutting where it will promote adventitious root initiation. Simultaneously, hydrolytic enzymes mobilise stored carbohydrates, especially sugar and starch that are transported to the rooting zone at the cutting base where it provides the necessary energy for cell division and differentiation (Krajnc *et al*., 2013; Steffens and Rasmussen, 2016). Sabatino *et al*. (2014) also attributed the higher rooting percentage observed in apical cuttings of *T. fruticans* with an intact apex to the possible basipetal transport of IAA in cuttings.

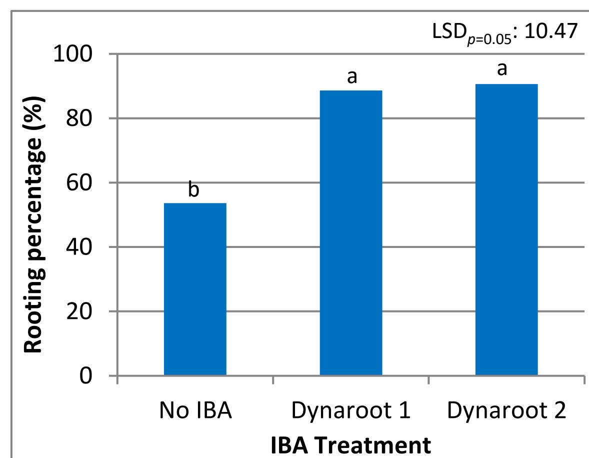


Figure 5.8. Effect of IBA treatment on rooting of *H. coddii* subsp. *barnardii* cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA.

Improvement in the rooting response of stem cuttings with exogenous auxin treatment is well documented. Application of Seradix® B No.2 rooting powder (0.3% IBA) also significantly improved the rooting percentage in *Lobostemon fruticosus* cuttings from 34.4% in the control to 47.8% in treated cuttings (Swarts *et al*., 2018). This rooting percentage, however, is much lower than the 90.7% rooting achieved in *H. coddii* subsp. *barnardii* cuttings with Dynaroot 2 (0.3% IBA). Salaš *et al*. (2012) reported a significant improvement in the rooting percentage of *Weigelia hybrida* cuttings from 8.1% in the control to 94.1% with the application of Rhizopon AA powder containing 1% IBA. The number of roots per cutting also increased from 1.96 roots on untreated to 3.84 roots on IBA treated cuttings. Improvement in the rooting

response with liquid applications of IBA was also reported. Carpenter and Cornell (1992) reported 100% rooting in cuttings of *H. rosa-sinensis* cv. Pink Versicolor when treated with 2500 ppm IBA solution compared to lower rooting (73%) of untreated cuttings. Only 1.7% untreated hardwood cuttings of *H. rosa-sinensis* (Yellow double hybrid) formed adventitious roots which was significantly increased to 45% when treated with 2000 mg L^{-1} IBA solution (Torkashvand and Shadparvar, 2012). Chowdhuri *et al*. (2017) observed low rooting percentages in softwood (34%) and semi-hardwood (51%) cuttings of *H. rosa-sinensis* with no IBA treatment that was improved to 67% and 87.3% respectively, with application of 3000 ppm IBA. Krajnc *et al*. (2013) reported that exogenous auxin stimulates the movement of carbohydrates in the leaves and upper stem to the base of the cutting where it provides energy and the carbon backbone for synthesis of other compounds such as proteins. In contrast, the application of exogenous auxin had an adverse effect on rooting in other species. Auxin did not improve the rooting response in *Lippia origanoides* cuttings. It significantly decreased from 83.3% in untreated cuttings to 43.3% and 36.7% in cuttings treated with 2000 and 4000 mg L^{-1} IBA solution, respectively (Herrera-Moreno *et al*., 2013). Razvi *et al*. (2014) also reported a higher rooting percentage (87.5%) in control cuttings of *Bauhinia purpurea* and reduced rooting of 60% and 50% when treated with 4000 and 6000 ppm IBA respectively. Auxin is required to stimulate the formation of root meristemoids in the induction phase, but excessive amounts of exogenous auxin can become inhibitory and block the outgrowth of root primordia after formation of the meristemoids (De Klerk *et al*., 1999; Saumya *et al*., 2014).

5.3.2.2.3 Effect of rooting medium

In each of the rooting media, more than 70% of *H. coddii* subsp. *barnardii* cuttings formed adventitious roots (Figure 5.9), although there were no significant differences between the means (Appendix C: Table 1.2). The highest rooting percentage (87.2%) was recorded for vermiculite followed by 82.4% in the sand and coco peat (S & CP) and 75.1% in the potting soil, vermiculite and sand (PS, V & S) media. The lowest rooting percentages were observed for cuttings in vermiculite and coco peat (V & CP) and potting soil and coco peat (PS & CP), 72.6% and 71.1% respectively (Figure 5.9).

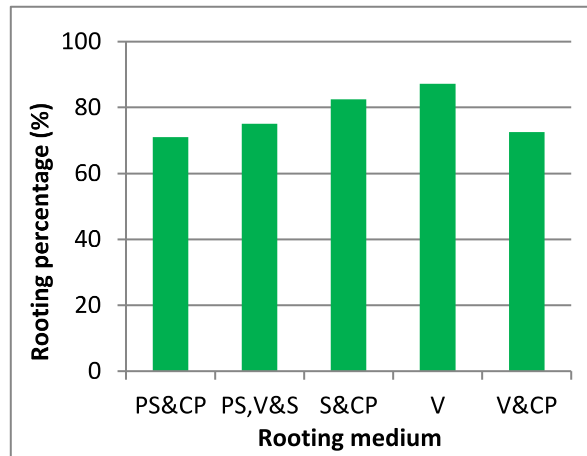


Figure 5.9. Effect of medium on rooting of *H. coddii* subsp. *barnardii* cuttings after four weeks. PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat. No significant difference between means.

Other plant species showed varying rooting responses depending on the rooting medium used. Lee *et al*. (2009) also found no significant differences in the rooting percentage (76.7–96.7%) of *Hydrangea serrata* var. *acuminata* cuttings rooted in four different media. The highest rooting percentage (96.7%) was found in a peat moss, perlite and vermiculite [1:1:1 (v/v/v)] mixture followed by 80% rooting in vermiculite, which was slightly lower than the 87.2% rooting of *H. coddii* subsp. *barnardii* cuttings in vermiculite. Cuttings achieved 76.7% rooting in a vermiculite and coco peat [1:1 (v/v)] mixture, which corresponds to the 72.6% rooting of *H. coddii* subsp. *barnardii* cuttings in this medium. Sardoei (2014) reported only 10% rooting when semi-hardwood cuttings of *Psidium guajava* were rooted in a 1:1 (v/v) mixture of sand and coco peat, unlike the 82.4% observed in *H. coddii* subsp. *barnardii* cuttings in the same medium. Shadparvar *et al*. (2011) reported 66.7% rooting in IBA treated *H. rosa-sinensis* cuttings rooted in a coco peat and perlite medium, while only 33.3% of cuttings rooted in a mixture of sand and perlite. The highest rooting percentage (40%) for cuttings of *H. rosa-sinensis* was obtained in a coco peat and perlite mixture, while only 13% of the cuttings rooted in sand (Torkashvand and Shadparvar, 2012). These rooting percentages were much lower than those attained for *H. coddii* subsp. *barnardii* cuttings.

The selection of the medium used for rooting of cuttings depends on the nature of the plant species and cutting type (Szczepaniak *et al*., 2016). A rooting medium with a well-balanced oxygen and water-holding capacity supports transpiration,

availability of oxygen, nutrient uptake, growth and aeration during adventitious root formation (Ofori *et al*., 1996; Mabizela *et al*., 2017). A fine-textured medium with small pores should be supplemented with a coarse-textured medium to improve porosity and ensure a good aerated environment around the base of the cutting (Szczepaniak *et al*., 2016; Mabizela *et al*., 2017). A well-aerated rooting medium with high porosity as in vermiculite allows the formed roots to penetrate easily into the medium resulting in an extensive root system, which also increases water and nutrient uptake (Rinaldi *et al*, 2017; Sedaghat *et al*., 2017; Magesa *et al*., 2018). On the other hand, if the porosity is too high the medium might not be able to retain enough water necessary for root induction, therefore, the aeration and water-holding capacity of the medium must be balanced to ensure optimal rooting (Ofori *et al*., 1996). For example, coco peat can be added to sand to improve the water holding capacity and nutrient absorption of the medium (Hume, 1949) as also performed in this study. Coco peat provides a good balance of water and air capacity to roots and has a good re-wetting capacity. Sand is infertile and is most often mixed with other compounds to increase drainage and air holding capacity. Vermiculite is added to media to retain water and minerals such as K, Ca and Mg. Composted softwood pine bark is a component of potting soil and has a high air holding capacity (Barrett *et al*., 2016; Farhan *et al*., 2018). These substrates either alone, or mixed in various combinations were suitable for rooting cuttings of *H. coddii* subsp. *barnardii*.

The media used in this study showed different degrees of compaction after root formation. Media containing potting soil as component, were loose and would crumble when removed from the plugs (Figure 5.10A & B). Vermiculite (Figure 5.10D) was also loosely compacted since most of the medium would fall off when the rooted cutting was removed from the seedling plugs. The sand and coco peat mixture (Figure 5.10C) compacted better, although the sand component easily fell off when handled. The vermiculite and coco peat mixture (Figure 5.10E) had a well-compacted root ball with little loss of the medium when removed from the seedling plug, which would make transplanting easier.

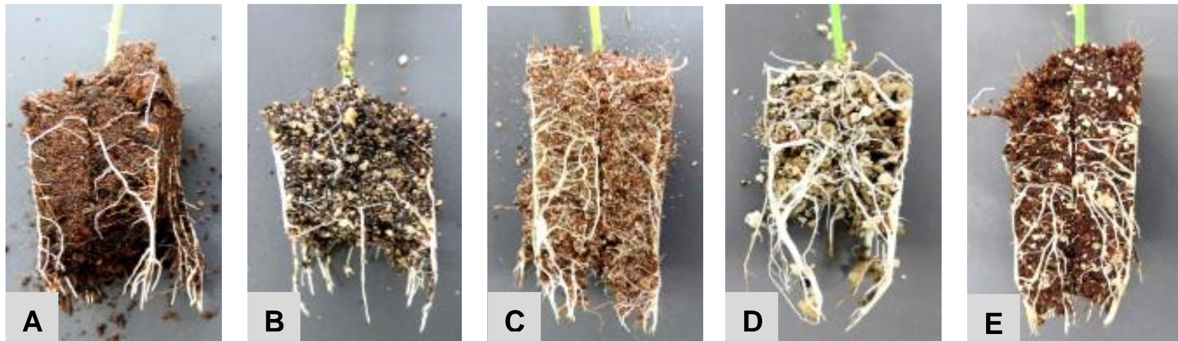


Figure 5.10. Compaction of rooting media after adventitious root formation on a basal cutting treated with Dynaroot 2 rooting powder. (A) Potting soil and coco peat [PS & CP]; (B) Potting soil, vermiculite and sand [PS, V & S]; (C) Sand and coco peat [S & CP]; (D) Vermiculite [V]; (E) Vermiculite and coco peat [V & CP].

5.3.2.3 Quantitative evaluation of adventitious root formation on cuttings

The cutting type, IBA treatment and rooting medium significantly affected the quantity of adventitious roots formed on cuttings (Appendix C: Table 1.3) which was evaluated by a root score system (Section 5.2.5).

5.3.2.3.1 Effect of cutting type

The results indicated that more roots per cutting were formed on basal cuttings of *H. coddii* subsp. *barnardii*. More than half (55%) of basal cuttings formed more than 10 roots per cutting (Score 5), while significantly less (42.4%) of the apical cuttings had a Score of 5 after four weeks. Furthermore, significantly less of the basal cuttings (19.1%) had fewer roots (2–10 roots, Score 4) than the 33.3% of the apical cuttings with 2–10 roots per cutting. In both types of cuttings, 22% of each did not root (Score 1), less than 1% had visible root primordia (Score 2) and less than 3% had a single root (Score 3) at this stage (Figure 5.11).

In contrast to these results, Sabatino *et al*. (2014) described significantly more roots (11.4) on apical cuttings of *T. fruticans* than the 6.1 roots on basal cuttings. Araya *et al*. (2007) also found significantly more roots (19.5) on apical cuttings of *A. phylicoides* than on basal cuttings, which had 12.6 roots. However, Rinaldi *et al*. (2017) reported better rooting on basal cuttings of *Dovyalis* sp. with an average of seven roots formed per cutting compared to an average of four roots on apical cuttings. According to Hartmann *et al*. (2011) basal cuttings could have higher carbohydrate levels than apical cuttings that will benefit root formation.

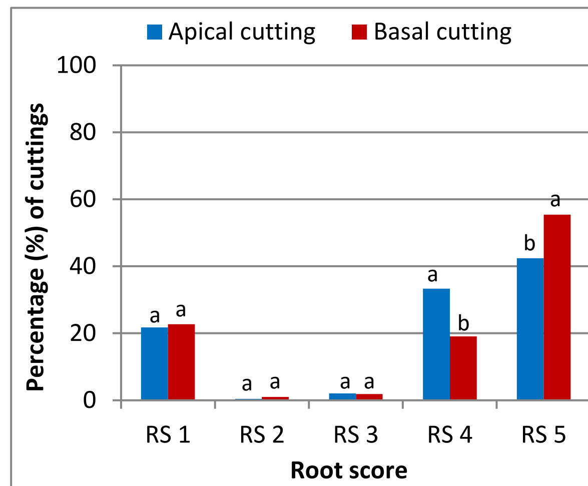


Figure 5.11. Effect of cutting type on the quantity of roots (root score) of cuttings after four weeks. For each root score, means (bars) with the same letters indicate no significant differences at the 5% level of significance. LSD: RS 4: 9.59; RS 5: 8.76. Root score (RS): [1] No roots; [2] Visible root primordia; [3] Single root; [4] 2–10 roots; [5] >10 roots.

5.3.2.3.2 Effect of IBA treatment

The application of IBA-containing rooting powder on cuttings of *H. coddii* subsp. *barnardii* improved the root quantity. Only 15.8% of untreated cuttings (control) had more than 10 roots per cutting (Score 5) which increased to 57.2% of cuttings treated with Dynaroot 1 and significantly improved to 73.7% with the application of Dynaroot 2 (Figure 5.12). In the control treatment, the highest percentage (46.1%) of cuttings had no roots (Score 1) followed by formation of 2–10 roots (Score 4) in 34.2% of these cuttings. Significant lower percentages of cuttings treated with Dynaroot 1 and Dynaroot 2 did not root, 11.3% and 9.3% respectively. In the IBA treatments and the control, less than 3% of cuttings had only visible root primordia (Score 2) or a single root (Score 3) (Figure 5.12).

An increase in the average number of roots on *H. rosa-sinensis* (Yellow double hybrid) cuttings, from 0.17 roots in the control to 2.83 and 2.75 roots treated with 2000 and 4000 mg L^{-1} BA solution respectively, was reported by Torkashvand and Shadparvar (2012). More roots (18) formed on stem cuttings of *T. fruticans* when treated with 0.5% IBA solution compared to only one root in the control cuttings (Sabatino *et al.*, 2014). Furthermore, the number of roots in softwood cuttings of *H. rosa-sinensis* also increased significantly from an average of two roots in the control to 15 roots with the application of 3000 ppm IBA (Chowdhuri *et al.*, 2017).

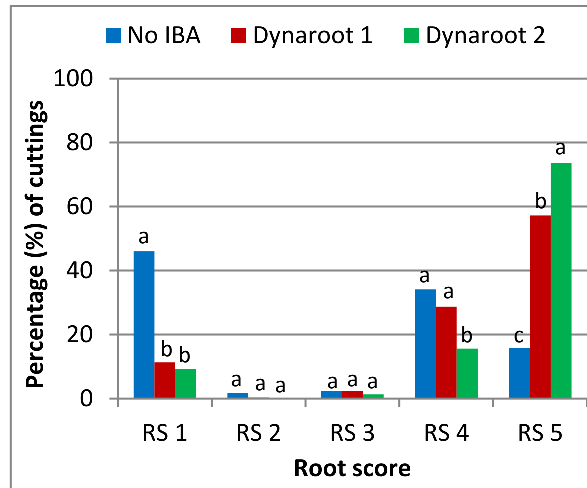


Figure 5.12. Effect of IBA treatment on the quantity of roots (root score) of cuttings after four weeks. For each root score, means (bars) with the same letters indicate no significant differences at the 5% level of significance. LSD: RS 1: 10.65; RS 4: 11.75; RS 5: 10.73. Root score (RS): [1] No roots; [2] Visible root primordia; [3] Single root; [4] 2–10 roots; [5] >10 roots. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA.

A higher IBA concentration (1 g L^{-1}) resulted in the highest average number of roots (25) per softwood cutting of *H. sabdariffa* compared to less roots (16) with the lower (0.5 g L^{-1}) IBA concentration, while the least roots (6) were formed on untreated cuttings (Govinden-Soulange *et al*., 2009) as also seen in *H. coddii* subsp. *barnardii*. In contrast, Herrera-Moreno *et al.* (2013) reported higher rooting percentages and a higher number of roots on untreated cuttings of *Lippia origanoides* compared to cuttings that received an IBA treatment (2000 and 4000 mg L^{-1}). Baul *et al.* (2011) reported no significant difference in the number of roots on untreated cuttings (2.4 roots) of *Litsea monopetala* and cuttings treated with 0.1, 0.2 and 0.4% IBA (2.5, 2.8 and 2.1 roots respectively).

5.3.2.3.3 Effect of rooting medium

The highest percentage (68.7%) of *H. coddii* subsp. *barnardii* cuttings rooted in vermiculite had more than 10 roots per cutting (Score 5) with 18% of these cuttings that had 2–10 roots (Score 4). After 4 weeks, only 12.8% of cuttings in this medium did not root (Score 1), whereas less than 1% of cuttings had only a single root (Score 3) (Figure 5.13). The same trend was observed for cuttings rooted in sand and coco peat (S & CP) and vermiculite and coco peat (V & CP), although the percentage of cuttings with Score 5 was slightly lower in S & CP (56.9%) and significantly lower in V & CP (51.9%). More cuttings in S & CP (17.6%) than in vermiculite (V) did not root, whereas double the percentage (26.9%) of cuttings in V & CP did not root (Figure

5.13). In rooting media that contained a mixture of potting soil and coco peat (PS & CP) and potting soil, vermiculite and sand (PS, V & S), 29% and 25% of cuttings respectively, did not form roots. These two media had a significantly lower percentage (around 33%) of cuttings with more than 10 roots per cutting (Score 5), while another third had 2–10 roots per cutting (Score 4). A significantly higher (5.8%) percentage of cuttings in the PS, V & S medium had only a single root compared to the 1–2% of cuttings in all the other media (Figure 5.13). Cuttings from all treatments that did not form roots or where root formation was delayed were likely to deteriorate and died off.

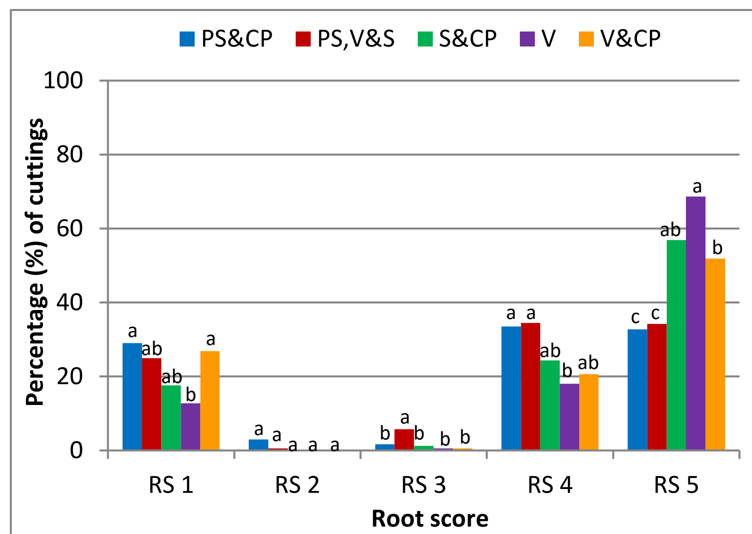


Figure 5.13. Effect of rooting medium on the quantity of roots (root score) of cuttings after four weeks. For each root score, means (bars) with the same letters indicate no significant differences at the 5% level of significance. LSD: RS 3: 3.45; RS 5: 13.85. Root score (RS): [1] No roots; [2] Visible root primordia; [3] Single root; [4] 2–10 roots; [5] >10 roots. Rooting media: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

Unlike in this study, Lee *et al*., (2009) reported the highest number of roots (93.8) per cutting in *H. serrata* var. *acuminata* cuttings rooted in a vermiculite and coco peat ([1:1 (v/v)] mixture and a lower number of roots per cutting (63.1) in vermiculite. The lowest number of roots (51.2) was obtained in a coco peat and perlite [1:1 (v/v)] mixture. In *Pelargonium sidoides*, Pholo *et al*. (2013) found a higher number of roots per cutting (18.5) in sand which differed significantly from the 11.4 roots in a sand and coco peat [2:1 (v/v)] mixture. Mesén *et al*. (1997) also found more roots per cutting (7.5) of *Cordia alliodora* rooted in sand than in sawdust with 3.5 roots. Araya *et al*. (2007) reported a higher number of roots (19) per cutting of *A. phylicoides* rooted in sand compared to 16 roots per cutting in a pine bark medium. Sand is a

porous medium that facilitates the proliferation of roots through the medium as also seen in this study for cuttings rooted in a sand and coco peat (S & CP) medium. A rooting medium that provides sufficient aeration and exchange of gases results in better root penetration and formation of a large number of roots on cuttings (Magesa *et al*., 2018). Media with a high water holding capacity such as sawdust is associated with high water uptake by cuttings that can increase rooting percentages. However, too much water might prevent sufficient oxygen diffusion to the cutting base leading to anoxia and subsequent rotting of cuttings (Mesén *et al*., 1997). Formation of a large number of roots on cuttings as seen in this study with the vermiculite (V), sand and coco peat (S & CP), and vermiculite and coco peat (V & CP) media will increase the survival of regenerated plants in pots or in the soil (Chowdhuri *et al*., 2017).

5.3.2.4 Position of origin of adventitious roots on cuttings

Adventitious roots formed at the wound edge (Position 1) in the control cuttings of *H. coddii* subsp. *barnardii* in all the media (Figure 5.7 I & II), whereas roots formed at the wound edge, at and above the lowest node, and sometimes at upper node(s) (Position 3) in cuttings treated with both Dynaroot 1 and 2 powder (Figure 5.7 III–VI). The position of origin of adventitious root formation on the cuttings was significantly affected by cutting type, IBA treatment and rooting medium (Appendix C: Table 1.4).

5.3.2.4.1 Effect of cutting type

In both apical and basal cuttings, around 30% of cuttings formed roots at the wound edge (Position 1). Furthermore, 28.9% of apical cuttings formed roots at the wound edge and lowest node (Position 2), while significantly less (13.5%) of the basal cuttings formed roots in this position. Most (55%) of the basal cuttings had root formation at the wound edge, at and above the lowest node, and sometimes at upper node(s) (Position 3) which was significantly higher than the 38.9% of the apical cuttings. Basal cuttings seemed to enable root formation on a greater surface of the cutting (Figure 5.14).

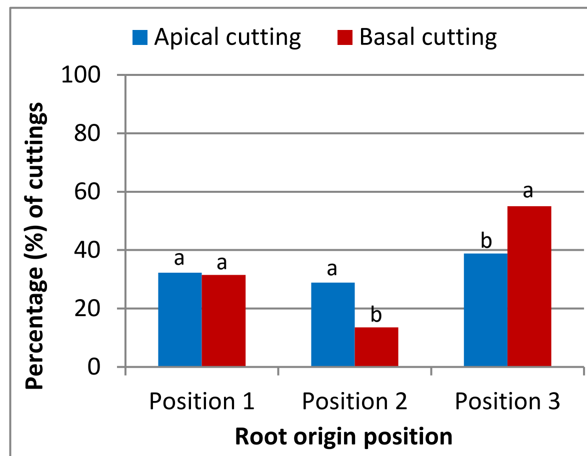


Figure 5.14. Effect of cutting type on the position of origin of adventitious roots on cuttings after four weeks. For each position of root origin, means (bars) with the same letters indicate no significant differences at the 5% level of significance. LSD: Position 2: 7.37; Position 3: 7.91. Position: [1] Roots at wound edge; [2] Roots at wound edge and lowest node; [3] Roots at wound edge, at and above the lowest node, and sometimes at upper node(s).

5.3.2.4.2 Effect of IBA treatment

In the absence of IBA, 99.6% of cuttings formed adventitious roots at the wound edge (Position 1). The application of rooting powder led to the formation of roots at the wound edge and lowest node (Position 2) and at the wound edge, at and above the lowest node, and sometimes at upper node(s) (Position 3) in most of the cuttings. The IBA strength, however, caused significant differences in the formation of roots at Position 2 and 3. Most of the IBA treated cuttings formed roots at Position 3, although Dynaroot 2 had a significantly more pronounced effect (78.2%) on root formation in this position than Dynaroot 1 (55.1%) (Figure 5.15).

In the control cuttings of *H. coddii* subsp. *barnardii* the adventitious roots appeared at the wound edge of the cutting which could be attributed to the movement of endogenous auxin (IAA) down the stem to the base of the cutting where it initiated adventitious root primordia (Haissig, 1974; Husen and Pal, 2007). The application of exogenous IBA affected the appearance of adventitious roots on the epidermis higher up on the *H. coddii* subsp. *barnardii* cuttings. The appearance of roots in Position 2 and 3 shows that application of talc powder containing IBA was suitable for the development of a strong adventitious root system in cuttings of *H. coddi* subsp. *barnardii*. The IBA present in the Dynaroot rooting powder was probably taken up into the stem at the exposed wounded surface of the *H. coddii* subsp.

barnardii cuttings, and moved higher up in the stem via the vascular system as reported by Geneve (2000). In contrast, cuttings of *Euonymus kiautschovicus* and *Dendranthema morifolium* formed adventitious roots only at the bottom of the cutting when treated with auxin containing talc powder (Geneve, 2000). It is reported that exogenous IBA can also increase hydrogen peroxide production, which promotes root formation (Davies and Hartmann, 1988; Steffens and Rasmussen, 2016).

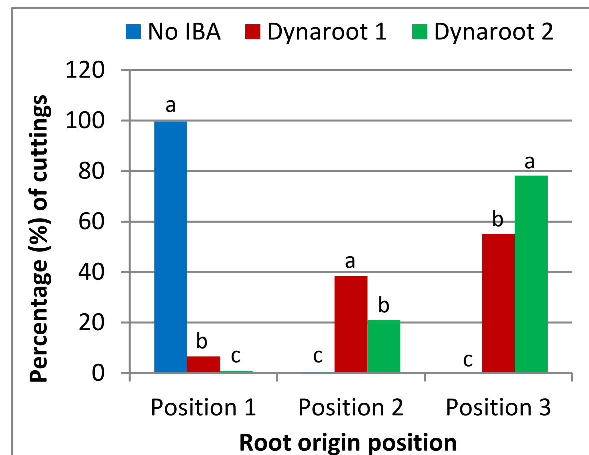


Figure 5.15. Effect of IBA treatment on the position of origin of adventitious roots on cuttings after four weeks. For each position of root origin, means (bars) with the same letters indicate no significant differences at the 5% level of significance. LSD: Position 1: 3.43; Position 2: 9.06; Position 3: 9.73. Position: [1]: Roots at wound edge; [2] Roots at wound edge and lowest node; [3] Roots at wound edge, at and above the lowest node, and sometimes at apical node(s). Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA.

5.3.2.4.3 Effect of rooting medium

The best rooting medium for induction of roots at Position 3 [(wound edge, at and above the lowest node, and sometimes at upper node(s)] was vermiculite (71.1%) followed by sand and coco peat (S & CP) and vermiculite and coco peat (V & CP) with significantly lower percentages, 55.8% and 47.8% respectively. Despite also containing coco peat, the most unsuitable rooting medium for forming roots in Position 3 was potting soil and coco peat (PS & CP) with 21.3%. Most of the cuttings in this medium (41.2%) formed roots at Position 2 (at the wound edge and lowest node). Almost 38% of these cuttings and cuttings in potting soil, vermiculite and sand (PS, V & S) formed roots at the wound edge which differed significantly from cuttings in vermiculite (V) and vermiculite and coco peat (V & CP) (Figure 5.16).

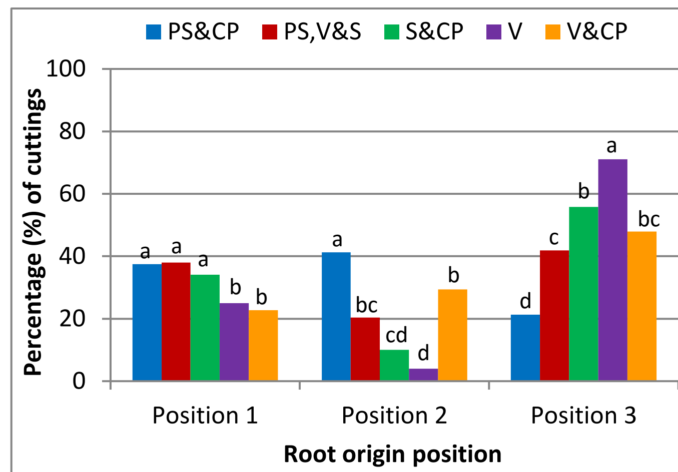


Figure 5.16. Effect of rooting medium on the position of origin of adventitious roots on cuttings after four weeks. For each position of root origin, means (bars) with the same letters indicate no significant differences at the 5% level of significance. LSD: Position 1: 4.43; Position 2: 11.69; Position 3: 12.55. Position: [1] Roots at wound edge; [2] Roots at wound edge and lowest node; [3] Roots at wound edge, at and above the lowest node, and sometimes at upper node(s). Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.2.5 Dry root biomass of cuttings

The fresh and dry root biomass of the *H. coddii* subsp. *barnardii* cuttings was significantly affected by the cutting type, IBA treatment and rooting medium (Appendix C: Table 1.5 and 1.6). Since the fresh and dry root biomass showed the same trend and significant differences, only the results for the dry root biomass are presented.

5.3.2.5.1 Effect of cutting type

Basal cuttings of *H. coddii* subsp. *barnardii* had a significantly higher dry root biomass (0.033 g) than the apical cuttings (0.027 g) (Figure 5.17A). De Souza *et al.* 2015 also found the highest dry root mass (0.04 g) on basal cuttings of *H. rosa-sinenis* cv. Snow Queen, while the apical cuttings had no dry root mass since only 3.3% of the cuttings rooted. In contrast, a significantly higher dry root mass (0.12 g) was obtained from apical cuttings *Lavandula dentata* compared to the 0.02 g dry root mass of basal cuttings (Bona *et al.*, 2012).

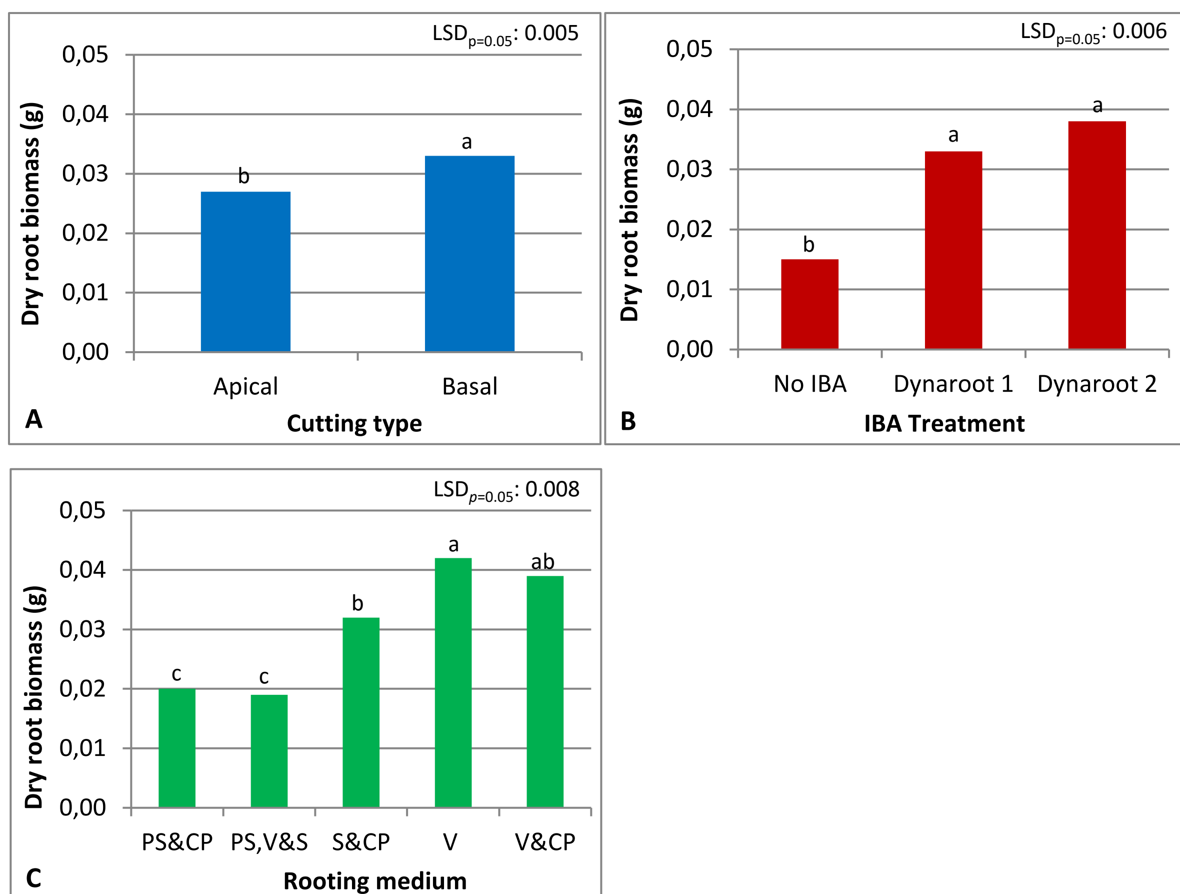


Figure 5.17. Effect of different factors on the dry root biomass of cuttings after four weeks. (A) Cutting type; (B) IBA treatment; (C) Rooting medium. Means (bars) with the same letter are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA.
PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.2.5.2 Effect of IBA treatment

The application of IBA on the *H. coddii* subsp. *barnardii* cuttings resulted in a significantly higher dry root biomass than in untreated cuttings with no significant difference in dry root biomass between Dynaroot 1 and Dynaroot 2 (Figure 5.17B). Baldotto *et al.* (2015) also reported a higher (1.07 g) dry root biomass in *H. rosa-sinensis* cuttings treated with 1000 mg L^{-1} (0.1%) IBA, than the dry root biomass of control cuttings (0.67 g).

5.3.2.5.3 Effect of rooting medium

The highest dry root biomass (0.042 g) was recorded for *H. coddii* subsp. *barnardii* cuttings rooted in vermiculite (V). This did not differ significantly from the dry root biomass for cuttings in the vermiculite and coco peat (V & CP) medium, but differed significantly from that of cuttings rooted in the sand and coco peat (S & CP) medium

(Figure 5.17C). These results correlate with the higher number of strong and thick roots formed in these media. When evaluating the rooting response in different media, as in this study, it is important to consider both parameters (number of roots and dry root biomass) when choosing the best medium. A stronger root system (higher dry root biomass), that can withstand transplanting better, is important for survival of plants. The lowest dry root biomass was recorded for cuttings rooted in media that contained potting soil [potting soil and coco peat (PS & CP) and potting soil, vermiculite and sand (PS, V & S)] which was significantly lower than the dry root biomass of cuttings rooted in the other three media (Figure 5.17C). In contrast to *H. coddii* subsp. *barnardii*, Lee *et al.* (2009) reported a significant higher dry root mass (35 g) in cuttings of *H. serrata* var. *acuminata* rooted in coco peat and vermiculite [1:1 (v/v)], while cuttings rooted in vermiculite had half the dry root biomass (17 g) of that. The lowest dry root biomass (14.7 g) was found in cuttings rooted in coco peat and perlite [1:1 (v/v)]. Herrera-Moreno *et al.* (2013) described no significant difference in the dry root biomass of cuttings of *Tagetes zypaquirensis* rooted in coco peat alone and in mixtures with coal slag [1:1 (v/v)], and coal slag and river sand [1:1 (v/v)].

H. coddii subsp. *barnardii* plants are herbaceous and since young plants were used as stock plants for cuttings, lignification might not have been present in basal cuttings yet. Basal cuttings were produced first on the stem and might have accumulated more carbohydrates, nitrogenous substances, amino acids and phenolic compounds than the apical cuttings before being excised (Govinden-Soulange *et al.*, 2009; De Souza *et al.*, 2015; Rinaldi *et al.*, 2017). Therefore, the better performance with regard to rooting parameters (root score, position of origin of roots and dry root mass) were observed in basal cuttings.

5.3.3 Growth responses of cuttings

Changes (mostly an increase) in growth parameters (length, number of nodes and basal diameter) of surviving rooted cuttings were observed at the end of the experimental period, four weeks after planting. Axillary bud sprouts were also observed in rooted cuttings.

5.3.3.1 Effect of cutting type

The type of stem cutting (apical and basal) had a significant effect on the increase in number of nodes of the cuttings, but not on the increase in length and basal diameter (Appendix C: Table 1.7–1.9).

The basal cuttings had a significantly higher increase (1.66) in the number of nodes than the apical cuttings with an increase of 1.23 nodes (Figure 5.18B). Although not significantly different, the basal cuttings increased more in length and basal diameter than the apical cuttings (Figure 5.18A & C). Qadri *et al.* (2018) also found no significant difference in the cutting diameter of apical (4.41 mm) and basal (4.74 mm) softwood cuttings of *Psidium guajava*.

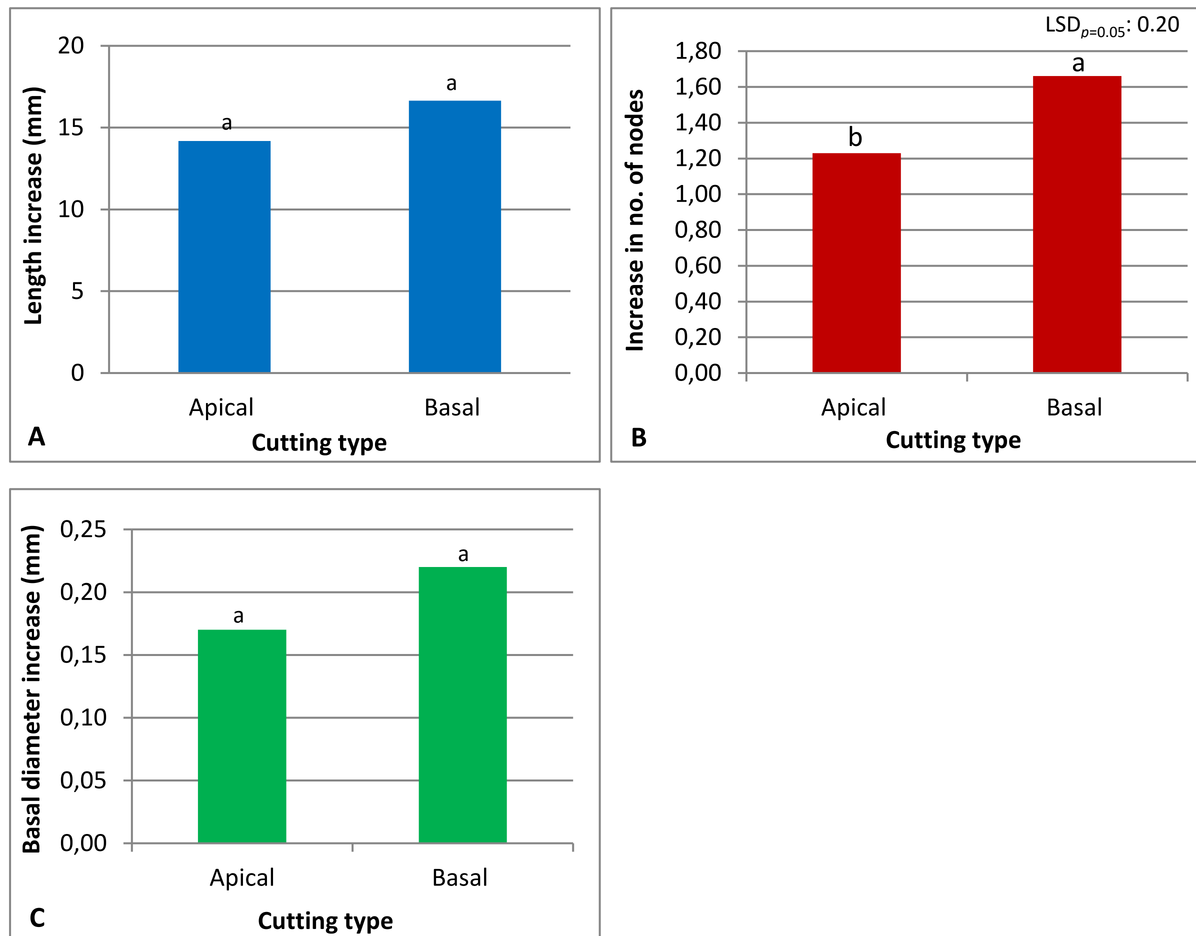


Figure 5.18. Effect of cutting type on growth parameters of cuttings. (A) Increase in length; (B) Increase in number of nodes; (C) Increase in basal diameter. Means (bars) with the same letter are not significantly different at the 5% level of significance.

5.3.3.2 Effect of IBA treatment

Treatment of cuttings with IBA-containing rooting powder caused highly significant differences in the increase in length, nodes and basal diameter of cuttings (Appendix C: Table 1.7–1.9). In the absence of IBA treatment, the lowest average increase in length (8.9 mm), number of nodes (0.9 nodes) and basal diameter (0.1 mm) were observed which differed significantly from values for cuttings treated with both Dynaroot 1 and 2 (Figure 5.19A, B & C). Cuttings treated with rooting powder showed a double increase in the length and number of nodes on cuttings (Figure 5.19A & B) and a 4–5 times increase in the basal diameter (Figure 5.19C) when compared to that of the control cuttings with no significant differences between IBA treatments.

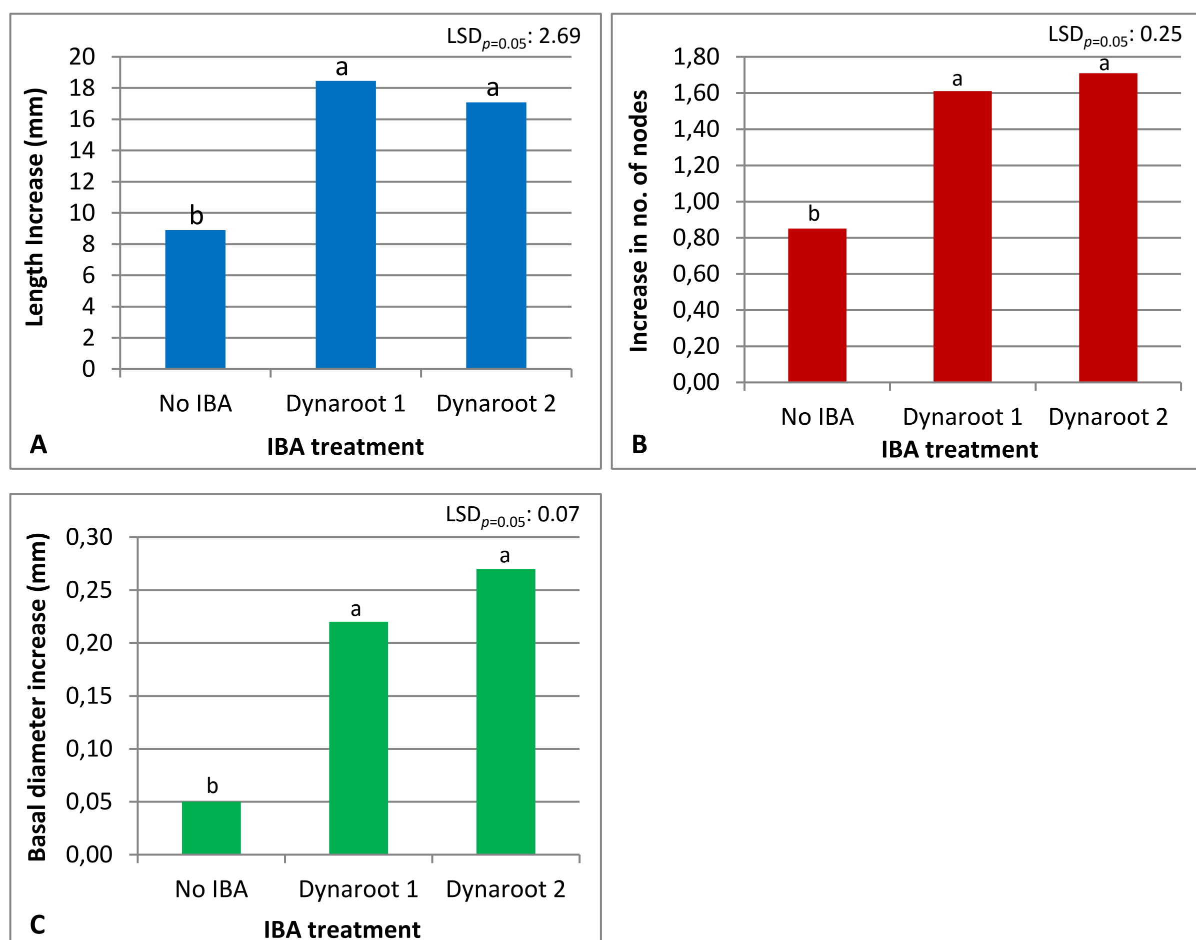


Figure 5.19. Effect of IBA treatment on growth parameters of cuttings. (A) Increase in length; (B) Increase in number of nodes; (C) Increase in basal diameter. Means (bars) with the same letter are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA.

Softwood cuttings of *H. sabdariffa* showed the highest increase in length (28 mm) with a lower (0.5 g L^{-1}) concentration of IBA, followed by untreated cuttings with 19 mm, while the length increase was reduced to 14 mm with a higher (1 g L^{-1}) IBA concentration. Hoque (2016) reported that the length of *Camellia sinensis* cuttings progressively increased with an increase in the IBA concentration. Control cuttings were the shortest (5.43 cm) compared to 12.70 cm and 12.98 cm with 4000 and 6000 ppm IBA treatments respectively. Unlike in *H. coddii* subsp. *barnardii*, the lower concentration (0.5 g L^{-1}) of IBA caused the highest increase (2.7 mm) in the basal diameter of *H. sabdariffa* cuttings, while the control cuttings also showed the lowest (1.4 mm) increase (Govinden-Soulange *et al.*, 2009). Baldotto *et al.* (2015) also reported the lowest increase in stem diameter (4.78 mm) in control cuttings of *H. rosa-sinensis* cuttings compared to the IBA treatment (1000 mg L^{-1}) with a 5.03 mm increase. Untreated (control) cuttings of *Dovyalis* sp. also had a lower stem diameter increase (1.2 mm) than the 1.3 mm increase in cuttings treated with 1666 mg L^{-1} IBA (Rinaldi *et al.*, 2017). It is evident that the application of IBA also improved growth parameters of cuttings in other plant species, but the response is dependent on the concentration. Growth parameters in *H. coddii* subsp. *barnardii* cuttings also increased with the application of IBA, although the concentration did not cause significant differences in the response.

5.3.3.3 Effect of rooting medium

All growth parameters were significantly affected by the rooting medium (Appendix C: Table 1.7–1.9). Cuttings rooted in vermiculite and coco peat (V & CP) and in vermiculite (V) showed the highest increase in length over the four week period, 21.5 mm and 19.1 mm respectively, with no significant difference between these two media (Figure 5.20A). The lowest increase in length (significant) was observed for cuttings rooted in media containing potting soil with 8.3 mm and 12.7 mm increase in potting soil, vermiculite and sand (PS, V & S) and potting soil and coco peat (PS & CP) media respectively (Figure 5.20A). The highest increase (significant) in number of nodes (2.1) was recorded for cuttings rooted in the vermiculite and coco peat (V & CP) medium, while the lowest increase (significant) was observed in potting soil, vermiculite and sand (PS, V & S) and potting soil and coco peat (PS & CP) media, 0.9 and 1.1 nodes respectively (Figure 5.20B). The increase in basal diameter was the highest (0.4 mm) in cuttings rooted in vermiculite, which differed significantly

from the increase in all other rooting media. The lowest increase was observed in cuttings rooted in potting soil, vermiculite and sand (PS, V & S) and potting soil and coco peat (PS & CP), 0.08 mm and 0.12 mm respectively (Figure 5.20C).

Sardoei and Rahbarian (2014) found no significant differences in the diameter (1–1.16 cm) of *Ficus benjamina* cuttings rooted in a variety of rooting media namely peat moss, coco peat and mixtures [2:1:1 (v/v/v)] of peat moss, sand and perlite and coco peat, sand and perlite. However, the diameter (31 mm) of *Rosmarinus officinalis* cuttings rooted in coco peat differed significantly from the diameter (53 mm) of cuttings in a peat moss, sand and perlite mixture [2:1:1 (v/v/v)] (Sardoei and Rahbarian, 2014).

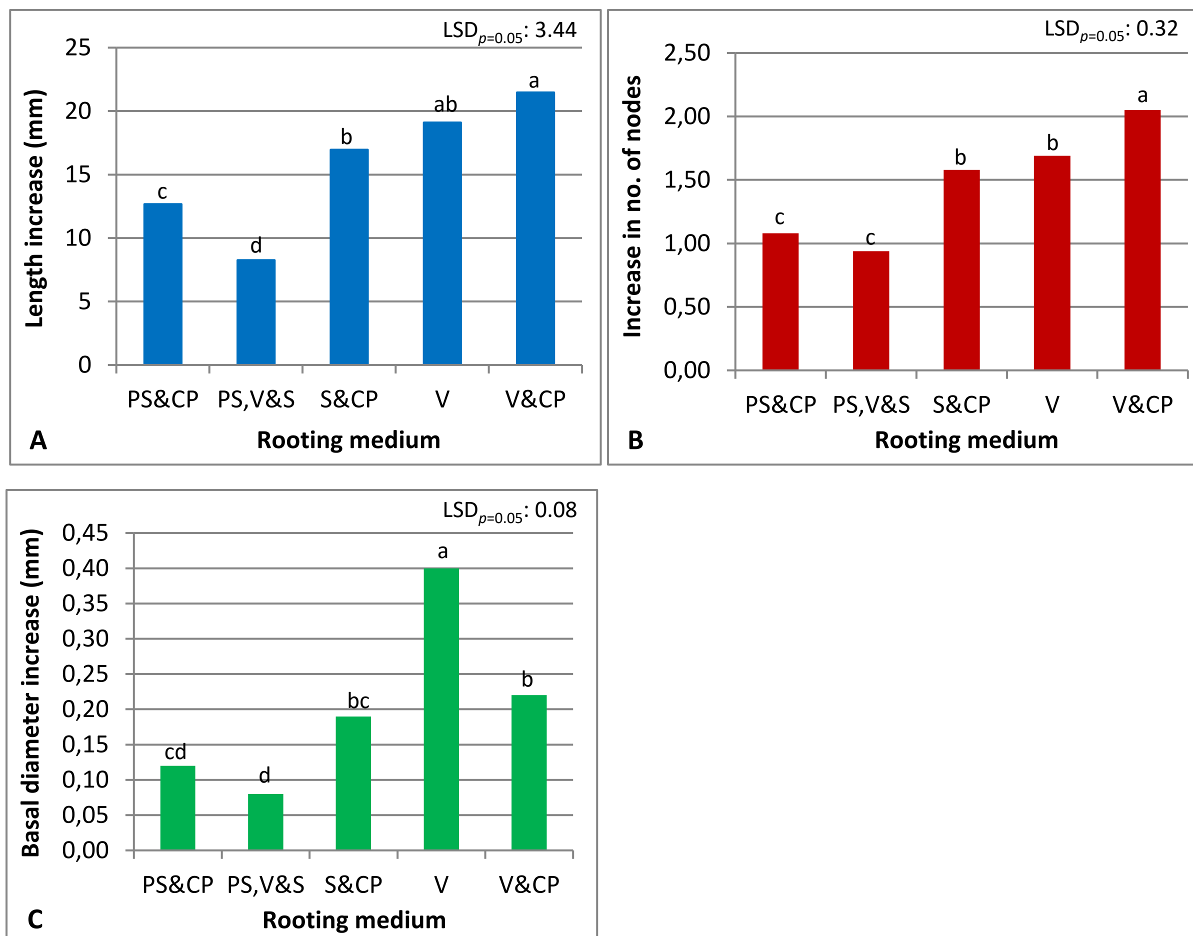


Figure 5.20. Effect of rooting medium on growth parameters of cuttings. (A) Increase in length; (B) Increase in number of nodes; (C) Increase in basal diameter. Means (bars) with the same letter are not significantly different at the 5% level of significance. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.3.4 Sprouting of axillary buds

Axillary buds started to sprout in some of the apical and basal cuttings in all the rooting media irrespective of the auxin treatment (Figure 5.21A). Opening of pre-existing flower buds were also visible on some of the cuttings and new flower buds formed in certain of the cuttings [basal, control, (PS, V & S); apical, Dynaroot 2, (V & CP)] (Figure 5.21B). The cutting type, IBA treatment and rooting medium had a significant effect on sprouting of axillary buds (Appendix C: Table 1.10).

