DETERMINATION OF DROUGHT STRESS TOLERANCE AMONG SOYBEAN VARIETIES USING MORPHOLOGICAL AND PHYSIOLOGICAL MARKERS

by

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DECLARATION

I declare that **DETERMINATION OF DROUGHT STRESS TOLERANCE AMONG SOYBEAN VARIETIES USING MORPHOLOGICAL AND PHYSIOLOGICAL MARKERS** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted by me before for any other degree at any institution.

Paseka Tritieth Mabulwana

Signature **Date**

DEDICATION

This is a special dedication to my late sister, Tinyiko Tercia Mabulwana-Mnisi for being a wonderful sister during her short and precious life on earth. *Ndza ku tsundzuka, ndza ku rhandza hinkwawo masiku ya ku hanya ka mina. Etlela hi ku rhula phyembye ra ka hina, madyondza ya Khalanga na n'wa Mulambya!*

TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHODS

CHAPTER 4: RESULTS

CHAPTER 5: DISCUSSION AND CONCLUSSIONS

CHAPTER 6: REFERENCES

APPENDICES

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

The aim of the study was to identify drought tolerant South African soybean cultivars for cultivation where water is a limited resource. Soybean [*Glycine max*. (L.) Merr] is one of the most important legumes in the world. A lot of attention has been focused on soybean cultivation in South Africa recently. Soybean production is mainly affected by several biotic and abiotic factors which reduce the yield and quality of the crop.

Six South African soybean cultivars (LS 677, LS 678, Mopanie, Sonop, Knap and Pan 1564) and two American cultivars (R01 416 and R01 581) were carefully studied for morphological and physiological markers which contribute to drought tolerance. The study was conducted at the University of Limpopo (Turfloop campus). Soybean plants were grown in a glasshouse in a randomised block design given same amounts of nutrients and differing amounts of water (limited and overwatering).

Data was collected at R3 growth stage by measuring several morphological (stem length, leaf surface area, flowers and seeds counts) and physiological (percentage chlorophyll, moisture content, total phenolics, total flavonoids, ureide content and antioxidant activity) parameters. An anatomical study was also carried out on the transverse sections of leaves, roots, leaf stalk and nodules.

The different cultivars reacted differently to the three water treatments. LS 678 produced the tallest plants whereas those of Pan 1564 were the shortest. Water stress affected plants by reducing the number of flowers produced, the leaf surface area as well as the relative leaf water content. The moisture content of the growth medium was reduced faster as the plants matured and it was also lowered by the limited water availability. Percentage chlorophyll is another trait which was affected by water limitation. Cultivars with high phenolic and flavonoids content were associated with high antioxidant activity and slightly yielded higher than the others.

The anatomical transverse sections of the roots and petioles have shown some secondary growth. The anatomy of the nodules of Mopani has shown some interesting differences in response to the three treatments. Limited water decreased

the size of the vascular tissue and sclerenchyma as a result altering the functionality of the nodule. The anatomy of Sonop's petiole had a thickened sclerenchymatous bundle sheath covering the phloem tissue. The sclerenchyma tissue is thought to guard against loss of water. The cross section of the leaf had a double layer of palisade mesophyll (upper surface) and only a single layer of spongy mesophyll (lower surface). In addition, the mesophyll and the epidermal cells of Mopani appeared much thicker.

In terms of yield, there was no cultivar which yielded the highest but Mopani yielded the lowest. Since Mopani was low yielding, the main focus of the discussion was on the features (morphological, physiological and anatomical) of Mopani which can be associated with drought susceptibility. Some of these features include reduced stem length, large leaf surface area, low relative leaf water content, low growth medium moisture content and low antioxidant activity.

CHAPTER 1: INTRODUCTION

1.1 Soybean cultivation in South Africa

Soybean [*Glycine max* (L.) Merr.] is considered to be a very important grain legume world-wide (Kumar *et al*., 2008; Ahmed *et al*., 2010). In South Africa, soybean production has been going on for more than two decades but has only become successful recently (Pschorn-Strauss and Baijnath-Pillay, 2004).

1.1.1 Soybean producing areas

Soybean cultivation in South Africa is widespread. Major soybean producing areas per province are the Mpumalanga highveld; the Free State highveld; areas around Pietermaritzburg in KwaZulu Natal province; the highveld of the Northwest province; the Westrand in Gauteng province; the Limpopo river valley and the southern parts of the Limpopo province. Soybean production by the other three provinces (Northern Cape, Eastern Cape and Western Cape) is minimal. From the year 2005, soybean production fluctuated but picked up significantly during the 2009-2010 season. This is according to the Department of Agriculture, Forestry and Fisheries (DAFF, 2010).

1.1.2 Soybean production and consumption

Singh and Singh (1992) indicated that major food crop producers are major consumers. They stated that the soybean is an exception to that rule as a result of its variety of forms of consumption (raw and processed). The form in which this crop is consumed is mainly determined by the area in which it is being used. South Africa exports a variety of high quality processed soybean products like soybean flour, textured soybean protein and soybean oil.

DAFF (2010) analysed the relationship between soybean production trends and consumption between the years 2000 and 2009 in South Africa. According to the profile, soybean consumption was higher than production in 2000, 2001, 2003, 2005 and 2007. With the year 2007 being the worst because about 360 000 tons of soybean were consumed whereas only 200 000 were produced. The total soybean

produced was more than that consumed during the years 2002, 2004, 2006, 2008 and 2009. A significant increase in soybean production was achieved in 2009, when more than 500 000 tons were produced while only about 300 000 tons were consumed.

1.1.3 Areas under soybean cultivation

According to DAFF (2010), though the area under soybean cultivation in South Africa fluctuated, it increased from 94 000 hectares during 1999/2000 to 238 000 ha in the 2008/2009 season. During the same period productivity increased from 1.6 tons/ha to 2.1 tons/ha. The fluctuations in area under soybean cultivation and tonnage produced are mainly affected by the weather and price forecasts.

1.1.4 Production under dryland and irrigation

In South Africa the soybean is cultivated both under dryland and irrigation. Expansion usually involves switching from other crops to soybean. During the 2008/09 season, suitable land (areas/zones) for ongoing cultivation (2 610 346 ha) and potential cultivation of soybean under both dry land and irrigation conditions were estimated to be 3.0 million ha which had a percentage growth of 15 percent from the previous season. Under dry land conditions, existing (2 449 254 ha) and potential soybean production was estimated to be 2 774 767 ha with a percentage growth of 13.3. Soybean production under irrigation was also estimated, with existing 161 092 ha and potential growth of 218 226 ha which is 35.5 percentage growth. Productivity under dryland ranges from 1.0 to 3.0 tons/ha while under irrigation is about 5.0 tons/ha (Blignaunt and Taute, 2010). The above information therefore serves as evidence that irrigation farming improves crop production and yield, provided that available water is sufficient.

1.2 Uses of soybean

Soybean is regarded as the most important protein source compared with wheat and maize (Kumar *et al*, 2008; Joyner *et al*, 2010). It is used for drinks (Joyner *et al*., 2010), food and animal feed all over the world (Kisman, 2003; Liu *et al*., 2003;

Goldflus *et al*., 2006; Malencic *et* al., 2007; Lobato *et al.,* 2008; Ahmed *et* al., 2010). This crop has very high oil and protein contents (Malencic *et al*., 2007; Kumar *et* al., 2008; Ahmed *et* al., 2010) which are important seed quality components in the economy (Marton, 2010). Because of these important uses, demand for soybean production has increased globally (Brown *et al*., 2005).

DAFF (2010) outlined the estimates of soybean utilisation in South Africa as follows: 25% of the total soybean produced is mainly processed to produce oil and oilcake, 60% is used for animal feed whereas only 20% is being used for human consumption. These estimates indicate that the majority (60%) of the soybean produced in South Africa is being utilised for animal feed. The same was pointed out by Pschorn-Strauss and Baijnath-Pillay (2004).

Soybean is also very useful in improving the soil as one of its most important agronomic characteristics is the capability to take atmospheric nitrogen and fix or convert it (Kumar *et al*., 2008) to a form more usable by the soybeans themselves (Purcell *et al*., 2000) and other plants (Ahmed *et al*., 2010; Marton, 2010; Mugendi *et al*., 2010). Nitrogen fixation in soybean is brought about by a mutualistic relationship between the soybean roots and *Bradyrhizobium japonicum* bacterium which forms nodules (swellings) in the roots. The bacterium aids the plant in fixing or converting atmospheric nitrogen into a form that is more usable by the plant (Ahmed *et al*., 2010).

1.3 Soybean export and import in South Africa

Pschorn-Strauss and Baijnath-Pillay (2004) reported South Africa as a soybean “net importer”. This is supported by DAFF (2010) which stated that the country is not doing so well in the export market. Soybean export was reported to be very poor (less than 8 500 tons per annum) between the years 2000 and 2008. A total of 599 435 tons were imported with only 2 800 tons exported. During the period 2001 to 2002 only 0.2 % soybean was exported and increased to 1.5 % in 2005. Although the export was still low, it was much better than during the other years. As a result of improved production trends, soybean export drastically increased in 2009 when a total of 161 620 tons were exported and only 1 495 tons imported (DAFF, 2010).

Soybean meal is one of the processed forms of soybean which is the major consumed product in South Africa. Unfortunately very little (100 000 tons) is produced in the country, as a result more than 90% of this popular soybean product is imported from Argentina (Esterhuizen, 2010). Based on the above information, one can therefore conclude that there is a relationship between soybean production and import - export. The total soybean production and consumption will determine how much can be exported or imported.

1.4 Need for cultivation area expansion

There is a need for the South African soybean industry to expand in order to meet the domestic demand. According to DAFF (2010), soybean production is by far less than consumption; hence more soybean is being imported to satisfy consumption needs. Since expansion involves switching from other crops to soybean, profitability will be a contributing factor. Improved production methods and high yielding cultivars are necessary to make a decision to switch or not to.

1.5 Motivation

Soybean has twice the amount of seed protein present in wheat and maize (Kumar *et al*., 2008). Due to its characteristics, soybean is used and appreciated in South Africa by consumers, the farming communities and commercial seed companies. However, South Africa does not produce enough soybean either under irrigation or dryland to meet the demand. This is compoded by water shortages in South Africa. Therefore more information about available cultivars is required.

The study will show which combinations of morphological and physiological characteristics confer drought tolerance in soybeans. Such characteristics can be investigated in other crops as well. The identified varieties can be used for cultivation in drier areas; for cultivation area expansion; and in breeding programs for drought tolerance. This project will address the problem of access to efficient and easily measurable physiological and morphological markers in soybean breeding programs to allow selection of soybean cultivars for growth in the drier areas of South Africa.

1.6 Research hypothesis

In order to determine which morphological and physiological characteristics confer drought tolerance to soybean, the following hypothesis was proposed: soybean cultivars that have a higher yield under limited water supply have similar morphological and physiological characteristics to those that have a lower yield.

1.7 Outcomes of the research project

The establishment of an experimental setup that can efficiently measure drought tolerance / sensitivity in plants will benefit crop producers to determine which of their crops are tolerant to drought. The identification of drought tolerant soybean varieties will enable farmers to cultivate soybeans in areas where water is scarce.

1.8 Aim

The study aimed to understand drought tolerance and susceptibility in soybean.

1.9 Objectives

Objectives of the research were to:

i. Establish and optimise growth conditions in a glass house that can be used to determine drought tolerance / susceptibility in soybeans.
ii. Identify soybean varieties that yield more under limiting water conditions.
iii. Identify soybean varieties that carry out nitrogen fixation under limiting water conditions.
iv. Select and morphologically/physiologically characterise South African soybean varieties with a potential for drought tolerance.

1.10 Dissertation outline

Chapter 1 - Introduction
This chapter basically focuses mainly on the production of soybean in South Africa. Areas within the country in which soybean is being produced are outlined.

Production and consumption trends of this crop as well as its export and import are discussed. Importance of soybean and outcomes of the study are also indicated in this chapter.

Chapter 2 - Literature survey

The morphology and life cycle of the soybean plant are detailed. Global concerns such as feeding the growing population and climate change (variability) are some of the main topics discussed in this chapter. World cultivation of soybean is indicated. Two forms of crop production namely; dryland (rainfed) and irrigation farming as well as factors affecting those form part of this chapter. Drought stress as one of the major environmental factors affecting crop production is introduced. Availability of water in South Africa as the main concern for irrigation agriculture is also part of the literature studied. Possible strategies of improving dryland agriculture are indicated. Effects of drought stress on soybean (growth and yield) and ways in which this crop adapts to water stress are also shown.

Chapter 3 - Research methodology

This chapter outlines how the research was designed; the materials used for the study as well as the procedures and protocols used for measuring each parameter. The type of data (morphological and physiological markers) collected and how it was analysed is indicated in this chapter.

Chapter 4 - Results

The findings of the research are presented in this chapter.

Chapter 5 - Discussion and conclusions.

In this chapter, research findings are discussed in comparison with previous work done on the same topic. Recommendations are also stated.

Chapter 6 - The literature cited in this study is acknowledged by the listing of references.

CHAPTER 2: LITERATURE REVIEW

2.1 The soybean plant

Soybean [*Glycine max* (L.) Merr.] (Family Fabaceae) is an annual seed legume with a broad variety of cultivars. The plant grows up to 61 to 91 cm in height. The leaves are trifoliate and the flowers are usually purple or yellow. It can bear as many as 100 to 150 pods containing yellow seeds. The pods usually contain two to three seeds per pod but some pods rarely have one seed especially the low yielding cultivars. The plant is covered with very soft tiny brown hairs. The growth of the plant from seed germination to seed maturity takes about sixteen weeks (Shurtleff and Aoyagi, 2009).

Figure 2.1: Morphology of soybean, Pan 1564 cultivar.

Soybean growth is designated by several vegetative and reproductive growth stages (Fehr *et al*., 1971). The number of nodes on the main stem indicates the vegetative stage of that particular plant e.g. a plant with three nodes is in vegetative stage 3 (V3), one with eight nodes is in V8 and so on. Flowering indicates the beginning of reproductive stages where the uppermost four nodes are considered. Reproductive stage 1 (R1) is the onset of flowering, when a flower forms at any of the four nodes; reproductive stage 2 (R2) is the formation of a flower below the uppermost node (of the four) when the leaf at the node has completely unrolled; reproductive stage 3 (R3) is the start of pod formation and the pod is about 0.5 cm long; reproductive stage 4 (R4) is when the first pod is about 2.0 cm long; reproductive stage 5 (R5) is the beginning of pod filling; reproductive stage 6 (R6) is at complete pod filling; reproductive stages 7 and 8 (R7 and R8) are maturation stages. R1 to R8 growth stages can take up to approximately 70 days (Fehr *et al*., 1971).

Soybean plants can be grouped according to their growth habits into basically two main types, determinate and indeterminate. The determinant varieties will flower at a certain time of the year, basically when the days begin to shorten. The latter usually complete their vegetative growth prior to flowering with a group of flowers (raceme) at the tip where the stem ends. Indeterminate varieties will continue to flower and put on fruit until the weather dictates that it is time to curtail plant growth, they continue to increase in length for some time after the onset of flowering (Liu *et al*., 2010).

Soybean maturity (flowering and ripening) usually takes about 90 to 100 days after planting date depending on the type of variety. Early cultivars can mature after about 75 days whereas it can take up to 200 days or more for the late varieties to mature (Shurtleff and Aoyagi, 2009). Unlike other legumes, soybeans are unique because of their built-in time clock. These plants are sensitive to short days (photoperiodism). Soybean maturity varies for different cultivars and is determined by the photoperiod, which is the length of day and night (Shurtleff and Aoyagi, 2009).

