EFFECTS OF APPLICATION OF PHYTOSTIM® BIOSTIMULANT ON GROWTH, YIELD, POSTHARVEST QUALITY, AND METABOLITES OF TWO AMARANTH SPECIES

BY

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DISSERTATION

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DECLARATION

I MAGWELE MAPULA PRECIOUS declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Agricultural Management in Plant Production is my original work, regardless of the acknowledgments given as citations or quotations throughout. This document has not previously been submitted by me for a degree at this or any other university; it is my work in design and execution, and all material contained herein has been duly acknowledged.

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DEDICATION

I would like to dedicate this dissertation to my late parents, Mother (Mosibudi) and Father (Thomas). My siblings, my sister Dikeledi Annah and my brother Mohlaume David Magwele.

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PUBLICATION'S CONTRIBUTION

Manuscripts drafted.

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Conferences

M.P Magwele, T.K Sathekge, S. Mpai, A.R Ndhlala. Effects of application of Phytostim® biostimulant on growth and yield of *Amaranthus species*. South African Association of Botanists. 48th Annual conference. The Ranch Protea hotel, Polokwane, South Africa. 17-20 January 2023.

M.P Magwele, T.K Sathekge, S. Mpai, A.R Ndhlala. Effects of Phytostim® biostimulant on postharvest quality attributes and nutrient components of *Amaranthus species*. 13th Faculty of Science and Agriculture, Postgraduate Research Day. 20-22 September 2023. Bolivia Lodge, Polokwane

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GLOSSARY

GBRCE	Green Biotechnologies Research Centre of Excellence
ALVs	African Leafy Vegetables
ELVs	Exotic Leafy Vegetables
Ca	Calcium
P	Phosphorus
Na	Sodium
Mg	Magnesium
Cu	Copper
Zn	Zinc
Fe	Iron
Mn	Manganese
N	Nitrogen
Se	Selenium
K	Potassium
CHO	Carbohydrates, Hydrogen and Oxygen
Uv	Ultraviolet
WHO	World Health Organization
ARC	Agricultural Research Centre
RCBD	Randomized Complete Block Design
Cm	Centimeter
%	Percentage
M	Meter
®	Registered
° C	Degree Celsius
cm^3	Cubic centimeter
V	Volume
Ml	Milliliter
H	Hours

ANOVA	Analysis of Variance
P	Probability
≤	Less or equal
≥	Greater or equal
LATS	Limpopo Agro-Food Technology Station
CRD	Complete Randomized Design
D	Days
DMRT	Duncan Multiple Range Test
SE	Standard error
G	Grams
$HN0_3$	Nitric acid
h°	Hue angle
ICPE	Coupled Plasma Emission Spectrometer
Ppm	Parts per million
HCL	Hydrochloric acid
µm	Micrometer
LC-MS	Liquid Chromatograph Mass Spectrometer
Min	Minutes
Rt	Retention time
m/z	Molar mass
UPLC	Ultra-Performance Liquid Chromatograph
PDA	Photodiode array
PCA	Principal Components Analysis
OPLS-DA	Orthogonal Partial Least Squares- Discriminant Analysis
AOAC	Association of Official Analytical Chemists
FAO	Food and Agricultural Organization
QTOF	Quadrupole Time of Flight
Mg/L	Miligrams per liter
Mg/kg	Miligrams per kilograms
His	Histidine
Ile	Isoleucine

Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Thr	Threonine
Try	Tryptophan
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
Glu	Glutamic acid
Gly	Glysine
Pro	Proline
Ser	Serine
DSI	Department of Science and Innovative
NRF	National Research Foundation
$C_2 H_5 OH$	Ethanol
$H_3 PO_4$	Phosphoric acid
H_2O	Water
ULWASA	University of Limpopo Women's Academic Solidarity Association

ABSTRACT

Amaranthus species are indigenous crops in South Africa, commonly consumed as leafy vegetables. It has been consumed for many decades by the rural community population for its nutritional and nutraceutical properties. However, the crop is still not commercialized due to limited productivity in the country. There is scant information on the cultivation of *Amaranthus species* and its improvement as compared to the exotic crops such as spinach and lettuce. Thus, it is pertinent to find ways of improving the crop in terms of yield, and postharvest quality attributes. This study was aimed at establishing the information on the effect of different concentrations of Phytostim® biostimulant on growth, yield, and postharvest quality of *Amaranthus cruentus* and *Amaranthus caudatus*. The objectives of this study was to (1) investigate the effects of different concentrations of Phytostim® biostimulant on growth and yield attributes, (2) to investigate the interactive effect of Phytostim® biostimulant concentrations and storage period on postharvest shelf-life quality attributes, nutritional components, and secondary metabolites of the two *Amaranthus species*. The different concentrations of Phytostim® biostimulant used in this study were 0, 0.5, 1, 1.5, 2.5, 3, 4.5, and 6% while untreated plants (0%) were used as control in all the objectives.

To achieve objective one: four-week-old seedlings of *Amaranthus species* were grown in a greenhouse condition following a Randomized Complete Block Design (RCBD) for a period of eight weeks (60 days). Different concentrations of Phytostim® biostimulant were foliar-applied after every 14-day until harvest. Growth and yield attributes (stem diameter, plant height, number of branches, number of leaves per plant, fresh and dried leaf mass per plant) data were collected at harvest. It was observed that Phytostim® biostimulant significantly affected ($p<0.05$) growth and yield attributes of amaranth. On the growth attributes, Phytostim® biostimulant increased the plant height by 97 cm and 110 cm at 1.5 % concentrations in both *Amaranth species*. While on the stem diameter increased by 13 mm and 17 mm. On the number of branches, it increased by 30 and 44. On yield parameter, highest biomass obtained was 74.23 g and 85.93 g per plant.

On the second objective: the harvested leafy vegetables of amaranth were separated into uniform bundles of 100 g weight in a well-ventilated punnet and stored at ambient temperature for 0, 3, and 6 days for assessment of weight loss, color, and visual quality. During these storage period, a total of 5 g per replicate was sampled at three days intervals and oven dried at 40 ºC for 72 h for analysis of nutrient components and secondary metabolites such as mineral elements, amino acids, protein, and phenolic compounds. It was observed that Phytostim® biostimulant and storage days significantly affected ($p<0.05$) some of postharvest quality and nutritional components of amaranth. Weight loss in 2.5% biostimulant was 20.85%, and 30.89% at day 6 in *A. cruentus and A.caudatus*. These in terms of color, the leaves that maintained a good color quality was at 0.5 % ($h° = 148.21$ and $a^* = -12.29$) for *A. cruentus*. At the end of storage period (6d) the leaves maintained a good color quality was at 2.5% ($h° = 124.14$ and $a^* = -10.66$) in *A. caudatus*. The obtained results revealed that weight loss%, color, and visual quality of amaranth were significantly ($p<0.05$) influenced by different concentrations of Phytostim® biostimulant and storage time. All the above-mentioned postharvest attributes were improved at 2.5% concentrations of Phytostim® biostimulant in comparison to control. Moreover, the study further revealed that different concentrations of Phytostim® biostimulant significantly ($p<0.05$) influenced the mineral elements (Mg, Ca, Fe, K, Zn, N, Cu, Se, and Mn), amino acids (His, Leu, Lys, Met, Phe, Thr, Val, Ala, Arg, Asp, Glu, Gly, Pro and Ser) and protein content of the studied leaves. The nutrients components were enhanced at 2.5% concentrations of Phytostim® biostimulant as compared to the control and other concentrations of Phytostim® biostimulant. Thus, it can be concluded that Phytostim® biostimulant can be recommended to be used effectively by farmers at 2.5% for up to 6 days or less in preserving the high-quality characteristics of *Amaranth species* during ambient temperature.

On the last stage of this study, the untargeted phenolic compounds were determined using the Liquid Chromatograph Mass Spectrometer (UPLC-MS). A total of 12 phenolic compounds were detected in the studied leaves. Phenolic compounds of the treated plants were significantly higher in comparison to the phenolic compound of the untreated

plants. Principle Component Analysis and Orthogonal Partial Least Squares-Discriminant Analysis showed that Phytostim® biostimulant is the main factor responsible for the variation in the studied crop. The major identified phenolic metabolites were members of the coumarin glucoside, glucuronic acid, and flavonoid-3-glycosides. Predominant phenolic compound quantified was rutin in both species. This objective spotted Phytostim® biostimulant concentrations at 3% to be the best for enhancing secondary metabolites. Therefore, overall recommendation these objectives suggest that Phytostim® biostimulant concentrations starting from 1.5% up to 3% could be used to improve the yield, nutrients, and secondary metabolites.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Amaranth is a genus belonging to the family of Amaranthaceae and has about 60 species including *Amaranthus cruentus* and *Amaranthus caudatus*. Majority of the species are harvested at the tender maturity stages and consumed by humans as leafy vegetables (Murphy and Tranel, 2018; Wafaa *et al*., 2020; Kongdang *et al*., 2021). The two species differs based on their morphological characters, whereby *A. caudatus* is characterized by central stem that grows at taproot system, with leathery leaves which differs from the *A. cruentus*. The leaves of *A. cruentus* are slippery with thick stems which grows straight or branched (Gerrano *et al*., 2017) as shown in figure 1.1. *Amaranthus species* is a popular indigenized food crop that grow in the wild and in conventional cropping fields and consumed largely by rural communities in most parts of the African continent. In South Africa, leaves and shoots of *Amaranthus species* are harvested, boiled, and consumed as a relish with porridge (pap) made from maize meal or sorghum meal, or rice. Although there is still scant information about the production rate of *Amaranthus species* within the South African food production industry, its consumption provides immense impact during dry seasons when stored dried leaves are being useful to combat food scarcity. In addition to its use as a source of food, some communities process the fresh aboveground parts for use in the *treatment* of high cholesterol and swelling of mouth or throat (Aderibigbe *et al*., 2022). Therefore, the crop serves as an important source of food and medicine, particularly in rural communities.

In 2020, the UNICEF (UNICEF, 2020) estimated that across the world, more than 10 million adults and more than 3 million children experienced severe food insecurity that leads to malnutrition. Without a doubt, the figures were increased by the outbreak of the COVID-19 epidemic, which was associated with rising inflation rates and economic deterioration (Ahn and Norwood, 2021). When human body is deprived of nutritious food, it become susceptible to attack by diseases such as high blood pressure, cancer, diabetes, arthritis, kidney disease, and cardiovascular diseases (Roser, and Ritchie,

2019). The most common approach used to mitigate food shortage and provide quality food is the cultivation of food crops along the application of agro-inputs such as biostimulants (Calvo *et al.,* 2014; Kathrin and Craigie, 2011; Bhupenchandra *et al*., 2022). African countries have seen an increase in the importation of biostimulants along an increase in companies that manufacture biostimulants (Raimi *et al*., 2017). The application of biostimulants of plant origin has gained momentum in vegetable production in particular to the amino acid and protein hydrolysed types (Mpai *et al*., 2022). They are described as biologically active compounds extracted from organic plant extracts or animal (Bulgari *et al*., 2019; Paul *et al*., 2019). An moringa based biostimulants can be applied either through foliar or drench to be absorbed through leaf or root systems respectively. They can also be applied as seed priming agent for improved germination (Sorrentino *et a*l., 2021). In fact, these moringa based biostimulants has been associated with enhanced crop yield, plant quality and can act as elicitors for mitigation of adaptation mechanisms during abiotic stress such as salinity or water stress (Abdelgawad *et al*., 2018; Paul *et al*., 2019). Phytostim® biostimulants is a moringa-based biostimulant constituted from 22 amino acids important for growth and developments of plants (Figure 1.2). It can be treated as alternative to other agricultural inputs that produce safe and nutritious food.

1.2 Problem statement

Amaranthus species are listed in the South African food database among 21 vegetables that improve food security (FAO, 2018). Yet, the crop is still underutilized and mostly grown by small-scale farmers whereby synthetic fertilizers are the main input for improved productivity (Mondal *et al*., 2019; Aderibigbe *et al*., 2022). Intriguingly, the addition of synthetic fertilizers to vegetables can result in the accumulation of non-nutritive compounds such as nitrites and nitrates (Mampholo *et al*., 2018), which cause ailments such as methemoglobinemia when taken in more than the daily acceptable volume (Clain, 2011). Furthermore, leafy vegetables grown with the application of higher quantities of some synthetic fertilizers after harvest affect the market by narrowing the shelf life and have adverse effects on the health of human beings (Rahiel *et al*., 2918). In fact, about

50% of leafy vegetables are exposed to post-harvest losses due to exposure to higher and improper quantities of synthetic fertilizers and other related factors such as poor handling (FAO, 2018).

While several studies have been conducted on *Amaranthus species*, their focus was on crop production using other agricultural practices such as synthetic fertilizers (Gogo *et al*., 2017; Mondal *et al*., 2019; Emmanuel and Babalola, 2022). Less attention has been given to research that focus on initiatives to extend the postharvest shelf life of *Amaranthus species* through assessing the effects of agro inputs on the nutritional components and the composition of metabolites. Furthermore, information regarding the involvement of biostimulants that are accessible to farmers, including Phytostim®, on improving growth, yield, and post-harvest qualities is limited. Therefore, there is a need for research that would contribute such information so as to help improve our understanding on the role that biostimulants play in improving the adaptability of food crops and importantly, their effects on the growth and quality of important food crops such as *Amaranthus species*.

A B

Figure 1.1: *Amaranthus species* (A) *A. cruentus* and (B) *A. caudatus* (Source: www.maltawild plant.com)

Figure 1.2: Phytostim® biostimulant (Source: Moringa products South Africa)

1.3 Rationale of the study

Leafy vegetables like amaranth play a fundamental role in the nutrition security and therefore health of humans as they are important sources of essential nutrient elements, such as dietary fiber, vitamins, gluten-free protein, and carbohydrates (Leakey *et al*., 2022). In addition, Baraniak, and Małgorzata (2022) stated that, species of amaranth are rich in phytonutrients such as antioxidants, carotenoids, and phenolic compounds, which making valuable in the pharmaceutical industry for the production of medicinal products widely used against atherosclerosis, stomach ulcers, tuberculosis, antiseptic, antifungal, and anti-inflammatory preparations. Moreover, the food crop is considered to exhibit low calories, which are important to balance or substitute food rich in fats (Kakimoto *et al*., 2016; Joshi and Verma,2020; Imathiu, 2021). The commercially available biostimulant, Phytostim®, is an agro-input that has the potential to be used as an alternative to synthetic fertilizers in the production of amaranth given that it has been manufactured using biological constituents that promote plant growth, yield, and quality. Currently, techniques such as cold storage and modified atmospheric packaging are used to preserve leafy vegetables' post-harvest shelf-life by altering the respiration and transpiration processes (Gogo *et al*., 2017; Jin *et al*., 2021). There is an urgent need to assess whether the supply

of agricultural inputs such as biostimulants can improve the productivity, nutritional value, secondary metabolites, and extend the shelf life of food crops including amaranth. Results from such research will likely promote adoption of the food crop and bringing their cultivation in mainstream commercial agriculture.

1.4 Purpose of the study

1.4.1 Aim

Development of information to improve growth, yield, postharvest quality and metabolites of *Amaranthus cruentus* and *Amaranthus caudatus* through the use of Phytostim® biostimulant.

1.4.2 Objectives

To achieve findings from chapter 3 to 5 the following objectives were addressed.

i. To determine the effect of foliar application of different concentrations of Phytostim® biostimulant on the growth and yield in *A. cruentus* and *A. caudatus*.
ii. To evaluate the interactive effect of different concentrations of Phytostim® biostimulant and storage period on post-harvest quality and nutritional components in *A. cruentus* and *A. caudatus.*
iii. To assess the interactive effect of different concentrations of Phytostim® biostimulant and storage period on secondary metabolites in *A. cruentus* and *A. caudatus*

1.5 Reliability, validity, and objectivity

For this study, reliability of data was assessed based on a statistical analysis using Statistix 10.0 software at the probability level of 5%, validity was achieved through replicating the treatments as well as control and repeating the experiments in time. Objectivity was achieved by ensuring that results are discussed based on observed evidence, relating the findings with other study findings which are coherent and incoherent to our studies and to eliminate all forms of subjectivity (Leedy and Ormrod, 2019).

1.6 Bias

Bias was minimized by ensuring that the error in each experiment was reduced through replications. Also, randomly assigning treatments within an appropriate research design reduced bias (Leedy and Ormrod, 2019).

1.7 Scientific contribution

The study intended to establish a particular species of the selected amaranth that performed under greenhouse condition by showing an increase on growth, yield, and quality. Also, to determine a specific concentration of Phytostim® that would reveal improved growth, yield, and quality of amaranth. The information that will be generated, especially the results, will be distributed among small-scale farmers and amaranth growers so as to educate them on an alternative agro-input that promote sustainable production of the food crop.

1.8 Structure of the dissertation

The subsequent chapters in the dissertation are presented as follows: Chapter 1, addresses the background, research problem, its impacts, the possible cause, possible solution, aim, objectives, reliability, bias, and scientific significance of the current research project. Chapter 2 focuses on the literature review which furnishes an overview of work done and works not done on the research problem, followed by chapter 3 which constitutes the research work on the effects of foliar application of different concentrations of Phytostim® biostimulant on growth and yield of the two *Amaranthus species*. Chapter 4 describes and discuss the results of the research work on the interactive effects of different concentrations of Phytostim® biostimulant on post-harvest quality and nutritional composition. Chapter 5 describes and discusses results on interactive effects of different concentrations of Phytostim® biostimulant on secondary metabolites of the selected species of amaranth. All research-based chapters will follow a format of peer reviewed research articles. The final chapter, which is chapter 6, presents the overall conclusion of the study, and recommendations for future research were made. In the citations and references, the Harvard style were used, with author-alphabet as approved by the Senate of the University of Limpopo.

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CHAPTER 2

LITERATURE REVIEW

2.1 Work done on the problem statement.

2.1.1 Some of the common African Leafy Vegetables (ALVs) in South Africa

The term African leafy vegetables refer to leafy vegetables that are indigenous to the African continent. These include various species of amaranth which contribute to food and nutrient security. Nevertheless, they are currently underutilized in most African countries, including South Africa. According to Maseko *et al*. (2017), South Africa is home to more than one hundred different leafy vegetables which are endemic to the country however few are widely cultivated and/or consumed and these include *Cleome gynandra* (spider plant), *Amaranthus spp*., *Citrullus lanatus* (bitter melon), *Corchorus olitorius* (jute mallow), *Vigna unguiculata* (cowpea), and *Cucurbita spp*. (pumpkin). In some South African languages, green vegetables are referred to morogo (Sesotho, sepedi), imifino (IsiZulu, IsiXhosa), and miroho in tshiVhenda. Already by these terms, one can tell that these are underutilized crops. That notwithstanding, they play a crucial role in food security and/or nutrition security in South African households. Their availability is highly variable and largely depend on factors such as time of the year, the status of poverty, distance availability of markets selling fresh produce, household income, and level of urbanization (Mabhaudhi *et al*., 2017). Table 2.1 shows a summary of various indigenous food crops in South Africa

2.1.2 Comparison of ALVs and exotic leafy vegetables (ELVs)

Scholarly studies report that in recent times, the consumption of ALVs has decreased while that of exotic vegetables has increased (Njume *et al*., 2014). This is intriguing because the ALVs carry nutrients whose concentration is higher and make a better contribution to the wellbeing of humans compared to ELVs. Njume *et al*. (2014) further

reported that, ALVs are largely widely utilized in rural and smallholder communities where they are harvested from the wild or cultivated backyard fields (Maseko *et al*., 2018). By contrast, most species of ELVs are produced mainly by the commercial breeders. While ALVs are considered as weed by commercial crop farmers, they serve as an alternative source of food by smallholder farmers (Maseko *et al*., 2018). In smallholder cropping systems, women usually do most of the gathering, cultivation, and harvesting of ALVs. When they germinate from cultivated fields, they are distinguishable from other nonedible weeds according to their usefulness. Table 2.2 below outline the difference of nutritious level between ALVs and ELVs, and clearly there is evidence of higher nutrients composition and contents in ALV than some of the ELV.

Table 2.1: Some of the African indigenous leafy vegetables (ALVs) commonly consumed in South Africa.

Common name	Scientific name	Image	Source
Nightshade	*Solanum scabrum*		*www.naturehomeopathy.com*
Cowpea	*Vigna unguiculata*		*Plantvillage.psu.edu*
Amaranth	*Amaranthus spp.*		*www.maltawild plant.com*

Moringa	*Moringa oleifera*		*www.healthydietbase.com*
Traditional pumpkin	*Cucurbita spp*		*Naturebring.com*
Cleome	*Cleome gyandra*		www.etsy.com
Gushe	*Corchorus spp*		*Pza.sanbi.org*

Table 2.2: Nutritional composition of African leafy vegetables and exotic leafy vegetables

Nutrients	African Leafy vegetables		Exotic leafy vegetables	
	Amaranth	Cowpea	Cabbage	Lettuce
Iron (mg)	8.9	39	0.7	0.86
Protein (g)	4.6	41	1.7	1.36
Moisture (%)	84.0	87.6	91.4	90.3
Calories	42		26	19
Carbohydrates (g)	8.2	6.8	6.0	2.8
Fiber (g)	1.8		1.2	1.3
Vitamin C	64		54	9.2
Calcium (mg)	410	548.5	47	36
Phosphorus (mg)	103	136.4	40	29
B-carotene (mg)	571.6	3662.9	100	120
Thiamine	0.05	0.07	0.04	
Riboflavin	0.4		0.1	

Folic acid (mg/100g)	112

Source: Njume, *et al* (2014)

2.1.3 Amaranth uses and their production status in South Africa

Amaranth species have been important in different parts of the world and at different times for thousands of years ago (Tesfay *et al.,* 2016). Production of amaranth was promoted for various reasons: food security, substantial crops to improve family income and earn foreign exchange, and for promotion of urban agriculture among others (Maseko *et al.,* 2018). Its leaves are often collected at tender, immature stages for preparation of vegetable relish.

The crop is regarded as an indigenous vegetable in Africa, where it is cooked alone or in conjunction with other leafy vegetables and consumed alongside other stable foods such as porridge. However, production of this crop remains low in South Africa because it is generally thought to grow naturally during the rainy season. Factors that limit the consumption of *Amaranthus species* include rapid urbanization and behavioral shift in the lifestyle of the rural African population; predominantly the misguided perception of indigenous vegetation is associated with poverty, thus, being avoided by children (Padulos *et al.,* 2013). This is a significant challenge because South African youngsters consume less than two-thirds of the necessary dietary limits. Figure 2.1 below denotes the production areas of *Amaranth species* in South Africa. From figure it shows that S2 (in lime) colour, it indicates that amaranth is highly consumed in these provinces.