Figure 5.21. (A) Sprouting of axillary buds (arrow) on cuttings after four weeks; (B) Flower bud that formed on a cutting rooted in vermiculite and coco peat.

5.3.3.4.1 Effect of cutting type

A significantly higher percentage (50.2%) of the basal cuttings had sprouted buds compared to the 39.1% of apical cuttings with bud sprouts (Figure 5.22A).

5.3.3.4.2 Effect of IBA treatment

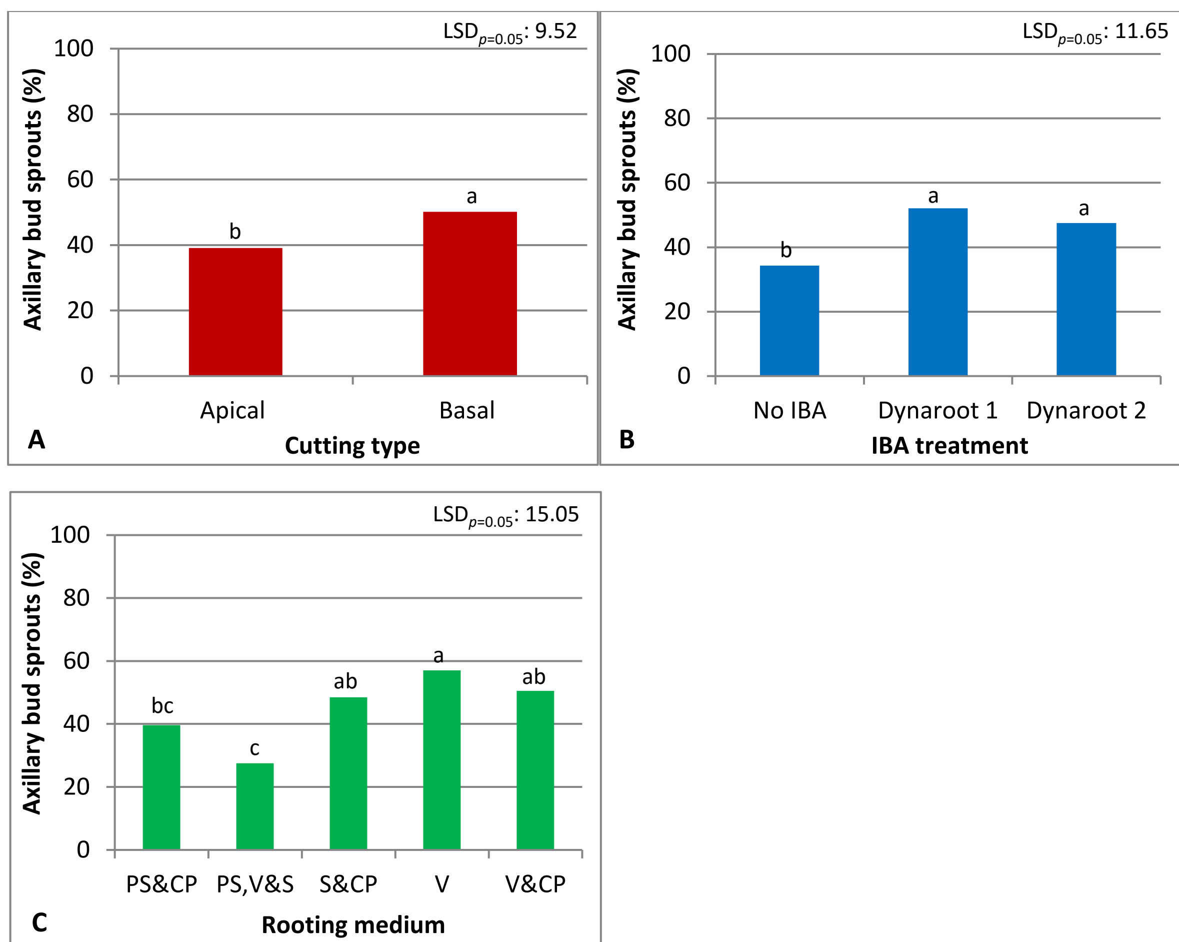
Significantly more cuttings treated with Dynaroot 1 and 2 had bud sprouts, 52.2% and 47.57% respectively, than the untreated cuttings (34.3%) (Figure 5.22B). Auxin treated cuttings of other plant species also had a better bud sprouting response than untreated cuttings. Treatment of *H. rosa-sinensis* (Yellow double hybrid) cuttings with 2000 and 4000 mg L^{-1} IBA solution also resulted in significantly more, 1.8 and 2.3 respectively, bud sprouts per cutting than the average 0.4 buds on the control cuttings (Torkashvand and Shadparvar, 2012). In *Moringa concanensis*, 100% of the softwood cuttings treated with 250 mg L^{-1} IBA had bud sprouts after 8 weeks, while 73% and 77% of cuttings treated with 50 and 400 mg L^{-1} IBA respectively, had bud sprouts. None of the control cuttings survived (Manokari *et al.*, 2016). In contrast, Razvi *et al.* (2014) reported more proliferated shoots (1.4) on untreated cuttings of

Bauhinia purpurea than the 1.1 shoots that proliferated on cuttings treated with 4000 and 6000 ppm IBA.

5.3.3.4.3 Effect of rooting medium

Significantly less of the *H. coddii* subsp. *barnardii* cuttings that rooted in a potting soil mixture medium (PS & CP and PS, V & S) had axillary bud sprouts, 39.7% and 27.5% respectively, than the cuttings rooted in vermiculite (57.1%). No significant difference in bud sprouting was observed between vermiculite (V), sand and coco peat (S & CP) and vermiculite and coco peat (V & CP) (Figure 5.22C). In *H. rosa-sinensis* (Yellow double hybrid) hardwood cuttings grown in a sand medium produced the lowest number of bud sprouts (0.8) compared to cuttings grown in coco peat, and coco peat and perlite medium, 1.4 and 1.9 bud sprouts respectively (Torkashvand and Shadparvar, 2012). Qadri *et al.* (2018) reported the highest percentage of bud sprouts on *Psidium guajava* cuttings rooted in silt medium (also highest rooting response), which differed significantly from the percentage of bud sprouts in the other rooting media (peat moss, sand and sawdust, topsoil, and bagasse and silt).

The high percentage of *H. coddii* subsp. *barnardii* cuttings with axillary bud sprouts was probably due to the high number of roots (Score 4 and 5, Section 5.3.2.4) and high root biomass of cuttings rooted in vermiculite (V), sand and coco peat (S & CP) and vermiculite and coco peat (V & CP). The production of cytokinins in newly formed adventitious roots is associated with the promotion of axillary bud outgrowth when transported upward in the stem via the xylem sap to the shoot (Hartmann *et al.*, 2011; Izadi and Zarei, 2014; Taiz *et al.*, 2015). Hansen and Kristensen (1990) reported a positive relationship between the number of roots formed on cuttings of *Schefflera arboricola* and *Stephanotis floribunda* and the quantity of and time to sprouting of axillary buds. Formation of numerous adventitious roots in a short time also resulted in faster and increased axillary bud break in *H. rosa-sinensis* which promoted vigorous post propagation growth and ensured high quality plants (Bertram, 1992).



5.22. Effect of different factors on sprouting of axillary buds on cuttings after four weeks. (A) Cutting type; (B) IBA treatment; (C) Rooting medium. Means (bars) with the same letters are not significantly different at the 5% level of significance. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

Morphological observations

The rooted cuttings in the various media had a similar morphological appearance (Figure 5.23A–E), except for those in the vermiculite medium where leaf chlorosis was observed (Figure 5.23D). Most plants grow the best in solid medium when the pH of the medium ranges from 5.5 to 7 (Hamza, 2008; Taiz and Zeiger, 2010). The high pH (9.19) recorded in the vermiculite medium used in this study may have negatively affected the availability of mineral elements to the roots, resulting in deficiency symptoms such as chlorosis.

Figure 5.23. Rooted cuttings in various media after four weeks. (A) Potting soil and coco peat [PS & CP); (B) Potting soil, vermiculite and sand [PS, V & S]; (C) Sand and coco peat [S & CP]; (D) Vermiculite [V] (arrow indicates chlorotic leaves); (E) Vermiculite and coco peat [V & CP].

5.3.4 Interactive effect of factors on rooting and cutting growth parameters

In the above results, the effect of a single factor on rooting and cutting growth parameters were presented indicating that basal cuttings of *H. coddii* subsp. *barnardii* treated with Dynaroot 2 rooting powder and rooted in vermiculite (V) and vermiculite and coco peat (V & CP) media gave the best results. However, it is also advisable to look at the interactions between different factors to determine the best vegetative propagation protocol for stem cuttings.

The interaction between the cutting type, IBA treatment, and rooting medium caused significant differences in survival and rooting of cuttings and in cutting growth parameters. The interactive effect of cutting type and rooting medium was significant for the increase in length and for Position 2 of origin of roots (Appendix C: Table 1.7). The interaction between the IBA treatment and rooting medium was significant for all the rooting and cutting growth parameters [survival, rooting of cuttings (including Root score 5 and position of origin), dry root mass, increase in length, the number of

nodes, basal diameter, axillary bud sprouting on cuttings] (Appendix C: Table 1.6–1.9). The interactive effect of cutting type and IBA treatment was only significant for the position of origin of adventitious roots, whereas the interactive effects of the three factors (cutting type, IBA treatment and rooting medium) were not significant for any of the parameters (Appendix C: Table 1.1–1.10). The interaction between cutting type and rooting medium, IBA treatment and rooting medium, and cutting type and rooting medium for Root score 5 and the position of origin of adventitious roots are not presented here.

5.3.4.1 Interactive effect of cutting type and rooting medium on the length of cuttings

Basal cuttings rooted in the potting soil, vermiculite and sand medium (PS, V & S), in vermiculite (V), and in the vermiculite and coco peat (V & CP) medium exhibited a greater increase in length than the apical cuttings in the same rooting media. In contrast, the rooted apical cuttings in the potting soil and coco peat (PS & CP) medium and in the sand and coco peat (S & CP) medium increased more in length than the basal cuttings (Figure 5.24). The difference in length increase between apical and basal cuttings was most evident in the vermiculite (V) and vermiculite and coco peat (V & CP) media. Basal cuttings increased the most in length (25.8 mm) in the vermiculite and coco peat (V & CP) medium followed by cuttings rooted in vermiculite with a length increase of 21.9 mm. The smallest differences in length increase were observed between cuttings rooted in the other three media, two of which contained potting soil (Figure 5.24).

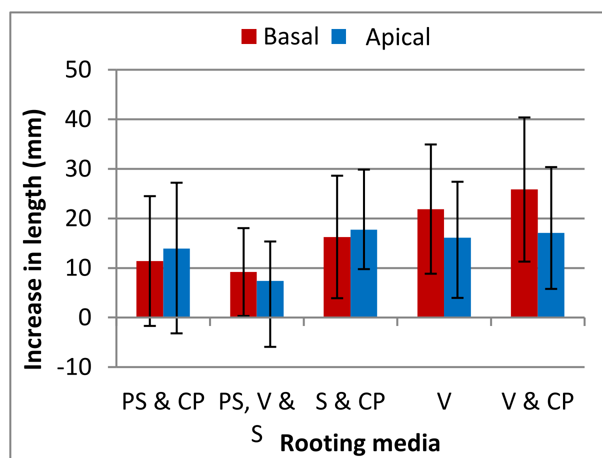


Figure 5.24. The interactive effect of cutting type and rooting medium on length of cuttings after four weeks. Vertical bars represent the standard error.Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.4.2 Interactive effect of IBA treatment and medium on rooting parameters

5.3.4.2.1 Survival of cuttings

The rooting medium and its interaction with the IBA treatment was significant for the survival of stem cuttings in the sand and coco peat (S & CP), vermiculite (V) and vermiculite and coco peat (V & CP) media (Figure 5.25). The mean survival percentage was significantly lower in untreated cuttings (control) rooted in these media. There was no significant interactive effect for survival of cuttings in the potting soil and coco peat (PS & CP) and potting soil, vermiculite and sand (PS, V & S) media. The lowest survival, 50% and 56.7% for untreated cuttings, was noted in the vermiculite and coco peat (V & CP) and vermiculite (V) media respectively (Figure 5.25) which might be due to drying out of the rooting medium that affected the rooting response (no or longer time to rooting) in untreated cuttings.

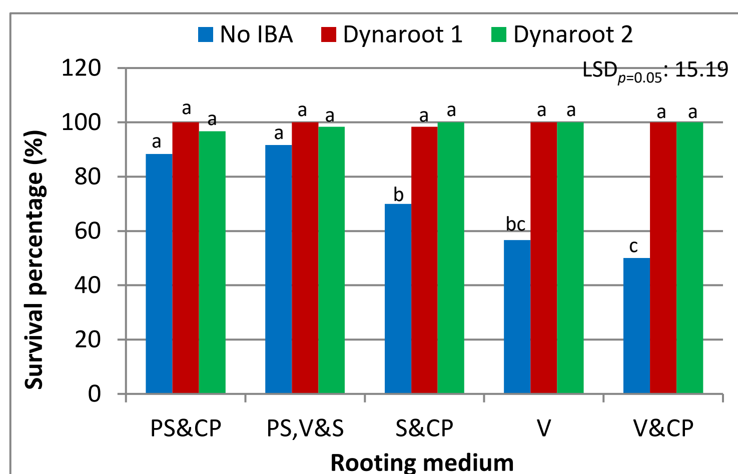


Figure 5.25. The interactive effect of IBA treatment and rooting medium on the survival of cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance.Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

The best survival rate (86.7%) for cuttings of *H. rosa-sinensis* (Yellow double hybrid) was achieved on cuttings treated with 2000 and 4000 mg L^{-1} IBA solution and rooted in a coco peat and perlite mixture followed by 73.3% survival of cuttings with the same IBA treatments in coco peat. The lowest survival rate for untreated cuttings was recorded in sand alone (13.3%) and in a sand and perlite (20%) medium (Torkashvand and Shadparvar, 2012).

5.3.4.2.2 Rooting percentage of cuttings

Significantly more cuttings treated with IBA rooting powder (both Dynaroot 1 and 2) rooted in potting soil, vermiculite and sand (PS, V & S), vermiculite (V) and vermiculite and coco peat (V & CP) media than the untreated (control) cuttings (Figure 5.26). Cuttings of *Azadirachta indica* treated with 500 mg L^{-1} IBA also rooted better (80%) in vermiculite than in sand (60%) (Gehlot *et al*., 2014). Cuttings of *H. coddii* subsp. *barnardii* treated with Dynaroot 1 and rooted in sand and coco peat (S & CP) had a significantly higher rooting percentage than the control cuttings (Figure 5.26).

Similarly, better rooting responses with IBA treatments in various rooting media, was also reported for other species. Untreated (control) cuttings of *Perovskia atriplicifolia* rooted in a perlite and sand medium showed a low rooting percentage (6.7%) compared to 41.3% rooting of cuttings treated with Rhizopon powder (1% IBA) in the same medium (Dumitraşcu, 2008). Shadparvar *et al*. (2011) reported no rooting in untreated (control) cuttings of *H. rosa-sinensis* rooted in a coco peat-perlite medium compared to 67.7% rooting in IBA treated (4000 mg L^{-1} IBA) cuttings in the same medium. In contrast, untreated cuttings of *Lippia origanoides* rooted in coco peat showed the highest rooting percentage (83.3%) which was significantly higher than the 36.6% rooting of cuttings treated with 4000 mg L^{-1} IBA in the same medium. This was also observed in cuttings rooted in mixtures of coco peat and coal slag [1:1 (v/v)] and coco peat, coal slag and river sand [1:1:1 (v/v/v)] (Herrera-Moreno *et al*., 2013).

No significant interaction for the rooting percentage in *H. coddii* subsp. *barnardii* cuttings was observed between Dynaroot 1 and Dynaroot 2 for any of the rooting media (Figure 5.26). In contrast, Shadparvar *et al*. (2011) reported a lower rooting percentage (27%) in cuttings of *H. rosa-sinensis* treated with a lower concentration (1000 mg L^{-1}) of IBA solution and rooted in a peat-perlite medium compared to 67.7% rooting in the same medium with a higher concentration (4000 mg L^{-1}) of IBA solution.

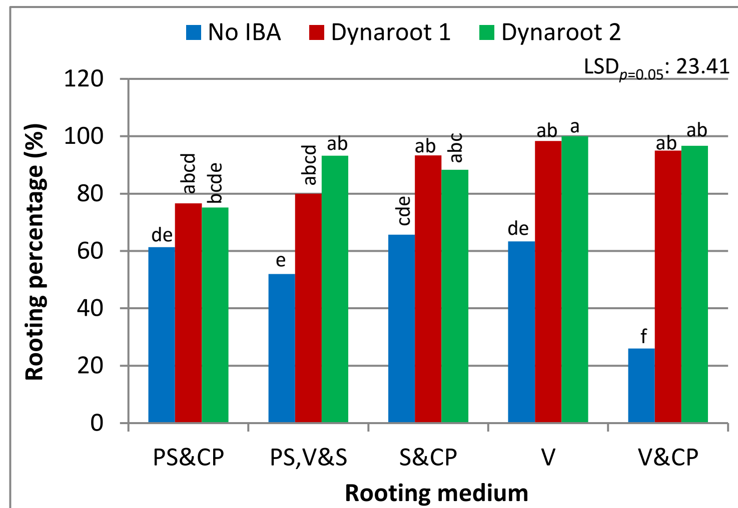


Figure 5.26. The interactive effect of IBA treatment and rooting medium on the rooting percentage of cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance.Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.4.2.3 Dry root biomass

The dry root biomass of control cuttings differed significantly from at least one IBA treatment in all the rooting media, except in potting soil and coco peat (PS & CP) and vermiculite (V) (Figure 5.27). Cuttings treated with Dynaroot 2 and rooted in vermiculite and coco peat (V & CP) had a significantly higher dry root biomass than the cuttings treated with Dynaroot 1. These cuttings also had the highest dry root biomass (0.055 g) (Figure 5.27). In all the other rooting media there was no significant difference in the dry root biomass of IBA treated cuttings (Dynaroot 1 and Dynaroot 2). In the vermiculite (V) medium the dry root biomass of untreated cuttings did not differ significantly from both IBA treatments, but it was significantly better than the control cuttings in all the other rooting media.

In *Lippia alba*, untreated and cuttings treated with 4000 mg L^{-1} IBA rooted in coco peat alone and in mixtures of coco peat and coal slag [1:1 (v/v)] and coco peat, coal slag and river sand [1:1:1 (v/v/v)] showed no significant difference in the dry root mass. However, cuttings treated with 2000 mg L^{-1} IBA and rooted in coco peat alone had a higher dry root mass than those in the other two media (Herrrera-Morena *et al*., 2013).

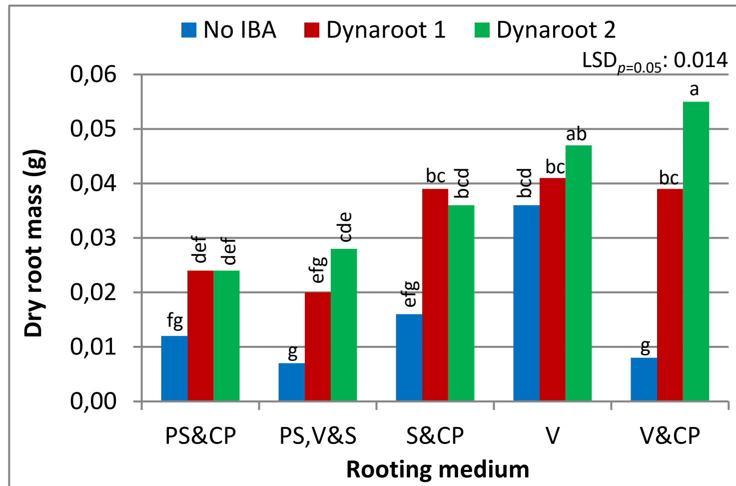


Figure 5.27. The interactive effect of IBA treatment and rooting medium on the dry root mass of cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA, Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.4.3 Interactive effect of IBA treatment and medium on cutting growth parameters

5.3.4.3.1 Length of cuttings

Rooted cuttings in all the rooting media increased in length depending on the IBA treatment. Application of Dynaroot 1 and 2 did not significantly affect the length increase in any of the rooting media, but the medium had a significant effect on the length increase in untreated cuttings (Figure 5.28). The lowest increase in length for all the rooting media, except vermiculite, was recorded for the control cuttings. In the potting soil and coco peat (PS & CP) and vermiculite and coco peat (V & CP) media the length increase in the control cuttings was significantly lower than that of both IBA treatments. No significant difference in length increase was observed between the control and two IBA treatments in the vermiculite (V) and between the control and Dynaroot 1 in potting soil, sand and vermiculite (PS, V & S), and Dynaroot 2 in sand and coco peat (S & CP) (Figure 5.28).

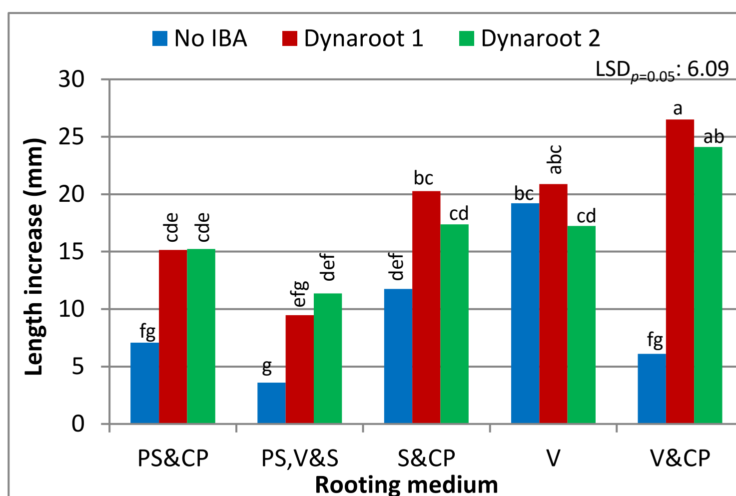


Figure 5.28. The interactive effect of IBA treatment and rooting medium on the length of cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.4.3.2 Number of nodes of cuttings

Cuttings that rooted showed an increase in the number of nodes in all the rooting media, but it was dependent on IBA treatment. The lowest increase in the number of nodes for all the rooting media was recorded for the untreated (control) cuttings (Figure 5.29). Both Dynaroot 1 and 2 did not significantly affect the increase in number of nodes in any of the rooting media.

The highest increase in the number of nodes (2.5) was observed for cuttings treated with Dynaroot 1 rooting power and rooted in vermiculite and coco peat (V & CP) medium. This differed significantly from the increase in node number in IBA treatments and the control in all the other rooting media, except for the cuttings treated with Dynaroot 2 rooting powder and rooted in the same medium (Figure 5.29).

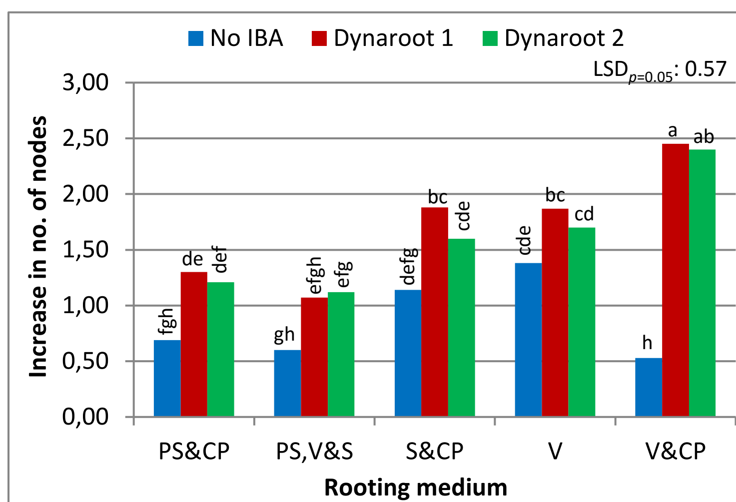


Figure 5.29. The interactive effect of IBA treatment and rooting medium on the number of nodes of cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

5.3.4.3.3 Basal diameter of cuttings

Cuttings from both IBA treatments and the control showed an increase in basal diameter in all the rooting media, although it was more pronounced in IBA treated cuttings (Figure 5.30). Untreated cuttings did not show a significant increase in basal diameter compared to both IBA treatments in the potting soil and coco peat (PS & CP) and potting soil, vermiculite and sand (PS, V & S) media, but it differed significantly in the other three rooting media (Figure 5.30). The IBA treatment (Dynaroot 1 and 2) did not significantly affect the increase in basal diameter in any of the rooting media. The highest increase in the basal diameter (0.5 mm) was for cuttings treated with Dynaroot 2 and rooted in vermiculite (V) medium which differed significantly from the increase in all the other treatments, except from the Dynaroot 1 treatment in vermiculite (Figure 5.30).

5.3.4.3.4 Sprouting of axillary buds

In the vermiculite and coco peat (V & CP) and potting soil and coco peat (PS & CP) media, a significantly lower percentage of untreated cuttings (control) had bud sprouts than cuttings treated with IBA rooting powder, either with both Dynaroot 1 and 2 (V & CP) or only with Dynaroot 1 (PS & CP) (Figure 5.31). In all the other rooting media the percentage of cuttings with bud sprouts was not significantly affected by the interactive effect of medium and IBA treatment. There was thus no

significant difference between cuttings with bud sprouts, irrespective of the absence or presence of IBA (Figure 5.31).

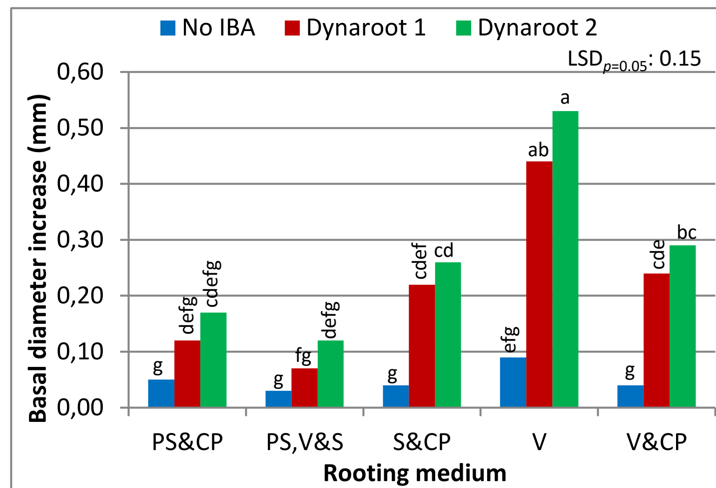


Figure 5.30. The interactive effect of IBA treatment and rooting medium on the basal diameter of cuttings after four weeks. Means (bars) with the same letters are not significantly different at the 5% level of significance.Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

Torkashvand and Shadparvar (2012) also reported a significantly lower number of sprouted buds on cuttings of *H. rosa-sinensis* (Yellow double hybrid) in the absence of IBA in a coco peat and perlite medium, whereas there was no significant difference in bud sprouts between the different IBA treatments (1000, 2000 and 4000 mg L^{-1} IBA) in this medium. In the sand medium, the interaction with 4000 mg L^{-1} IBA caused a significantly higher number (1.7) of bud sprouts on cuttings than the interaction with untreated cuttings (no bud sprouts) and the lower (2000 mg L^{-1}) IBA concentration (0.6 bud sprouts).

In the current study, no significant interactions between cutting type and auxin treatment for any of the parameters were observed. This was also noted by De Souza *et al.* (2015) who found no significant interactions between the cutting type and auxin treatment for any of the rooting parameters when different types of *H. rosa-sinensis* cuttings were treated with various concentrations of IBA.

Despite the fact that the ANOVA analysis showed no significant interactive effect between cutting type, IBA treatment and rooting medium for the rooting percentage (Appendix C, Table 1.2), it was found that apical and basal cuttings of *H. coddii*

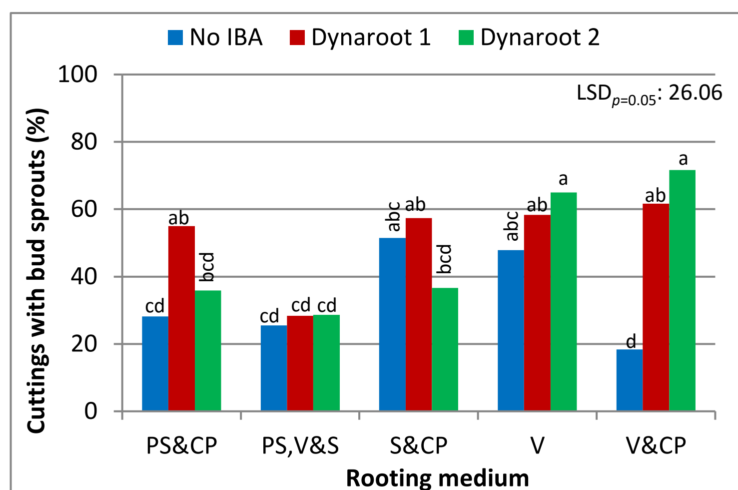


Figure 5.31 The interactive effect of IBA treatment and rooting medium on the percentage of cuttings with sprouted axillary buds. Means (bars) with the same letter are not significantly different at the 5% level of significance. Dynaroot 1: 0.1% IBA, Dynaroot 2: 0.3% IBA. Rooting medium: PS & CP: potting soil and coco peat; PS, V & S: potting soil, vermiculite and sand; S & CP: sand and coco peat; V: vermiculite; V & CP: vermiculite and coco peat.

subsp. *barnardii* treated with both Dynaroot 1 and Dynaroot 2 rooting powder and rooted in vermiculite (V) and vermiculite and coco peat (V & CP) media resulted in 90–100% rooting. Rooting percentages of 85–95% were observed in apical cuttings treated with Dynaroot 1 and in basal cuttings treated with Dynaroot 1 and Dynaroot 2 in sand and coco peat (S & CP), and in apical and basal cuttings treated with Dynaroot 2 and rooted in potting soil, vermiculite and sand (PS, V & S) medium. The lowest rooting (70–83%) for IBA treated cuttings (both Dynaroot 1 and 2), was observed in apical and basal cuttings rooted in potting soil and coco peat (PS & CP).

Even though vermiculite gave a high rooting percentage and numerous roots per cuttings, the weakness for its use as rooting medium for cuttings of *H. coddii* subsp. *barnardii* is the nutrient deficiency that was observed on rooted cuttings after four weeks of incubation. If vermiculite alone is used for rooting purposes it is recommended that a nutrient solution should be given to the rooted cuttings from the third week onwards (after rooting). From the results obtained, it therefore might be better to combine the vermiculite with an organic product such as coco peat. The combination of vermiculite and coco peat in this study also gave good rooting responses and the medium compacted well after adventitious root development, which would make transplanting easier.

5.3.5 Correlation between different variables

The initial length of *H. coddii* subsp. *barnardii* cuttings varied from 70–125 mm with 7–9 nodes and a basal diameter of 1.22–2.68 mm. Pearson's correlation (Appendix C: Table 2) were performed to determine the relationship between the initial length, number of nodes and basal diameter of cuttings and the same parameters, including the fresh and dry root biomass, at the end of the experiment. The *p*-values (Appendix C: Table 3) indicated that most of the correlation coefficients are significant, except for those between the initial cutting length and the harvest basal diameter and fresh root mass at the end of the experiment (Appendix C: Table 3). Although the *p*-values indicated significant relationships, the Pearson's correlation coefficients showed weak to moderate correlations between most of the variables. This could be due to the large sample size (*n*=810) where small correlations may reach statistical significance. The scatter plots in Appendix C also showed the weak linear relationships.

None of the initial variables (length, number of nodes and basal diameter) had a correlation with the fresh or dry root biomass of the *H. coddii* subsp. *barnardii* cuttings since the correlation coefficients are negligible (Appendix C: Table 6). Different initial lengths (5–13 cm) of *Lavandula dentata* cuttings also had no relationship with harvest parameters such as rooting percentage, number of roots per cutting and root length. No relationship between cutting length (5–10 cm) and fresh and dry root mass was observed, although the longer cuttings (13 cm) had a slightly higher fresh and dry root mass (Bona *et al*., 2012). Castellanos-Castro and Bonfil (2013) also indicated no significant correlation between the initial size (length and diameter) of stem cuttings of *Bursera glabrifolia* and the number of roots formed on cuttings. However, Naidu and Jones (2009) found that cutting length affected the rooting performance of *Eucalyptus* cuttings. The shortest cuttings (5 cm) had the lowest rooting percentage, fewer roots and leaves and less dry shoot and root mass. Filho *et al*. (2014) also indicated that shorter (5–14 cm) cuttings of *Hylocereus undatus* had a lower dry root mass (3.2 g) than longer (17–20 cm) cuttings where the dry root mass was 4 g.

In *H. coddii* subsp. *barnardii*, the correlation coefficients for the initial basal diameter and the cutting length and number of nodes at harvest, as well as the correlation

coefficient between the initial nodes and the harvest basal diameter were also negligible (Appendix C: Table 2, Table 6). A weak positive correlation was observed between the initial cutting length and the number of nodes on cuttings when harvested (r=0.455, p<.0001), while a moderate positive correlation was observed between initial node number and the harvest length of cuttings (r=0.551, p<.0001) (Appendix C: Table 2, Table 6). A strong positive correlation and linear relationship were found between the initial cutting length and the harvest length (r=0.726, p<.0001). There were moderate positive correlations between the initial number of nodes and basal diameter and these parameters at harvest time, (r=0.681, p<.0001) and (r=0.649, p<.0001) respectively (Appendix C: Table 2, Table 6). Weak to moderate positive correlations between harvest cutting variables and the fresh and dry root mass, and between harvest length and basal diameter, as well as between the basal diameter and the number of nodes at harvest were observed. A strong positive correlation between cutting length and number of nodes at harvest (r=0.766, p<0.0001) and a very high/strong positive relationship between the fresh root mass and the dry root mass (r=0.958, p<.0001) was observed (Appendix C: Table 2, Table 6). The correlation between initial cutting length and harvest length of cuttings could be expected since cuttings that rooted showed an increase in length and then also an increase in the number of nodes, therefore the strong correlation between harvest length and number of nodes at harvest. The strong relationship between the fresh and dry root biomass could also be foreseen and many authors only use one of the two parameters when reporting results since the same trend is often found.

The p-values (Appendix C: Table 5) for Spearman's ranked correlation for most of the correlation coefficients (Appendix C: Table 4) were significant, although the correlation coefficient values were small, which could be attributed to the large sample size (n=667). A weak positive correlation between rooting percentage and axillary bud sprouts (r_s=0.437, p<.0001), and between root score and axillary bud sprouts (r_s=0.426, p<.0001) was found (Appendix C: Table 4, Table 6). Formation of adventitious roots on cuttings would promote the sprouting of axillary buds through the action of cytokin produced in the roots (Hansen and Kristensen, 1990) as also indicated by this correlation. Furthermore, there was a strong positive monotonic relationship between the rooting percentage and the root score (r_s=0.725, p<.0001), and a moderate positive correlation between the root score and the root origin

(r_s=0.599, p<.0001). These correlations could have been expected since rooted cuttings of *H. coddii* subsp. *barnardii* would have a certain quantity of adventitious roots (root score) which originated somewhere on the cutting (root origin position).

The *p*-values of Spearman's ranked correlation indicated that there was no significant relationship between the presence of flower buds on *H. coddii* subsp. *barnardii* cuttings at the beginning of the experiment and the survival and rooting percentage of cuttings, root score, and root origin at the end of the experiment (Appendix C: Table 5). This indicates that the presence of flower buds on the cuttings did not affect rooting. There was a significant relationship between the initial flower bud presence and the axillary bud sprouts, but the correlation coefficient is negligible (r_s=0.225, p<.0001) (Appendix C: Table 4, 5 & 6). In most plants, especially the easily-rooted species, cuttings can be taken from shoots that are in the vegetative stage (without flower buds) or in the reproductive stage (with flower buds), but in hard-to-root species the presence of flower buds can be detrimental to rooting (Biran and Halevy, 1973; DeVier and Geneve, 1997; Hartmann *et al*., 2011). Cuttings of *H. coddii* subsp. *barnardii* formed adventitious roots despite the presence of flower buds on cuttings at the beginning of the experiment. In contrast, the rooting percentage in *Dahlia* cuttings decreased significantly from 77% in cuttings with a removed terminal flowering bud to 42% in cuttings with a terminal flowering bud (Biran and Halevy, 1973). Cuttings of *Chrysanthemum* sp. taken from a reproductive plant with flowers showed a lower rooting percentage and lesser roots than cuttings taken from vegetative plants (DeVier and Geneve, 1997). Rasmussen *et al.* (2015) found that adventitious root formation was inhibited when stem meristems in plants changed from the vegetative phase to the reproductive phase, even before flower appearance. The flowering stimulus can have an antagonistic effect on rooting, while flower buds and flowers can also act as a competing sink for metabolites which can be detrimental to rooting of cuttings (DeVier and Geneve, 1997; Hartmann *et al*., 2011).

In conclusion, the results from this study revealed that both apical and basal stem cuttings of *H. coddii* subsp. *barnardii* can be used for establishment of new plants, although some of the rooting and cutting parameters were better with basal cuttings. The application of exogenous IBA (0.1% and 0.3%) in the form of rooting hormone

powder (Dynaroot 1 and 2) resulted in high rooting and survival percentages and the formation of numerous roots per cutting which was more pronounced with Dynaroot 2 . Vermiculite (V), vermiculite and coco peat (V & CP) and sand and coco peat (S & CP) rooting media gave the best responses in terms of rooting and cutting growth parameters. Furthermore, the rooted cuttings would be ready for transplanting at 21–28 days after establishment. Figure 5.32 below, presents an overview of vegetative propagation of *H. coddii* subsp. *barnardii* by stem cuttings.

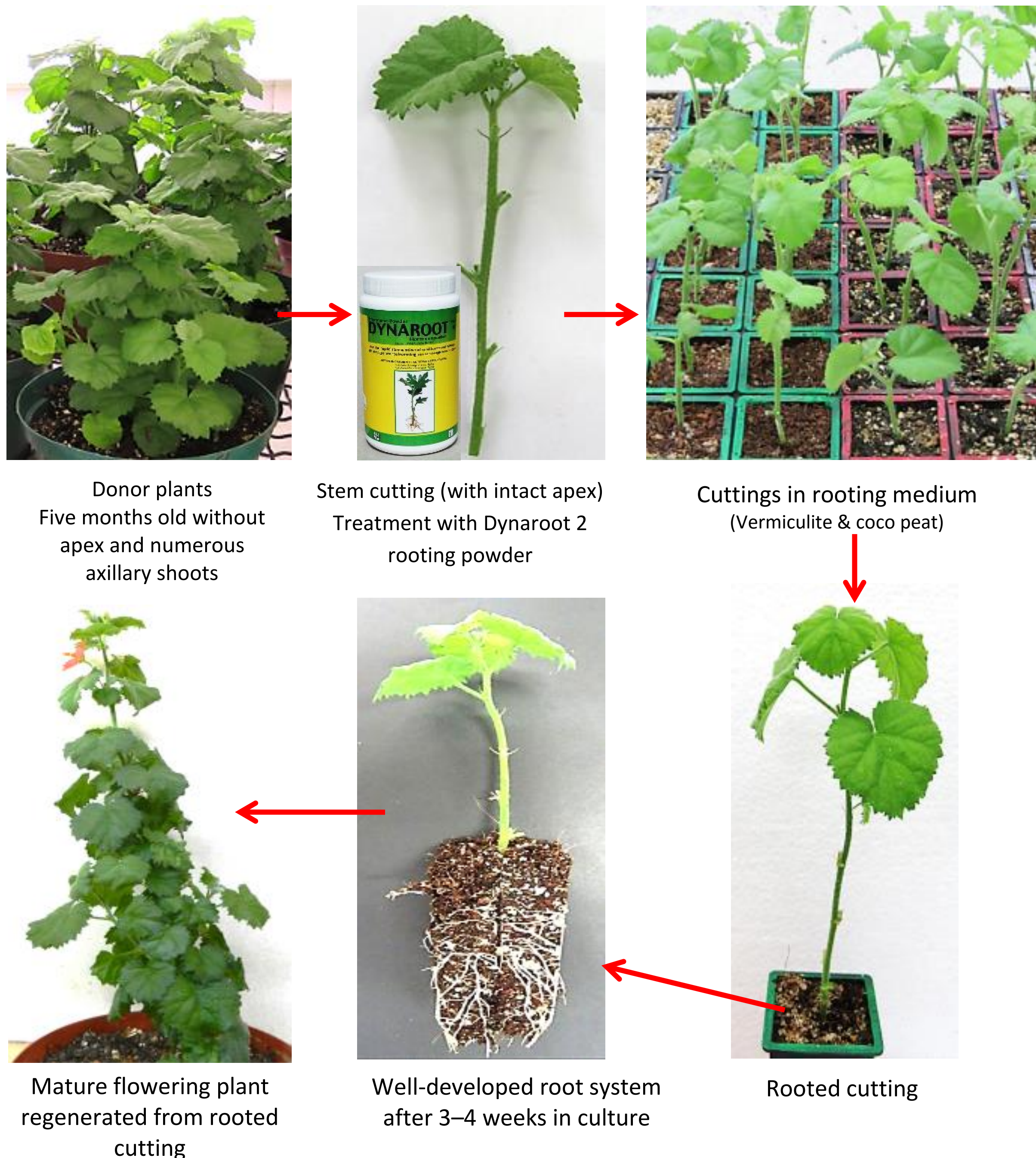


Figure 5.32. An overview of vegetative propagation of *H. coddii* subsp. *barnardii* by stem cuttings.

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Chapter 6

CHAPTER 6

In vitro culture of *Hibiscus coddii* subsp. *barnardii*

6.1 Introduction

Hibiscus coddii subsp. *barnardii* can be successfully propagated *in vivo* by seeds and shoot cuttings as described in this study (Chapter 4 and 5), although only one plant per seed and/or per cutting was produced, resulting in a low regeneration efficiency. *In vitro* micropropagation offers the potential to produce a large number of plants from one aseptic explant in a short time. Production of plants is also independent of seasonal changes and plant material can be stored for long periods, needing little maintenance between subcultures (Thiart, 2003). On the other hand, special facilities and advanced skills are required, which can inflate production costs. Low survival of regenerated plants during acclimatisation, and undesirable genetic variations can also occur (Rout *et al*., 2006; Christensen *et al*., 2008; Bhalla *et al*., 2009; Kumar and Reddy, 2011).

Micropropagation generally involves four stages, namely establishment *in vitro* of an axenic shoot culture from a disinfected explant (Stage I) and shoot multiplication with various subcultures in the absence or presence of plant growth regulators (PGRs) in Stage II. This is followed by *in vitro* or *ex vitro* rooting of *in vitro* grown shoots (Stage III), and finally *ex vitro* acclimatisation and hardening-off of regenerated plantlets in soil culture (Stage IV). Shoot culture is the most commonly used micropropagation technique for shoot multiplication. Shoot induction from axillary buds on single and multiple nodal explants and from shoot tips forms the basis. Adventitious shoots formed directly on explants such as stem internodes, leaves and petioles, and indirectly from callus are also used for shoot multiplication, although indirect organogenesis can increase somaclonal variations (Thiart, 2003; Rout *et al*., 2006; Iliev *et al*., 2010; Hartmann *et al*., 2011; Kumar and Reddy, 2011).

Shoot cultures were used in micropropagation of several *Hibiscus* species such as *H. acetosella*, *H. cannabinus*, *H. moscheutos*, *H. sabdariffa*, and *H. syriacus*. In most instances *in vitro* germinated seedlings served as aseptic explant source for shoot culture, although no such information was found for *H. coddii* subsp. *barnardii*

(Jenderek and Olney, 2001; Sakhanokho, 2008; Govinden-Soulange *et al*., 2009; Sakhanokho and Kelley, 2009; Ayadi *et al*., 2011). Explants such as shoot apices, cotyledons, cotyledonary petioles, hypocotyls, leaves, and nodal stem segments obtained from *in vitro* grown seedlings were used for the initiation and multiplication of shoots, mostly on Murashige and Skoog (MS) medium (Khatun *et al*., 2003; Herath *et al*., 2004; Airò *et al*., 2009; Bhalla *et al*., 2009; Ayadi *et al*., 2011; Sultana *et al*., 2016a). Direct regeneration of adventitious shoots from leaf and internodal stem segments of *H. cannabinus* seedlings and indirect regeneration through callus formation was reported (McLean *et al*., 1992; Samanthi *et al*., 2013). Mature plants of *H. moscheutos*, *H. rosa-sinensis* and *H. sabdariffa* growing naturally or cultivated in a greenhouse, also served as explant source for shoot culture after surface disinfection of the plant material (West and Preece, 2004; Christensen *et al*., 2008; Dar *et al*., 2012; Manokari *et al*., 2016).

Different types, concentrations and combinations of PGRs are used in micropropagation of plants. In general, cytokinins are used to induce multiple shoot formation, whereas auxins induce adventitious roots (Gaba, 2005; Kane, 2005; Kumar and Reddy, 2011). 6-Benzyl-aminopurine (BAP) is reported to be the most effective cytokinin for shoot induction since it can cause proliferation of more axillary buds in a shorter period of time (Herath *et al*., 2004; Gaba, 2005; Sakhanokho, 2008). It was also used in micropropagation studies of several *Hibiscus* species, but the shoot response depended on the concentration, plant species and explant type (Agrawal *et al*., 1997; Herath *et al*., 2004; Bhalla *et al*., 2009). Zapata *et al*. (1999) reported shoot initiation after three weeks on 91.7% of *H. cannabinus* shoot apex explants on MS medium with 0.1 mg L^{-1} BAP. Shoot induction on nodal explants of *H. sabdariffa* was also reported in the presence of varying concentrations of BAP (0.1–2 mg L^{-1}). However, the percentage of explants with proliferated shoots significantly reduced with an increasing BAP concentration, from 73% with 0.1 mg L^{-1} BAP to 33% with 2 mg L^{-1} BAP (Govinden-Soulange *et al*., 2009). In contrast, a higher BAP concentration (17.74 µM) resulted in 100% shoot regeneration efficiency and the highest number of shoot buds (33.2) per shoot apex explant of *H. sabdariffa* as compared to only 2.7 shoots per explant with 8.87 µM BAP (Gómez-Leyva *et al*., 2008). Other cytokinins such as thidiazuron (TDZ) and kinetin (Kin) were also used for shoot induction in *Hibiscus* species with varying results. Srivatanakul *et al*. (2000)

reported the highest number of shoots (6.7) per shoot apex explant of *H. cannabinus* cv. Tainung 1 with 1 µM TDZ compared to lesser shoots (4.2) per explant with a higher TDZ concentration (20 µM). On the other hand, the use of TDZ in shoot apex explants of *H. sabdariffa* did not induce bud sprouts, resulted in vitrified callus formation, and deformed apical buds (Gómez-Leyva *et al.*, 2008). Cytokinins are also used in combination to increase shoot multiplication efficiency. The best shoot induction response (3.7 shoots/explant) on shoot apex explants of *H. acetosella* was obtained with a combination of 8.9 µM BAP and 0.6 µM TDZ, whereas only 1.3 shoots were attained with 13.3 µM BAP alone (Sakhanokho, 2008). Manokari *et al.* (2016) reported improved shoot numbers, 8.2 shoots per nodal explant of *H. sabdariffa*, with a combination of BAP and Kin (0.5 mg L^{-1} each) compared to only 3.3 and 2.1 shoots respectively when BAP and Kin (0.5 mg L^{-1}) were used alone.

Combinations of cytokinins and auxins are also used for shoot induction, and depending on the ratio shoots, roots or callus can be induced on explants (Gaspar *et al.*, 1996; Gaba, 2005; Hartmann *et al.*, 2011). The highest adventitious shoot regeneration (59.5%) from cotyledonary petioles of *H. cannabinus* was obtained with the combination of 5 mg L^{-1} BAP and 0.5 mg L^{-1} Indole-3-acetic acid (IAA), whereas lower BAP (1 mg L^{-1}) combined with 0.5 mg L^{-1} IAA resulted in no shoot regeneration (Khatun *et al.*, 2003). A combination of 1 µM TDZ and 0.5 µM 2,4-Dichlorophenoxyacetic acid (2,4-D) improved shoot formation in shoot apex explants of *H. cannabinus* cv. Everglades 71 to 80% as compared to 60% with 20 µM TDZ alone (Srivatanakul *et al.*, 2000). Reduction in shoot regeneration efficiency in the presence of some PGRs was also reported. The lowest response (32.2%) in nodal explants of *H. cannabinus* was obtained with the combination of 0.5 mg L^{-1} BAP and 1 mg L^{-1} 1-Naphthaleneacetic acid (NAA) (Ayadi *et al.*, 2011).

Some *Hibiscus* plants responded better on media without PGRs. The highest shoot induction (90.5%) on nodal explants of *H. cannabinus* was obtained on PGR-free medium, whereas 1 mg L^{-1} BAP significantly reduced the shoot induction response to 69.4% (Ayadi *et al.*, 2011). Significantly higher shoot formation (81%) and number of shoots (3.2) per nodal explant of *H. sabdariffa* were also reported on BAP-free

medium in comparison with only 33% shoot formation and 1.2 shoots per explant with 2 mg L^{-1} BAP (Govinden-Soulange *et al.*, 2009).