2.2 The need to feed a growing population

Mankind is faced by a very serious fundamental challenge of feeding a drastically increasing global population. The areas that are struggling to produce enough food are prone to environmental challenges such as overpopulation, poverty, drought and

climate change. Such areas or landscapes are said to be vulnerable to the environment (Rockstrom, 2003).

Overpopulation is defined by Young (2005) as 'population in excess of the capacity of land to supply its food needs and expresses itself locally in terms of farm size'. Ali and Talukder (2008) indicated that the world population (6000 million) is expected to increase by 30 % (8100 million) by the year 2030. The world population is increasing faster while the resources available for supporting and fulfilling its needs are being depleted. According to Lutz and Qiang (2002), global population has drastically increased during the 20th century and further population growth is expected. On the other hand, land is limited, the earth cannot be expanded; and the conversion of pristine land into agricultural land works against conservation and principles of ecosystems. Carr (2004) stated that deforestation and conversion of forest to agricultural activities indicate an increasing population density which implies that farming is a major human activity which transforms the land and therefore negatively affecting the environment.

In his review on population growth, Young (2005) stated that population growth results in many challenges such as poverty, pollution, inequality, deforestation and depletion of natural resources. The increasing global population will create environmental degradation resulting in a serious demand for food, water, energy as well as shelter. Rapid population growth also affects negatively the economic development and sustainability of natural resources (Young, 2005).

Africa among other continents is considered to be one of the poorest as a result of its weak economy which leads to insufficient food supply. Another problem is the vulnerability of the continent to climate change which is predicted to be more frequent and extreme. As a result, the continent, specifically the west part of it is facing poverty. The uneven production and distribution of food in the continent also contribute to the hungry expanding population (Huntingford *et al.*, 2005).

Conserving resources is becoming very important in order to feed and sustain the growing population. Farmers and researchers are striving to satisfy the need for abundant and inexpensive food to meet the challenge of feeding the growing

population from a degrading area of land (Minnesota Agri-growth Council, 2009). With the increasing global population, production of resources especially food has to be increased to feed the population. Developing cheaper but more healthy and nutritious foods such as soybeans, corn, wheat and rice can help alleviate the world hunger. The soybean in particular can be grown in a variety of areas as it is easy to grow, manage and harvest. It can also produce a high yield within a short period of time (Joyner *et al*., 2010). Babovic and Milic (2006) presented experimental evidence demonstrating that irrigation farming system can serve as a tool to improve food crop production hence feeding the increasing, hungry population and alleviating poverty.

2.3 World cultivation of soybean

Global soybean production is more than twice as much compared to that of all the other grain legumes (Marton, 2010). The United States of America (USA), China, Brazil, Indonesia, Japan, Korea and Argentina are the major soybean producing countries (Ahmed *et al*., 2010). During the year 2003, global soybean production according to Chianu *et* al. (2008) was as follows: the USA produced 34 % of the total world production, Brazil 28 %, Argentina 18%, China and India both produced 9%, Paraguay 2% and the rest of the countries contributed only 5% of the total global soybean production collectively (Chianu *et al*., 2008).

According to Chianu *et* al. (2008), global soybean production reached a maximum of 180 million tons during the 1999/2000 season which increased to 190.1 million metric tons in 2003. Joyner *et al*. (2010) reported an increased world-wide production of soybean of 210.9 million metric tons in 2009. Moreover, the USA improved from 34 % production (Chianu *et al*., 2008) to 38% (Oz *et al*., 2009) in 2009.

2.4 Cultivation of soybean in Africa

The main soybean cultivating countries in the African continent include Nigeria, Uganda, Zimbabwe as well as South Africa (Chianu *et al*., 2008). According to Shurtleff and Aoyagi (2009) of the total soybean produced in Africa during the season 2008/2009, Nigeria contributed 39 %, South Africa 35 %, Uganda 14 %,

Zimbabwe 6 %, Egypt and Zambia being the least soybean producing countries contributing 3 % each.

2.5 Cultivation of soybean in South Africa

South Africa is the second largest soybean producer in Africa according to Shurtleff and Aoyagi (2009). Soybean production in South Africa is mainly under dry land farming with the total annual yield of up to 3 tons per hectare. The crop is produced in all provinces with the Mpumalanga province being the top producer contributing more than 40% of the total soybean produced in the country (DAFF, 2010).

2.6 Climate variability and weather changes

Climate change, often referred to as global warming is one of the greatest challenges the world is faced with. It causes some serious implications to the global weather. Global warming is responsible for frequent drought and floods as well as poverty and poor health challenges (DEAT, 2004; Vohland and Barry, 2009).

Global warming is caused by alarming concentrations of greenhouse gases in the atmosphere. Carbon dioxide (CO_2) is the most important greenhouse gas. Burning of fossil fuel increases the concentration of CO_2 in the atmosphere which eventually increases the temperatures of the globe. Although overpopulation may increase the emission of greenhouse gases, the future estimations of the actual rates of CO_2 emission are somewhat difficult because other factors such as economic growth and technology improvement may contribute towards global warming (Huntingford *et al*., 2005).

Climate change influences or rather controls crop production. The quantity and quality of food crops produced depends on the variability and intensity of the climatic aspects (rainfall, drought). The success of a certain crop in a particular area depends on the climatic variability experienced by that particular location (Huntingford *et al*., 2005).

Climate change and variability is likely to negatively impact the world agricultural industry which plays a vital role in feeding the growing population. This phenomenon threatens the sustainability and predictability of the global population communities especially the vulnerable sub-Saharan region of the African continent (Mwiturubani and van Wyk, 2010).

Africa is vulnerable to global warming because of its low capacity to adapt to environmental changes. This is due to many contributing factors such as poverty, over population, floods and drought as well as the available agricultural production systems. These factors rely mainly on rainfall. Floods and droughts are becoming frequently extreme and severe as a result of climate change. As a result, the continent is facing a very serious challenge of scarcity of resources which eventually lead to competition for available resouces (Mwiturubani and van Wyk, 2010).

The spatial and temporal variability of rainfall due to global warming is a very crucial aspect which has a serious influence on the operation of water sources. South Africa has an annual average rainfall below the global average. According DWAF (2000), South Africa has an average annual rainfall of 500 mm compared to 800 mm global average. It is therefore of imperative importance that the authorities (water management) plan for possible future variations in rainfall as a result of climate change.

2.7 Water availability / scarcity in South Africa

Water is one of the most important necessities for everyday life but it also becomes a limiting resource for people, animals and the environment because of its scarcity (Krausman *et al*., 2006). Its availability for both agricultural and domestic purposes depends on the growing competition as well as availability of water resources (Ali and Talukder, 2008).

Water is a scarce resource in South Africa. Its availability is physically limited whereas human utilisation or demand for fresh water is increasing with the growing population. Metcalf-Wallach (2008) stated that South Africa is one of the countries with limited natural water resources. The country is said to have few rivers and a

major portion of its water is utilised for agricultural activities (Metcalf-Wallach, 2008). According to Wallace *et al*. (2003) increasing water demand above any other factors (climate change / variability, rainfall timing) is a dominating culprit of water scarcity.

South Africa does not have enough water sources and the average annual rainfall is far less than the global average. Water availability at its source and the type of facility used to withdraw / transport it will determine its supply to where it is being utilised. The well-known water sources include rainfall, reservoirs, rivers, lakes, dams and ground water (Metcalf-Wallach, 2008).

Global warming is associated with increasing temperatures which causes a rise in evaporation rates posing a strain on water sources (Metcalf-Wallach, 2008). Vohland and Barry (2009) reviewed *in situ* rainwater harvesting (RWH) as a promising practice to combat the issue of water scarcity and land degradation due to climate change in sub-Saharan Africa. The *in situ* RWH practices are considered to be effective since they promote more productivity in agriculture by providing stability, restoration and resilience to global warming. This involves artificial collection of rainwater for storage (underground and aboveground) to be utilised mainly in agriculture (Vohland and Barry, 2009).

Effective management and use of water can also be approached by genetic improvement of crops thus increasing crop water productivity and reducing environmental problems. Genetic improvement of crops includes developing drought tolerant plants that use less water, therefore playing a big role in saving water (Ali and Talukder, 2008).

Griffin and Mjelde (2000) suggested that water management also has a huge impact on the problem of water unavailability in relation to drought stress. Wallace *et al*. (2003) pointed out that environmental issues such as water quality and protection of the ecosystem are always of greater concern where water is at least available and are surely becoming a problem in areas where water is scarce.

As a result of water scarcity, there is a competition for water resources between the increasing human population and the other sectors including the ecosystems. Water

conservation and management practices therefore need to be improved to ensure sufficient supply of fresh water to all sectors and consumers. In addition to the improvement in water management, there should also be an effort in the improvement of crop productivity under dryland conditions.

2.8 Crop production and yield under dryland / rain-fed farming compared to irrigation

According to Browman (2003) dryland comprises about 40 percent of the global land area. In some of his work Browman included the lands which received more than 2,000 mm rainfall as dryland. Despite the broad definition of dryland, in general, lands that receive very limited precipitation are regarded as dryland.

Agricultural development in dryland involves intensive hard work but the ecology in dryland are thought to be more tolerant to water stress than in moist areas. Batterbury (2001) pointed out that farming in dryland is a life of intensive labour but pays off at the end of the day. He also indicated the hardships that are involved in dryland farming as a result of climate change which results in variability and instability of the environment. Babovic and Milic (2006) indicated that dryland farming results in reduced total yield of food crops grown on a large scale area.

Irrigation is used in agriculture to improve the yield of food crops as compared to dryland or rain fed farming. Babovic and Milic (2006) provided experimental evidence demonstrating that irrigation improved yield to about two times that of farming in dryland. Climate change, especially rainfall patterns will determine the effectiveness of irrigation in agriculture. When precipitation is enough, irrigation becomes less effective but under drier conditions, irrigation becomes more effective therefore increasing crop yield than dryland (rainfed) farming (Babovic and Milic, 2006).

Future improvement of crop production management must consider an interdisciplinary approach which will include all causative agents of environmental instability. To achieve this goal, inputs are needed from “climate scientist, agricultural scientists and extension specialists” who will work closely together to strive for improvement and stability in food crop production (Stone and Meinke, 2005).

2.8.1 Other factors affecting crop production

The variability of yield quantity and quality of food crops is affected at large and local spatial scales by several biotic and abiotic factors (Porter and Semenov, 2005). The agricultural industry can be affected by factors such as water availability in the soil (soil moisture), evaporation of the earth's surface and humidity in the atmosphere. These factors are subject to local and international variations and are mainly influenced by climate variability and rainfall patterns world-wide (Huntingford *et al*., 2005). Climate change is the dominating factor which directly affects the quality of crop production (Stone and Meinke, 2005) by increasing global temperature and altering precipitation (Porter and Semenov, 2005).

2.8.1.1 Irrigation methods and equipment

Irrigation methods and equipment are determined by several factors including: the type and components of the soil surface, type of crops and water availability. According to Babovic and Milic (2006) "Center pivot and lateral move" systems are suitable for irrigating relatively large areas unlike small irrigation methods which can be used to irrigate small areas or agricultural fields. Factors responsible for the price of irrigation equipment include but are not limited to the size and type of equipment as well as availability and distance of water reservoir (Babovic and Milic, 2006).

Babovic and Milic (2006) stated that irrigation farming methods are currently efficient and will continue to increase crop production in the future. He pointed out that these farming methods had increased the area from 50 to 250 million hectares. Irrigation is therefore considered to be the future of the agricultural industry. Besides increasing crop production and improving the economy by increasing profit, irrigation also provides stable and favourable conditions for farming practices (Babovic and Milic, 2006). Irrigation methods are only effective where water is available.

2.8.1.2 Weeds

Any plant (wild or common) growing where it is not wanted and is in competition with cultivated plants is considered a weed. Weeds reduce crop production especially in

dry land farming by competing with crops mainly for water. The competition between weeds and crops is not only for water but they can compete for other resources such as light, nutrient supply as well as the available space for growth. The competition therefore decreases the yield because the weeds out-compete the crops causing them not to have enough resources necessary for growth. Weed control is very crucial mainly for crops produced under dry land farming to prevent low yields as a result of competition. Commonly used methods to control weeds include manual removal (hand picking), tillage and herbicides (Unger and Howell, 1999).

2.8.1.3 Drought stress

Drought stress refers to a situation where the demand of water for consumption is higher than the availability thereof. Water is a vital resource for life. Many organisms including plants and animals are altered by unsuitable and variable rainfall patterns. Drought stress in frequent and extreme episodes causes extensive loss for the agricultural industry. Water deficit decreases crop yield and therefore increase damage to the agricultural industry and the economy at large (Babovic and Milic, 2006). Water availability for irrigation is significant for optimum food crop production by the farming communities. Drought stress is therefore the most detrimental and prevalent form of environmental stress (Zidenga, 2006).

2.8.1.3.1 Effects of drought stress on plant morphology

Plants undergoing water deficiency reduce growth rate of leaves and cells (Purwanto, 2003). Drought stress causes plants to undergo morphological, physiological and biochemical changes which inhibit plant growth and may eventually lead to death (Cellier *et al*., 1998).

Environmental factors such as water unavailability have a negative impact on the growth of plants. Drought causes water deficit which is mainly responsible for reduction of plant growth and yield (Kisman, 2003; Zidenga, 2006; Hufstetler *et al*., 2007). Water stress during the vegetative stages of plant growth is a dominating factor for reduced growth and yield (Mirakhori *et al*., 2009). Kisman (2003) reported

that plants adjust to drought stress by reducing the size of leaves while increasing water use efficiency (reduce loss of water).

2.8.1.3.2 Effects of drought stress on plant physiology

Drought stress affects major physiological processes such as translocation, gaseous exchange, transpiration as well as photosynthesis (Kisman, 2003). Water deficit is known to increase water use efficiency (Purwanto, 2003); increase concentration of solutes in the soil which results in an osmotic flow of water from the cells increasing the solutes concentration in the cells (Zidenga, 2006). Furthermore, water stress lowers water potential, disrupting membranes and vital metabolic processes like photosynthesis (Zidenga, 2006).

Carbon dioxide from the atmosphere enters the leaves through open stomata to be "fixed" and utilised by the plant. The open stomata not only allow carbon dioxide to enter but it also allows water to escape in the form of vapour. As a result plants need to devise means (open and close stomata) in which they acquire enough carbon dioxide yet retaining sufficient water for their wellbeing (Huntingford *et al*., 2005).

Galle *et al*. (2007) reported that water stress reduces the level of carbon dioxide fixation in plants by closing stomatal openings and lowering "mesophyll conductance" therefore limiting the process of photosynthesis. Closure of stomata as a result of water stress is the main factor altering or preventing the vital process of photosynthesis. Under moderate water stress, the effect on photosynthesis is moderate and can be repaired. Unfortunately that is not the case with extreme drought where the limitation of photosynthesis is severe and cannot be repaired (Galle *et al*., 2007). An example of intense damage caused by drought stress in plants is the degradation of lipid membranes. This damage is severe and plants cannot recover from it (Gigon *et al*., 2004).

Water stress is also known to induce oxidative stress (Blokhina *et al*., 2003) which leads to the formation of reactive oxygen species (ROS). Although non-stressed plants produce ROS at low levels, increasing stress levels promote elevated amounts of ROS. Examples of ROS are hydrogen peroxide, hydroxyl radical, singlet

oxygen and superoxide anion. These derivatives of oxygen are very toxic and can disrupt the electron transport chain (Mittler *et al*., 2004) and some ROS are the causative agents of damage in essential cellular components such as lipids, nucleic acids, carbohydrates and proteins (Zidenga, 2006).