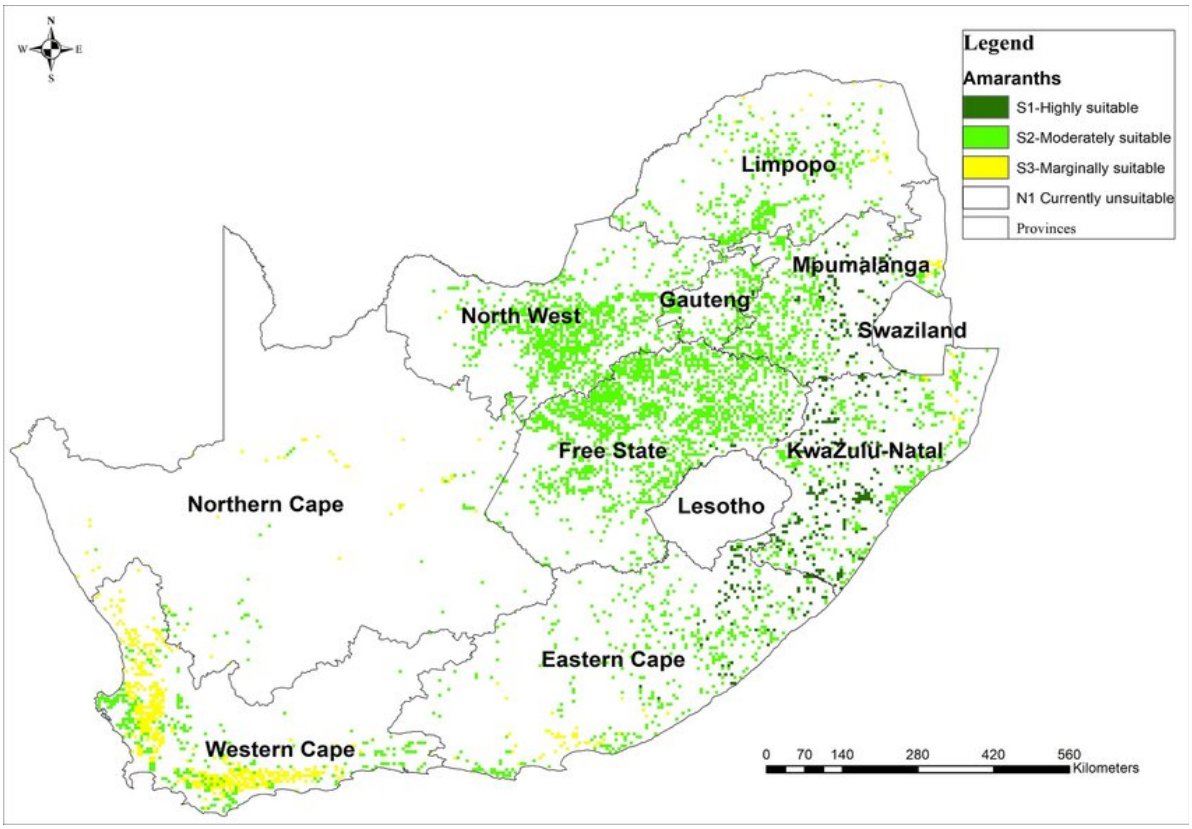


Figure 2.1. *Amaranth species* production areas in South Africa. [Source: Obianuju and Olubukola, (2022)]

2.1.4 Nutritional value of difference species of amaranths

Off late, some species of the amaranths genus are receiving more attention from researchers partly due to their superior nutritional value compared to widely consumed grain crops, elevating their contribution and value among policy makers and the public, as significant contributors of cheap, alternative, and better-quality food. For instance, amaranth contain two-fold higher content of lysine compared to that in wheat grain and three-fold greater content relative to that of maize grain (Adhikary *et al*., 2020). The fresh leaves of amaranth exhibit protein content (dry matter) that range from 17.5 to 38.3% as well as up to 5% lysine (Ayodele and Shittu, 2013). Furthermore, fresh leaves of the leafy vegetable reveal three-fold higher levels of vitamin A and vitamin C, calcium, and niacin compared to that in fresh leaves of spinach (Kamal *et al*., 2022). When the nutritional value of fresh leaves of amaranth was compared to that of lettuce, the former exhibited

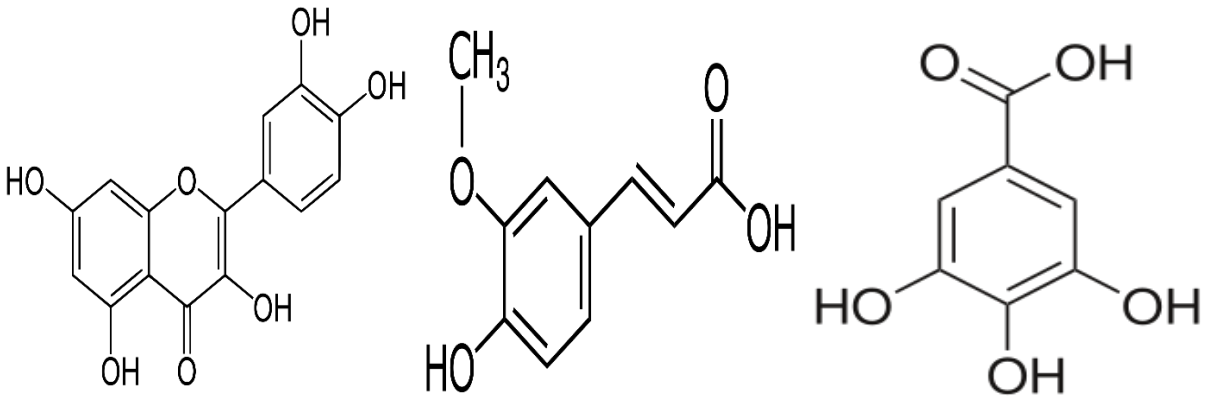
18 times more vitamin A, 13 times more vitamin C, 20 times more calcium, and 7 times more iron (Guillet, 2004). Without a doubt, the fresh aboveground parts of the leafy food crop are highly nutritious, and it can grow well on marginal lands given its ability to withstand hot and dry conditions. In addition to their value to the food industry, *Amaranth species* are recognized to have therapeutic qualities. Similar to most ALVs, amaranth is rarely cultivated in South Africa since it grows naturally (Mavengahama *et al*., 2013).

2.1.5. Secondary metabolites composition of amaranths

The World Health Organization's (WHO) global initiative program encourages people to eat traditional vegetables because they contain non-nutritional bioactive components with health-promoting and protective characteristics. Of the components are secondary metabolites commonly known as phytochemicals whose function include the prevention of human body from contracting chronic diseases and they possess color, aroma, and flavor. Also, consumption of food with non-nutrients helps prevent a variety of diseases such as cancer, cardiovascular and many other diseases compared the few whose prevention is attributed to the consumption of food crops that contain conventional micro- and macronutrients. Phytochemicals provide multiple health benefits to humans largely due to the antioxidants that they contain. The consumption of indigenous vegetables does not only resolve challenges related to nutrition security but is reported to benefit the human body with benefits associated with their being chemo-preventative and cardio-preventative as well as ability to protect against oxidation of free radicals. Phytochemicals are classified into distinct categories such as polyphenols, alkaloids, nitrogen-containing compounds, organosulphur compounds, phytosterols, and carotenoids. The variation between these classes is based on the nature of subsequent groups attached to them.

2.1.5 (a) Phenolic compounds

Plant phenols are bioactive substances that form a significant part of our daily diet. They reportedly offer benefits due to their antioxidant, anti-tumoral, antiviral, and antibiotic properties (Cock *et al*., 1996). They embrace a range of substances that posse's aromatic rings with one or more hydroxyl substances, and they are derived from the shikimate and phenylpropanoid pathways (Robards *et al*., 2009). Phenolics are preset in all plant tissues. Plant phenols also benefit plants through defense against attach by pests, diseases, and predators. Their metabolic origin is via the pentose phosphate, shikimate pathway, and phenylpropanoid metabolism (Eghadami and Sadeghi, 2010). Figure 2.2 below demonstrate the chemical structure of some phenolic compounds such as phenolic acids and flavonoids present in amaranths.

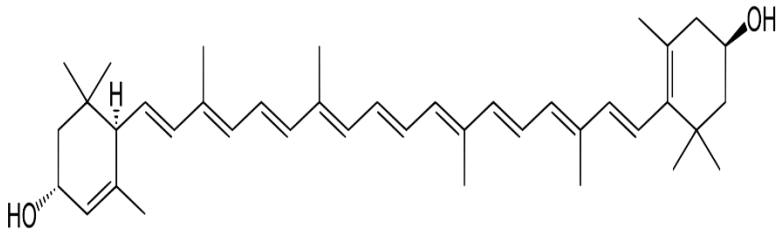


Quercetin ferullic acid gallic acid

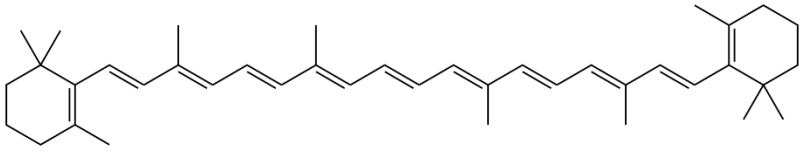
Figure 2.2: Chemical structure of some phenolic acids and flavonoids present in amaranths. (Source: www.researchgate.net)

2.1.5 (b) Carotenoids profiles

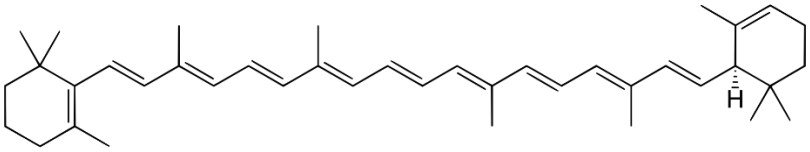
Carotenoids are organic plant pigments that are found in the chloroplasts and chromoplasts of plants while others are found in photosynthetic organisms including some fungi and bacteria (Mortensen, 2006). So far, more than 750 carotenoids have been identified in nature and their main function is the coloration of plants (i.e., yellow, orange, and red colors). In green plants, the color of carotenoids is masked by the more dominant pigment, chlorophyll, and their concentration increase and becomes visible only when plant growth results from the degradation of chlorophyll (Zhang *et al*., 2011). However, only 50 carotenoids are reported to be absorbed and metabolized by the human body. Hence, fruits and vegetables that contain high quantities of carotenoids have been associated a decrease in the risk of various age-related diseases mostly cancer and sight (eye) diseases (Krinsky and Johnson, 2005). Carotenoids are categorized into hydrocarbons, beta-carotene, alpha-carotene, lycopene, xanthophylls, or oxygen-containing carotenoids, beta-cryptoxanthin, lutein, and zeaxanthin (Krinsky and Johnson, 2005). Kiokias *et al.* (2016) also defined carotenoids as isopropanoid compounds that are biosynthesized by tail-to-tail linkages of two C20 geranylgeranyl diphosphate molecules. Which produces the parent C40 carbon skeleton from where all the individuals' variations are derived. Figure 2.3 presents various chemical structure of carotenoids commonly found in amaranth.



Lutein



Beta-carotene



Alpha-carotene

Figure 2.3: Illustrate the chemical structure of various carotenoids of which (lutein) and (β-carotene) are precursors. (Source: www.researchgate.net)

2.1.6 Overview of plant biostimulants as tool for greener solution

The globe is currently grappling with the momentous challenge of an increasing human population which has put pressure on agricultural land. For example, an increase in human population decreases croplands through mining activities and land degradation (Shackleton *et al*., 2020; Santpoort, 2020). Furthermore, it has led to an increased demand for food in a unit area and has also resulted in a shift of cultivation to extreme marginal areas and types of soils. Normally, crop farmers achieve higher crop yields through the use of synthetic fertilizers, however, their continuous application is associated with increased costs for the farmers (Jewell *et al.,* 2010). Pollution of water bodies, low efficiency and other negative impacts, all which render their usage as non-eco-friendly. Given that most inorganic fertilizers are imported by developing countries, their accessibility is a challenge and the occurrence of unpredictable and high frequency of extreme weather events have a negative effect on the efficacy. Plant and Soil Scientists are constantly looking for solutions that involve crop production through the use of agricultural inputs that are ecofriendly and accessible. Such approaches are promoted as alternatives that can be used to produce food that would feed the ever-rising human population as pressure on agroecosystem and socio-economic factors become severe

(Zhang *et al*., 2011). The severity of the challenges is caused by the unpredictability of extreme climate-related events, shrinking of agricultural lands, depletion of natural resources, poor soil nutrition, and reduced crop responses to agrochemicals.

According to Du Jardin (2015), biostimulants may be any substance or mixture of substances of natural origin which improves the condition of crops without causing adverse side effects when applied in small quantities. Moreover, the author further identified that enzymes, proteins, amino acids, micronutrients, and other compounds may be used as a biostimulants, including phenols, salicylic acids, humic and fulvic acids, or protein hydrolases.

Battacharyya and Colla *et al*. (2015) found that the addition of biostimulants to soils accelerates the rate of desorption of bound plant nutrients. Also, when applied to the plant, growth media or seeds, they positively alter the physiological processes of plants and promote the growth, yield, and quality of plants (Gaiero *et al*., 2013; Zhao *et al*., 2018). Furthermore, Bulgari *et al*. (2015) identified that the supply of plants with biostimulants alter their anatomical and physiological properties. Physiologically, they promote various biological activities including photosynthesis, the synthesis of nucleic acid, respiration, antioxidant, and chlorophyll production, and increased metabolism (Bulgari *et al*., 2015). They affect the anatomy of plants through interacting with the environment by promoting the activity of enzymes through the action of phytohormones (Bulgari *et al*., 2015; Moloto *et al*., 2021). In addition, some biostimulants promote the growth of endophytic and non-endophytic organisms that interact with phytohormones (Manzotti *et al*., 2020). Without a doubt, there is sufficient evidence that the application of biostimulants increase the growth, yield, and quality of plants along the promotion of plant tolerance and recovery from abiotic stress, promotion of nutrient assimilation, translocation, and promotion of efficient water use (Bulgari *et al*., 2019). The benefits of biostimulants include being able to promote plant growth and development across all growth stages of a plant's life cycle, from germination post-harvest (Manzotti *et al*., 2020).

2.1.7 Impact of plant -based biostimulants on the growth and yield in leafy vegetables.

According to Tarantino *et al*. (2018), the activity of biostimulants may be described as multifaceted due to its broad spectrum of functionality. This explain n why some food producers have developed interest in agricultural inputs that are manufactured through biological processes and whose application led to the attainment of the largest quantity of healthiest-looking products because such draws consumers' attention, and above all, has multiple health benefits to mankind's body systems (Fawzy, 2012). Of noteworthy, attainment of higher yield depends on the type of biostimulants used, the rate, the method of application, and the plant variety (Milic *et al*., 2018). Previous studies showed that biostimulants affect crop growth and yield positively by increasing size, influencing their metabolic and enzymatic processes. Fawzy (2012) described an increase in the growth and yield of biostimulants-supplied cucumber as associated with an increase in the average length and diameter attained and the biostimulant contained components including humic acids, nitrogen, amino acids, and auxins.

Given the fact that scholarly literature showing elongation and increase in the diameter of biostimulants-supplied vegetables is abound, it seems obvious that most of these agro-inputs elicit the same response. A study conducted by Chaski, and Petropoulos, (2022) showed that the application of biostimulants derived from seaweed extracts mixed with amino acids exhibited the highest weight, leaf weight, as well as chlorophyll content in lettuce grown under induced drought conditions compared to that established with full irrigation. Jain *et al*. (2020), showed that the use of Moringa leaf extracts (MLE) as fertilizer improved the growth and yield of young plants, increased the resistance to diseases and pests, enhanced leaf duration, number of roots, and generally enhanced yield by 20% and 35%. Table 2.3 below shows the summary of effects of biostimulant on growth and yield.

Table 2.3: outlines impact of different plant based biostimulant on growth and yield.

Biostimulants	Growth and yield parameter	Crop	References
Humic acids and amino acids	Leaf length and diameter	Cucumber	Fawzy, 2012
Seaweed extract and amino acids	Plant height and leaf weight	Lettuce	Chaski, and Petropoulos, (2022)
Moringa leafy extracts	Leaf length, roots, and biomass	Tomato	Jain *et al.,* 2020

2.1.8 Impact of plant -based biostimulants on shelf life of leafy vegetables.

The main constraint to increased production especially to sellers of ALVs is the high perishability in the fresh form (Smith and Eyzaguirre, 2007). Another major constraint is that they are seasonal (Vorster *et al*., 2005). Therefore, there is a need to develop and promote appropriate processing techniques to minimize post-harvest losses and such could ensure regular supplies of leafy vegetables from the production areas to consumers. The quality of harvested vegetables deteriorates which leads to losses which occur during the harvesting and handling chain, for example, mechanical damage, and pathological infections play a role in increasing crop loss after harvest (Chakraborty and Chattopadhyay, 2018). Postharvest quality is essential because it is a stage when a food product is marketed and if it is healthy, it has advantages in that it attracts consumers and assumed to improve human health. According to De Diego and Spíchal (2022), there are categories of plant biostimulants that contain compounds which reduce decay and improve the quality of a product by delaying the onset of senescence during storage. Therefore, their application to food produce at the preharvest stage can improve product shelf life, since they can alter, promote, or inhibit plant physiological and morphological processes when applied at a very low concentration (Perezjmenez *et al*., 2015). Several studies report that the supply of some plant biostimulants retard the occurrence of several

postharvest changes and overall, enhance the quality of fruits and vegetables when exposed to various storage conditions such as cold storage, modified atmospheric packaging, and controlled atmosphere storage (Alsawmah *et al*., 2018; Chakraborty and Chattopadhyay, 2018; Hasan *et al*., 2019). However, so far, there is dearth of published literature especially on the use of biostimulants to enhance the postharvest quality of food produce such as leafy vegetables. Postharvest effects of biostimulants involve increasing the flexibility of cell walls at the same time extend the shelf-life of fruits and vegetables for consumption and facilitate their storage. A study conducted by miceli *et al*. (2021) indicated that preharvest treatments with *E. maxima* extract were effective in delaying leaf senescence and extending the shelf-life of fresh-cut leaf lettuce. Another study conducted by Cristofano *et al*. (2021) showed that protein hydrolysates improved the shelf life of nightshade. Nevertheless, there is limited information on the effects of biostimulants on preharvest properties of indigenous leafy vegetables, including extending the shelf life of especially *Amaranthus species*.

2.1.9 Impact of plant-based biostimulants on nutritional composition of leafy vegetables.

Food that exhibits high nutritional quality is desirable largely because it contribute to the maintenance of health and nutritional well-being of humans. In particular, the nutritional well-being is the driving force for the development and maximization of human genetic potential (Radhika *et al*., 2011). Therefore, health-conscious people insist on eating and/or buying dietary quality of food that improve the quality of their diet since it helps maintain the overall health and fitness. On the other hand, when such food is made available to rural communities, it addresses deep-rooted malnutrition. The option of buying nutritiously health food is costly of poor rural households, therefore, another approach that can help address malnutrition is the diversification of the fertilizer program. A study conducted by Du Jardin (2015) reveals that the inclusion of seaweed extracts within a fertilizer program enhanced the nutritional quality of crops through increased the accumulation of both macro- and micronutrients. In another study, the concentration of main macronutrients (nitrogen, phosphorus, and potassium) in grain of *Glycine max* was significantly enhanced by the addition of seaweed extract at varying dilutions of 10, 12.5, and 15% (v/v) when compared to the control (Rathore *et al.*, 2017).

2.1.10 Impact of plant-based biostimulants on the secondary metabolites of leafy vegetables.

Indigenous leafy vegetables have the potential to prevent different diseases, infections, and condition due to the phytochemicals compound that consist of antioxidants, anti-inflammatory and anti-biotic properties (Ahmad and Aslam, 2016). Interestingly, antioxidants also have a physiological effect on humans and tend to function differently within the human body by protecting it against diseases (Kia *et al*., 2018). Only few biostimulants have been reported to influence the secondary metabolite content of plants. For example, Nardi *et al*. (2016) showed that protein hydrolysates improved the production of secondary metabolites of Cleome plant including phenols and antioxidants and further increased flavonoid biosynthesis. A significant increase was observed in the phenolic content of the *Phaseolus vulgaris* treated with Kelpak® while untreated plants had no increase in phenolic content (Kocira *et al*., 2018). However, flavonoid content was significantly enhanced in both treated plants and untreated plants. The polyphenol content of potato tubers was significantly increased by the Kelpak® application (Ramírez *et al*., 2014). Research on the effect of biostimulants on the phytochemistry of plants has been conducted, however, limited research has been conducted on indigenous plants such as *Amaranth species*. Table 2.4 shows a summary of effects of different plant based biostimulants on secondary metabolites.

Table 2.4: Impact of different plant- based biostimulants on secondary metabolites.

Biostimulants	Plant	Metabolites	References
Protein hydrolysis	Cleome	Phenolic acids, flavonoids, and antioxidants	Nardi *et al.,* 2016
Kelpak®	*Phaseolus vulgaris*	Phenolic acids and flavaniods	Kocira *et al.,* 2018).
Kelpak®	Potato	Phenolic acids	Ramírez *et al.,* 2014).

2.2 Work not done on the Research problem.

Various studies have been conducted on the impact of biostimulants on plant growth, yield, nutritional composition, and secondary metabolites of amaranths and other African leafy vegetables (ALVs). However, scholarly researchers then to use other biostimulants such as seaweed extract, and protein hydrolyses among other biostimulants. Currently, no studies have been conducted on Phytostim® biostimulants since it is a relatively newly developed product. Hence, there is rarely published literature on its ability to affect the growth, yield, and nutritional quality of crops. Even though biostimulants promote plant growth and nutrition, their efficacy differs with plant species and environmental conditions, as well as the type of biostimulants used. *Amaranthus species* is among underutilized species of important value in food security, especially in rural communities. With the increase in malnutrition across the world, optimizing yield, biochemical content, and mineral elements of amaranths remains of utmost importance, especially in food-insecure regions. This, therefore, demands more studies using pre-harvest organic sustainable agricultural input to prolong the shelf life of ALVs such as amaranth and be stored for small scale farmers with less cost effects.

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CHAPTER 3

EFFECTS OF PHYTOSTIM® BIOSTIMULANT ON GROWTH AND YIELD OF *AMARANTHUS CAUDATUS* AND *AMARANTHUS CRUENTUS*

ABSTRACT

Amaranthus species are indigenous crops to South Africa, and commonly consumed as leafy vegetables to improve food security. This study aimed at evaluating the impact of supplying different concentrations of Phytostim® biostimulant on the growth and yield components in *Amaranth cruentus* and *Amaranth caudatus*. A completely randomized block design was used to establish a greenhouse trial which consisted of eight treatments based on different concentrations of Phytostim® biostimulant (0, 0. 5; 1; 1; 2.5; 3; 4.5; and 6%), with the untreated sample being a control (0%). Each treatment was replicated eight times and the biostimulant was applied as foliar at 14-day intervals. At the four-leaf stage, seedlings of *A. cruentus* and *A. caudatus* were transplanted in a mixture of growth medium consisting of Hygromix growth medium, pasteurized loam, and sandy soil at a ratio of 2:1:1. Data for vegetative growth and yield parameters including (stem diameter, plant height, and number of branches per plant, number of leaves per plant, fresh and dried leaf mass per plant) were collected at termination day after eight weeks. Data was subjected to one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test was used for separating means using the Statistix 10.0 software package. The results revealed that all the growth and yield-related parameters were highly influenced by the application of 1.5% followed by 1 and 2.5% biostimulant concentrations, respectively. These concentrations performed better in comparison to untreated samples and other biostimulant concentrations. Application of 1.5% concentrations can be recommended for improved growth and yield components in *Amaranthus species* to a level resulting in net economic benefits.