Auxins such as IAA, Indole-3-butyric acid (IBA) and NAA are used alone, or in combination with cytokinins (BAP and Kin) to induce adventitious root formation on micropropagated shoots (Vardja and Vardja, 2001; Kane, 2005; Govinden-Soulange *et al.*, 2009). Shoots of *H. rosa-sinensis* rooted the best (72%) when cultured on MS medium with 2.85 µM IAA compared to 30% rooting with lower IAA (1.42 µM) and 15% in the absence of IAA (Airò *et al.*, 2009). Supplementation of MS medium with 0.5 mg L^{-1} IBA resulted in root formation in 95% of adventitious shoots of *H. syriacus*, whereas only 20% of shoots in IBA-free medium rooted (Yang *et al.*, 1995). In contrast, shoot explants of *H. cannabinus* showed the highest rooting (91%) on PGR-free medium, while only 17.1% rooting was found on explants in the presence of 2 mg L^{-1} BAP & 1 mg L^{-1} IBA (Ayadi *et al.*, 2011).

The transplanting of regenerated plantlets to a soil medium are hindered by the viability of the *in vitro* formed adventitious root system. Researchers therefore also study rooting of microshoots *ex vitro*. Adventitious roots formed *ex vitro* are longer and thicker, more flexible and, in general, improve the survival rate of plants. This method also saves time, labour and production costs (Yan *et al.*, 2010; Hartmann *et al*, 2011; Patel *et al.*, 2014; Ranaweera *et al.*, 2013). Hemphill *et al.* (1998) reported a low *in vitro* rooting response (22%) on microshoots of *Gossypium hirsitum* when cultured with 1 µM IBA, whereas 89% of microshoots rooted *ex vitro* in soil after treatment with Rootone® hormone rooting powder containing 0.2% (w/w) 1-Naphthaleneacetamide (NAD). Successful *ex vitro* rooting of microshoots was also reported for other plant species such as *Camellia sinensis*, *Basella alba*, and *Aerva lanata* when auxins, such as IBA and NAA, were used to treat microshoots (Ranaweera *et al.*, 2013; Shekhawat and Manokari, 2016; Shekhawat *et al.*, 2016).

Acclimatisation of *in vitro* regenerated plantlets is an important micropropagation stage and low survival of plants is common (De Klerk, 2002; Kane, 2005; Iliev *et al.*, 2010). The plants face several challenges such as reduced photosynthetic capacity, malfunctioning stomata and poor water retention capacity due to the lack of a waxy cuticle on the leaves (Kane, 2005; Rout *et al.*, 2006; Iliev *et al.*, 2010). These plants

must therefore be gradually exposed to environmental conditions by keeping them at high humidity, while also exposing them to a higher light intensity. This is often done by covering plantlets with transparent plastic bags for the first week or two, and then gradually removing the bags, where after the plants are hardened-off in an uncontrolled environment (De Klerk, 2002; Hartmann *et al*., 2011).

The development of an efficient *in vitro* propagation protocol for *H. coddii* subsp. *barnardii* would allow for fast, large-scale production that is currently limited by seasonal availability of plants. An extensive literature survey indicates that to date and to the author's best knowledge, no other research on *in vitro* propagation of *H. coddii* subsp. *barnardii* has been published. This study aimed to investigate the efficiency of *in vitro* seed germination for seedling production and their suitability as an explant source for shoot multiplication, rooting and plant regeneration using various PGRs. Acclimatisation and hardening-off of the regenerated plants were also studied.

6.2 Materials and methods

6.2.1 General requirements for *in vitro* cultures

All *in vitro* experiments were performed in a specialised plant tissue culture laboratory at the Department of Biodiversity (UL). Disinfection and inoculation procedures were performed under aseptic conditions in a laminar flow cabinet. The working area and hands were intermittently disinfected with 70% ethanol prior and during the performance of experiments to avoid contamination. All instruments, glassware, distilled water and culture media were sterilised for 20 minutes at 121°C in an autoclave (100 kPa). The dissecting instruments were sterilised in a glass bead steriliser (Keller Steri 350) prior to the excision and inoculation of sterile plant material (Beyl, 2005).

6.2.2 Composition and preparation of culture media

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) containing macronutrients, micronutrients, an iron source, and vitamins was prepared from stock solutions as indicated in table 6.1. Sucrose (3%) was used as carbon source (Franklin and Dixon, 1994). All chemicals (plant cell culture tested) used for *in vitro* cultures were from Sigma®. The full strength MS medium was supplemented with various concentrations and combinations of cytokinins and auxins depending on the plant growth regulator (PGR) treatment. MS medium without PGRs was used as a control. The pH of the medium was adjusted to 5.8 with 1 N NaOH or HCl prior to the addition of agar [0.1% (w/v)] or Gelrite® [0.3% (w/v)] as solidifying agent. The medium was dispensed into Magenta™ B-cap glass culture vessels (20–30 ml in each) and autoclaved at 121°C for 20 minutes.

6.2.3 Culture growth conditions

All *in vitro* cultures (seed, shoot and root) were maintained in a growth room under controlled conditions at 24±2°C with a 16-hour photoperiod at 55–60 μmol m^{-2} s^{-1} provided by a mixture of cool-white (Phillips and Osram) and Gro-lux (Sylvania) fluorescent tubes.

Table 6.1. Composition and preparation of MS medium adopted from Franklin and Dixon (1994).

Constituent	Molarity in medium	Concentration of stock solution (mg L^{-1})	Volume of stock per litre of medium (ml)
Major inorganic nutrients			
NH_4NO_3	2.06×10^{-2}	33 000	
KNO_3	1.88×10^{-2}	38 000	
$CaCl_2.2H_2O$	3.00×10^{-3}	8 800	50
$MgSO_4.7H_2O$	1.50×10^{-3}	7 400	
KH_2PO_4	1.25×10^{-3}	3 400	
Trace elements			
KI	5.00×10^{-6}	166	
H_3BO_3	1.00×10^{-4}	1 240	
$MnSO_4.4H_2O$	9.99×10^{-5}	4 460	
$ZnSO_4.7H_2O$	2.00×10^{-5}	1 720	5
$Na_2MoO_4.2H_2O$	1.00×10^{-6}	50	
$CuSO_4.5H_2O$	1.00×10^{-7}	5	
$CoCl_2.6H_2O$	1.00×10^{-7}	5	
Iron source			
$FeSO_4.7H_2O$	1.00×10^{-4}	5 560	5
$Na_2EDTA.2H_2O$	1.00×10^{-4}	7 460	
Vitamins			
myo-Inositol	4.90×10^{-4}	20 000	
Nicotinic acid	4.66×10^{-6}	100	
Pyridoxine-HCl	2.40×10^{-6}	100	5
Thiamine-HCl	3.00×10^{-7}	100	
Glycine	3.00×10^{-5}	400	
Carbon source			
Sucrose	8.80×10^{-2}		Added as solid (30 g L^{-1})
Solidifying agent			Added as solid
Gelrite®			3 g L^{-1}
Agar			1 g L^{-1}

6.2.4 Seed germination

6.2.4.1 Plant material

The open flowers of mature plants of *H. coddii* subsp. *barnardii* grown in a greenhouse were hand-pollinated and the ripe fruit capsules with seeds were collected. Seeds were removed from the capsules and stored in paper bags at ambient temperature until further use.

6.2.4.2 Seed scarification and disinfection

Chemical scarification of seeds was performed with 98% sulfuric acid (H_2SO_4) for 30 minutes. After scarification, the seeds were first rinsed under running tap water and then with distilled water to remove the H_2SO_4 and hair debris from the seeds. The scarified seeds were surface disinfected, first with 70% ethanol for 90 seconds and then for 25 minutes with 50% (v/v) commercial bleach (Jik®) solution containing 1.75% active sodium hypochlorite (NaOCl), and 2–3 drops of added Tween 20. The seeds were rinsed three times with sterile, distilled water after each disinfection step to remove the disinfectant.

6.2.4.3 Seed cultures

In order to test *in vitro* germination, scarified and disinfected seeds were aseptically inoculated on various types of sterile cultures as follows:

a. Filter paper bridges

Moist filter paper (Whatman No. 1) bridges were placed in Magenta™ B-cap glass bottles layered with distilled water (15 ml).

b. MS medium

Five different strengths [¼, ½, ¾, full (1), 1¼] of PGR-free MS medium containing 3% (w/v) sucrose and solidified with 0.1% (w/v) agar or 0.3% (w/v) Gelrite®.

c. Gelrite® (control)

Solid medium containing only Gelrite® (12 g L^{-1}) dissolved in distilled water was used as a control.

All seed cultures were kept under controlled growth conditions (Section 6.2.3) for germination.

6.2.5 Seedling development

In a pilot study, seeds germinated on filter paper bridges were aseptically transferred after radicle protrusion (1–2 mm) to ½ and full strength MS medium for further seedling development. Seeds germinated directly in the different strengths of MS media and in the Gelrite® medium remained in the culture bottles for further seedling development. Seedlings from the Gelrite® culture were used as a control. All seedlings were kept under controlled growth conditions (Section 6.2.3) for up to seven weeks to monitor the seedling development.

6.2.6 Experimental design for seed germination and seedling development

The seed germination experiment on filter paper bridges had three replicates with 120 seeds each. The experimental unit was 10 seeds per culture bottle. Seed germination and seedling development on the different strengths of MS media followed a randomised complete block design (RCBD) with six treatments replicated four times. The six treatments were Gelrite® (control) and five strengths of MS media [¼, ½, ¾, full (1), and 1¼]. The experimental unit was 10 seeds per treatment. Cultures for seedling development on ½ and full strength MS media (after subculture from filter paper) was a pair-wise design with 60 replicates. The experimental unit was one seedling in a culture bottle.

6.2.7 Data collection for seed germination and seedling development

6.2.7.1 Seed germination

In all the cultures (filter paper bridges, MS medium, and Gelrite®), seed germination was regarded as protrusion of the radicle (1–2 mm) through the seed coat (Hartmann *et al*., 2011). Germination was recorded daily until no further germination was observed (14 days). Germination indices were calculated as follows:

i. Final germination (%) (FGP):
(Rehman *et al*., 2014)

$$FGP = \frac{No\ of\ seeds\ that\ germinated}{Total\ no\ of\ seeds} x100$$

ii. Mean germination time (MGT):

$$MGT = \frac{\sum Dn}{\sum n}$$

where *n* is the number of seeds with protruded radicle on day D and day D is the number of days counted from the beginning of germination (Ellis and Roberts, 1981).

iii. Germination rate index (GRI):

$$GRI = \frac{\#of\ seeds\ with\ radicle}{Days\ of\ first\ count} \pm - - \mp \frac{\#of\ seeds\ with\ radicle}{Days\ of\ final\ count}$$

(Al-Mudaris, 1998; Ranal and De Santana, 2006)

6.2.7.2 Seedling development

The development of seedlings derived from different seed cultures (filter paper, MS medium, and Gelrite®) was evaluated with respect to growth, survival and morphology for a period up to seven weeks in order to select the best culture medium for *in vitro* seedling growth. The number of nodes on the main stem of the

seedlings was counted weekly over a period of five weeks whilst the seedlings remained in the culture bottles (non-destructive method). The number of surviving seedlings was recorded after five weeks. Visible changes in the morphological appearance of seedlings developed on the different strengths of MS media and Gelrite® cultures were also monitored and photographed weekly for seven weeks.

6.2.8 Data analysis for seed germination and seedling development

All collected data were analysed with the SAS® Version 9.3 statistical software (SAS Institute, 2011). Data was subjected to analysis of variance (ANOVA) using the linear models procedure (PROC GLM). The means of the treatments were separated with T tests (LSD) at the 5% level of significance (Snedecor and Cochran, 1989). The Shapiro-Wilk's test was performed on the standardised residuals to test for deviations from normality (Shapiro and Wilk, 1965).

6.2.9 Shoot culture

6.2.9.1 Explant source and preparation

Different explant sources were tested in order to select the most suitable explant for *in vitro* shoot culture.

6.2.9.1.1 *In vivo* grown plants

a. Donor plants

A pilot study was conducted to evaluate the suitability of *H. coddii* subsp. *barnardii* plants growing in nature and grown *in vivo* from seeds under controlled environmental conditions (Section 6.2.11) for 4–5 months as donors of explants for *in vitro* culture.

b. Surface disinfection

In a pilot study, the effect of surface disinfection on the survival of explants obtained from the donor plants was tested. Axillary shoot cuttings (100–150 mm) obtained from the plants were used as an explant source for *in vitro* shoot culture. Cuttings were first washed for 5–10 minutes in soapy water and thoroughly rinsed with tap water where after cuttings were surface disinfected in 70% ethanol for 60 seconds. Further disinfection with 25% [0.875% (w/v) NaOCl] commercial bleach (Jik®) solution for 25 minutes and with 50% [1.75% (w/v) NaOCl] bleach solution for 15

minutes were tested. A few (2–3) drops of Tween 20 were added to the bleach solutions. In order to remove the disinfectant, the cuttings were rinsed three times with sterile distilled water after each disinfection step. The cuttings were further trimmed to nodal explants with 1–4 nodes and inoculated on PGR-free MS medium.

6.2.9.1.2 *In vitro* produced seedlings

The suitability of *in vitro* produced seedlings as an explant source for shoot culture was also tested. Disinfection of these explants was not required since they derived from an aseptic culture.

a. Seedlings as an explant source for axillary shoot induction

Five weeks old *in vitro* seedlings grown on PGR-free MS medium were used for this experiment. The culture bottle was opened in a laminar flow cabinet and the apex of the seedling was aseptically excised using sterile forceps and scissors, while the seedling remained in the same culture bottle without subculturing. After the excision procedure, the culture bottle was closed and cultures were further kept under controlled growth conditions (Section 6.2.3) to observe the effect of apex removal of the seedling on axillary shoot proliferation.

b. Seedlings as a source for nodal shoot explants

In contrast to the procedure described above (Section 6.2.9.1.2 a), in this experiment 5–6 week old seedlings (Figure 6.1A.1) were removed from the culture bottles. Each seedling was aseptically dissected in a Petri dish lined with sterile distilled water to prevent wilting of the explants. The roots of the seedling were removed and the remaining main shoot of the seedling was split into an upper (with apex) (Figure 6.1B) and lower part (with cotyledon node and piece of hypocotyl) (Figure 6.1C) which are referred to as apical and basal seedling shoot explants respectively. Each explant had 3–4 nodes (Figure 6.1B & C). Small explants (1–2 nodes) were also dissected (Figure 6.1D).

6.2.9.1.3 *In vitro* produced axillary shoots as an explant source

Primary axillary shoots derived from seedlings (Section 6.2.9.1.2 a) were also tested as an explant source for shoot multiplication. Axillary shoots (3–4 nodes) were excised from the seedling (Figure 6.1E) including the nodal part of the seedling's

stem. The axillary shoots (Figure 6.1F) were divided into two groups, one where the apex was removed (Figure 6.1G) and one where the apex was retained (Figure 6.1H). These are further referred to as axillary shoot explants with apex and without apex.

6.2.9.2 Factors affecting *in vitro* shoot induction

6.2.9.2.1 Effect of explant type, size and position of inoculation

Large (3–4 nodes, Figure 6.1B & C) and small (1–2 nodes, Figure 6.1D) apical and basal shoot explants derived directly from seedlings (Section 6.2.9.1.2 b) were inoculated on PGR-free MS medium either in a vertical or horizontal position. Single primary axillary shoots (30–50 mm) of varying thickness, 1–2 mm (Figure 6.1J) and 1 mm (Figure 6.1K), and clumps of short (<10 mm) primary axillary shoots (Figure 6.1L) derived from this experiment, were also used as an explant source for shoot multiplication. The single shoots were dissected in the same way as described in Section 6.2.9.1.3 to obtain large (3–4 nodes) and small (1–2 nodes, Figure 6.1M) axillary shoot explants with and without an apex.

6.2.9.2.2 Effect of plant growth regulators

a. Effect of BAP concentration

i. Basal seedling shoot explants (Figure 6.1C) were cultured on MS medium with various concentrations of BAP ranging from 0.50–4 mg L^{-1} (Table 6.2).

ii. Based on the outcome of the above experiment (i), the effect of lower concentrations (0.25–1 mg L^{-1}) of BAP on shoot induction was studied on apical (Figure 6.1B) and basal shoot explants (Figure 6.1C) derived from seedlings (Section 6.2.9.1.2 b), and on primary axillary shoots, with and without an apex (Sections 6.2.9.1.3 & 6.2.9.2.1, Figure 6.1G, H, J, K, L, M) (Table 6.2).

In all experiments, MS medium without BAP served as a control. Newly developed axillary shoots from each culture were subcultured individually, or as a clump of short axillary shoots, to PGR-free MS medium and MS medium supplemented with various concentrations (0.25–1 mg L^{-1}) of BAP. Rooted explants and axillary shoots (individual or clump) were transferred to pots with moist vermiculite and acclimatised under controlled conditions (6.2.11).

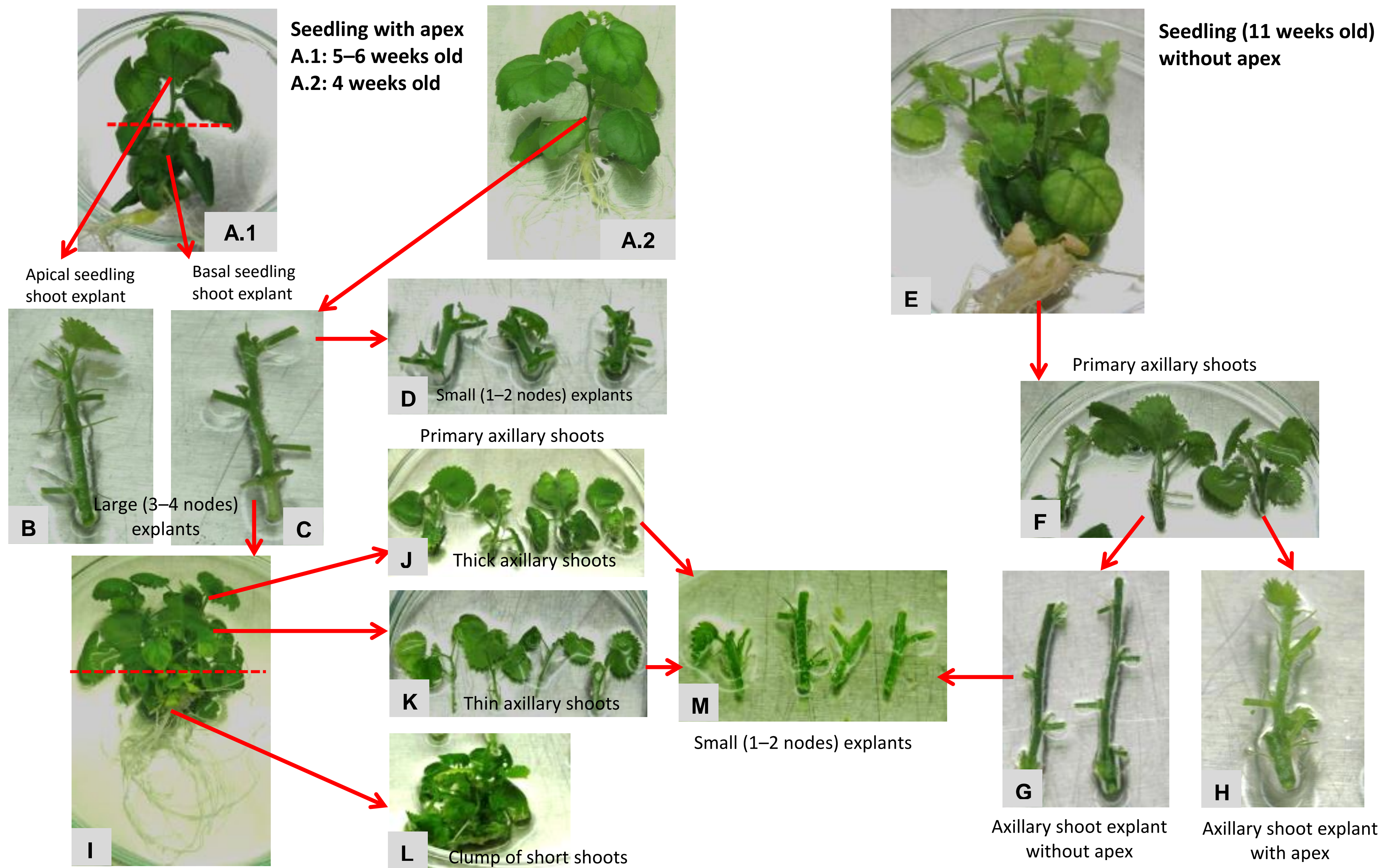

Figure 6.1. *In vitro* grown seedlings and primary axillary shoots of *H. coddii* subsp. *barnardii* served as explant source.

Table 6.2. Various types of explants obtained from seedlings and primary axillary shoots of *H. coddii* subsp. *barnardii* and various BAP concentrations used for shoot induction.

	Explant source		
	Seedling		Primary axillary shoots
BAP ($mg\ L^{-1}$)	**Basal shoot explant**	**Apical (with apex) & basal shoot explants**	**Large and small (with & without apex) shoot explants**
0.00	x	x	x
0.25		x	x
0.50	x	x	x
0.75		x	x
1.00	x	x	x
2.00	x		
3.00	x		
4.00	x		

b. Effect of BAP and auxins

Based on the preliminary results from the above BAP experiments (Section 6.2.9.2.2 a) and literature (Ayadi *et al.*, 2011), further experiments were performed to evaluate the effect of explant source and type and PGRs on *in vitro* shoot induction. Two types of explants with four nodes were cultured on MS medium supplemented with different concentrations of BAP alone and in combination with NAA and IBA (Table 6.3). One type of explant was obtained from the lower (basal) part of a seedling as described in Section 6.2.9.12 b and is further referred to as seedling explants. For this experiment young (4 week old) seedlings (Figure 6.1A.2) were used as explant source to compare their response with the older (5-6 weeks) seedlings used in the other two experiments (Section 6.2.9.2.1 & 6.2.9.2.2 a). The other type of explant was obtained from excised primary axillary shoots that proliferated after apex removal of seedlings (Section 6.2.9.1.3, Figure 6.1G). These explants (without an apex) are further referred to as axillary shoot explants. All explants were inoculated vertically with one node submerged in the medium and three nodes with pre-existing axillary buds (one per node) remaining above the medium.

Table 6.3. Combination of BAP and auxins for *in vitro* shoot induction on basal seedling shoot explants and axillary shoot explants without an apex.

Treatments	BAP (mg L^{-1})	IBA (mg L^{-1})	NAA (mg L^{-1})
T1	0.00	0.00	0.00
T2	0.50	–	–
T3	1.00	–	–
T4	0.50	–	0.50
T5	1.00	–	0.50
T6	0.50	0.50	–
T7	1.00	0.50	–

(*n*=20 explants/treatment and 60 axillary buds/treatment)

6.2.9.3 Experimental design for shoot culture

6.2.9.3.1 *In vivo* grown plants

For the surface disinfection experiment there were five explants replicated three times for each bleach concentration (25% and 50%). The culture bottles were randomly arranged in the growth room.

6.2.9.3.2 Seedlings as an explant source for axillary shoot induction

For this experiment, 15–20 culture bottles containing one seedling per bottle were used. Seedling cultures were randomly arranged in the growth room during the seedling development over a period of five to six weeks for axillary shoot induction. The experiment was repeated four times.

6.2.9.3.3 Seedlings as a source for nodal shoot explants

Twenty seedling shoot explants of each type (apical and basal) were used for this experiment with one explant inoculated per bottle and the experiment was repeated twice. For the position of the explant experiment, ten basal seedling shoot explants were used for each position, vertical and horizontal, with one explant per bottle. The experiment was repeated twice. The culture bottles for all experiments were arranged at random in the growth room.

6.2.9.3.4 Effect of BAP concentration on shoot induction

In the experiment with the higher BAP concentrations and the basal seedling shoot explants, the five BAP (0.50–4 mg L^{-1}) treatments and the control were replicated 12

times. For the lower BAP concentrations (0.25–1 mg L^{-1}) there were 10 replicates for each of the five BAP treatments and the control for both apical and basal seedling shoot explants. For the axillary shoots, the replicates of the five BAP treatments and the control were determined by availability of the axillary shoots. One to three explants were inoculated in a culture vessel and the vessels were arranged randomly in the growth room.

6.2.9.3.5 Effect of BAP and auxins on shoot induction

The experiment was a RCBD with seven treatments (Table 6.3) and 20 explants per treatment replicated five times. The experimental unit was two bottles (one explant per bottle) and the experiment was repeated twice.

6.2.9.4 Data collection and analysis for shoot culture

6.2.9.4.1 *In vivo* grown plants

After surface disinfection, the percentage of contaminated cultures and surviving explants were recorded weekly.

6.2.9.4.2 Seedlings as an explant source for axillary shoot induction

The number of axillary shoots per seedling developed over a period of five weeks was recorded and the cultures were photographed weekly.

6.2.9.4.3 Seedlings as a source for nodal shoot explants

The percentage of explants that showed axillary bud sprouting, shoot proliferation, rooting, callus formation and survival were recorded over a period of four weeks. Data were used for qualitative evaluation and are supported by photographs.

6.2.9.4.4 Effect of BAP concentration on shoot induction

For these experiments, the parameters described in Section 6.2.9.4.3 above were observed and recorded.

6.2.9.4.5 Effect of BAP and auxins on shoot induction

In seedling and axillary shoot explants (three axillary buds per explant), the axillary bud sprouting, shoot proliferation and survival of each bud were monitored (n=60 buds/treatment). Rooting, callus formation and size of the callus were recorded per

explant (n=20 explants/treatment). Cultures were evaluated weekly and data were collected for four weeks. For ease of reference and recording of data, the following qualitative five class and three class descriptive ranking scales were used:

a. Bud sprouting and shoot proliferation

1: Pre-existing axillary bud.
2: Bud sprout 1–2 mm.
3: Short axillary shoot at least 5 mm with visible leaves.
4: Short axillary shoot >5 mm <10 mm with at least 2 well-developed open leaves.
5: Axillary shoot 15–25 mm with at least 2 nodes.
(Based on Niedz *et al.*, 2007)

b. Callus size

1: Callus diameter 1–5 mm (small).
2: Callus diameter 5–10 mm (medium).
3: Callus diameter >10 mm (large).
(Based on Niedz *et al.*, 2007 and modified according to McLean *et al.*, 1992)

Data analysis was performed with the SAS® Version 9.3 statistical software package (SAS Institute, 2011). The frequency procedure was used to test for associations between different variables. For each trait, units were classed (scored) on a linear ordinal scale. Contingency RxC frequency tables were formed to test for association between PGR treatment and axillary bud sprouting, shoot proliferation and callus formation, including the size of callus (Snedecor and Cochran, 1989). Pearson's chi-square test (χ^2) test was performed to test for association (patterns) and where significant evidence was found, graphs were constructed to demonstrate differences in patterns (Cochran, 1952).

6.2.10 Root culture

6.2.10.1 Plant material

Primary axillary shoots (5–6 weeks old) that proliferated on *in vitro* seedlings after apex removal (Section 6.2.9.1.2 a, Figure 6.1F), were used as microcuttings for both the *in vitro* and *ex vitro* rooting experiments. The young plants (11 weeks old) were removed from the culture bottles and the roots were cut off. In addition, the axillary shoots (4–5 nodes) were excised from the main stem (Section 6.2.9.1.3). The leaves were trimmed retaining the upper one or two leaves and the apical bud was left intact (Figure 6.1H).

6.2.10.2 *In vitro* rooting

For this experiment, the microcuttings were randomly selected and inoculated on full strength MS medium supplemented with different types (IBA and NAA) and concentrations (0.5 and 1 mg L^{-1}) of auxins. MS medium without auxin served as a control.

6.2.10.3 *Ex vitro* rooting

For this experiment, the axillary shoots (microcuttings) were divided into two groups. One group of microcuttings derived from the lower three nodes of the main seedling stem and is further referred to as basal cuttings (Figure 6.2A). Another group of microcuttings derived from the upper three nodes of the main stem, and is further referred to as apical cuttings (Figure 6.2B). The base of each type of cutting was dipped in Dynaroot^{TM} No. 1 rooting hormone powder (0.1% IBA) and the excess powder was tapped off. Control cuttings did not receive the hormonal treatment. The cuttings were planted in small plastic pots (50 mm diameter and length) containing moist sterile vermiculite (Figure 6.2C) and acclimatised under controlled environmental conditions as described in 6.2.11. The vermiculite was kept moist by regular mist spraying with tap water.

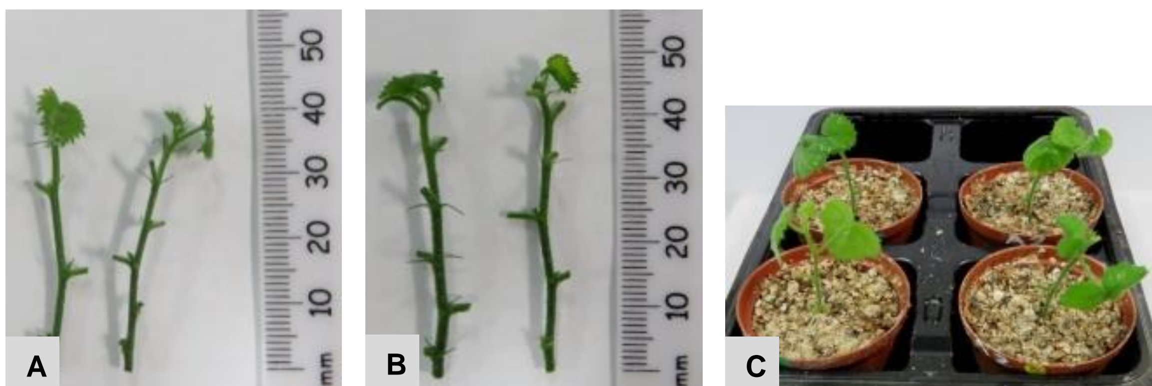

Figure 6.2. Axillary shoots (microcuttings) taken from seedlings grown *in vitro*. (A) Basal cuttings; (B) Apical cuttings; (C) Cuttings directly after placement in moist, sterile vermiculite.

6.2.10.4 Experimental design for root culture

6.2.10.4.1 *In vitro* rooting

Four auxin treatments namely 0.5 mg L^{-1} IBA, 1 mg L^{-1} IBA, 0.5 mg L^{-1} NAA, 1 mg L^{-1} NAA, and the control (0 mg L^{-1} auxin) were tested. The five treatments were replicated ten times in a RCBD in the growth room (Section 6.2.3). The experimental unit was one axillary shoot per bottle. The experiment was repeated twice.

6.2.10.4.2 *Ex vitro* rooting

There were four treatments namely basal cuttings without hormone powder (Basal –IBA), basal cuttings treated with hormone powder (Basal +IBA), apical cuttings without hormone powder (Apical –IBA), and apical cuttings treated with hormone powder (Apical +IBA). The four cuttings used for the treatments came from the same seedling and were placed together in a block. There were ten randomised blocks (replicates) with one cutting as an experimental unit. The experiment was performed twice.

6.2.10.5 Data collection and analysis for root culture

6.2.10.5.1 *In vitro* rooting

Formation of adventitious roots and callus for each microcutting were recorded weekly for six weeks.

6.2.10.5.2 *Ex vitro* rooting

The rooting of each of the microcuttings was recorded weekly for six weeks. A cutting was considered to have rooted if there was resistance when the cutting was pulled upwards. The survival of each rooted cutting (plant) was also recorded. The rooted and acclimatised plants were further monitored for survival and formation of flower buds and flowers.

Data for both *in vitro* and *ex vitro* rooting experiments were analysed with the GenStat64-bit Release 18.2 (PC/Windows 8) statistical package (VSN International, 2016) to test for treatment effects per week. An analysis of variance (ANOVA) was used to determine if the PGR treatment had an effect on *in vitro* rooting and callus formation, and in *ex vitro* rooting to test the effect of cutting position and IBA treatment on rooting percentage. Fisher's protected and unprotected least significant

difference (LSD) test, at the 5% and 10% level of significance, was performed to distinguish between different means (Williams and Abdi, 2010).

6.2.11 *Ex vitro* establishment of regenerated plants

a. Acclimatisation

Acclimatisation of *in vitro* and *ex vitro* cultures were performed under controlled conditions in a growth room at 24°C±2°C with a 16 hour photoperiod at 150–200 µmol m^{-2} s^{-1} provided by a mixture of cool-white (Phillips and Osram) and Gro-lux (Sylvania) fluorescent tubes. *In vitro* produced seedlings and regenerated plantlets were removed from the culture vessels. The excess MS medium was carefully washed from the roots with tap water where after the plants were placed in plastic pots (diameter 120 mm) filled with moist vermiculite. Each regenerated plantlet was covered with a transparent plastic bag and was gradually exposed to lower humidity by perforating the bag after the first week and by increasing the number of holes weekly. Bags were completely removed after 2–3 weeks. *Ex vitro* rooted cuttings were acclimatised in the same way. Survival of acclimatised plants was monitored. After 2–3 weeks the acclimatised plants were transferred to plastic pots (diameter 180 mm) that contained a mixture of potting soil, vermiculite and sand [5:3:1 (v/v/v)] and maintained in the growth room for a further one to two weeks. Rooted cuttings from the *ex vitro* experiment (Section 6.3.10.3) were transferred to the same potting conditions at 11 weeks.

b. Hardening-off

All plants were hardened-off in a greenhouse under uncontrolled environmental conditions. Plants were watered with tap water three times per week and received a nutrient solution [Nulandis® Dr Fisher's Multifeed Classic 19:8:16 (43)] once per week. Survival and flowering of the plants were monitored for a further 12 weeks.

6.3 Results and Discussion

6.3.1 Surface disinfection of scarified seeds

The selected method for disinfection of chemically scarified seeds of *H. coddii* subsp. *barnardii* using 70% ethanol (90 s) and 50% (v/v) commercial bleach (Jik®) solution containing 1.75% active NaOCl for 25 minutes, proved suitable for establishment of aseptic conditions for *in vitro* germination of all seed cultures since no microbial contaminations were observed. Other authors reported a similar effective seed disinfection procedure with ethanol and NaOCl of several *Hibiscus* species, such as *H. cannabinus*, *H. sabdariffa* and *H. moscheutos*, although the time of disinfection and the concentration of the disinfectants varied with the species (Herath *et al.*, 2004; Gómez-Leyva *et al.*, 2008; Sakhanokho and Kelley, 2009; Ayadi *et al.*, 2011). Disinfection of *Grewia tenax* seeds with 70% ethanol (30 s) and 20% (v/v) Clorox® (containing 5.25% NaOCl) commercial bleach solution for 15 minutes also resulted in contamination-free seed cultures (Daffalla *et al.*, 2016). In contrast, disinfection of *Coscinium fenestratum* seeds with 70% ethanol (60 s) and higher concentration of Clorox® solution (75%) for 3 minutes resulted in 40% microbial contamination of seed cultures. It also reduced the viability of the remaining seeds (Warakagoda and Subasinghe, 2014).

6.3.2 Seed germination

Germination (protrusion of the radicle) of *H. coddii* subsp. *barnardii* seeds in all three seed cultures [filter paper, MS medium (different strengths) and Gelrite®] started from the first two days after inoculation (Figure 6.3A, B & C). Germination responses of the different seed cultures were evaluated by assessing the following germination parameters: final germination (%) (FGP), mean germination time (MGT) and the germination rate index (GRI) (Table 6.4A, B, & C). The ANOVA analysis showed no statistical differences between the three different cultures for any of the germination parameters (Appendix D: Table 1). However, it showed significant differences between the different strengths of MS media and the Gelrite® cultures with respect to the MGT and the GRI, but not to the FGP (Appendix D: Table 2).

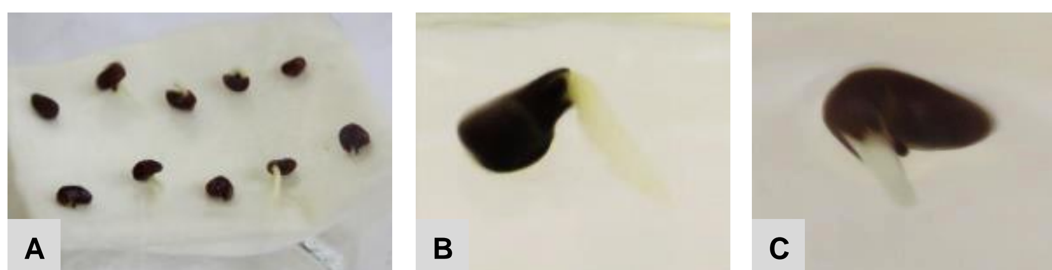

Figure 6.3. *In vitro* germination (protrusion of the radicle) of *H. coddii* subsp. *barnardii* seeds two days after inoculation on (A) Filter paper bridges; (B) Full strength MS medium; (C) Gelrite® (1.2%).

Final germination percentage (FGP)

Scarified and disinfected seeds showed high (85–97.5%) FGPs in all three seed cultures, although no significant differences were found between the seed cultures (Table 6.4A, B & C). The high FGPs in these cultures can be attributed to the even and steady exposure of seeds to moisture throughout the germination process. Seeds on the filter paper bridges attained 96.4% germination (Table 6.4A). Similar high seed germination percentages (≥90%) on moist filter paper were reported by Agrawal *et al*. (1997) for *G. hirsitum* and by Gupta *et al*. (2017) for *Gymnocladus assamicus*. Seeds of *H. coddii* subsp. *barnardii* attained 85–97.5% germination on all tested strengths of MS media, and the Gelrite® cultures (Table 6.4B & C). Ayadi *et al*. (2011) also reported high germination (100%) within two to three days when scarified seeds of *H. cannabinus* were germinated on full strength MS medium. Furthermore, Daffalla *et al*. (2016) reported 100% germination of *G. tenax* seeds within 14 days when cultured on water agar and ½ and full strength MS media. Contrary to the high FGPs recorded for *H. coddii* subsp. *barnardii* seeds on ½ (97.5%) and full (95%) strength of MS media (Table 6.4B), Gupta *et al.* (2017) reported only 24% and 17% germination of *G. assamicus* seeds on ½ and full strength MS media, respectively. Only 25–30% of *G. hirsitum* seeds germinated on full strength MS medium (Agrawal *et al*., 1997). The low germination percentage recorded in these studies was ascribed to possible impurities in the agar or sensitivity of the seeds to the high salt content of the MS medium (Agrawal *et al*., 1997; Abdellatef and Khalafalla, 2007; Gupta *et al*., 2017). Preliminary seed germination experiments of *H. coddii* subsp. *barnardii* also showed poorer (20–30%) germination responses on MS medium solidified with agar as compared to the Gelrite® that was selected as solidifying agent for all *in vitro* cultures of this study.

Table 6.4. Germination responses of *H. coddii* subsp. *barnardii* seeds on various aseptic cultures.

Seed culture	Germination parameters		
	FGP (%)	MGT (days)	GRI (No. of germinated seeds/day)
A. Filter paper bridges	96.40	1.78	6.71
B. MS culture medium strengths	**FGP (%)**	**MGT (days)**	**GRI (No. of germinated seeds/day)**
¼ MS	95.00	1.72[a]	6.67[ab]
½ MS	97.50	2.17[ab]	6.01[b]
¾ MS	97.50	2.83[abc]	5.34[bc]
Full MS	95.00	3.47[bc]	4.60[cd]
1¼ MS	85.00	4.01[c]	3.60[d]
C. Gelrite® (1.2%)	97.50	1.55[a]	7.74[a]
F pr.	0.282	0.008	0.000
LSD	–	1.35	1.36

FGP: final germination (%); MGT: mean germination time in days; GRI: germination rate index (number of normally germinated seeds per day). Means with the same letter within a column are not significantly different at the 5% level of significance.

Mean germination time (MGT)

Although not significantly different the MGT (1.78 days) of *H. coddii* subsp. *barnardii* seeds on filter paper cultures, was shorter than the times recorded for most of the MS media strengths (Appendix D: Table 1; Table 6.4A & B). Gupta *et al.* (2017) also found a shorter MGT and higher FGP for seeds of *G. assamicus* germinated on sterile moist filter paper than for seeds germinated on different strengths of MS media.

The lowest MGT (1.55 days) for *H. coddii* subsp. *barnardii* seeds was recorded on the Gelrite® culture which differed significantly from the MGT for seeds on full and 1¼, but not from ¼, ½, and ¾ strengths of MS media (Table 6.4B & C). Daffalla *et al.* (2016) also reported a faster germination time for *G. tenax* seeds placed on water agar (control medium) which differed significantly from the MGT on full strength, but not from ½ strength MS medium. The MGT for *H. coddii* subsp. *barnardii* seeds increased with an increase in the strength of MS medium. Seeds cultured on ¼, ½, and ¾ strengths germinated faster than seeds on full and 1¼ strengths of MS media. The lowest MGT (1.72 days) was obtained on ¼ strength, which differed significantly

from the MGT for seeds on full (3.47 days) and 1¼ (4 days) strengths of MS media (Table 6.4B). Seeds of *G. tenax* on ½ strength MS medium also germinated significantly faster (4.8 days) than the seeds on full strength medium (6.2 days) (Daffalla *et al.*, 2016). This might suggest that a lack or lower concentration of macro- and microelements is not detrimental to seed germination and that the good germination response of *H. coddii* subsp. *barnardii* seeds was mainly related to water availability as also reported by Koné *et al.* (2015) for *Vigna subterranea*. The delay in germination of *H. coddii* subsp. *barnardii* seeds cultured on full and 1¼ strength of MS media could be ascribed to the high salt concentration in MS medium as compared to other culture media with a lower salt content used for *in vitro* propagation (Beyl, 2005; Hartmann *et al.*, 2011). Solanki and Siwach (2012) observed no germination when seeds of *Aconitum heterophyllum* were cultured on cotton wool moistened with MS medium or on solid MS medium, while seeds only germinated on cotton wool moistened with distilled water.

Germination rate index (GRI)

The GRI for seeds of *H. coddii* subsp. *barnardii* followed the same trend as the MGT. A significantly higher number of seeds germinated per day on ¼ and ½ MS media strengths than on full and 1¼ strengths of MS media (Table 6.4B & C). The highest GRI (7.74 seeds/day) was obtained for the Gelrite® culture that differed significantly from the GRI for all strengths of MS media except the ¼ strength (Table 6.4B & C). Likewise, Daffalla *et al.* (2016) reported that culturing seeds of *G. tenax* on water agar medium with no added nutrients accelerated the germination rate. The GRI for *H. coddii* subsp. *barnardii* seeds on filter paper cultures was higher than the GRI for all the MS medium strengths, but not higher than the GRI for the Gelrite® culture (Table 6.4A, B & C), although no significant differences were found between the three seed cultures.

Good germination responses were achieved in all three *in vitro* seed cultures. These cultures provided a constant moisture level without the need for watering seeds as required for *in vivo* germination in soil cultures. It also reduced the time for obtaining viable seedlings as also reported by Roni *et al.* (2018). Seeds can therefore be considered as a good starting material for *in vitro* establishment of *H. coddii* subsp. *barnardii* seedlings, which could serve as an aseptic explant source for shoot culture.

6.3.3 Seedling development

The development of *H. coddii* subsp. *barnardii* seedlings derived from three different seed cultures (filter paper, MS medium and Gelrite®) was studied in order to select the best culture medium for production of *in vitro* seedlings as an aseptic explant source.

6.3.3.1 Seedlings developed from seeds germinated on filter paper

The results of a pilot study showed that seeds germinated on filter paper bridges (Figure 6.4A) could develop into well-established seedlings after transfer to ½ and full strengths of MS media (Figure 6.4B & C). The ANOVA analysis showed that the medium strength did not have a significant effect on the number of nodes and the survival percentage of these seedlings from week 2–5 (Appendix D: Table 3). The seedlings in both strengths of MS media cultures showed an increase in the number of nodes on the main stem from three to nine nodes over a period of 5 weeks. The seedlings on the two MS medium strengths also attained a high (98.3%) survival percentage after five weeks (Table 6.5).

Table 6.5. The number of nodes and survival percentage of seedlings developed from seeds germinated on filter paper and transferred to ½ and 1 (full) strength MS medium.

	Number of nodes				Survival %
MS medium strength	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 5**
½ MS	3.50	5.97	7.60	9.17	98.33
Full MS	3.12	5.62	7.13	8.87	98.33
F pr.	0.063	0.135	0.190	0.446	0.753
LSD	–	–	–	–	–

No significant differences at the 5% level of significance.

Morphological observations

The cotyledons of seedlings grown on both ½ and full strengths of MS media appeared in the first week and remained dark green up to the fourth week of culture. From the fifth week onwards, yellowing of the cotyledons was visible in seedlings grown on ½ strength MS medium [Figure 6.4B (c)], while this symptom was not observed for seedlings on full strength MS medium [Figure 6.4C (c)]. The first true leaves appeared on both strengths of MS media in the second week [Figure 6.4B & C (a & b)]. The lower leaves of seedlings grown on ½ strength MS medium showed

severe symptoms of interveinal chlorosis from the fifth week [Figure 6.4B (c & d)], whereas leaves of seedlings on full strength MS medium remained green [Figure 6.4C (c & d)]. A well-developed root system was established on seedlings from both MS medium strengths. The tap root developed lateral roots from the second week onwards and callus formation at the base of the seedling was initiated from the fourth week onwards on full strength MS medium [Figure 6.4C (c & d)].

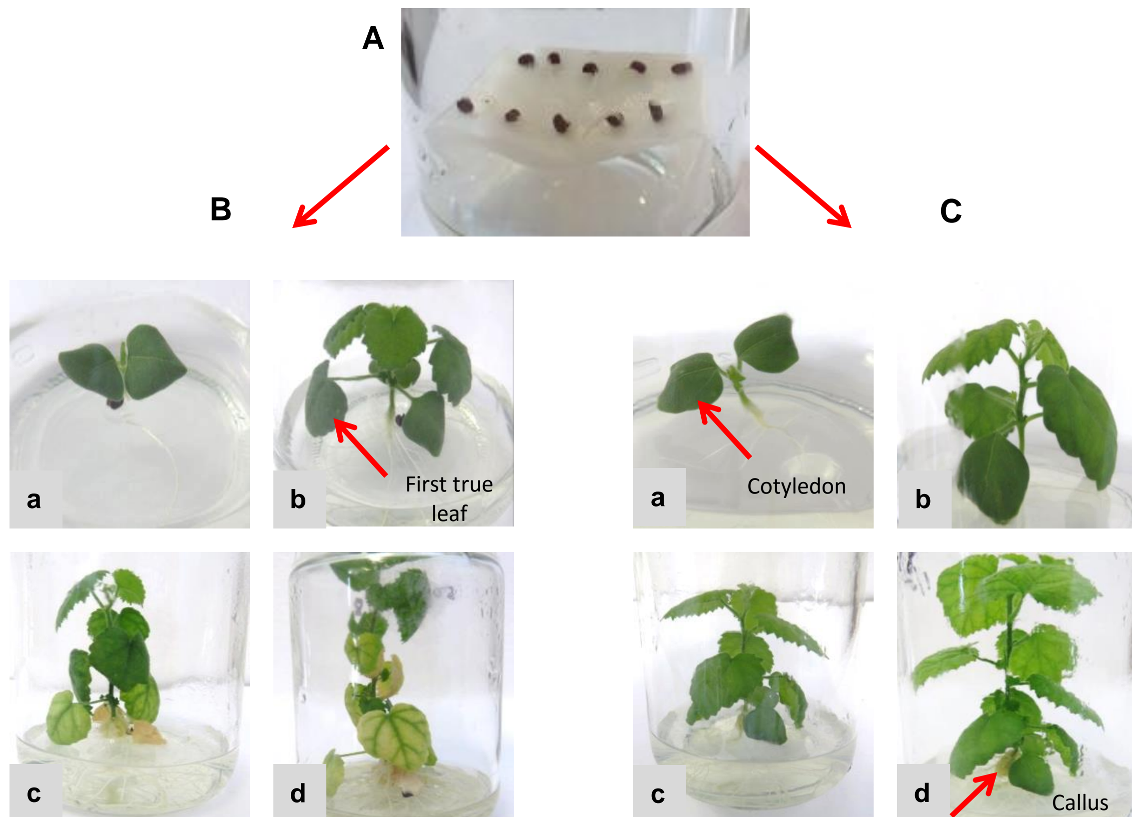

Figure 6.4. Seedling development of *H. coddii* subsp. *barnardii* seeds germinated on (A) Filter paper bridges and transferred to (B) ½ strength MS medium and (C) Full strength MS medium at (a) Week 1; (b) Week 3; (c) Week 5; (d) Week 7 of development.

In the present study, it was observed that the faster germination of seeds on filter paper bridges facilitated seedling development after the transfer of germinated seeds on full strength MS medium. Such an approach could be useful for *in vitro* establishment of seedlings in plant species where it is difficult to germinate seeds directly on tissue culture media. Sekhukhune et al. (2018) reported similar observations for *in vitro* germination of *Actinidia arguta* and *A. chinensis* seeds.

6.3.3.2 Seedlings developed from seeds germinated directly on MS medium and Gelrite

The ANOVA analysis showed that different MS medium strengths and Gelrite® had a significant effect on the number of nodes (week 2–5) of seedlings and on the survival percentage of the seedlings at five weeks (Appendix D: Table 4). *In vitro* grown *H. coddii* subsp. *barnardii* seedlings showed a progressive increase in the number of nodes on all strengths of MS media over a period of 5 weeks (Table 6.6). The highest number (8.42–8.48) of nodes per seedling at week 5 was recorded on the lower (¼, ½ and ¾) strengths of MS media with no significant difference between these treatments. However, it was significantly higher than the number of nodes on full strength MS medium (7.67 nodes) and Gelrite® which had the least number (2.33) of nodes (Table 6.6). Two week old seedlings of *G. tenax* cultured on water agar (control) also had the lowest number of nodes, whereas in contrast to our findings, the most nodes were recorded on seedlings cultured on full strength MS medium (Daffalla *et al*., 2016).

The MS medium strength and Gelrite® (control) treatment also influenced the survival of seedlings. The best seedling survival (100%) at week 5 was observed on higher strengths (¾, full, and 1¼) of MS media, while significantly less (86.3%) seedlings survived on the lowest strength (¼) of MS medium. None of the seedlings on the Gelrite® culture survived after four weeks which could be due to the lack of nutrients in the medium (Table 6.6).

Table 6.6. The number of nodes and survival percentage of seedlings developed from seeds germinated directly on different strengths of MS medium and on Gelrite®.

Treatment	Number of nodes				Survival %
	Week 2	Week 3	Week 4	Week 5	Week 5
¼ MS	3.32^{a}	5.79^{a}	7.22^{a}	8.48^{a}	86.33^{b}
½ MS	3.29^{a}	5.39^{ab}	6.95^{a}	8.46^{a}	96.67^{ab}
¾ MS	3.19^{ab}	5.04^{bc}	6.68^{ab}	8.42^{a}	100.00^{a}
1 (full) MS	2.78^{c}	4.37^{d}	6.04^{b}	7.67^{b}	100.00^{a}
1¼ MS	2.90^{bc}	4.51^{cd}	6.16^{b}	7.80^{ab}	100.00^{a}
Gelrite®	1.37^{d}	2.20^{e}	2.33^{c}	2.33^{c}	0.00^{c}
F pr.	<.0001	<.0001	<.0001	<.0001	<.0001
LSD	0.32	0.59	0.68	0.74	12.18

Means with the same letter within a column are not significantly different at the 5% level of significance.