2.8.1.3.3 Effects of drought stress on soybean growth rate

Water deficiency decreases growth of soybean leaves (Purwanto, 2003), roots, main stem height, internode length/number of nodes, number of flowers (Desclaux *et al*., 2000), leaf area, leaf area index and leaf weight (Kisman, 2003), increasing water use efficiency (Purwanto, 2003). Borges (2004) indicated that water stress also causes soybeans to abort leaves, pods and flowers. Drought stress shortens reproductive stages of soybean plants hastening flowering and pod formation (Desclaux *et al.*, 2000). Kisman (2003), reported that the effect of water stress on growth of soybean depends on two factors, i.e. growth stage during which the stress is induced and the degree of stress induced.

2.8.1.3.4 Effects of drought stress on soybean yield

Soybean, like many crops is negatively affected by lack of water mainly in the form of rain. Drought stress is the most important factor responsible for low yield in soybean crops (Purwanto, 2003). Whenever water supply is not efficient, nutrient supply to all plant organs is lowered (Kokubun *et al*., 2001). Water stress can also affect soybean yield by decreasing the number of pods per plant, number of seeds per pod, total weight per seed (Hall and Twidwell, 2002; Borges, 2004), as well as symbiotic nitrogen fixation (Serraj, 2003). Serraj *et al.* (1999) indicated that water deficit leads to lower soybean yield by affecting mainly the sensitive symbiotic nitrogen fixation process.

Soybean production can also be affected by high temperature, low yielding varieties, poor seed quality, weed competition (Hungria and Vargas, 2000), uneven rain distribution, soil pH, insects, diseases, weeds as well as nutrient availability in the soil (Purwanto, 2003). In soybean, drought is the greatest threat to profitability and too often a crop with great promise ends up with poor yield because of dry weather.

Drought stress occurring during the different stages of development reduces soybean yields (Lobato *et al.,* 2008), by aborting younger pods and stems (Hall and Twidwell, 2002; Liu *et al.,* 2003).

Furthermore, drought stress decreases soybean yield by the inhibition of essential processes like photosynthesis (Pelleschi *et al*., 1997; Kokubun *et al*., 2001), nitrogen fixation, photosynthetic gaseous exchange and osmoregulation (Pelleschi *et al*., 1997) thus, accelerating abortion rates (Kokubun *et al*., 2001). Dybing *et al.* (1986) indicated that drought induces shedding of flowers and pods. Insufficient water supply can also alter the metabolism of sugars (sucrose and hexoses), thus, causing an increase in solute concentration leading to starch depletion (Pelleschi *et al*., 1997; Liu *et al*., 2003; Sweeney *et al*., 2003).

2.9 Strategies to improve crop productivity under dry land / rain-fed farming

2.9.1 Cultivation practices

Dryland agriculture is not easy due to limited and variable precipitation. The spatial, unpredictable rainfall patterns as a result of climate change lead to limited water availability for the farming community. Some cultivation practices can be employed to better crop yield and production in dryland farming (Unger and Howell, 1999).

2.9.1.1 Double cropping

Double cropping is a sustainable agricultural practice in which more than one crop is grown on the same ground during the same period of time. Irrigation helps increase and stabilise crop production and also promote double cropping. It helps to naturally promote soil quality. Double cropping has an advantage of increasing crop and land productivity which help in boosting the economy and feed the ever-growing hungry population (Babovic and Milic, 2006).

2.9.1.2 Tillage

Tillage simply means agricultural preparation of the soil (land) for growing crops. Tillage can also mean leaving plant residue to rot on the surface of the soil. The soil

manipulation result in achieving optimum environmental conditions for plant establishment and growth enhancing optimum crop production. The land/seedbed can be prepared by means of several methods including; digging, shoveling, picking and hoeing. These practices are very effective in reducing soil erosion (by wind and water), runoff and water evaporation. Tillage promotes water infiltration and moisture retention. This practice promotes water conservation as it allows the soil to absorb more water (precipitation or irrigation) while loosing very little via the process of evaporation (Unger and Howell, 1999). Tillage practices are very effectively appropriate and therefore can be used by the agricultural industry to improve food crop production.

2.9.2 Improved varieties / cultivars

Plants can be mainly improved by using two methods namely: conventional plant breeding and plant biotechnology. Both the methods involve changing genetic composition of plants to improve them to suit human needs.

2.9.2.1 Conventional plant breeding

Conventional plant breeding involves changing the genetic composition to improve varieties. Cultivars are improved for tolerance to environmental stress factors (drought, salinity, high temperatures), diseases (pests) and also to improve the yield and quality of developed varieties. This plant breeding method involves crossing two plants (male and female) to combine the desired traits from both parents [Organisation for Economic Co-operation and Development (OECD), 1993].

Conventional plant breeding is regarded as an extremely important tool but it also has some limitations. A cross between two parents may result in the progeny inheriting a mixture of genes (both desirable and negative traits). As a result of this mixture of genes, plant breeders end up back crossing the progeny which is labour intensive, time consuming and also requires sophisticated equipment and techniques (OECD, 1993).

2.9.3 Biotechnology

Abiotic stress such as water deficit, high temperatures and salinity are some of the factors which negatively affect the agricultural industry by lowering crop quality and production. Plant genetic transformation for stress tolerance and resistance improves plants for cultivation in drier areas (Zhang *et al*., 2000). Genetic engineering is emerging as a very successful tool for the agricultural community. The practice involves the ability to insert a DNA segment into an organism which will alter its genetic makeup. Enzymes are used to remove a DNA segment (from another organism) which codes for a desired trait (for example, drought resistance) and incorporate it into that of a host. Single celled organisms like bacteria (*Agrobacterium tumefaciens*) are mainly used for genetic manipulation of plants. In the farming industry, crops are mainly improved for resistance against abiotic stresses such as drought, salinity and extreme temperatures. Genetically improved cultivars can help in increasing crop yield production in drylands (Hu *et al*., 2006).

Genetic modification (GM) differs from conventional plant breeding mainly because instead of mixing a lot of genes from two sexual parents, only a desired specific gene will be isolated and inserted into a plant of interest. This plant breeding technique is convenient and time friendly. GM avoids random mixture of negative (undesirable) genes and also allows genetic diversity since it allows a mixture of genes even in organisms which are not closely related. GM is therefore viewed as an imperative tool to solve global environmental challenges (Jauhar, 2006).

Both conventional breeding and biotechnology require prior knowledge of cultivars or organisms with desired traits. Screening and selection of cultivars or organisms for traits is therefore a necessary step preceding breeding.
Water is scarce. It can negatively impact soybean yield / productivity. Therefore there is a need to develop ways of assessing cultivars for tolerance to drought to improve output or yield.

2.10 How plants adapt to drought

Drought stress is a complex process which negatively affects plant growth and reproduction. Plants respond to drought stress differently. The type of mechanism used by plants to adapt to dry conditions depends on the type of plant, the growth stage during which the stress occurs and the intensity of the stress (Izanloo *et al*., 2008).

Plants possess several adaptive traits to endure periods of drought. Certain plants, including soybean have devised mechanisms (escape, tolerance, avoidance) which are induced by stressors like drought to survive under low water conditions. Plants escape drought stress by shortening their life cycle therefore maturing earlier. Another mechanism used by plants to adapt to drought stress is avoidance where water loss is reduced and absorption is increased. Drought tolerance is a very complex mechanism which involves several aspects including osmotic adjustment and increased antioxidant activity (Yoshimura *et* al., 2008). These plants use a series of morphological, physiological, cellular and molecular processes to respond to drought stress (Cellier *et al*., 1998; Shinozaki and Yamaguchi-Shinozaki, 2007).

2.10.1 Morphological adaptations

Heschel and Riginos (2005) reported that plants can respond to water stress by reducing their leaf sizes and which will help enable them to maintain high water potential. Geophytes survive water deficiency by losing all their vegetative parts (die) during drought periods and then rise again when water becomes available (Zidenga, 2006). The morphological features associated with drought stress in plants include but are not limited to reduced leaf surface area as well as flowers and pod abortion (Kisman, 2003).

2.10.2 Physiological adaptations

Plants can escape drought stress by rapidly increasing their growth rate to reach their maturity stage before the stress becomes intense. They can also adapt to water stress physiologically by absorbing more water while reducing the rate of water loss

via transpiration. Transpiration rate can be decreased by lowering stomatal conductance and reducing the leaf surface area. Plants can also survive water deficit by osmotic adjustment (maintaining turgor pressure) during drier periods (Izanloo *et al*., 2008).

Generally, plants use two mechanisms namely: non-enzymatic enzymatic and pathways to scavenge damaging ROS (Masoumi *et al*., 2011). Non-enzymatic mechanisms employ several secondary metabolites to prevent formation of or scavenge ROS. Enzymatic pathways involve the use of different enzymes to eliminate the unwanted toxins (Blokhina *et al*., 2003).

2.10.2.1 Production of antioxidants (non-enzymatic mechanism)

Antioxidants are secondary metabolites which are produced by stressed plants. They are mainly produced as a form of defensive mechanisms against oxidative stress (caused by free radicals) and animals (herbivores) and pests. Examples of antioxidants include flavonoids, tannins, phenolics, ascorbic acid and glutathione. The type of secondary metabolites produced by a plant depends on the variety of a plant (Stajner *et al*., 2009).

Many foods such as fruits, vegetables and grains contain antioxidants. Antioxidants are capable of delaying, retarding or minimising the development of rancidity, thus maintaining nutritional quality and increasing the shelf life of products (Maisuthisakul *et al*., 2005). Like most plants, soybeans use antioxidant systems such as ascorbic acid, and phenolic compounds to scavenge or prevent the formation of ROS (Sakihama *et al.*, 2002; Blokhina *et al*., 2003; Zidenga, 2006).

2.10.2.2 Production of radical scavenging enzymes (enzymatic mechanism)

The enzymes involved in scavenging ROS include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). These enzymes help the plants by preventing the formation of or quenching toxic compounds minimizing the oxidative damage caused (Mittler *et al*., 2004). The enzymes basically catalyze the conversion of toxic ROS to less harmful substances (Yordanov *et al*., 2003).

SOD is a key enzyme which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. CAT is responsible for catalyzing the conversion of hydrogen peroxide into oxygen and water. The latter is very important because of its highest turnover number which is the ability to carry out millions of reactions in a second. GPX is involved in the reduction of lipid hydroperoxides to alcohol and it also reduces free hydrogen peroxide to water (Masuomi *et al*., 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Research design

The research was undertaken at the University of Limpopo (Turfloop campus). Soybean plants were grown (for 16 weeks) in a glass house; given same amounts of nutrients but differing amounts of water (limited and overwatering). Measurements were carried out on different parameters to see the effect of water limitation on the growth and yield of the different soybean cultivars.

3.1.1 Selection of soybean varieties with potential to drought tolerance

Data (unpublished) from the Agricultural Research Counsel (ARC) was used to select six soybean cultivars namely: Mopani, Sonop, Knap, Pan 1564, LS 677 and LS 678 that are high or low yielding in warmer areas of South Africa. These were selected to represent three drought tolerant and three drought susceptible cultivars. Two imported cultivars (R01 416F and R01 581F) were included for comparison as they are known to be drought tolerant.

3.1.2 Plant establishment

Soybean plants (from each of the selected varieties) were grown in vermiculite in plastic pots in a glasshouse in a randomised block design. Before sowing, the seeds were inoculated with the nitrogen-fixing bacteria *Bradyrhizobuim japonicum*. The plants were supplied with equal amounts of nutrient solution (3.1.3) and different amounts of distilled water starting at R1 growth stage shown on Figure 2 below. The controls were watered to saturation (until water leaks at the bottom of the pots). There were three pots per treatment with each pot containing three plants.

Figure 3.1: Soybean plants at R1 growth stage.

3.1.3 Treatments

All soybean plants were given an equal amount (300 ml) of nitrogen free nutrient solution (Appendix D) once a week and varying amounts of water (distilled) twice a week. Treatments were started at R1 growth stage.

Treatment A (control): Soybean plants were watered to saturation – until water leaks out through the small holes at the bottom of the plastic pots. The plants were given 600 ml twice a week. The total volume of watering per week was 1500 ml.

Treatment B (experimental): Half (300 ml) of the volume given in A twice a week. The total watering added up to 900 ml.

Treatment C (experimental): A quarter (150 ml) of the volume given in A twice a week adding up to the total of 600 ml watering per week.

3.2 Data collection

The following determinations and observations were made on each soybean plant from each treatment at R3 growth stage. All observations were made in duplicates on both control and treatments.

3.2.1 Plant height

The height of all plants was measured in centimetres from just above the level of the vermiculite to the tip of the plant. A long wooden ruler (1 metre) was used to measure the height of the soybean plants.

3.2.2 Flower and seed counts

The number of flowers and pods produced or aborted and the numbers of seeds per pod were counted directly from each plant in all treatments and controls.

3.2.3 Percentage chlorophyll

Percentage chlorophyll (calculated as percentage of chlorophyll absorbance over total pigment absorbance) in the leaves was measured on the youngest fully expanded leaf using the Minolta Chlorophyll meter (SPAD-502 Minolta) which measures chlorophyll fluorescence directly from the leaves without removing them from the plants. Data was collected every second week starting from two weeks after commencement of the treatments.

3.2.4 Growth medium moisture content

A Theta moisture probe (type ML1) was dipped into the growth medium (vermiculite) to determine the water content of the growth medium. The moisture content was measured every second week after the onset of treatments.

3.2.5 Leaf surface area (LSA)

Three leaves per plant from all treatments were traced on a blank paper; the leaf traces were cut out and weighed on an electronic balance in triplicates and the mean was used for calculations. A 5.00 cm x 5.00 cm square of blank paper was weighed. The formula below was used to estimate the leaf surface area of each leaf:

$$LSA = \frac{25.00\ cm^2 \times \text{mass of leaf trace}}{\text{mass of } 25.00\ cm^2 \text{ paper}}$$

3.2.6 Relative leaf water content (RLWC)

The middle leaflet of the trifoliate (three per plant per treatment) was detached from the plants and weighed immediately to determine the fresh weight. The leaflets were completely immersed in distilled water for 24 hours. After 24 hours, water was blotted from the leaflets and the leaflets were re-weighed to determine the saturated weight. They were then dried in an oven at 60°C for 24 hours. When the leaflets were completely dry they were weighed three times until a constant weight (dry weight) was achieved. The following formula was used to calculate the leaf relative water content:

$$LRWC = \frac{Fm - Dm}{Sm - Dm}$$

Where:

Fm =fresh mass

Sm = saturated mass and

Dm = dry mass

3.3 Sampling for physiological analysis

Two leaflets per plant from each treatment were harvested and immediately frozen in liquid nitrogen and stored at -70 ºC.

The samples collected were used to determine total phenolics, total flavonoids, ureides contents as well as antioxidant activity which were analysed spectrophotometrically. The analyses were done in duplicates on each leaf collected.

3.3.1 Determination of total phenolics

Total phenolics were determined spectrophotometrically according to the Folin-Ciocalteau method (Torres *et al.* 1987).

3.3.1.1 Phenolic extraction

Frozen plant leaf material was ground to a fine powder in liquid nitrogen. A 100 mg mass of the powder was weighed out in duplicates into 150 ml Erlenmeyer flasks and 15 ml of methanol added. The flasks were stoppered and phenolics extracted on a shaker for two hours. The extracts were filtered into 50 ml volumetric flasks through Whatman No. 1 filter paper. The residue was washed three times with 10 ml volumes of methanol and the extracts made to 50 ml volume with methanol.

3.3.1.2 Phenolic analysis

A 500 µl volume of each extract was pipetted in triplicates into 10 ml volumetric flasks and 5.0 ml of distilled water was added. Folin-Ciocalteau (0.5 ml) was added to the mixture, mixed thoroughly and allowed to stand for five minutes at room temperature. A volume of 1.50 ml of 20 % sodium carbonate was added and the extracts made to final volume with distilled water. The extracts were mixed thoroughly and incubated at 50 °C for two hours. The mixture was vortexed and absorbance read at 765 nm using Varian Cary IE UV-Visible Spectrophotometer.