Keywords: Phytostim® biostimulant, *Amaranthus caudatus*, *Amaranthus cruentus*, growth and yield parameters.

3.1 INTRODUCTION

Amaranthus species are among important indigenous food crops that are indigenous and able to adapt to marginal soils of most African conditions and add to the nutritional diversity of especially rural communities (Maseko *et al*., 2017). Despite the importance role in improving the nutrition of humans, there is dearth data on their production under cropping fields in South Africa. Therefore, there is a need for initiatives that can encourage the cultivation of the important food crop, especially through the use of cheap and environmentally friendly agricultural inputs such as biostimulants. The use of technologically innovative in food production has great potential to contribute to the sustainable development of plant production and this explain why such approaches attract interest from stakeholders, the fertilizer industry, researchers, and farmers. The intention of using innovations that are based on agricultural technologies largely result in the improvement of the growth and yield on various crops while reducing negative environmental problems associated with the use of especially inorganic fertilizers. One such innovation is the use of plant-based biostimulants as agricultural inputs. In the past years, South Africa has experienced an increase in companies that import, manufacture, and sell biostimulants (Raimi *et al*., 2017). In general, the application of plant biostimulants to plants is aimed at improving their yield and quality, also, the products elicit mechanisms that improve the ability of plants to adapt to biotic and abiotic stress conditions (Mpai *et al*., 2022). Of plant biostimulants is Phytostim® which is manufactured using extract of the Moringa tree and prepared of 22 amino acids that are reportedly vital for plant growth. Broadly, plant growth refers to an increase in the volume or mass of a plant volume with or without the formation of new structures such as organs, tissues, cells, or cell organelles (Albersheim *et al*., 2010). The process of growth is usually associated with development cell, tissue specialization, and reproduction. Whereas plant yield is a measurement concerned with the quantification of products towards or during the harvesting period (Albersheim *et al*., 2010). Usually, an increase in yield is associated with an improve in the quality of plants, be it vegetables or fruits. Markedly improved quality of especially food crops is particularly essential to producers and consumers alike as it allows for the attainment of health-looking food products.

Objective of the study

The objective of this chapter was to investigate whether different concentrations of Phytostim® biostimulant would have an effect on the growth and yield of *A. cruentus* and *A. cruentus.*

3.2 MATERIALS AND METHODS

3.2.1 Description of the study area

Experiments for *A. cruentus* and *A. caudatus* were conducted simultaneously under greenhouse conditions at the Green Biotechnologies Research Centre of Excellence (GBRCE), University of Limpopo, South Africa (23°53''10'S, 29°44''15'E) during spring (September – November) 2022. The greenhouse structure had an area of 2000 m^2 (100 m × 20 m) in size, with thermostatically activated fans on the north-facing wall and the wet wall on the south-facing side for moderating inside temperatures. Day/night ambient temperature range from 20/25°C, with the top part of the structure covered with a 35% radiation-allowing green net.

3.2.2 Treatments and research design

The experiment was laid out in a randomized complete block design (RCBD) with eight treatments (0, 0.5, 1, 1.5, 2.5, 3, 4.5, and 6%) of Phytostim® biostimulant, replicated eight times and untreated (0) was used as control (figure 3.1).

(a) (b)

Figure 3.1: Experimental layout (a) *A. cruentus* and (b) *A. caudatus.*

3.2.3 Properties of Phytostim® biostimulant

It is a plant derived biostimulant extracted through enzymatic hydrolysis of proteins in *Moringa oleifera* Lam. crude extract. The enzymatic hydrolysis procedure was also performed to separate the insoluble residues of amino acids compounds following similar procedures to those described by Paul *et al*., (2019). The final product of Phytostim® biostimulant contained 22 aminogram made-up of valine (323.8 mg/L), isoleucine (246.6 mg/L), leucine (437.4 mg/L), phenylalanine (259.2 mg/L),glutamic acid (507.6 mg/L), aspartic acid (315.0 mg/L), glycine (244.6 mg/L), serine (269.2 mg/L), threonine (249.9 mg/L), alanine (365.2 mg/L) and proline (222.1 mg/L) which constitutes 70% of the active ingredients (Mpai *et al*., 2022).

3.2.4 Procedure and cultural practices

The biostimulant was prepared by diluting selected concentrations of Phytostim® biostimulant with distilled water. Seeds of *A. cruentus and A. caudatus* were obtained from the ARC-VIMP, Pretoria, South Africa. The seeds were planted and raised in a medium sterile 200 polystyrene seedling trays filled with Hygromix (Hygrotech, Pretoria, South Africa) under greenhouse conditions and irrigated up to infiltration filed capacity till they sprout. At the two-leaf stage, seedlings were hardened-off for a week outside the greenhouse prior planting. Twenty-centimeter plastic pots were arranged on greenhouse benches at intra- and inter- row spacing of (20 x 40 cm). After 4 weeks of sowing at early whorl stage, uniform *A. cruentus* and *A. caudatus* seedlings were transplanted directly into 20 cm plastic pots each containing approximately 6880 cm^3 growing mixture of steam pasteurized (300 °C for 45 minutes) loam soil, sand, and Hygromix at a ratio of 2:1:1 v/v/v. Each pot contained one plant per drip hole, irrigated with 250 mL of water as reported by Mpai *et al.*, 2022. Treatments was applied eight days after transplanting to allow for adaptation, then repeated at 14-day intervals as foliar application until harvest. Pests, and diseases were managed in accordance to crop specifications (Farha *et al.,* 2018). Phytostim® biostimulant was used as an organic fertilizer. Therefore, no other fertilizers were applied during the growth season. At harvest, the plants were uprooted, excess soil were removed from the roots using paper towels.

3.2.4 Data collection

The vegetative growth parameters, stem diameter, plant height, number of branches and chlorophyll index were measured randomly per treatment. Stem diameter was measured using a digital Vernier caliper. Plant height was measured from the soil level in the pot to the tip of the leaf flag using a ruler stick. Numbers of branches per tree were counted.

The yield parameters were measured at the termination point of the experiment at harvest. During termination, fresh leaf, fresh root, and dry mass, and the number of leaves were measured per treatment. The fresh mass of leaves and roots was weighed on a weighing scale whereas the dry mass of leaves and roots were oven dried at 40 °C for 72 h and masses were recorded. Leaves were randomly counted and recorded.

3.2.5 Data analysis

The data obtained for different plant parameters were statistically analyzed using one-way analysis of variance (ANOVA) through the Statistix 10.0 software to observe significant differences. The significance of the differences among the treatment means was evaluated by the Duncan's Multiple Range Test at a 5% level of probability.

3.3 RESULTS AND DISCUSSION

3.3.1. Effect of Phytostim® biostimulant treatments on growth attributes of *Amaranth species.*

In this research chapter, the aim was to assess whether different concentrations of Phytostim® biostimulant on *Amaranth species* alter their growth attributes including plant height, stem diameter, and number of branches. Results indicated that the application of different concentrations of Phytostim® biostimulant significantly ($p<0.05$) affected the aforementioned growth attributes. For example, the application of 1.5% Phytostim® biostimulant improved the most of the selected growth attributes of studied crop as it outperformed the other concentrations.

Figure 3.2 demonstrate that different concentrations of Phytostim® biostimulant had significant ($p<0.05$) effects on the height of *Amaranth species*. From the results, it can be observed that foliar-spraying 1.5% of Phytostim® biostimulant was more effective in increasing the height, three-folds greater than the control of the studied crop. The results

further demonstrated that using 0.5; 1; 1.5; 2.5; and 3% of Phytostim® biostimulant resulted in two-folds taller plants than that used as controls. By contrast, the application of 4.5 and 6% of Phytostim® biostimulant reduced plant height. Overall, the results showed that plants of *A. caudatus* were markedly taller compared to that of *A. cruentus*. The significantly higher height of *A. caudatus* could be attributed to genetic variability between the species. According to Shireen *et al.* (2018) taller food crops are considered desirable as they have better ability to absorb nutrients and capture sunlight for photosynthesis.

Furthermore, figure 3.3 illustrate that different concentrations of Phytostim® biostimulant had significant ($p<0.05$) effects on the stem diameter of the selected *Amaranth species*. In fact, the treated plants recorded enhanced the stem diameter of the plants as compared to the untreated plants (control). The results on stem diameter showed a similar trend to that of the plant height, showed on Figure 3.3a for *A. cruentus*. However, figure 3.3b show no significant difference between the treated and untreated plants of *A. caudatus* on stem diameter. According to Ding *et al*. (2023), stem diameter is among important agronomic traits that predict lodging resistance and yield and both parameters can attribute to variations in the two *Amaranth species*. In addition, it could also be attributed to the genetic variability to adapt to marginal growing conditions. The results could confirm that the species are sensitive to climatic conditions (Veronical *et al*., 2021).

The results shown in Figures 3.4 and 3.5 also demonstrate that different concentrations of the Phytostim® biostimulant had significant effects ($p<0.05$) on the number of branches and chlorophyll index of the *Amaranth species*, respectively. The trend observed on plant height was also observed on number of branches and chlorophyll index. According to Simson (2023), branches play a crucial role in the growth, development, and overall function of a plant as they provide support for leaves and other structures, facilitate the distribution of water, nutrients, and hormones, and contribute to the overall shape and architecture of the plant. Furthermore, Simson (2023) reported that during the growth and development of branches, hormones, particularly auxins and cytokinins, which control the initiation and elongation of branches, play a crucial role. Other management practices such as exposure to light conditions, addition of mineral nutrients, and pruning practices

can also impact branch growth and branching patterns (Raza *et al*.,2020). This means that Phytostim® biostimulant play a significant role in enhancing the growth of amaranth partly because it is made from *Moringa oleifera* that constitutes of plant growth regulators such as auxins and cytokinins. Based on Kalaji *et al.* (2017), chlorophyll is a good indicator of plant health and is an indicator of the growth and development of plants. The results further demonstrate that the growth varied based on the concentrations of the biostimulant that was applied and between the selected species.

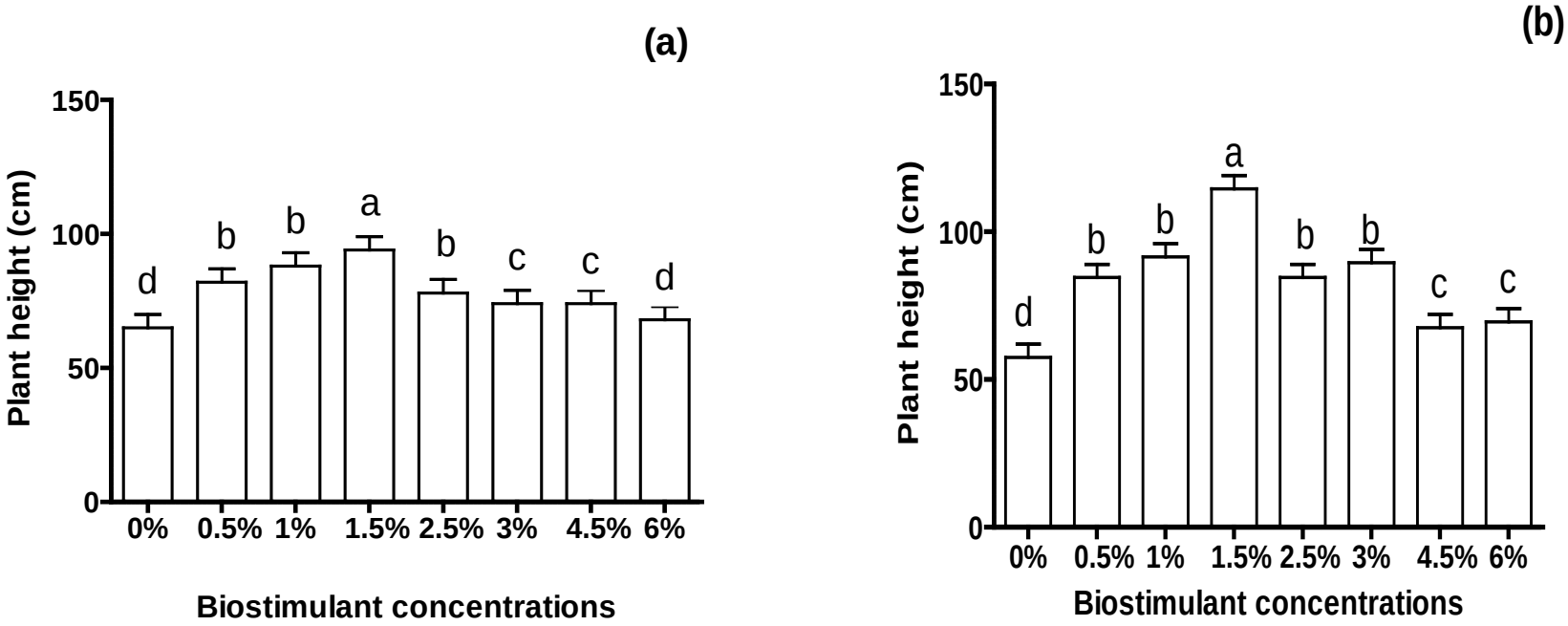


Figure 3.2: Effects of different concentrations of Phytostim® biostimulant on plant height (a) *A. cruentus* and (b) *A. caudatus*. Bars (± SE) with different letters are significantly different at ($p<0.05$).

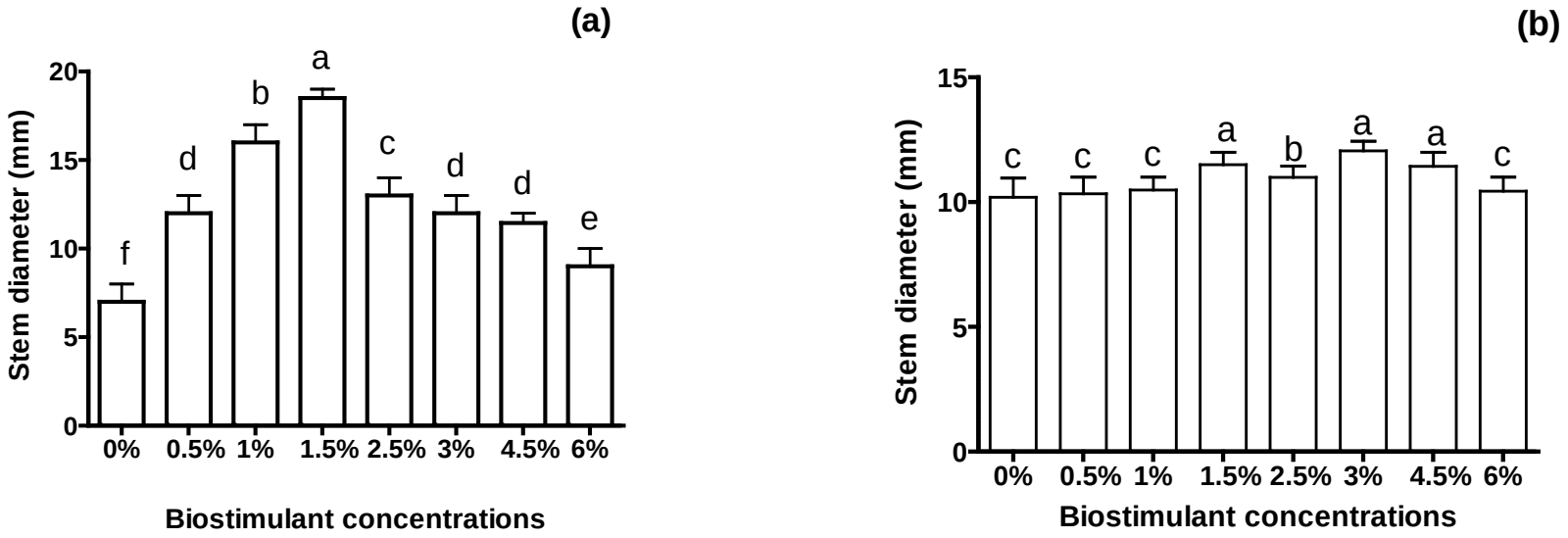


Figure 3.3: Effects of different concentrations of Phytostim® biostimulant on stem diameter (a) *A. cruentus* and (b) *A. caudatus*. Bars (± SE) with different letters are significantly different at ($p<0.05$).

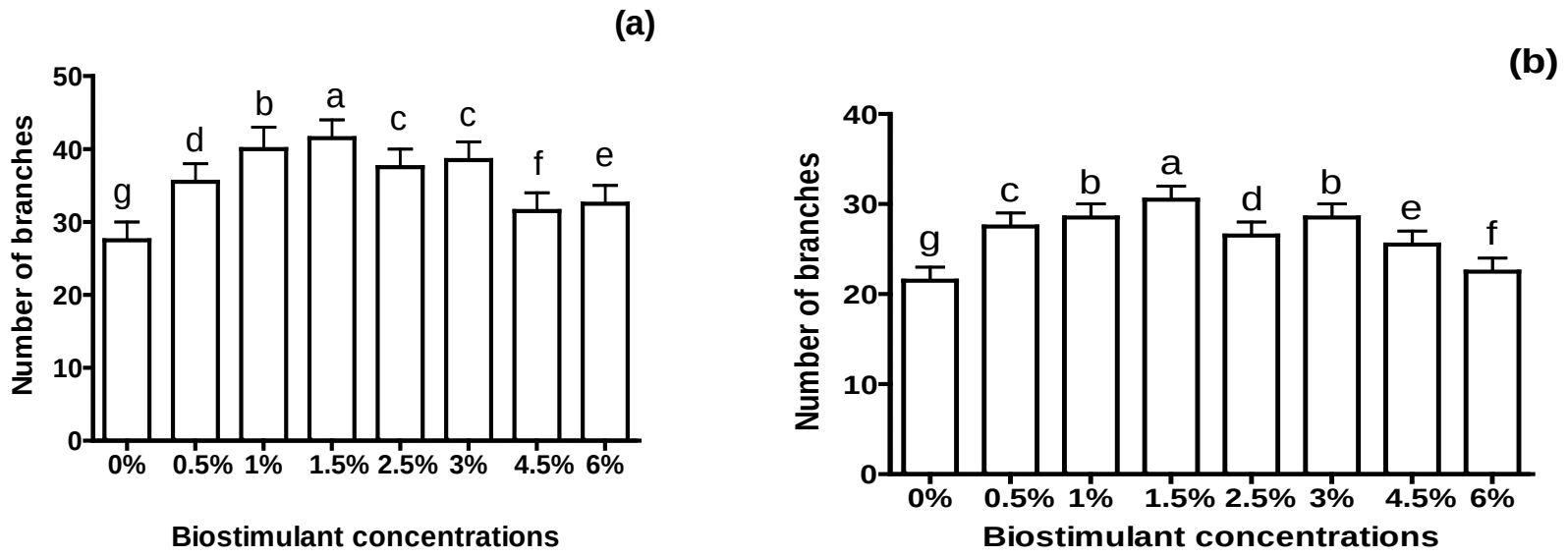


Figure 3.4: Effects of different concentrations of Phytostim® biostimulant on number of branches (a) *A. cruentus* and (b*) A. caudatus*. Bars (± SE) with different letters are significantly different at ($p<0.05$)

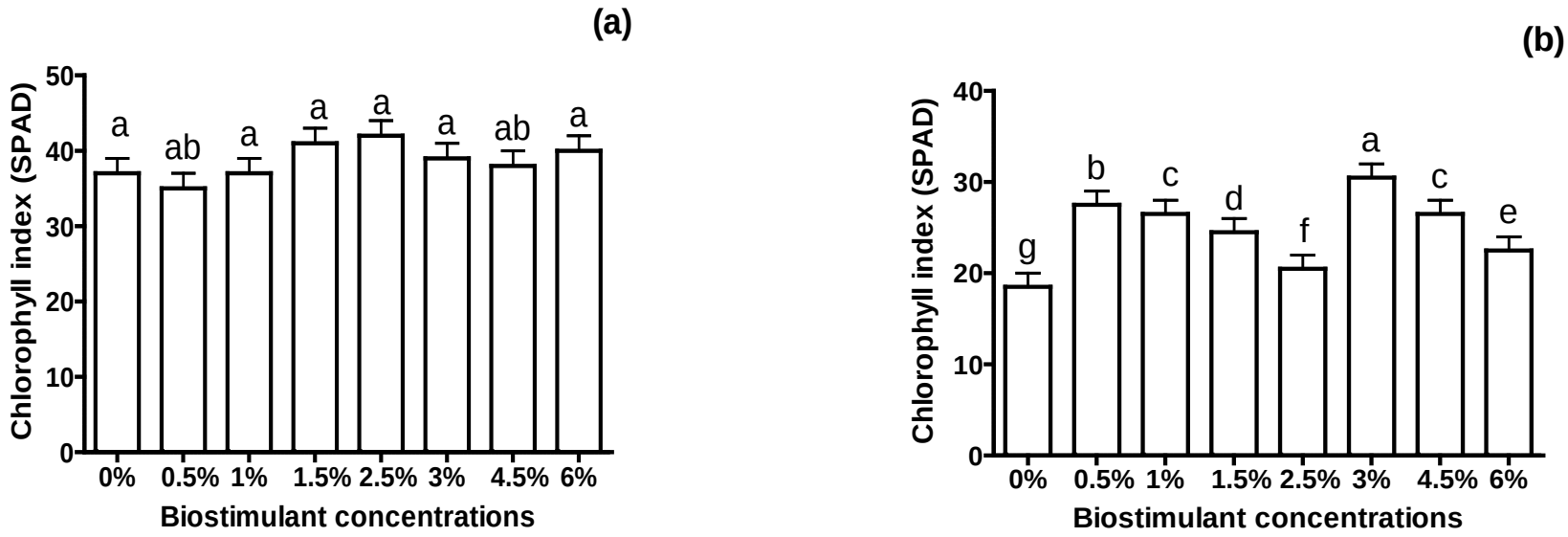


Figure 3.5: Effects of different concentrations of Phytostim® biostimulant on chlorophyll index (a) *A. cruentus* and (b) *A. caudatus*. Bars (± SE) with different letters are significantly different at ($p<0.05$).

3.3.2 Effect of Phytostim® biostimulant treatments on yield of *Amaranth species*.

The selected different concentrations Phytostim® biostimulant significantly affected ($p<0.5$) the yield attributes of the two *Amaranth species*, however, some of the yield attributes were not significantly affected ($p>0.05$). Table 3.1 show a summary of the effects of the application of Phytostim® biostimulant, on parameters determined during the harvest of *A. cruentus*. The results indicate that shoot biomass, aerial mass, leaf length, leaf width, root mass, and number of leaves were significantly affected by the application of Phytostim® biostimulant since it improved most of the yield parameters positively. In contrast, the root length and dry weight of roots were unaffected by the application of Phytostim® biostimulant. Table 3.2 illustrate that supplying different concentrations of Phytostim® biostimulant affected the yield of *A. caudatus*. It denotes that biomass, aerial mass and dry leaves were significantly influenced by the application of different concentrations of Phytostim® biostimulant. Lastly, the leaf length, leaf width, root length, roots mass and number of leaves did not differ significantly ($p>0.05$).