Morphological observations

The cotyledons of seedlings started to appear in the first week of culture on all strengths of MS media and Gelrite® (control) followed by the first true leaves. Well-developed seedlings established after two weeks without morphological differences between the treatments. However, from the third week onwards seedlings on the Gelrite® culture (without nutrients) showed visible morphological changes such as yellowing of the cotyledons and interveinal leaf chlorosis [Figure 6.5A (b)] followed by further deterioration and subsequent death of seedlings [Figure 6.5A (c & d)]. This could be due to the depletion of nutrients from the cotyledons and complete lack of nutrients in the medium since plants cannot complete their life cycle without essential nutrients (Taiz *et al*., 2015). Although the germination of seeds on the Gelrite® medium was faster, it was detrimental to seedling development after 2 to 3 weeks in culture. This shows that seedlings of *H. coddii* subsp. *barnardii* require nutrients for their growth and survival in *in vitro* culture.

The strength of MS medium also affected seedling development. Seedlings grown on lower (¼ and ½) strengths of MS media showed symptoms of senescence such as yellowing of cotyledons from the second and third week onwards and interveinal chlorosis on the lower leaves of the seedlings from the fourth week onwards [Figure 6.5B & C (c & d)]. Cotyledons of seedlings grown on higher (¾, full and 1¼) strengths of MS media remained green up to the seventh week [Figure 6.5D–F (c & d)]. Seedlings grown on ¾ strength of MS medium showed symptoms of interveinal chlorosis on the lower leaves at a later stage (fifth week onwards) [Figure 6.5D(c)] that are associated with deficiency of essential mobile elements such as Mg. This could be due to depletion of nutrients in the yellow cotyledons and the relocation of mobile elements from older to younger leaves (Taiz and Zeiger, 2010; McCauley *et al*., 2011). These responses indicated that the lower strengths (¼, ½, and ¾) of MS media are not sustainable for seedling development.

Seedlings grown on higher (full and 1¼) strengths of MS media had well-developed leaves and thicker stems after 5–7 weeks in culture, although symptoms of interveinal chlorosis appeared on the younger (upper) leaves of seedlings from the fifth week onwards which was more pronounced on the 1¼ MS medium strength [Figure 6.5E & F (c & d)]. Such symptoms might be associated with deficiency of

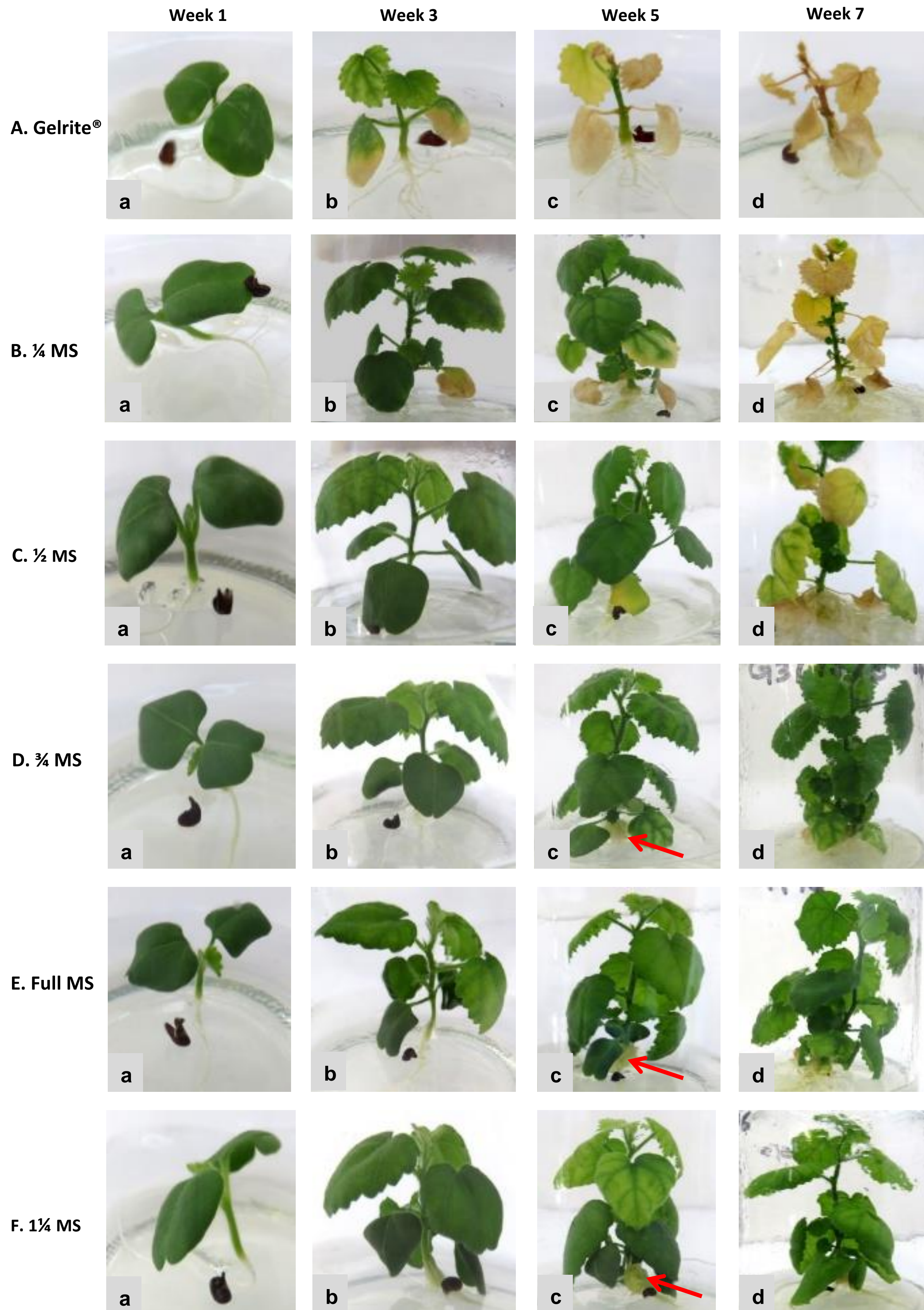

Figure 6.5. Seedling development on Gelrite® and different strengths of MS media. (A) Gelrite® (control); (B) ¼ MS; (C) ½ MS; (D) ¾ MS; (E) Full MS; (F) 1¼ MS in (a) Week 1; (b) Week 3; (c) Week 5; (d) Week 7 of development. Arrows indicate callus formation.

essential immobile nutrients such as Fe and Ca that cannot be readily mobilised from the older leaves (Taiz and Zeiger, 2010; McCauley *et al.*, 2011). These results show that seedlings cannot be sustained on these media strengths for more than 6–7 weeks. A well-developed root system consisting of a taproot and lateral roots developed from the second week onwards on seedlings cultured on all MS medium strengths (Figure 6.5B–F). Higher strengths (¾, 1 and 1¼) of MS medium caused callus formation at the base of seedlings from the fourth week onwards [Figure 6.5D–F (c)]. Further studies are required to clarify this response.

Although the best performance in terms of germination parameters was found for seeds germinated on filter paper, Gelrite®, and lower (¼ and ½) strengths of MS media, these cultures proved unsustainable for *in vitro* seedling establishment. However, these types of cultures could be suitable for germination studies as also recommended by Daffalla *et al.* (2016) for water agar medium. Based on the above results, full strength MS medium was selected as the most suitable for *in vitro* production of seedlings, which can also serve as an explant source for further *in vitro* multiplication studies. This is in accordance with research on *Hibiscus* and other members of the Malvaceae family where full strength MS medium was also used for *in vitro* seed germination and development of seedlings that were used as an aseptic explant source (Khatun *et al.*, 2003; Sakhanokho, 2008; Ayadi *et al.*, 2011; Daffalla *et al.*, 2016; Sultana *et al.*, 2016a).

6.3.4 Shoot culture

Successful regeneration of plantlets through micropropagation depends on various factors including the explant source, the explant type and the use of suitable PGRs for induction of multiple shoots (Christensen *et al.*, 2008; Sultana *et al.*, 2016a). The effect of these factors on micropropagation of *H. coddii* subsp. *barnardii* is discussed in this section.

6.3.4.1 Explant source and disinfection

6.3.4.1.1 Donor plants from nature

Preliminary studies on surface disinfection of nodal explants obtained from *H. coddii* subsp. *barnardii* plants collected from nature showed high incidences of bacterial contamination, irrespective of the concentration of the disinfectant and duration of the treatment, although no fungal infection was observed. Disinfection with 70% ethanol (60 seconds) followed by 25% bleach solution [Jik® 3.5 % (w/v) NaOCl] for 25 minutes and 50% bleach solution for 15 minutes resulted in 85% and 80% contamination of *in vitro* cultures respectively, and high mortality (80%) of the explants. In contrast, Dar *et al*. (2012) reported 88% survival and no contamination of nodal explants of *H. rosa-sinensis* collected from an outside garden when surface-disinfected with 50% (v/v) Clorox® [5.25% (w/v) NaOCl] bleach solution containing higher concentration (2.63%) of NaOCl. However, microbial contaminations of *in vitro* cultures are found to occur more readily when the explant source is taken directly from field grown plants (Daud *et al*. 2012). Disinfection of nodal explants is considered to be more difficult since the attachment of the petiole to the stem at an angle creates a V-shaped trough wherein contaminants can be trapped making it more difficult to sterilise nodal explants with axillary buds (Dar *et al*., 2012).

6.3.4.1.2 *In vivo* grown donor plants

Plants propagated *in vivo* from seeds under controlled environmental conditions were also investigated as an explant source, since explants derived from physiologically pre-conditioned plants are considered less prone to microbial contamination in *in vitro* cultures (Hartmann *et al*. 2011). West and Preece (2004), Christensen *et al*. (2008) and Manokari *et al*. (2016) used pre-conditioned plants of *H. moscheutos*, *H. rosa-sinensis* and *H. sabdariffa* grown in pots in a greenhouse as donor plants. Surface disinfection of *H. coddii* subsp. *barnardii* nodal shoot explants with 70% ethanol (60 seconds) and 50% (v/v) commercial bleach (Jik®) solution containing 1.75% active NaOCl for 15 minutes substantially reduced bacterial contamination (20%) of cultures as compared to the explants from naturally grown plants (85%). However, this treatment caused severe bleaching and necrosis of the explants, as well as death of pre-existing axillary buds, from the second week onwards, thus resulting in only 15% explant survival by the third week. Disinfection of explants with lower concentration [25% (v/v) bleach (Jik®) solution containing

0.875% active NaOCl] for 25 minutes slightly improved the survival (35%), although browning of the explants was also observed at three weeks of culture. Bhalla *et al.* (2009) also reported extensive bleaching of some *H. rosa-sinensis* nodal explants when surface disinfected with 2.63% NaOCl solution prepared from Clorox® bleach. Disinfection with other sterilants [80% ethanol (20 s) and 0.1% mercuric chloride ($HgCl_2$) solution for 5 minutes] was also detrimental to *Lawsonia inermis* shoot tip explants, which showed necrosis and died after six days in culture (Shiji and Siril, 2018). Manokari *et al.* (2016) found low contamination (2–5%) of *H. sabdariffa* explants when disinfected with $HgCl_2$ for 3½ minutes but reported blackening of the explants with 5 minutes of disinfection. On the other hand, Christensen *et al.* (2008) and Airò *et al.* (2009) successfully disinfected the explants of *H. rosa-sinensis* (>90% explants free of visual contamination) with 1–1.5% NaOCl for 20 minutes. From literature, it appears that the success of the surface disinfection procedure may depend on the plant tissue and type of explants.

The results of our study showed that nodal shoot explants derived from naturally grown and *in vivo* propagated *H. coddii* subsp. *barnardii* plants seem not suitable as explant source for the establishment of an aseptic *in vitro* culture due their sensitivity to the surface disinfection treatment and high mortality. Henceforth, the suitability of *in vitro* produced seedlings as explant source was further studied.

6.3.4.2 *In vitro* produced seedlings

The explant source is one of the important determinants of successful *in vitro* cultures (Bhalla *et al.*, 2009; Yildiz, 2012). Results of this study showed that nodal explants obtained from *H. coddii* subsp. *barnardii* plants collected from nature and from *in vivo* grown plants are unsuitable as explant source for *in vitro* shoot culture. The suitability of *in vitro* produced seedlings as explant source was further studied.

6.3.4.2.1 Axillary shoot induction on *in vitro* grown seedlings

In vitro grown seedlings, similarly to *in vivo* grown plants (Chapter 4), exhibited strong apical dominance resulting in the development of a single stem without proliferation of axillary shoots (Figure 6.6A). The effect of removal of the apex on axillary shoot induction of *in vitro* grown seedlings was studied. The excision of the apex (Figure 6.6B) from five-week-old seedlings was aseptically performed directly in

the culture vessel without the need of transplanting to a new culture medium. This procedure proved successful for axillary shoot induction over a short period, resulting in axillary bud sprouts in the first week (Figure 6.6C) followed by shoot proliferation and elongation from the third week onwards (Figure 6.6D). An average of 5.65 ±1.04 primary axillary shoots (40–50 mm in length) per seedling developed after five to six weeks of culture (Figure 6.6E). This response could be attributed to removal of the seedling apex, which is known to release the suppressing effect of endogenous auxin on the axillary buds and to promote outgrowth of the axillary shoots (Gómez-Leyva *et al*., 2008; Hartmann *et al*., 2011).

The procedure described above proved to be time, cost and labour effective for generating aseptic axillary shoots. Such shoots can be used as explants for further *in vitro* shoot culture and as microshoots for *in vitro* and *ex vitro* rooting purposes. To the best of our knowledge, this is the first report on such technique for *in vitro* axillary shoot induction of *Hibiscus* species.

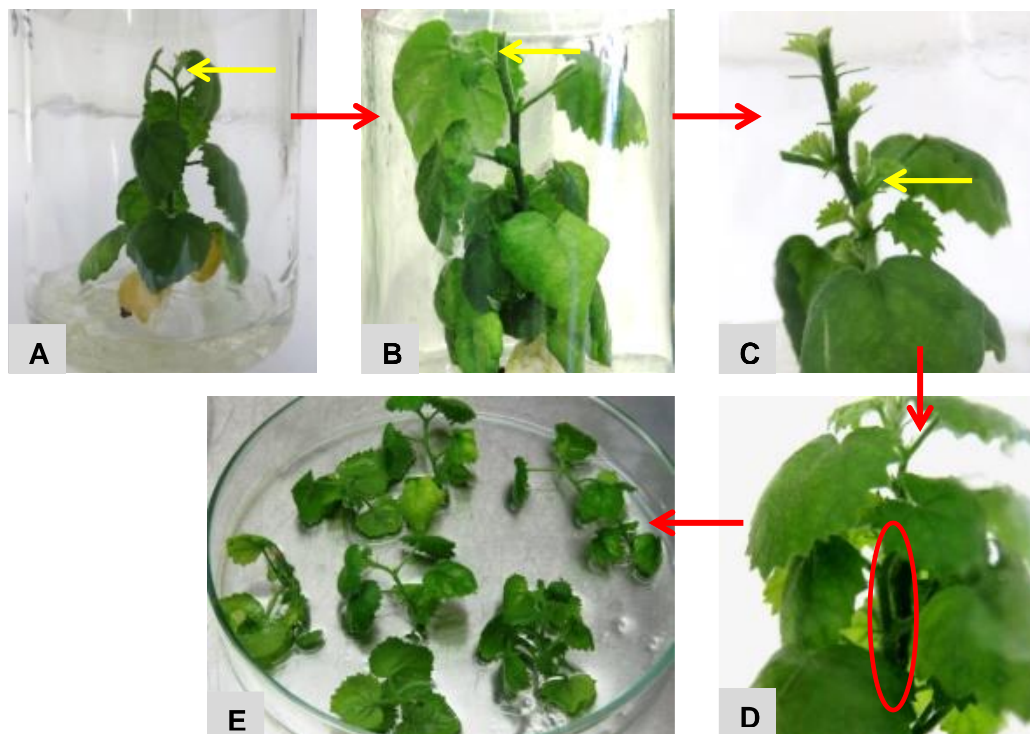

Figure 6.6. Axillary bud sprouting, shoot proliferation and elongation on *in vitro* grown seedlings after removal of the apex. (A) Five-week-old seedling with an intact apex (arrow) and no axillary shoots; (B) Five-week-old seedling with removed apex (arrow); (C) Sprouting of axillary buds (arrow) on the main stem after one week; (D) Proliferation and elongation of axillary shoots after five weeks; (E) Primary axillary shoots obtained from one seedling after six weeks in culture without subculturing.

6.3.4.2.2 Seedlings as a source for nodal shoot explants

Five to six week old *in vitro* grown *H. coddii* subsp. *barnardii* seedlings also proved suitable as a source of various types (apical and basal) and sizes (small, 1–2 nodes; large 3–4 nodes) of shoot explants. The effect of type, size and inoculation position of explants cultured on PGR-free medium was investigated to select the best explant for *in vitro* shoot culture. Shoot apex, nodal segment, hypocotyl, cotyledon, and leaf explants from *in vitro* grown seedlings of other *Hibiscus* species such as *H. sabdariffa* and *H. cannibinus* were used for shoot induction with varying results (Zapata *et al*., 1999; Gómez-Leyva *et al*., 2008; Govinden-Soulange *et al*., 2009; Khatun *et al*., 2003; Samanthi *et al*., 2013; Sultana *et al*., 2016a).

Survival of the *H. coddii* subsp. *barnardii* shoot explants in *in vitro* cultures depended on the size of the explant. Small explants (1–2 nodes) obtained from both the upper (apical) and lower (basal) part of the seedling did not survive after three to four weeks in culture (Figure 6.7) and proved not suitable for further studies on shoot multiplication. Larger explants (90%) remained viable (with green stems) after four weeks in culture. Kane (2005) and Yildiz (2012) reported that larger shoot explants have more axillary buds that can proliferate into shoots, grow faster and can survive better. Therefore, the shoot induction response of larger shoot explants (3–4 nodes) with respect to type of explant (apical and basal) and inoculation position (vertical and horizontal) was studied.

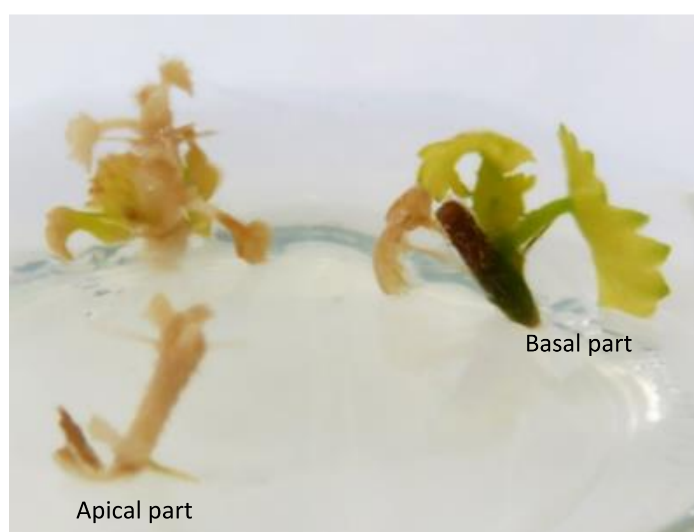

Figure 6.7. Small explants (1–2 nodes) obtained from the apical and basal part of seedlings and cultured on PGR-free MS medium died after three to four weeks in culture.

Sprouting (1–2 mm) of pre-existing axillary buds occurred in 100% of both apical and basal shoot explants [Figure 6.8A, B & C (a)] from the first week of culture onto fresh PGR-free MS medium. However, primary axillary shoot proliferation and elongation were only observed on basal [Figure 6.8B & C (b)] and not on apical [Figure 6.8A (b)] seedling shoot explants after 2–3 weeks of culture. The suppression of axillary shoot proliferation observed in the apical seedling shoot explants could be ascribed to apical dominance exerted by the presence of an apex in contrast to the basal shoot explants without an apex, as also reported by other studies (Bhalla *et al*., 2009; Hartmann *et al*., 2011). After four weeks of culture, an average of 3–5 primary axillary shoots (15–25 mm) were recorded on rooted basal seedling shoot explants of *H. coddii* subsp. *barnardii*, irrespective of the inoculation (vertical or horizontal) position [Figure 6.8B & C (b)]. Herath *et al*. (2004) also successfully used the basal part of *H. cannabinus* seedlings as explant for shoot induction. Manokari *et al*. (2016) reported a better shoot induction response on nodal explants of *H. sabdariffa* than on apical shoot explants. Only a few short (<10 mm) axillary shoots, which started to yellow and subsequently died after four weeks (Figure 6.8D), developed on *H. coddii* subsp. *barnardii* basal seedling shoot explants that did not root. This suggests that more frequent subculturing onto fresh MS medium could alleviate the problem (Bhalla *et al*., 2009; Hartmann *et al*., 2011; Manokari *et al*., 2016). Addition of cytokinin to the medium might also delay senescence of explants (Vardja and Vardja, 2001; Bhalla *et al*., 2009).

Adventitious roots formed on both apical and basal shoot explants of *H. coddii* subsp. *barnardii* on PGR-free MS medium after three weeks in culture (Figure 6.9A & B), but the response varied depending on the explant type and the inoculation position. More (40%) of the vertically oriented basal shoot explants (Figure 6.9B) formed adventitious roots than the horizontally oriented (15%) explants (Figure 6.9C), whereas only 8% of the apical shoot explants (Figure 6.9A) rooted. Ayadi *et al*. (2011) also reported adventitious root formation on nodal explants of *H. cannabinus* after three weeks of culture on BAP-free medium, although the rooting response (91%) was better than in *H. coddii* subsp. *barnardii*. In plants, endogenous auxin produced by young stem tips and leaves is reported to induce adventitious roots in cuttings (Hartmann *et al*., 2011; Veraplakorn, 2016).

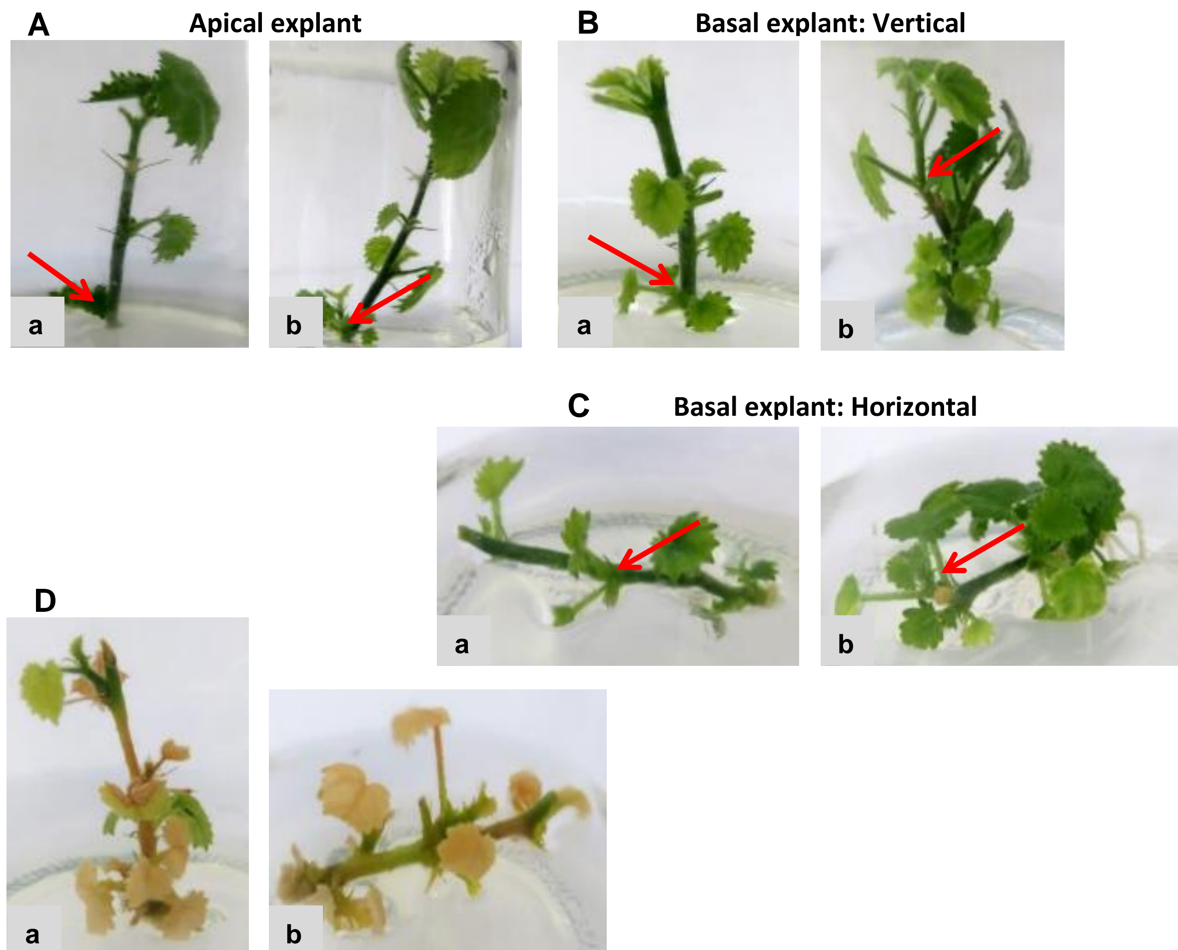

Figure 6.8. Examples of (A) Apical shoot explant inoculated vertically; (B) Basal shoot explant inoculated vertically; (C) Basal shoot explant inoculated horizontally. (a) Axillary bud sprouting (arrow) after one week and (b) Shoot proliferation (arrow) after four weeks in culture; (D) Yellowing of proliferated axillary shoots and leaves and subsequent death of basal shoot explants without adventitious root formation: (a) Vertically; (b) Horizontally inoculated explant.

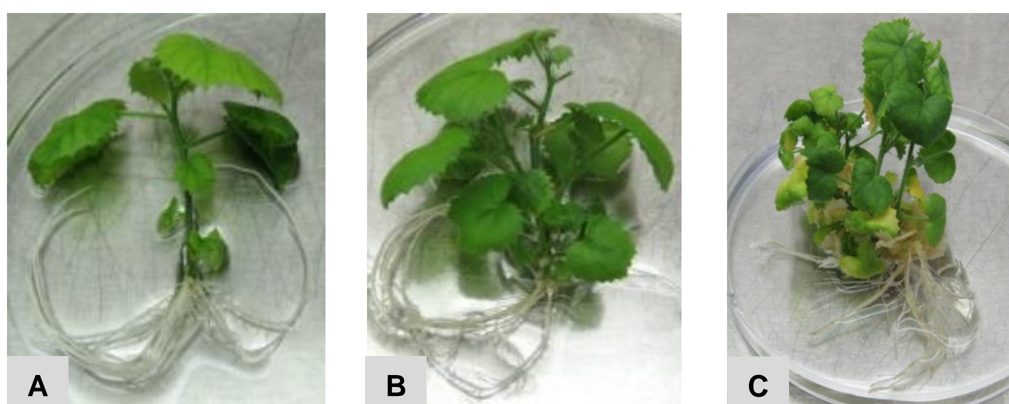

Figure 6.9. Examples of adventitious root formation on different seedling shoot explants after three weeks of culture on PGR-free MS medium. (A) Apical shoot explant inoculated vertically; (B) Basal shoot explant inoculated vertically; (C) Basal shoot explant inoculated horizontally.

According to Bhalla *et al.* (2009) direct root formation on PGR-free medium is ideal for acclimatisation in soil since it eliminates the additional step for root induction with rooting hormones and thereby shortens the plant regeneration process.

Callus formation is considered as a natural response of plant tissue to wounding (Bhalla *et al.*, 2009; Ikeuchi *et al.*, 2013). Such response was observed at the cut end of basal shoot explants of *H. coddii* subsp. *barnardii* that did not root and it was more distinct in horizontally (Figure 6.10B) than in vertically placed explants (Figure 6.10A). Both vertically and horizontally oriented explants of *Eucalyptus gunnii* formed callus and it was also more pronounced on the cut ends of horizontally placed explants (Hervé *et al.* (2001).

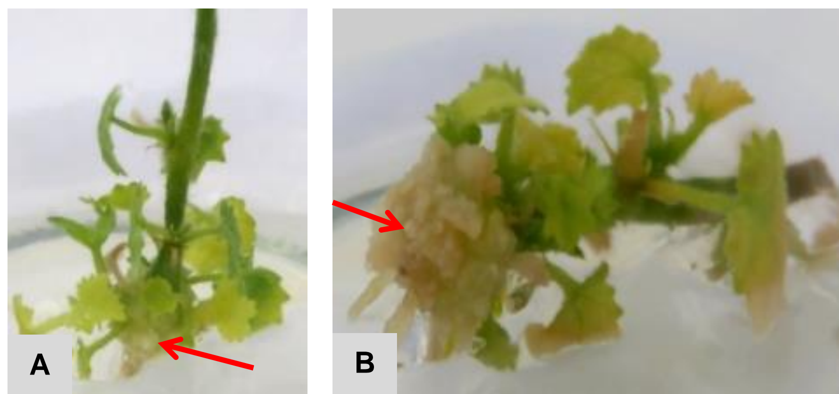

Figure 6.10. Callus formation (arrows) on basal seedling shoot explants inoculated in different positions after four weeks in culture. (A) Vertical position; (B) Horizontal position.

This study suggests that large (3–4 nodes) basal shoot explants obtained from *in vitro* grown seedlings of *H. coddii* subsp. *barnardii* and cultured on PGR-free medium in a vertical position are suitable for the establishment of shoot cultures. Such explants (Figure 6.11A), can give rise to 4–5 single primary axillary shoots (30–50 mm) of varying thickness [2–3 mm (thick) and 1 mm (thin)] (Figure 6.11B & D) and a clump of short (5–10 mm) primary axillary shoots (Figure 6.11C) that could be used as a secondary explant source for shoot multiplication and for plant regeneration from *ex vitro* rooted shoots. Regenerated plantlets of *H. coddii* subsp. *barnardii* from PGR-free medium were successfully acclimatised.

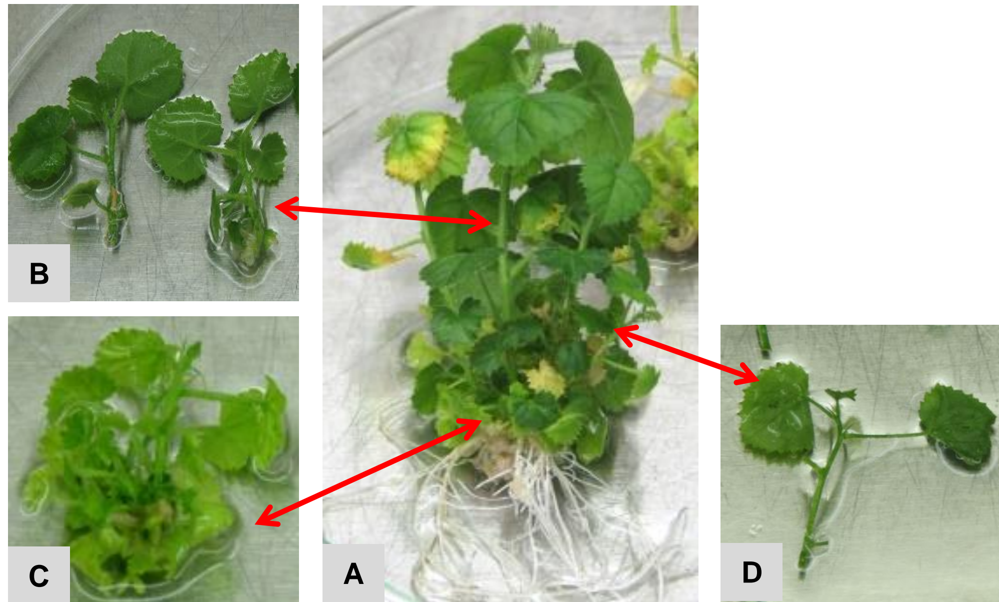

Figure 6.11. Examples of primary axillary shoots induced on rooted basal seedling shoot explants, which can serve as secondary explant source for shoot multiplication or for plant regeneration. (A) Rooted basal explant; (B) Long (30–50 mm) and thick (2–3 mm) axillary shoots; (C) Clump of short (5–10mm) axillary shoots on piece of original explant; (D) Thin (1 mm) axillary shoots.

6.3.4.3 Effect of plant growth regulators and explant source on shoot induction

6.3.4.3.1 Effect of BAP concentration

Cytokinins are used in micropropagation of plants to enhance shoot multiplication (Gaba, 2005; Abdellatef and Khalafalla, 2007). Christensen *et al*. (2008) selected BAP as cytokinin for multiple shoot induction in *H. rosa-sinensis* since kinetin had little effect when compared to BAP, while TDZ caused excessive callus formation and death of the shoots. Studies of other *Hibiscus* species also reported the use of BAP for *in vitro* shoot induction, although the response was dependent on species and explant type (Zapata *et al*., 1999; Herath *et al*., 2004; Gómez-Leyva *et al*., 2008; Bhalla *et al*., 2009; Sami *et al*., 2016). Even though vertically oriented basal seedling shoot explants (Section 6.3.4.2.2.) of *H. coddii* subsp. *barnardii* had the highest shoot regeneration efficiency (up to 40%) on PGR-free MS medium, the effect of BAP on *in vitro* shoot induction of different shoot explants from various sources (seedlings and axillary shoots) was also researched.

a. <u>BAP concentration (0.50–4 mg L^{-1})</u>

A pilot screening was conducted to evaluate the effect of a broad range of five BAP concentrations (0.50, 1, 2, 3, 4 mg L^{-1}) on the shoot induction response of basal seedling shoot explants of *H. coddii* subsp. *barnardii*.

Sprouting of pre-existing axillary buds (3–4/explant) occurred on 100% of basal shoot explants after one week of culture on MS medium, irrespective of the presence or absence of BAP (Figure 6.12A (a–d), Week 1). The best bud sprout response, 96% and 82% in nodal explants of *H. sabdariffa* was observed with 1 mg L^{-1} and 0.50 mg L^{-1} BAP respectively, whereas no bud sprouting was reported on BAP-free medium (Manokari *et al*., 2016).

The best shoot induction response in *H. coddii* subsp. *barnardii* was in the BAP-free MS medium where 70% of the explants had 3–4 shoots that elongated up to 15–25 mm after four weeks of culture [Figure 6.12B (a)]. Govinden-Soulange *et al*. (2009) also reported the highest axillary shoot formation (81%) and the highest number of shoots (3.2) per nodal explant of *H. sabdariffa* cultured on PGR-free MS medium, whereas the lowest shoot induction response (33%) was reported with 2 mg L^{-1} BAP. In contrast, 8.8 µM BAP resulted in the highest number of shoots (11) per basal shoot explant of *H. cannabinus* compared to only two shoots per explant produced on BAP-free medium (Herath *et al*., 2004). Nodal explants of *H. rosa-sinensis* produced 1.94 shoots per explant when cultured on MS medium with 15 µM BAP compared to less shoots (1.14) on BAP-free medium (Bhalla *et al*., 2009), which may suggest that the shoot induction response varies depending on species and explant type.

Proliferation of short shoots (<5 mm) was observed on 30% and 20% of *H. coddii* subsp. *barnardii* explants with lower concentrations of BAP, 0.5 and 1 mg L^{-1} respectively [Figure 6.12B (b & c)], whereas higher concentrations (2–4 mg L^{-1}) of BAP did not promote shoot proliferation [Figure 6.12B (d)] in any of the basal shoot explants. High concentrations of BAP may cause ethylene production that negatively affects shoot regeneration and elongation by restricting lengthening of internodes (Khan *et al*., 2015). Manokari *et al*. (2016) also reported a reduction in *H. sabdariffa* shoot length from 26 mm to 11 mm with an increasing concentration of BAP (1.50–3 mg L^{-1}).

Adventitious root formation on the basal shoot explants was initiated from the second week of culture on BAP-free medium and 67% of these explants rooted after four weeks (Figure 6.13A). However, root formation was not observed in any of the BAP (0.50–4 mg L^{-1}) treatments. In accordance, no root formation was found on nodal explants of *H. sabdariffa* in the presence of 0.5–3 mg L^{-1} BAP (Manokari *et al.*, 2016). Sakhanokho (2008) reported that lower concentrations of BAP (4.4 µM) promoted root formation (up to 82.2%) in shoot apex explants of *H. acetosella* as compared to 55.6% rooting on PGR-free medium, but higher BAP concentrations (8.9 and 13.3 µM) also resulted in less root formation, 46.7% and 40% respectively.

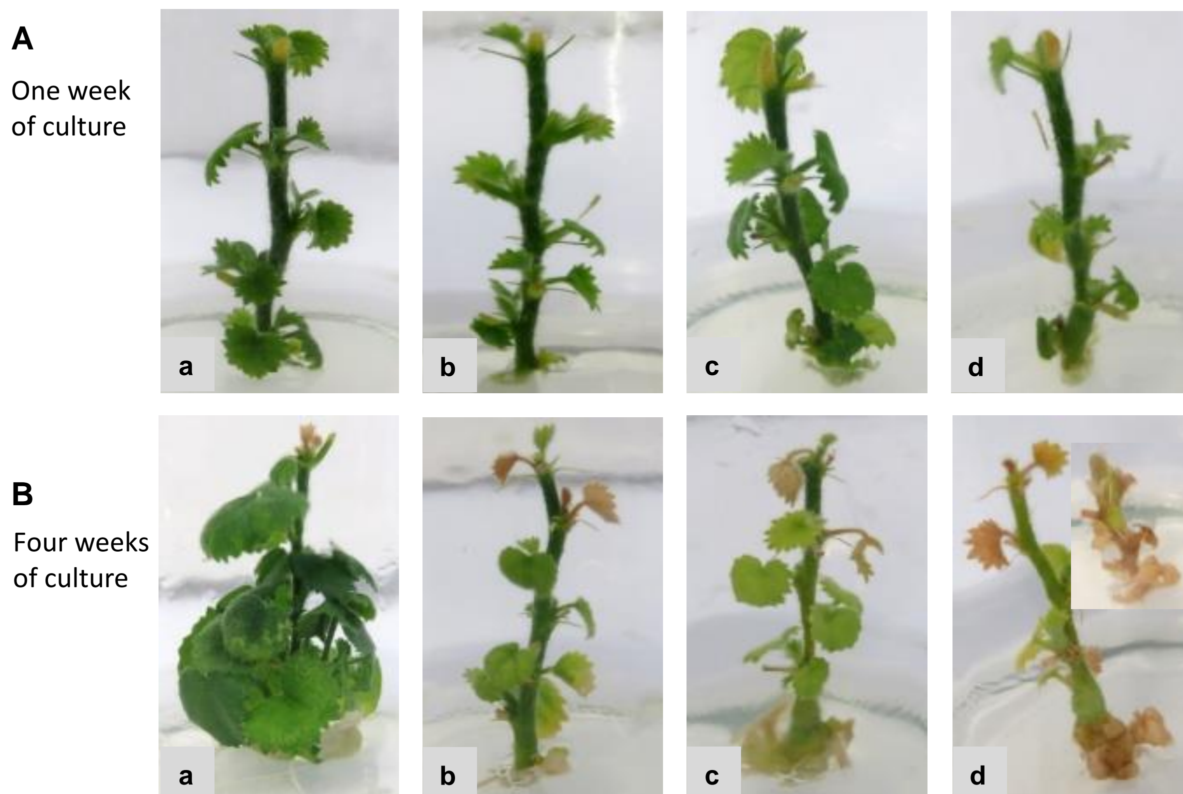

Figure 6.12. Examples of the effect of various BAP concentrations (0.50–4 mg L^{-1}) on sprouting of axillary buds and shoot elongation on basal seedling shoot explants after (A) One week and (B) Four weeks of culture. (a) Control; (b) 0.50 mg L^{-1} BAP; (c) 1 mg L^{-1} BAP; (d) 4 mg L^{-1} BAP.

Figure 6.13. (A) Adventitious root formation on basal seedling shoot explant after 3 weeks of culture on BAP-free MS medium; (B) Callus formation on basal seedling shoot explant after 4 weeks of culture on MS medium supplemented with 3 mg L^{-1} BAP.

All BAP treatments resulted in callus induction at the cut end of the *H. coddii* subsp. *barnardii* basal shoot explants (Figure 6.13B) and the percentage of explants with callus (50–75%) correlated with the increase in the BAP concentration In contrast, only 25% of the control explants had callus. Manokari *et al*. (2016) found no callus formation on nodal explants of *H. sabdariffa* cultured on lower concentrations of BAP(0.50 and 1 mg L^{-1}), but reported a high frequency of callus formation with 2.5 and 3 mg L^{-1} BAP.

All (100%) of the *H. coddii* subsp. *barnardii* explants in the BAP-free MS medium remained viable, whereas the increase in the BAP concentration adversely affected the viability. Explants on media with higher BAP concentrations (2, 3 and 4 mg L^{-1}) showed signs of tissue necrosis (browning of explant and leaves) from the third week onwards, which resulted in the death of 75%, 83% and 92% of explants respectively [Figure 6.12B (d), Week 4]. This response could be linked to the excessive callus formation on explants from these BAP treatments that could lead to restriction of nutrient supply and subsequent death of explants. Reduction in survival rate of shoot explants from other *Hibiscus* species was also associated with higher concentrations of BAP in *in vitro* cultures. Christensen *et al*. (2008) reported high survival (95% and 93.9%) of nodal explants of *H. rosa-sinensis* in the presence of lower BAP concentrations, 0.22 and 2.2 μM respectively. It significantly reduced to 65% survival with a higher (22 μM) BAP concentration. Bhalla *et al*. (2009) reported a 20% decrease in the viability of nodal explants of *H. rosa-sinensis* when cultured on MS medium supplemented with 10, 15 and 20 μM BAP.

b. BAP concentration (0.25–1 mg L^{-1})

Due to the poor shoot induction response of basal seedling shoot explants to higher concentrations (2, 3 and 4 mg L^{-1}) of BAP, the effect of lower concentrations (0.25, 0.50, 0.75 and 1 mg L^{-1}) of BAP on various explant types was examined. Apical and basal shoot explants obtained from different sources as described in Section 6.3.4.2.1 and 6.3.4.2.2 were used.

Apical and basal seedling shoot explants

In this experiment, the best bud sprouting and shoot induction response was also observed on BAP-free medium, although the response varied between explant types. Sprouting of pre-existing axillary buds (3–4/explant) occurred on 100% of both apical and basal shoot explants after one week of culture [Table 6.7, Figure 6.14A & B (a), Week 1]. All sprouted buds proliferated into shoots after four weeks of culture, although the shoot length varied with the explant type and the position of the shoots on the explant. Basal shoot explants (100%) yielded an average of 3–4 shoots (15–25 mm in length) per explant [Figure 6.14B (a), Week 4]. Similarly, Ayadi *et al.* (2011) reported a high shoot induction response (90.5%) on seedling nodal explants of *H. cannabinus* cultured on PGR-free medium. Nodal seedling explants of *H. sabdariffa* also gave the best (81%) shoot regeneration response with 3.2 shoots per explant in the absence of BAP (Govinden-Soulange *et al.*, 2009). In contrast, apical shoot explants of *H. coddii* subsp. *barnardii* formed 1–3 axillary shoots that were short (<10 mm) on the upper part of the explant and longer (10–15 mm) at the base [Figure 6.14A (a), Week 4]. The longer shoot at the base of apical shoot explants of *H. coddii* subsp. *barnardii* could be due to the decrease in the suppressing effect of auxin further away from the apex (Prusinkiewicza *et al.*, 2009).

Supplementation of the MS medium with low BAP concentrations did not improve shoot induction in *H. coddii* subsp. *barnardii* as compared to the control (Table 6.7). The highest bud sprouting (90% and 70%) and shoot induction response (80% and 60%) was observed for basal shoot explants in the presence of lower concentrations (0.25 and 0.50 mg L^{-1}) of BAP, although the proliferated shoots (5–10 mm) did not develop further [Table 6.7, Figure 6.14B (b & c), Week 4]. Reduction in bud sprouting (60%) and shoot proliferation (50%) response on basal shoot explants occurred with the increase in the BAP concentration to 0.75 and 1 mg L^{-1} (Table 6.7).

Table 6.7. The effect of BAP (0.25–1 mg L^{-1}) on shoot induction, rooting response and callus formation of apical and basal shoot explants obtained from seedlings.

	Apical shoot explant BAP concentration (mg L^{-1})					**Basal shoot explant BAP concentration (mg L^{-1})**				
	0	**0.25**	**0.50**	**0.75**	**1**	**0**	**0.25**	**0.50**	**0.75**	**1**
Response	*(%) Explants*					*(%) Explants*				
Bud sprouts (after one week)	100	70	40	30	20	100	90	70	60	60
Proliferated shoots (after four weeks)	100	60	20	10	10	100	80	60	50	50
Adventitious roots	30	0	0	0	0	20	0	0	0	0
Callus formation	30	40	40	50	50	50	70	80	80	90

Only short (<5 mm) shoots formed on basal shoot explants from these treatments that did not develop further [Figure 6.14B (d & e), Week 4]. Zapata *et al.* (1999) reported 85% and 91.7% shoot regeneration on shoot apex explants of *H. cannabinus* cultured on BAP-free medium and medium with 0.1 mg L^{-1} BAP respectively, whereas shoot regeneration was inhibited in the presence of higher BAP concentration (1 mg L^{-1}). Sami *et al.* (2016) reported the highest number of shoots (3.3) per nodal shoot explant of *H. syriacus* with a lower BAP concentration (0.20 mg L^{-1}). Shoot length in *H. rosa-sinensis* cv. Cassiopeia Wind Yellow was also reduced to 2 mm with higher BAP (22 µM) compared to 5 mm shoots obtained with a lower BAP concentration (2.2 µM) (Christensen *et al.*, 2008). Shoot length in *H. cannabinus* also reduced from 80 mm on BAP-free medium to 44 mm in the presence of 1 mg L^{-1} BAP (Ayadi *et al.*, 2011).

The shoot induction response also varied between *H. coddii* subsp. *barnardii* explant types in all BAP treatments. Similar to the control, bud sprouting and shoot proliferation responses were less pronounced in apical shoot explants than in basal shoot explants. An increasing BAP concentration negatively affected the shoot induction response of the apical shoot explants. The lowest bud sprouting (20–30%) and shoot proliferation (10%) was recorded for apical shoot explants on 0.75 and 1 mg L^{-1} BAP (Table 6.7). The short (<5 mm) proliferated shoots on apical shoot explants on all BAP concentrations did not develop further [Figure 6.14A (b–e), Week 4]. Different shoot responses were also reported for shoot apex and nodal explants obtained from axillary shoots of *G. hirsitum*. Nodal explants gave the best shoot induction response with 0.3 µM BAP, whereas shoot apex explants required

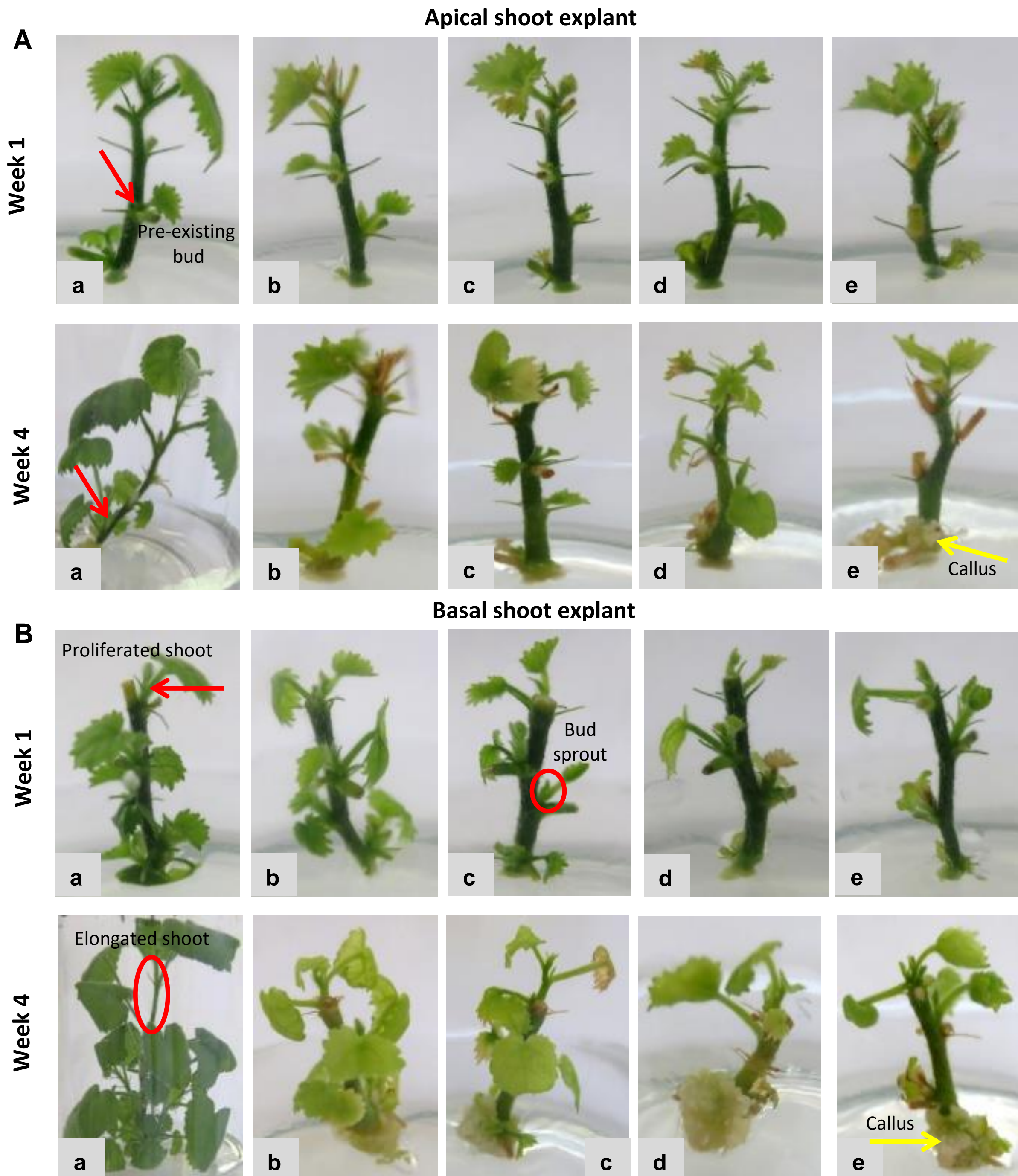

Figure 6.14. Examples of the effect of various BAP concentrations on axillary bud sprouting, shoot proliferation and callus formation on (A) Apical; (B) Basal seedling shoot explants after one week and four weeks of culture. (a) Control; (b) 0.25 mg L^{-1}; BAP (c) 0.50 mg L^{-1} BAP; (d) 0.75 mg L^{-1} BAP; (e) 1 mg L^{-1} BAP.

<u>Apical explants</u>: Control: Upper shoots <5 mm, no further development; Lowest shoot (arrow) 10–15 mm.
BAP (0.25–1 mg L^{-1}): Shoots <5 mm, no further development. Callus diameter: 1–5 mm.
<u>Basal explants</u>: Control: Shoots 15–25 mm; BAP (0.25 & 0.50 mg L^{-1}): Shoots 5–10 mm, no further development; BAP (0.75 & 1 mg L^{-1}): Shoots <5 mm, no further development. Callus diameter: 5–10 mm.

3.3 times more BAP (1 μM) for shoot induction. In contrast to *H. coddii* subsp. *barnardii*, both types of *G. hirsitum* explants did not form shoots when cultured on BAP-free medium (Hemphill *et al*., 1998). BAP also did not improve shoot induction in shoot apex explants of *H. acetosella* where one shoot per explant was produced on both BAP-free medium and medium with 4.4 μM BAP (Sakhanokho, 2008). Results of the present study showed that the best shoot induction response on both apical and basal shoot explants of *H. coddii* subsp. *barnardii* was obtained on BAP-free medium, although basal shoot explants performed better.