3.3.1.3 Phenolic standard curve

Gallic acid (0.200 g) was dissolved in methanol and made to a final volume of 100 ml to make a stock solution of 2000 mg/l. A dilution series of 0.00, 2.00, 4.00, 6.00, 8.00, 10.00, 12.00 and 14.00 mg/l was made into test tubes in duplicates. Folin-Ciocalteau reagent was used to make a preparation as above (3.3.1.2) and absorbance read at 765 nm. A standard curve was plotted from the values and total phenolics of the extracts were calculated from the curve as gallic acid equivalents.

3.3.2 Determination of total flavonoids

Total flavonoids were determined as described by Marinova *et al (*2005).

3.3.2.1 Flavonoid analysis

An aliquot of 500 µl of the extract (3.3.1.1) was pipetted into a 10 ml volumetric flask. A volume of 2.0 ml distilled water was added followed by a 1.5 ml of 5 % sodium nitrate and mixed well. The extracts were incubated for five minutes at room temperature. A volume of 0.15 ml of 10 % aluminium chloride was added and the extracts were incubated again for six minutes. A volume of 1.0 ml of 1M sodium hydroxide was added and distilled water was used to make the extracts to 10 ml volume. The solutions were thoroughly mixed and absorbance read at 510 nm. The model Varian Cary IE UV-Visible Spectrophotometer was used.

3.3.2.2 Flavonoid standard curve

Catechin (0.200 g) was dissolved in methanol and made to a final volume of 100 ml to make a stock solution of 2000 mg/l. A dilution series of 0.00, 2.00, 4.00, 6.00, 8.00, 10.00, 12.00 and 14.00 mg/l was prepared in duplicates in test tubes. Further preparations were done as in (3.3.2.1) above and absorbance read at 510 nm. A standard curve was plotted from the values and total flavonoids of the extracts were calculated from the curve as catechin equivalents.

3.3.3 Determination of antioxidant activity

The 2.2-Diphenyl-1-picrylhydrazyl (DPPH) method (Odhav *et al*., 2007) was used to determine the antioxidant activicty.

3.3.3.1 Antioxidant assay

A volume of 2.5 ml of the plant extracts in (3.3.1.1) was pipetted into a 150 ml flask. A 1.0 ml volume of 0.3 mM DPPH (in methanol) was added and thoroughly mixed.

Methanol (2.5 ml) was used as a blank or negative control. The extracts were incubated at room temperature for thirty minutes and absorbance read at 518 nm.

3.3.3.2 Antioxidant standard

A volume of 1.0 mM of ascorbic acid was prepared then a 2.5 ml volume was used like the plant extract above as a positive control and absorbance measured at 518 nm. Scavenging capacity was determined using the formula below:

% Scavanging capacity = 100 – [Abs. of sample – Abs. of blank] X 100/Abs. of positive control.

3.3.4 Extraction and quantification of ureides

Ureides were extracted as described by Van Heerden *et al*. (2008).

3.3.4.1 Ureide extraction from leaves and nodules

Frozen material was ground in liquid nitrogen and the ureides (allantoin and allantoic acid) were extracted with 1.0 ml of 0.2 m NaOH. Samples were boiled for 20 minutes in 2.0 ml microfuge tube to convert all allantoin to allantoic acid. Ice was used to cool down the samples to room temperature. Samples were centrifuged at 10 000 xg for 10 minutes. A volume of 350 µl of distilled water was added to 50 µl of the extracts. The ureide content was determined by following the procedure below (3.3.4.3).

3.3.4.2 Allantoin standard curve (0.0 – 8.0 µg)

Allantoin standard solution (0.1µg/µl) was prepared fresh on the day of use. A standard series of 0.0, 0.50, 1.00, 1.50, 2.00, 4.00, 6.00 and 8.0 ng/µl range was used for the preparation of the standard curve.

A volume of 80 µl reagent A was added to each tube containing 400 µl of diluted plant extract (above) or allantoin standards. The extracts were vortexed and boiled for 10 minutes. Reagent E (160 µl) was added and extracts vortexed and boiled for two minutes. Ice was used to briefly cool the extracts to room temperature. A volume

of 360 µl reagent F was added and extracts vortexed and then incubated at room temperature for 10 minutes. The extracts were briefly centrifuged just prior to measuring the absorbance if necessary. The absorbance was measured at 525 nm.

3.4 Anatomical investigation

An anatomical study was carried out on petioles, roots and nodule samples for each treatment of the eight different soybean varieties according to Rajan (2003).

3.4.1 Preservation

Plant samples were collected and immediately immersed in the preservative formalin acetic acid (FAA) in small vials. The specimens were preserved to stop all the metabolic processes. The plant materials were left in the preservative fluid for a day.

3.4.2 Dehydration

The specimen were removed from the preservative and thoroughly washed in distilled water. The dehydration method involved seven stages during which the samples were immersed in the necessary fluid for a day. An ethyl alcohol series of 50 %, 70%, 90% and 100 % was used for stages 1 to four respectively. For the fifth stage, a mixture of 1:1 ratio of absolute alcohol and xylol was used. For stage 6, the specimens were placed in a mixture of 25 % absolute alcohol and 75 % xylol. On the last stage (day 7), the plant tissues were dipped in to absolute xylol solution for 24 hours. After the seventh day, the plant material was completely dehydrated and looked transparent.

3.4.3 Infiltration

The purpose of this step was to ensure that the paraffin wax completely entered into the plant tissues so that the material can be easily cut. Two to four pieces of paraffin wax were added into the vials containing the specimen daily for five days. The vials were transferred into a hot air oven with the temperature adjusted to the melting point of the wax. Few pieces of wax were added daily until all the xylol in the vials

was replaced by paraffin wax. This was achieved by ensuring there was no smell of xylol when the vials were smelt.

3.4.4 Paraffin embedding

Match boxes were used. Glycerine was smeared into the inner surface of the boxes. Melted paraffin was poured into the boxes and the contents of the vials were emptied into the boxes. The plant materials were quickly arranged in proper order before the paraffin solidified. The preparations were left to cool for eight hours. After the paraffin had solidified, the blocks in which the specimen were embedded were removed from the box and cut into suitable pieces.

3.4.5 Tissue sectioning

A sliding microtome (Reichert Austria Nr 307198) was used. The microtome was set at 15 µm. The paraffin blocks were fixed to wooden 'riders'. This was done by heating the top of the riders and the base of the paraffin block and fixed then allowed to cool at room temperature. The tissue blocks were placed on the stage with the large part of wax below and the knob was tightened. The blade was cleaned by immersing it in xylol and wiped. All the knobs (blade holder, block stage) were tightened. The tissues were cut by sliding the microtome. As the microtome was slid, the cylinder holding the rider moved to expose the block to the knife edge. The cut sections were transferred to the water bath at 50^{O} using a fine brush. Glass slides were immersed in to the water to allow the sections to cling on them. The slides were allowed to stand overnight to allow the sections to stick to the slides.

3.4.6 Staining

The slides with the paraffin ribbons adhering to them (with plant sections) were immersed in a coupling jar containing xylol for ten minutes for surface decalcification. The paraffin dissolved in xylol and only the cut sections remained on the slides. The slides were kept in 50:50 xylol and absolute alcohol and passed through a down grade series of alcohol of 100%, 90%, and 70%, washed in distilled water to remove traces of alcohol and then transferred to Saffranin (which was used as the main

stain) for 15 to 30 minutes. After washed in distilled water, the slides were observed under a light microscope and were overstained. Distilled water was used to destain the specimen for 30 minutes. They were passed through an upgrade series of alcohol (70%, 90%, and 100%) for dehydration. The slides were stained with Fast green (counter stain) for two minutes. Oil of clove was used to wash off the excess stain. The specimens were then transferred to xylol.

3.4.7 Mounting

Glycerine jelly B was used as a mounting medium. Suitable drops of mounting medium were placed on the specimen (on top of the slides). A glass coverslip was held at the edges and allowed to touch the edge of the mounting medium at an angle of 45^{O}. A needle was used to slowly lower the coverslip on the mounting medium to avoid trapping air bubbles. Excess mounting medium was wiped off using a paper towel. The slides were allowed to dry before they were observed under a light microscope.

3.5 Seed dry mass

At growth stage R8 the remaining plants (ready for harvest) were harvested individually, the seeds were dried at 60 °C for twenty four hours and weighed until a constant mass was obtained.

3.6 Data analysis

Data for the different parameters (analyses) obtained above were used for statistical analysis. One-way Analysis of Variance (ANOVA) was used to determine if there were significant differences among the cultivars in the different treatments. Between-treatment variances were compared with within-treatment variances at the $P<0.05$ level of confidence. When within-treatment variances are larger than the between-treatment variances, it shows that there are real differences among the cultivars rather than chance differences. The program Statistical Package for Social Sciences (SPSS), PASW (Predictive Analytical Software) 18 was used for the analysis.

CHAPTER 4: RESULTS

4.1 Plant height

As indicated in Table 4.1, plant height was assessed on the basis of stem length measured in centimetres. Water limitation affected soybean growth by decreasing the stem length from the A to the C treatments. The tallest stems were those of the cultivar LS 678 in the A treatment and the shortest were those of Pan 1564 from the C treatments. On average, stem length was decreased by 5.0 % in the B treatments and by 15.4 % in the C treatments. However, water limitation did not have much effect on the plant height of the two cultivars R01 416F and R01 581F. The height of R01 581F fluctuated among the treatments. The stem lengths of 31.2 cm and 31.4 cm were measured on both A and C treatments of the cultivar R01 581F respectively whereas the plants on B treatment grew up 30.3 cm. The plant height of the cultivar R01 416F was 26.4 cm for treatment A which dropped and remained at 23 cm for both treatments B and C. The effect of water stress was more pronounced on LS 678 where the height dropped from 50.1 cm in treatment A to 39.2 cm in treatment B and further reduced to 27.3 cm in treatment C with reduced water availability.

Figure 4.1: Soybean cultivars LS 678 and Pan 1564 treatments A, B and C at R3 growth stage.

Table 4.1 Average stem length (cm) of the cultivars from different treatments at R3 growth stage

Cultivar	Treatment A (1500 ml watering)	Treatment B (900 ml watering)	Treatment C (600 ml watering)
Pan 1564	23.4 +/- 0.28	22.1 +/- 0.14	21.6 +/- 0.77
Knap	39.2 +/- 0.42	37.7 +/- 0.56	36.6 +/- 0.14
Mopani	33.2 +/- 0.35	32.1 +/- 0.21	25.8 +/- 0.14
Sonop	40.3 +/- 0.28	37.2 +/- 0.07	35.3 +/- 0.42
LS 677	32.5 +/- 0.28	31.6 +/- 0.28	28.3 +/- 0.14
LS 678	50.1 +/- 0.56	39.2 +/- 0.28	27.3 +/- 0.07
R01581F	31.2 +/- 0.84	30.3 +/- 0.21	31.4 +/- 0.07
R01416F	26.4 +/- 0.35	23.4 +/- 0.49	23.2 +/- 0.21

Values are means of six samples for A & B and nine samples for C+/- standard deviation

4.2 Number of flowers, Leaf Surface Area and Relative Leaf water content

Table 4.2 below shows the number of flowers, LSA and RLWC of all the eight soybean cultivars at R3 growth stage. Water stress reduced all the three parameters for the six South African cultivars (Pan 1564, Sonop, Mopani, Knap, LS 677 and LS 678) whereas the two American cultivars (R01 416 and R01 581) showed a different trend. Cultivar R01 416F had a mean of 10 flowers at treatment A which decreased to 7 under B treatment, however the number of flowers increased to the mean of 8 under reduced water treatment. A different trend was observed in the same cultivar for the LSA where the B treatment has shown the largest LSA of 44 cm^2 which is far higher than both A (28 cm^2) and C (24 cm^2). All the other seven cultivars (Pan 1564, Sonop, Mopani, Knap, LS 677, LS 678 and R01 581) responded to drought stress by decreasing the LSA. Furthermore, cultivar Mopani has the largest LSA where A has 114 cm^2, B 66 cm^2 and C 39 cm^2.

Reduced water levels also had a negative effect on the RLWC R01 581F, Knap, Sonop, Mopani and Pan 1564. The RLWC of these two cultivars, LS 677 and LS 678 decreased for B treatment then increased under water stress. The American cultivar R01 416F had the same RLWC of 89 % for both treatment A and B which was reduced to 75 % under water stress.

Table 4.2 The average number of flowers, LSA and RLWC measured at R3 growth stage

Cultivar	No. flowers	LSA (cm^2)	RLWC (%)
Pan 1564 A	10 +/- 4.24	46 +/- 3.88	94 +/- 2.57
B	9 +/- 3.53	29 +/- 2.81	83 +/-1.72
C	7 +/- 1.41	10 +/-1.44	65 +/- 0.89
Knap A	10 +/- 0	42 +/-0.36	83 +/- 2.16
B	8 +/- 3.53	29 +/- 1.53	69 +/- 1.57
C	6 +/- 0	21 +/- 0	60 +/- 0.42
Mopani A	9 +/- 2.82	114 +/- 2.14	77 +/- 0.76
B	8 +/- 0.70	66 +/- 0.81	74 +/- 1.52
C	6 +/- 2.82	39 +/- 1.57	61 +/- 0.71
Sonop A	9 +/- 0.70	49 +/- 0.56	90 +/- 0.73
B	6 +/- 5.65	38 +/- 3.81	84 +/- 1.43
C	5 +/- 1.41	19 +/- 2.40	80 +/- 2.14
LS 677 A	8 +/- 2.12	45 +/- 3.14	94 +/- 0.24
B	8 +/- 0.70	36 +/- 0.72	80 +/- 1.36
C	6 +/- 0	21 +/- 0.19	88 +/- 1.47
LS 678 A	10 +/- 2.12	38+/- 1.36	88 +/- 1.13
B	8 +/- 0	25 +/- 0.17	88 +/- 0.32
C	7 +/- 4.24	16 +/- 3.98	75 +/- 2.81
R01 581F A	8 +/- 1.41	58 +/- 1.57	88 +/- 1.33
B	10 +/- 1.41	40 +/- 1.68	82 +/- 0.93
C	8 +/- 0.70	15 +/- 0.92	77 +/- 0.72
R01 416F A	10 +/- 1.41	28 +/- 1.64	89 +/- 1.49
B	7 +/- 0.70	44 +/- 0.71	89 +/- 0.65
C	8 +/- 0.70	24 +/- 0.63	75 +/- 0.68

Values are means of six samples for A & B and nine samples for C+/- standard deviation

4.3 Moisture content of the growth medium

Generally, limited water levels decreased the moisture content of the vermiculite (growth medium) of all the cultivars studied as shown on Table 4.3 below. Furthermore, the moisture content within the treatment was reduced with time. Bigger plants loose more water and transpired more than smaller plants.