When supplied at higher or non-recommended rates, inorganic fertilizers can have detrimental effects on crops, soils, and water bodies. Therefore, the development of products that can be used for co-application along inorganic fertilizers of alone, to support the growth of plants is crucial to mitigate the negative impact associated with improper application or inorganic fertilizers. In particular, the use of natural plant products such as *Moringa oleifera* extracts to develop plant biostimulants including Phytostim®, offer alternative and environmentally friendly technology. However, there is scanty information whether the application of some of these products as agricultural inputs improve the growth and yield of plants. The results revealed that supplying Phytostim® significantly ($p<0.05$) affected the growth and yield of the *Amaranth species*. Even though, some of the measured variables were not significantly affected. Phytostim® biostimulants supplied at 1.5% significantly improved the growth of *A. cruentus* and *A. caudatus*, as shown by the height, stem diameter, number of branches, and chlorophyll index (Figures 3.2 to 3.5) respectively. The observed response could be attributed to the effects of phytohormones, which are widely reported as present in most plant biostimulants and improve the growth and yield of plants by triggering physiological process of plant (Soliman and Hamed,

2019; Stasio *et al*., 2017). In agreement with our findings, Mpai *et al*. (2022) found that Phytostim® significantly improved the growth and yield of lettuce cultivars when applied at 3%. Abdalla (2013) found that a adding a biostimulant made using extracts of *Moringa oleifera* significantly improved the rockets (*Eruca vesicaria subsp. Sativa*) when supplied at 2%, resulting in an overall increase in height. Several researchers have indicated that extracts of *Moringa oleifera* contain the plant hormone zeatin, which is involved in numerous vital plant physiological processes (Mehmood *et al*., 2021; Mashamaite *et al*. 2022; Yuniate *et al*., 2022). Similarly, Toscano *et al* (2021) reported that the application of biostimulants made from extract of *Moringa oleifera* lead to a significant increase in the growth and yield of the baby leaves, cultivar kale, and broccoli. The current results on the effects of the biostimulant made using extracts of *Moringa oliefera* on *A. cruentus* and *A. caudatus,* concur with that from previous studies. As shown on biostimulants derived from extracts of seaweed, smoke-derived bioactive compounds also stimulate the growth and productivity of many horticultural crops. Kulkarni *et al*., (2019) reported that seaweed extracts significantly affected the growth and yield of spinach.

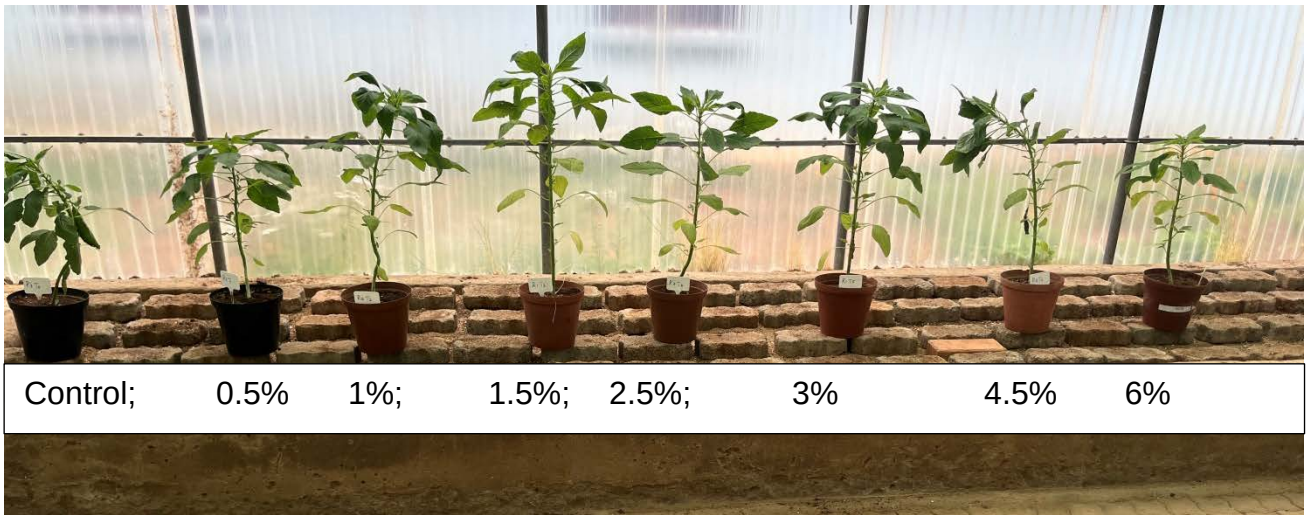
It is therefore not surprising that Phytostim® biostimulants, which is also a concentrate from extracts of *Moringa oleifera* that contain amino acids, produced similar results to that of other biostimulants derived from extracts of *Moringa oleifera* which emphasizes the notion that it stimulates growth and development as observed in *A. cruentus* and *A. caudatus*. Furthermore, the 22 essential amino acid molecules present in the Phytostim ® biostimulants play a direct role in supporting plant growth and development and are also responsible for the improvement of crop growth and yield parameters. This is because, amino acids participate in maintaining the structure of proteins required for cell division, cell differentiation, and growth. Also, they can be converted into polyamines and enlarge by entering the hormone structures allowing nitrogen movement between the cell and organs.

CONCLUSION

Results of this study indicate that Phytostim® biostimulant as a foliar spray on *A. cruentus* and *A. caudatus* had significant positive effects on the growth and yield parameters such

as plant height, stem diameter, number of branches, number of leaves etc. It can also be concluded that the plants treated with Phytostim® biostimulant at lowest concentrations from 0.5 to 3% were of good growth and yield as compared to the untreated plants and Phytostim® biostimulant at concentrations from 4.5 and 6%. Findings from the present study, further indicate that these biostimulant should be applied at the lowest concentrations less than 3% as they give good quality results.

(a)



(b)

Figure 3.6: Effect of Phytostim® biostimulant on growth of *Amaranth species* (a) *A. cruentus* and (b) *A. caudatus*

Table 3.1: Effects of different concentrations of Phytostim® biostimulant at harvest on *A. cruentus*.

Treatments (%)	Biomass (g)	Aerial mass (g)	Root mass (g)	Leaf length (cm)	Leaf width (cm)	Root length (cm)	Dry roots (g)	Number. of leaves
0	61.53 ± 3.1^{b}	45.73 ± 0.49^{e}	16.13 ± 0.95^{a}	14.00 ± 2.12^{c}	6.50 ± 0.35^{bc}	24.00 ± 1.89^{a}	9.33 ± 1.41^{a}	28.17 ± 2.99^{d}
0.5	71.03 ± 3.41^{a}	60.60 ± 0.49^{ab}	10.43 ± 0.68^{c}	14.33 ± 2.12^{bc}	6.16 ± 0.35^{c}	19.83 ± 1.89^{a}	9.33 ± 1.41^{a}	36.27 ± 2.99^{b}
1	74.23 ± 3.41^{a}	64.00 ± 0.49^{a}	10.23 ± 0.35^{c}	15.33 ± 2.12^{b}	7.26 ± 0.49^{b}	22.17 ± 1.89^{a}	10.00 ± 1.41^{a}	33.47 ± 2.99^{c}
1.5	73.16 ± 6.18^{a}	59.59 ± 0.89^{b}	13.66 ± 0.66^{b}	16.42 ± 3.85^{a}	8.65 ± 2.26^{a}	31.74 ± 3.43^{a}	10.17 ± 2.55^{a}	38.03 ± 5.44^{a}
2.5	63.63 ± 4.28^{b}	56.67 ± 0.62^{bc}	6.97 ± 0.44^{f}	13.67 ± 2.66^{cd}	6.33 ± 3.46^{c}	19.83 ± 2.89^{a}	8.67 ± 1.77^{a}	24.53 ± 3.75^{e}
3	49.96 ± 3.41^{d}	42.50 ± 0.49^{f}	7.47 ± 0.35^{e}	13.50 ± 2.12^{cd}	6.67 ± 3.31^{bc}	22.33 ± 1.89^{a}	9.67 ± 1.41^{a}	19.90 ± 2.90^{g}
4.5	58.33 ± 3.41^{c}	50.00 ± 0.49^{d}	8.67 ± 0.48^{d}	13.33 ± 2.19^{c}	6.33 ± 0.59^{c}	21.83 ± 1.89^{a}	10.33 ± 1.41^{a}	21.80 ± 2.99^{f}
6	65.60 ± 3.41^{b}	55.03 ± 0.49^{bc}	10.56 ± 0.35^{c}	14.50 ± 2.12^{bc}	6.33 ± 2.45^{c}	19.17 ± 1.89^{a}	9.33 ± 1.41^{a}	25.80 ± 2.99^{e}
F-Statistics	0.00**	0.00**	0.04*	0.00**	0.01**	0.07ns	0.99ns	0.01**

Value (Mean ± SE) with different letters on each column are significantly different at ns= not significant at ($p>0.05$), * = ($p<0.05$), ** = ($p<0.01$).

Table 3.2: Effects of different concentrations of Phytostim® biostimulant at harvest on *A. caudatus*.

Treatments (%)	Biomass (g)	Aerial mass (g)	Root mass (g)	Leaf length (cm)	Leaf width (cm)	Root length (cm)	Dry roots (g)	Number. of leaves
0	65.26 ± 2.73^{d}	56.33 ± 1.97^{c}	8.30 ± 1.48^{a}	16.33 ± 1.31^{a}	7.00 ± 0.56^{a}	23.50 ± 2.78^{a}	9.00 ± 0.97^{h}	24.40 ± 5.41^{a}
0.5	65.50 ± 3.45^{d}	54.03 ± 2.49^{d}	10.97 ± 1.87^{a}	15.83 ± 1.66^{a}	7.16 ± 0.71^{a}	25.17 ± 3.51^{a}	10.00 ± 1.23^{e}	26.80 ± 6.83^{a}
1	60.97 ± 2.90^{e}	51.73 ± 2.09^{e}	9.23 ± 1.57^{a}	18.33 ± 1.39^{a}	8.16 ± 0.60^{a}	25.17 ± 2.95^{a}	9.67 ± 1.03^{f}	23.47 ± 5.70^{a}
1.5	72.93 ± 3.45^{b}	61.43 ± 2.44^{b}	11.50 ± 1.87^{a}	18.17 ± 1.66^{a}	8.33 ± 0.71^{a}	23.50 ± 3.51^{a}	11.67 ± 1.23^{c}	28.90 ± 6.38^{a}
2.5	85.93 ± 5.00^{a}	71.17 ± 3.61^{a}	14.77 ± 2.71^{a}	16.33 ± 2.41^{a}	7.16 ± 8.11^{a}	21.33 ± 5.10^{a}	15.00 ± 1.78^{a}	38.60 ± 9.91^{a}
3	76.57 ± 2.40^{ab}	65.20 ± 1.73^{b}	11.37 ± 1.30^{a}	18.17 ± 1.15^{a}	7.33 ± 7.23^{a}	28.00 ± 2.45^{a}	10.33 ± 0.85^{d}	34.73 ± 4.76^{a}
4.5	66.20 ± 5.00^{c}	56.13 ± 3.61^{c}	10.17 ± 2.71^{a}	15.67 ± 2.40^{a}	7.66 ± 8.11^{a}	18.17 ± 5.10^{a}	9.33 ± 1.78^{d}	28.17 ± 9.91^{a}
6	75.53 ± 2.73^{ab}	63.90 ± 1.97^{b}	11.63 ± 1.48^{a}	15.50 ± 1.31^{a}	7.63 ± 7.83^{a}	21.33 ± 2.78^{a}	12.00 ± 0.97^{b}	31.57 ± 5.41^{a}
F-statistics	0.01**	0.04*	0.06ns	0.93ns	0.83ns	0.41ns	0.03*	0.07ns

Value (Mean ± SE) with different letters on each column are significantly different at ns= not significant at ($p>0.05$), * = ($p<0.05$), ** = ($p<0.01$).

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CHAPTER 4:

INTERACTIVE EFFECTS OF DIFFERENT CONCENTRATIONS OF PHYTOSTIM® BIOSTIMULANT AND STORAGE ON POSTHARVEST QUALITY AND NUTRITION OF *AMARANTHUS CAUDATUS* AND *AMARANTHUS CRUENTUS*

ABSTRACT

The study was aimed at investigating the effects of different concentrations Phytostim® biostimulant and storage period interaction on postharvest quality and nutritional status of *A. cruentus* and *A. caudatus*. The experiments were laid out following the factorial treatments (8 x 3) arranged in a Completely Randomized Design (CRD). Each treatment was replicated three times. An equal weight of 100 g was packed in ventilated punnet and stored at an ambient temperature for 0,3 and 6 days. The postharvest quality attributes (weight loss%, color, and visual quality) were accessed. A total 5 g of sample per biostimulant concentrations and storage time (0, 3, and 6 d) were sampled, oven dried at 40 °C for 72 h and kept for the analysis of nutrients (minerals, amino acids, and proteins). The obtained results revealed that weight loss%, color, and visual quality of amaranth were significantly ($p<0.05$) influenced by different concentrations of Phytostim® biostimulant and storage time. All the above-mentioned postharvest attributes were maintained at 2.5% concentration of Phytostim® biostimulant in comparison to control. Moreover, the study further revealed that different concentrations of Phytostim® biostimulant and storage time significantly ($p<0.05$) influenced the mineral elements (Mg, Ca, Fe, K, Zn, N, Cu, Se, and Mn), amino acids (His, Leu, Lys, Met, Phe, Thr, Val, Ala, Arg, Asp, Glu, Gly, Pro and Ser) and protein content of the studied leaves. The nutrients components were enhanced at 2.5% concentration of Phytostim® biostimulant as compared to the control and other concentrations of Phytostim® biostimulant. Thus, it can be concluded that Phytostim® biostimulant can be recommended to be used effectively by farmers at 2.5% or less in preserving the high-quality characteristics of *Amaranth species* during ambient temperature.

Keywords: *Amaranthus species*, Phytostim® biostimulant, postharvest quality attributes, nutrients components and ambient temperature.

4.1 INTRODUCTION

One of the greatest challenges in agriculture, besides enhancing food production, is to provide quality of food and essential nutrients necessary for humans to maintain good health (Ngoroyemoto et al., 2020). Leafy vegetables are high-value crops that have a high nutritive value as they are rich sources of essential minerals (macro and micronutrients) elements (Kwenin *et al.,* 2011). In addition to high nutritional value they provide with protein, fiber, amino acids, and dietary energy. The essential minerals vary from species to species (Asyira *et al.,* 2016). Asyira *et al*. (2016) further stated that, macro minerals such as Mg, Ca, P, K, Na are required in large qualities and micro minerals such as Zn, Fe, Cu, Se, Mn are required in small quantities. They play essential role in health, balanced diet required for normal metabolic activities of the human body and growth (Berto *et al.,* 2015; Pereira and Dantas, 2016). While proteins are major macronutrients that are large with complex molecules composed of various compositions of amino acids (Methionine, Leucine, Tryptophan etc.). They play critical roles in cellular functions, structure, and regulations of metabolic activities. In addition, they have great stability during processing. However, their profiles in green leafy vegetables are highly dependent on the application of external synthetic fertilizers or organic soil amendments (Maseko *et al*., 2017).

Leafy vegetables are highly perishable when stored in fresh form due to various biological and environmental factors with temperature playing a central role (Ambuko *et al.,* 2017). This may lead to their nutritional quality to be reduced (Barrett and Lloyd, 2012). Therefore, proper cultivation practices and input that helps in prolonging shelf life is necessary. The use of nitrogen fertilizers at 120kg/ha which were commercially recommended for lettuce production were reported detrimental to postharvest shelf file through increased browning color and phytonutrients of lettuce (Mampholo *et al.,* 2018). While the use of Phytostim® biostimulant were reported to effectively enhance the lettuce yield and secondary metabolites (Mpai *et al.,* 2022). The main problem arising from this background is based on identifying the correct concentrations for pre-harvest application of Phytostim® biostimulant on *Amaranth species*, which will retain the quality and nutrients for more than that of the control. The study aimed at investigating if Phytostim®

biostimulant will escalate the shelf life of *A. cruentus* and *A. caudatus* during the storage and maintain good nutritional components.

Objective of the study

The objective of this chapter was to determine the interactive effect of different concentrations of Phytostim® biostimulant and storage time on post-harvest quality and nutritional components in *A. cruentus* and *A. caudatus.*

4.2 MATERIALS AND METHODS

4.2.1 Experimental sites, Treatments, and Design

The experiment was carried out at the Green Biotechnologies Research Centre of Excellence (GBRCE), at the University of Limpopo, Limpopo Province of South Africa (23°53′10″S, 29°44′15″E) under the storage condition at ambient temperature for postharvest quality attributes. While the analysis of nutrient components was conducted at Agro-Food Technology Station (LATS) laboratory. The experiment was laid out following 8 x 3 factorial treatments (eight treatments for Phytostim® biostimulant and three for storage interval) arranged in a Complete Randomized Design (CRD). Phytostim® biostimulant treatments (0, 0.5, 1, 1.5, 2.5, 3, 4.5, and 6%) and storage period for (0, 3 and 6 days) replicated three times.

4.2.2 Procedures and Materials

Leafy amaranth vegetables were harvested 60 days (8 weeks) after transplanting, early in the morning for reducing moisture loss, and immediately moved to the laboratory setting. The leafy vegetables (leaves) were then separated into uniform bundles of approximately equal weight of 100 g. Then the harvested fresh leaves of *A. cruentus* and *A. caudatus* were packed in a well-ventilated punnet film (15 x 10 cm), stored at ambient temperature for 0, 3 and 6 days. During storage, post-harvest qualities (color changes, weight loss, and visual quality) were assessed at an interval of three days (0, 3, and 6 d). A total of 5 g per replicate was sampled and oven dried at 40 ºC for 72 h for analysis of nutrient components.

4.2.3 Data collection

Determination of weight loss: The weight loss was calculated as the difference between initial weight at 0 day (at the beginning of the experiment) and final weight (after removal at the storage condition), weighed at different days at the storage condition following formula quantified by (AOAC, 2003).

Weight loss (%) = $\frac{(intial\ weight - final\ weight)}{(initial\ weight)}\ x\ 100$

Determination of color: Color was measured non-destructively using a portable colorimeter (Chroma Meter, Konica Minolta Sensing Inc., Japan) which was calibrated with a white calibration card. The CIELAB color space; a* (-value mean greenness and + value means redness, and h° (colour intensity >180°; means yellowness; <180° means green) were recorded on the three readings on the equatorial region of the leafy vegetables. In the CIE color system, negative a* values describe the intensity of green color. Whereas hue angle (h°) represents the changes in aging as explained by McGuire (1992).

Visual quality evaluation: Visual quality of the leafy vegetables was assessed based on the observation. Quality was scored on scale of 1 to 10, with a score of 10 given for excellent and fresh appearance, 8 for good, 6 for fair (limit of marketability), 4 for fair (usable but not saleable), and 2 for unusable (Sun *et al.,* 2021).

Determination of mineral composition: Before, analysis approximately 0.10 g dried materials were digested in 40 mL of 4% nitric acid (HNO_3), followed by placing the container on a vortex to allow for complete wetting of the mixture. The materials were magnetically stirred, thereafter incubated in a 95 °C water-bath for 90 minutes, allowed to cool down at room temperature, filtered, decanted into 50 mL tubes which were covered with a foil and then selected nutrient elements were analyzed. The analysis of macro mineral elements such as Mg, K, and Ca and trace elements such as Mn, Se, Cu, Fe, and Zn were determined using an Inductively Coupled Plasma Emission Spectrometer (ICPE-9000, Shimadzu) (Huang and Schulte, 1985). Nitrogen (N) were determined using TruSpec. The instrument was calibrated using standard solutions of elements of trace elements (concentrations range 0.1 to 1 ppm) and macro (concentrations range 10 50

ppm). The concentrations of elements in the samples were determined from the linear calibration of the standard and expressed as parts per million (ppm).

Determination of amino acids: Amino acid analysis was performed according to Grobbelaar as described by Mpai *et al*. (2018), using a fresh pulp of the sample. A volume of 100 g was vortexed with 6 N HCl 0,5 mL with the resulting mixture held in an oven at 110 °C for 18 h and after cooling, centrifuged and filtered. The resulting filtrate was dried using a speed vacuum and reconstituted in a borate buffer (70 µL) for derivatization. Samples were derivatized using an accQ-Tag Ultra amino acid kit and the sample was analyzed twice. The derivatizations kit contains five vials of each of the following: AccQ-Tag derivatizing agent (6-aminoquinolyl-N- hydroxysuccinimidyl carbamate (AQC), dry acetonitrile for preparing the AQC, and sodium borate buffer (0.2 M, pH 8.8) to be used in the derivatization reaction. 54 Initially, the samples were undiluted and then diluted 10 times to quantify the amino acids that are present in higher concentrations. The derivatization process was performed by adding 10 µL aliquot of the prepared undiluted sample (which contained 20 µL/L norvaline in 80 µL of the sample) to the 20 µL of AQC, vortexed and held in the oven at 55 °C for 10 min. Thereafter, the vials were cooled, and the samples were ready for the Ultra Performance Liquid Chromatograph (UPLC) analysis. Amino acid separation and detection were performed using a Waters Aquity Ultra Performance Liquid Chromatograph (UPLC) fitted with a photodiode array (PDA) detector. An aliquot of 1 µL of sample was injected into the mobile phase which conveys the derivatized amino acids onto a Waters Ultra Tag C18 column (2.1 x 50 mm x 1.7 µm) held at 60 °C. The gradient was set up and commenced with 99.95 eluent A (water) and 1 % eluent B (acetonitrile). The total run time was 9.5 min, and the run flow rate was 0.7 mL.

Determination of proteins: The Bradford assay was used for protein determination as described by (Ernst and Zor, 2010). Briefly, 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL 95% ethanol (C2H5OH). Thereafter, 100 mL of 85% phosphoric acid (H3PO4) was carefully added under stirring, before H2O was added to a total volume of 1 L. The solution was filtered and kept at 4°C. For the measurements, 100 µL extract and 5 mL Bradford solution were mixed and incubated for 5 min. A standard curve was made

of BSA (0, 0.0625, 0.125, 0.25, 0.5 and 1 g L^{-1}) and absorbance was read at 595 nm. Then nitrogen was calculated and converted to percentage by simply multiplying with a constant protein factor of 6.25.

4.2.4 Statistical analysis

Experimental data were statistically analyzed using two-way analysis of variance (ANOVA) to determine the effects of different concentrations of Phytostim® biostimulant and storage period interaction on postharvest quality and nutritional status of amaranth. The mean and standard error value were computed in Microsoft excel 2019 using data to test statistical significance of difference amongst control and treatments (Phytostim® biostimulant concentrations) of each storage days (0, 3, and 6 d). Duncan Multiple Range (DMRT) was used to separate treatment means. All the analyses were performed using Statistix 10.0 software package.