The rooting response of *H. coddii* subsp. *barnardii* was low and occurred on apical (30%) and basal (20%) shoot explants only on BAP-free medium. No root formation on both types of explants on all the BAP treatments was observed (Table 6.7). Similarly, Ayadi *et al*. (2011) reported 91% rooting of *H. cannabinus* nodal explants on BAP-free medium. In contrast, Sakhanokho (2008) indicated less root formation (55.6%) on *H. acetosella* explants on BAP-free medium than on medium with 4.4 μM BAP (82.2%).

Callus formation occurred on apical and basal shoot explants of *H. coddii* subsp. *barnardii* that did not root and the frequency increased with an increase in the BAP concentration. The percentage of explants with callus ranged from 40–50% on apical and 70–90% on basal shoot explants in the presence of BAP. The highest callus formation (90%) was observed on basal shoot explants cultured on medium with 1 mg L^{-1} BAP. Apical (30%) and basal (50%) shoot explants on the BAP-free medium exhibited the lowest frequency of callus formation (Table 6.7). Zapata *et al*. (1999) also reported callus formation on all shoot apex explants of *G. hirsitum* when cultured on medium with 1 mg L^{-1} BAP. Smaller callus (1–5 mm diameter) formed on *H. coddii* subsp. *barnardii* apical shoot explants, while larger callus (5–10 mm) formed on the basal shoot explants (Table 6.7, Figure 6.14A & B, Week 4). Yellowing and senescence of leaves were visible on explants that did not form roots or had callus, although the stems of explants were still green [Figure 6.14A & B (e), Week 4] which could be due the presence of BAP in the medium that is reported to delay senescence (Vardja and Vardja, 2001; Gaba, 2005).

Primary axillary shoot explants

Primary axillary shoots produced directly *in vitro* on seedlings after apex removal (Section 6.3.4.2.1) and on rooted basal seedling shoot explants cultured on PGR-free medium (Section 6.3.4.2.2) were also used for this experiment. Individual axillary shoot explants (with and without apex) of different sizes (small and large) responded differently when cultured on BAP-free MS medium and in the presence of BAP. Small explants (1–2 nodes) did not survive after 3–4 weeks in culture regardless of the explant source and type of medium used for culturing. These explants proved not suitable for further studies on shoot multiplication. Larger explants (3–4 nodes) from thicker shoots (1–2 mm diameter) showed higher (>80%) survival after 3–4 weeks in culture.

Similar responses in terms of bud sprouting, shoot proliferation and adventitious root formation were observed in large (3–4 nodes) axillary shoot explants irrespective of the source. Sprouting of pre-existing buds was suppressed on axillary shoot explants with an apex (Figure 6.15A) probably due to apical dominance exerted by the apex. Removal of the apex resulted in bud sprouting (3–4 buds/explant) on 100% of explants on BAP-free medium (Figure 6.15B) and on 80% of explants in the presence of 0.25 mg L^{-1} BAP (Figure 6.15C) after one week of culture. Secondary axillary shoot proliferation (3–4/explant, 15–20 mm in length) developed from sprouted buds on 80% of explants after four weeks of culture on BAP-free medium (Figure 6.15E).

Suppression of bud sprouting was observed with an increasing BAP concentration where 50% of explants had sprouted buds on MS medium containing 0.50 and 0.75 mg L^{-1} BAP and only 40% of explants on 1 mg L^{-1} BAP (Figure 6.15D).

No shoot proliferation occurred on *H. coddii* subsp. *barnardii* explants in the presence of all (0.25–1 mg L^{-1}) concentrations of BAP (Figure 6.15F). Shoot regeneration efficiency in *H. cannabinus* shoot explants was negatively affected by an increasing BAP concentration (4.4, 8.8 and 22 µM) and reduced from 100% in BAP-free medium to 56.7% with 22 µM BAP (Herath *et al.*, 2004). This contradicts findings of Christensen *et al.* (2008) who described the highest number of proliferated secondary axillary shoots (2.2) on primary axillary shoot explants of

H. rosa-sinensis cv. Cassiopeia Wind Yellow with 2.2 μM BAP compared to only 1.1 shoot per explant on BAP-free medium. This could be attributed to the difference in plant species response, especially between domesticated and wild plant species.

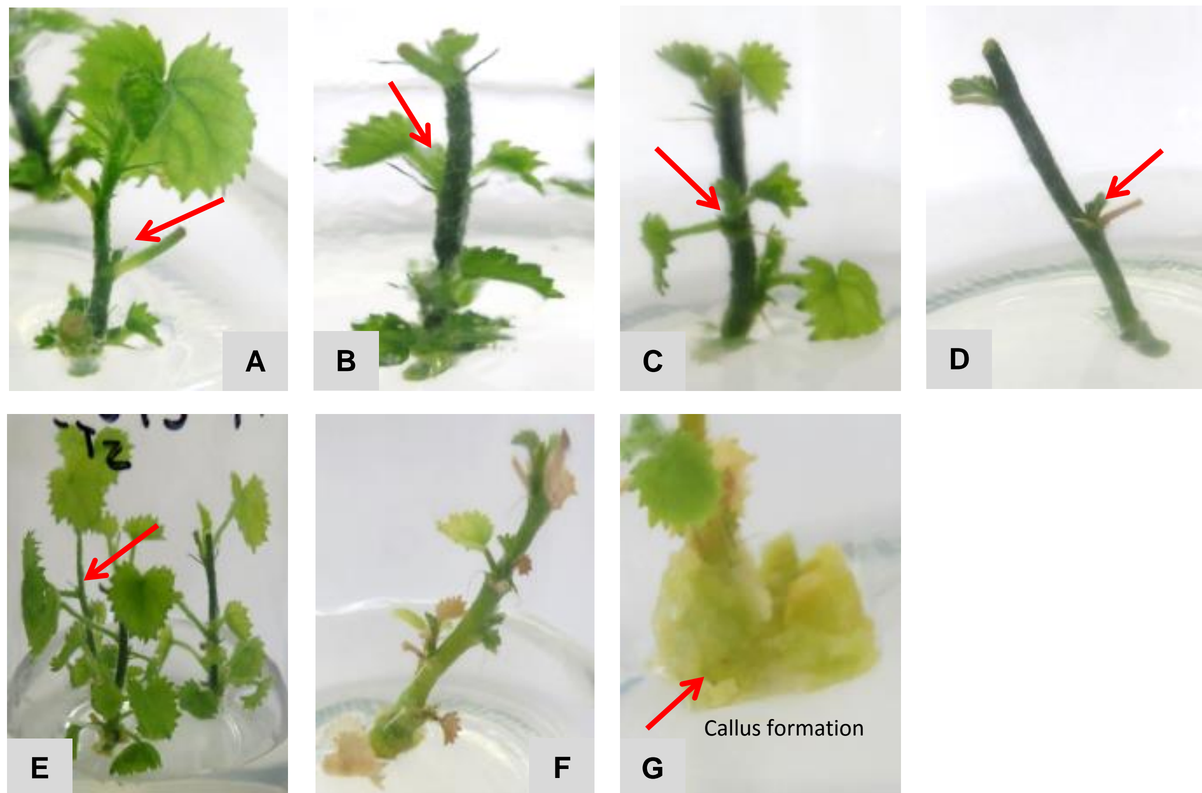

Figure 6.15. Examples of bud sprouting and shoot proliferation on primary axillary shoot explants. (A) Suppression of bud sprouting in explants with an apex. Sprouting of pre-existing axillary buds in explants without an apex after one week of culture on (B) BAP-free medium; (C) Medium with 0.25 mg L^{-1} BAP; (D) Medium with 1 mg L^{-1} BAP. (E) Elongated secondary axillary shoots on BAP-free medium; (F) No shoot elongation on medium with 1 mg L^{-1} BAP; (G) Callus formation on explants with 1 mg L^{-1} BAP.

Adventitious roots only formed on *H. coddii* subsp. *barnardii* axillary shoot explants [with apex (30%) and without apex (40%)] in the absence of BAP. Regenerated plants from rooted explants were suitable for transfer to a soil culture medium and were successfully acclimatised. Formation of adventitious roots on the explants seemed imperative for further development and survival since proliferated secondary axillary shoots on the explants without roots deteriorated and died off. The presence of BAP (0.25–1 mg L^{-1}) promoted callus induction on non-rooted explants and the frequency (50–80%) increased with an increasing BAP concentration (Figure 6.15G). The least callus formation (30%) was observed in the absence of BAP.

Further subculturing of the secondary axillary shoots of *H. coddii* subsp. *barnardii* onto fresh MS medium (with and without BAP) did not improve shoot multiplication. Tertiary axillary shoots developed on only 20% of secondary axillary shoot explants on BAP-free medium only after formation of adventitious roots, suggesting low shoot regeneration efficiency. No shoot regeneration was observed on explants in the presence of all BAP concentrations (0.25–1 mg L^{-1}) and explants died after 3–4 weeks in culture. The low regeneration efficiency could be due to the inability of plant tissues to rejuvenate. The shortage or oversupply of nutrients and/or PGRs in the media could also be contributing factors (Iliev *et al.*, 2010). A decline in the shoot regeneration efficiency with subsequent subculturing of shoots of *H. rosa-sinensis* was also reported by Christensen *et al.* (2008).

Clumps of short (10–15 mm) primary axillary shoots that remained on the original explant responded differently depending on the presence or absence of BAP in the medium. The shoots on the clumps (Figure 6.16A) elongated when transferred to BAP-free MS medium (Figure 6.16B) whereas those on all BAP concentrations did not develop further and died. After three weeks of culture, 45% of these clumps formed adventitious roots (Figure 6.16C) and the primary axillary shoots (4–6) increased in length up to 40–50 mm. These rooted clumps of shoots were successfully acclimatised in moist vermiculite and developed into mature plants (Figure 6.16D). This suggests that the presence of a piece of the initial explant that remained on the clump is beneficial for survival of axillary shoots and that clumps of short axillary shoots can be used for plant regeneration. Similarly, subculturing of sub-divided clumps of *H. cannabinus* on PGR-free medium resulted in shoot elongation and formation of adventitious roots (Srivatanakul *et al.*, 2000).

Figure 6.16. Example of shoot elongation and adventitious root formation on a clump of short primary axillary shoots on BAP-free MS medium after three weeks of culture. (A) Clump with short axillary shoots before culture; (B) Elongated axillary shoots; (C) Adventitious root formation on clump; (D) Acclimatised plant after three weeks.

6.3.4.3.2 Effect of BAP and auxins

The poor shoot induction response of *H. coddii* subsp. *barnardii* explants to various BAP concentrations alone necessitated the need to study the effect of BAP in combination with other plant growth regulators (auxins). Auxins may counteract the inhibitory effect of BAP on shoot elongation and regeneration observed in the other two experiments, although the chances of callus formation are also increased (Kane, 2005; Khan *et al*., 2015). It has been reported that combinations of cytokinin and auxin had a positive effect on multiple shoot induction of *Hibiscus* species and the response may vary with species and explant type (Yang *et al*., 1995; Zapata *et al*., 1999; Ayadi *et al*., 2011). Based on our research and a literature survey, the effect of BAP (0.5 and 1 mg L^{-1}) alone, and in combination with IBA and NAA (0.5 mg L^{-1}), on shoot induction of *H. coddii* subsp. *barnardii* basal seedling and basal axillary shoot explants was studied.

Statistical analysis showed that the PGR treatments and the control had a significant association with bud sprouting, shoot proliferation, rooting and callus formation while

the response also varied with the explant type (Appendix D: Table 5 and 6). The PGR treatment (different concentrations and combinations of BAP, IBA and NAA) and the control caused significant distribution pattern differences for bud sprouting and shoot proliferation on both types of explants (Figure 6.17). The highest (58.3%) frequency of bud sprouting (Class 2) from pre-existing axillary buds and the lowest percentage (6.7%) of proliferated short shoots (Class 3) was attained on axillary shoot explants after one week of culture on PGR-free medium [Figure 6.17E, Figure 6.18B (a), Week 1]. An opposite response was observed in seedling explants, where a low bud sprouting frequency (36.7%) was accompanied by proliferation of higher frequencies [46.7% (Class 3) and 5% (Class 4)] of short axillary shoots [Figure 6.17A, Figure 6.18A (a), Week 1]. Both seedling and axillary shoot explants retained the bud-sprouting trend on PGR-free medium and showed similar shoot proliferation responses after four weeks of culture. The highest frequencies, 41.7% and 38.3% of short axillary shoots (Class 3 and 4 respectively) were attained on basal seedling explants. This indicates a decrease in the sprouted buds from 36.7% (week 1) to 11.7% (week 4) and an increase in the proliferation of new short shoots (Class 3). The percentage of shoots from Class 4 increased almost 8-fold (from 5 to 38.3%) showing elongation of already proliferated shoots, although this length renders them unsuitable for subculture and shoot multiplication at this stage. Only a small percentage (6.7%) of longer shoots (Class 5) developed after four weeks [Figure 6.17D, Figure 6.18A (a), Week 4]. A similar trend was observed in axillary shoot explants where the majority (45%) of sprouted shoots were short (Class 3) and a very low frequency (3.3%) was longer shoots (Class 5) [Figure 6.17H, Figure 6.18B (a), Week 4]. This response indicates poor shoot regeneration efficiency of shoot explants derived from young seedlings (4 weeks) in contrast to the other two experiments (Section 6.3.4.2.2 & 6.3.4.3.1) where shoot proliferation occurred in 100% of shoot explants derived from older seedlings (5–6 weeks). Sultana *et al.* (2016a) also reported no shoot regeneration on hypocotyl explants of 10-day-old seedlings of *H. cannabinus* cultured on PGR-free medium. It also shows that the response may depend on plant species and the type and age of the explant.

The PGR treatments caused similar frequency distributions in bud sprouting in both seedling and axillary shoot explants of *H. coddii* subsp. *barnardii* at week 1 (Figure 6.17A & E). The lowest frequency of bud sprouting (Class 2) was observed in the

presence of BAP alone. On seedling explants, the least bud sprouts (20%) were observed with 1 mg L^{-1} BAP [Figure 6.17A, Figure 6.18A (c), Week 1], whereas the presence of 0.5 mg L^{-1} BAP caused the lowest (21.7%) frequency of bud sprouts on axillary shoot explants [Figure 6.17E, Figure 6.18B (b), Week 1]. The combination of 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA resulted in the highest (45%) frequency of bud sprouting on seedling explants [Figure 6.17A, Figure 6.18A (d), Week 1]. In axillary shoot explants, the best (38.3%) bud sprout response was observed with the combination of 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA [Figure 6.17E, Figure 6.18B (f), Week 1].

Low shoot regeneration efficiency in *H. coddii* subsp. *barnardii* was observed for all PGR treatments and showed dependence on the explant type. The lower concentration of BAP (0.5 mg L^{-1}) alone and in combination with both NAA and IBA (0.5 mg L^{-1}) resulted in the highest (40–43.3%) proliferation of short shoots (Class 3) on *H. coddii* subsp. *barnardii* seedling explants after 4 weeks of culture [Figure 6.17D, Figure 6.18A (b, d & f), Week 4]. In contrast, the higher BAP concentration (1 mg L^{-1}) alone or in combination with NAA and IBA (0.5 mg L^{-1}) had lower frequencies (18.3–25%) of short shoots (Class 3) on seedling explants at this stage [Figure 6.1 D, Figure 6.18A (c, e & g), Week 4]. Ayadi *et al*. (2011) reported better shoot regeneration efficiency in seedling nodal explants of *H. cannabinus* with (1 mg L^{-1}) BAP alone (69.4%), but also a 32.2% reduction in regeneration with a combination of 0.5 mg L^{-1} BAP and 1 mg L^{-1} NAA.

Axillary shoot explants of *H. coddii* subsp. *barnardii* also showed low (16.7–25%) shoot proliferation efficiency (Class 3) for all PGR treatments except for the combination of 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA where the lowest (3.7%) shoot proliferation was attained (Figure 6.17H). Zapata *et al*. (1999) found similar results for *H. cannabinus* with low percentages (<10%) of shoot regeneration from shoot apex explants in the presence of 1 mg L^{-1} BAP alone or in combination with NAA (0.1 and 1 mg L^{-1}), whereas the lower BAP concentration (0.1 mg L^{-1}) resulted in 91.7% shoot regeneration. The highest number of shoots (2.2) on axillary shoot explants of *H. rosa-sinensis* cv. Cassiopeia Wind Yellow was reported with 2.2 µM BAP compared to significantly less shoots (1.1) on PGR-free medium (Christensen *et al*., 2008). The culture of axillary shoot explants of *H. rosa-sinensis* in the presence of

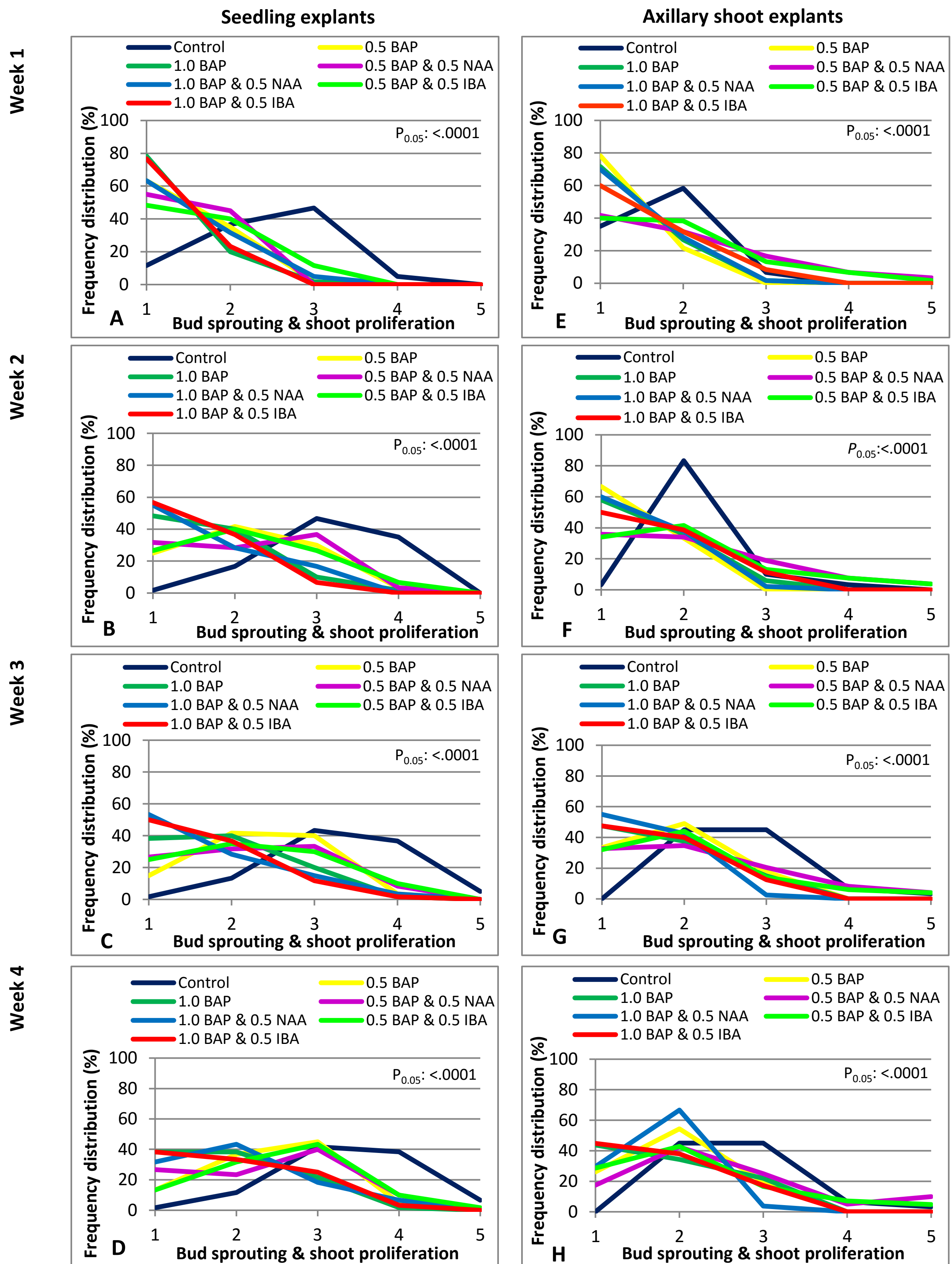

Figure 6.17. Effect of BAP and auxins on axillary bud sprouting and shoot elongation on basal seedling (A–D) and basal axillary shoot (E–H) explants from Week 1 to Week 4.

Bud sprout & shoot proliferation class: [1] Pre-existing axillary bud; [2] Bud sprout 1–2 mm; [3] Short axillary shoot at least 5 mm with visible leaves; [4] Short axillary shoot >5 mm <10 mm with at least 2 well-developed open leaves; [5] Longer axillary shoots 15-25 mm with at least 2 nodes.

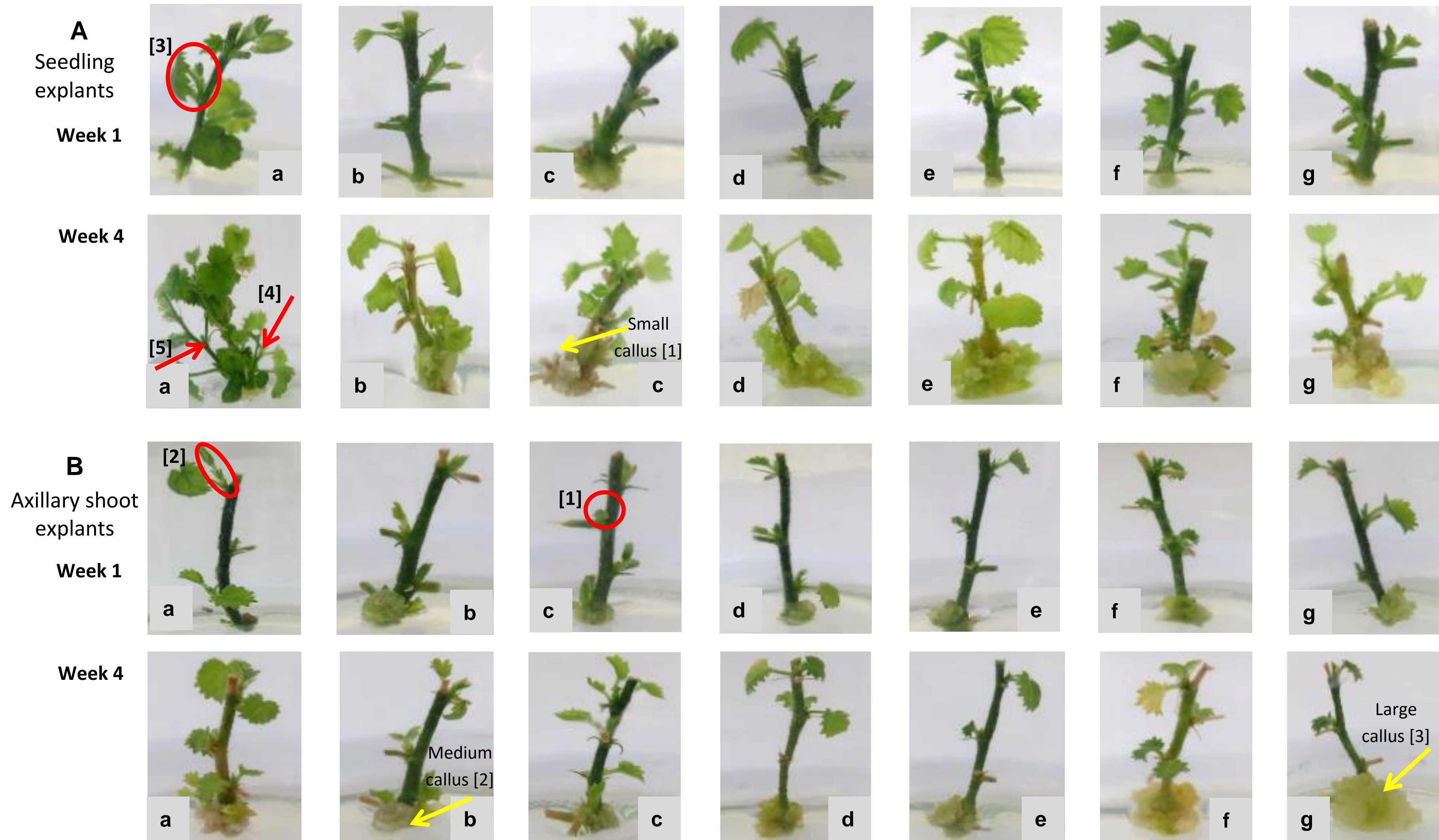

Figure 6.18. Examples of the effect of BAP and auxins on axillary bud sprouting, shoot elongation and callus formation on different shoot explants after one week and four weeks of culture. (A) Seedling explants; (B) Axillary shoot explants: (a) Control (no PGRs); (b) 0.5 mg L^{-1} BAP; (c) 1 mg L^{-1} BAP; (d) 0.5 mg L^{-1} BAP & 0.5 mg L^{-1} NAA; (e) 1 mg L^{-1} BAP & 0.5 mg L^{-1} NAA; (f) 0.5 mg L^{-1} BAP & 0.5 mg L^{-1} IBA; (g) 1 mg L^{-1} BAP & 0.5 mg L^{-1} IBA.

Bud sprout & shoot proliferation class: [1] Pre-existing axillary bud; [2] Bud sprout 1–2 mm; [3] Short axillary shoot at least 5 mm with visible leaves; [4] Short axillary shoot >5 mm <10 mm with at least 2 well-developed open leaves; [5] Longer axillary shoots 15-25 mm with at least 2 nodes. Callus size: [1] Small: diameter 1–5 mm; [2] Medium: diameter 5–10 mm; [3] Large: diameter >10 mm.

0.40 mg L^{-1} BAP and 0.1 mg L^{-1} IAA achieved the best response (4.9 shoots per explant) in comparison to PGR-free medium where less than two shoots per explant were observed (Airò *et al.*, 2009). Sultana *et al.* (2016a) found that shoot regeneration was also dependent on explant type. Hypocotyl explants of *H. cannabinus* showed the highest shoot regeneration response (34.9%) compared to 19.4% regeneration in the cotyledon explants in the presence of the same concentration of BAP (2 mg L^{-1}). Very poor (≤10%) or no elongation of proliferated short shoots in both explants of *H. coddii* subsp. *barnardii* occurred in all the PGR treatments after four weeks of culture (Figure 6.17D & H), which suggests the inhibitory effect of PGRs on *in vitro* shoot regeneration in this plant.

Bud and shoot viability in *H. coddii* subsp. *barnardii* depended on the explant type and the PGR treatment. In axillary shoot explants, the highest BAP concentration (1 mg L^{-1}) alone and in combination with 0.5 mg L^{-1} IBA and NAA resulted in the lowest survival (45–48%) after four weeks of culture (results not shown), whereas the control was not affected. The bud and shoot viability in seedling explants were not affected negatively irrespective of the presence or absence of PGRs, although the short shoots formed in this culture was not suitable for excision and further shoot multiplication. Further studies are required to test the effect of frequent subculture (2–3 weeks) on shoot elongation and survival. From the results of this study (similarly to Section 6.3.4.3.1), it is evident that the PGR-free medium was the best for shoot induction of both *H. coddii* subsp. *barnardii* explants. The BAP alone, and in combination with NAA and IBA, did not improve the shoot induction response that may indicate the recalcitrant nature of the plant in *in vitro* culture in the presence of PGRs.

The *in vitro* rooting response of both types of *H. coddii* subsp. *barnardii* explants was poor, regardless of the PGR treatment. After four weeks of culture only 10% of the seedling explants cultured on PGR-free medium, formed adventitious roots. In contrast, Ayadi *et al.* (2011) reported high rooting (91%) of *H. cannabinus* shoots in the absence of PGRs followed by 77% rooting in shoots with 0.5 mg L^{-1} BAP and less root formation (57.2%) with 1 mg L^{-1} BAP. A combination of 0.1 mg L^{-1} BAP and NAA each resulted in 78% rooting of regenerated shoots of *H. cannabinus*, while

higher concentrations of both PGRs (1 mg L^{-1}) drastically reduced rooting to 8.3% (Zapata *et al*., 1999).

Instead of roots, friable callus was formed at the base of most *H. coddii* subsp. *barnardii* explants and the response depended on the explant type and PGR treatment. Significant associations between the PGR treatments and the percentage of explants with callus were observed (Appendix D: Table 5 and 6). Initial callus formation started in the first week of culture on axillary, and in the second week on seedling shoot explants in most PGR treatments and the control (Table 6.8).

In axillary shoot explants, the lower concentration (0.5 mg L^{-1}) of BAP alone, and combined with NAA and IBA (0.5 mg L^{-1}), and 1 mg L^{-1} BAP in combination with 0.5 mg L^{-1} IBA resulted in the highest callus formation (50–70%) after one week of culture. No callus was formed in the absence of PGRs during the first two weeks (Table 6.8). BAP (1 mg L^{-1}) alone or in combination with 1 mg L^{-1} NAA also caused callus formation on shoot apex explants of *G. hirsitum* (Zapata *et al*., 1999). In seedling explants of *H. coddii* subsp. *barnardii* the highest incidence of callus formation was noted with 1 mg L^{-1} BAP alone (55%) and combined with 0.5 mg L^{-1} IBA (75%), and in 0.5 mg L^{-1} BAP combined with 0.5 mg L^{-1} IBA (60%). Low (10%) percentage of explants in PGR-free medium had callus at the base after two weeks of culture (Table 6.8). The highest percentage of callus induction (71.4%) in hypocotyl explants of *H. cannabinus* was reported with higher concentrations of BAP (7.5 mg L^{-1}) and NAA (2 mg L^{-1}) (Sultana *et al*., 2016b). Callus formation on both seedling and axillary shoot explants of *H. coddii* subsp. *barnardii* progressively increased in all PGR treatments and the control after four weeks. Higher percentage (85–100%) of both types of shoot explants from all PGR treatments [Table 6.8, Figure 6.18A & B (b–g), Week 4] had callus at the base than in the absence of PGRs where callus formation was less in both axillary (60%) and seedling (85%) shoot explants [Table 6.8, Figure 6.18A & B (a), Week 4]. In the absence of PGRs, no callus formation was found on *H. cannabinus* explants (Sultana *et al*., 2016b).

Table 6.8. Effect of BAP alone or combined with NAA and IBA on callus formation of seedling and axillary shoot explants of *H. coddii* subsp. *barnardii*.

Treatment	**% Explants with callus**			
Seedling explants	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Control	0	10	80	85
0.5 mg L^{-1} BAP	5	40	80	90
1.0 mg L^{-1} BAP	0	55	70	85
0.5 mg L^{-1} BAP & 0.5 mg L^{-1} NAA	0	30	100	100
1.0 mg L^{-1} BAP & 0,5 mg L^{-1} NAA	0	25	95	100
0.5 mg L^{-1} BAP & 0.5 mg L^{-1} IBA	0	60	100	100
1.0 mg L^{-1} BAP & 0.5 mg L^{-1} IBA	0	75	100	100
$P_{0.05}$	***0.418***	***0.000***	***0.004***	***0.064***
Axillary shoot explants	**% Explants with callus**			
	Week 1	**Week 2**	**Week 3**	**Week 4**
Control	0	0	30	60
0.5 mg L^{-1} BAP	50	75	95	100
1.0 mg L^{-1} BAP	30	70	90	100
0.5 mg L^{-1} BAP & 0.5 mg L^{-1} NAA	60	60	60	100
1.0 mg L^{-1} BAP & 0.5 mg L^{-1} NAA	45	45	75	100
0.5 mg L^{-1} BAP & 0.5 mg L^{-1} IBA	70	75	80	100
1.0 mg L^{-1} BAP & 0.5 mg L^{-1} IBA	60	60	70	95
$P_{0.05}$	***0.000***	***<.0001***	***0.000***	***<.0001***

Callus is formed as a natural wound response when cells around the damaged area divide rapidly to form a layer of cells over the wound (Bhalla *et al.*, 2009; Ikeuchi *et al.*, 2013). Callus tissue competes with axillary meristems of explants for nutrients in the medium and compromises the shoot regeneration ability of explants (Chen *et al.*, 2010). This could explain the low shoot regeneration in both seedling and axillary shoot explants of *H. coddii* subsp. *barnardii* explants. The formed callus was found to be non-regenerative and prevented further shoot development. In contrast, Jeon *et al.* (2009) noted the formation of adventitious shoots from callus in the presence of 0.5 mg L^{-1} BAP and 0.01 mg L^{-1} NAA in *H. syriacus*. Development of adventitious shoots from callus formed on cotyledon petiole explants (60%) and seedling leaf explants (69%) of *H. cannabinus* when cultured on MS medium supplemented with varying combinations and concentrations of BAP, IAA, IBA and NAA was also reported (Khatun *et al.*, 2003; Samanthi *et al.*, 2013). McLean *et al.* (1992) however, reported only 3–6% regeneration of adventitious shoots from callus formed on internodal explants of *H. cannabinus*. Further *in vitro* studies of *H. coddii* subsp. *barnardii* could focus on the indirect regeneration of plantlets from callus, although

such regeneration through adventitious shoots could induce somaclonal variations in the regenerated plants (Iliev *et al*., 2010; Hartmann *et al*, 2011; Samanthi *et al*., 2013).

The PGR treatments caused a significant association with the size of the callus formed at the base of *H. coddii* subsp. *barnardii* seedling and axillary shoot explants (Appendix D: Table 5 and 6). Different patterns of frequency distributions were found in seedling (Figure 6.19A) and axillary (Figure 6.19B) shoot explants after four weeks of culture. Although a high percentage of callus formation was observed on both explant types cultured on PGR-free medium (Table 6.8), the formed callus was mostly small [Figure 6.18A & B (a), Week 4]. Small callus (Class 1) was formed on 64% of the seedling explants (Figure 6.19A), whereas 100% of the axillary shoot explants had small callus after four weeks of culture (Figure 6.19B). McLean *et al*. (1992) also reported the smallest-sized callus on internodal explants of *H. cannabinus* cultured on MS medium without PGRs. Seedling explants of *H. coddii* subsp. *barnardii* cultured on 0.5 mg L^{-1} and 1 mg L^{-1} BAP alone formed mostly small-sized (44% and 53% respectively) and medium-sized (Class 2) callus, 41% and 44% respectively [Figure 6.19A, Figure 6.18A (b & c), Week 4]. Sié *et al*. (2010) indicated that 0.1 mg L^{-1} BAP alone also caused small callus (2.8 mm diameter) on 38% of hypocotyl explants of *H. sabdariffa*. The highest frequency (70%) of *H. coddii* subsp. *barnardii* explants with medium-sized callus was observed on axillary shoot explants in the presence of 0.5 mg L^{-1} and 1 mg L^{-1} BAP [Figure 6.19B, Figure 6.18B (b & c), Week 4].

Both concentrations of BAP (0.5 and 1 mg L^{-1}) combined with 0.5 mg L^{-1} NAA and with 0.5 mg L^{-1} IBA led to high frequencies of medium and large-sized (Class 3) callus in seedling explants of *H. coddii* subsp. *barnardii* with less than 20% of explants with small callus in all these PGR treatments [Figure 6.19A, Figure 6.18A (d–g), Week 4]. A combination of higher concentrations of BAP (3 mg L^{-1}) and NAA (1 mg L^{-1}) caused large-sized callus (7.4 mm diameter) in 97% of *H. cannabinus* hypocotyl explants (Sié *et al*., 2010). The largest sized callus on internodal explants of *H. cannabinus* was achieved with 1 mg L^{-1} BAP combined with higher concentrations of NAA (1 mg L^{-1} and 3 mg L^{-1}) (McLean *et al*., 1992). The seedling explant of *H. coddii* subsp. *barnardii* was excised with a piece of hypocotyl attached

to it, which could explain the larger sized callus formed on high percentages of these explants. The highest percentage of callus formation (60.5%) was also reported on hypocotyl explants of *H. cannabinus* (Sultana *et al*., 2016b). In axillary shoot explants of *H. coddii* subsp. *barnardii*, the four PGR combinations caused an almost equal frequency distribution between small, medium and large-sized callus with less formation (30–35%) of large callus [Figure 6.19B, Figure 6.18B (d–g), Week 4] than large-sized callus in seedling explants (35–65%).

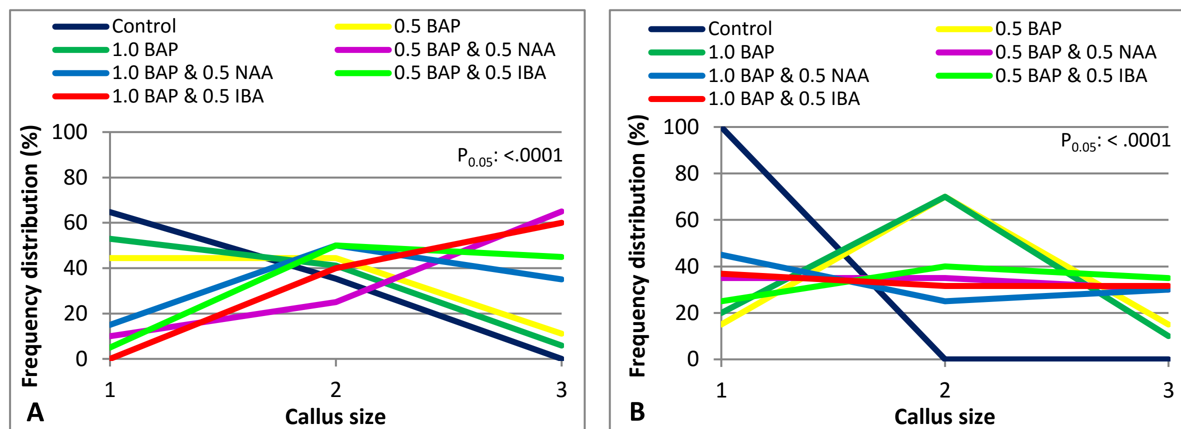

Figure 6.19. Effect of BAP alone or combined with NAA and IBA on the size of callus formed after four weeks on (A) Seedling explant; (B) Axillary shoot explants of *H. coddii* subsp. *barnardii*.

Callus size: [1] Small- diameter 1–5 mm; [2] Medium-diameter 5–10 mm; [3] Large: diameter >10 mm.

6.3.5 Root culture

6.3.5.1 *In vitro* rooting

Adventitious root formation on shoots produced during *in vitro* culture is essential for successful development and survival of plantlets *ex vitro* (Hartmann *et al*., 2011; Shekhawat *et al*., 2015; Islam *et al*., 2017). The ANOVA analysis showed that the auxin treatments (0.5 and 1 mg L^{-1} IBA and NAA) had no significant effect on the rooting percentage and callus formation at the base of *H. coddii* subsp. *barnardii* microcuttings (Appendix D: Table 7). However, developmental variations in these responses were observed over a period of 6 weeks and are presented below.

Formation of adventitious roots on microcuttings of *H. coddii* subsp. *barnardii* was first initiated from the second week of culture only in the presence of 1 mg L^{-1} NAA and reached the maximum of 15% after four weeks (Figure 6.20). It has been

reported that higher concentrations of NAA stimulate natural ethylene which may be inhibitory to rooting in some *Hibiscus* species (De Klerk and Hanecakova, 2008; Tolera, 2016). Christensen *et al.* (2008) found that a lower NAA concentration (2.7 μM) improved rooting to 45.3%, whereas 21.1% and 6% rooting was reported with 8.1 μM and 16.2 μM NAA respectively. However, our results showed that a lower concentration of NAA (0.5 mg L^{-1}) led to the lowest rooting percentage (5%) after 5 weeks (Figure 6.20) that may be due to species specificity as also reported by Jenifer *et al.* (2012).

Similar to the NAA treatments, the best rooting response (20%) was obtained with a higher concentration of IBA (1 mg L^{-1}) while only 10% of microcuttings rooted in the presence of lower IBA (Figure 6.20). Supplementation of the medium with a high IBA concentration (2.5 mg L^{-1}) enhanced the *in vitro* rooting response of shoots of *H. sabdariffa* 4-fold to 86.2% compared to 28.1% in medium with 1.5 mg L^{-1} IBA (Govinden-Soulange *et al.*, 2009). In contrast, Yang *et al.* (1995) and Sultana *et al.* (2016a) reported the highest rooting (95%) on microshoots of *H. cannabinus* and *H. syriacus* on MS medium supplemented with 0.5 mg L^{-1} IBA and only 20% rooting on IBA-free medium. The adventitious roots induced on microcuttings of *H. coddii* subsp. *barnardii* in the presence of NAA were thicker and shorter (Figure 6.21B & C) as compared to roots formed in the IBA treatment and the control (Figure 6.21A, D & E).

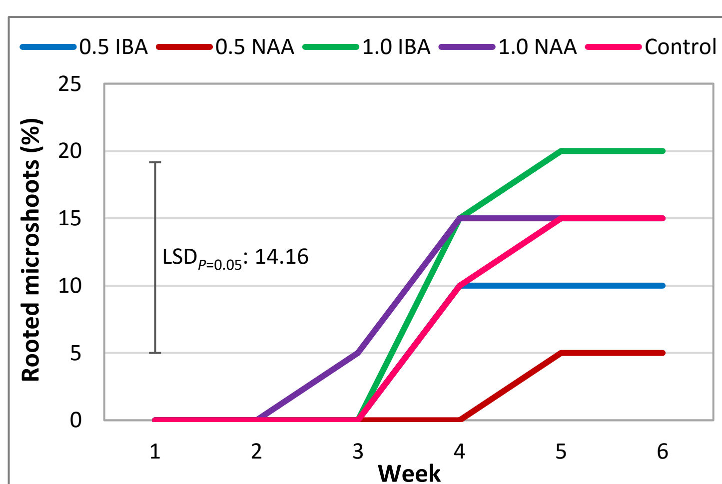

Figure 6.20. Weekly i*n vitro* rooting response of *H. coddii* subsp. *barnardii* microcuttings cultured on MS medium in the presence of various concentrations of NAA and IBA.
F pr.: Treatment: 0.578; Week:<.001 (ANOVA used to compare means and relative variance between them).

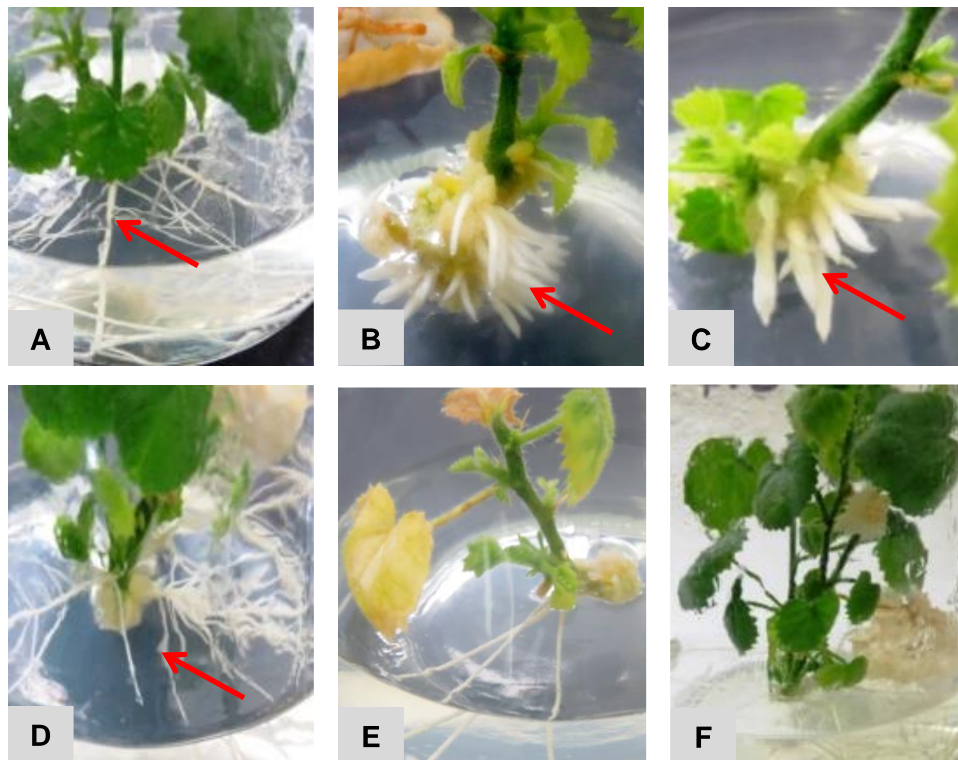

Figure 6.21. Adventitious root formation at five weeks on microcuttings cultured on MS medium supplemented with (A) No auxins (control) with long, thin roots; (B) 0.5 mg L^{-1} NAA; (C) 1 mg L^{-1} NAA with short, thick roots; (D) 0.5 mg L^{-1} IBA; (E) 1 mg L^{-1} IBA with long, thin roots; (F) Rooted microcutting (control) ready for transplant at six weeks.

Ali *et al*. (2009) also described the formation of short roots on microcuttings of *Olea europaea* cv. Moraiolo with 0.5 mg L^{-1} NAA compared to longer and better developed roots in the presence of IBA. It is reported that NAA inhibits root elongation *in vitro* since high concentrations stimulates ethylene production which adversely affects root growth (Ali *et al*., 2009; Taiz *et al*., 2015).

The results of this experiment indicate that the addition of exogenous NAA and IBA to the MS medium did not significantly increase the rooting response of microcuttings of *H. coddii* subsp. *barnardii* which was similar to the response in the absence of auxin where 15% of cuttings rooted after five weeks (Figure 6.20). Although exogenous auxin might be necessary for the induction of adventitious root formation, it can suppress the later development of root primordia in some plant species (De Klerk *et al*., 1999; Yan *et al*., 2014). It may also disrupt the mobilisation of total auxin (endogenous and exogenous) in shoots with an apex that in turn will suppress root formation (Srivatanakul *et al*., 2000). *H. coddii* subsp. *barnardii* plants exhibit strong

apical dominance, which suggests high levels of endogenous auxin in the microcuttings with an apex. The solid culture medium and reduced gas diffusion in the closed culture vessels may also lead to accumulation of ethylene at the base of microcuttings, which could suppress root growth and elongation (De Klerk, 2000; De Klerk, 2002). Further studies will be required to test the effect of subculturing of microcuttings from auxin treatments to PGR-free MS medium after 3–4 weeks of culture when root formation was initiated. The effect of less potent auxins such as IAA, either alone or in combination with NAA and/or IBA, on *in vitro* rooting can also be tested for further improvement of the rooting response as suggested by Tolera (2016).

The addition of exogenous auxin to the culture medium may also induce callus formation on microcuttings (Khatun *et al.*, 2003). Both concentrations (0.5 and 1 mg L^{-1}) of NAA resulted in the highest percentage of callus formation (65–70%) on *H. coddii* subsp. *barnardii* microcuttings followed by 1 and 0.5 mg L^{-1} IBA with 60% and 45% respectively, after six weeks of culture. The lowest percentage of callus formation was in the absence of auxin (control) where only 25% of the microcuttings had callus (Figure 6.22, Figure 6.23). Sultana *et al.* (2016b) reported no callus formation on explants of *H. cannabinus* on PGR-free medium, but 44.2% of explants formed callus when 1 mg L^{-1} BAP was combined with 1 mg L^{-1} NAA. Higher concentrations of both NAA and BAP (2 mg L^{-1} and 7.5 mg L^{-1} respectively) resulted in 71.4% callus induction. No callus was formed on microcuttings of *Lippia rotundifolia* grown in MS media supplemented with varying NAA concentrations (0.11, 0.22, 0.33, 0.44 µM) (De Resende *et al.*, 2015).

It has been reported that adventitious roots produced *in vitro* are fragile and easily breakable during handling (Yan *et al.*, 2010). The thicker roots formed on microcuttings of *H. coddii* subsp. *barnardii* in the presence of NAA (Figure 6.21 B & C) could be advantageous when rooted cuttings are transplanted from *in vitro* to soil cultures since these roots are stronger and less prone to breakage as compared to thinner roots from the IBA treatments and the control (Figure 6.21A, D & E). Another drawback of *in vitro* root formation is the dying-off of the adventitious root system after transplanting the regenerated plantlets to soil requiring formation of new roots (Kane, 2005; Ranaweera *et al.*, 2013). Christensen *et al.* (2008) suggested that *ex*

vitro rooting of microcuttings of *Hibiscus* species could be an alternative for unsuccessful *in vitro* rooting responses since it would reduce the time and cost of propagation.

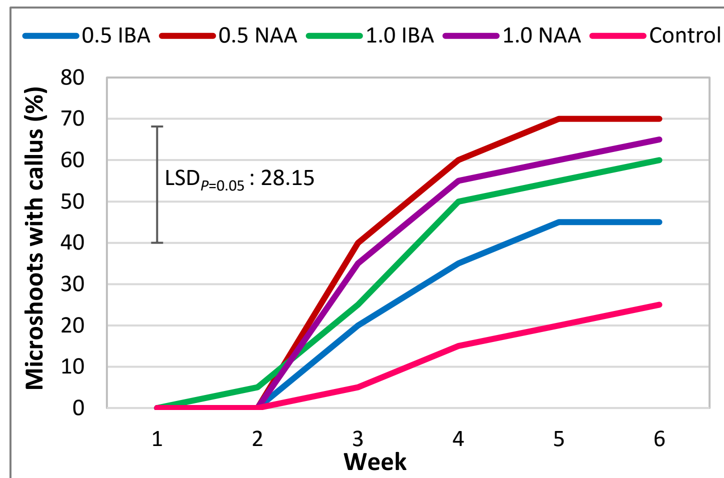

Figure 6.22. Weekly callus formation on microcuttings of *H. coddii* subsp. *barnardii* cultured on MS medium in the presence of various concentrations of NAA and IBA.
F pr.: Treatment: 0.131; Week:<.001 (ANOVA used to compare means and relative variance between them).

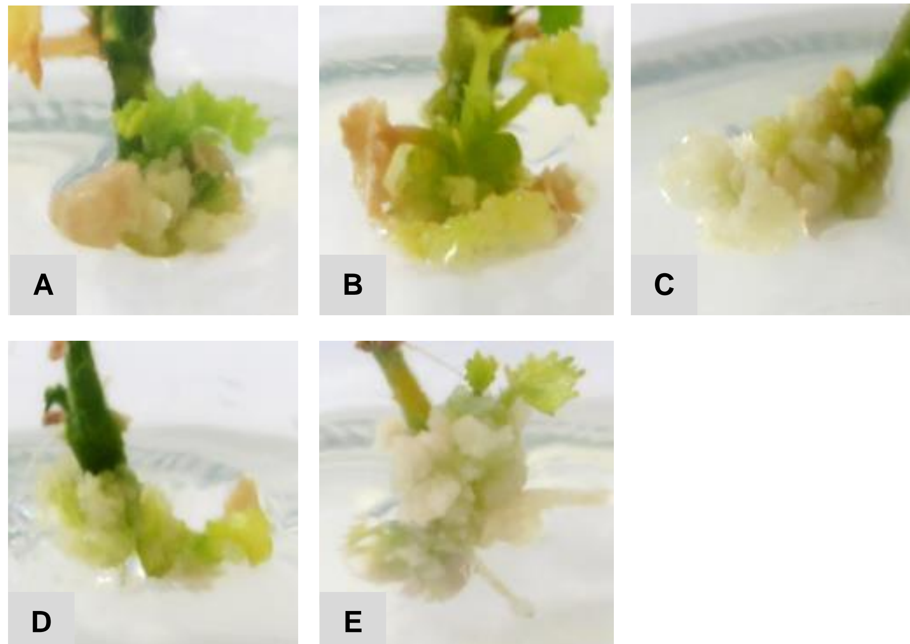

Figure 6.23. Examples of callus formation on *in vitro* cultured microcuttings of *H. coddii* subsp. *barnardii* after four weeks of culture in the presence of various concentrations of NAA and IBA. (A) No auxins (control); (B) 0.5 mg L^{-1} NAA; (C) 1 mg L^{-1} NAA; (D) 0.5 mg L^{-1} IBA; (E) 1 mg L^{-1} IBA.