Table 4.3 Growth medium moisture content measured at two weeks intervals after the onset of the treatments

Cultivar	% Moisture (week 2)	% Moisture (week 4)	% Moisture (week 6)	% Moisture (week 8)
Pan 1564 A	34.1	32.2	29.3	25.4
B	26.3	24.3	20.2	19.5
C	11.6	10.2	8.3	7.3
Knap A	22.2	20.3	19.1	15.2
B	13.5	11.2	10.2	9.3
C	8.1	62.3	4.3	3.2
Mopani A	31.6	28.1	24.2	22.1
B	18.1	16.2	15.2	11.2
C	8.2	6.3	5.2	4.3
Sonop A	25.6	20.2	20.1	16.6
B	12.1	11.1	9.2	6.2
C	11.2	9.2	7.3	4.1
LS 677 A	24.4	21.5	20.2	19.1
B	17.4	14.2	12.1	8.2
C	9.2	7.5	5.4	4.5
LS 678 A	31.3	27.2	23.3	20.3
B	22.3	21.5	19.2	17.2
C	10.2	9.2	6.1	4.1
R01 581F A	33.1	31.5	23.3	21.3
B	28.2	23.2	21.1	19.1
C	8.5	7.5	5.3	4.5
R01 416F A	33.3.	31.2	23.1	21.4
B	28.6	23.3	21.3	19.2.
C	8.3	7.1	5.1	4.4

4.4 Percentage chlorophyll

The effect of water deficit on percentage chlorophyll showed a similar trend, decreased with increasing water stress, with that of relative leaf water content. Percentage chlorophyll was also negatively affected by reduced water levels (Table 4.4). This was true for all the studied soybean cultivars except LS 677, LS 678, R01 416F and R01 581F where the percentage chlorophyll fluctuated among the treatments.

For the cultivar LS 677, treatment B had percentage chlorophyll of 43 which was higher than both A (39) and C (41) (Table 4.4). It seems the cultivar was sensitive to both too much and too little water available.

As indicated in Table 4.4, for the first data (second week) collected, the percentage chlorophyll of the cultivar LS 678 was 41 under the A treatment which dropped and remained at 38 for both B and C treatments. During the fourth week, the chlorophyll content was constant (35 %) for both treatments A and B which was reduced to 33 % in treatment C. For the sixth week, the chlorophyll content decreased with lowering water levels. Data for the eighth week indicated that the percentage chlorophyll dropped from 36 (A treatment) to 30 (B treatment) which increased to 31 under treatment C.

The two American cultivars R01 416F and R01 581F showed no trend, the values for the percentage chlorophyll fluctuated between treatments with little variations among the treatments as well as the cultivars themselves.

Table 4.4 Mean percentage chlorophyll measured at two weekly intervals

Cultivar	% chlorophyll (week 2)	% chlorophyll (week 4)	% chlorophyll (week 6)	% chlorophyll (week 8)
Pan 1564 A	41.2	36.1	36.2	35.1
B	40.3	34.2	34.3	35.4
C	35.2	30.3	33.3	32.2
Knap A	42.1	36.3	37.2	35.1
B	39.3	33.3	34.4	30.2
C	33.6	31.2	30.3	26.3
Mopani A	38.5	30.2.	28.2	26.5
B	34.2	28.1	25.2	23.4
C	31.3	25.5	24.4	21.2
Sonop A	38.2	30.2	28.1	26.3
B	34.1	28.4	25.4	23.2
C	31.5	25.2	24.2	21.1
LS 677 A	39.6	38.1	37.3	36.2
B	43.4	34.2	32.4	33.3
C	41.2	33.3	30.2	31.2
LS 678 A	41.1	35.4	36.3	36.1
B	38.5	35.5	32.1	30.2
C	38.4	33.2	33.2	31.3
R01 581F A	36.1	31.1	30.4	30.2
B	31.2	33.2	31.5	28.1
C	38.3	35.3	32.3	31.2
R01 416F A	37.6	34.3	31.2	29.3
B	39.2	30.2	32.1	29.2
C	33.4	30.5	30.3	28.1

4.5 Physiological analysis

4.5.1 Total phenolics and total flavonoids

The concentration of total phenolics is generally higher than that of total flavonoids. The following soybean cultivars LS 677, LS 678, and R01 416F showed a higher phenolic content than the others. Cultivars R01 581F, Mopani and Sonop had higher phenolic contents under limited water conditions. Pan 1564 produced higher concentration of phenolics under treatment B.

4.5.2 Antioxidant activity

As indicated in Table 4.5, reduced water availability increased the antioxidant activity of Mopani, Knap, LS 677 and LS 678 while the antioxidant of activity of R01 416, R01 581, Sonop and Pan fluctuated with only slight differences among the three treatments. Furthermore, Sonop was shown to have the highest antioxidant activity of 75 % (A treatment), 70 % (B treatment) and 77 % (C treatment).

4.5.3 Ureide content

The seven cultivars namely; Pan 1564, Mopani, LS 677, LS 678, R01 416F and R01 581F indicated a higher concentration of ureides in the leaves under treatment B. However, cultivar Knap has reacted slightly different whereby the ureides concentration increased with decreasing water availability (Table 4.5).

Table 4.5 Results of physiological analysis

Cultivar	Total phenolics (µg/g fresh)	Total flavonoids (µg/g fresh)	Non-flavonoid phenolics (µg/g fresh)	Antioxidant activity (% of gallic acid	Ureides content in leaves (µg/g fresh)
Pan 1564 A	27.301 +/- 0.06	20.813	6.488	36.178 +/- 2.77	0.206 +/- 1.02
B	38.571 +/- 0.03	16.428	22.143	47.454 +/- 0.07	0.239 +/- 0.21
C	35.704 +/- 0.05	14.076	21.628	46.886 +/- 0.92	0.175 +/- 0.72
Knap A	24.49 +/- 0.10	19.943	4.547	38.786 +/- 0.17	0.217 +/- 0.39
B	46.309 +/- 0.04	20.67	25.639	49.913 +/- 0.09	0.26 +/- 0.01
C	30.511 +/- 0.06	20.194	10.317	53.339 +/- 1.23	0.294 +/- 0.95
Mopani A	26.614 +/- 0.08	19.067	7.547	42.643 +/- 0.17	0.228 +/- 0.09
B	24.682 +/- 0.14	18.692	5.99	44.251 +/- 0.61	0.304 +/- 0.73
C	32.915 +/- 0.03	14.588	18.327	45.465 +/- 0.49	0.272 +/- 0.38
Sonop A	24.955 +/- 0.07	15.521	9.434	75.885 +/- 0.24	0.277 +/- 0.51
B	24.741 +/- 0.02	14.791	9.95	70.42 +/- 1.62	0.3 +/- 1.52
C	28.516 +/- 0.11	16.61	11.906	77.173 +/- 1.51	0.221 +/- 1.69
LS 677 A	30.715 +/- 0.02	19.652	11.063	30.958 +/- 0.40	0.28 +/- 0.12
B	48.535 +/- 0.04	19.433	29.102	64.134 +/- 0.86	0.365 +/- 0.18
C	56.163 +/- 0.02	18.45	37.713	75.494 +/- 0.68	0.224 +/- 0.67
LS 678 A	33.066 +/- 0.03	19.857	13.209	26.326 +/- 0.77	0.157 +/- 0.84
B	64.011 +/- 0	19.693	44.318	35.693 +/- 0.75	0.293 +/- 0.77
C	52.331 +/- 0.02	19.034	33.297	61.665 +/- 1.50	0.174 +/- 1.70
R01 581F A	29.553 +/- 0.03	19.338	10.215	47.774 +/- 0.32	0.195 +/- 0.24
B	25.314 +/- 0.02	17.88	7.434	42.093 +/- 0.10	0.388 +/- 0.08
C	41.379 +/- 0	17.199	24.18	46.226 +/- 0.31	0.143 +/- 0.37
R01 416F A	65.088 +/- 0	19.227	45.861	47.601 +/- 1.29	0.219 +/- 0.38
B	59.694 +/- 0	18.391	41.303	45.616 +/- 0.05	0.365 +/- 0.05
C	33.457 +/- 0.07	17.928	15.529	49.112 +/- 0.89	0.155 +/- 1.17

Values are means of six samples for A & B and nine samples for C+/- standard deviation

4.6 Anatomy

Anatomical structures of the eight soybean cultivars in all the three treatments were studied based on the transverse sections of the root, the nodule, the petiole and the leaf.

4.6.1 Roots

In general, roots had the vascular system at the centre called a compact central stele. Protoxylem and protophloem were arranged in an alternating manner around the stele. Metaxylem was located on the inner side of the protoxylem. Covering the stele was a thick layer of cells called the endodermis. The cortex was found outside the stele and was composed of a ground tissue, mainly parenchyma tissue.

At the centre of the root was an extensive xylem (Figure 4.2). The xylem arches were not clearly defined and could not be counted. The xylem seemed to have undergone secondary growth and had pushed the cortex to the outside. The extensive xylem could be an adaptation for growth under limiting water conditions since herbaceous plants do not undergo secondary growth.

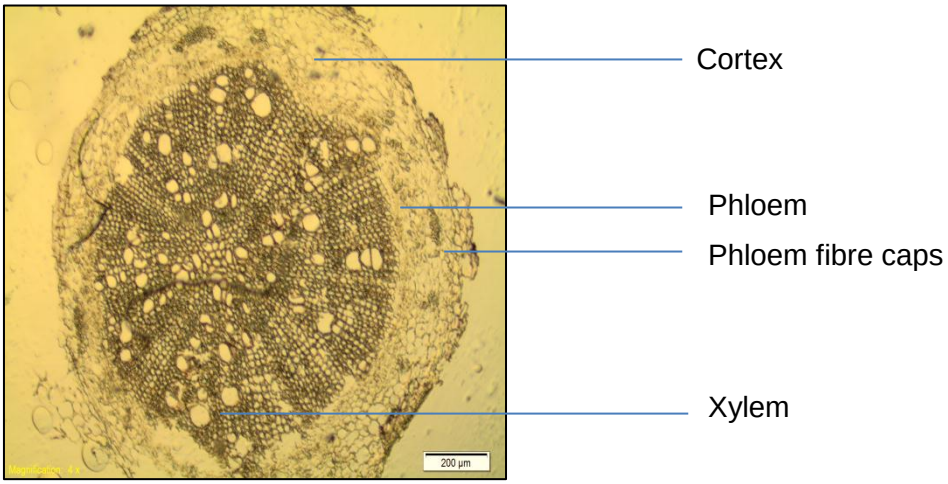

Figure 4.2: Anatomy of the root of soybean cultivar R01 581F treatment A.

For the cultivar R01 581F under treatment A, a pentarch of primary xylem was clearer than that of the cultivar R01 414F. Some secondary thickenings were also observed. The cortex was much smaller and composed of parenhyma tissue.

4.6.2 Nodules

Below the outermost layer (cork) of the nodule was a layer of sclerenchymatous tissue. The vascular system was present in the form of vascular bundles which surrounded bacteria infected parenchyma (large mass) found at the centre of the

nodule (Figure 4.3). The nodule also had uninfected parenchyma tissue located towards the outer parts of the nodule.

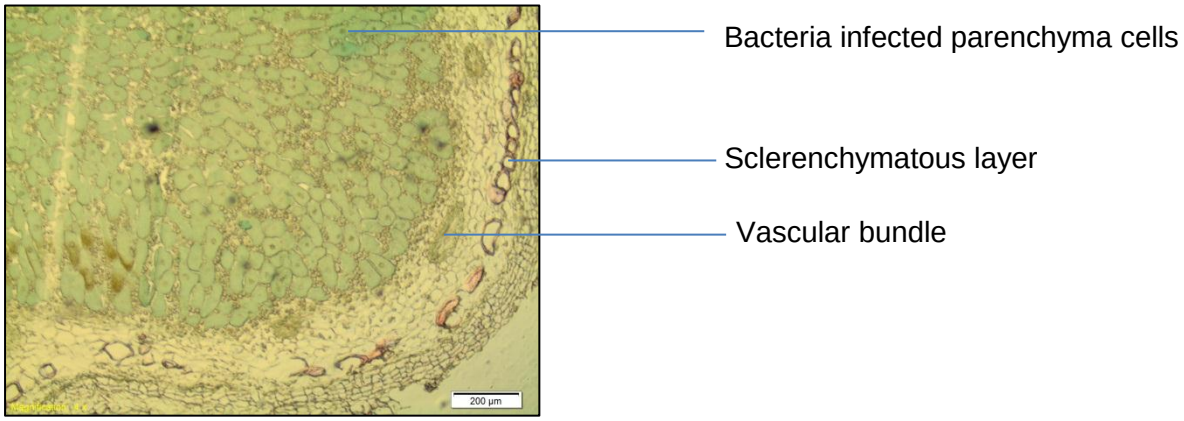

Figure 4.3: Nodule anatomy of the soybean cultivar R01 581F under treatment B.

The sclerenchyma cells of the cultivar R01 581F treatment A were bigger than those of the same cultivar in treatment B. The infected parenchyma cells were medium sized, irregular and located far below the layer of sclerenchyma tissue. The sclerenchyma cells of the nodule in treatment B of the same cultivar were smaller than those in treatment A. The infected parenchyma cells were also irregular but are much closer to the sclerenchyma tissue.

Soybean cultivar LS 677 treatment A showed large and more thickened sclerenchyma tissue. The infected parenchyma cells were not of the same size, the ones on the inside were smaller and roundish whereas the ones towards the outside were bigger and irregular in shape.

For the cultivar Mopani, there were various differences on the anatomy of the nodule within the three treatments. The nodule in treatment A appeared to be unique. The cells of sclerenchyma were very big and some were even clumped together at some points. The infected parenchyma cells were bigger, irregular and very few. The vascular system was present in the form of only one but larger vascular bundle.

Under treatment B, the nodule of Mopani cultivar showed a numerous number of irregular, loosely packed infected parenchyma cells. On the other hand, the sclerenchyma cells were smaller and less thickened. The vascular bundles were also smaller and many.

Under treatment C, the anatomy of the nodule of Mopani cultivar was similar to that of treatment B. Infected parenchyma cells at the centre of the nodule were much smaller and roundish whereas those towards the outside were bigger and some were a little elongated. Sclerenchyma cells were smaller than those in B treatment of the same cultivar (Figure 4.4).

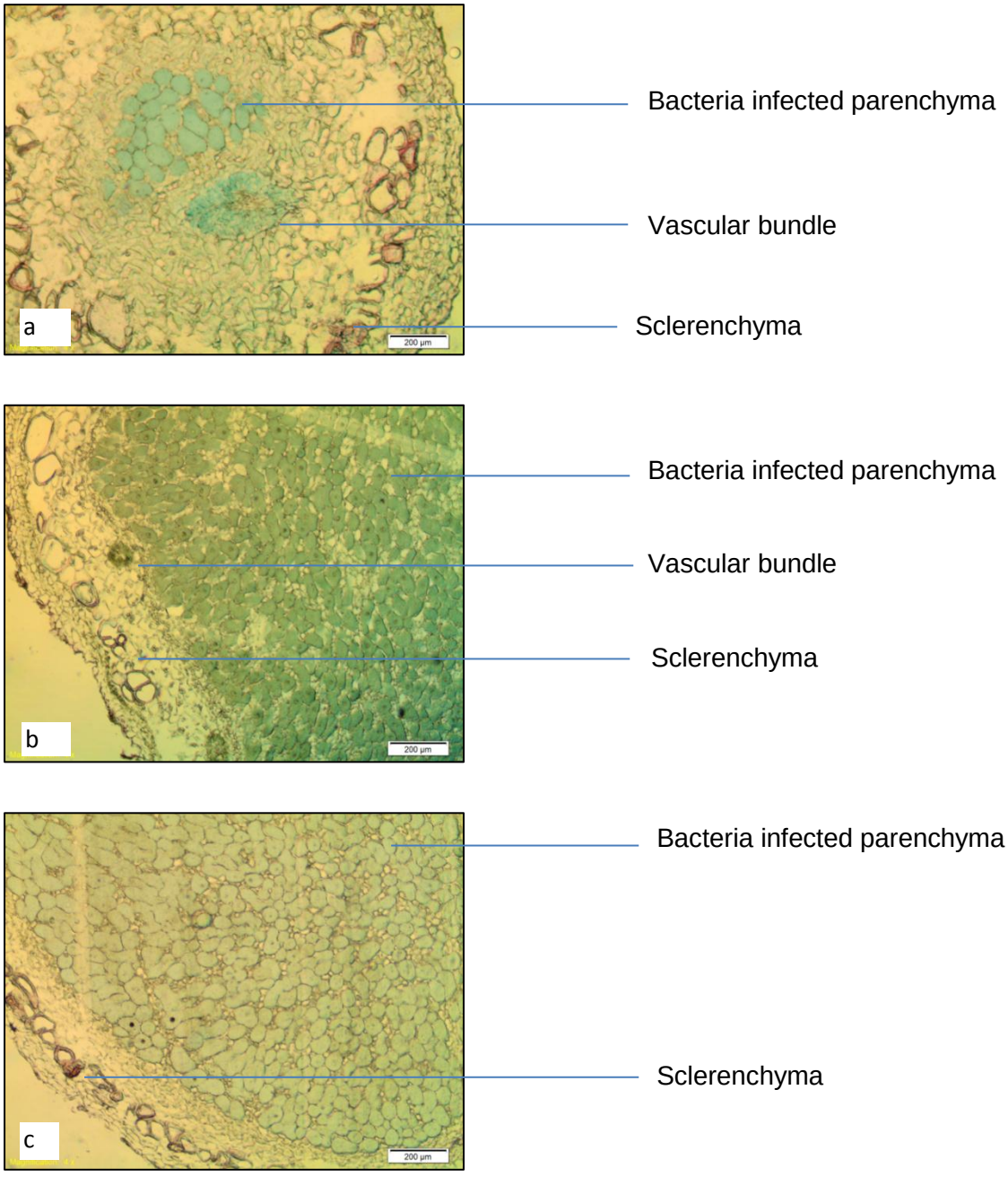

Figure 4.4: Soybean nodule anatomy of Mopani cultivar under treatment A (a), treatment B (b) and treatment C (c).