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of different concentrations of Phytostim® biostimulant and storage period on postharvest quality attributes of *Amaranth species.*

4.3.1.1 Weight loss

The different concentrations of Phytostim® biostimulant and storage period interaction significantly affected ($p \leq 0.05$) the weight loss of *Amaranth species.* It was observed in general that the weight loss progressively increased as the storage period increased (Figure 4.1). The untreated samples had higher weight loss as compared to the treated samples. The control resulted in a higher weight loss of 39.15% (*A. cruentus*) as compared treated samples while the lower weight loss was 20.81% at the concentrations of 2.5%. In *A.caudatus*, the untreated plants had higher weight loss of 46% and the lower weight loss was 34.26% obtained at the concentrations of 0.5% of Phytostim® biostimulant The overall results, indicated that at day three , weight loss percentage were between (25 to 30%) in the untreated samples and (15 to 25%) at different concentrationss of Phytostim® biostimulant. Whereas, at day six the weight loss percentage were between (35 to 40%) in untreated samples and (25 to 30%) in treated samples. The untreated samples and treated samples with 3 to 6% and control showed a clear increase of weight loss up to 40%. Leafy vegetables become unmarketable if they encounter a moisture loss of more than 15% of the original fresh mass (Charles *et al.,* 2017). The results shows that the leaves of both studied species treated with lower concentrations (0.5% to 3%) maintained moisture up to over 70% at day six of storage.

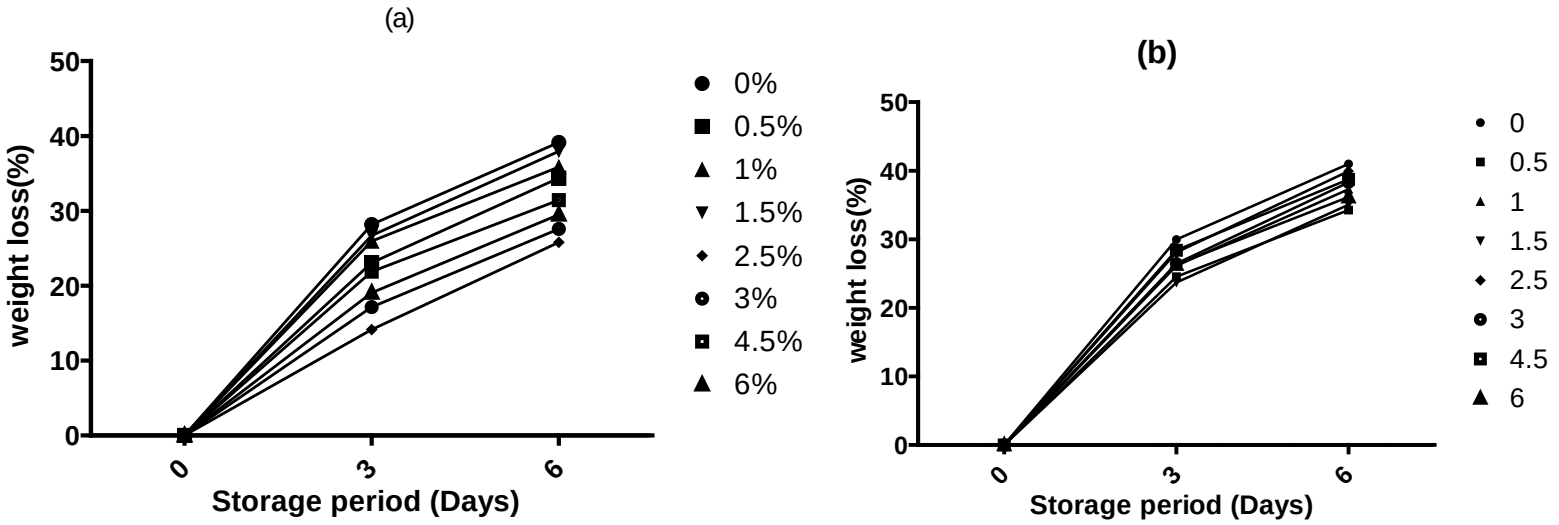

Figure 4.1: Effects of different concentrations of Phytostim® biostimulant and storage period interaction on weight loss (a) *A. cruentus* and (b) *A. caudatus.* Data in the interaction was analyzed and means were separated using Duncan Multiple Range Test (DMRT) at the probability level of 5%.

4.3.1.2 Color

The interaction between different concentrations of Phytostim® biostimulant and storage period significantly affected ($p<0.05$) some of the color coordinates in the studied *Amaranth species*. Figure 4.3 shows h° values in *A. cruentus*, which were significantly affected by interaction between Phytostim® biostimulant and storage period. Whilst a* coordinates were not influenced by Phytostim® biostimulant and storage period interaction. In *A. caudatus* both a* and h° values were not influenced by Phytostim® biostimulant and storage period interaction. The results further demonstrated that in *A. cruentus*, at day 0, hue angle (h°) of plant samples treated different concentrations of Phytostim® biostimulant between 3, 4.5 and 6% had higher values (180° < 270°) meaning there were light yellow in color as compared to control. And those that treated with different concentrations of Phytostim® biostimulant between (0.5 to 2.5%) ranged from (130° < 180°) meaning that they were dark green. While at day three, hue angle (h°) of (0.5 to 2.5%) concentrations of Phytostim® biostimulant reduced gradually as the leafy vegetables' color changed from the initial dark green color (>130°) to a lighter shade of green (<120°).Whereas hue angle (h°) of (3, 4.5 and 6%) concentrations of Phytostim® biostimulant also changed as the storage period increases from the initial light yellow color (>180°) to yellow color (<270°). By the end of the storage, leafy vegetables of (0.5, 1, 1.5 and 2.5%) concentrations of Phytostim® biostimulant further reduced from a lighter shade green (<120°) to light green (<100°). while (3, 4.5 and 6%) concentrations of Phytostim® biostimulant reduced from yellow color (<200°) to blue color (180°) meaning that at the end storage they got spoilt. In *A. caudatus* hue angle initially at day 0, the plant samples color was dark green (>130°) to a lighter shade of green (<120°). When storage period increased the hue angle (h°) reduced slowly with storage period, however there was no notable significance difference on the color.

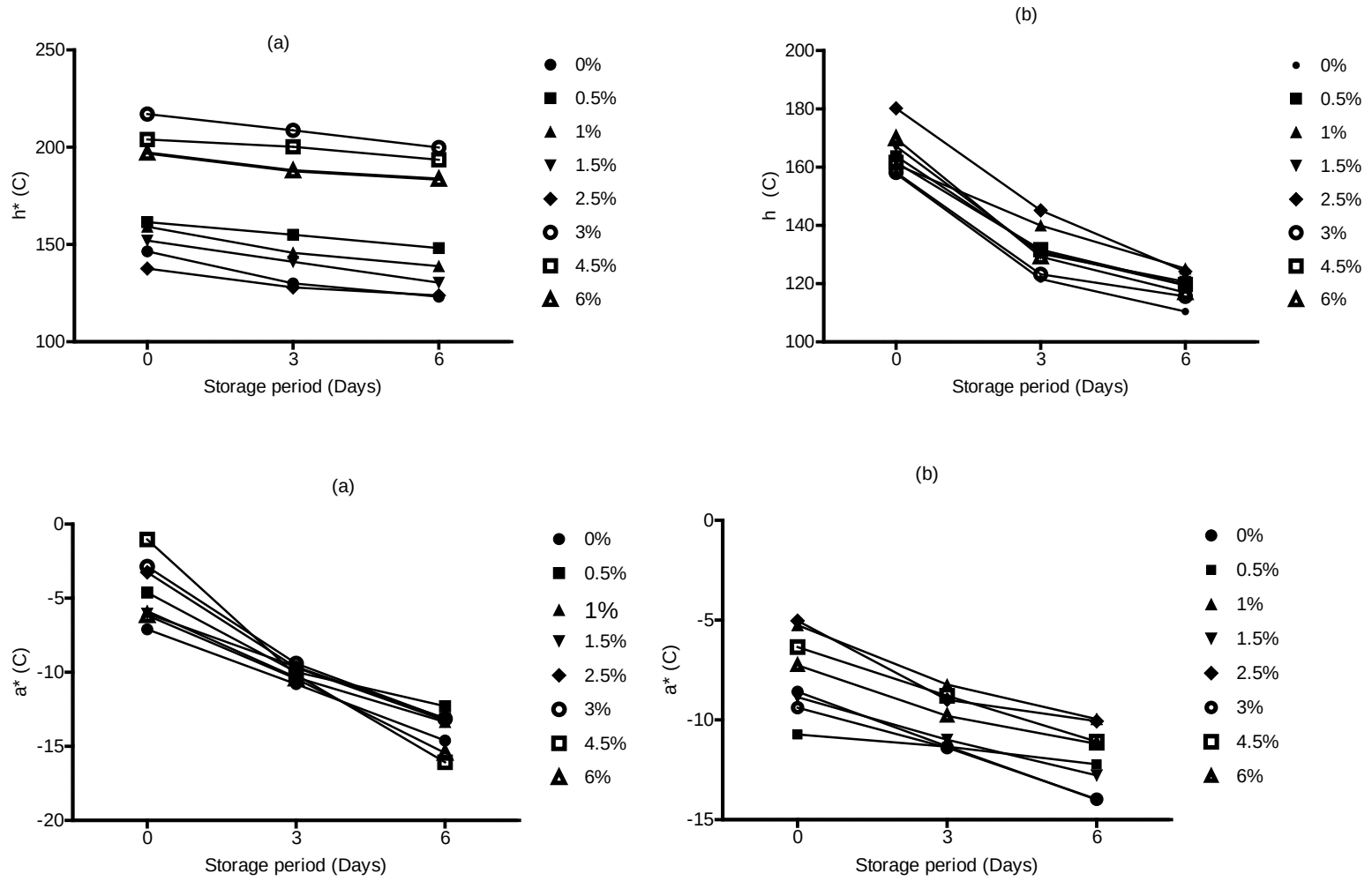

Figure 4.2: Effects of different concentrations of Phytostim® biostimulant and storage period interaction on color change (a) *A. cruentus* and (b) *A. caudatus*. Data in the interaction was analyzed and means were separated using Duncan Multiple Range Test (DMRT) at the probability level of 5%.

4.3.1.4 Visual quality

Visual quality of the leafy vegetable decreased when the storage period increased. Initially at day zero in *A. cruents*, Phytostim® biostimulant with concentrations from (0.5 to 2.5%) were ranged between (10-9) meaning that they were excellent and fresh appearance. Phytostim® biostimulant with concentrations from (3 to 6%) were ranged between from (8-9) meaning that they were good. At the end of the storage, leafy vegetables applied at Phytostim® biostimulant concentrations from (0.5 to 2.5%) decreased sharply from (10-4) scores which denotes that even after the storage the vegetables were usable but not saleable. Moreover, the leafy vegetables applied at Phytostim® biostimulant concentrations from (3 to 6%) at the end of the storage gradually declined from 10 to 2 which indicates that they were unusable. The same trend observed in *A. cruentus* was also observed *A. caudatus*. However, 1% followed by 2.5% performed better in maintaining the color at the end of the storage in both *Amaranth species*.

Table 4.1: Interaction effects of different concentrations of Phytostim® biostimulant and storage period on *Amaranth species* on visual quality.

		Scores																			
		A. Cruentus										*A. Caudatus*									
Treatments		10	9	**8**	7	6	5	4	3	2	1	10	9	8	7	6	5	4	3	2	1
Day 0	0	X										X									
	0.5	X										X									
	1	X										X									
	1.5	X										X									
	2.5	X										X									
	3			X								X									
	4.5			X								X									
	6			X								X									
Day 3	0					X									X						
	0.5			X										X							
	1				X									X							
	1.5			X											X						
	2.5			X											X						
	3				X									X							
	4.5					X										X					
	6					X											X				

Day 6	0						x																	
	0.5			X										X										
	1			X										X										
	1.5				X											X								
	2.5			X										X										
	3						X										x							
	4.5						X												X					
	6						X																x	

Significance level at ($p<0.05$). Visual quality scores ranged from 1 to 10. Excellent and fresh appearance (10-9), Good (8-7), fair but limit of marketability (6-5), fair and usable but not saleable (4-3), unusable (2-1).

4.3.2 Effects of different concentrations of Phytostim® biostimulants and storage period interaction on the nutritional components of *Amaranth species.*

4.3.2.1 Mineral elements

Interaction effects of Phytostim® biostimulants and storage period on the mineral elements of *Amaranth species* was investigated. And the results demonstrated a significant effect ($p<0.05$) on mineral elements including calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), selenium (Se), zinc (Zn) and nitrogen (N). As reported by Sarker *et al*., (2020), these mineral were also reported in twelve green amaranth genotypes. The observed variation showed that the highest mineral elements were observed in biostimulant-treated plants in comparison to the untreated plants. The highest macro mineral contents quantified in *A. cruentus* were found to be calcium (253.33 mg/L) in the plants treated with 0.5% biostimulants at day zero, and this were two fold higher than the untreated samples (117.33 mg/L).In addition, the content of calcium reduced to 230.67 mg/L and 116.00 mg/L as the same 0.5% treated samples at day three and yet no further change was observed in up to day six. At the same time (0.5% biostimulant and 0 day), potassium (143.33 mg/L), and magnesium (70.27 mg/L) were also leading in the treated plants as compared to the untreated plants. Whereas in *A. caudatus*, the plant treated with 1% biostimulants at day zero showed to have high macro minerals in calcium (148.67 mg/L), followed by potassium (102.00 mg/L), and magnesium (48.80 mg/L). In the untreated plants (0%), obtained high macro mineral was (97.05 mg/L). At day three and day six, the macro mineral elements maintained the same value until the end of the storage (6d). This shows that treated plants were two to three folds higher than the untreated plants.

Moreover, the trace (micro) elements found in the *Amaranth species* were iron, selenium, manganese, zinc, and copper. In *A. cruentus*, the high micro mineral was found to be selenium (36.63 mg/L), followed by zinc (34.23 mg/L), iron (24.73 mg/L), manganese (11.39 mg/L), and copper (0.74 mg/L) at 1% biostimulants at day zero. In control, the high micro mineral elements obtained were (26.97 mg/L). Whereas in *A. caudatus*, the high micro mineral was found to be selenium (34.47 mg/L), followed by zinc (31.67 mg/L), iron (22.50 mg/L), manganese (10.54 mg/L) and copper (0.70 mg/L) at 3% biostimulants. In

control was found to be (24.73 mg/L) which shows that in both species treated were one-fold higher than the untreated plants. At day six and three, the same trend as observed in macro mineral elements was also observed in micro minerals. According to Jimenez-Aguiar and Grusak, the contents of K, Ca, and Mg were higher than those found in spinach, kale, black nightshade and spider flower. These could be attributed by the contents of the amino-acid based biostimulant which encompasses 22 amino acids which act as elicitors for improving growth and developments of plants (Mokgalabone *et al*., 2023). Therefore is possible to suggest that the consumption 50 g of sample treated with 0.5% could unsure the 1000 mg for adults ‘adequate intake of Ca, according to the National academy of medicine.

Table 4.2: Effects of different concentrations of Phytostim® biostimulants and storage period interaction on the mineral elements of *A. cruentus*.

		Mineral elements units (mg/L)								
Treatments (%)	Days	Ca	Cu	N	Fe	K	Mg	Mn	Se	Zn
0	0	117.33±0.27^{f}	0.62±0.02^{b}	2.74±0.39^{e}	18.33±0.96^{c}	73.57±0.68^{d}	34.03±2.47^{f}	6.62±0.48^{d}	26.97±1.19^{c}	25.07±1.06^{c}
	3	116.00±0.47^{f}	0.60±0.01^{b}	2.22±0.06^{e}	17.47±0.52^{c}	71.33±0.27^{d}	32.43±0.03^{f}	5.64±0.46^{d}	23.07±0.36^{c}	24.07±0.41^{c}
	6	116.00±0.47^{f}	0.60±0.01^{b}	2.22±0.06^{e}	17.47±0.52^{c}	71.33±0.27^{d}	32.43±0.03^{f}	5.64±0.46^{d}	23.07±0.36^{c}	24.07±0.41^{c}
0.5	0	235.33±0.98^{a}	0.52±0.03^{c}	3.00±0.1^{d}	17.40±1.27^{d}	143.33±0.72^{a}	70.27±0.14^{a}	10.05±0.86^{a}	17.20±1.19^{d}	16.83±1.04^{d}
	3	230.67±2.37^{a}	0.51±0.02^{c}	2.97±0.03^{d}	16.17±0.89^{d}	143.20±1.18^{a}	69.97±3.29^{a}	11.41±0.73^{a}	17.93±0.63^{d}	15.93±0.73^{d}
	6	230.67±2.37^{a}	0.51±0.02^{c}	2.97±0.03^{d}	16.17±0.89^{d}	143.20±1.18^{a}	69.97±3.29^{a}	11.41±0.73^{a}	17.93±0.63^{d}	15.93±0.73^{d}
1	0	182.67±0.98^{c}	0.74±0.03^{a}	3.73±0.8^{b}	24.73±1.32^{a}	62.70±0.46^{f}	47.47±0.14^{d}	8.39±0.89^{c}	36.63±0.92^{a}	34.23±1.08^{a}
	3	180.33±3.16^{c}	0.71±0.05^{a}	3.52±0.71^{b}	23.37±2.01^{a}	61.47±0.05^{f}	46.33±2.19^{d}	8.64±2.25^{c}	35.73±1.39^{a}	30.70±1.28^{a}
	6	180.33±3.16^{c}	0.71±0.05^{a}	3.52±0.71^{b}	23.37±2.01^{a}	61.47±0.05^{f}	46.33±2.19^{d}	8.64±2.25^{c}	35.73±1.39^{a}	30.70±1.28^{b}
1.5	0	178.33±1.91^{d}	0.51±0.03^{c}	4.95±0.03^{a}	15.87±1.05^{e}	102±0.47^{b}	50.03±0.18^{c}	10.24±0.82$_{a}$	11.71±1.41^{e}	12.19±1.33^{e}
	3	173.33±0.98^{d}	0.51±0.01^{c}	3.54±0.02^{a}	15.40±0.49^{e}	102.33±0.27^{b}	46.30±2.12^{c}	9.37±0.48^{a}	10.63±0.38^{e}	11.13±0.28^{e}
	6	173.33±0.98^{d}	0.51±0.01^{c}	3.54±0.02^{a}	15.40±0.49^{e}	102.33±0.27^{b}	46.30±2.12^{c}	9.37±0.48^{a}	10.63±0.38^{e}	11.13±0.28^{e}
2.5	0	163.00±1.25^{e}	0.45±0.02^{d}	3.73±0.01^{a}	13.07±1.01^{f}	98.13±0.44^{c}	42.13±3.24^{e}	11.22±0.76^{a}	9.91±1.05^{f}	10.28±0.99^{f}
	3	161.33±0.54^{e}	0.41±0.01^{d}	3.69±0.9^{a}	13.60±0.54^{f}	97.67±0.27^{c}	40.40±2.90^{e}	11.24±0.58^{a}	8.23±0.19^{f}	10.37±0.28^{f}
	6	161.33±0.54^{e}	0.41±0.01^{d}	3.69±0.9^{a}	13.60±0.54^{f}	97.67±0.27^{c}	40.40±2.90^{e}	11.24±0.58^{a}	8.23±0.19^{f}	10.37±0.28^{f}
3	0	195.33±0.72^{b}	0.65±0.03^{b}	3.44±2.1^{c}	22.00±1.31^{b}	65.27±0.52^{e}	55.37±0.14^{b}	9.75±0.81^{b}	30.00±1.23^{b}	28.00±1.29^{b}
	3	191.33±0.34^{b}	0.63±0.01^{b}	2.95±01.61^{c}	20.67±0.07^{b}	63.63±2.79^{e}	52.83±0.62^{b}	9.81±1.47^{b}	29.77±1.23^{b}	27.53±1.02^{b}
	6	191.33±0.34^{b}	0.63±0.01^{b}	2.95±01.61^{c}	20.67±0.07^{b}	63.63±2.79^{e}	52.83±0.62^{b}	9.81±1.47^{b}	29.77±1.23^{b}	27.53±1.02^{b}

Macro minerals: Magnesium (Mg), Calcium (Ca). Nitrogen (N), Potassium (K). Micro minerals: Manganese (Mn), Copper (Cu), Selenium (Se), Zinc (Zn), Iron (Fe). Value (Mean± SE) followed by different letters on each column based on treatments were significantly different at ($p<0.05$), according to DMRT.

Table 4.3: Effects of different concentrations of Phytostim® biostimulants and storage period interaction on the mineral elements of *A. caudatus*.