6.3.5.2 *Ex vitro* rooting

Primary axillary shoots obtained from the apical and basal position on the main stem of *in vitro* grown seedlings of *H. coddii* subsp. *barnardii* proved suitable as microcuttings for *ex vitro* rooting in vermiculite medium. The statistical analysis for the main effects showed that exogenous IBA had a significant effect on the rooting percentage of microcuttings, whereas rooting was not significantly affected by the position of the axillary shoots on the seedling (cutting type). However, the interactive effect of IBA treatment and cutting type was significant for the rooting percentage in some of the weeks (Appendix D: Table 8).

The application of IBA-containing rooting powder on microcuttings resulted in a significantly higher rooting percentage (70.4%) than in the untreated cuttings (28.3%) (Figure 6.24A). The position from where the microcutting was taken from the seedling (cutting type) did not have a significant effect on rooting percentage (Figure 6.24B) indicating that axillary microshoots irrespective of their position on the seedling can be used for *ex vitro* rooting.

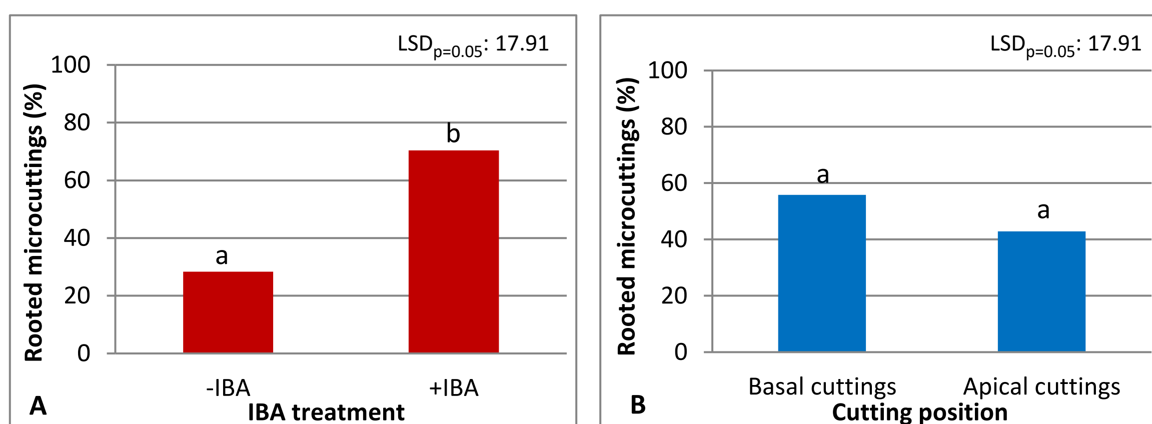

Figure 6.24. Effect of (A) IBA treatment; (B) Cutting type on *ex vitro* rooting percentage of *in vitro* produced microcuttings of *H. coddii* subsp. *barnardii*. Means (bars) with the same letters are not significantly different at the 5% level of significance.

The treatment of microcuttings with the lowest strength of commercial rooting hormone Dynaroot No.1 (0.1% IBA) promoted rooting in both types of cuttings after two weeks of culture. However, the response of the basal cuttings was significantly higher (90%) than the apical cuttings where a lower percentage of rooting (50%) was attained at this stage. The IBA-treated cuttings reached the maximum rooting after three weeks of culture with 100% of the basal and 80% of the apical cuttings that

rooted. The position of the cutting had no significant effect on rooting of IBA treated cuttings after six weeks (Figure 6.25). These results show that the commercial Dynaroot No.1 rooting hormone powder can be successfully used for *ex vitro* rooting of *H. coddii* subsp. *barnardii* microcuttings.

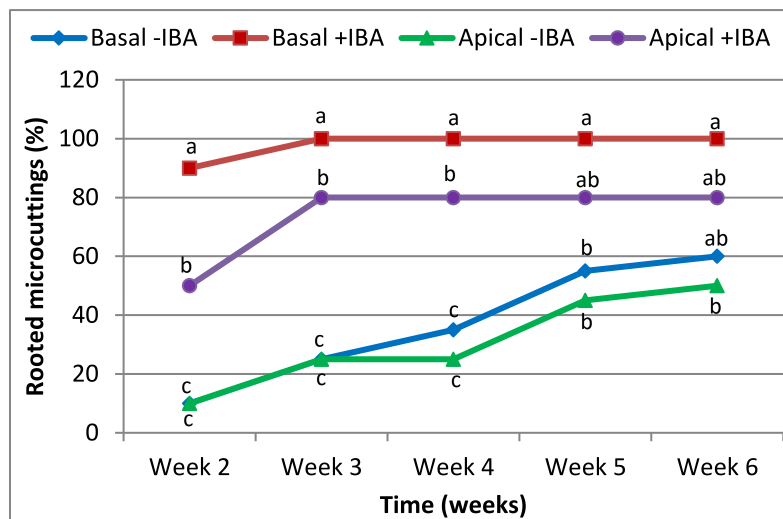

Figure 6.25. Effect of cutting position and IBA treatment on e*x vitro* rooting of microcuttings obtained from *in vitro* grown seedlings of *H. coddii* subsp. *barnardii*. Values with same letters within a week indicate no significant difference at 10% significance level. (ANOVA used to compare means and relative variance between them). Basal: Taken from lower three nodes on main stem of seedling; Apical: Taken from the upper three nodes on main stem of seedling; - IBA: No Dynaroot hormone powder; +IBA: Dynaroot No. 1 hormone root powder.

Similar results were reported for *ex vitro* rooting of other plant species when different brands of commercial rooting hormones were used. High percentage (89%) of adventitious root formation was attained on microcuttings of *G. hirsitum* treated with Rootone® hormone powder containing 0.2% Nicotinamide adenine dinucleotide (NAD) and rooted *ex vitro* in soil (Hemphill *et al*., 1998). Benmahioul *et al.* (2012) reported 78.6% rooting in microshoots of *Pistacia vera* when shoots were dipped in Rhizopon® AA rooting powder (2% IBA) and planted in a peat-perlite-vermiculite (80:15:5%) mixture. Other studies also reported successful *ex vitro* rooting when microcuttings were exposed to a short treatment with IBA solutions containing various concentrations of the hormone. Ranaweera *et al.* (2013) attained 100% rooting of *Camellia sinensis* microcuttings when treated with lower IBA concentration (50 mg L^{-1}) for three hours and planted in a mixture of coir dust, top soil and sand (1:1:1 v/v). Shekhawat *et al.* (2015) reported 97% rooting of *Passiflora foetida* microcuttings in Soilrite® medium after a five minute treatment with 300 mg L^{-1} IBA, whereas no rooting was observed in untreated microcuttings. *In vitro* produced

microcuttings of *Lawsonia inermis* were successfully rooted (100%) when treated with 0.1 mg L^{-1} IBA solution for five minutes and planted in a soil: sand (1:2) mixture (Shiji and Siril, 2018). Further studies on the effect of short treatments with various concentrations of liquid IBA on *ex vitro* rooting of *H. coddii* subsp. *barnardii* microcuttings can be done in order to select the best treatment and optimisation of the protocol.

Only 10% of the untreated microcuttings (both apical and basal) of *H. coddii* subsp. *barnardii* formed roots at two weeks, which differed significantly from the IBA treatment. A maximum of 60% of the basal and 50% of the apical (50%) cuttings rooted after six weeks that did not differ significantly from each other (Figure 6.25).

An average of 35% of the untreated microcuttings that did not root, died (browning and tissue necrosis) during the course of the experiment which could be due to the soft nature of *in vitro* grown cuttings. Such cuttings are prone to wilting and drying out when planted in the *ex vitro* rooting medium. Thiart (2003) recommends a faster working procedure and keeping the cuttings covered with plastic bags for a longer time during acclimatisation to alleviate the problem. Vermiculite proved to be a suitable rooting medium for *ex vitro* rooting of microcuttings of *H. coddii* subsp. *barnardii* resulting in high rooting percentages. Shiji and Siril (2018) reported that a more aerated rooting media allows for sufficient gas exchange that could be a contributory factor to successful *ex vitro* adventitious root formation since they observed 100% rooting of IBA-treated (90 mg L^{-1} for 5 minutes) *Lawsonia inermis* microcuttings in a soil:sand (2:1) mixture.

There was an initial difference in root morphology of treated and untreated cuttings. The control cuttings produced short, thick roots after 2–3 weeks of culture in contrast to the long, thin roots developed on the microcuttings treated with Dynaroot 1 [Figure 6.26A & B (a)], but this difference disappeared with time. A compact ball of adventitious roots developed on all types of cuttings after 11 weeks of culture and the regenerated plants showed vigorous growth [Figure 6.26A & B (b & c)]. Other studies also reported that *ex vitro* formed roots are stronger and not so prone to breaking and have a better vascular system than *in vitro* produced roots. Such roots

are not easily damaged during transfer to soil which increase plant survival (Iliev *et al.*, 2010; Ranaweera *et al.*, 2013).

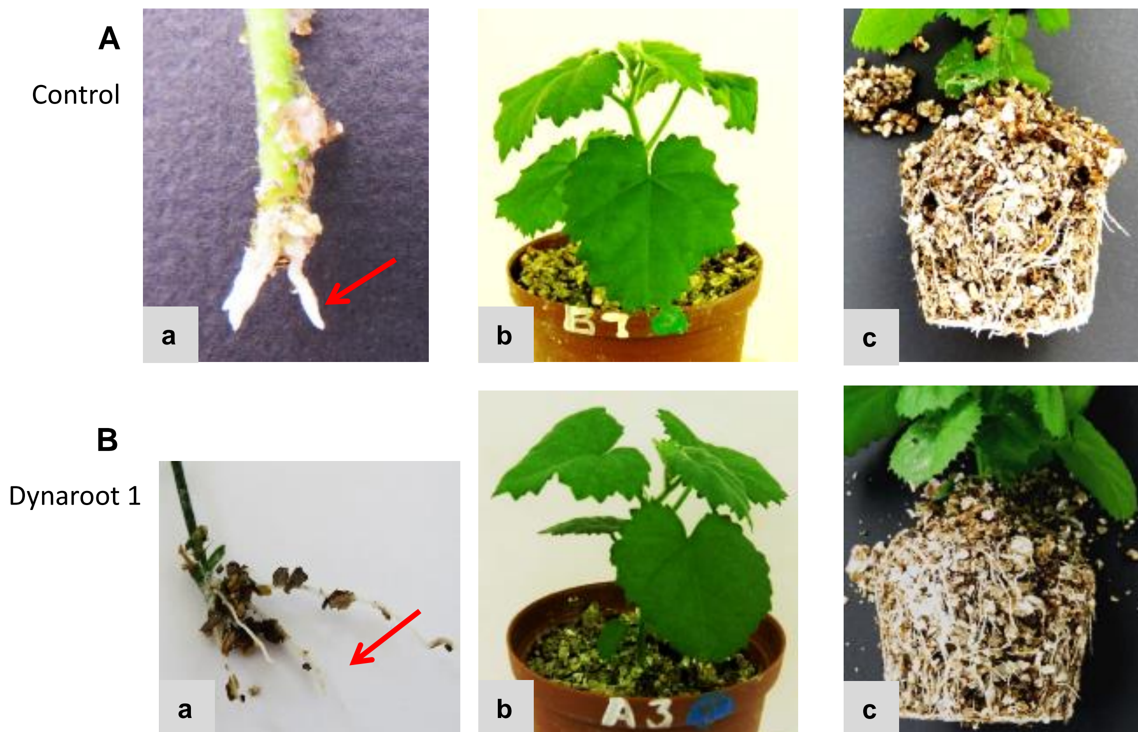

Figure 6.26. *Ex vitro* rooting in vermiculite of basal microcuttings obtained from *in vitro* grown seedlings. (A) Control microcutting (no IBA); (B) Cutting treated with Dynaroot No.1 rooting hormone powder. (a) Adventitious root formation after 2–3 weeks; (b) Rooted microcutting (regenerated plant) after 6 weeks of culture; (c) Well-developed adventitious root system at 11 weeks.

Young well-developed plants [Figure 6.27A & B (c)] obtained from *ex vitro* rooted microcuttings from all treatments were transferred to a potting soil mix (5 potting soil:3 vermiculite:1 sand) at 11 weeks. Plant with an intact apex [Figure 6.27A (a)] elongated and showed strong apical dominance [Figure 6.27A (d)]. The apex of some microcuttings from all treatments (38% in total) died two to three weeks after planting the cutting in vermiculite [Figure 6.27B (a)]. This was not detrimental to the plant, but rather led to the proliferation of numerous secondary axillary shoots from the base of the rooted microcutting which elongated further [Figure 6.27B (b)]. Flower buds and flowers (Figure 6.27A (d)] started to form on the main shoot of plants with an intact apex (18 weeks old) followed by the formation of flower buds on axillary shoots in plants without an apex (19–20 weeks old) (Figure 6.27B (d)].

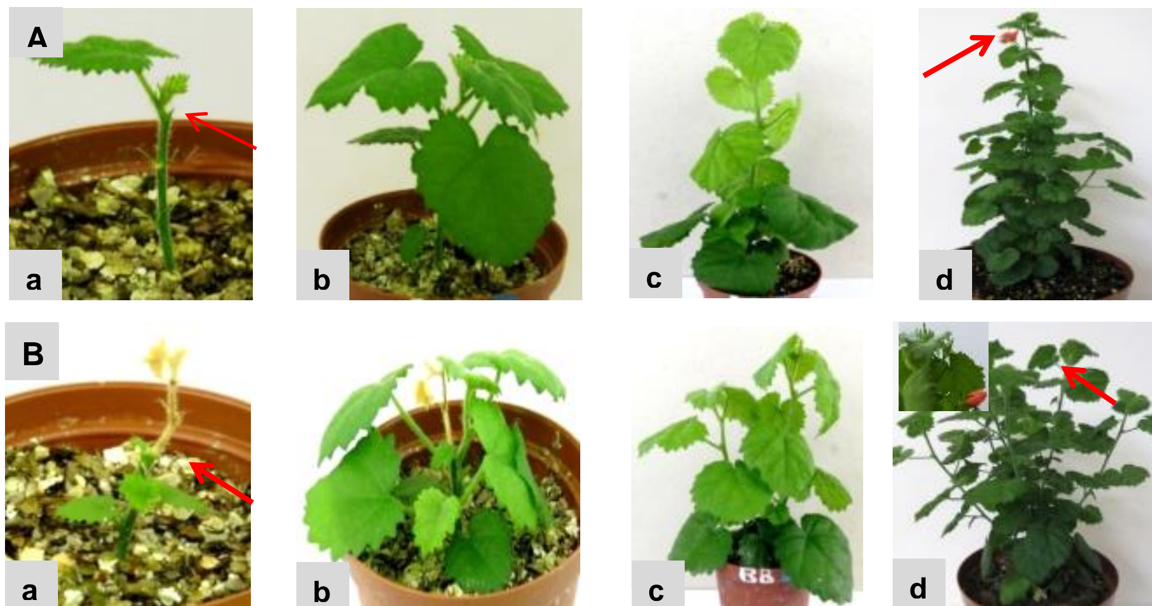

Figure 6.27. Further development of basal microcuttings treated with Dynaroot No.1 rooting hormone powder. (A) Rooted microcutting with an intact apex; (B) Rooted microcutting of which the apex died. (a) Intact and dead apex at 3 weeks; (b) Rooted microcuttings (regenerated plant) at 6 weeks; (c) Young plants were transplanted at 11 weeks to a potting soil mixture; (d) Hardened-off plants formed flower buds and had open flowers at 18 weeks.

There was no significant difference between treatments with respect to the percentage of surviving rooted plants (Figure 6.28). Relatively good average plant regeneration efficiency of 64% was attained for *ex vitro* rooted microcuttings from all treatments. The regeneration efficiency of *ex vitro* rooted microcuttings depends on species type. Microcuttings of *Pyrus elaeagrifolia* rooted *ex vitro* attained lower regeneration efficiency (55%) than *H. coddii* subsp. *barnardii* (Aygun and Dumanoglu, 2015). Benmahioul *et al.* (2012) reported higher regeneration (81.5%) of *ex vitro* rooted microcuttings of *Pistacia vera*.

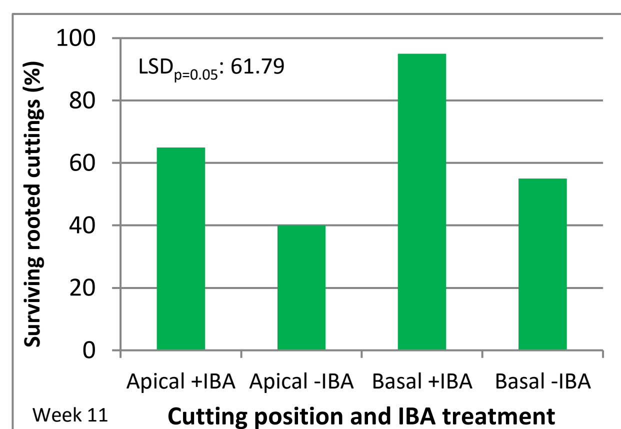

Figure 6.28. Survival percentage of *ex vitro* rooted microcuttings after 11 weeks.

Results showed that treatment of *H. coddii* subsp. *barnardii* microcuttings with IBA-containing rooting hormone powder resulted in fast and high rooting percentages irrespective of the position from where the cuttings were taken from the seedling. *Ex vitro* rooting decreases the cost, resources and labour involved with *in vitro* rooting procedures and it also reduces the time for rooting and acclimatisation of rooted plantlets (Hartmann *et al*., 2011; Ranaweera *et al*., 2013; Shekhawat *et al*., 2015; Shiji and Siril, 2018). The *ex vitro* rooting procedure as described here has the potential to generate six to eight *H. coddii* subsp. *barnardii* plants from one *in vitro* produced seedling in 4–5 months. Further studies could look at the production of *in vitro* seedlings in cheaper substrates such as sand and vermiculite supplemented with a nutrient solution as also suggested by Yan *et al*. (2010) for *Siratia grosvenorii*. The seedlings will grow faster than *in vivo* produced seedlings and removal of the apex would result in proliferation and elongation of axillary shoots that could be used as microcuttings in 4–5 weeks.

6.3.6 *Ex vitro* establishment of regenerated plantlets

a. Acclimatisation

Acclimatisation is an important stage of micropropagation and low survival of regenerated plantlets due to reduced photosynthetic capacity, malfunctioning stomata and poor water retention capacity, is a common problem (De Klerk, 2000; Kane, 2005; Iliev *et al*., 2010; Hartmann *et al*., 2011). These plants can therefore not be transferred directly to uncontrolled conditions or to the open field, but need to be gradually acclimatised to conditions of reduced humidity and increased light intensity (Thiart, 2003; Rout *et al*., 2006; Hartmann *et al*., 2011).

In vitro produced seedlings and plantlets of *H. coddii* subsp. *barnardii* were successfully acclimatised over a period of 2–3 weeks in moist vermiculite under controlled conditions (Section 6.3.11.1). Yang *et al*. (1995) also successfully acclimatised regenerated plantlets of *H. syriacus* in moist vermiculite, whereas plantlets of *H. sabdariffa* were acclimatised in a perlite and Sunshine® (1:1) medium covered with transparent cellophane (Gómez-Leyva *et al*., 2008). Perforated transparent plastic bags were used to cover *H. coddii* subsp. *barnardii* plants in the first few weeks of acclimatisation for gradual exposure from high humidity conditions in the culture vessel to a lower humidity and high light intensity in the open

environment (De Klerk, 2002; Hartmann *et al.*, 2011). Khatun *et al.* (2003) also used perforated plastic bags to acclimatise regenerated plantlets of *H. cannabinus* and achieved 95% survival. Similarly, high (92–100%) survival of acclimatised seedlings [Figure 6.29A (b)] and plantlets of *H. coddii* subsp. *barnardii* regenerated from rooted primary shoot explants [Figure 6.29B (b)] on PGR-free medium was achieved after 3–4 weeks. However, only 60% of plants originating from the *in vitro* rooting experiment (Section 6.3.5.1) survived the acclimatisation. The low survival in these plantlets could be due to dying-off of *in vitro* formed roots when transplanted to a solid medium causing the need for new root development as reported by Kane (2005) and Ranaweera *et al.* (2013). In contrast, 100% of *ex vitro* rooted plants were successfully acclimatised. This could be due to the direct rooting of microcuttings in a solid medium, which results in stronger and more flexible roots. Simultaneous rooting and acclimatisation improve the survival rate of plants (Benmahioul *et al.*, 2012; Ranaweera *et al.*, 2013)

b. <u>Hardening-off</u>

Plants of *H. coddii* subsp. *barnardii* transferred to a mixture of potting soil, vermiculite and sand [5:3:1 (v/v/v)] responded well to hardening-off under uncontrolled conditions in a greenhouse (Section 6.3.11.2) with 80–100% survival of plants. Acclimatised plants of *H. sabdariffa* were successfully hardened-off (100% survival) in pots with soil kept in a greenhouse for four weeks (Gómez-Leyva *et al.*, 2008). Sami *et al.* (2016) also reported high survival (96%) of acclimatised *H. syriacus* plants transferred to a mixture of peat and sand (1:1) and hardened-off in a greenhouse. In contrast, McLean *et al.* (1992) indicated only 38% survival of acclimatised *H. cannabinus* plants after transfer to a peat and sand (2:1) medium. *In vitro* generated mature plants of *H. coddii* subsp. *barnardii* flowered 2–3 months after the transplant and no phenotypic variation was observed between these plants [Figure 6.29A & B (c)] and plants obtained from nature and through conventional propagation by cuttings.

Figure 6.29. Examples of acclimatised and hardened-off plants. (A) Seedling grown *in vitro* on 1¼ MS medium strength; (B) Rooted basal seedling explant (a) Prior transplant; (b) After acclimatisation at three weeks; (c) Hardened-off plants at three months with flower buds and flowers (arrows).

Results of this study show that seedlings of *H. coddii* subsp. *barnardii* could be produced *in vitro* (Figure 6.30). Removal of the seedling apex at five weeks induced 5–8 axillary shoots per seedling within 5–6 weeks. These axillary shoots could be used successfully for *ex vitro* rooting (Figure 6.30). The basal part (including cotyledon node and piece of hypocotyl) of seedlings was more responsive in *in vitro* culture in terms of bud sprouting and shoot proliferation than axillary shoots without an apex. The best shoot induction and rooting response was noted in explants cultured on PGR-free medium (Figure 6.30). Compared to *in vivo* propagation, *in vitro* cultures could be beneficial for faster production of seedlings and plants all year round in limited space with low maintenance of plant material.

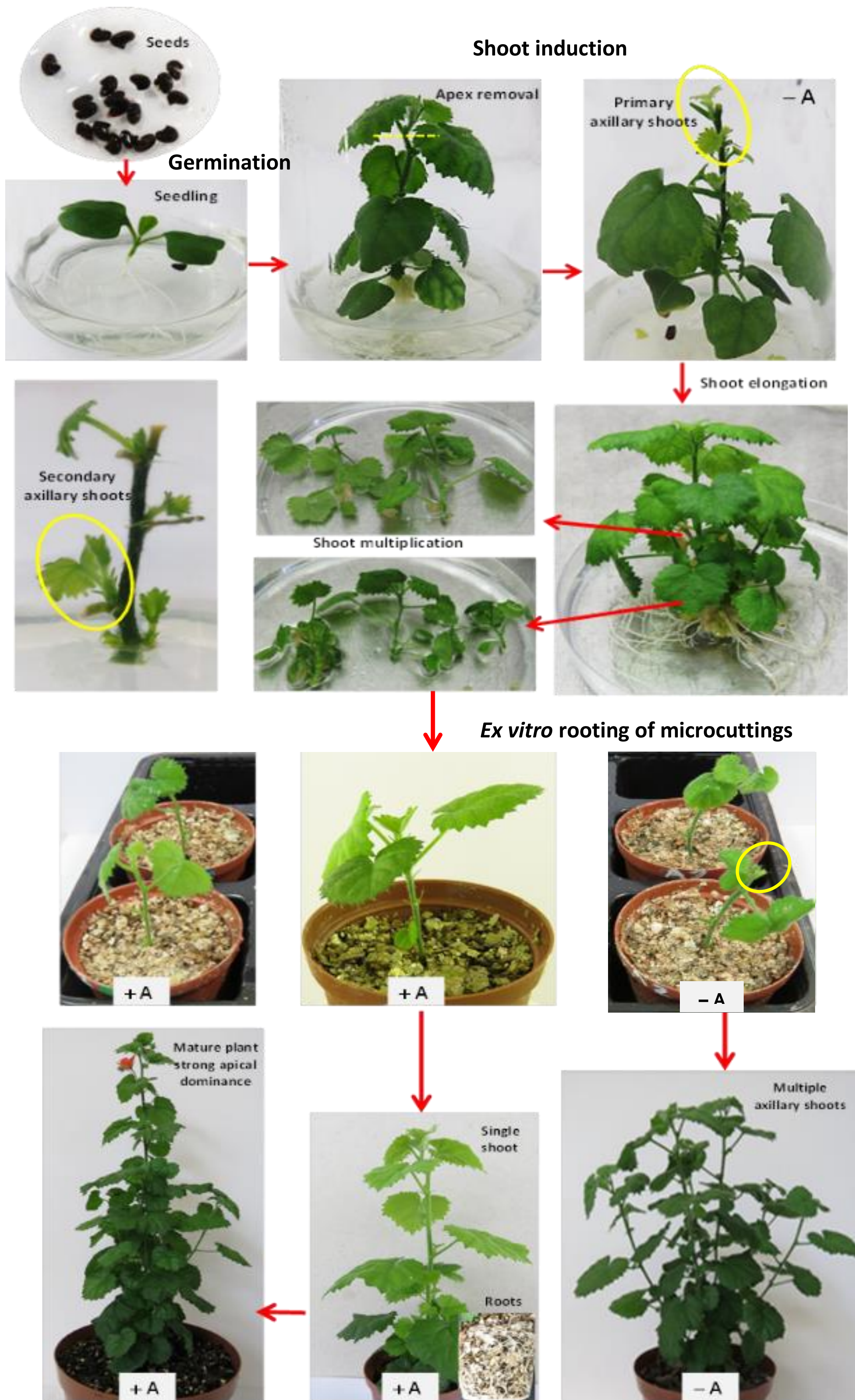

Figure 6.30. An overview of *in vitro* culture of *H. coddii* subsp. *barnardii.*

6.4 References

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Chapter 7

CHAPTER 7
General conclusions and Recommendations

The Sekhukhuneland endemic *Hibiscus coddii* subsp. *barnardii* could be effectively propagated *in vivo* by means of seeds and stem cuttings. *In vitro* culture can also be used for faster production of seedlings and of plants by *ex vitro* rooting of microcuttings. Large-scale production of plants for *ex situ* conservation and for commercial supply is thus possible.

The results obtained from the study led to the following conclusions:

Characterisation of *Hibiscus coddii* subsp. *barnardii*

Wild plants of *H. coddii* subsp. *barnardii* are mainly restricted to rocky outcrops and this habitat association is most probably due to physical factors such as wind, which traps seeds in rock crevices where they are able to germinate. Plants grow in soils with high levels of Mg, Fe, Ca, Ni and Cr. Some of the plant species found near *H. coddii* subsp. *barnardii* in nature, such as *Heteropyxis natalenis*, *Karomia speciosa*, *Mundulea sericea* subsp. *sericea* and *Scadoxus puniceus,* are already grown by indigenous nurseries, while others such as *Aneilema longirrhizum* and *Triaspis glaucophylla* have potential as new ornamentals.

***In vivo* propagation of *Hibiscus coddii* subsp. *barnardii* by seeds**

Seed germination

Chemical scarification with 98% sulfuric acid for 30 minutes significantly improved seed germination and seedling emergence of *H. coddii* subsp. *barnardii*. The best temperature for seed germination and seedling development is 25°C.

Seedling and plant development

Strong, healthy seedlings can be produced within 12 weeks from seeds germinated preferably in a commercial seedling mix and provided with sufficient nutrients. Plants grown in pots also require adequate nutrients to prevent deficiency symptoms and ensure the most optimal growth. Plants show strong apical dominance with a single stem. Removal of the apex from the main stem of 4–5 month old plants promoted

outgrowth of axillary shoots suitable as cuttings for vegetative propagation. This also resulted in a bushy plant with flowers formed all over the plant.

***In vivo* propagation of *Hibiscus coddii* subsp. *barnardii* by stem cuttings**

Apical and basal cuttings can both be used for vegetative propagation, although basal cuttings gave better rooting responses. The application of Dynaroot™ No.1 (0.1% IBA) and Dynaroot™ No.2 (0.3% IBA) rooting hormone powder resulted in good rooting responses (89–91%) and led to formation of numerous roots per cutting which was more pronounced with Dynaroot 2. The best rooting and cutting growth parameters were observed in vermiculite medium alone and in combination with coco peat, followed by a coco peat and sand mixture. Seven to eight regenerated plants can be obtained from one *in vivo* grown plant within six months.

In vitro* culture of *Hibiscus coddii* subsp. *barnardii

Seed culture

Seeds could be used as suitable starting material for *in vitro* establishment of seedlings. Scarified seeds germinated the most readily on lower strengths (¼, ½, ¾) of Murashige and Skoog (MS) medium, although these media were not suitable for further seedling development. Seedling development was best supported on full MS medium.

Shoot culture

In vitro grown seedlings provided aseptic uniform explant material. Excision of the apex of 4–5 week old seedlings while it remained in the culture vessel, resulted in the outgrowth of numerous (up to 8) axillary shoots. Large (3–4 nodes) basal nodal explants obtained directly from *in vitro* grown seedlings and cultured on PGR-free MS medium in a vertical position was the most suitable for generating axillary shoots and for plant regeneration. The use of plant growth regulators (PGRs) such as 6-Benzylaminopurine (0.25–4 mg L^{-1}) alone and combined with 0.5 mg L^{-1} IBA and 1-Naphtalene-acetic acid (NAA) resulted in low shoot regeneration efficiency irrespective of the explant source and type.

Root culture

In vitro rooting of microcuttings derived from *in vitro* grown seedlings with removed apex proved ineffective in the presence of 0.5 and 1 mg L^{-1} IBA and NAA. *Ex vitro* rooting of microcuttings proved successful for plant regeneration (up to 8 plants per seedling) within four months and mature flowering plants were produced within 5–6 months.

Ex vitro establishment of regenerated plants

Successful acclimatisation of *in vitro* produced seedlings and plantlets of *H. coddii* subsp. *barnardii* under controlled environmental conditions was achieved by using perforated transparent plastic bags. Plants transferred to a mixture of potting soil, vermiculite and sand [5:3:1(v/v/v)] responded well to hardening-off under uncontrolled conditions in a greenhouse with 80–100% survival of plants.

Compared to *in vivo* propagation, *in vitro* cultures could be beneficial for faster production of seedlings and plants all year round in limited space with low maintenance of plant material.

Future directions and recommendations for further research

The research reported here revealed numerous fields of study that could be pursued in future for *H. coddii* subsp. *barnardii*. These include:

i. Phenology of plants in nature including possible pollinators.
ii. Heavy metal tolerance of plants with specific reference to Ni and Cr.
iii. Seed viability studies for horticultural and conservation purposes.
iv. Anatomical studies of the water gap structure and the mechanism through which the seed coat becomes permeable to water.
v. Direct and indirect adventitious shoot culture for *in vitro* multiplication.
vi. Polyploidisation studies to increase the flower size.
vii. Microsatellite analysis of regenerated plants to ensure fidelity to mother stock.
viii. Market research amongst plant growers and nursery owners to test the acceptance and suitability of *H. coddii* subsp. *barnardii* plants for the South African horticulture landscape.

Appendices

APPENDICES

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Appendix A

APPENDIX A: Data and statistical analysis for Chapter 3

Table 1. Temperature and rainfall data in Potlake Nature Reserve (2015, 2016, 2017).

Month	2015			2016			2017		
	Min temp °C	Max temp °C	Rainfall mm	Min temp °C	Max temp °C	Rainfall mm	Min temp °C	Max temp °C	Rainfall mm
January	16	32	52	13	38	26	13	32	57.5
February	15	32	12	15	36	56	13	27	54
March	16	29	-	18	36	212	15	31	15
April	12	33	-	18	31	12	14	25	41
May	11	31	-	13	26	61	5	23	55
June	11	27	-	11	27	1.5	4	21	-
July	10	29	-	8	21	-	9	22	3
August	15	32	-	12	23	-	15	25	-
September	17	30	13	13	26	-	12	27	-
October	11	31	39	20	34	27	15	29	64.5
November	14	31	73	17	32	108	14	30	66
December	19	34	89	13	32	79	14	32	137.5
Total			**278**			**582.5**			**493.5**

Table 2. Collection points of soil samples at the three selected sites in Sekhukhuneland.

Sample	Site 1 D4190 Apèl Road		Site 2 Potlake (1st rocky outcrop)		Site 3 Potlake (2nd rocky outcrop)	
	Location	Altitude (m)	Location	Altitude(m)	Location	Altitude (m)
1 +Plant	24°17'35.2"S 29°47'33.7"E	751	24°15'18.9"S 29°54'44.2"E	826	24°15'18.0"S 29°54'22.8"E	840
1 –Plant	24°17'34.8"S 29°47'34.0"E	750	24°15'19.3"S 29°54'44.1"E	825	24°15'18.5"S 29°54'22.9"E	834
2 +Plant	24°17'34.4"S 29°47'30.4"E	754	24°15'17.5"S 29°54'44.8"E	830	24°15'16.8"S 29°54'23.9"E	838
2 –Plant	24°17'35.2"S 29°47'30.4"E	748	24°15'17.2"S 29°54'44.9"E	828	24°15'15.7"S 29°54'24.2"E	830
3 +Plant	24°17'36.3"S 29°47'32.1"E	754	24°15'17.2"S 29°54'41.2"E	829	24°15'17.6"S 29°54'21.6"E	836
3 –Plant	24°17'36.5"S 29°47'31.8"E	753	24°15'17.5"S 29°54'41.1"E	829	24°15'17.9"S 29°54'21.7"E	836
4 +Plant	24°17'35.6"S 29°47'33.8"E	754	24°15'19.0"S 29°54'42.0"E	824	24°15'17.6"S 29°54'24.4"E	835
4 –Plant	24°17'35.1"S 29°47'34.4"E	750	24°15'19.2"S 29°54'42.0"E	824	24°15'18.5"S 29°54'24.7"E	831
5 +Plant	24°17'34.5"S 29°47'31.1"E	749	24°15'17.3"S 29°54'43.9"E	830	24°15'16.6"S 29°54'22.5"E	839
5 –Plant	24°17'35.4"S 29°47'30.9"E	749	24°15'16.9"S 29°54'44.0"E	828	24°15'16.0"S 29°54'21.7"E	831
6 +Plant	24°17'34.8"S 29°47'32.4"E	756	24°15'18.8"S 29°54'42.7"E	827	24°15'17.5"S 29°54'23.8"E	837
6 –Plant	24°17'34.1"S 29°47'32.6"E	750	24°15'19.1"S 29°54'42.6"E	824	24°15'17.8"S 29°54'23.8"E	837

+Plant: soil collected at the plant. –Plant: soil collected away from the plants.

Table 3. Measurements of plants in their natural habitat at Site 2 and Site 3.

Plant	Site 2 Height (mm)	Site 2 No of branches	Site 3 Height (mm)	Site 3 No of branches
1	600	14	530	8
2	1050	13	500	10
3	670	3	420	4
4	800	9	780	4
5	480	5	750	10
6	830	6	550	4
7	850	5	670	6
8	690	7	450	4
9	700	8	770	12
10	670	6	470	6
11	980	10	520	6
12	700	8	700	6
13	830	3	870	10
14	420	3	600	4
15	760	8	570	9
16	500	4	580	5
17	500	3	400	6
18	820	11	600	5
19	970	7	660	6
20	870	5	700	8
21	450	7	900	7
22	940	6	550	5
23	700	6	600	3
24	1070	10	400	9
25	750	5	590	7
AVG	**744,00**	**6,88**	**605,20**	**6,56**
STD	**184,48**	**3,02**	**137,54**	**2,38**

Leaves	Site 2 Width mm	Site 2 Length mm	Site 3 Width mm	Site 3 Length mm
1	32	33	31	30
2	30	25	35	32
3	25	25	30	30
4	35	29	30	29
5	40	40	27	30
6	25	28	32	30
7	22	25	30	25
8	25	26	35	30
9	25	27	36	30
10	31	30	30	30
11	25	20	37	32
12	34	32	40	40
13	30	27	37	40
14	31	28	32	32
15	28	26	30	31
16	21	25	30	29
17	27	22	45	45
18	30	33	35	35
19	47	45	35	32
20	45	35	32	31
21	28	25	35	32
22	32	30	45	40
23	30	25	33	32
24	30	24	27	27
25	40	38	37	33
26	34	32	36	32
27	32	31	30	31
28	33	30	45	42
29	32	31	40	37
30	32	30	37	32
AVG	**31,03**	**29,23**	**34,47**	**32,70**
STD	**6,04**	**5,36**	**4,95**	**4,56**

Soil analyses: ICPE method

Table 4. Different mineral elements, percentage C and pH of soil samples collected from three sites in Sekhukhuneland during four periods. Soil samples were taken in pairs (at the plant and away from the plant). [GenStat64-bit Release 18.2 (PC/Windows 8) software].

Analysis of variance (ANOVA)

Variate (Concentration mg L^{-1})

Source	DF	Type I SS	Mean square	F value	Pr > F
Ca					
SITE	2	25357.82	12678.91	69.23	<.001 **
PAIR	1	3678.22	3678.22	40.96	<.001 **
K					
SITE	2	21.7045	10.8522	18.17	<.001 **
PAIR	1	14.2676	14.2676	47.38	<.001 **
Mg					
SITE	2	43251.7	21625.9	8.42	<.001 **
PAIR	1	14652.3	14652.3	17.30	<.001 **
P					
SITE	2	54.40	27.20	1.22	0.302 N.S.
PAIR	1	7.44	7.44	0.70	0.407 N.S.
S					
SITE	2	3.952	1.976	0.92	0.404 N.S.
PAIR	1	0.830	0.830	0.19	0.662 N.S.
B					
SITE	2	0.08365	0.04183	1.24	0.297 N.S.
PAIR	1	0.05546	0.05546	2.33	0.132 N.S.
Cr					
SITE	2	160.248	80.124	7.94	<.001 **
PAIR	1	119.403	119.403	19.77	<.001 **
Cu					
SITE	2	0.06501	0.03251	1.59	0.212 N.S.
PAIR	1	0.03618	0.03618	1.69	0.199 N.S.
Fe					
SITE	2	2848.9	1424.5	2.60	0.082 N.S.
PAIR	1	427.6	427.6	2.16	0.147 N.S.
Mn					
SITE	2	8.360	4.180	2.13	0.127 N.S.
PAIR	1	8.650	8.650	5.98	0.017 *
Mo					
SITE	2	1.57616	0.78808	31.06	<.001 **
PAIR	1	0.00006	0.00006	0.00	0.966 N.S.
Na					
SITE	2	479.193	239.596	118.75	<.001 **
PAIR	1	0.011	0.011	0.01	0.918 N.S.
Zn					
SITE	2	0.8038	0.4019	1.62	0.207 N.S.
PAIR	1	0.8251	0.8251	6.71	0.012 *

Source	DF	Type I SS	Mean square	F value	Pr > F
Percentage C					
SITE	2	26.668	13.334	4.74	0.012 *
PAIR	1	105.444	105.444	65.30	<.001 **
pH KCl					
SITE	2	2.5812	1.2906	16.75	<.001 **
PAIR	1	18.2350	18.2350	181.33	<.001 **

N.S.: Not significant; *Significant (p=0.001–0.05); **Highly significant (p<.001) at the 5% level of significance.

Table 5. Mineral element concentration, percentage C and pH of soil samples collected from three sites in Sekhukhuneland.

Element (mg L^{-1})	Site 1 Apèl Road	Site 2 Potlake	Site 3 Potlake	LSD	*P*<0.05
Ca	38.27^{a}	8.170^{b}	12.590^{b}	5.53	**
K	2.39^{a}	1.579^{b}	1.559^{b}	0.32	**
Mg	19.22^{b}	61.630^{a}	38.720^{b}	20.69	**
P	6.59a	5.130a	5.550a	–	N.S.
S	6.27a	6.360a	5.970a	–	N.S.
B	0.30a	0.355a	0.349a	–	N.S.
Cr	7.87^{b}	10.443^{a}	9.352^{a}	1.30	**
Cu	0.26a	0.216a	0.219a	–	N.S.
Fe	23.80a	33.000a	23.300a	–	N.S.
Mn	4.37a	4.870a	4.340a	–	N.S.
Mo	0.17^{c}	0.342^{b}	0.416^{a}	0.07	**
Na	6.71^{a}	2.834^{b}	2.851^{b}	0.58	**
Zn	0.63a	0.505a	0.445a	–	N.S.
%C	4.39	3.400	4.210	0.69	*
pH (KCl)	7.39^{b}	7.655^{a}	7.696^{a}	0.11	**

N.S.: Not significant; *Significant (p=0.001–0.05); **Highly significant (p<.001) Means with the same letter within a row are not significantly different at the 5% level of significance.

Table 6. Mineral element concentration, %C and pH of soil samples collected at the plants (+Plant) and away from the plants (–Plant).

Element (mg L^{-1})	+Plant	–Plant	LSD	*P*<0.05
Ca	14.620^{a}	24.730^{b}	3.16	**
K	2.158^{a}	1.529^{b}	0.18	**
Mg	29.800^{a}	49.900^{b}	9.70	**
P	5.980	5.530	–	N.S.
S	6.120	6.270	–	N.S.
B	0.315	0.355	–	N.S.
Cr	10.130^{a}	8.310^{b}	0.82	**
Cu	0.216	0.248	–	N.S.
Fe	28.400	25.000	–	N.S.
Mn	4.770^{a}	4.280^{b}	0.40	*
Mo	0.307	0.309	–	N.S.
Na	4.141	4.123	–	N.S.
Zn	0.449^{a}	0.601^{b}	0.12	*
%C	4.850^{a}	3.140^{b}	0.42	**
pH (KCl)	7.225^{a}	7.937^{b}	0.11	**

N.S.: Not significant; *Significant (p=0.001–0.05); **Highly significant (p<.001). Means with the same letter within a row are not significantly different at the 5% significance level.

Soil analyses: XRF method

Table 7. Major oxides in soils collected from three sites in Sekhukhuneland at the plants (+Plant) and away from the plants (–Plant).

SITE 1				+ Plant				
Weight %	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**Avg**	**STD**
SiO_2	40,88	41,21	38,13	41,44	40,97	41,84	40,75	1,33
TiO_2	0,42	0,41	0,48	0,43	0,36	0,37	0,41	0,04
Al_2O_3	16,91	17,55	14,64	16,57	17,66	17,58	16,82	1,15
Fe_2O_3	5,41	5,49	6,24	5,59	5,29	5,44	5,58	0,34
MnO	0,11	0,11	0,10	0,11	0,11	0,11	0,11	0,00
MgO	2,58	2,78	2,54	2,81	2,89	2,87	2,75	0,15
CaO	8,39	9,16	7,45	7,74	9,32	0,87	7,16	3,17
Na_2O	0,67	1,16	0,46	0,55	1,03	0,87	0,79	0,28
K_2O	0,68	0,46	0,61	0,73	0,44	0,72	0,61	0,13
P_2O_5	0,52	0,45	0,57	0,67	0,44	0,58	0,54	0,09
SITE 1				**– Plant**				
Weight %	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**Avg**	**STD**
SiO_2	37,18	43,70	39,10	40,19	43,05	44,01	41,21	2,80
TiO_2	0,42	0,47	0,43	0,45	0,34	0,34	0,41	0,06
Al_2O_3	14,32	20,44	14,04	14,73	19,69	18,23	16,91	2,89
Fe_2O_3	4,99	4,56	5,58	5,25	4,63	4,35	4,89	0,47
MnO	0,11	0,09	0,12	0,12	0,09	0,09	0,10	0,02
MgO	2,76	2,84	3,39	3,26	2,81	2,82	2,98	0,27
CaO	11,19	10,23	10,91	10,47	10,90	10,37	10,68	0,38
Na_2O	0,59	1,43	0,31	0,62	1,32	1,30	0,93	0,48
K_2O	0,45	0,39	0,46	0,46	0,36	0,37	0,42	0,05
P_2O_5	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
SITE 2				**+ Plant**				
Weight %	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**Avg**	**STD**
SiO_2	48,99	45,07	39,10	44,58	50,78	46,35	45,81	4,05
TiO_2	0,40	0,20	0,44	0,33	0,30	0,42	0,35	0,09
Al_2O_3	4,11	2,45	14,04	4,07	4,32	3,87	5,48	4,25
Fe_2O_3	11,04	9,47	5,58	12,76	10,65	11,87	10,23	2,53
MnO	0,22	0,22	0,10	0,20	0,00	0,19	0,16	0,09
MgO	15,60	25,77	3,39	17,39	22,89	15,91	16,83	7,75
CaO	1,28	3,81	10,91	1,44	2,18	1,55	3,53	3,73
Na_2O	1,29	0,00	0,00	0,00	0,16	0,00	0,24	0,52
K_2O	0,38	0,16	0,46	0,33	0,44	0,36	0,36	0,11
P_2O_5	0,39	0,41	5,58	0,43	0,00	0,45	1,21	2,15
SITE 2				**– Plant**				
Weight %	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**Avg**	**STD**
SiO_2	48,77	40,97	46,44	54,12	42,37	43,46	46,02	4,87
TiO_2	0,28	0,37	0,30	0,26	0,24	0,25	0,28	0,05
Al_2O_3	2,55	17,66	3,67	3,75	2,87	2,10	5,43	6,02
Fe_2O_3	10,80	5,29	11,76	10,14	10,29	10,04	9,72	2,26
MnO	0,18	0,08	0,19	0,22	0,17	0,20	0,17	0,05
MgO	21,45	2,89	18,91	23,15	24,48	18,99	18,31	7,87
CaO	1,30	9,32	1,72	1,21	3,06	1,24	2,98	3,19

SITE 2				– Plant (continue)				
Weight %	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**Avg**	**STD**
Na_2O	0,00	1,03	0,00	0,13	0,00	0,00	0,19	0,41
K_2O	0,23	0,44	0,26	0,27	0,25	0,21	0,28	0,08
P_2O_5	0,36	0,44	0,43	0,00	0,66	0,35	0,37	0,21
SITE 3				**+ Plant**				
Weight %	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**	**Avg**	**STD**
SiO_2	41,86	46,44	46,49	44,56	41,50	44,58	44,24	2,16
TiO_2	0,32	0,30	0,30	0,42	0,29	0,33	0,33	0,05
Al_2O_3	3,53	3,67	3,67	4,74	3,54	4,07	3,87	0,47
Fe_2O_3	12,24	11,76	11,39	13,75	11,90	12,76	12,30	0,85
MnO	0,23	0,19	0,18	0,20	0,20	0,20	0,20	0,02
MgO	15,66	18,91	19,75	14,61	15,47	17,39	16,97	2,06
CaO	1,70	1,72	1,56	1,54	2,06	1,44	1,67	0,22
Na_2O	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
K_2O	0,34	0,26	0,29	0,47	0,35	0,33	0,34	0,07
P_2O_5	0,48	0,43	0,47	0,49	0,52	0,43	0,47	0,04
SITE 3				**– Plant**				
Weight %	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**	**Avg**	**STD**
SiO_2	46,93	36,34	45,46	46,80	45,07	32,79	42,23	6,09
TiO_2	0,29	0,41	0,35	0,28	0,20	0,20	0,29	0,08
Al_2O_3	3,49	3,32	4,12	3,16	2,45	1,75	3,05	0,83
Fe_2O_3	10,87	9,97	10,21	10,35	9,47	10,61	10,25	0,49
MnO	0,22	0,17	0,16	0,22	0,17	0,16	0,18	0,03
MgO	20,81	12,22	16,10	0,00	25,77	26,40	16,88	9,92
CaO	1,71	4,01	2,29	2,19	3,81	4,79	3,13	1,23
Na_2O	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
K_2O	0,27	0,34	0,39	0,24	0,16	0,16	0,26	0,09
P_2O_5	0,49	0,55	0,75	0,48	0,41	0,42	0,52	0,13

Table 8. Trace elements in soils collected from three sites in Sekhukhuneland at the plants (+Plant) and away from the plants (–Plant).

SITE 1				+ Plant				
ppm	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**Avg**	**STD**
Cu	27	24	40	35	25	32	31	6
Ni	114	119	129	117	119	127	121	6
Zn	49	40	70	61	40	62	54	13
Cr	411	350	511	430	403	405	418	53
V	91	103	99	95	97	89	96	5
Rb	24	13	29	27	15	21	22	6
Sr	260	257	233	255	268	252	254	12
Y	8	6	9	9	6	7	8	1
Zr	60	56	75	61	45	45	57	11

SITE 1				– Plant				
ppm	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**Avg**	**STD**
Cu	23	16	27	19	29	14	21	6
Ni	102	91	111	98	96	85	97	9
Zn	37	27	44	38	29	29	34	7
Cr	274	251	279	291	237	226	260	26
V	104	79	114	103	94	87	97	13
Rb	15	13	17	17	10	10	14	3
Sr	336	284	327	325	279	294	308	25
Y	8	79	7	8	0	7	18	30
Zr	77	37	72	83	0	58	55	31
SITE 2				**+ Plant**				
ppm	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**Avg**	**STD**
Cu	24	0	27	0	0	0	9	13
Ni	866	910	111	11160	1150	960	2526	4245
Zn	111	77	44	92	107	141	95	33
Cr	8000	3850	279	5850	5370	12090	5907	3970
V	74	41	114	56	47	86	70	27
Rb	19	11	17	46	18	39	25	14
Sr	21	22	327	21	29	30	75	124
Y	5	0	7	0	0	3	3	3
Zr	0	28	72	51	66	112	55	39
SITE 2				**– Plant**				
ppm	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**Avg**	**STD**
Cu	0	25	0	0	30	0	9	14
Ni	931	119	938	818	11000	874	2447	4202
Zn	87	40	97	95	100	100	87	23
Cr	6360	403	4010	6420	5340	7170	4951	2481
V	67	97	45	63	45	67	64	19
Rb	29	15	18	13	29	11	19	8
Sr	13	268	17	13	45	9	61	102
Y	0	6	0	0	0	0	1	2
Zr	55	45	44	51	39	52	48	6
SITE 3				**+ Plant**				
ppm	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**	**Avg**	**STD**
Cu	32	0	27	0	0	0	10	15
Ni	1230	938	967	1300	971	1160	1094	155
Zn	105	97	103	116	105	92	103	8
Cr	6090	4010	6280	7340	3680	5850	5542	1413
V	59	45	54	70	58	56	57	8
Rb	20	18	18	28	21	46	25	11
Sr	28	17	21	30	30	21	25	6
Y	0	0	5	5	0	0	2	3
Zr	56	44	54	77	42	51	54	13
SITE 3				**– Plant**				
ppm	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**	**Avg**	**STD**
Cu	26	36	76	0	0	0	23	30
Ni	977	1270	929	1010	910	1590	1114	267
Zn	99	94	328	101	77	102	134	96

SITE 3				– Plant (continue)				
ppm	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**	**Avg**	**STD**
Cr	7590	4070	6370	7660	3850	7650	6198	1803
V	61	50	63	48	41	44	51	9
Rb	32	18	24	13	11	10	18	9
Sr	24	36	54	20	22	42	33	13
Y	61	0	63	0	0	0	21	32
Zr	50	82	53	46	28	40	50	18

Table 9. Checklist of plant species from study site localities in Sekhukhuneland and that grow in close vicinity of *H. coddii* subsp. *barnardii*.