4.6.3 Leaf stalk (petiole)

The anatomy of a cross section of a petiole was similar to that of a stem. It had the epidermis which was the covering layer under which the cortex (ground tissue) was located. Pith occupied the centre of the petiole. The xylem occurred in the form of a ring surrounding the pith. Unlike in other eudicot petioles, the vascular bundles were not clearly defined and the arrangement was similar to that in the root (Figure 4.2). This looked like an adaptation to limiting water conditions. The phloem occured as a ring surrounding the xylem (Figure 4.5). The phloem was protected by a layer of phloem fibre caps to add strength to the phloem.

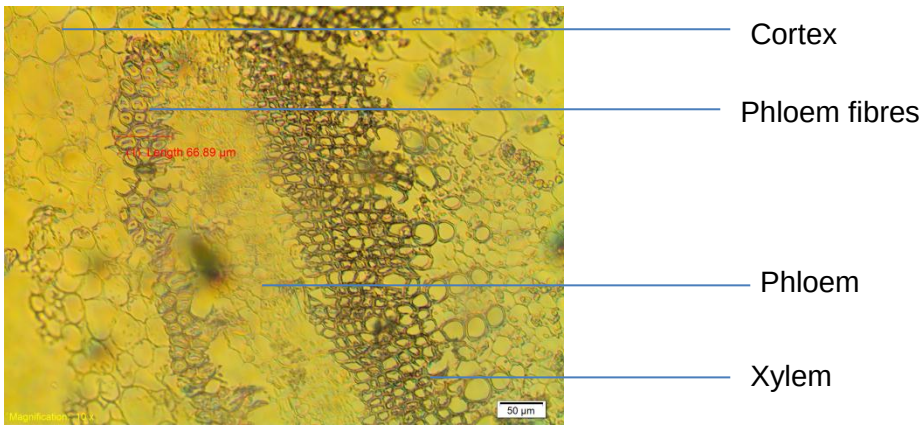

Figure 4.5: A cross section of the petiole of R01 581F treatment B.

4.6.4 Leaves

Like most leaves, the anatomy of soybean leaf dermal tissue was composed of both adaxial (upper) and abaxial (lower) epidermis. Usually the upper epidermis was thicker and had a thicker cuticle than the lower epidermis. More stomata were located on the lower epidermis. Below the upper epidermis, palisade mesophyll cells were located. These were elongated, closely packed parenchyma cells containing chloroplasts. Spongy mesophyll cells were found above the lower epidermis; they appeared smaller and had lots of air spaces between them to allow air movement. They also contained chloroplasts. A midrib (midvein) which is a big vascular bundle was found at the centre of the leaf. Small vascular bundles were also found on the network of the leaf.

The midvein of the cultivar R01 581F treatment B displayed medium sized xylem vessels below which a bundle sheath (sclerenchymatous) was located. This is illustrated in figure 4.6 below. The palisade mesophyll cells appeared to be thin, elongated and loosely packed whereas the spongy mesophylls were small with huge air spaces between them. Both types of mesophyll contained chloroplasts

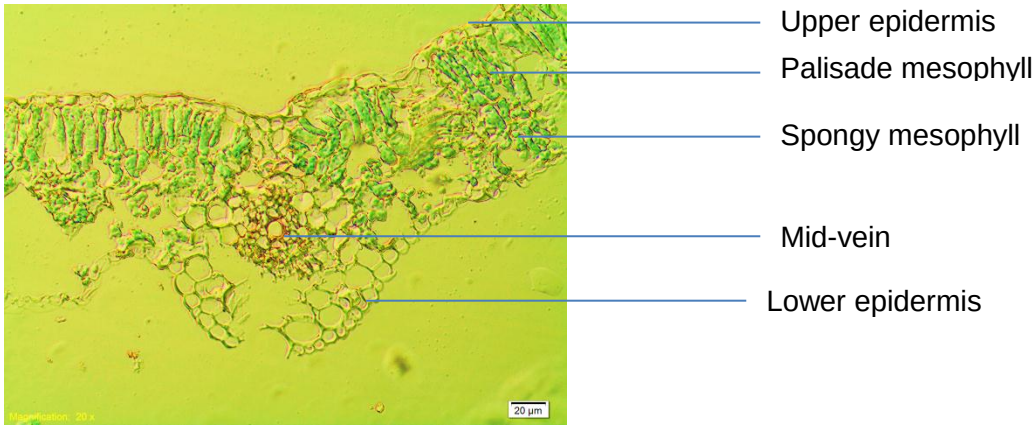

Figure 4.6: The cross section of the leaf anatomy of the cultivar R01 581F treatment B.

A big midvein was seen in the leaf of soybean cultivar LS 677 treatment B. Protoxylem vessels were located towards the inside (to the upper epidermis) and Metaxylem vessels to the outside (lower epidermis). There were lots of big parenchyma cells on both sides of the midrib. The bundle sheath was thick and composed of several layers of sclerenchyma cells. Both upper and lower epidermal layers were thick. Palisade mesophyll cells were thicker, shorter, and more compact containing more chloroplasts. The spongy mesophylls were also thicker with big air spaces and also contained more chloroplasts.

The C treatment of the cultivar LS 677 showed a big difference on the size of xylem vessels in the midvein. The protoxylem vessels were far smaller than those in treatment B but the arrangement was the same, protoxylem to the inside and Metaxylem to the outside. The midvein as a whole was also smaller than that of treatment B but the Metaxylem xylem vessels appeared to be a bit bigger. The big parenchyma cells were also observed on both sides of the midvein. The bundle sheath was also far smaller than that in treatment B. Both mesophyll cells were far

apart and contained fewer chloroplasts. Both epidermal layers were much thicker than in treatment B.

The anatomy of the cultivar Sonop A treatment was similar to that of the cultivar LS 677 C treatment but Sonop was much a bigger specimen. The sizes and arrangement of xylem vessels were also similar to LS 677 C cultivar. A layer of large parenchyma cells was observed above the midvein above which there were smaller parenchyma cells. The upper epidermis was much thicker than the lower one. Mesophyll cells were very small (thin), loosely arranged with fewer chloroplasts and very big air spaces between them especially the spongy mesophylls.

A difference was observed between the leaf anatomies of Knap A treatment and Mopanie A. The epidermal layers in Knap cultivar were thin. The palisade mesophylls were elongated, evenly distributed, had some air spaces in between and contained fewer chloroplasts. It was the same case with spongy mesophyll. Both upper and lower epidermises of Mopani cultivar were thicker and both contained a thick cuticle. Palisade mesophylls appeared shorter, broad, and compact with more chloroplasts. Spongy mesophylls were also bigger and closely packed with smaller air spaces and more chloroplasts.

4.7 Yield

The yield was assessed in terms of the mass of the total number of seeds produced at R8 growth stage. Pan 1564 and Sonop show the same yielding trend among the three treatments, where treatment A yielded the highest (11.4 g) followed by C (10.6 g) then B (10.2 g). In cultivars Knap, LS 677, R01 581F and R01 416F treatment B yielded the highest, followed by treatment A and C which gave the lowest yield. Mopani and LS 678 show a different trend altogether as the yield decreased with the decreasing water availability. The results are displayed in Figure 4.7 below.

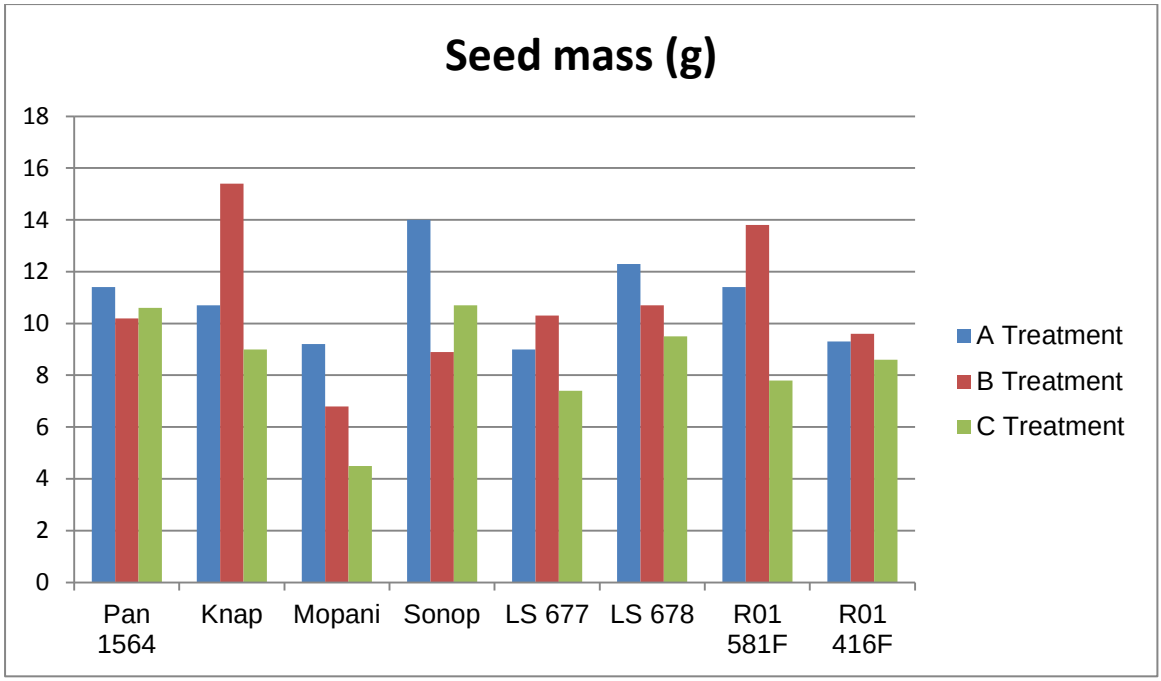

Figure 4.7: Grain yields of the eight soybean cultivars at R8 growth stage under the three treatments.

One way analysis of variance of the yield under limited water supply showed that there was a significant difference in yield among the different cultivars in the under watering treatment. The P value is 0.001 at 95 % level of confidence (Table 4.6). Since this value was less than 0.05 the null hypothesis was rejected. That is, the different cultivars yielded differently under water limiting conditions and had different morphological and physiological traits.

Table 4.6 One way analysis of variance of the cultivar yields under the different water treatments

Treatment		Sum of Squares	df	Mean Square	F	Sig.
Over Watering	Between Groups	127.584	7	18.226	2.858	.016
	Within Groups	255.067	40	6.377		
	Total	382.651	47			
Correct Watering	Between Groups	312.628	7	44.661	10.197	.000
	Within Groups	175.188	40	4.380		
	Total	487.816	47			
Under Watering	Between Groups	256.084	7	36.583	3.958	.001
	Within Groups	591.545	64	9.243		
	Total	847.629	71			

However, the above table does not show which cultivars yield higher than the others. The descriptive statistics table (Appendix A) showed that Sonop was the highest yielding cultivar under limiting water supply. However, the Bonferroni multiple comparison statistics (Appendix B) showed that Sonop yielded significantly higher than only one cultivar among the seven, namely, Mopani. A look again at the Descriptive statistics (Appendix A) showed that Mopani was the lowest yielding cultivar. The Multiple comparisons statistics (Appendix B) indicated that Mopani yielded significantly lower than three of the seven. On this basis, the discussion was focused on characteristics that are shown by the low yielding cultivar and how these might contribute to low yield rather than characteristics associated with high yield. In other words, there is no significantly high yielding cultivar but a significantly low yielding cultivar under limited water supply.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

The aim of the experiment was to identify soybean cultivars that yield higher than the others under limited water supply and also to identify the morphological and physiological characteristics that contribute to the higher yield under such conditions.

5.1 Plant height

Among the eight soybean cultivars studied, six (South African) were negatively affected by reduced water availability whereas water stress did not show much effect on the other two (American) cultivars (Figure 4.7). The effect of water deficit on soybean stem length was also reported by Desclaux *et al*. (2000) who found similar results resembled by the six South African soybean cultivars (Sonop, Mopani, Knap, Pan 1564, LS 677, LS 678) used for the current study. The two American cultivars (R01 416F and R01 581F) were included in this study for comparison as they were registered for improved yield and nitrogen fixation under drought stress by Chen *et al*. (2007). The improvement of these two cultivars is said to have an attribute to their height not being directly affected by water stress. Stem length influences yield in soybean in the sense that flowers are borne at the nodes. The more the nodes produced the greater the potential of producing more flowers and pods.

For Mopani (which yielded significantly lower than the others), the stem length decreased by 3.3 % in treatment B and 22.3 % under treatment C whereas in Sonop (which yielded higher) the length decreased by 7.7 % in treatment B and only 12 % in treatment C. There is a difference of 9.5 cm between Sonop and Mopanie under limiting water availability which is thought to have contributed towards Mopani having the lowest yield. Furthermore, Mopani was better adapted to the change of water availability from treatment A to B where the stem length was reduced by only 3.3 % but the same cannot be said under low water levels (22.3 % reduction of stem length). A different pattern was observed for Sonop, plant height was more reduced under B treatment (7.7 %) and a lower percentage of 12.4 in treatment C. From this realisation, it can then be concluded that the more the stem length is reduced, the lower the plant will yield. Therefore it is concluded that reduced stem length as a result of limited water availability can be used as a morphological marker to identify some low yielding soybean cultivars.

5.2 Flowers, leaf surface area, relative leaf water content and growth medium moisture content

The numbers of flowers and leaf surface area (LSA) were reduced by drought stress in most cultivars. These results were in accordance with Borges, (2004). Reducing the LSA is one of the mechanisms used by plants to reduce transpiration rate which may lead to extreme drying.

There were not many differences between the two cultivars namely Sonop and Mopani in terms of the number of flowers. They both produced nine flowers in treatment A; interestingly the numbers were higher in Mopani for both treatments B and C. From the higher numbers of flowers, one would expect Mopani to yield higher than Sonop but that did not prove to be the case. The cause of low yield in Mopani might be due to production of fewer seeds per pod.