Mineral elements units (mg/ L)										
Treatments (%)	Days	Ca	Cu	N	Fe	K	Mg	Mn	Se	Zn
0	0	97,05±0.47^{f}	0.55±0.02^{b}	2.09±0.07^{b}	16.97±1.05^{ab}	53.33±1.95^{e}	33.27±7.88^{d}	7.06±2.09^{c}	24.73±0.97^{b}	22.40±0.90^{b}
	3	90.03±0.02^{f}	0,50±0.08^{b}	1,46±0.23^{b}	11.05±0.05^{ab}	50,86±0.85^{e}	23,33±0.00^{d}	6.45±0.99^{c}	22,60±0.01^{b}	19,98±0.52^{b}
	6	90.03±0.02^{f}	0,50±0.08^{b}	1,46±0.23^{b}	11.05±0.05^{ab}	50,86±0.85^{e}	23,33±0.00^{d}	6.45±0.99^{c}	22,60±0.01^{b}	19,98±0.52^{b}
0.5	0	124.67±0.27^{d}	0.43±0.00^{c}	3.007±0.03^{a}	11.83±0.35^{c}	84.70±0.05^{c}	37.70±1.88^{c}	7.93±0.38^{c}	8.18±0.14^{e}	8.82±0.90^{e}
	3	115.00±0.33^{d}	0,40±0.02^{c}	2,81±0.09^{a}	10.33±0.10^{c}	79,80±0.06^{c}	37,52±0.81^{c}	5.66±0.09^{c}	6.23±0.04^{e}	5.89±0.00^{e}
	6	115.00±0.33^{d}	0,40±0.02^{c}	2,81±0.09^{a}	10.33±0.10^{c}	79,80±0.06^{c}	37,52±0.81^{c}	5.66±0.09^{c}	6.23±0.04^{e}	5.89±0.00^{e}
1	0	148.67±0.54^{a}	0.49±0.02^{c}	3.45±0.96^{a}	16.63±0.75^{ab}	102±0.94^{b}	47.87±2.93^{a}	10.18±0.59^{a}	13.00±0.70^{c}	13.17±0.69^{c}
	3	144.2±0.09^{a}	0.43±0.04^{c}	2.15±0.13^{a}	11.5±0.44^{ab}	83.2±0.56^{b}	39.41±0.18^{a}	9.58±0.44^{a}	12.7±0.88^{c}	11.9±0.22^{c}
	6	144.2±0.09^{a}	0.43±0.04^{c}	2.15±0.13^{a}	11.5±0.44^{ab}	83.2±0.56^{b}	39.41±0.18^{a}	9.58±0.44^{a}	12.7±0.88^{c}	11.9±0.22^{c}
1.5	0	126.00±0.00^{c}	0.44±0.01^{f}	2.67±0.03^{b}	12.33±0.63^{b}	77.83±0.07^{d}	39.40±2.49^{b}	8.25±0.52^{b}	9.76±0.36^{e}	10.05±0.35^{d}
	3	119.14±0.73^{c}	0.28±0.05^{f}	1.33±0.19^{b}	12.12±0.88^{b}	63.91±0.56^{d}	39.16±0.35^{b}	6.89±0.78^{b}	5.96±0.45^{e}	9.55±0.23^{d}
	6	119.14±0.73^{c}	0.28±0.05^{f}	1.33±0.19^{b}	12.12±0.88^{b}	63.91±0.56^{d}	39.16±0.35^{b}	6.89±0.78^{b}	5.96±0.45	9.55±0.23^{d}
2.5	0	129.33±0.98^{b}	0.46±0.02^{d}	3.39±0.04^{a}	13.37±0.72^{b}	139.00±0.94^{a}	48.80±3.44^{a}	9.24±0.57^{b}	12.93±0.71^{d}	10.57±0.73^{d}
	3	101.00±0.47^{b}	0,41±0.00^{d}	2.88±0.66^{a}	10.75±0.96^{b}	124.41±0.61^{a}	39.98±0.00^{a}	6.11±0.21^{b}	10.23±0.48^{d}	9.91±0.65^{d}
	6	101.00±0.47^{b}	0,41±0.00^{d}	2.88±0.66^{a}	10.75±0.96^{b}	124.41±0.61^{a}	39.98±0.00^{a}	6.11±0.21^{b}	10.23±0.48^{d}	9.91±0.65^{d}
3	0	106.33±0.72^{e}	0.70±0.00^{a}	2.04±0.05^{b}	22.50±0.33^{a}	52.87±0.55^{e}	33.47±2.80^{d}	10.54±0.78^{a}	34.47±0.14^{a}	31.67±0.15^{a}
	3	102.39±0.22^{e}	0.65±0.00^{a}	1.87±0.42^{b}	19.33±0.04^{a}	39.72±0.78^{e}	28.07±0.47^{d}	9.66±0.11^{a}	30.21±0.48^{a}	27.91±0.48^{a}
	6	102.39±0.22^{e}	0.65±0.00^{a}	1.87±0.42^{b}	19.33±0.04^{a}	39.72±0.78^{e}	28.07±0.47^{d}	9.66±0.11^{a}	30.21±0.48^{a}	27.91±0.48^{a}

Macro minerals: Magnesium (Mg), Calcium (Ca). Nitrogen (N), Potassium (K). Micro minerals: Manganese (Mn), Copper (Cu), Selenium (Se), Zinc (Zn), Iron (Fe). Value (Mean± SE) followed by different letters on each column based on treatments were significantly different at ($p<0.05$), according to DMRT.

4.3.2.2 Amino acids composition

Approximately 16 amino acids, both essential and non-essential were quantified in the Amaranth species. The essential amino acids including histidine (His), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), and valine (Val) were detected. Non-essential amino acids such as alanine (Ala), arginine (Arg), asparagine (Asp), glutamic acid (Glu), glycine (Gly), proline (Pro), and serine (Ser) were detected. Interaction effects of Phytostim® biostimulants and storage period significantly affected ($p<0.05$) the amino acids composition of *Amaranth species* as shown in (Table 4.4 to Table 4.7). Similar amino acids profiles were detected in Okra and amaranth species (Mokgalabone *et al.,* 2023; Jahan *et al.,* 2022). There was no clear trend observed within the storage days. The obtained results illustrated that, the higher essential amino acid detected was leucine and non-essential amino acids was alanine in both Amaranth species. These results are in contrast with those reported by Jahan et al., (2022), describing glutamic and lysine as the predominant amino acid in *Amaranth tricolor*. Reasons related to such differences could be attributed to genomic and morphological variation of the species, since the studied species in this work were green pigmented, whilst the reported species were red pigmented.

In *A. cruentus*, higher level of essential amino acid was leucine (1.54 mg/kg) which were found at 1% of Phytostim® biostimulants at day zero. While control exhibited highest essential amino acid of 1.08 mg/kg at day zero. At day three, it reduced to 1.01 mg/kg and 1.05 mg/kg respectively. The highest non-essential amino acid found, was alanine, glutamic acid and arginine at 1% of Phytostim ® biostimulants at day zero. While in control, it was found to be 1.84 mg/kg. At day three it was reduced to 1.82 mg/kg and 0, 76 mg/kg. Both essential and non-essential amino acid maintained the value from day three until the end of the storage (6d). Furthermore, the overall results indicated that in each different concentration of Phytostim® biostimulants at 0%, high essential amino acid were leucine (1.1 mg/kg) and low was histidine (0.41 mg/kg). At 0.5%, high essential amino acid was alanine (1.98 mg/kg) and lower was methionine (0.31 mg/kg). At 1%, high essential amino acids were alanine (2.85 mg/kg) and lower were methionine (0.31 mg/kg).

At 1.5%, the highest amino acids were alanine (1.72 mg/kg) and the lowest were histidine (0.23 mg/kg). At 2.5%, the highest amino acid was alanine (1.93 mg/kg) and lowest was methionine (0.23 mg/kg). At 3%, the highest amino acid obtained was alanine (2.15 mg/kg) and lowest was isoleucine (0.72 mg/kg).

Whereas in *A. caudatus*, higher levels of essential amino acid were leucine (1.15 mg/kg) at 3% Phytostim® biostimulants at day zero. At day three it was reduced to (1. 06 mg/kg). While control it was found to be 1.09 mg/kg, when the storage increases at day three it was reduced to 0. 96 mg/kg. The higher level of non-essential amino acid found was alanine (2.30 mg/kg) at 0% at day zero, then reduced to 1. 6 mg/kg. Arginine and glutamic acid were higher as well. Similar trend as observed in *A. cruentus* from day three till the end of storage (6d) was also observed in *A. caudatus*. In each concentration of Phytostim® biostimulants at 0%, high amino acid obtained was arginine (1.72 mg/kg), and low was methionine (0.22 mg/kg). At 0.5%, high alanine (1.68 mg/kg) and low tryptophan (0.2 mg/kg). At 1%, high alanine (1.86 mg/kg) and low methionine (0.22 mg/kg). At 1.5%, High alanine: 1.72 mg/kg) and low tryptophan: 0.4 mg/kg). At 2.5%, high (alanine: 1.91 mg/kg) and low (tryptophan: 0.1 mg/kg). At 3%, high (alanine: 1.90 mg/kg) and low (methionine: 0.27 mg/kg). The results clearly indicate that alanine appeared to be predominant in all the concentration of Phytostim® biostimulants. Thus, since alanine amino acids are well known for numerous health benefits, they can offer good supplement for dietary. Therefore, the crop can be a reliable source for the provision of amino acids, and its consumption needs to be encouraged to improve the balanced diet. Amino acids respond to nitrogen or fertilization application. In this case, amino acid based biostimulant contained 22 amino acids including glutamic acid and arginine: the nitrogen donors for Proline. Amino acids such as glutamic acid were reported that their roles in plants leaves were associated with transportation nitrogen compounds.

Table 4.4: Interaction effects of Phytostim® biostimulants and storage period on the essential amino acids of *A. cruentus*.

		Units' mg/kg							
Treatments	Days	His	Leu	Val	Phe	Thr	Lys	Met	Try
0	0	0.40±0.00^{b}	1.08±0.33^{d}	0.79±0.92^{b}	0.28±0.33^{c}	0.93±0.01^{b}	0.70±0.23^{b}	0.58±0.72^{a}	0.70±0.45^{b}
	3	0.37±0.09^{b}	1.05±0.01^{d}	0.78±0.55^{b}	0.08±0.02^{c}	0.70±0.00^{bc}	0.15±0.99^{bc}	0.29±0.28^{a}	0.22±0.79^{b}
	6	0.37±0.09^{b}	1.05±0.01^{d}	0.78±0.55^{b}	0.08±0.02^{c}	0.7±0.00^{bc}	0.15±0.99^{bc}	0.29±0.28^{a}	0.22±0.79^{b}
0.5	0	0.41±0.00^{b}	1.10±0.48^{d}	0.70±0.00^{ab}	1.05±0.00^{a}	0.7±0.23^{d}	0.74±0.00^{b}	0.31±0.03^{c}	0.44±0.22^{c}
	3	0.38±0.22^{b}	1.08±0.77^{d}	0.69±0.44^{ab}	1.01±0.56^{a}	0.65±0.02^{cd}	0.66±0.11^{b}	0.30±0.42^{c}	0.30±0.31^{c}
	6	0.38±0.22^{b}	1.08±0.77^{d}	0.69±0.44^{ab}	1,01±0.56^{a}	0.65±0.02^{cd}	0.66±0.11^{b}	0.30±0.42^{c}	0.30±0.31^{c}
1	0	0.66±0.78^{a}	1.54±0.45^{a}	1.07±0.17^{c}	1.57±0.74^{a}	1.24±0.05^{a}	1.00±0.10^{a}	0.31±0.00^{c}	0.86±0.99^{a}
	3	0.32±0.01^{a}	1.01±0.33^{a}	0.65±0.28^{c}	0.95±0.00^{a}	0.84±0.78^{ab}	0.97±0.45^{a}	0.28±0.03^{c}	0.14±0.88^{a}
	6	0.32±0.01^{a}	1.01±0.33^{a}	0.65±0.28^{c}	0.95±0.00^{a}	0.84±0.78^{ab}	0.97±0.45^{a}	0.28±0.03^{c}	0.14±0.88^{a}
1.5	0	0.27±0.99^{d}	1.23±0.88^{c}	0.66±0.25^{d}	0.97±0.01^{b}	0.89±0.42^{d}	0.60±0.05^{c}	0.44±0.23^{b}	0.35±0.89^{d}
	3	0.26±0.00^{d}	1.21±0.01^{c}	0.58±0.85^{d}	0.88±0.99^{b}	0.64±0.00^{cd}	0.55±0.01^{c}	0.34±0.00^{b}	0.22±0.02^{d}
	6	0.26±0.00^{d}	1.21±0.01^{c}	0.58±0.85^{d}	0.88±0.99^{b}	0.64±0.00^{cd}	0.55±0.01^{c}	0.34±0.00^{b}	0.22±0.02^{d}
2.5	0	0.37±0.11^{c}	1.31±0.00^{b}	0.79±0.14^{b}	1.10±0.03^{b}	0.87±0.57^{c}	1.09±0.03^{a}	0.23±0.22^{d}	0.45±0.01^{c}
	3	0.32±0.01^{c}	1.26±0.57^{b}	0.72±0.33^{b}	0.99±0.46^{b}	0.73±0.04^{bc}	1.06±0.00^{a}	0.19±0.28^{d}	0,19±0.72^{c}
	6	0.32±0.01^{c}	1.26±0.57^{b}	0.72±0.33^{b}	0.99±0.46^{b}	0.73±0.04^{bc}	1.06±0.00^{a}	0.19±0.28^{d}	0.19±0.72^{c}
3	0	0.29±0.61^{d}	1.37±0.44^{b}	0.86±0.00^{a}	1.22±0.66^{a}	0.72±0.91^{e}	1.10±0.00^{a}	0.32±0.65^{c}	0.17±0.00^{e}
	3	0.28±0.00^{d}	1.15±0.01^{b}	0.64±0.03^{a}	0.7±0.02^{ab}	0.01±0.99^{e}	0.28±0.00^{bc}	0.27±0.43^{c}	0.10±0.52^{e}
	6	0.28±0.00^{d}	1,.5±0.01^{b}	0.64±0.03^{a}	0.7±0.02^{ab}	0.01±0.99^{e}	0.28±0.00^{bc}	0.27±0.43^{c}	0.10±0.52^{e}

Histidine (His), Leucine (Leu), valine (Val), phenylalanine (Phe), threonine (Thr), lysine (Lys), methionine (Met), tryptophan (Try). Value (Mean± SE) followed by different letters on each column based on species were significantly different at ($p<0.05$), according to DMRT.

Table 4.5: Interaction effects of Phytostim® biostimulants and storage period on the non-essential amino acids of *A. cruentus.*

Treatments (%)	Days	Gly	Ser	Pro	Ala	Asp	Glu	Arg	Ile
0	0	0.84±0.44^{c}	0.81±0.03^{c}	0.74±0.23^{b}	1.84±0.00^{d}	0.98±0.78^{b}	0.84±0.78^{d}	1.48±0.45^{e}	0.60±0.01^{c}
	3	0.81±0.00^{c}	0.77±0.03^{c}	0.70±0.22^{b}	0.76±0.04^{d}	0.66±0.00^{b}	0.77±0.05^{d}	1.37±0.47^{e}	0.58±0.89^{c}
	6	0.81±0.00^{c}	0.77±0.03^{c}	0.70±0.22^{b}	0.76±0.04^{d}	0.66±0.00^{b}	0.77±0.05^{d}	1.37±0.47^{e}	0.58±0.89^{c}
0.5	0	0.96±0.83^{b}	1.08±0.72^{b}	0.76±0.07^{b}	1.98±0.71^{c}	0.92±0.00^{b}	1.04±0.44^{c}	1.57±0.99^{d}	0.65±0.02^{c}
	3	0.89±0.06^{b}	1.06±0.04^{b}	0.68±0.00^{b}	1.91±0.12^{c}	0.85±0.33^{b}	1.01±0.27^{c}	1.55±0.07^{d}	0.64±0.00^{c}
	6	0.89±0.06^{b}	1.06±0.04^{b}	0.68±0.00^{b}	1.91±0.12^{c}	0.85±0.33^{b}	1.01±0.27	1.55±0.07^{d}	0.64±0.00^{c}
1	0	1.22±0.01^{a}	1.09±0.91^{b}	0.93±0.78^{a}	2.85±0.66^{a}	1.12±0.99^{a}	1.15±0.07^{b}	2.07±0.37^{a}	0.86±0.47^{a}
	3	0.80±0.01^{a}	0.93±0.66^{b}	0.71±0.11^{a}	1.82±0.52^{a}	0.80±0.02^{a}	0.96±0.08^{b}	1.49±0.09^{a}	0.57±0.59^{a}
	6	0.80±0.01^{a}	0.93±0.66^{b}	0.71±0.11^{a}	1.82±0.52^{a}	0.80±0.02^{a}	0.96±0.08^{b}	1.49±0.09^{a}	0.57±0.59^{a}
1.5	0	0.75±0.05^{d}	0.68±0.51^{d}	0.70±0.46^{b}	1.72±0.04^{e}	0.87±0.37^{c}	1.08±0.78^{c}	1.31±0.77^{f}	0.62±0.33^{c}
	3	0.72±0.00^{d}	0.65±0.02^{d}	0.60±0.00^{b}	1.66±0.03^{e}	0.82±0.04^{c}	0.97±0.99^{c}	1.25±0.64^{f}	0.62±0.23^{c}
	6	0.72±0.00^{d}	0.65±0.02^{d}	0.60±0.00^{b}	1.66±0.03^{e}	0.82±0.04^{c}	0.97±0.99^{c}	1.25±0.64^{f}	0.62±0.23^{c}
2.5	0	0.95±0.72^{b}	0.94±0.78^{c}	0.74±0.88^{b}	1.93±0.06^{c}	1.04±0.00^{a}	1.00±1.23^{c}	1.70±0.55^{b}	0.57±0.04^{d}
	3	0.93±0.00^{b}	0.07±0.08^{c}	0.71±0.12^{b}	1.01±0.22^{c}	0.48±0.49^{a}	0.88±0.00^{c}	1.67±0.01^{b}	0.39±0.39^{d}
	6	0.93±0.00^{b}	0.07±0.08^{c}	0.71±0.12^{b}	1.01±0.22^{c}	0.48±0.49^{a}	0.88±0.00^{c}	1.67±0.01^{b}	0.39±0.39^{d}
3	0	0.99±0.22^{b}	1.22±0.45^{a}	0.74±0.82^{b}	2.15±0.79^{b}	1.00±0.21^{a}	1.28±1.78^{a}	1.68±0.33^{c}	0.72±0.79^{b}
	3	0.86±0.78^{b}	0.99±0.01^{a}	0.66±0.45^{b}	1.73±0.05^{b}	0.84±0.88^{a}	0.97±0.06^{a}	1.41±0.19^{c}	0.62±0.07^{b}
	6	0.86±0.78^{b}	0.99±0.01^{a}	0.66±0.45^{b}	1.73±0.05^{b}	0.84±0.88^{a}	0.97±0.06^{a}	1.41±0.19^{c}	0.62±0.07^{b}

Glycine (Gly), serine (Ser), proline (Pro), alanine (Ala), asparagine (Asp), glutamic acid (Glu), isoleucine (Ile). Value (Mean± SE) followed by different letters on each column based on species were significantly different at ($p<0.05$), according to DMRT.

Table 4.6: Interaction effects of Phytostim® biostimulants and storage period on the essential amino acids of *A. caudatus*.

Treatment (%)	days	His	Leu	Val	Phe	Thr	Lys	Met	Try
0	0	0.32±0.01^{a}	1.09±0.47^{d}	0.84±0.00^{a}	1.15±0.41^{a}	0.82±0.44^{a}	1.28±0.34^{a}	0.22±0.45^{c}	0.49±0.24^{a}
	3	0.28±0.55^{a}	0.96±0.47^{d}	0.71±0.78^{a}	0.88±0.89^{a}	0.60±0.00^{a}	0.91±0.08^{a}	0.09±0.23^{c}	0.36±0.44^{a}
	6	0.28±0.55^{a}	0.96±0.47^{d}	0.71±0.78^{a}	0.88±0.89^{a}	0.60±0.00^{a}	0.91±0.08^{a}	0.09±0.23^{c}	0.36±0.44^{a}
0.5	0	0.27±0.99^{d}	1.23±0.88^{a}	0.66±0.25^{c}	0.97±0.01^{b}	0.89±0.42^{a}	0.60±0.05^{c}	0.44±0.23^{a}	0.35±0.89^{c}
	3	0.09±0.25^{d}	0.91±0.00^{bc}	0.54±0.09^{c}	0.23±0.07^{b}	0.44±0.05^{a}	0.54±0.11^{c}	0.25±0.58^{a}	0.15±0.52^{c}
	6	0.09±0.25^{d}	0.91±0.00^{bc}	0.54±0.09^{c}	0.23±0.07^{b}	0.44±0.05^{a}	0.54±0.11^{c}	0.25±0.58^{a}	0.15±0.52^{c}
1	0	0.25±0.89^{b}	1.04±0.66^{e}	0.65±0.46^{c}	0.90±0.04^{b}	0.72±0.07^{b}	1.03±0.23^{a}	0.22±0.45^{c}	0.37±0.47^{b}
	3	0.11±0.03^{b}	0.95±0.09^{e}	0.63±0.01^{c}	0.45±0.02^{b}	0.58±0.00^{b}	0.91±0.78^{a}	0.05±0.00^{c}	0.26±0.99^{b}
	6	0.11±0.03^{b}	0.95±0.09^{e}	0.63±0.01^{c}	0.45±0.02^{b}	0.58±0.00^{b}	0.91±0.78^{a}	0.05±0.00^{c}	0.26±0.99^{b}
1.5	0	0.34±0.44^{a}	1.10±0.32^{c}	0.71±0.73^{b}	1.08±0.01^{a}	0.83±0.55^{a}	1.12±0.99^{a}	0.28±0.01^{b}	0.40±0.99^{a}
	3	0.31±0.23^{a}	1.02±0.22^{c}	0,70±0.57^{b}	1.01±0.04^{a}	0.81±0.08^{a}	0.81±0.01^{a}	0.22±0.52^{b}	0.27±0.43^{a}
	6	0.31±0.23^{a}	1.02±0.22^{c}	0.70±0.57^{b}	1.01±0.04^{a}	0.81±0.08^{a}	0.81±0.01^{a}	0.22±0.52^{b}	0.27±0.43^{a}
2.5	0	0.29±0.56^{b}	1.13±0.12^{b}	0.73±0.76^{b}	1.02±0.56^{a}	0.67±0.89^{c}	0.92±0.45^{b}	0.30±0.32^{a}	0.19±0.12^{d}
	3	0.23±0.45^{b}	1.12±0.00^{b}	0.70±0.03^{b}	0.91±0.85^{a}	0.59±0.05^{c}	0.21±0.00^{b}	0.30±0.00^{a}	0.15±0.00^{d}
	6	0.23±0.45^{b}	1.12±0.00^{b}	0.70±0.03^{b}	0.91±0.85^{a}	0.59±0.05^{c}	0.21±0.00^{b}	0.30±0.00^{a}	0.15±0.00^{d}
3	0	0.27±0.24^{b}	1.15±0.47^{a}	0.61±0.00^{c}	0.88±0.22^{c}	0.78±0.90^{b}	1.13±0.00^{a}	0.27±0.77^{b}	0.39±0.98^{b}
	3	0.21±0.88^{b}	1.06±0.07^{a}	0.51±0.26^{c}	0.74±0.15^{c}	0.69±0.56^{b}	0.93±0.41^{a}	0.17±0.13^{b}	0.28±0.33^{b}
	6	0.21±0.88^{b}	1.06±0.07^{a}	0.51±0.26^{c}	0.74±0.15^{c}	0.69±0.56^{b}	0.93±0.41^{a}	0.17±0.13^{b}	0.28±0.33^{b}

Histidine (His), Leucine (Leu), valine (Val), phenylalanine (Phe), threonine (Thr), lysine (Lys), methionine (Met), tryptophan (Try). Value (Mean± SE) followed by different letters on each column based on species were significantly different at ($p<0.05$), according to DMRT.

Table 4.7: Interaction effects of Phytostim® biostimulants and storage period on the non-essential amino acids of *A. caudatus*.