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Abrus laevigatus E.Mey.	Fabaceae	Shrub		x	x
Acalypha indica L. var. *indica*	Euphorbiaceae	Forb	x	x	x
Achyranthes sicula (L.) All.*	Amaranthaceae	Forb		x	x
Adenia fruticosa Burtt Davy subsp. *fruticosa*	Passifloraceae	Shrub	x		
Aloe cryptopoda Baker	Asphodelaceae	Succulent	x		x
Amaranthus praetermissus Brenan	Amaranthaceae	Forb	x	x	x
Aneilema sp.	Commelinaceae	Forb	x		x
Aneilema longirrhizum Faden	Commelinaceae	Forb		x	x
Aptosimum lineare Marloth & Engl. var. *lineare*	Scrophulariaceae	Forb	x	x	x
Aristida congesta Roem. & Schult. subsp. *barbicollis* (Trin. & Rupr.) De Winter	Poaceae	Grass	x	x	x
Asparagus buchananii Baker	Asparagaceae	Shrub	x		
Asparagus laricinus Burch.	Asparagaceae	Shrub		x	x
Asparagus suaveolens Burch.	Asparagaceae	Shrub	x	x	x
Balanites maughamii Sprague	Zygophyllaceae	Tree		x	x
Barleria crossandriformis C.B.Clarke	Acanthaceae	Forb		x	x
Barleria elegans S.Moore ex C.B.Clarke	Acanthaceae	Shrub		x	x
Blepharis aspera Oberm.	Acanthaceae	Shrub	x		
Blepharis subvolubilis C.B.Clarke	Acanthaceae	Shrub		x	
Boscia albitrunca (Burch.) Gilg & Gilg-Ben.	Brassicaceae	Tree	x	x	x
Boscia foetida Schinz subsp. *rehmanniana* (Pestal.) Toelken	Brassicaceae	Tree	x	x	x
Canthium armatum (K.Schum.) Lantz	Rubiaceae	Shrub	x	x	x
Cardiospermum corindum L.	Sapindaceae	Climber	x	x	x
Ceratotheca triloba (Bernh.) Hook.f.	Pedaliaceae	Forb	x	x	x
Cheilanthes involuta (Sw.) Schelpe & N.C.Anthony var. *involuta*	Pteridaceae	Fern	x		
Cheilanthes involuta (Sw.) Schelpe & N.C.Anthony var. *obscura* (N.C.Anthony) N.C.Anthony	Pteridaceae	Fern			x
Chenopodium album L.*	Chenopodiaceae	Forb	x		

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Cleome angustifolia Forssk. subsp. *diandra* (Burch.) Kers	Brassicaceae	Forb		x	
Clerodendrum ternatum Schinz	Lamiaceae	Shrub	x		
Coccinia rehmannii Cogn.	Cucurbitaceae	Climber	x	x	x
Combretum apiculatum Sond. subsp. *apiculatum*	Combretaceae	Tree		x	x
Combretum molle R.Br. ex G.Don	Combretaceae	Tree		x	
Commelina erecta L.	Commelinaceae	Forb	x	x	x
*Commicarpus pilosu*s (Heimerl) Meikle	Nyctaginaceae	Forb	x		
Commiphora marlothii Engl.	Burseraceae	Tree	x	x	x
Commiphora pyracanthoides Engl.	Burseraceae	Tree	x	x	x
Commiphora tenuipetiolata Engl.	Burseraceae	Tree	x	x	x
Corallocarpus triangularis Cogn.	Cucurbitaceae	Climber	x		
Corbichonia decumbens (Forssk.) Exell	Molluginaceae	Forb	x	x	x
Croton gratissimus Burch. var. *gratissimus*	Euphorbiaceae	Tree	x	x	x
Croton menyhartii Pax	Euphorbiaceae	Tree	x	x	x
Cucumis melo L. subsp. *agrestis* (Naudin) Pangalo	Cucurbitaceae	Climber		x	x
Cyperus fulgens C.B.Clarke	Cyperaceae	Forb	x		
Cyphostemma sulcatum (C.A.Sm.) J.J.M.van der Merwe	Vitaceae	Climber	x	x	x
Dactyloctenium aegyptium (L.) Willd.	Poaceae	Grass	x		
Dichrostachys cinerea (L.) Wight & Arn. subsp. *africana* Brenan & Brummitt	Fabaceae	Tree	x	x	x
Dicoma tomentosa Cass.	Asteraceae	Forb	x		
Digitaria velutina (Forssk.) P.Beauv.	Poaceae	Grass	x		
Diospyros lycioides Desf. subsp. *lycioides*	Ebenaceae	Shrub			x
Dipcadi glaucum (Burch. ex Ker Gawl.) Baker	Hyacinthaceae	Geophyte	x		
Diplotaxis muralis (L.) DC.*	Brassicaceae	Forb			x
Ehretia rigida (Thunb.) Druce subsp. *nervifolia* Retief & A.E.van Wyk	Boraginaceae	Shrub	x	x	x
Endostemon tenuiflorus (Benth.) M.Ashby	Lamiaceae	Forb	x		
Enneapogon scoparius Stapf	Poaceae	Grass	x		

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Eragrostis biflora Hack. ex Schinz	Poaceae	Grass		x	x
Eragrostis curvula (Schrad.) Nees	Poaceae	Grass		x	x
Eragrostis racemosa (Thunb.) Steud.	Poaceae	Grass	x		
Euphorbia ingens E.Mey. ex Boiss.	Euphorbiaceae	Succulent	x	x	x
Euphorbia neopolycnemoides Pax & K.Hoffm.	Euphorbiaceae	Succulent	x	x	x
Evolvulus alsinoides (L.) L.	Convolvulaceae	Forb	x	x	x
Felicia clavipilosa Grau subsp. *transvaalensis* Grau	Asteraceae	Forb	x	x	x
Ficus abutilifolia (Miq.) Miq.	Moraceae	Tree	x	x	x
Flaveria bidentis (L.) Kuntze*	Asteraceae	Forb	x		
Flueggea virosa (Roxb. ex Willd.) Voigt subsp. *virosa*	Phyllanthaceae	Shrub	x	x	x
Geigeria burkei Harv. subsp. *burkei*	Asteraceae	Forb	x	x	x
Gisekia africana (Lour.) Kuntze var. *africana*	Gisekiaceae	Forb	x	x	x
Gisekia africana (Lour.) Kuntze var. *decagyna* Hauman	Gisekiaceae	Forb	x	x	x
Gloriosa superba L.	Colchicaceae	Geophyte			x
Grewia flava DC.	Malvaceae	Shrub	x	x	x
Grewia flavescens Juss.	Malvaceae	Shrub	x	x	x
Grewia monticola Sond.	Malvaceae	Shrub	x	x	x
Heliotropium ciliatum Kaplan	Boraginaceae	Forb	x	x	x
Heliotropium lineare (A.DC.) Gürke	Boraginaceae	Forb	x	x	x
Hermannia glanduligera K.Schum.	Malvaceae	Forb		x	
Hermannia modesta (Ehrenb.) Mast.	Malvaceae	Forb		x	
Hermbstaedtia odorata (Burch.) T.Cooke var. *odorata*	Amaranthaceae	Forb		x	x
Heteropogon contortus (L.) Roem. & Schult.	Poaceae	Grass	x	x	x
Heteropyxis natalensis Harv.	Heteropyxidaceae	Tree	x		
Hibiscus coddii Exell subsp. *barnardii* (Exell) Leistner & P.J.D.Winter	Malvaceae	Forb	x	x	x
Hibiscus micranthus L.f. var. *micranthus*	Malvaceae	Forb	x		
Hibiscus sidiformis Baill.	Malvaceae	Forb		x	x

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Hibiscus vitifolius L. subsp. *vulgaris* Brenan & Exell	Malvaceae	Forb			x
Holubia saccata Oliv.	Pedaliaceae	Forb	x		
Imperata cylindrica (L.) Raeusch.	Poaceae	Grass		x	
Indigofera circinnata Benth. ex Harv.	Fabaceae	Shrub	x	x	
Indigofera lydenbergensis N.E.Br.	Fabaceae	Forb	x	x	x
Indigofera tristoides N.E.Br.	Fabaceae	Forb	x	x	x
Indigofera vicioides Jaub. & Spach var. *vicioides*	Fabaceae	Forb		x	x
Ipomoea obscura (L.) Ker Gawl. var. *obscura*	Convolvulaceae	Creeper	x		
Justicia odora (Forssk.) Vahl	Acanthaceae	Forb	x		
Justicia protracta (Nees) T. Anderson subsp. *protracta*	Acanthaceae	Forb	x		
Kalanchoe brachyloba Welw. ex Britten	Crassulaceae	Forb	x		
Karomia speciosa (Hutch. & Corbishley) R.Fern.	Lamiaceae	Tree		x	x
Kewa bowkeriana (Sond.) Christenh.	Kewaceae	Forb	x		
Kleinia longiflora DC.	Asteraceae	Forb	x	x	x
Kohautia caespitosa Schnizl. subsp. *brachyloba* (Sond.) D.Mantell	Rubiaceae	Forb		x	
Kyphocarpa angustifolia (Moq.) Lopr.	Amaranthaceae	Forb		x	x
Lantana rugosa Thunb.	Verbenaceae	Shrub	x	x	x
Ledebouria apertiflora (Baker) Jessop	Hyacinthaceae	Geophyte	x		
Leonotis pentadentata J.C. Manning & Goldblatt	Lamiaceae	Forb	x	x	x
Limeum argute-carinatum Wawra ex Wawra & Peyr. var. *argute-carinatum*	Molluginaceae	Forb	x	x	x
Limeum sulcatum (Klotzsch) Hutch. var. *sulcatum*	Molluginaceae	Forb	x	x	x
Lycium cinereum Thunb.	Solanaceae	Shrub	x		
Maytenus albata (N.E.Br.) E.Schmidt bis & Jordaan	Celastraceae	Shrub			x
Melhania rehmannii Szyszyl.	Malvaceae	Shrub	x		x
Melinis repens (Willd.) Zizka subsp. *repens*	Poaceae	Grass	x	x	x
Monsonia glauca R.Knuth	Geraniaceae	Forb	x		
Mundulea sericea (Willd.) A.Chev. subsp. *sericea*	Fabaceae	Shrub	x	x	x

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Ochna inermis (Forssk.) Schweinf.	Ochnaceae	Tree	x		
Ocimum filamentosum Forssk.	Lamiaceae	Forb	x	x	x
Opuntia ficus-indica (L.) Mill.*	Cactaceae	Tree	x		
Pancratium tenuifolium Hochst. ex A.Rich.	Amaryllidaceae	Forb		x	x
Panicum maximum Jacq.	Poaceae	Grass	x	x	x
Pappea capensis Eckl. & Zeyh.	Sapindaceae	Tree	x		x
Pavetta zeyheri Sond. subsp. *zeyheri*	Rubiaceae	Tree		x	
Pegolettia senegalensis Cass.	Asteraceae	Forb	x	x	x
Peliostomum leucorrhizum E.Mey. ex Benth.	Scrophulariaceae	Forb			x
Pellaea calomelanos (Sw.) Link var. *calomelanos*	Pteridaceae	Fern	x	x	x
Phyllanthus incurvus Thunb.	Phyllanthaceae	Forb	x	x	x
Phyllanthus loandensis Welw. ex Müll.Arg.	Phyllanthaceae	Forb		x	
Phyllanthus maderaspatensis L.	Phyllanthaceae	Forb	x	x	x
Portulaca kermesina N.E.Br.	Portulacaceae	Forb		x	
Portulaca quadrifida L.	Portulacaceae	Forb	x		
Pouzolzia mixta Solms var. *mixta*	Urticaceae	Tree			x
Premna mooiensis (H.Pearson) W.Piep.	Lamiaceae	Tree	x		
Pristimera longipetiolata (Oliv.) N.Hallé	Celastraceae	Tree		x	x
Ptaeroxylon obliquum (Thunb.) Radlk.	Rutaceae	Tree	x	x	x
Pupalia lappacea (L.) A.Juss. var. *lappacea*	Amaranthaceae	Forb	x	x	
Rotheca myricoides (Hochst.) Steane & Mabb.	Lamiaceae	Shrub		x	
Ruellia patula Jacq.	Acanthaceae	Forb	x		
Ruttya ovata Harv.	Acanthaceae	Tree		x	x
Sansevieria hyacinthoides (L.) Druce	Ruscaceae	Forb	x	x	x
Sarcostemma viminale (L.) R.Br. subsp. *viminale*	Apocynaceae	Climber		x	
Scadoxus puniceus (L.) Friis & Nordal	Amaryllidaceae	Geophyte		x	
Schkuhria pinnata (Lam.) Kuntze ex Thell*	Asteraceae	Forb	x		

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Sclerocarya birrea (A.Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro	Anacardiaceae	Tree		x	x
Searsia engleri (Britten) Moffett	Anacardiaceae	Tree	x	x	
Seddera suffruticosa (Schinz) Hallier f.	Convolvulaceae	Forb	x	x	x
Senegalia burkei (Benth.) Kyal. & Boatwr.	Fabaceae	Tree			x
Senegalia mellifera (Vahl) Seigler & Ebinger subsp. *detinens* (Burch.) Kyal. & Boatwr.	Fabaceae	Tree	x	x	x
Senegalia senegal (L.) Britton var. *leiorhachis* (Brenan) Kyal. & Boatwr.	Fabaceae	Tree			
Senna italica Mill. subsp. *arachoides* (Burch.) Lock	Fabaceae	Shrub	x		
Sericorema remotiflora (Hook.f.) Lopr.	Amaranthaceae	Shrub		x	x
Sesamum triphyllum Welw. ex Asch. var. *triphyllum*	Pedaliaceae	Forb	x		
Sida cordifolia L. subsp. *cordifolia*	Malvaceae	Shrub	x	x	x
Solanum campylacanthum Hochst. ex A.Rich. subsp. *panduriforme* (Drège ex Dunal) J.Samuels	Solanaceae	Forb	x		
Solanum tomentosum L. var. *tomentosum*	Solanaceae	Forb	x		
Sporobolus stapfianus Gand.	Poaceae	Grass		x	x
Sterculia rogersii N.E.Br.	Malvaceae	Tree		x	x
Tephrosia purpurea (L.) Pers. subsp. *leptostachya* (DC.) Brummitt var. *leptostachya*	Fabaceae	Forb	x		
Tinospora fragosa (I.Verd.) I.Verd. & Troupin	Menispermaceae	Climber		x	x
Tragia dioica Sond.	Euphorbiaceae	Forb	x		
Tragus berteronianus Schult.	Poaceae	Grass	x	x	x
Triaspis glaucophylla Engl.	Malpighiaceae	Shrub	x		
Tribulus terrestris L.	Zygophyllaceae	Forb	x	x	x
Tricliceras glanduliferum (Klotzsch) R.Fern.	Turneraceae	Forb			x
Turraea obtusifolia Hochst.	Meliaceae	Shrub		x	x
Urochloa mosambicensis (Hack.) Dandy	Poaceae	Grass	x	x	
Vachellia grandicornuta (Gerstner) Seigler & Ebinger	Fabaceae	Tree			x
Vachellia tortilis (Forssk.) Gallaso & Banfi subsp. *heteracantha* (Burch.) Kyal. & Boatwr.	Fabaceae	Tree	x	x	x
Vangueria infausta Burch. subsp. *infausta*	Rubiaceae	Tree			x
Vangueria madagascariensis J.F.Gmel.	Rubiaceae	Tree	x		x

Species name	Family name	Growth form	Site 1	Site 2	Site 3
Vepris reflexa I.Verd.	Rutaceae	Tree		x	x
Ximenia americana L. var. *microphylla* Welw. ex Oliv.	Olacaceae	Shrub	x	x	
Zinnia peruviana (L.) L.*	Asteraceae	Forb	x	x	x
Ziziphus mucronata Willd. subsp. *mucronata*	Rhamnaceae	Tree		x	x
* Naturalised plant species					

Appendix B

APPENDIX B: Data and statistical analysis for Chapter 4

A. Seed germination

Table 1. Effect of chemical scarification with various sulfuric acid concentrations and different scarification times on seed germination and seedling emergence indices. [GenStat64-bit Release 17.1 (PC/Windows 7)].

Analysis of variance (ANOVA)

Variate	d.f.	s.s.	m.s.	v.r	F pr.
Final germination %	13	47257.1	3635.2	19.07	<.001 **
Mean germination time	13	1083.41	83.34	1.38	0.235 N.S.
Germination index	13	279.841	21.526	11.41	<.001 **
Contamination %	13	1583.3	121.8	1.08	0.418 N.S.
Final emergence %	13	52047.6	4003.7	27.43	<.001 **
Mean emergence time	13	369.52	28.42	0.63	0.806 N.S.
Emergence index	13	41.66640	3.20511	35.33	<.001 **
Seedlings with first leaves	13	70885.0	5453.0	5.37	<.001 **
Survival of seedlings	13	67189.0	5168.0	4.99	<.001 **

N.S.: Not significant; *Significant (p=0.001–0.05); **Highly significant (p<.001)

Table 2. Effect of scarification with 98% sulfuric acid for various durations on seed germination and seedling emergence indices (SAS Version 9.3 statistical software).

Analysis of variance (ANOVA)

Source	DF	Type I SS	Mean square	F value	Pr > F
Final germination %					
Trial	1	38.57143	38.57143	0.20	0.6605 N.S.
Treatment	9	67965.71429	7551.74603	38.27	<.0001 **
Trial*Treatment	9	654.28571	72.69841	0.37	0.9442 N.S.
Mean germination time					
Trial	1	11.1896458	11.1896458	1.16	0.2871 N.S.
Treatment	9	123.9621157	13.7735684	1.43	0.2049 N.S.
Trial*Treatment	9	147.4081768	16.3786863	1.70	0.1175 N.S.
Germination rate index					
Trial	1	0.3767668	0.3767668	0.18	0.6705 N.S.
Treatment	9	407.9027657	45.3225295	22.06	<.0001 **
Trial*Treatment	9	4.3788035	0.4865337	0.24	0.9871 N.S.
GT_{50}					
Trial	1	13.9083634	13.9083634	7.87	0.0096 *
Treatment	7	371.6169797	53.0881400	30.03	<.0001 **
Trial*Treatment	7	281.7418449	40.2488350	22.77	<.0001 **
Final emergence %					
Trial	1	1190.47619	1190.47619	3.69	0.0611 N.S.
Treatment	9	55971.42857	6219.04762	19.28	<.0001 **
Trial*Treatment	9	1211.90476	134.65608	0.42	0.9190 N.S.
Mean emergence time					
Trial	1	31.3393601	31.3393601	2.19	0.1460 N.S.
Treatment	9	265.0242300	29.4471367	2.06	0.0544 N.S.
Trial*Treatment	9	287.2364792	31.9151644	2.23	0.0374 *
Emergence rate index					
Trial	1	1.25744048	1.25744048	3.32	0.0752 N.S.
Treatment	9	58.73849143	6.52649905	17.22	<.0001 **
Trial*Treatment	9	0.94222857	0.10469206	0.28	0.9780 N.S.
ET_{50}					
Trial	1	0.21741775	0.21741775	0.60	0.4480 N.S.
Treatment	7	18.70920254	2.67274322	7.33	0.0001 **
Trial*Treatment	7	1.57635328	0.22519333	0.62	0.7361 N.S.

N.S.: Not significant; *Significant (p=0.001–0.05); **Highly significant (p<.001)

Table 3. Effect of temperature on seed germination and seedling emergence after 336 hours of incubation (SAS Version 9.3 statistical software).

Analysis of variance (ANOVA)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Final germination %	4	38.1964560	9.5491140	0.87	0.5018 N.S.
Mean germination time	4	26763.46422	6690.86605	21.47	<.0001 **
Germination rate index	4	3.51557600	0.87889400	42.63	<.0001 **
GT_{50}	4	27056.88847	6764.22212	17.34	<.0001 **
Final emergence %	4	32253.99826	8063.49956	429.24	<.0001 **
Mean emergence time	4	83358.03906	20839.50977	59.99	<.0001 **
Emergence rate index	4	0.65513600	0.16378400	89.18	<.0001 **
ET_{50}	3	38083.64547	12694.54849	27.27	<.0001 **

N.S.: Not significant; **Highly significant (*p*<.0001)

B. Effect of apex removal and nutrients on biometric parameters and flowering.

Table 4. Daily minimum and maximum temperature and relative humidity (R.H.) in the greenhouse for the duration of the plant development trials.

Week	Day	Min. temp °C	Max. temp °C	Min. R.H.	Max. R.H.
1	1	20,2	35,0	23,1	55,4
	2	18,6	29,1	42,8	82,3
	3	16,8	25,5	48,1	80,8
	4	16,9	26,7	42,1	79,5
	5	16,2	29,8	35,7	79,7
	6	15,3	33,6	28,2	79,0
	7	16,4	33,1	26,1	79,9
2	1	16,6	34,9	17,8	75,2
	2	18,9	29,4	34,3	74,6
	3	16,8	32,4	25,3	82,6
	4	17,9	30,2	26,1	83,2
	5	14,9	31,9	12,8	73,9
	6	15,9	34,2	18,0	67,9
	7	16,6	32,3	28,7	67,5
3	1	16,8	30,2	33,9	83,1
	2	16,7	31,5	30,3	81,6
	3	19,1	28,0	41,1	65,1
	4	19,7	30,3	41,0	88,1
	5	18,2	29,2	41,3	87,8
	6	16,8	30,7	32,9	84,8
	7	19,0	34,9	28,7	89,1
4	1	17,2	27,4	49,6	92,0
	2	17,2	27,3	45,5	88,1
	3	18,1	27,7	43,4	93,6
	4	17,8	28,4	52,1	91,9
	5	18,9	34,5	34,0	92,6
	6	19,4	32,2	39,1	80,2
	7	18,2	30,9	40,4	82,9
5	1	18,2	30,6	40,1	86,5
	2	17,8	31,8	34,3	86,9
	3	19,0	31,1	38,7	80,9
	4	18,1	30,8	26,5	83,0
	5	17,6	31,4	39,0	85,9
	6	16,6	31,1	34,4	80,1
	7	18,9	29,7	39,0	90,2
6	1	17,0	29,7	38,7	82,4
	2	17,5	29,1	34,7	84,5
	3	16,1	30,3	35,2	81,2
	4	18,1	26,5	52,0	81,4
	5	19,2	30,6	37,5	90,6
	6	18,7	31,7	45,6	92,2
	7	18,9	31,5	41,7	89,3
7	1	19,3	34,5	34,5	90,6
	2	19,2	29,9	54,5	93,1
	3	18,2	20,7	86,8	92,6
	4	16,7	22,2	75,7	87,2
	5	17,1	25,5	49,8	95,0
	6	17,9	28,7	55,8	94,5
	7	17,1	29,0	48,2	94,6
8	1	16,4	20,1	66,1	86,1
	2	16,0	27,0	50,2	90,0
	3	17,8	31,5	31,1	93,0
	4	16,8	33,9	24,8	88,6
	5	16,6	29,2	35,0	83,0
	6	15,7	26,4	46,4	84,6
	7	16,2	31,7	37,1	89,7
Average		**17.56**	**30.05**	**38.93**	**84.39**

Table 5. Effect of treatment and location on biometric parameters from week 0 to week 8. [GenStat64-bit Release 18.2 (PC/Windows 8).

Analysis of variance (ANOVA)

Week 0

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant height					
Location	1	40.1	40.1	0.03	0.896 N.S.
Treatment	3	3374.4	1124.8	1.48	0.227 N.S.
Treatment.Location	3	2538.3	846.1	1.11	0.350 N.S.
Number of nodes					
Location	1	0.009	0.009	0.02	0.910 N.S.
Treatment	3	9.312	3.104	1.74	0.165 N.S.
Treatment.Location	3	0.884	0.295	0.17	0.919 N.S.
Basal diameter					
Location	1	0.2527	0.2527	0.33	0.666 N.S.
Treatment	3	0.1340	0.0447	0.44	0.726 N.S.
Treatment.Location	3	0.0634	0.0211	0.21	0.891 N.S.

N.S.: Not significant; *Significant (*p*=0.001–0.05); **Highly significant (*p*<.001).

Week 2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant height					
Location	1	76442.	76442.	13.65	0.168 N.S.
Treatment	3	343790	114597.	56.95	<.001 **
Treatment.Location	3	10235.	3412.	1.70	0.175 N.S.
Number of nodes					
Location	1	47.580	47.580	592.11	0.026 *
Treatment	3	781.598	260.533	96.45	<.001 **
Treatment.Location	3	39.455	13.152	4.87	0.004 *
Basal diameter					
Location	1	0.1209	0.1209	15.33	0.159 N.S.
Treatment	3	0.5364	0.1788	1.58	0.201 N.S.
Treatment.Location	3	0.1944	0.0648	0.57	0.635 N.S.
No of axillary shoots 10–80 mm					
Location	1	32.143	32.143	14.06	0.166
Treatment	3	176.857	58.952	13.68	<.001 **
Treatment.Location	3	38.571	12.857	2.98	0.036 *
No of axillary shoots ≥90 mm					
Location	1	1.080	1.080	0.34	0.666 N.S.
Treatment	3	21.027	7.009	2.14	0.102 N.S.
Treatment.Location	3	12.312	4.104	1.25	0.297 N.S.

N.S.: Not significant; *Significant (*p*=0.001–0.05), **Highly significant (*p*<.001).

Week 4

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant height					
Location	1	117781.	117781.	45.24	0.094 N.S.
Treatment	3	669791.	223264.	90.60	<.001 **
Treatment.Location	3	6026.	2009.	0.82	0.489 N.S.
Number of nodes					
Location	1	1.080	1.080	1.00	0.500 N.S.
Treatment	3	3186.955	1062.318	351.24	<.001 **
Treatment.Location	3	13.384	4.461	1.48	0.228 N.S.
Basal diameter					
Location	1	0.7313	0.7313	193.85	0.046 *

Treatment	3	5.7479	1.9160	8.67	<.001 **
Treatment.Location	3	1.5670	0.5223	2.36	0.078 N.S.
No of axillary shoots 10–80 mm					
Location	1	18.080	18.080	11.98	0.179 N.S.
Treatment	3	205.384	68.461	14.99	<.001 **
Treatment.Location	3	47.670	15.890	3.48	0.020 *
No of axillary shoots ≥90 mm					
Location	1	33.223	33.223	148.84	0.052
Treatment	3	368.027	122.676	30.76	<.001 **
Treatment.Location	3	23.098	7.699	1.93	0.132 N.S.

N.S.: Not significant; *Significant (p=0.001–0.05), **Highly significant (p<.001).

Week 6

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant height					
Location	1	218154.	218154.	65.45	0.078 N.S.
Treatment	3	1130012.	376671.	123.80	<.001 **
Treatment.Location	3	16561.	5520.	1.81	0.151 N.S.
Number of nodes					
Location	1	7.509	7.509	841.00	0.022 *.
Treatment	3	7771.170	2590.390	703.53	<.001 **
Treatment.Location	3	99.384	33.128	9.00	<.001 **
Basal diameter					
Location	1	0.8349	0.8349	359.51	0.034 *
Treatment	3	17.3321	5.7774	27.04	<.001 **
Treatment.Location	3	0.2849	0.0950	0.44	0.722 N.S.
No of axillary shoots 10–80 mm					
Location	1	77.223	77.223	2.05	0.388 N.S.
Treatment	3	348.455	116.152	25.16	<.001 **
Treatment.Location	3	42.670	14.223	3.08	0.032 *
No of axillary shoots ≥90 mm					
Location	1	175.000	175.000	6.72	0.234 N.S.
Treatment	3	851.750	283.917	50.70	<.001 **
Treatment.Location	3	97.929	32.643	5.83	0.001 *

N.S.: Not significant; *Significant (p=0.001–0.05), **Highly significant (p<.001).

Week 8

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant length					
Location	1	257760.	257760.	73.43	0.074 N.S.
Treatment	3	1704872.	568291.	180.92	<.001 **
Treatment.Location	3	22422.	7474.	2.38	0.076 N.S.
Number of nodes					
Location	1	1.750	1.750	3.06	0.330N.S.
Treatment	3	13457.750	4485.917	959.95	<.001 **
Treatment.Location	3	257.750	85.917	18.39	<.001 **
Basal circumference					
Location	1	0.9602	0.9602	129.86	0.056 N.S.
Treatment	3	26.3907	8.7969	39.24	<.001 **
Treatment.Location	3	0.6786	0.2262	1.01	0.393 N.S.
No of axillary shoots 10–80 mm					
Location	1	180.036	180.036	4.12	0.292 N.S.
Treatment	3	592.893	197.631	38.31	<.001 **
Treatment.Location	3	24.179	8.060	1.56	0.205 N.S.
No of axillary shoots ≥90 mm					
Location	1	182.580	182.580	4.84	0.272 N.S.
Treatment	3	1069.741	356.580	65.36	<.001 **
Treatment.Location	3	100.955	33.652	6.17	<.001 **

N.S.: Not significant; *Significant (p=0.001–0.05), **Highly significant (p<.001).

Table 6. The interactive effect of treatment and location on biometric parameters of *H. coddii* subsp. *barnardii* plants on a bi-weekly basis.

	Plant height (mm)		No. of nodes		Basal diameter (mm)		No. axillary shoots (10-80 mm)		No. of axillary shoots ≥90 mm	
	Growth room	Greenhouse	Growth room	Greenhouse	Growth room	Greenhouse	Growth room	Greenhouse	Growth room	Greenhouse
Week 0										
+A+N	105.3	107.8	14.71	14.71	1.85	1.76				
+A-N	104.4	96.4	14.79	14.57	1.87	1.82				
-A+N	98.3	86.5	14.00	14.00	1.86	1.69				
-A-N	98.6	111.2	14.43	14.71	1.90	1.84				
F pr.	0.350		0.919		0.891					
Week 2										
+A+N	217.80 b	287.10 a	18.14 b	20.93 a	2.86	2.65	8.29 c	6.64 d	2.00	1.29
+A-N	209.00 bc	281.10 a	18.50 b	20.64 a	2.61	2.61	8.14 cd	6.64 d	1.00	0.64
-A+N	121.70 d	148.80 d	14.00 c	14.00 c	2.60	2.55	11.14 a	9.07 bc	2.07	1.43
-A-N	120.90 d	161.30 cd	14.43 c	14.71 c	2.62	2.62	9.29 bc	10.21 ab	1.50	2.43
F pr.	0.175; Tmt <.001 (23.87)		0.004 (1.076)		0.635		0.036 (1.502)		0.297	
Week 4										
+A+N	335.90 b	394.40 a	26.50 a	25.79 a	3.62 a	3.44 ab	10.00 b	10.71 ab	5.07 b	2.79 cd
+A-N	251.70 c	341.50 b	22.79 c	24.00 b	3.10 c	3.12 bc	8.07 c	6.57 e	1.71 de	0.86 e
-A+N	171.80 ef	223.70 cd	14.00 d	14.00 d	3.59 a	3.04 c	12.14 a	9.57 bcd	7.07 a	5.64 b
-A-N	142.80 f	202.00 de	14.43 d	14.71 d	2.90 c	2.95 c	9.57 bc	9.71 b	3.14 cd	3.36 c
F pr.	0.489; Tmt<.001 (26.41)		0.228; Tmt<.001 (0.925)		0.078; Tmt<.001 (0.250); Loc 0.046 (0.148)		0.020 (1.481)		0.132; Tmt<.001 (1.063)	
Week 6										
+A+N	432.60 b	507.60 a	34.21 a	32.50 b	4.25 a	3.98 ab	12.93 ab	12.71 abc	7.50 b	4.36 cde
+A-N	270.60 cd	401.00 b	25.71 d	29.21 c	3.41 c	3.27 c	9.07 cef	6.93 bdf	2.50 dfg	1.14 eg
-A+N	237.30 d	312.40 c	14.00 e	14.00 e	4.10 ab	3.84 b	13.21 a	9.79 abde	12.00 a	6.79 bc
-A-N	159.10 e	231.70 d	14.43 e	14.71 e	3.21 c	3.19 c	11.00 cd	10.14 abde	4.36 bd	4.07 bdef
F pr.	0.151; Tmt<.001 (29.35)		<.001 (1.251)		0.722; Tmt<.001 (0.246); Loc 0.034 (0.116)		0.032 (6.261)		0.001 (4.203)	
Week 8										
+A+N	510.60 b	593.00 a	41.64 a	37.71 b	4.65 a	4.36 ab	16.64 a	13.86 ab	9.21b	6.36 bc
+A-N	275.00 de	419.90 c	28.64 d	33.29 c	3.58 c	3.49 c	10.29 bdef	7.43 cdf	2.79 cef	1.43 df
-A+N	305.70 d	384.90 c	14.00 e	14.00 e	4.58 a	4.20 b	13.43 bc	9.93 acdefg	13.00 a	7.50 abc
-A-N	167.20 f	244.50 e	14.43 e	14.71 e	3.43 c	3.44 c	11.50 bd	10.50 acde	4.86 cd	4.36 bde
F pr.	0.076; Tmt<.001 (29.82)		<.001 (1.438)		0.393; Tmt <.001 (0.252)		0.205; Tmt<.001 (1.209)		<.001 (5.840)	

F pr. for Treatment (Tmt) x Location (Loc) is shown. If one or both of the individual factors was significant, it is also shown. LSD is shown in brackets.
For each week, the same letters within a column and a row indicates no significant difference.

Table 7. Chi-square tables for flower distribution frequencies

The tables were created with the frequency procedure of SAS Version 9.3 statistical software to indicate the association between factors (trial, location, treatment) and flower formation for different time periods.

There were no flower buds or flowers at week 0.

Week 2

Table of TRIAL by Flowers

Trial	Flowers			
Frequency	FBAp	FBFAp	NFBF	Total
1	20	6	30	56
2	20	1	35	56
Total	40	7	65	112

Statistic	DF	Value	Prob
Chi-Square	2	3.9560	0.1383 N.S.
Likelihood Ratio Chi-Square	2	4.3474	0.1138
Mantel-Haenszel Chi-Square	1	0.2492	0.6176
Phi Coefficient		0.1879	
Contingency Coefficient		0.1847	
Cramer's V		0.1879	

Table of LOCATION by Flowers

Location	Flowers			
Frequency	FBAp	FBFAp	NFBF	Total
Glasshouse	22	3	31	56
Growth	18	4	34	56
Total	40	7	65	112

Statistic	DF	Value	Prob
Chi-Square	2	0.6813	0.7113 N.S.
Likelihood Ratio Chi-Square	2	0.6825	0.7109
Mantel-Haenszel Chi-Square	1	0.4885	0.4846
Phi Coefficient			0.0780
Contingency Coefficient			0.0778
Cramer's V			0.0780

Table of TREATMENT by Flowers

Treatment	Flowers			
Frequency	FBAp	FBFAp	NFBF	Total
MTMN	0	0	28	28
MTWN	0	0	28	28
WTMN	21	2	5	28
WTWN	19	5	4	28
Total	40	7	65	112

Statistic	DF	Value	Prob
Chi-Square	6	83.7868	<.0001 **
Likelihood Ratio Chi-Square	6	104.5237	<.0001
Mantel-Haenszel Chi-Square	1	59.6699	<.0001
Phi Coefficient		0.8649	
Contingency Coefficient		0.6542	
Cramer's V		0.6116	

Week 4

Table of TRIAL by Flowers

Trial	Flowers					
Frequency	FBAp	FBAs	FBFAp	FBFApFBAs	NFBF	Total
1	5	0	22	1	28	56
2	13	1	12	0	30	56
Total	18	1	34	1	58	112

Statistic	DF	Value	Prob
Chi-Square	4	8.5657	0.0729 N.S.
Likelihood Ratio Chi-Square	4	9.5097	0.0495
Mantel-Haenszel Chi-Square	1	0.7806	0.3770
Phi Coefficient		0.2765	
Contingency Coefficient		0.2665	
Cramer's V		0.2765	

Table of LOCATION by Flowers

Location	Flowers					
Frequency	FBAp	FBAs	FBFAp	FBFApFBAs	NFBF	Total
Glasshouse	10	0	17	0	29	56
Growth	8	1	17	1	29	56
Total	18	1	34	1	58	112

Statistic	DF	Value	Prob
Chi-Square	4	2.2222	0.6950 N.S.
Likelihood Ratio Chi-Square	4	2.9953	0.5586
Mantel-Haenszel Chi-Square	1	0.0637	0.8007
Phi Coefficient		0.1409	
Contingency Coefficient		0.1395	
Cramer's V		0.1409	

Table of TREATMENT by Flowers

Treatment	Flowers					
Frequency	FBAp	FBAs	FBFAp	FBFApFBAs	NFBF	Total
MTMN	0	0	0	0	28	28
MTWN	0	1	0	0	27	28
WTMN	8	0	17	0	3	28
WTWN	10	0	17	1	0	28
Total	18	1	34	1	58	112

Statistic	DF	Value	Prob
Chi-Square	12	105.4100	<.0001 **
Likelihood Ratio Chi-Square	12	138.8242	<.0001
Mantel-Haenszel Chi-Square	1	66.0648	<.0001
Phi Coefficient		0.9701	
Contingency Coefficient		0.6963	
Cramer's V		0.5601	

Week 6

Table of TRIAL by Flowers

Trial	Flowers						
Frequency	FBAp	FBAs	FBFAp	FBFApFBAs	FBFApNN	NFBF	Total
1	1	9	17	1	9	19	56
2	0	8	16	0	9	23	56
Total	1	17	33	1	18	42	112

Statistic	DF	Value	Prob
Chi-Square	5	2.4701	0.7810 N.S.
Likelihood Ratio Chi-Square	5	3.2433	0.6625
Mantel-Haenszel Chi-Square	1	0.6772	0.4106
Phi Coefficient		0.1485	
Contingency Coefficient		0.1469	
Cramer's V		0.1485	

Table of LOCATION by Flowers

Location	Flowers						
Frequency	FBAp	FBAs	FBFAp	FBFApFBAs	FBFApNN	NFBF	Total
Glasshouse	0	11	20	0	7	18	56
Growth	1	6	13	1	11	24	56
Total	1	17	33	1	18	42	112

Statistic	DF	Value	Prob
Chi-Square	5	6.7015	0.2438 N.S.
Likelihood Ratio Chi-Square	5	7.5178	0.1849
Mantel-Haenszel Chi-Square	1	3.1094	0.0778
Phi Coefficient		0.2446	
Contingency Coefficient		0.2376	
Cramer's V		0.2446	

Table of TREATMENT by Flowers

Treatment	Flowers						
Frequency	FBAp	FBAs	FBFAp	FBFApFBAs	FBFApNN	NFBF	Total
MTMN	0	0	0	0	0	28	28
MTWN	0	17	0	0	0	11	28
WTMN	0	0	7	0	18	3	28
WTWN	1	0	26	1	0	0	28
Total	1	17	33	1	18	42	112

Statistic	DF	Value	Prob
Chi-Square	15	210.9264	<.0001 **
Likelihood Ratio Chi-Square	15	208.4088	<.0001
Mantel-Haenszel Chi-Square	1	35.2928	<.0001
Phi Coefficient		1.3723	
Contingency Coefficient		0.8082	
Cramer's V		0.7923	

Week 8

Table of TRIAL by Flowers

Trial	Flowers						
Frequency	FBAs	FBFAp	FBFApFBAs	FBFApNN	FBFAs	NFBF	Total
1	6	12	5	11	8	14	56
2	6	10	4	11	8	17	56
Total	12	22	9	22	16	31	112

Statistic	DF	Value	Prob
Chi-Square	5	0.5833	0.9888 N.S.
Likelihood Ratio Chi-Square	5	0.5842	0.9887
Mantel-Haenszel Chi-Square	1	0.3487	0.5549
Phi Coefficient		0.0722	
Contingency Coefficient		0.0720	
Cramer's V		0.0722	

Table of LOCATION by Flowers

Location	Flowers						
Frequency	FBAs	FBFAp	FBFApFBAs	FBFApNN	FBFAs	NFBF	Total
Glasshouse	4	16	0	11	10	15	56
Growth	8	6	9	11	6	16	56
Total	12	22	9	22	16	31	112

Statistic	DF	Value	Prob
Chi-Square	5	15.9110	0.0071 *
Likelihood Ratio Chi-Square	5	19.5954	0.0015
Mantel-Haenszel Chi-Square	1	0.0259	0.8721
Phi Coefficient		0.3769	
Contingency Coefficient		0.3527	
Cramer's V		0.3769	

Table of TREATMENT by Flowers

Treatment	Flowers						
Frequency	FBAs	FBFAp	FBFApFBAs	FBFApNN	FBFAs	NFBF	Total
MTMN	0	0	0	0	0	28	28
MTWN	12	0	0	0	16	0	28
WTMN	0	3	0	22	0	3	28
WTWN	0	19	9	0	0	0	28
Total	12	22	9	22	16	31	112

Statistic	DF	Value	Prob
Chi-Square	15	293.5953	<.0001 **
Likelihood Ratio Chi-Square	15	273.2923	<.0001
Mantel-Haenszel Chi-Square	1	48.1364	<.0001
Phi Coefficient		1.6191	
Contingency Coefficient		0.8508	
Cramer's V		0.9348	

Appendix C

APPENDIX C: Data and statistical analyses for Chapter 5

Table 1. The effect of factors (cutting type, IBA treatment, rooting medium) on various rooting and cutting parameters after four weeks of culture. (SAS Version 9.3 statistical software).

Analysis of variance (ANOVA)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
1.1 Survival of cuttings					
Media	4	2411.11111	602.77778	3.49	0.01288 *
Cutting	1	40.00000	40.00000	0.23	0.6322 N.S.
Media*Cutting	4	571.11111	142.77778	0.83	0.5137 N.S.
IBA	2	15686.66667	7843.33333	45.40	<.0001 **
Media*IBA	8	5902.22222	737.77778	4.27	0.0004 *
Cut*IBA	2	20.00000	10.00000	0.06	0.9438 N.S.
Media*Cut*IBA	8	1168.88889	146.11111	0.85	0.5668 N.S.
1.2 Rooting percentage of cuttings					
Media	4	3434.11111	858.52778	2.09	0.0933 N.S.
Cutting	1	25.60000	25.60000	0.06	0.8036 N.S.
Media*Cutting	4	1780.51111	445.12778	1.09	0.3724 N.S.
IBA	2	25980.00000	12990.00000	31.67	<.0001 **
Media*IBA	8	7449.55556	931.19444	2.27	0.0347 *
Cut*IBA	2	133.06667	66.53333	0.16	0.8507 N.S.
Media*Cut*IBA	8	2246.48889	280.81111	0.68	0.7033 N.S.
1.3 Root Score					
Root Score 1					
Media	4	3355.28889	838.82222	1.98	0.1100 N.S.
Cutting	1	17.77778	17.77778	0.04	0.8385 N.S.
Media*Cutting	4	1936.44444	484.11111	1.14	0.3464 N.S.
IBA	2	25597.42222	12798.71111	30.16	<.0001 **
Media*IBA	8	6977.91111	872.23889	2.06	0.0554 N.S.
Cut*IBA	2	141.95556	70.97778	0.17	0.8464 N.S.
Media*Cut*IBA	8	2415.15556	301.89444	0.71	0.6803 N.S.
Root Score 2					
Media	4	117.5111111	29.3777778	1.30	0.2817 N.S.
Cutting	1	5.8777778	5.8777778	0.26	0.6123 N.S.
Media*Cutting	4	60.1777778	15.0444444	0.66	0.6192 N.S.
IBA	2	52.8666667	26.4333333	1.17	0.3183 N.S.
Media*IBA	8	270.3555556	33.7944444	1.49	0.1799 N.S.
Cut*IBA	2	33.7555556	16.8777778	0.75	0.4790 N.S.
Media*Cut*IBA	8	98.3555556	12.2944444	0.54	0.8192 N.S.
Root Score 3					
Media	4	342.7333333	85.6833333	3.21	0.0188 *
Cutting	1	1.1111111	1.1111111	0.04	0.8390 N.S.
Media*Cutting	4	171.4444444	42.8611111	1.61	0.1846 N.S.
IBA	2	18.7555556	9.3777778	0.35	0.7049 N.S.
Media*IBA	8	64.1333333	8.0166667	0.30	0.9628 N.S.
Cut*IBA	2	95.5555556	47.7777778	1.79	0.1757 N.S.
Media*Cut*IBA	8	82.8888889	10.3611111	0.39	0.9224 N.S.
Root Score 4					
Media	4	4006.844444	1001.711111	1.94	0.1160 N.S.
Cutting	1	4536.900000	4536.900000	8.78	0.0044 *
Media*Cutting	4	846.488889	211.622222	0.41	0.8009 N.S.
IBA	2	5451.288889	2725.644444	5.28	0.0078 *
Media*IBA	8	5043.822222	630.477778	1.22	0.3034 N.S.
Cut*IBA	2	40.266667	20.133333	0.04	0.9618 N.S.
Media*Cut*IBA	8	4880.844444	610.105556	1.18	0.3260 N.S.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Root Score 5					
Media	4	16899.11111	4224.77778	9.80	<.0001 **
Cutting	1	3815.51111	3815.51111	8.85	0.0043 *
MediaxCutting	4	1162.04444	290.51111	0.67	0.6126 N.S.
IBA	2	53419.48889	26709.74444	61.98	<.0001 **
MediaxIBA	8	7311.28889	913.91111	2.12	0.0481 *
CutxIBA	2	744.42222	372.21111	0.86	0.4269 N.S
MediaxCutxIBA	8	3529.02222	441.12778	1.02	0.4289 N.S.
2.4 <u>Root Origin</u>					
Position 1					
Media	4	3616.5366	904.1341	21.96	<.0001 **
Cutting	1	8.3912	8.3912	0.20	0.6535 N.S.
MediaxCutting	4	245.7158	61.4289	1.49	0.2178 N.S.
IBA	2	158455.6084	79227.8042	1924.24	<.0001 **
MediaxIBA	8	775.1569	96.8946	2.35	0.0303 *
CutxIBA	2	364.5641	182.2821	4.43	0.0167 *
MediaxCutxIBA	8	212.2489	26.5311	0.64	0.7369 N.S.
Position 2					
Media	4	15024.39918	3756.09980	13.09	<.0001 **
Cutting	1	5095.62590	5095.62590	17.76	<.0001 **
MediaxCutting	4	3215.53543	803.88386	2.80	0.0349 *
IBA	2	19038.22519	9519.11260	33.17	<.0001 **
MediaxIBA	8	9849.27990	1231.15999	4.29	0.0005 *
CutxIBA	2	1994.01598	997.00799	3.47	0.0382 *
MediaxCutxIBA	8	2182.70728	272.83841	0.95	0.4839 N.S.
Position 3					
Media	4	23152.31506	5788.07876	17.50	<.0001 **
Cutting	1	5502.10310	5502.10310	16.63	0.0002 *
MediaxCutting	4	2217.15895	554.28974	1.68	0.1694 N.S.
IBA	2	84961.33507	42480.66753	128.42	<.0001 **
MediaxIBA	8	13253.98662	1656.74833	5.01	0.0001 *
CutxIBA	2	2846.91160	1423.45580	4.30	0.0185 *
MediaxCutxIBA	8	2375.57070	296.94634	0.90	0.5252 N.S.
2.5 <u>Fresh root mass</u>					
Media	4	8.16258854	2.04064714	15.83	<.0001 **
Cutting	1	0.93649878	0.93649878	7.26	0.0092 *
MediaxCutting	4	0.59381824	0.14845456	1.15	0.3416 N.S.
IBA	2	7.68977635	3.84488818	29.82	<.0001 **
MediaxIBA	8	2.69664169	0.33708021	2.61	0.0163 *
CuttingxIBA	2	0.04594022	0.02297011	0.18	0.8373 N.S.
MediaxCuttingxIBA	8	0.91593573	0.11449197	0.89	0.5323 N.S.
2.6 <u>Dry root mass</u>					
Media	4	0.07044547	0.01761137	14.19	<.0001 **
Cutting	1	0.00741228	0.00741228	5.97	0.0176 *
MediaxCutting	4	0.00318092	0.00079523	0.64	0.6355 N.S.
IBA	2	0.06919270	0.03459635	27.88	<.0001 **
MediaxIBA	8	0.02273252	0.00284157	2.29	0.0332 *
CuttingxIBA	2	0.00016798	0.00008399	0.07	0.9346 N.S.
MediaxCuttingxIBA	8	0.00849587	0.00106198	0.86	0.5585 N.S.
2.7 <u>Increase in cutting length</u>					
Media	4	16993.69659	4248.42415	17.85	<.0001 **
Cutting	1	934.66069	934.66069	3.93	0.0523 *
MediaxCutting	4	3543.20400	885.80100	3.72	0.0092 *
IBA	2	12647.77602	6323.88801	26.57	<.0001 **
MediaxIBA	8	5814.43939	726.80492	3.05	0.0062 *
CuttingxIBA	2	60.49486	30.24743	0.13	0.8809 N.S.
MediaxCuttingxIBA	8	1420.50378	177.56297	0.75	0.6508 N.S.

Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
2.8 Increase in number of nodes					
Media	4	136.7646073	34.1911518	16.58	<.0001 **
Cutting	1	35.4232306	35.4232306	17.18	0.0001 *
MediaxCutting	4	15.7312826	3.9328206	1.91	0.1213 N.S.
IBA	2	81.6099557	40.8049779	19.79	<.0001 **
MediaxIBA	8	39.3741785	4.9217723	2.39	0.0268 *
CuttingxIBA	2	5.4565971	2.7282985	1.32	0.2742 N.s.
MediaxCuttingxIBA	8	7.7197021	0.9649628	0.47	0.8736 N.S.
2.9 Increase in basal circumference					
Media	4	9.71076964	2.42769241	16.91	<.0001 **
Cutting	1	0.23503571	0.23503571	1.64	0.2058 N.S.
MediaxCutting	4	0.24169469	0.06042367	0.42	0.7929 N.S.
IBA	2	6.44269002	3.22134501	22.44	<.0001 **
MediaxIBA	8	2.63671366	0.32958921	2.30	0.0328 *
CuttingxIBA	2	0.09650309	0.04825155	0.34	0.7159 N.S.
MediaxCuttingxIBA	8	0.22163097	0.02770387	0.19	0.9909 N.S.
2.10 Secondary axillary bud sprouts on cuttings					
Media	4	9405.93333	2351.48333	4.63	0.0026 *
Cutting	1	2744.54444	2744.54444	5.40	0.0237 *
MediaxCutting	4	1461.40000	365.35000	0.72	0.5827 N.S.
IBA	2	5169.62222	2584.81111	5.08	0.0092 *
MediaxIBA	8	9064.60000	1133.07500	2.23	0.0380 *
CutxIBA	2	652.02222	326.01111	0.64	0.5303 N.S.
MediaxCutxIBA	8	1580.20000	197.52500	0.39	0.9225 N.S.

N.s.: Not significant; *Significant (p=0.0001–0.05); **Highly significant (p<.0001)

Correlation between different variables

The correlations between variables were determined with the XLSTAT (version 2015.04.36025) software.