Mopani is the only cultivar with the highest LSA in all three treatments. Due to this, Mopani was prone to drought stress water loss through the leaves. The inability to reduce leaf surface area therefore can be concluded to be another morphological trait which contributes to low yield in soybean. More resources are invested in vegetative growth than in reproductive growth.

Water stress reduced the relative leaf water content (RLWC). Furthermore, there are differences among the three treatments. Higher RLWC under drought treatments were observed on some soybean cultivars. This may be mainly due to such cultivars having the ability to absorb more water from the growth medium or they might have the ability to reduce the transpiration rate. Another possibility is that the retention of water was related to a tolerance strategy, where sugars and or other osmolytes accumulate under water stress. This would lower the osmotic potential of the cells and subsequently cause more water to be retained.

RLWC is very high in all treatments for Sonop as compared to Mopani. The reason behind this finding is thought to be the large LSA in Mopani. Mopani absorbs water from the growth medium and loose it through the leaves (high transpiration rate as a result of LSA) whereas Sonop absorbs water and minimize loss thereof by means of smaller LSA. As a result, RLWC remains higher in Sonop. Therefore, low RLWC can be associated with low yielding soybean cultivars.

As the level of drought stress became intense, the level of water in the growth medium also decreased as a result the plants do not receive enough water to carry on with the metabolic activities. Moreover, the plants also lose more of their water through the process of transpiration (Lobato *et al*., 2008). During dry seasons, moisture content in the ground (growth medium) decreases and become insufficient for the plant to carry on with its daily life metabolic activities.

The growth medium moisture content of Mopani was higher than that of Sonop under treatments A and B, whereas that of Sonop was higher in treatment C. This realisation suggests that Mopani absorbed a lot of water under limited water conditions which in turn was lost through the leaves as a result of the large LSA. On the other hand, Sonop showed a higher moisture content of the growth medium which means it adapted better to lower water availability. As a result Sonop yielded higher than Mopani. Therefore, it is concluded that a large surface are under limiting water conditions is another trait that can be associated with lower yielding soybean cultivars.

5.3 Percentage chlorophyll

Chlorophyll content is a key factor which affects the rate of photosynthesis therefore controlling growth rate of plants. The percentage chlorophyll was higher in well watered treatments than in stressed ones. A reason for this might be destruction of chlorophyll molecules as a result of lipid peroxidation (Hassanzadeh *et al*., 2009) induced by reactive oxygen species. Hassanzadeh *et al*. (2009) indicate that higher yielding sesame genotypes had higher chlorophyll content than low yielding ones.

It was noted that Mopani had lower percentage chlorophyll than Sonop in all treatments. Another interesting observation is that the percentage chlorophyll was more reduced with decreasing water available in Mopani whereas in Sonop the effect was not apparent. Reduced percentage chlorophyll therefore is another character which is an indication of water stress sensitive cultivars. Reduced chlorophyll content in soybean leaves under water deficit was also reported recently by Masoumi *et al*. (2011).

5.4 Phenolics

Phenolic compounds are secondary metabolites produced by plants. There are many types of phenolics with diverse functions. The different types of phenolic compounds include but are not limited to isoflavones, benzoic acids, phenolic acids and flavonoids (Michalak, 2006). Phenolics possess many biological functions such as antioxidant activity and anticarcinogenic functions which are known to promote human health (Marinova *et al*., 2005).

With regard to the phenolic compounds, cultivars Knap B treatment, LS 677 (B and C), R01 416F (A and B) as well as R01 581F (C) showed high levels of total phenolics. A higher antioxidant activity was also observed on these cultivars. Similar results were also reported by Malencic *et al*. (2007).

Both cultivars Mopani and Sonop produced higher phenolic contents under water deficit but that of Mopani was higher than that produced by Sonop under treatment C. Total phenolics produced by Mopani were highest under the C treatment followed by A then lastly B. A different pattern was observed in Sonop where there were not many differences among treatments A and B but treatment C has shown the highest concentration of phenolic compounds accompanied by a high antioxidant activity.

5.5 Flavonoids

Flavonoids are one of the examples of phenolic compounds which are essential health promoters. Like other phenolics, they have antioxidant activities which relieve oxidative stress by removing ROS by non-enzymatic mechanisms. Although phenolic compounds like flavonoids are naturally synthesized by plants, their accumulation is mainly induced by several stress factors like drought and temperature extremes (Sakihama *et al*., 2002).

According to the results obtained in this study, soybean cultivar Pan 1564 showed the highest concentration of flavonoids under the over watered treatment. For the cultivar Knap, the total flavonoids were higher in all the treatments; this was true also for the antioxidant activity and total phenolics. Malencic *et al*. (2007) reported similar findings on their investigation of phenolic content and antioxidant properties of soybean seeds.

Mopani showed a higher concentration of flavonoids than Sonop and the concentration decreased with the decreasing water availability. Sonop in treatment C showed the highest flavonoid concentration than the other two treatments. In addition to that, the concentration was also higher than that of Mopani under water stress. This suggests that the higher concentration of total flavonoids under water deficit increases the ability of plants to tolerate water stress and produce better.

5.6 Antioxidant activity

Antioxidants include both enzymatic (SOD, glutathione) and non-enzymatic (phenolics, flavonoids, tannins). The function of both these antioxidant systems is to scavenge unwanted ROS caused by oxidative stress (Sakihama *et al*., 2002). According to Malencic *et al*. (2007), the DPPH activity only measures the non-enzymatic antioxidant activity.

With regards to DPPH scavenging activity, the cultivars that showed high concentration of phenolics also has high antioxidant activity except for the cultivar Sonop which had the highest antioxidant activity but with lower concentration of total phenolics and total flavonoids. Results also showed that water stress increased the antioxidant activities of most of the soybean varieties.

The antioxidant activity of Sonop was remarkably higher than that of Mopani. For Mopani, the antioxidant activity slightly increased with an increasing water limitation. On the other hand, treatment C of Sonop has shown the highest antioxidant activity followed by A then C treatment. Since Sonop had the highest antioxidant activity, it can then be concluded that cultivars with high antioxidant activity are adapted to higher yield than those with lower antioxidant activities under limited water availability.

Since antioxidant activities are different and complex, it is therefore essential to study and understand the mechanisms involved in soybean plants in order to evaluate and interpret the results obtained properly.

5.7 Ureides

Ureides (allantoin and allantoic acid) are a form of fixed nitrogen which are exported from the nodules to other plant parts. Ureides are a product of nitrogen fixation. Drought stress can alter nodule functionality which means that transportation of ureides to the shoots and sugars to the nodule may be impaired. This interference with the metabolic activities of the nodule may result in accumulation of the ureides which may eventually lead to feedback inhibition of the nitrogen fixation process (Purcell *et al*., 2000).

The concentration of ureides was determined from the leaves at R3 growth stage. Most soybean cultivars showed higher ureide concentrations under the B treatment followed by treatment C and lastly A which was the lowest. In fewer cases ureides concentration increased with increasing water stress. This shows that conditions under B treatment are suitable for nitrogen fixation. Under treatment A, the amount of water applied might be too high and thus limited the availability of oxygen which is required in moderate amounts for nitrogen fixation. Ladrera *et al*. (2007) found that nitrogen fixation was inhibited in the soybean cultivar 'Bixoli' which is sensitive to drought stress.

There were not many differences with respect to ureide concentration in the leaves of Mopani and those of Sonop. Another similarity shared by these two cultivars is the trend of ureide concentration within the three treatments. Treatment B showed a high ureide concentration in both cultivars which suggests that maximum nitrogen fixation occurs when plants are watered sufficiently. Too much watering (treatment A) and limited water supply (treatment C) are not favourable for the process of nitrogen fixation. Knap had the highest ureide content under limited water availability (Table 4.5) which meant that it could carry out nitrogen fixation under water stress.

5.8 Anatomy

5.8.1 Roots

There were no major differences on root anatomy among the roots from the different cultivars. They all showed a xylem structure that seemed to have undergone secondary growth though soybean is a herbaceous plant. The presence of

secondary growth was also reported in the mid-eighties by Russin and Evert (1984) who studied the morphology and anatomy of the *Populus deltoides* leaf.

5.8.2 Nodules

The layer of sclerenchyma tissue helps in reducing the amount of oxygen entering the nodule, thus creating an anaerobic condition. The enzyme nitrogenase is responsible for fixing atmospheric nitrogen and requires an anaerobic environment which is achieved by the bacteroids together with the sclerenchyma tissue. The role of the vascular bundles is to transport sugars into the nodule and nitrogenous compounds out of the nodule.

Clear variations on the anatomy of the nodule were observed on the cultivar R01 581F under different treatments. The sclerenchyma cells were bigger in size and more thickened under treatment A and smaller under the B treatment. This meant that drought stress reduced the size of sclerenchyma and therefore allowing oxygen to pass through into the centre of the nodule where nitrogen fixation was taking place. As a result the enzyme nitrogenase would have been affected and so would be the process of nitrogen fixation.

The anatomy of the nodule did not show noticeable changes in all treatment for the cultivar Sonop. However, this cannot be said for Mopani cultivar. Under treatment A, Mopani has shown a distinct nodule anatomy. The layer of sclerenchyma was large and thickened with fewer infected parenchyma cells and one large vascular bundle enclosed. This environment was conducive for nitrogen fixation. The sclerenchymatous layer ensured an anaerobic condition for the bacteria *Rhizobium sp.* to function optimally. On the other hand, the large vascular system facilitated transportation of water, dissolved minerals and food substances throughout the nodule. The nodule anatomies of treatments B and C were different from what was observed on A. The infected cells were numerous but smaller than those of A. The sclerenchyma cells and vascular bundles had reduced size with decreasing water availability. Water stress affected nodule anatomy by decreasing the size of sclerenchyma and infected cells, as a result the functional efficiency of the nodule was lowered. Water deficit also reduced the size of the vascular bundles in the nodules which might have reduced the surface area of the transport system.

5.8.3 Petioles

The arrangement and size of xylem vessels in some cultivars for example R01 581F showed not much differences. This can be translated as uniform transport mainly of water under different treatments. This was not the case in some cultivars like Sonop where the xylem vessels were reduced in size which indicated that water stress reduced the size of the vessels. Petioles of some cultivars like Sonop were more strengthened with about three to four layers of phloem fibre caps which could be explained as a form of drought tolerant mechanism.

5.8.4 Leaves

Soybean leaves survive water stress during which water uptake from the roots is often curtailed due to insufficient water supply. Transpiration rate during this time has to be reduced in order for the soybean plants to avoid or even tolerate desiccation. This was achieved by the presence of a thick cuticle layer on both the upper and lower epidermis.

Water deficit was responsible for reduction of the vascular tissue (mainly xylem vessels) in the midvein. Mesophyll cells became thicker under drought stress. Both the upper and lower epidermis also became thicker under limited water conditions except for the cultivar Mopanie (Figure 4.7, l) which showed a very thick mesophyll and epidermis also under the control treatment. The same results were also reported by Makbul *et al*. (2011).

The anatomical structures of Mopani and Sonop were clearly different. The upper epidermis of Sonop was thicker than the lower epidermis whereas for Mopani both the lower and upper epidermis were thicker. In addition to that, a thick cuticle layer was seen on both the epidermal tissues. The other difference between the two cultivars was the size of the mesophyll cells. In Sonop, the mesophyll cells were thinner with large air spaces whereas those of Mopani were thicker and compact. These anatomical variations can be linked to the large surface area. Mopani had a large surface which allowed it to accumulate more water, as a result the thick epidermis and cuticle compensate for the water loss. Another difference is the sclerenchymatous bundle sheath of the mid-vein which was much thicker in Sonop. A very interesting observation was the absence of stomata on both the lower and

upper epidermis of the soybean cultivars studied. A further investigation needs to be carried out to evaluate the mechanisms of gaseous exchange in soybeans where stomata are not seen.

5.9 Yield

Drought stress has been reported to be a major abiotic stress factor which reduce plant yield (Keyvan, 2010). Low water levels in the soil causes a reduction of water potential in plants which causes the plant cells to be hypertonic. To continue plant development and growth under water stress, plants need to adapt to the high solute concentration through osmoregulation (Keyvan, 2010).

In this research, soybean yield of several cultivars was assessed in terms of weight of one hundred seeds measured in grams. Cultivars Pan 1564, Knap, LS 677, LS 678, R01 416F and R01 581F showed the highest yield under limited water levels. Mopanie was the lowest yielding cultivar with a seed weight of 4.5 g under the drought treatment.

Grain yield (mass) was generally reduced under drought stressed treatments when compared to the control and well watered treatments. An effect of water deficit stress on soybean grain yield was also reported by Masoumi *et al*. (2011).

5.10 Conclusions

The more the stem length is reduced, the lower the plant will yield. Therefore, reduced stem length as a result of limited water availability can be used as a morphological marker to identify some low yielding soybean cultivars.

The inability to reduce leaf surface area under limited water availability can be concluded to be another morphological trait which contributes to low yield in soybean. Low RLWC can be associated with low yielding soybean cultivars.

Reduced percentage chlorophyll is another character which is an indication of water stress sensitive cultivars. The higher the concentration of total flavonoids under water deficit the better the cultivar will yield.

Cultivars with high antioxidant activity yield higher than those with lower antioxidant activities. Too much or limited water supply is not favourable for the process of nitrogen fixation.

CHAPTER 6: REFERENCES

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APPENDICES

APPENDIX A: Yield descriptive statistics table

Descriptives

Yield

Treatment		N	Mean	Std. Deviation	Std. Error
Over Watering	PAN	6	11.46483	3.832703	1.564694
	MOPANIE	6	9.20433	.864457	.352913
	SONOP	6	14.06867	2.550470	1.041225
	KNAP	6	10.75883	1.348587	.550559
	LS 677	6	9.04250	.641586	.261927
	LS 678	6	12.30083	1.160001	.473568
	RO1 416F	6	9.36283	.769204	.314026
	RO1 581F	6	11.40450	4.990393	2.037319
	Total	48	10.95092	2.853334	.411843
Correct Watering	PAN	6	10.24300	3.147297	1.284878
	MOPANIE	6	6.80050	2.162272	.882744
	SONOP	6	8.99450	.979898	.400042
	KNAP	6	15.47500	2.345826	.957680
	LS 677	6	10.39267	1.189336	.485545
	LS 678	6	10.70700	.911615	.372165
	RO1 416F	6	9.63200	2.914553	1.189861
	RO1 581F	6	13.82617	1.803726	.736368
	Total	48	10.75885	3.221655	.465006
Under Watering	PAN	9	10.61344	3.740662	1.246887
	MOPANIE	9	4.50111	1.476327	.492109

	SONOP	9	10.73811	2.792012	.930671
	KNAP	9	9.00867	3.746735	1.248912
	LS 677	9	7.40011	2.552188	.850729
	LS 678	9	9.56167	3.063444	1.021148
	RO1 416F	9	8.65700	3.888579	1.296193
	RO1 581F	9	7.88122	2.217720	.739240
	Total	72	8.54517	3.455204	.407200

Descriptives

Yield

Treatment		95% Confidence Interval for Mean		Minimum	Maximum
		Lower Bound	Upper Bound		
Over Watering	PAN	7.44266	15.48701	6.864	16.285
	MOPANIE	8.29714	10.11152	8.440	10.910
	SONOP	11.39211	16.74522	11.429	17.650
	KNAP	9.34358	12.17409	9.431	12.457
	LS 677	8.36920	9.71580	8.139	9.850
	LS 678	11.08349	13.51818	10.712	14.104
	RO1 416F	8.55560	10.17006	8.363	10.340
	RO1 581F	6.16740	16.64160	6.283	16.838
	Total	10.12239	11.77944	6.283	17.650
Correct Watering	PAN	6.94011	13.54589	6.430	13.915
	MOPANIE	4.53133	9.06967	4.370	9.045
	SONOP	7.96616	10.02284	8.207	10.774
	KNAP	13.01321	17.93679	12.050	17.523