Treatments (%)	Days	Gly	Ser	Pro	Ala	Asp	Glu	Arg	Ile
0	0	1.03±0.89^{a}	0.94±0.42^{b}	0.78±0.09a	2.30±0.04^{a}	0.94±0.55^{b}	1.23±0.45^{b}	1.72±0.78^{a}	0.74±0.00^{a}
	3	0.80±0.05^{a}	0.77±0.22^{b}	0.58±0.55^{a}	1.60±0.78^{a}	0.81±0.00^{b}	0.81±0.03^{b}	0.89±0.00^{a}	0.61±0.01^{a}
	6	0.80±0.05^{a}	0.77±0.22^{b}	0.58±0.55^{a}	1.60±0.78^{a}	0.81±0.00^{b}	0.81±0.03^{b}	0.89±0.00^{a}	0.61±0.01^{a}
0.5	0	0.84±0.66^{e}	0.77±0.55^{d}	0.56±0.33^{c}	1.68±0.22^{d}	0.83±0.56^{c}	1.02±0.21^{d}	1.20±0.49^{f}	0.57±0.23^{c}
	3	0.80±0.23^{e}	0.23±0.34^{d}	0.53±0.55^{c}	1.51±0.05^{d}	0.61±0.02^{c}	0.82±0.77^{d}	1.13±0.66^{f}	0.44±0.01^{c}
	6	0.80±0.23^{e}	0.23±0.34^{d}	0.53±0.55^{c}	1.51±0.05^{d}	0.61±0.02^{c}	0.82±0.77^{d}	1.13±0.66	0.44±0.01^{c}
1	0	0.82±0.56^{f}	0.76±0.50^{d}	0.70±0.77^{a}	1.86±0.71^{c}	0.79±0.04^{d}	1.03±0.00^{d}	1.47±0.89^{d}	0.62±0.64^{b}
	3	0.74±0.58^{f}	0.66±0.69^{d}	0.66±0.03^{a}	1.57±0.08^{c}	0.68±0.44^{d}	0.92±0.37^{c}	1.43±0.48^{d}	0.56±0.02^{b}
	6	0.74±0.58^{f}	0.66±0.69^{d}	0.66±0.03^{a}	1.57±0.08^{c}	0.68±0.44^{d}	0.92±0.37^{d}	1.43±0.48^{d}	0.56±0.02^{b}
1.5	0	0.92±0.99^{b}	0.88±0.07^{c}	0.70±0.29^{a}	2.18±0.01^{a}	0.95±0.64^{b}	1.35±0.99^{a}	1.65±0.66^{b}	0.67±0.02^{b}
	3	0.89±0.01^{b}	0.25±0.05^{c}	0.68±0.38^{a}	1.62±0.06^{a}	0.82±0.00^{b}	0.86±0.89^{a}	1.37±0.04^{b}	0.61±0.07^{b}
	6	0.89±0.01^{b}	0.25±0.05c	0.68±0.38^{a}	1.62±0.06^{a}	0.82±0.00^{b}	0.86±0.89^{a}	1.37±0.04^{b}	0.61±0.07^{b}
2.5	0	0.87±0.34^{c}	1.07±0.11^{a}	0.66±0.19^{b}	1.91±0.33^{b}	1.02±0.23^{a}	1.05±0.78^{d}	1.36±0.34^{e}	0.57±0.77^{c}
	3	0.79±0.78^{c}	0.99±0.01^{a}	0.59±0.22^{b}	1.26±0.25^{b}	0.98±0.72^{a}	0.77±0.06^{d}	1.09±0.33^{e}	0.47±0.01^{c}
	6	0.79±0.78^{c}	0.99±0.01^{a}	0.59±0.22^{b}	1.26±0.25^{b}	0.98±0.72^{a}	0.77±0.06^{d}	1.09±0.33^{e}	0.47±0.01^{c}
3	0	0.85±0.41^{d}	0.73±0.86^{e}	0.69±0.03^{b}	1.90±0.29^{b}	0.80±0.08^{c}	1.18±1.04^{c}	1.53±0.21^{c}	0.70±0.04^{a}
	3	0.77±0.05^{d}	0.44±0.00^{e}	0.36±0.08^{b}	1.25±0.00^{b}	0.29±0.78^{c}	0.95±0.03^{c}	1.34±0.58^{c}	0.46±0.02^{a}
	6	0.77±0.05^{d}	0.44±0.00^{e}	0.36±0.08^{b}	1.25±0.00^{b}	0.29±0.78^{c}	0.95±0.03^{c}	1.34±0.58^{c}	0.46±0.02^{a}

Glycine (Gly), serine (Ser), proline (Pro), alanine (Ala), asparagine (Asp), glutamic acid (Glu), isoleucine (Ile). Value (Mean± SE) followed by different letters on each column based on species were significantly different at ($p<0.05$), according to DMRT.

4.3.2.3 Protein content

Interaction effects of Phytostim® biostimulants and storage period significantly ($p<0.05$) the protein content of *Amaranth species*. The treated plants showed to be higher in comparison to the untreated plants. In *A. cruentus* at day zero, high protein content found was 27% at 0.5% concentrations of Phytostim® biostimulants. While at day three it was reduced to 25% and the same value was maintained until the end of the storage (6d). initially, control exhibited the high protein content of 24%. When the storage period increases, it was reduced to 19%. In *A. caudatus* at day zero, the high level of protein content obtained was 47% at 1% concentrations of Phytostim® biostimulants. At day three and day six, its protein content was reduced to 41% until the end of storage time. Whilst control resulted in higher protein content of 31% and gradually reduced with the increase of storage up to 27%. The obtained results demonstrate that were two to three folds higher than untreated plants as shown (Figure 4.3). This means that Phytostim® biostimulants played a very huge role in enhancing protein content of *Amaranth species*.

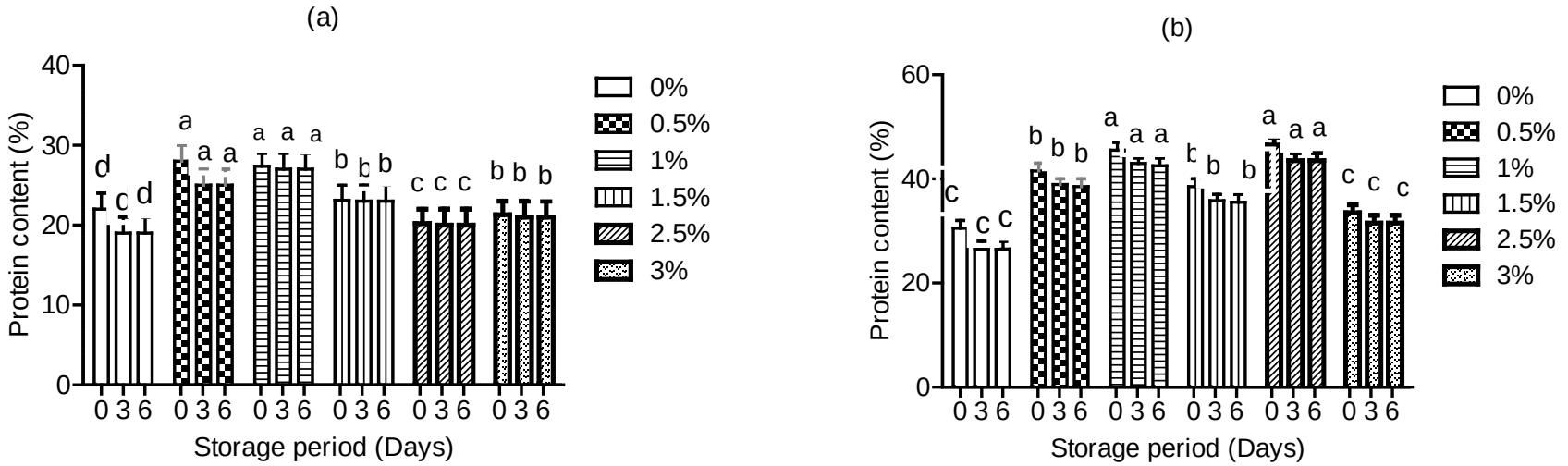

Figure 4.3: Effects of different concentrations of Phytostim® biostimulants on protein content of (a) *A. cruentus*, and (b) *A. caudatus*. Bars (± SE) with different letters are significantly different at ($p<0.05$).

In the present study, we investigated to check if Phytostim® biostimulant treatments will escalate the amaranth shelf life of retailers when stored at ambient temperature. The application Phytostim® biostimulant escalated the retailer's shelf life when stored at ambient temperature. Phytostim® biostimulant and storage period intersection had significant different ($p \leq 0.05$) on weight loss, color, and visual quality of *Amaranth species* (*A. cruentus* and *A. caudatus*). Weight loss after harvest is one of the common challenges farmers face especially if the commodity is to be sold by weight (Bhowmik and Pan, 1992). Plant treated with Phytostim® biostimulant had lower moisture loss and weight loss as compared to the untreated plant. The reduction in moisture loss and weight loss after treating it with Phytostim® biostimulant may be because of active ingredients such moringa leaf extracts and amino acids as permeable barrier against oxygen and carbon dioxide and thereby reducing respiration, water loss, and oxidation reaction rates (Kamel, 2014). In addition, it could be related to the abilities of the compounds contained in Phytostim® to improve various physiological processes such as changing the structure of cell walls and overcoming biotic and abiotic stresses (Stirk *et al.,* 2014; Bradford *et al*., 2020). It was observed in (Figure 4.1) that treating amaranth vegetables by 2.5% of Phytostim® biostimulant resulted in least percentage of weight loss comparing to other treatments. This is agreement with the study conducted by the (Abdalla, 2011). This supports that biostimulants contain the natural antioxidants that make the various crops overcome environmental stresses (Ghebreslassie, 2003). Another study conducted by Foline *et al*. (2011), is also coherent with our findings. Moisture loss of fresh vegetables is primarily due to transpiration and respiration. Transpiration is a mechanism in which water is lost due to differences in the vapor pressure of water in the atmosphere and the transpiring surface. Respiration causes weight reduction because a carbon atom is lost from the vegetables each time a carbon dioxide molecule is produced from an absorbed oxygen molecule and evolved into the atmosphere (Gharezi *et al*., 2012). The high weight loss of control vegetables was more evident as the shelf life of the retailers escalated.

Furthermore, loss of water from the vegetables could lead to loss of color. Color, it is an attribute influencing consumer attractiveness and product purchasing. According to Park

et al. (2018), found that green color loss is associated with loss of water, chlorophyll degradation and changes in carotenoids (carotenoids data not presented in the current study). In this study, Phytostim® biostimulant and storage period intersection influenced color at ($p \leq 0.05$). Although some of the color attributes were not influenced at ($p \geq 0.05$). The a* coordinates value was not influenced by Phytostim® biostimulant and storage period intersection in both amaranth species. This explains that a* coordinate (greenness) maintained the color throughout the storage. The hue angle (h^o) intensity of color showed decrease. This result might be a good indicator of how biostimulants affects leafy vegetable color, storage life, and quality of the leafy vegetables in general. The plants treated with Phytostim® biostimulant from 0.5 to 2.5% remained green up to 6d without showing signs of decaying such as having a foul odor, becoming slimy, and turning yellow color as compared to the untreated plants. The untreated plants started losing their fresh form on 3d of the storage. The results explain that the application of Phytostim® biostimulant delayed the vegetables from deteriorating. Moreover, it validates the endorsements made on the labeling of Phytostim® biostimulant it works best when applied at a concentration of less than 3% on leafy vegetables.

According to Ladele *et al*. (2016), mineral elements are important constituents of various elements and enzymes in our body which are involved in metabolism. Their absence has substantial consequences in health and their deficiency can cause variety of diseases. In addition, the predominant mineral elements found in the studied crop are essential in our bodies since they can act as an antioxidant, helping to protect cells from damage caused by free radicals. It also plays a role in thyroid function and supports the immune system. This means that the levels of heavy metals found in the studied crop are within the regulatory limits, suggesting that they may not impose any health risk.

Furthermore, the obtained results indicate that treated plants were characterized by overall higher minerals than the untreated plants (control). Amongst the two species, *A. cruentus* had higher minerals as compared to the *A. cruentus*. The treatments had positive effects on the mineral elements of the plants as their application enhanced the contents of macro and microelements. Macro minerals (Mg, Ca, K, and N) required for plant growth and development were found to be higher. Micro minerals (Cu, Fe, Se, Zn

and Mn) were also found to be higher. This improvement could be ascribed to the fact that Phytostim® compounds are made of leaves of moringa, rich in minerals. Therefore, when applied to the crops, the nutrients uptake is increased. The accumulation of the nutrients in amaranth were significantly higher due to the fertilization with Phytostim® biostimulants. In addition, the researcher Abdalla (2011) also found that, Moringa leaf extract, applied at rates of 2% or 3%, enhanced the leaves' photosynthetic pigments, total protein, total sugar, phytohormones (auxins, cytokinins and gibberellins) and several essential mineral nutrients, such as N, P, K, Ca, Fe and Mg of the rockets plants.

CONCLUSION

From the above-mentioned results, it can be concluded that 2.5% of Phytostim® biostimulant were the most successful in preserving the high-quality characteristics of *Amaranth species* during ambient temperature. Applying Phytostim® biostimulant treatments at 2.5% has performed better as compared to the control and other treatments concentrations as it resulted in the least amount of leafy vegetable deterioration and maintained the high moisture level. Thus, the current findings demonstrate that employing Phytostim® as a promising and effective natural compound to replace synthetic fertilizer inhibit the development of postharvest deterioration and enhances the quality and storability of leafy vegetables at doses of 2.5% or less. The benefits of these natural compounds for customers will be greater.

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CHAPTER 5

INTERACTIVE EFFECTS OF DIFFERENT CONCENTRATIONS OF PHYTOSTIM® BIOSTIMULANT AND STORAGE ON SECONDARY METABOLITES OF AMARANTHUS CAUDATUS AND AMARANTHUS CRUENTUS

ABSTRACT

Amaranthus species is an underutilized indigenous plant with well-known sources of different secondary metabolites including phenolic compounds. Less attention has been given to crop, though it has pharmaceutical benefits. The study was aimed at investigating the effects of different concentrations of Phytostim® biostimulants and storage period interaction on secondary metabolites of *A. cruentus* and *A. caudatus.* In this study, ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) analysis was used for metabolomic finger printing while phenolic compounds were assessed using Quadrupole time-of-flight (QTOF) mass spectrometer (MS). Among 55 identified untargeted phenolic compounds from *Amaranthus species* at different concentrations of Phytostim® biostimulant and storage period interaction. Members of phenolic compounds including organic acids, flavonoids, phenolic acids, glucuronic acid derivatives, and Coumarin glycosides were detected. The obtained results revealed that phenolic compounds were significantly ($p<0.05$) affected by the different concentrations of Phytostim® biostimulant and storage period interaction. Principal component analysis (PCA and Orthogonal partial least squares discriminant analysis (OPLS-DA) demonstrated a clear variation between the different concentrations of Phytostim® biostimulant and storage period. Whereby a 3% concentration of Phytostim® biostimulant at the end of the storage were effective in stimulating the phenolic compounds of *Amaranthus species* in comparison to control (0%).

Keywords: *Amaranth species*, Phenolic compounds, Phytostim® biostimulant, UHPLC-MS, PCA, OPLS-DA.

5.1 INTRODUCTION

Metabolomics have gained interest in the crop production studies recently. This is due to their association with abiotic factors such as storage, light, drought and salinity which creates a clear change in the metabolome profile. Indigenous plants are well-known sources of different secondary metabolites commonly known as phytochemicals (Pfukwa *et al*., 2020). Plant secondary metabolites are organic compounds produced by plants as a part of the defense mechanism response against environmental stresses triggered by microbial pathogens, mechanical wounding and direct exposure to UV or visible light (Madala *et al.,* 2014). However, they are not directly involved in their development, growth, and reproduction (Van Wyk, 2020). Their daily consumption in plants, fruits, vegetables, juices, and beverages leads to a balanced diet and health benefits, such as antioxidant, cardiovascular, antithrombotic, anti-inflammatory, and antitumor ones (Rana *et al.,* 2022). Phenolic compounds are of possible pharmacological value and have been reported to have antioxidative and anticarcinogenic effects. Flavonoids have long been recognized to possess anti-inflammatory, antiallergenic, antiviral and antiproliferative activities. Food materials are characterized by their nutritional and health properties, which are directly linked to metabolites composition. Additionally, chemical metabolites, such as polyphenolic compounds, influence food properties, color, taste, health, and nutritional quality (Nemzer *et al.,* 2021).

Metabolomics is a notion for describing the analyses of a comprehensive metabolite's characterization on plants, fruits, and vegetables. It offers a holistic understanding of metabolites and is an essential tool applied to demonstrate a potential relationship change in parameters and their response to an environmental stress (Cuber-Leon *et al.,* 2014). Metabolite profiling (targeted analysis) and metabolite fingerprinting (untargeted analysis) are fast-growing technologies for phenotyping and diagnostic analyses of plants (Mishra *et al*., 2017; Goudoum *et al.,* 2021). They allow the identification of the most important compounds (or groups of compounds) underlying differences between

genotypes or phenotypes (Mishra *et al.,* 2017). Analytical platforms, such as ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS), have been widely used in plant science for metabolomics applications to identify and quantify compounds (Chen *et al.,* 2013; Zheng *et al.,* 2020). Most discrimination methods use analytical tools such as chromatography, spectrophotometry, and chemometrics. Principal component analysis (PCA), hierarchy cluster analysis (HCA), and Orthogonal partial least squares discriminant analysis (OPLS-DA) are among the most widely used chemometric methods for determining differences between food samples. Furthermore, for the classification of food samples using the data generated by ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) analyses, supervised chemometric methods, multivariate calibration model partial least squares discriminant analysis (PLS-DA) has been extensively used to differentiate metabolites variation in nightshade, chinese cabbage, and pumpkin leaves treated with drying, fermentation, and cooking methods, respectively (Managa *et al.,* 2020). Yet such result based on different concentrations of Phytostim® biostimulant and postharvest storage is still lacking and thus the motive of this study was to determine effects of Phytostim® biostimulant and storage period on the secondary metabolites of amaranth.

The objective of the study

The objective of this chapter was to investigate the effect of different concentrations of Phytostim® biostimulant and storage period interaction on secondary metabolites of *Amaranthus species*.

5.2 MATERIALS AND METHODS

5.2.1. Sample preparations.

Harvested leaves of *A. cruentus* and *A. caudatus* were oven dried at 40° C for 72 h. The dried samples were pulverized into a fine powder using a blender. Then 5 g of samples were packed into a sealed plastic bag based on the treatments (Chapter 4). The pulverized samples were then transported to the University of Stellenbosch for analysis.

5.2.2 Extraction of plant materials for free polar metabolites

For the extraction of plant materials, a 1 g sample was accurately weighed into a 50 mL centrifuge tube with a screwcap and mixed with 10 mL of 50% methanol/1% formic acid. Thereafter, the samples were vortexed for 1 min, followed by extraction in an ultrasonic bath for 1 h. Then, 2 mL of the sample was withdrawn and centrifuged at 14 000 rpm for 5 min. The clear supernatant was then transferred into 1.5 mL glass vials for analysis.

5.2.3 Untargeted metabolites analysis using LC-MS

The untargeted polar metabolites profiling of *Amaranth species* were carried out using a Waters Synaptic G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to the Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) was used for high-resolution UPLC-MS analysis. Column elution and rate flow were used for MS prior Photodiode Array (PDA) allowing simultaneous collection of UV and MS spectra. The MS was operating in a negative ion electrospray mode and Nitrogen was used for the desolvation gas. The following parameters were then set extraction cone, 4 V, capillary voltage, 2500 V, sampling cone voltage, 45 V; source temperature, 100°C; desolvation temperature, 350°C and desolvation gas flow, 500 L/ h. Data were acquired by scanning over a range of *m/z* 100 to 1500 in the resolution mode and MSE mode. Separation was achieved on a Waters HSS T3, 2.1 × 100 mm, 1.7 µm column. An injection volume of 2 µL was used. Mobile phases A and B consisted of 0.1% formic acid and acetonitrile respectively. The gradient started at 100% solvent A for 1 min and changed to 28% B over 22 min in a linear way. It then went to 40% B over 50 s and a wash step of 1.5 min at 100%, followed by re-equilibration to initial conditions for 4 min. The flow rate was 0.3 mL/min, and the column temperature was maintained at 55°C.

5.2.4 Statistical analysis

The overall secondary-metabolite fingerprints were considered as an untargeted profile matrix to construct the models. Chemometric data analysis was performed using SIMCA ver 13.0 software to create an unsupervised PCA and supervised OPLS-DA models to observe clear clustering between different concentrations of Phytostim® biostimulant at different storage days within the two studied species (*A. cruentus* and *A. caudatus*).

Supervision was set to demonstrate similarities and differences of metabolites in T1, T2, T3, T4, and T5 at a 95% confidence interval. Observed differences at ($p<0.05$) were considered statistically significant according to the Duncan multiple range test.

5.3 RESULTS AND DISCUSSION

5.3.1 Tentative identification of untargeted metabolites in *Amaranthus species* at different concentrations of Phytostim® biostimulant during the storage days.

A total of 55 metabolites were detected in amaranth leaf samples. The identified metabolites were divided into 20 metabolite groups with the highly concentrated groups identified as phenolic acids (6), fatty acids (7), flavonoids (3), coumarin glycosides (2), glucuronic acid derivatives (7), and other (6) unknowns as depicted in (table 5.1). The detected members of phenolic acid were caffeic acid-3-0-glucuronide which eluted (rt: 16.52 with *m/z*: 457.1338), Ptelatoside-A which eluted (rt: 16.52 with *m/z*: 413.1422), ferulic acid (rt: 16.52 with *m/z*: 367.0665), 3-5-caffeoyl quinic acid (rt: 14.74 with *m/z*: 411.1319), 3-4-5-trihydroxyoxane-2-carboxylic acid (rt: 9.44 with *m/z*: 371.0591). The member of glucuronic acid derivatives was tentatively identified as 2-O-caffeoyl glucaric acid (others not mentioned in the table). Also had isomer detected at (rt: 7,173 with *m/z*: 371, 0603); (rt: 7,812 with *m/z*: 371, 0600); (rt: 8,385 with *m/z*: 371, 0617). Moreover, the results further corroborate the presence of coumarin glycosides tentatively identified as chlorogenic acid, detected at (rt: 13,14 with *m/z:* 353, 0497). Other phenolic compounds present in this crop were found to be members of flavonoids, tentatively identified as rutin which was detected at (rt: 16,52 with *m/z*: 609, 1453); Quercetin 3-galactoside (rt: 16,528 with *m/z*: 463, 0850); and Astragalin 7-rhamnoside (rt: 16,52 with *m/z*: 593, 1499). Lastly, highly concentrated metabolites were members of fatty acids including cyanidin 3-0-glucoside which eluted (rt: 16.52 with m/z: 837.3103), 6-ferulolyglucoside (rt: 15.65 with m/z: 473.1670), 7-epi-12-hydroxy jasmonic acid glucoside detected as isomer at (rt: 12.41 with m/z: 387,1646), (rt: 12.80 with m/z: 387.1646) and (rt: 13,35 with m/z: 387.1646), cis-3-hexenyl b primeveroside detected at (rt: 15.28 with m/z: 393.1764) and rt: (15.65 with m/z: 393.1764). Our observation regarding the changes of phenolic compounds content such as flavanoids during the amaranth confirmed the trends previously reported by Kalinova and Dadakova (2009). These authors determined the flavanoids contents in

leaves, flowers, stems, and seeds of six *Amaranthus species* (*A. caudatus*, A. *hypochondriacus*, *A. hybrid*, *A. retroflexus*, *A. cruentus*, and *A. tricolor*) and found that the flavanoids content in leaves was related to the developmental stage of the crop and that it usually increased with plant aging.

Table 5.1: LC-MS tentative identification assignment of untargeted metabolites compounds contained in *Amaranthus species* leaves subjected to different concentrations of Phytostim® biostimulant exposed on different days.