Table 2. Correlation matrix (Pearson)

Variables	InitLength	InitDiam	InitNodes	HarvLength	HarvDia	HarvNodes	FRootMass	DRootMass
InitLength	**1**	**0,079**	**0,539**	**0,726**	0,046	**0,455**	0,063	**0,076**
InitDiam	**0,079**	**1**	0,064	**0,150**	**0,649**	**0,135**	**0,096**	**0,078**
InitNodes	**0,539**	0,064	**1**	**0,551**	**0,161**	**0,681**	**0,247**	**0,248**
HarvLength	**0,726**	**0,150**	**0,551**	**1**	**0,346**	**0,766**	**0,530**	**0,535**
HarvDiam	0,046	**0,649**	**0,161**	**0,346**	**1**	**0,354**	**0,481**	**0,480**
HarvNodes	**0,455**	**0,135**	**0,681**	**0,766**	**0,354**	**1**	**0,575**	**0,569**
FRootMass	0,063	**0,096**	**0,247**	**0,530**	**0,481**	**0,575**	**1**	**0,958**
DRootMass	**0,076**	**0,078**	**0,248**	**0,535**	**0,480**	**0,569**	**0,958**	**1**

Table 3. p-values for Pearson's correlation matrix.

Variables	InitLength	InitDiam	InitNodes	HarvLength	HarvDiam	HarvNodes	FRootMass	DRootMass
InitLength	**0**	**0,017**	**0,000**	**0,000**	0,187	**0,000**	0,073	**0,030**
InitDiam	**0,017**	**0**	0,056	**< 0,0001**	**< 0,0001**	**0,000**	**0,006**	**0,027**
InitNodes	**< 0,0001**	0,056	**0**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**< 0,0001**
HarvLength	**< 0,0001**	**<**	**< 0,0001**	**0**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**< 0,0001**
HarvDiam	0,187	**<**	**< 0,0001**	**< 0,0001**	**0**	**< 0,0001**	**< 0,0001**	**< 0,0001**
HarvNodes	**< 0,0001**	**0,000**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**0**	**< 0,0001**	**< 0,0001**
FRootMass	0,073	**0,006**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**0**	**< 0,0001**
DRootMass	**0,030**	**0,027**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**< 0,0001**	**0**

For both tables: Values in bold are different from 0 with a significance level alpha=0.05

Table 4. Correlation matrix (Spearman)

Variables	InitFlBuds	Survival%	Rooting%	RootScore	RootOrigin	AxBudSprouts
InitFlBuds	**1**	0,038	0,066	0,053	-0,053	**0,225**
Survival%	0,038	**1**				
Rooting%	0,066		**1**	**0,725**		**0,437**
RootScore	0,053		**0,725**	**1**	**0,599**	**0,426**
RootOrigin	-0,053			**0,599**	**1**	0,000
AxBudSprouts	**0,225**		**0,437**	**0,426**	0,000	**1**

Table 5. *p*-values for Spearman's correlation matrix.

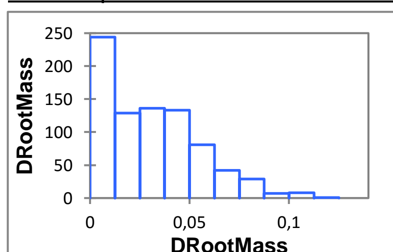
Variables	InitFlBuds	Survival%	Rooting%	RootScore	RootOrigin	AxBudSprouts
InitFlBuds	**0**	0,251	0,059	0,134	0,170	**0,000**
Survival%	0,251	**0**				
Rooting%	0,059		**0**	**< 0,0001**		**< 0,0001**
RootScore	0,134		**< 0,0001**	**0**	**< 0,0001**	**< 0,0001**
RootOrigin	0,170			**< 0,0001**	**0**	0,999
AxBudSprouts	**< 0,0001**		**< 0,0001**	**< 0,0001**	0,999	**0**

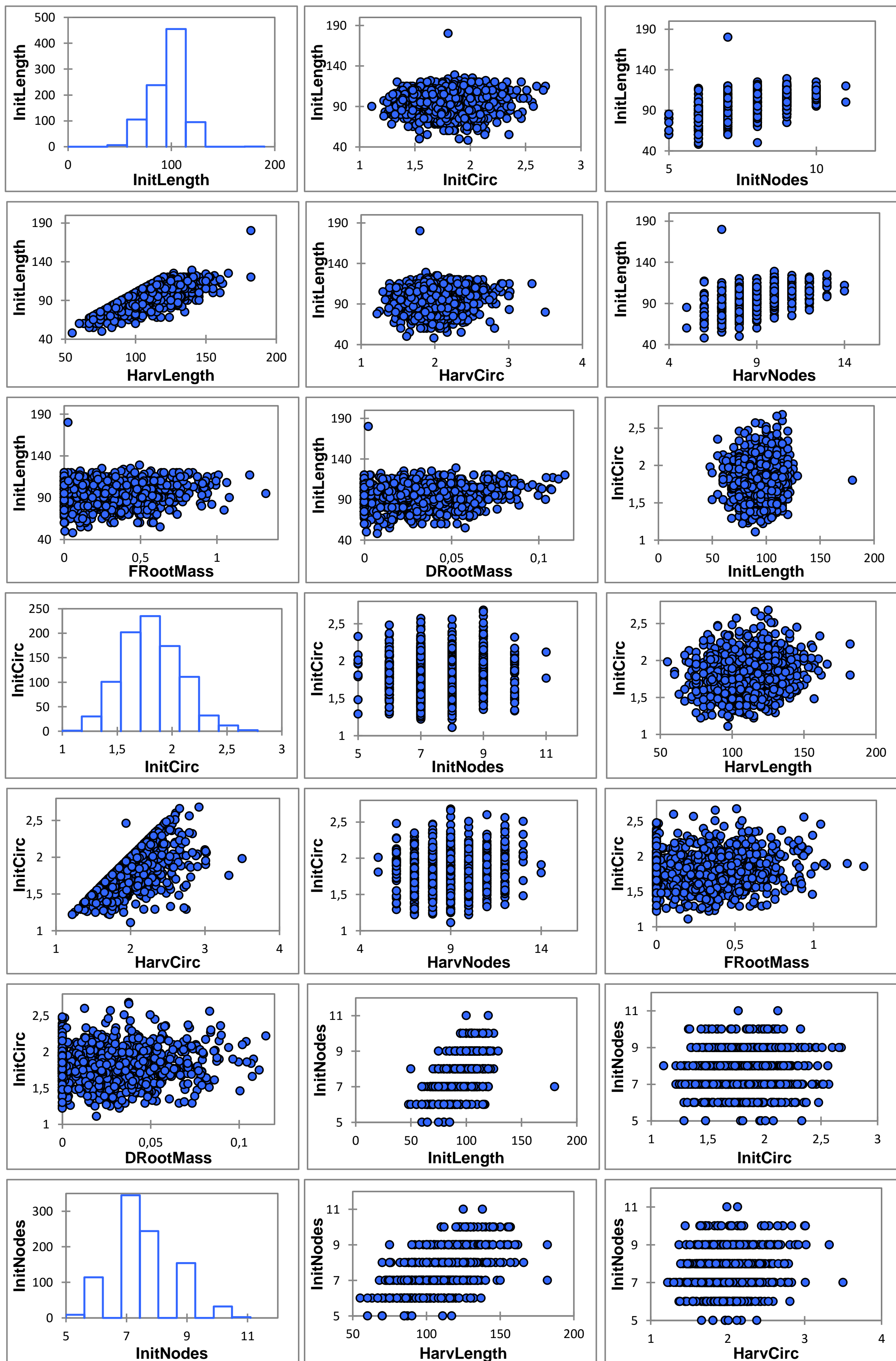
For both tables: Values in bold are different from 0 with a significance level alpha=0,05

Table 6. Rule of thumb for interpreting the size of a correlation coefficient according to Manaka (2012).

Size of the correlation	Interpretation
0.90 to 1.00 (-0.90 to -1.00)	Very high/strong positive (negative)
0.70 to 0.90 (-0.70 to -0.90)	High/Strong positive (negative) correlation
0.50 to 0.70 (-0.50 to -0.70)	Moderate positive (negative) correlation
0.30 to 0.50 (-0.30 to -0.50)	Low/Weak positive (negative) correlation
0.00 to 0.30 (-0.00 to -0.30)	Negligible correlation

Scatter plots for Pearson's correlation

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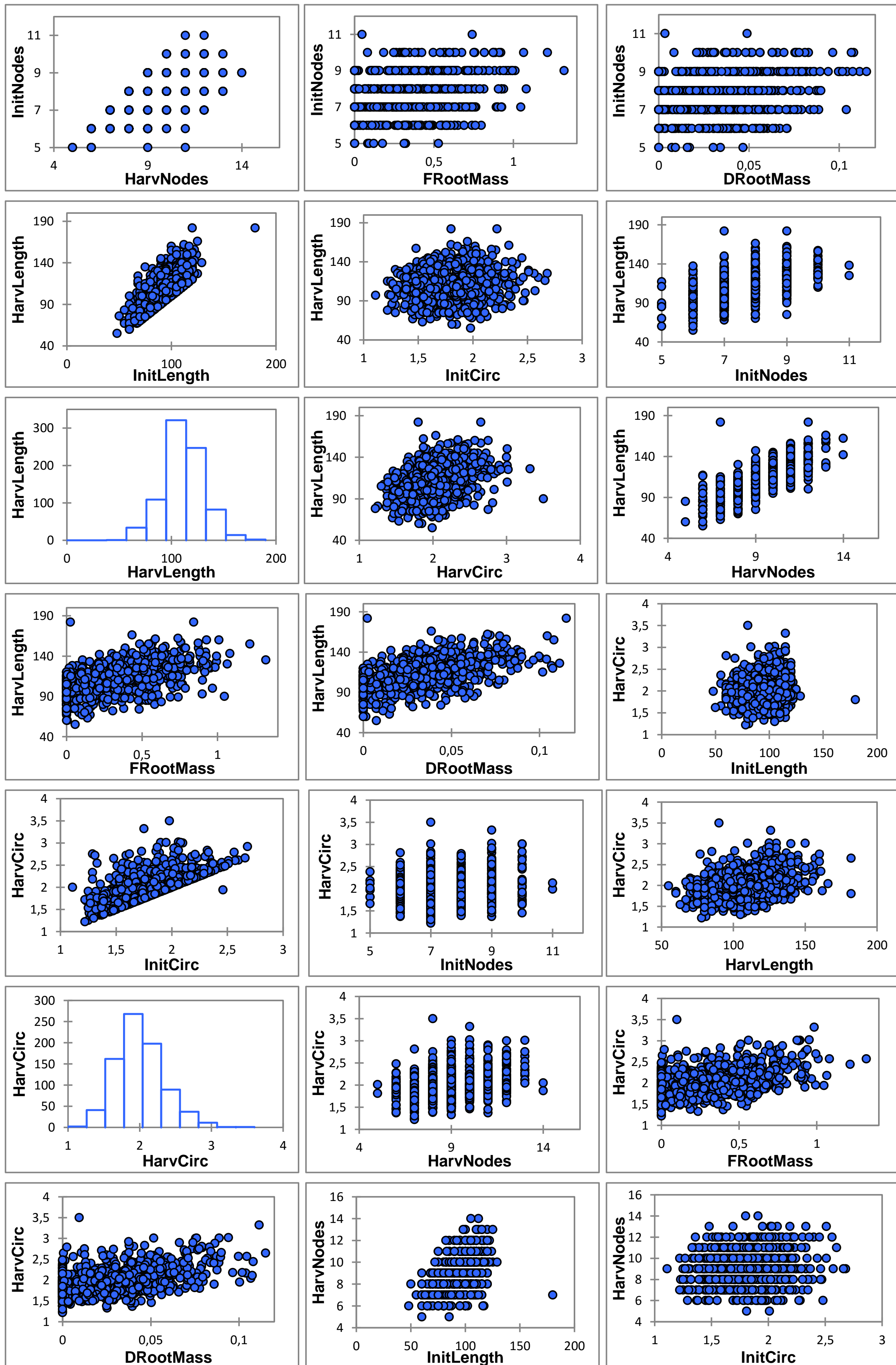

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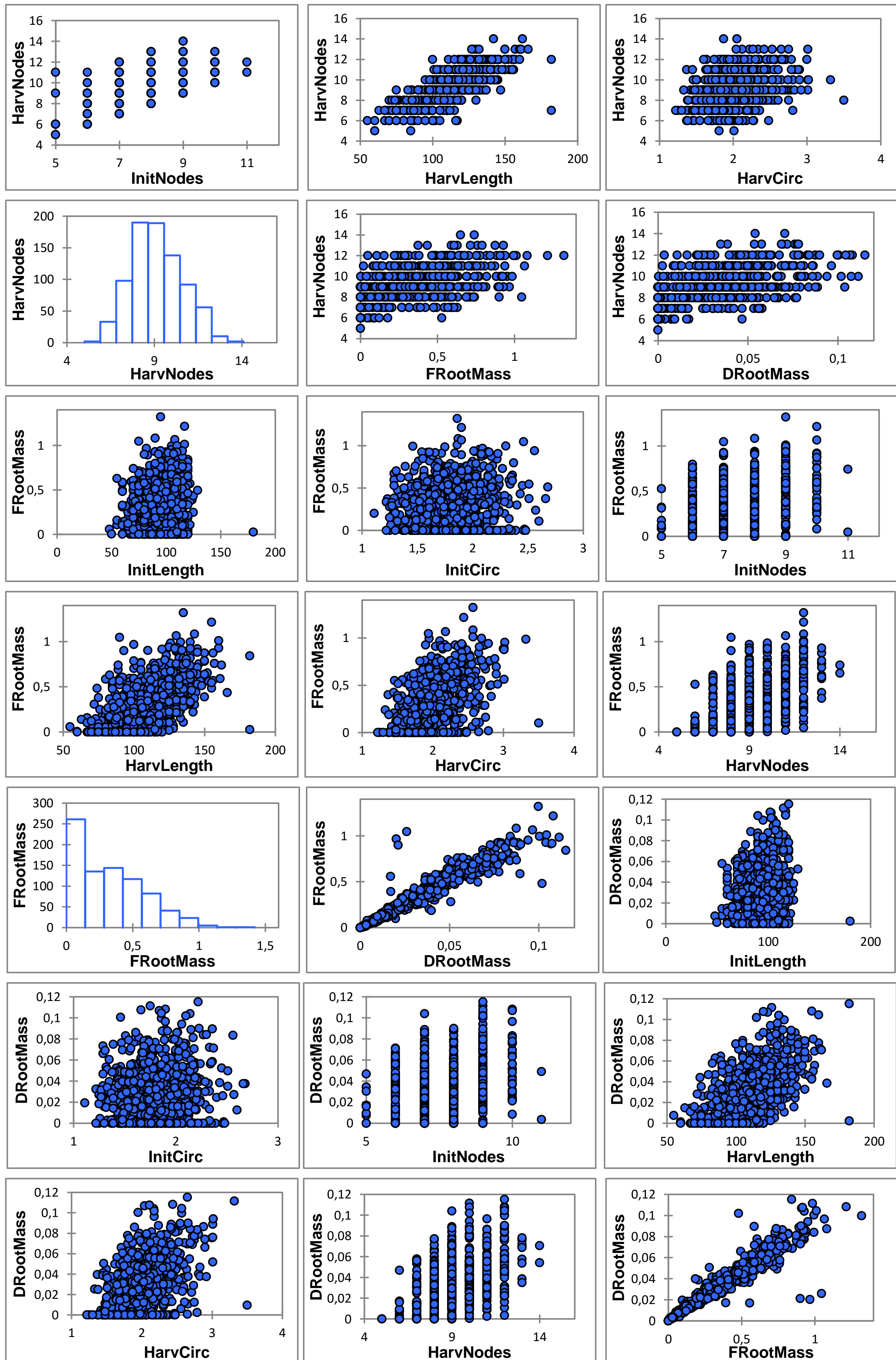
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Appendix D

APPENDIX D: Data and statistical analyses for Chapter 6

Table 1. Seed germination on filter paper bridges, different strengths of MS medium and on Gelrite®. (SAS Version 9.3 statistical software).

Analysis of variance (ANOVA)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Final germination percentage (FGP)					
Treatment	6	479.6340741	79.9390123	1.42	0.2569 N.S.
Mean germination time (MGT)					
Treatment	6	21.25066019	3.54177670	2.12	0.0967 N.S.
Germination rate index (GRI)					
Treatment	6	46.60482500	7.76747083	2.31	0.0740 N.S.

N.S.: Not significant at the 5% level of significance.

Table 2. Seed germination on different strengths of MS medium and on Gelrite®.

Analysis of variance (ANOVA)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Final germination percentage (FGP)					
Treatment	5	470.8333333	94.1666667	1.40	0.2815 N.S.
Mean germination time (MGT)					
Treatment	5	19.34847083	3.86969417	4.86	0.0077 *
Germination rate index (GRI)					
Treatment	5	43.67182083	8.73436417	10.67	0.0002 *

N.S.: Not significant; *Significant (p=0.0001–0.05) at the 5 % level of significance.

Table 3. Seedling development of seeds germinated on filter paper bridges and transferred to half and full MS medium.

Analysis of variance (ANOVA)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Number of nodes per week					
Week 2					
Model	6	1.33500000	0.22250000	4.38	0.0633 N.S.
Error	5	0.25416667	0.05083333		
Corrected Total	11	1.58916667			
Week 3					
Model	6	0.81166667	0.13527778	2.85	0.1354 N.S.
Error	5	0.23750000	0.04750000		
Corrected Total	11	1.04916667			
Week 4					
Model	6	1.34000000	0.22333333	2.29	0.1901 N.S.
Error	5	0.48666667	0.09733333		
Corrected Total	11	1.82666667			
Week 5					
Model	6	0.98666667	0.16444444	1.16	0.4456 N.S.
Error	5	0.71000000	0.14200000		
Corrected Total	11	1.69666667			

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Survival percentage at Week 5					
Model	6	66.6666667	11.111111	0.56	0.7529 N.S.
Error	5	100.0000000	20.000000		
Corrected Total	11	166.6666667			

N.S.: Not significant at the 5 % level of significance.

Table 4. Seedling development on different strength of MS medium and on Gelrite®.

Analysis of variance (ANOVA)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Number of nodes per week					
Week 2					
Model	7	8.26087222	1.18012460	39.09	<.0001**
Error	10	0.30188889	0.03018889		
Corrected Total	17	8.56276111			
Week 3					
Model	7	24.27716667	3.46816667	32.54	<.0001**
Error	10	1.06583333	0.10658333		
Corrected Total	17	25.34300000			
Week 4					
Model	7	48.97597222	6.99656746	49.65	<.0001**
Error	10	1.40925556	0.14092556		
Corrected Total	17	50.38522778			
Week 5					
Model	7	86.92812222	12.41830317	75.32	<.0001**
Error	10	1.64878889	0.16487889		
Corrected Total	17	88.57691111			
Survival percentage at Week 5					
Model	7	23828.16667	3404.02381	75.93	<.0001**
Error	10	448.33333	44.83333		
Corrected Total	17	24276.50000			

**Highly significant (*p*<.0001) at the 5 % level of significance

Table 5. The association between treatment and different shoot induction parameters per week for seedling explants. The tables were created with the frequency procedure of SAS Version 9.3 statistical software.

Week 1

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	7 11.67	22 36.67	28 46.67	3 5.00	0 0.00	60
0.5 BAP	38 63.33	21 35.00	1 1.67	0 0.00	0 0.00	60
1.00 BAP	47 78.33	12 20.00	1 1.67	0 0.00	0 0.00	60
0.5 BAP & 0.5 NAA	33 55.00	27 45.00	0 0.00	0 0.00	0 0.00	60
1.00 BAP & 0.5 NAA	38 63.33	19 31.67	3 5.00	0 0.00	0 0.00	60
0.5 BAP & 0.5 IBAP	29 48.33	24 40.00	7 11.67	0 0.00	0 0.00	60
1.00 BAP & 0.5 IBAP	46 76.67	14 23.33	0 0.00	0 0.00	0 0.00	60

Total	238	139	40	3	0	420

Statistic	DF	Value	Prob
Chi-Square	18	166.6573	<.0001**
Likelihood Ratio Chi-Square	18	143.5025	<.0001
Mantel-Haenszel Chi-Square	1	28.8699	<.0001
Phi Coefficient		0.6299	
Contingency Coefficient		0.5330	
Cramer's V		0.3637	

Table of Treatment by Callus

Treatment	Callus		
Frequency Row Pct	0	1	Total
Control	20 100.00	0 0.00	20
0.5 BAP	19 95.00	1 5.00	20
1.00 BAP	20 100.00	0 0.00	20
0.5 BAP & 0.5 NAA	20 100.00	0 0.00	20
1.00 BAP & 0.5 NAA	20 100.00	0 0.00	20
0.5 BAP & 0.5 IBAP	20 100.00	0 0.00	20
1.00 BAP & 0.5 IBAP	20 100.00	0 0.00	20
Total	139	1	140

Statistic	DF	Value	Prob
Chi-Square	6	6.0432	0.4184 NS
Likelihood Ratio Chi-Square	6	3.9355	0.6854
Mantel-Haenszel Chi-Square	1	2.2500	0.1336
Phi Coefficient		0.2078	
Contingency Coefficient		0.2034	
Cramer's V		0.2078	

Week 2

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	1 1.67	10 16.67	28 46.67	21 35.00	0 0.00	60
0.5 BAP	15 25.00	25 41.67	18 30.00	2 3.33	0 0.00	60
1.00 BAP	29 48.33	24 40.00	6 10.00	1 1.67	0 0.00	60
0.5 BAP & 0.5 NAA	19 31.67	17 28.33	22 36.67	2 3.33	0 0.00	60
1.00 BAP & 0.5 NAA	33 55.00	17 28.33	10 16.67	0 0.00	0 0.00	60
0.5 BAP & 0.5 IBAP	16 26.67	24 40.00	16 26.67	4 6.67	0 0.00	60
1.00 BAP & 0.5 IBAP	34 56.67	22 36.67	4 6.67	0 0.00	0 0.00	60
Total	147	139	104	30	0	420

Statistic	DF	Value	Prob
Chi-Square	18	158.4512	<.0001**
Likelihood Ratio Chi-Square	18	152.4939	<.0001
Mantel-Haenszel Chi-Square	1	5.0977	0.0240
Phi Coefficient		0.6142	
Contingency Coefficient		0.5234	
Cramer's V		0.3546	

Table of Treatment by Callus

Treatment	Callus		
Frequency Row Pct	0	1	Total
Control	18 90.00	2 10.00	20
0.5 BAP	12 60.00	8 40.00	20
1.00 BAP	9 45.00	11 55.00	20
0.5 BAP & 0.5 NAA	14 70.00	6 30.00	20
1.00 BAP & 0.5 NAA	15 75.00	5 25.00	20
0.5 BAP & 0.5 IBAP	8 40.00	12 60.00	20
1.00 BAP & 0.5 IBAP	5 25.00	15 75.00	20
Total	81	59	140

Statistic	DF	Value	Prob
Chi-Square	6	24.9592	0.0003
Likelihood Ratio Chi-Square	6	26.8185	0.0002
Mantel-Haenszel Chi-Square	1	3.8466	0.0498
Phi Coefficient		0.4222	
Contingency Coefficient		0.3890	
Cramer's V		0.4222	

Table of Treatment by Callus Class

Treatment	Callus class		
Frequency Row Pct	1	2	Total
Control	2 100.00	0 0.00	2
0.5 BAP	7 87.50	1 12.50	8
1.00 BAP	11 100.00	0 0.00	11
0.5 BAP & 0.5 NAA	6 100.00	0 0.00	6
1.00 BAP & 0.5 NAA	5 100.00	0 0.00	5
0.5 BAP & 0.5 IBAP	12 100.00	0 0.00	12
1.00 BAP & 0.5 IBAP	15 100.00	0 0.00	15
Total			

Statistic	DF	Value	Prob
Chi-Square	6	6.4849	0.3711 NS
Likelihood Ratio Chi-Square	6	4.1097	0.6618
Mantel-Haenszel Chi-Square	1	2.3640	0.1242

Phi Coefficient	0.3315
Contingency Coefficient	0.3147
Cramer's V	0.3315

Week 3

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	1 1.67	8 13.33	26 43.33	22 36.67	3 5.00	60
0.5 BAP	9 15.00	25 41.67	24 40.00	2 3.33	0 0.00	60
1.00 BAP	23 38.33	24 40.00	12 20.00	1 1.67	0 0.00	60
0.5 BAP & 0.5 NAA	16 26.67	19 31.67	20 33.33	5 8.33	0 0.00	60
1.00 BAP & 0.5 NAA	32 53.33	17 28.33	9 15.00	2 3.33	0 0.00	60
0.5 BAP & 0.5 IBAP	15 25.00	21 35.00	18 30.00	6 10.00	0 0.00	60
1.00 BAP & 0.5 IBAP	30 50.00	22 36.67	7 11.67	1 1.67	0 0.00	60
Total	126	136	116	39	3	420

Statistic	DF	Value	Prob
Chi-Square	24	150.1233	<.0001**
Likelihood Ratio Chi-Square	24	140.8972	<.0001
Mantel-Haenszel Chi-Square	1	3.3239	0.0683
Phi Coefficient		0.5979	
Contingency Coefficient		0.5131	
Cramer's V		0.2989	

Table of Treatment by Callus

Treatment	Callus		
Frequency Row Pct	0	1	Total
Control	4 20.00	16 80.00	20
0.5 BAP	4 20.00	16 80.00	20
1.00 BAP	6 30.00	14 70.00	20
0.5 BAP & 0.5 NAA	0 0.00	20 100.00	20
1.00 BAP & 0.5 NAA	1 5.00	19 95.00	20
0.5 BAP & 0.5 IBAP	0 0.00	20 100.00	20
1.00 BAP & 0.5 IBAP	0 0.00	20 100.00	20
Total	15	125	140

Statistic	DF	Value	Prob
Chi-Square	6	19.2640	0.0037*
Likelihood Ratio Chi-Square	6	22.9326	0.0008
Mantel-Haenszel Chi-Square	1	0.0741	0.7854
Phi Coefficient		0.3709	
Contingency Coefficient		0.3478	
Cramer's V		0.3709	

Table of Treatment by Callus Class

Treatment	Callus class			
Frequency Row Pct	1	2	3	Total
Control	15 93.75	1 6.25	0 0.00	16
0.5 BAP	11 68.75	4 25.00	1 6.25	16
1.00 BAP	11 78.57	3 21.43	0 0.00	14
0.5 BAP & 0.5 NAA	11 55.00	9 45.00	0 0.00	20
1.00 BAP & 0.5 NAA	13 68.42	6 31.58	0 0.00	19
0.5 BAP & 0.5 IBAP	10 50.00	10 50.00	0 0.00	20
1.00 BAP & 0.5 IBAP	9 45.00	11 55.00	0 0.00	20
Total	80	44	1	125

Statistic	DF	Value	Prob
Chi-Square	12	20.7231	0.0546 NS
Likelihood Ratio Chi-Square	12	19.5613	0.0759
Mantel-Haenszel Chi-Square	1	3.3860	0.0658
Phi Coefficient		0.4072	
Contingency Coefficient		0.3771	
Cramer's V		0.2879	

Week 4

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	1 1.67	7 11.67	25 41.67	23 38.33	4 6.67	60
0.5 BAP	8 13.33	22 36.67	27 45.00	3 5.00	0 0.00	60
1.00 BAP	23 38.33	23 38.33	13 21.67	1 1.67	0 0.00	60
0.5 BAP & 0.5 NAA	16 26.67	14 23.33	24 40.00	6 10.00	0 0.00	60
1.00 BAP & 0.5 NAA	19 31.67	26 43.33	11 18.33	4 6.67	0 0.00	60
0.5 BAP & 0.5 IBAP	8 13.33	19 31.67	26 43.33	6 10.00	1 1.67	60
1.00 BAP & 0.5 IBAP	23 38.33	20 33.33	15 25.00	2 3.33	0 0.00	60
Total	98	131	141	45	5	420

Statistic	DF	Value	Prob
Chi-Square	24	129.7636	<.0001**
Likelihood Ratio Chi-Square	24	122.8484	<.0001
Mantel-Haenszel Chi-Square	1	3.9732	0.0462
Phi Coefficient		0.5558	
Contingency Coefficient		0.4858	
Cramer's V		0.2779	

Table of Treatment by Callus

Treatment Frequency Row Pct	Callus 0	Callus 1	Total
Control	3 15.00	17 85.00	20
0.5 BAP	2 10.00	18 90.00	20
1.00 BAP	3 15.00	17 85.00	20
0.5 BAP & 0.5 NAA	0 0.00	20 100.00	20
1.00 BAP & 0.5 NAA	0 0.00	20 100.00	20
0.5 BAP & 0.5 IBAP	0 0.00	20 100.00	20
1.00 BAP & 0.5 IBAP	0 0.00	20 100.00	20
Total	8	132	140

Statistic	DF	Value	Prob
Chi-Square	6	11.9318	0.0635 NS
Likelihood Ratio Chi-Square	6	14.5091	0.0244
Mantel-Haenszel Chi-Square	1	0.2962	0.5863
Phi Coefficient		0.2919	
Contingency Coefficient		0.2802	
Cramer's V		0.2919	

Table of Treatment by Callus Class

Treatment Frequency Row Pct	Callus class 1	Callus class 2	Callus class 3	Total
Control	11 64.71	6 35.29	0 0.00	17
0.5 BAP	8 44.44	8 44.44	2 11.11	18
1.00 BAP	9 52.94	7 41.18	1 5.88	17
0.5 BAP & 0.5 NAA	2 10.00	5 25.00	13 65.00	20
1.00 BAP & 0.5 NAA	3 15.00	10 50.00	7 35.00	20
0.5 BAP & 0.5 IBAP	1 5.00	10 50.00	9 45.00	20
1.00 BAP & 0.5 IBAP	0 0.00	8 40.00	12 60.00	20
Total	34	54	44	133

Statistic	DF	Value	Prob
Chi-Square	18	60.1448	<.0001**
Likelihood Ratio Chi-Square	18	67.7829	<.0001
Mantel-Haenszel Chi-Square	1	2.4227	0.1196
Phi Coefficient		0.6725	
Contingency Coefficient		0.5580	
Cramer's V		0.3883	

Table 6. The association between treatment and different shoot induction parameters per week for axillary shoot explants. The tables were created with the frequency procedure of SAS Version 9.3 statistical software.

Week 1

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	21 35.00	35 58.33	4 6.67	0 0.00	0 0.00	60
0.5 BAP	47 78.33	13 21.67	0 0.00	0 0.00	0 0.00	60
1.00 BAP	43 71.67	16 26.67	1 1.67	0 0.00	0 0.00	60
0.5 BAP & 0.5 NAA	25 41.67	19 31.67	10 16.67	4 6.67	2 3.33	60
1.00 BAP & 0.5 NAA	42 70.00	17 28.33	1 1.67	0 0.00	0 0.00	60
0.5 BAP & 0.5 IBAP	24 40.00	23 38.33	8 13.33	4 6.67	1 1.67	60
1.00 BAP & 0.5 IBAP	36 60.00	19 31.67	5 8.33	0 0.00	0 0.00	60
Total	238	142	29	8	3	420

Statistic	DF	Value	Prob
Chi-Square	24	84.5328	<.0001**
Likelihood Ratio Chi-Square	24	85.9440	<.0001
Mantel-Haenszel Chi-Square	1	0.1464	0.7020
Phi Coefficient		0.4486	
Contingency Coefficient		0.4093	
Cramer's V		0.2243	

Table of Treatment by Callus

Treatment	Callus		
Frequency Row Pct	0	1	Total
Control	20 100.00	0 0.00	20
0.5 BAP	10 50.00	10 50.00	20
1.00 BAP	14 70.00	6 30.00	20
0.5 BAP & 0.5 NAA	8 40.00	12 60.00	20
1.00 BAP & 0.5 NAA	11 55.00	9 45.00	20
0.5 BAP & 0.5 IBAP	6 30.00	14 70.00	20
1.00 BAP & 0.5 IBAP	8 40.00	12 60.00	20
Total	77	63	140

Statistic	DF	Value	Prob
Chi-Square	6	27.0707	0.0001*
Likelihood Ratio Chi-Square	6	34.7174	<.0001
Mantel-Haenszel Chi-Square	1	11.4616	0.0007
Phi Coefficient		0.4397	
Contingency Coefficient		0.4025	
Cramer's V		0.4397	

Week 2

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	2 3.33	50 83.33	6 10.00	2 3.33	0 0.00	60
0.5 BAP	38 66.67	19 33.33	0 0.00	0 0.00	0 0.00	57
1.00 BAP	29 58.00	18 36.00	3 6.00	0 0.00	0 0.00	50
0.5 BAP & 0.5 NAA	19 35.85	18 33.96	10 18.87	4 7.55	2 3.77	53
1.00 BAP & 0.5 NAA	27 60.00	17 37.78	1 2.22	0 0.00	0 0.00	53
0.5 BAP & 0.5 IBAP	18 33.96	22 41.51	7 13.21	4 7.55	2 3.77	45
1.00 BAP & 0.5 IBAP	22 50.00	17 38.64	5 11.36	0 0.00	0 0.00	44
Total	155	161	32	10	4	362

Statistic	DF	Value	Prob
Chi-Square	24	101.5322	<.0001**
Likelihood Ratio Chi-Square	24	118.4832	<.0001
Mantel-Haenszel Chi-Square	1	2.8420	0.0918
Phi Coefficient		0.5296	
Contingency Coefficient		0.4680	
Cramer's V		0.2648	

Table of Treatment by Callus

Treatment	Callus		
Frequency Row Pct	0	1	Total
Control	20 100.00	0 0.00	20
0.5 BAP	5 25.00	15 75.00	20
1.00 BAP	6 30.00	14 70.00	20
0.5 BAP & 0.5 NAA	8 40.00	12 60.00	20
1.00 BAP & 0.5 NAA	11 55.00	9 45.00	20
0.5 BAP & 0.5 IBAP	5 25.00	15 75.00	20
1.00 BAP & 0.5 IBAP	8 40.00	12 60.00	20
Total	63	77	140

Statistic	DF	Value	Prob
Chi-Square	6	33.9394	<.0001**
Likelihood Ratio Chi-Square	6	41.8910	<.0001
Mantel-Haenszel Chi-Square	1	23.2741	<.0001
Phi Coefficient		0.4924	
Contingency Coefficient		0.4417	
Cramer's V		0.4924	

Week 3

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	0 0.00	27 45.00	27 45.00	4 6.67	2 3.33	60
0.5 BAP	17 33.33	25 49.02	9 17.65	0 0.00	0 0.00	51
1.00 BAP	19 47.50	15 37.50	6 15.00	0 0.00	0 0.00	40
0.5 BAP & 0.5 NAA	16 32.65	17 34.69	10 20.41	4 8.16	2 4.08	49
1.00 BAP & 0.5 NAA	22 55.00	17 42.50	1 2.50	0 0.00	0 0.00	40
0.5 BAP & 0.5 IBAP	16 32.00	22 44.00	7 14.00	3 6.00	2 4.00	50
1.00 BAP & 0.5 IBAP	19 47.50	16 40.00	5 12.50	0 0.00	0 0.00	40
Total	109	139	65	11	6	330

Statistic	DF	Value	Prob
Chi-Square	24	79.0848	<.0001**
Likelihood Ratio Chi-Square	24	102.2903	<.0001
Mantel-Haenszel Chi-Square	1	4.9505	0.0261
Phi Coefficient		0.4895	
Contingency Coefficient		0.4397	
Cramer's V		0.2448	

Table of Treatment by Callus

Treatment	Callus		
Frequency Row Pct	0	1	Total
Control	14 70.00	6 30.00	20
0.5 BAP	1 5.00	19 95.00	20
1.00 BAP	2 10.00	18 90.00	20
0.5 BAP & 0.5 NAA	8 40.00	12 60.00	20
1.00 BAP & 0.5 NAA	5 25.00	15 75.00	20
0.5 BAP & 0.5 IBAP	4 20.00	16 80.00	20
1.00 BAP & 0.5 IBAP	6 30.00	14 70.00	20
Total	40	100	140

Statistic	DF	Value	Prob
Chi-Square	6	27.7900	0.0001*
Likelihood Ratio Chi-Square	6	28.2724	<.0001
Mantel-Haenszel Chi-Square	1	13.2137	0.0003
Phi Coefficient		0.4455	
Contingency Coefficient		0.4070	
Cramer's V		0.4455	

Table of Treatment by Callus Class

Treatment	Callus Class			
Frequency Row Pct	1	2	3	Total
Control	6 100.00	0 0.00	0 0.00	6
0.5 BAP	4 21.05	14 73.68	1 5.26	19
1.00 BAP	7 38.89	10 55.56	1 5.56	18
0.5 BAP & 0.5 NAA	0 0.00	9 75.00	3 25.00	12
1.00 BAP & 0.5 NAA	6 40.00	6 40.00	3 20.00	15
0.5 BAP & 0.5 IBAP	2 12.50	9 56.25	5 31.25	16
1.00 BAP & 0.5 IBAP	2 14.29	6 42.86	6 42.86	14
Total	27	54	19	100

Statistic	DF	Value	Prob
Chi-Square	12	36.2014	0.0003*
Likelihood Ratio Chi-Square	12	39.1280	0.0001
Mantel-Haenszel Chi-Square	1	3.2426	0.0717
Phi Coefficient		0.6017	
Contingency Coefficient		0.5156	
Cramer's V		0.4254	

Week 4

Table of Treatment by Bud Class

Treatment	Bud and shoot proliferation class					
Frequency Row Pct	1	2	3	4	5	Total
Control	0 0.00	27 45.00	27 45.00	4 6.67	2 3.33	60
0.5 BAP	12 26.09	25 54.35	9 19.57	0 0.00	0 0.00	46
1.00 BAP	14 43.75	11 34.38	7 21.88	0 0.00	0 0.00	32
0.5 BAP & 0.5 NAA	7 17.50	17 42.50	10 25.00	2 5.00	4 10.00	40
1.00 BAP & 0.5 NAA	8 29.63	18 66.67	1 3.70	0 0.00	0 0.00	27
0.5 BAP & 0.5 IBA	12 28.57	18 42.86	7 16.67	3 7.14	2 4.76	42
1.00 BAP & 0.5 IBA	13 44.83	11 37.93	5 17.24	0 0.00	0 0.00	29
Total	66	127	66	9	8	276

Statistic	DF	Value	Prob
Chi-Square	24	69.0809	<.0001**
Likelihood Ratio Chi-Square	24	85.9123	<.0001
Mantel-Haenszel Chi-Square	1	5.3518	0.0207
Phi Coefficient		0.5003	
Contingency Coefficient		0.4474	
Cramer's V		0.2501	

Table of Treatment by Callus

Treatment Frequency Row Pct	Callus 0	Callus 1	Total
Control	8 40.00	12 60.00	20
0.5 BAP	0 0.00	20 100.00	20
1.00 BAP	0 0.00	20 100.00	20
0.5 BAP & 0.5 NAA	0 0.00	20 100.00	20
1.00 BAP & 0.5 NAA	0 0.00	20 100.00	20
0.5 BAP & 0.5 IBAP	0 0.00	20 100.00	20
1.00 BAP & 0.5 IBAP	1 5.00	19 95.00	20
Total	9	131	140

Statistics for Table of Treatment by Callus

Statistic	DF	Value	Prob
Chi-Square	6	44.4105	<.0001**
Likelihood Ratio Chi-Square	6	31.9471	<.0001
Mantel-Haenszel Chi-Square	1	18.4213	<.0001
Phi Coefficient		0.5632	
Contingency Coefficient		0.4907	
Cramer's V		0.5632	

Table of Treatment by Callus Class

Treatment Frequency Row Pct	Callus class 1	Callus class 2	Callus class 3	Total
Control	12 100.00	0 0.00	0 0.00	12
0.5 BAP	3 15.00	14 70.00	3 15.00	20
1.00 BAP	4 20.00	14 70.00	2 10.00	20
0.5 BAP & 0.5 NAA	7 35.00	7 35.00	6 30.00	20
1.00 BAP & 0.5 NAA	9 45.00	5 25.00	6 30.00	20
0.5 BAP & 0.5 IBAP	5 25.00	8 40.00	7 35.00	20
1.00 BAP & 0.5 IBAP	7 36.84	6 31.58	6 31.58	19
Total	47	54	30	131

Statistic	DF	Value	Prob
Chi-Square	12	41.1339	<.0001**
Likelihood Ratio Chi-Square	12	44.2894	<.0001
Mantel-Haenszel Chi-Square	1	8.7405	0.0031
Phi Coefficient		0.5604	
Contingency Coefficient		0.4888	
Cramer's V		0.3962	

Table 7. *In vitro* rooting: The effect of IBA treatment on rooting percentage and callus formation of *in vitro* produced axillary shoots used as microcuttings (at the 5% level of significance). [GenStat 64-bit Release 18.2 (PC/Windows 8) statistical software].

Analysis of variance (ANOVA)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
%Rooting					
Treatment	4	426.67	106.67	0.81	0.578 N.S.
Treatment x Week	20	433.33	21.67	0.84	0.647 N.S.
%Callus					
Treatment	4	6393.3	1598.3	3.41	0.131 N.S.
Treatment x Week	20	3366.7	168.3	1.42	0.202 N.S.

Table 8. *Ex vitro* rooting: The effect of treatment and cutting type on rooting percentage (per week) of *in vitro* produced axillary shoots used as microcuttings. [GenStat 64-bit Release 18.2 (PC/Windows 8) statistical software.]

Analysis of variance (ANOVA) (10% level of significance)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
%Rooting					
Week 2					
POSITION	1	800.00	800.00	16.00	0.028* (11.77)
HORMONE	1	7200.00	7200.00	144.00	0.001* (11.77)
POSITION.HORMONE	1	800.00	800.00	16.00	0.028* (16.64)
Week 3					
POSITION	1	200.00	200.00	6.00	0.092N.S.(9.61)
HORMONE	1	8450.00	8450.00	253.50	<.001 ** (9.61)
POSITION.HORMONE	1	200.00	200.00	6.00	0.092N.S.
(13.59)					
Week 4					
POSITION	1	450.00	450.00	13.50	0.035 *
HORMONE	1	7200.00	7200.00	216.00	<.001 **
POSITION.HORMONE	1	50.00	50.00	1.50	0.308 N.S.
Week 5					
POSITION	1	450.0	450.0	1.69	0.285
HORMONE	1	2450.0	2450.0	9.19	0.056
POSITION.HORMONE	1	50.0	50.0	0.19	0.694
Week 6					
POSITION	1	1012.5	1012.5	2.67	0.201 N.S.
HORMONE	1	2112.5	2112.5	5.57	0.099 N.S.
POSITION.HORMONE	1	112.5	112.5	0.30	0.624 N.S.

(LSD is shown in brackets)

After 6 weeks

Analysis of variance (ANOVA) (5% level of significance)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
%Rooting					
POSITION	1	2002.08	2002.08	5.27	0.105 N.S.
HORMONE	1	21252.08	21252.08	55.95	0.005 *
POSITION.HORMONE	1	752.08	752.08	1.98	0.254 N.S.
%Survival of rooted cuttings					
POSITION	1	1012.5	1012.5	2.67	0.201 N.S.
HORMONE	1	2112.5	2112.5	5.57	0.099 N.S.
POSITION.HORMONE	1	112.5	112.5	0.30	0.624 N.S.

Appendix E

APPENDIX E: Research Outputs

Journal article

Du Plessis, H.J., Kleynhans, R., Nikolova, R.V., Egan, B.A. 2019. Factors affecting seed propagation of *Hibiscus coddii* subsp. *barnardii*: a new potential ornamental plant. *Propagation of Ornamental Plants* 19(2): 38–47.

Conference presentations

Oral presentation

Du Plessis, H.J., Kleynhans, R., Nikolova, R.V., Egan, B.A. 2017. Effect of chemical scarification on seed germination of *Hibiscus coddii* subsp. *barnardii*. Combined Congress 2017, ATKV Klein-Kariba, Bela-Bela, 23–26 January 2017.

Poster presentation

Du Plessis, H.J., Nikolova, R.V. 2014. An endemic *Hibiscus* from Sekhukhuneland, South Africa with ornamental potential. The 29th International Horticultural Congress, Brisbane Convention and Exhibition Centre, Queensland, Australia, 77–22 August 2014.

Propagation of Ornamental Plants
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FACTORS AFFECTING SEED PROPAGATION OF *HIBISCUS CODDII* SUBSP. *BARNARDII*: A NEW POTENTIAL ORNAMENTAL PLANT

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Abstract

Hibiscus coddii subsp. *barnardii*, an endemic South African species, has potential as an ornamental flowering plant. It produces small, hard seeds that are difficult to germinate. The effect of scarification with 25%, 50%, and 98% sulphuric acid (H_2SO_4) for different durations (5-40 min), and the effect of temperature (15°C, 20°C, 25°C, 30°C, and 35°C) on seed germination on both moist filter paper and vermiculite were studied. Scarification of seeds in 98% H_2SO_4 for 30 min resulted in the highest final germination on filter paper (88.6%) and vermiculite (80%) cultures which was significantly different from seeds scarified for 5 min (52.9%) and the non-scarified seeds (< 5%). Scarified seeds cultured on filter paper attained 92.7-96% germination for all temperature treatments with no significant difference between the treatments. Seeds in vermiculite cultures incubated at 15°C failed to germinate while 83.3-94% seedling emergence was attained for cultures kept at 20-35°C. However, temperatures above 25°C (30°C and 35°C) had a detrimental effect on seedling survival. The best performance with regard to seed germination and subsequent seedling development (90% survival) was obtained when seeds were scarified with 98% H_2SO_4 for 30 min and germinated at 25°C.

Key words: scarification, seed germination, seedling emergence, sulphuric acid

INTRODUCTION

Ornamental plants that have either attractive foliage, or flowers, or both, constitute a significant part of the South African horticultural industry. Consumers and growers are constantly on the lookout for new plants that might be introduced onto the market, and indigenous plants are preferred as these are already adapted to local growing conditions (Bester et al. 2009, Kleynhans and Spies 2011, Middleton 2012). One such potential ornamental plant is *Hibiscus coddii* Exell subsp. *barnardii* (Exell) Leistner & P. J. D. Winter (family Malvaceae), an endemic subspecies from Sekhukhuneland, Limpopo province, South Africa. Plants are mainly found on rocky outcrops of the northern dry mixed bushveld vegetation type where they are exposed to a wide range of temperatures from 15°C to 35°C in summer months (Siebert 2001, Van Wyk and Smith 2001, Craib 2003, Mucina and Rutherford 2006).

The plant (Fig. 1A) is a perennial herb (0.6-1.5 m high) with hairy leaves and bright red flowers (Fig. 1B) during the summer season (December to March). Small (3-5 mm long), hard seeds with white cotton-like hairs (Fig. 1C) are produced after pollination (Retief and Herman 1997, Leistner et al. 2005).

The hard seed coat of *Hibiscus* species and other members of the Malvaceae family, imposes physical seed dormancy, which can prevent or slow down seed germination (Chachalis et al. 2008). Deposition of water-repelling substances such as cutin, suberin and waxes on the seed coat during seed maturation, affects its ability to take up water and prevents germination even when environmental conditions are favourable (Poljakoff-Mayber et al. 1992, Baskin et al. 2000, Gama-Arachchige et al. 2013, Geneve et al. 2018). The exact mechanism for uplifting physical dormancy under natural conditions is not well known for plant species with this type of dormancy, although factors such as fire, drying, freezing alternated with thawing, passage through the gut of animals, as well as high and widely fluctuating temperatures have been reported (Baskin et al. 2000, Hartmann et al. 2011, Baskin and Baskin 2014, Erickson et al. 2016). Craib (2003) reported that natural reproduction of *H. coddii* subsp. *barnardii* takes place

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EFFECT OF CHEMICAL SCARIFICATION ON SEED GERMINATION OF *HIBISCUS CODDII* SUBSP. *BARNARDII*

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INTRODUCTION

In the search for new ornamentals, indigenous plants are preferred as these are already adapted to local growing conditions (Middleton, 2012). One such potential ornamental plant is the Sekhukhuneland endemic *Hibiscus coddii* subsp. *barnardii*. This perennial, herbaceous plant with bright, red-orange flowers would be suitable for growing as a pot plant or small shrub, especially in sunny rockeries (Craib, 2003). The small, hard seeds produced by the plant are difficult to germinate, therefore, the effect of different sulfuric acid (H_2SO_4) concentrations and duration of scarification on seed germination was studied.

MATERIALS AND METHODS

Seeds were incubated in H_2SO_4 (98%, 50% and 25%) for 10, 20, 30 and 40 minutes respectively. The concentration of H_2SO_4 that resulted in the highest germination percentage was further used to determine the effect of duration of scarification on germination. Seeds were incubated in 98% H_2SO_4 for five to 40 minutes with five minute increments. Non-scarified seeds and seeds imbibed in distilled water were used as the controls. Scarified and non-scarified seeds were germinated on moist filter paper bridges and in moist vermiculite under controlled conditions. Radicle protrusion (2 mm) on filter paper and appearance of cotelydons above the vermiculite were considered as seed germination. All treatments were repeated three times with ten seeds per treatment. Various germination parameters, such as germination percentage (GP) and germination index (GI) were calculated. Data was analysed with the SAS and GenStat packages.

RESULTS AND DISCUSSION

The best germination response on both filter paper and vermiculite cultures was observed in seeds scarified with 98% H_2SO_4. Parameters such as GP and EI were significantly different from all other H_2SO_4 treatments and the controls. For seeds germinated on moist filter paper, duration of scarification (10 to 40 minutes) in 98% H_2SO_4 showed no significant differences in GP and EI, except for the five minutes treatment and the controls. For GP and EI, a similar trend was observed for seeds germinated in vermiculite.

CONCLUSION

Scarification with 98% sulfuric acid (from 10 to 40 minutes) significantly improved seed germination and seedling emergence of *H. coddi* subsp. *barnardii*.

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Keywords: germination index, germination percentage, ornamental plants, scarification, sulfuric acid

An Endemic Hibiscus from Sekhukhuneland, South Africa with Ornamental Potential

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Keywords: Plant endemism, horticulture market, germination, seedling emergence, shoot cuttings, 3-(indol-3-yl)-butyric acid

Abstract

South Africa is known world-wide for its rich floral biodiversity. Many of South Africa's indigenous plants are grown and commercialised by other countries. Recent market research, amongst plant nursery owners and growers in South Africa, showed the need of introducing new plants from the wild to the horticulture market. Sekhukhuneland, located in the Limpopo and Mpumalanga provinces, is a recognised centre of plant endemism. Some of the plants occurring in this drought-prone region are little known but have the potential to become popular garden or pot plants due to their attractive flowers and/or drought-tolerance. One such endemic plant is *Hibiscus coddii* subsp. *barnardii* which in nature is a virgate branched perennial herb (0.6–1.5 m high) with hairy leaves. Bright orange-red flowers are produced during the late summer season for about ten weeks. Our studies showed that the plant can be propagated by seeds and cuttings under controlled conditions at 24±2°C and a 16 hour photoperiod of 160–200 $\mu mol.m^{-2}s^{-1}$. It took an average of three weeks for seedling emergence and up to ten weeks for the plant to reach a flowering stage. Under these growth conditions the plant exhibited strong apical dominance with numerous nodes and axillary buds. Axillary shoot growth was induced during flowering and/or removal of the apex. Axillary shoot cuttings (at least 10 cm long) rooted within ten to fourteen days using commercial auxin rooting powder (Seradix®B No. 1). Plants regenerated from cuttings taken from a flowering plant formed flowers faster (within 5–6 weeks) than plants developed from seeds.