	LS 677	9.14453	11.64080	8.890	12.258
	LS 678	9.75032	11.66368	9.256	11.676
	RO1 416F	6.57336	12.69064	5.805	13.047
	RO1 581F	11.93327	15.71906	11.750	16.630
	Total	9.82338	11.69433	4.370	17.523
Under Watering	PAN	7.73812	13.48877	4.510	15.850
	MOPANIE	3.36631	5.63592	1.920	7.025
	SONOP	8.59198	12.88424	8.275	16.290
	KNAP	6.12867	11.88866	6.065	18.570
	LS 677	5.43833	9.36190	5.160	12.600
	LS 678	7.20689	11.91644	5.705	14.047
	RO1 416F	5.66797	11.64603	4.910	15.395
	RO1 581F	6.17653	9.58591	5.743	12.360
	Total	7.73323	9.35710	1.920	18.570

APPENDIX B: Bonferroni multiple comparison statistics table on yield

Multiple Comparisons

Yield

Bonferroni

Treatment	(I) Cultivar	(J) Cultivar	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Over Watering	PAN	MOPANIE	2.260500	1.457930	1.000	-2.61963	7.14063
		SONOP	-2.603833	1.457930	1.000	-7.48397	2.27630
		KNAP	.706000	1.457930	1.000	-4.17413	5.58613
		LS 677	2.422333	1.457930	1.000	-2.45780	7.30247
		LS 678	-.836000	1.457930	1.000	-5.71613	4.04413
		RO1 416F	2.102000	1.457930	1.000	-2.77813	6.98213
		RO1 581F	.060333	1.457930	1.000	-4.81980	4.94047
	MOPANIE	PAN	-2.260500	1.457930	1.000	-7.14063	2.61963
		SONOP	-4.864333	1.457930	.052	-9.74447	.01580
		KNAP	-1.554500	1.457930	1.000	-6.43463	3.32563
		LS 677	.161833	1.457930	1.000	-4.71830	5.04197
		LS 678	-3.096500	1.457930	1.000	-7.97663	1.78363
		RO1 416F	-.158500	1.457930	1.000	-5.03863	4.72163
		RO1 581F	-2.200167	1.457930	1.000	-7.08030	2.67997
	SONOP	PAN	2.603833	1.457930	1.000	-2.27630	7.48397
		MOPANIE	4.864333	1.457930	.052	-.01580	9.74447
		KNAP	3.309833	1.457930	.802	-1.57030	8.18997
		LS 677	5.026167*	1.457930	.038	.14603	9.90630

		LS 678	1.767833	1.457930	1.000	-3.11230	6.64797
		RO1 416F	4.705833	1.457930	.070	-.17430	9.58597
		RO1 581F	2.664167	1.457930	1.000	-2.21597	7.54430
	KNAP	PAN	-.706000	1.457930	1.000	-5.58613	4.17413
		MOPANIE	1.554500	1.457930	1.000	-3.32563	6.43463
		SONOP	-3.309833	1.457930	.802	-8.18997	1.57030
		LS 677	1.716333	1.457930	1.000	-3.16380	6.59647
		LS 678	-1.542000	1.457930	1.000	-6.42213	3.33813
		RO1 416F	1.396000	1.457930	1.000	-3.48413	6.27613
		RO1 581F	-.645667	1.457930	1.000	-5.52580	4.23447
	LS 677	PAN	-2.422333	1.457930	1.000	-7.30247	2.45780
		MOPANIE	-.161833	1.457930	1.000	-5.04197	4.71830
		SONOP	-5.026167*	1.457930	.038	-9.90630	-.14603
		KNAP	-1.716333	1.457930	1.000	-6.59647	3.16380
		LS 678	-3.258333	1.457930	.870	-8.13847	1.62180
		RO1 416F	-.320333	1.457930	1.000	-5.20047	4.55980
		RO1 581F	-2.362000	1.457930	1.000	-7.24213	2.51813
	LS 678	PAN	.836000	1.457930	1.000	-4.04413	5.71613
		MOPANIE	3.096500	1.457930	1.000	-1.78363	7.97663
		SONOP	-1.767833	1.457930	1.000	-6.64797	3.11230
		KNAP	1.542000	1.457930	1.000	-3.33813	6.42213
		LS 677	3.258333	1.457930	.870	-1.62180	8.13847
		RO1 416F	2.938000	1.457930	1.000	-1.94213	7.81813
		RO1 581F	.896333	1.457930	1.000	-3.98380	5.77647

	RO1 416F	PAN	-2.102000	1.457930	1.000	-6.98213	2.77813
		MOPANIE	.158500	1.457930	1.000	-4.72163	5.03863
		SONOP	-4.705833	1.457930	.070	-9.58597	.17430
		KNAP	-1.396000	1.457930	1.000	-6.27613	3.48413
		LS 677	.320333	1.457930	1.000	-4.55980	5.20047
		LS 678	-2.938000	1.457930	1.000	-7.81813	1.94213
		RO1 581F	-2.041667	1.457930	1.000	-6.92180	2.83847
	RO1 581F	PAN	-.060333	1.457930	1.000	-4.94047	4.81980
		MOPANIE	2.200167	1.457930	1.000	-2.67997	7.08030
		SONOP	-2.664167	1.457930	1.000	-7.54430	2.21597
		KNAP	.645667	1.457930	1.000	-4.23447	5.52580
		LS 677	2.362000	1.457930	1.000	-2.51813	7.24213
		LS 678	-.896333	1.457930	1.000	-5.77647	3.98380
		RO1 416F	2.041667	1.457930	1.000	-2.83847	6.92180
Correct Watering	PAN	MOPANIE	3.442500	1.208263	.193	-.60192	7.48692
		SONOP	1.248500	1.208263	1.000	-2.79592	5.29292
		KNAP	-5.232000*	1.208263	.003	-9.27642	-1.18758
		LS 677	-.149667	1.208263	1.000	-4.19409	3.89476
		LS 678	-.464000	1.208263	1.000	-4.50842	3.58042
		RO1 416F	.611000	1.208263	1.000	-3.43342	4.65542
		RO1 581F	-3.583167	1.208263	.142	-7.62759	.46126
	MOPANIE	PAN	-3.442500	1.208263	.193	-7.48692	.60192
		SONOP	-2.194000	1.208263	1.000	-6.23842	1.85042
		KNAP	-8.674500*	1.208263	.000	-12.71892	-4.63008
		LS 677	-3.592167	1.208263	.139	-7.63659	.45226

	LS 678	-3.906500	1.208263	.069	-7.95092	.13792
	RO1 416F	-2.831500	1.208263	.677	-6.87592	1.21292
	RO1 581F	-7.025667*	1.208263	.000	-11.07009	-2.98124
SONOP	PAN	-1.248500	1.208263	1.000	-5.29292	2.79592
	MOPANIE	2.194000	1.208263	1.000	-1.85042	6.23842
	KNAP	-6.480500*	1.208263	.000	-10.52492	-2.43608
	LS 677	-1.398167	1.208263	1.000	-5.44259	2.64626
	LS 678	-1.712500	1.208263	1.000	-5.75692	2.33192
	RO1 416F	-.637500	1.208263	1.000	-4.68192	3.40692
	RO1 581F	-4.831667*	1.208263	.007	-8.87609	-.78724
KNAP	PAN	5.232000*	1.208263	.003	1.18758	9.27642
	MOPANIE	8.674500*	1.208263	.000	4.63008	12.71892
	SONOP	6.480500*	1.208263	.000	2.43608	10.52492
	LS 677	5.082333*	1.208263	.004	1.03791	9.12676
	LS 678	4.768000*	1.208263	.009	.72358	8.81242
	RO1 416F	5.843000*	1.208263	.001	1.79858	9.88742
	RO1 581F	1.648833	1.208263	1.000	-2.39559	5.69326
LS 677	PAN	.149667	1.208263	1.000	-3.89476	4.19409
	MOPANIE	3.592167	1.208263	.139	-.45226	7.63659
	SONOP	1.398167	1.208263	1.000	-2.64626	5.44259
	KNAP	-5.082333*	1.208263	.004	-9.12676	-1.03791
	LS 678	-.314333	1.208263	1.000	-4.35876	3.73009
	RO1 416F	.760667	1.208263	1.000	-3.28376	4.80509
	RO1 581F	-3.433500	1.208263	.197	-7.47792	.61092
LS 678	PAN	.464000	1.208263	1.000	-3.58042	4.50842

		MOPANIE	3.906500	1.208263	.069	-.13792	7.95092
		SONOP	1.712500	1.208263	1.000	-2.33192	5.75692
		KNAP	-4.768000*	1.208263	.009	-8.81242	-.72358
		LS 677	.314333	1.208263	1.000	-3.73009	4.35876
		RO1 416F	1.075000	1.208263	1.000	-2.96942	5.11942
		RO1 581F	-3.119167	1.208263	.381	-7.16359	.92526
	RO1 416F	PAN	-.611000	1.208263	1.000	-4.65542	3.43342
		MOPANIE	2.831500	1.208263	.677	-1.21292	6.87592
		SONOP	.637500	1.208263	1.000	-3.40692	4.68192
		KNAP	-5.843000*	1.208263	.001	-9.88742	-1.79858
		LS 677	-.760667	1.208263	1.000	-4.80509	3.28376
		LS 678	-1.075000	1.208263	1.000	-5.11942	2.96942
		RO1 581F	-4.194167*	1.208263	.035	-8.23859	-.14974
	RO1 581F	PAN	3.583167	1.208263	.142	-.46126	7.62759
		MOPANIE	7.025667*	1.208263	.000	2.98124	11.07009
		SONOP	4.831667*	1.208263	.007	.78724	8.87609
		KNAP	-1.648833	1.208263	1.000	-5.69326	2.39559
		LS 677	3.433500	1.208263	.197	-.61092	7.47792
		LS 678	3.119167	1.208263	.381	-.92526	7.16359
		RO1 416F	4.194167*	1.208263	.035	.14974	8.23859
Under Watering	PAN	MOPANIE	6.112333*	1.433170	.002	1.43992	10.78475
		SONOP	-.124667	1.433170	1.000	-4.79708	4.54775
		KNAP	1.604778	1.433170	1.000	-3.06764	6.27719
		LS 677	3.213333	1.433170	.796	-1.45908	7.88575
		LS 678	1.051778	1.433170	1.000	-3.62064	5.72419

	RO1 416F	1.956444	1.433170	1.000	-2.71597	6.62886
	RO1 581F	2.732222	1.433170	1.000	-1.94019	7.40464
MOPANIE	PAN	-6.112333*	1.433170	.002	-10.78475	-1.43992
	SONOP	-6.237000*	1.433170	.001	-10.90942	-1.56458
	KNAP	-4.507556	1.433170	.071	-9.17997	.16486
	LS 677	-2.899000	1.433170	1.000	-7.57142	1.77342
	LS 678	-5.060556*	1.433170	.022	-9.73297	-.38814
	RO1 416F	-4.155889	1.433170	.143	-8.82831	.51653
	RO1 581F	-3.380111	1.433170	.600	-8.05253	1.29231
SONOP	PAN	.124667	1.433170	1.000	-4.54775	4.79708
	MOPANIE	6.237000*	1.433170	.001	1.56458	10.90942
	KNAP	1.729444	1.433170	1.000	-2.94297	6.40186
	LS 677	3.338000	1.433170	.645	-1.33442	8.01042
	LS 678	1.176444	1.433170	1.000	-3.49597	5.84886
	RO1 416F	2.081111	1.433170	1.000	-2.59131	6.75353
	RO1 581F	2.856889	1.433170	1.000	-1.81553	7.52931
KNAP	PAN	-1.604778	1.433170	1.000	-6.27719	3.06764
	MOPANIE	4.507556	1.433170	.071	-.16486	9.17997
	SONOP	-1.729444	1.433170	1.000	-6.40186	2.94297
	LS 677	1.608556	1.433170	1.000	-3.06386	6.28097
	LS 678	-.553000	1.433170	1.000	-5.22542	4.11942
	RO1 416F	.351667	1.433170	1.000	-4.32075	5.02408
	RO1 581F	1.127444	1.433170	1.000	-3.54497	5.79986
LS 677	PAN	-3.213333	1.433170	.796	-7.88575	1.45908

	MOPANIE	2.899000	1.433170	1.000	-1.77342	7.57142
	SONOP	-3.338000	1.433170	.645	-8.01042	1.33442
	KNAP	-1.608556	1.433170	1.000	-6.28097	3.06386
	LS 678	-2.161556	1.433170	1.000	-6.83397	2.51086
	RO1 416F	-1.256889	1.433170	1.000	-5.92931	3.41553
	RO1 581F	-.481111	1.433170	1.000	-5.15353	4.19131
LS 678	PAN	-1.051778	1.433170	1.000	-5.72419	3.62064
	MOPANIE	5.060556*	1.433170	.022	.38814	9.73297
	SONOP	-1.176444	1.433170	1.000	-5.84886	3.49597
	KNAP	.553000	1.433170	1.000	-4.11942	5.22542
	LS 677	2.161556	1.433170	1.000	-2.51086	6.83397
	RO1 416F	.904667	1.433170	1.000	-3.76775	5.57708
	RO1 581F	1.680444	1.433170	1.000	-2.99197	6.35286
RO1 416F	PAN	-1.956444	1.433170	1.000	-6.62886	2.71597
	MOPANIE	4.155889	1.433170	.143	-.51653	8.82831
	SONOP	-2.081111	1.433170	1.000	-6.75353	2.59131
	KNAP	-.351667	1.433170	1.000	-5.02408	4.32075
	LS 677	1.256889	1.433170	1.000	-3.41553	5.92931
	LS 678	-.904667	1.433170	1.000	-5.57708	3.76775
	RO1 581F	.775778	1.433170	1.000	-3.89664	5.44819
RO1 581F	PAN	-2.732222	1.433170	1.000	-7.40464	1.94019
	MOPANIE	3.380111	1.433170	.600	-1.29231	8.05253
	SONOP	-2.856889	1.433170	1.000	-7.52931	1.81553
	KNAP	-1.127444	1.433170	1.000	-5.79986	3.54497

	LS 677	.481111	1.433170	1.000	-4.19131	5.15353
	LS 678	-1.680444	1.433170	1.000	-6.35286	2.99197
	RO1 416F	-.775778	1.433170	1.000	-5.44819	3.89664

*. The mean difference is significant at the 0.05 level.

APPENDIX C: Reagents used for ureides analysis

A. 0.5 N NaOH (stored at room temperature).

B. 0.65 N HCl (stored at room temperature).

C. 0.33 % Phenylhydrazine solution (prepared on the day of use).

Reagents B and C were added together in a 1:1 ratio to form reagent E on the day of the assay.

D. 1.67 % KFeCn (prepared on day of use).

On the day of the assay, a 5.0 ml volume of reagent D was added to 20.0 ml concentrated HCl to form reagent F.

APPENDIX D: Nitrogen-free nutrient medium

Stock	Aliquot (for 1 litre of nutrient solution)
1 M KCl	10 ml
1 M $CaCl_2$	10 ml
1 M $MgSO_4$	2 ml
1 M KH_2PO_4	2 ml
Micronutrients	2 ml
FeEDTA	2 ml

One litre micronutrient stock solution contains the following nutrients:

2.86 g H_3BO_3

1.81 g $MnCl_2.4H_2O$

0.11 g $ZnCl_2$

0.05 g Cu $Cl_2.2H_2O$

0.025 g $Na_2MoO_4.2H_2O$

One litre of the FeEDTA stock solution contains:

25.0 g $FeSO_4.7H_2O$

34.0 g Na_2EDTA