Retention time (Rt) in min	Experimental *m/z* [M-H]	Chemical formula	Tentative identification	References
Phenolic acids				
14.741	411.13190	C25H24O12	3-5 caffeoyl quinic acid	Online database
16.528	413.14227	C19H26O10	Ptelatoside A	Emad *et al.,* 2022
16.528	457.13382	C20H26O12	3-5-caffeic acid-3-0-glucuronide	Ramabulana *et al.,* 2021
14.914	305.10602	C13H22O6S	Pinitol diacetonide	Online databse
9.442	371.05914	C15H16O11	3,4,5-trihydroxyoxane-2-carboxylic acid	Gooseen *et al.,* 2018
16.528	367.06659	C10H10O4	Ferulic acid	Online database
Coumarin glycosides				
13.148	353.04971	C15H14O10	Chlorogenic acid	Pereira *et al.,* 2018; Masike *et al.,* 2017.
Glucuronic acid derivatives				
7.173	371.06039	C15H16O11	2-O-caffeoylglucaric acid	Yasir *et al.,* 2016, Masike *et al.,* 2016.
7.812	371.06006	C15H16O11	2-O-caffeoylglucaric acid	Yasir *et al.,* 2016, Masike *et al.,* 2016.
8.385	371.06177	C15H16O11	2-O-caffeoylglucaric acid	Yasir *et al.,* 2016, Masike *et al.,* 2016.
Flavonoids				
16.528	609.14539	C27H30O16	Rutin	Patel, 2019

16.528	463.08508	C21H20O12	Quercetin 3-galactoside	Chen *et al.,*2016; Masike *et al.,* 2017
16.528	593.14990	C27H30O15	Astragalin 7-rhamnoside	Zhang *et al.,* 2018
Fatty acids				
16.52	837.3703		Cyanidin- 3-0-glucoside	Online database
12.41	387.1646		7-epi-12-hydroxyjasmonic acid glucoside	Online database
12.80	387.1646		7-epi-12-hydroxyjasmonic acid glucoside	Online database
13.35	387.1646		7-epi-12-hydroxyjasmonic acid glucoside	Online database
15.28	473.1670		6-ferulolyglucoside	Online database
15.65	393.1764		Cis-3-hexenyl b primeveroside	Yasir *et al.,* 2016, Masike *et al*., 2016.
15.75	393.1764		Cis-3-hexenyl b primeveroside	Yasir *et al.,* 2016, Masike *et al.,* 2016.

5.3.2 Quantification of phenolic compounds in *Amaranthus species* at different concentrations of Phytostim® biostimulant during the storage days.

The different concentrations of Phytostim® biostimulants and storage period interaction significantly affected ($p<0.05$) the phenolic compounds of *Amaranthus species*. The content of individual phenolic compounds in different Phytostim® biostimulants and storage period interaction is presented in Table 5.2. The observed results indicated that phenolic compounds gradually increased with the storage. However, some of the phenolic compounds exhibited insignificant changes ($p>0.05$) during the subsequent days of the storage (3, and 6 d). The results further demonstrate that 3% of Phytostim® biostimulants performed best as compared to the control and the other concentrations of Phytostim®

biostimulants. Which means that Phytostim® biostimulants promoted an increase in the phenolic compounds of *Amaranthus species*.

Out of the four tentatively identified phenolic acids including 3-5-caffeoly quinic acid, pteletoside A, ferulic acid and caffeic acid-3-0-glucuronide in *A. cruentus*, had significantly ($p<0.05$) increased with the storage at 0; 1; 1.5; 2.5 and 3%. Whereas 1% had significantly decreased with the storage. In *A. caudatus*, 0; 0.5; 1; and 1.5% had significantly increased with the storage while 2.5 and 3% had significantly decreased. While tentatively identified Flavonoids indicated that Rutin and Astragalin 7-rhamnoside increased in all treatments of both *Amaranthus species* whereas Quercetin 3-galactoside showed to gradual decrease in all treatments of both *Amaranthus species*. The phenomenon could be attributed to the faster loss of water and higher enzyme activity which accelerated the oxidation of the phenolic compounds (Deng *et al.,* 2018).

The results suggest that 0.5% biostimulants showed to decrease in most phenolic compounds of both *Amaranth species* while 3% biostimulants showed to increase the phenolic compounds of the studied crop better than the other concentrations. The results of this study demonstrate that Phytostim® biostimulants contributed towards the increase of the phenolic compounds as its phenolic compounds were higher than those in control (0% biostimulant). According to Emad *et al*. (2020), reported that the availability of the phenolic compounds in plant species depends on the skeletal phenolic chain as dictated by the plant genetics. Therefore, the obtained results in the individual polyphenolic compounds of the studied crop vary. For instances, significance difference ($p>0.05$) observed in the individual of phenolic acids and Flavonoids shows that, in *A. cruentus*, 3-5-caffeoly quinic acid had significantly higher constituent average of (631.03 mg/kg) at 3%. Ptelatoside A had highest constituent average of (670.31 mg/kg) at 0%. ferulic acid had higher constituent average of (296.76 mg/kg) at 0%. 2,5-dihydroxyphenoxy3, had higher constituent average of (1920.03 mg/kg) at 3%. Rutin, had higher constituent average of (645.73 mg/kg) at 3%. Astragalin 7-rhamnoside, constituent of (126.14mg/kg) at 3%. and Quercetin 3-galactosidem had higher constituent average of (48.06 mg/kg) at 2.5%. Whereas in *A. caudatus*, 3-5-caffeoly quinic acid had significantly higher constituent average of (152,90 mg/kg) at 0%. Ptelatoside A, the highest was observed in

1.5% (321,60 mg/kg). Ferulic acid highest was observed in 3% (199.72 mg/kg). 2,5-dihydroxyphenoxy3, the highest was observed in 3% (100,66 mg/kg). Rutin, the highest was observed in 3% (1011,60 mg/kg. Astragalin 7-rhamnoside, the highest was observed in 1% (207,38 mg/kg). Quercetin 3-galactoside, the highest was observed in 1% (148,86 mg/kg). Moreover, the results of this study coherent with the study conducted by Schröter *et al*. (2018), who found that most of the identified compounds (Quercetin 3-galactoside, caffeic acid, and rutin) were detected previously in leaves, seeds, and other aerial parts of *A. caudatus*. Flavonoids acids were also found in stems of *A. spinosus*.

Table 5.2: Quantification of some phenolic compound found in *Amaranthus cruentus*.

		Phenolic compounds (mg/kg)						
Treatments		3-5-caffeoly quinic acid	Ptelatoside A	Ferulic acid	Caffeic acid-3-0-glucuronide	Rutin	Quercetin 3-galactoside	Astragalin 7-rhamnoside
Day 0	T0	345.12±5.46^{d}	516.27±0.23^{a}	163.02 ±1.25^{b}	1012.65±3.66^{e}	261.70±1.22^{c}	102.59±11.02^{a}	106.39±7.90^{b}
	T1	189.88±0.30^{e}	70.40±0.89^{e}	119.34±11.23^{c}	1412.96±7.88^{b}	249.26±0.78^{c}	12.99±0.89^{e}	29.20±9.48^{e}
	T2	542.22±7.29^{b}	260.34±1.25^{d}	109.54±10.45^{d}	1307.10±1.65^{c}	476.14±1.58^{a}	26.50±1.12^{d}	66.00±6.55^{c}
	T3	542.20±1.20^{b}	260.30±1.00^{d}	109.50±9.48^{d}	1307.10±1.98^{c}	476.10±1.56^{a}	26.50±1.12^{d}	66.00±6.55^{c}
	T4	507.35±0.89^{c}	306.70±0.00^{c}	104.41±5.88^{d}	1263.43±0.88^{d}	330.40±2.11^{b}	35.15±2.45^{c}	59.29±2.45^{d}
	T5	600.74±1.44^{a}	437.31±0.00^{b}	189.69±7.23^{a}	1775.00±1.29^{a}	397.53±0.47^{b}	55.74±1.13^{b}	121.98±9.48^{a}
Day 3	T0	474.29±2.78^{c}	642.28±1.89^{a}	175.40±2.56^{b}	1295.40±1.25^{c}	270.52±0.90^{d}	33.06±0.36^{b}	38.21±1.89^{d}
	T1	265.90±0.04^{d}	80.59±2.45^{d}	142.56±5.11^{e}	996.88±1.32^{d}	348.85±1.65^{c}	13.49±0.10^{e}	30.48±4.77^{e}
	T2	585.09±0.03^{b}	371.51±0.00^{c}	155.90±5.00^{d}	1459.26±2.52^{b}	569.07±1.13^{a}	30.65±0.75^{c}	67.93±3.54^{b}
	T3	465.90±1.33^{c}	300.60±0.03^{c}	142.60±4.78^{e}	996.90±1.54^{d}	584.80±1.09^{a}	33.50±3.25^{b}	69.50±2.48^{b}
	T4	538.09±3.99^{b}	354.07±8.44^{c}	166.82±9.22^{c}	1427.35±2.28^{b}	370.65±0.89^{c}	38.49±1.02^{a}	61.91±3.99^{c}
	T5	630.15±8.99^{a}	437.77±1.66^{b}	196.57±4.89^{a}	1833.61±2.87^{a}	420.59±2.44^{b}	25.25±0.98^{d}	123.84±7.66^{a}
Day 6	T0	555.06±5.00^{d}	670.31±1.22^{a}	186.76±3.45^{b}	1496.91±1.19^{b}	331.14±2.32^{d}	25.93±3.33^{e}	25.37±1.55^{e}
	T1	374.23±0.03^{f}	93.63±0.05^{d}	162.38±2.78^{d}	198.43±3.52^{d}	359.07±1.27^{e}	20.00±1.20^{f}	37.85±2.33^{d}
	T2	600.52±0.74^{c}	466.60±0.00^{b}	179.17±1.22^{c}	1498.35±1.02^{b}	593.49±2.02^{b}	36.00±2.48^{c}	73.80±2.01^{b}
	T3	400.50±0.09^{e}	345.60±0.89^{c}	179.71±1.03^{c}	686.35±1.15^{c}	598.50±2.09^{b}	40.00±0.67^{b}	79.75±0.88^{b}
	T4	626.67±1.22^{b}	486.54±0.33^{b}	199.48±2.23^{a}	1444.26±1.65^{b}	471.45±2.40^{c}	48.06±2.52^{a}	63.44±2.02^{c}
	T5	631.01±5.77^{a}	492.41±1.33^{b}	199.72±1.00^{a}	1920.03±4.25^{a}	645.73±1.77^{a}	30.20±3.53^{d}	126.14±1.68^{a}

Values were expressed as (means ± SE) in columns with different letter (s) differ significantly ($p \leq 0.05$) according to the Duncan multiple range test.

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Table 5.3: Quantification of some phenolic compound found in *Amaranthus caudatus*

		Phenolic compounds (mg/kg)						
	Treatments	3-5-caffeoly quinic acid	Ptelatoside A	Ferulic acid	Caffeic acid-3-0-glucuronide	Rutin	Quercetin 3-galactoside	Astragalin 7-rhamnoside
Day 0	T0	98.49±0.10^{d}	136.20±0.20^{c}	32.13±0.40^{c}	48.98±0.06^{e}	694.78±0.00^{c}	135.28±6.45^{d}	143.46±0.40^{d}
	T1	128.24±0.8^{a}	338.92±0.90^{a}	56.94±0.60^{a}	94.72±2.48^{b}	764.04±0.45^{b}	455.96±7.80^{a}	295.46±0.06^{a}
	T2	104.35±0.77^{c}	281.94±1.33^{b}	22.07±0.90^{d}	72.35±0.92^{c}	629.01±0.80^{d}	247.44±1.45^{c}	209.72±0.10^{c}
	T3	108.49±0.31^{c}	266.88±1.55^{b}	50.19±1.22^{b}	102.10±0.30^{a}	602.28±0.30^{e}	318.09±0.90^{b}	207.38±1.10^{c}
	T4	105.74±0.78^{c}	271.20±1.67^{b}	28.46±0.40^{d}	79.78±2.03^{c}	591.85±0.00^{f}	224.63±0.10^{c}	216.67±0.92^{b}
	T5	118.15±0.00^{b}	92.31±0.80^{d}	16.64±0.01^{e}	52.47±1.11^{d}	818.24±0.40^{a}	63.06±1.00^{e}	52.13±1.88^{e}
Day 3	T0	100.36±0.80^{e}	154.60±1.89^{d}	39.36±0.70^{c}	63.78±0.50^{d}	702.93±0.20^{d}	94.69±0.70^{c}	151.67±0.77^{c}
	T1	138.19±0.60^{a}	251.64±3.45^{c}	58.67±1.00^{a}	73.70±0.60^{c}	852.69±0.89^{b}	225.65±0.40^{a}	228.73±1.28^{a}
	T2	128.40±1.23^{b}	309.60±5.66^{a}	34.54±0.00^{d}	78.73±0.10^{a}	772.96±1.66^{c}	103.21±1.23^{b}	159.41±0.90^{b}
	T3	113.36±2.22^{d}	298.24±2.36^{b}	54.54±0.00^{b}	54.54±0.00	621.05±0.40^{f}	67.81±1.77^{d}	117.69±1.00^{d}
	T4	116.33±1.00^{d}	127.56±2.77^{d}	28.30±0.50^{e}	46.85±1.78^{e}	664.72±2.02^{e}	29.85±0.28^{f}	35.31±2.76^{e}
	T5	122.53±0.50^{c}	108.90±1.00^{e}	25.34±0.80^{f}	74.52±3.66^{b}	943.30±1.48^{a}	32.87±0.99^{e}	35.49±0.01^{e}
Day 6	T0	110.16±0.30^{f}	116.01±0.90^{f}	49.20±1.00^{c}	81.36±0.60^{b}	734.01±0.80^{d}	90.65±1.44^{c}	159.54±0.90^{b}
	T1	145.99±1.56^{b}	313.92±0.60^{b}	58.12±0.20^{a}	70.32±0.82^{d}	929.04±0.70^{b}	148.86±1.21^{b}	205.71±2.43^{a}
	T2	138.49±3.56^{c}	266.88±1.44^{c}	50.19±0.60^{c}	78.55±1.22^{c}	902.28±0.90^{b}	318.09±0.31^{a}	207.38±3.66^{a}
	T3	128.33±3.33^{d}	321.60±2.27^{a}	54.15±0.78^{b}	54.75±3.61^{e}	784.66±0.77^{c}	31.54±0.40^{e}	57.62±0.11^{c}
	T4	121.73±0.07^{e}	126.76±0.27^{e}	30.75±0.55^{d}	37.07±2.05^{f}	756.70±0.21^{d}	14.26±0.02^{e}	20.43±0.60^{d}
	T5	152.90±0.05^{a}	137.43±0.60^{d}	25.65±0.04^{e}	100.66±0.85^{a}	1011.60±0.20^{a}	72.65±0.10^{d}	50.65±0.02^{c}

Values were expressed as (means ± SE) in columns with different letter (s) differ significantly ($p \leq 0.05$) according to the Duncan multiple range test.

5.3.3 Multivariate Analysis

Phenolic compounds present in the *Amaranthus species* vary based on the species and treatments. To highlight metabolome fingerprint change induced by Phytostim® biostimulant and storage days. The multivariate analysis method was employed to generate metabolome features simultaneously and then identify relationship patterns between them. The PCA (unsupervised) based on UPLC–Q-TOF/MS was applied to illustrate understanding of metabolites clustering pattern of *Amaranthus species* at different concentrations of Phytostim® biostimulant and storage days. The PCA (unsupervised) based on UPLC–Q-TOF/MS as depicted in (Fig 5.1) demonstrated clear clustering according to the species, though there was no clear variation amongst the different concentrations of Phytostim® biostimulant interaction with the storage days. However, the results further show that phenolic compounds that are in PC1 (57.8%) are distinct from PC2 (14.6%). *Amaranth caudatus* applied at concentrations of 3% (T5) followed by 2.5% (T4) outperformed best as they stimulated the phenolic compounds. The results further demonstrated that at the end of the storage (6d) there was an inhabitant of the phenolic compound at T4 while T5 stimulated the phenolic compound until the end of storage (6d). This could be ascribed to the damage to the cell structure and the presence of molecular oxygen due to their catalyzed oxidation.

Additionally, a supervised (OPLS-DA) model was generated to differentiate between different concentrations of Phytostim® biostimulant and storage days (Fig 5.2). The results shown below demonstrated good model statistics with predictive ability (Q2 cum value: 72%) that was above 50%. The discrimination of the samples in two clusters indicates the aroma components in this leafy vegetable were different based on the concentrations of Phytostim® biostimulant and storage days. However, the observed clustering of samples in supervised (OPLS-DA) and unsupervised (PCA) plots shows that there were similar trends of metabolite profiles in the studied crop (*Amaranth species*).

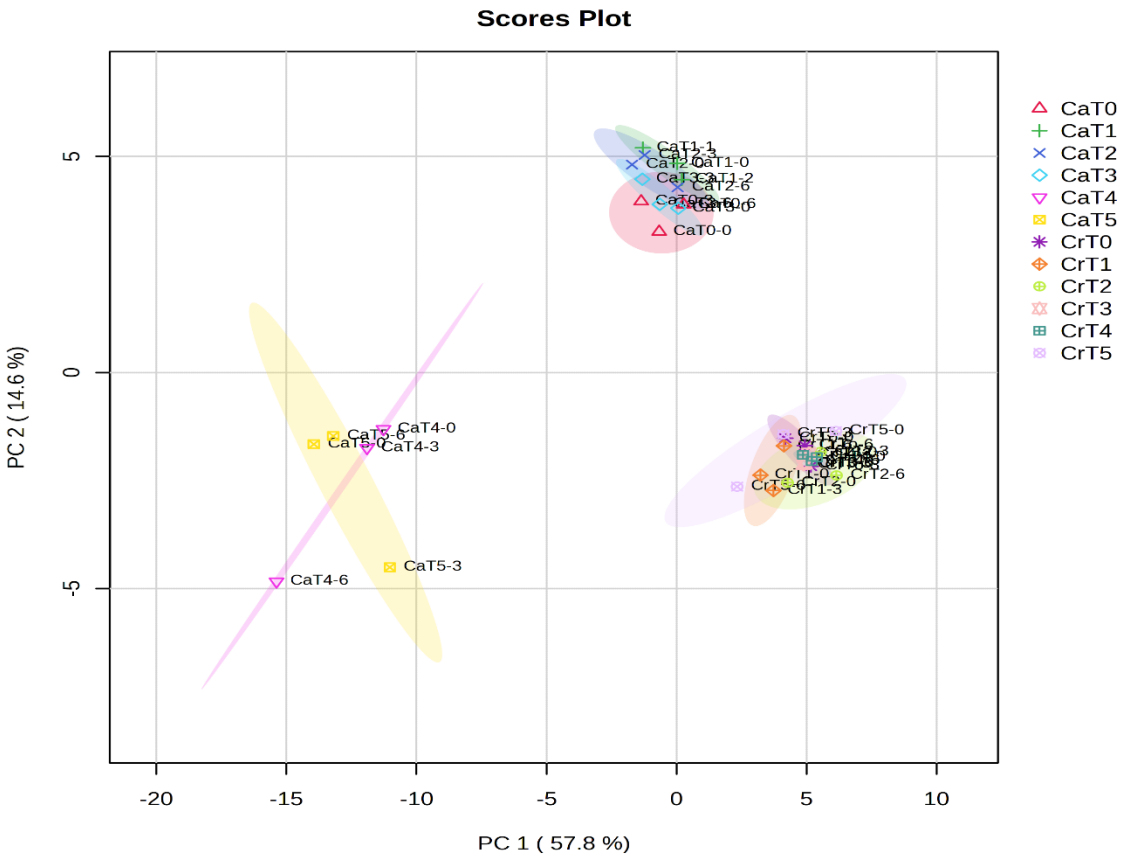
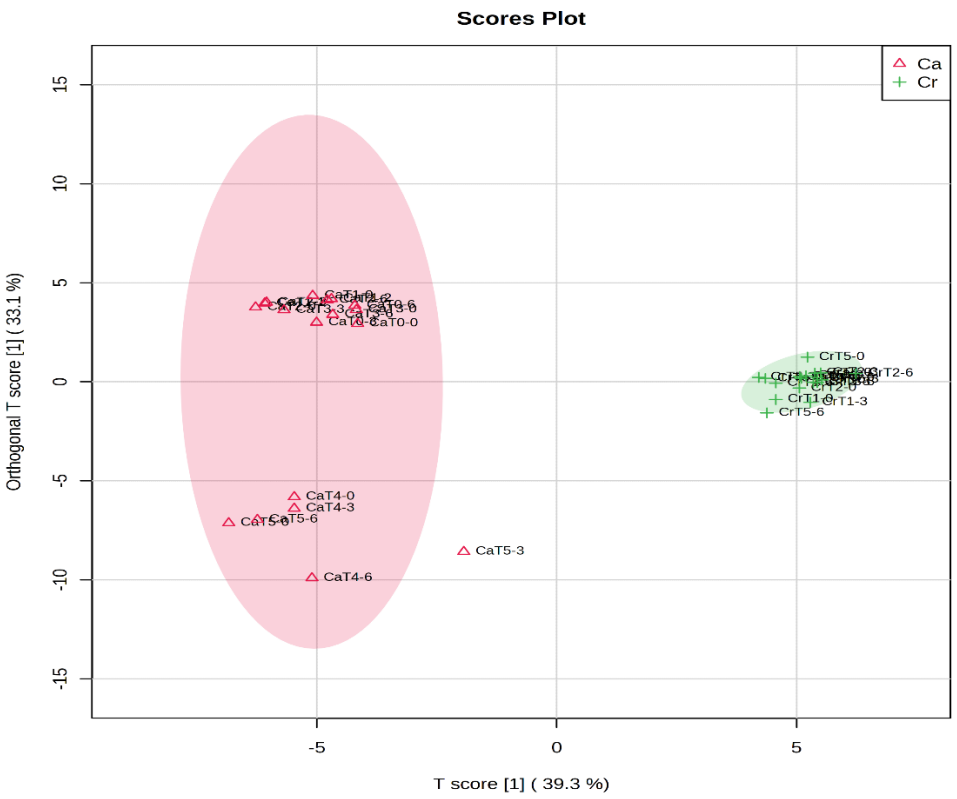


Figure 5.1: Score plot of principal component analysis (unsupervised) based on UPLC–Q-TOF/MS. Ca= *Amaranthus caudatus*; Cr= *Amaranthus cruentus*; T= Phytostim® biostimulant at T0=0%, T1=0.5%, T2=1%, T3=1.5%, T4=2.5% and T5=3%.



B H 6

Figure 5.2: Score plot of orthogonal partial least squares discriminant analysis of UPLC–Q-TOF/MS spectra of the leaves of *Amaranthus species* (supervised). Ca= *Amaranthus caudatus*; Cr= *Amaranthus cruentus*; T= Phytostim® biostimulant at T0=0%, T1=0.5%, T2=1%, T3=1.5%, T4=2.5% and T5=3%.

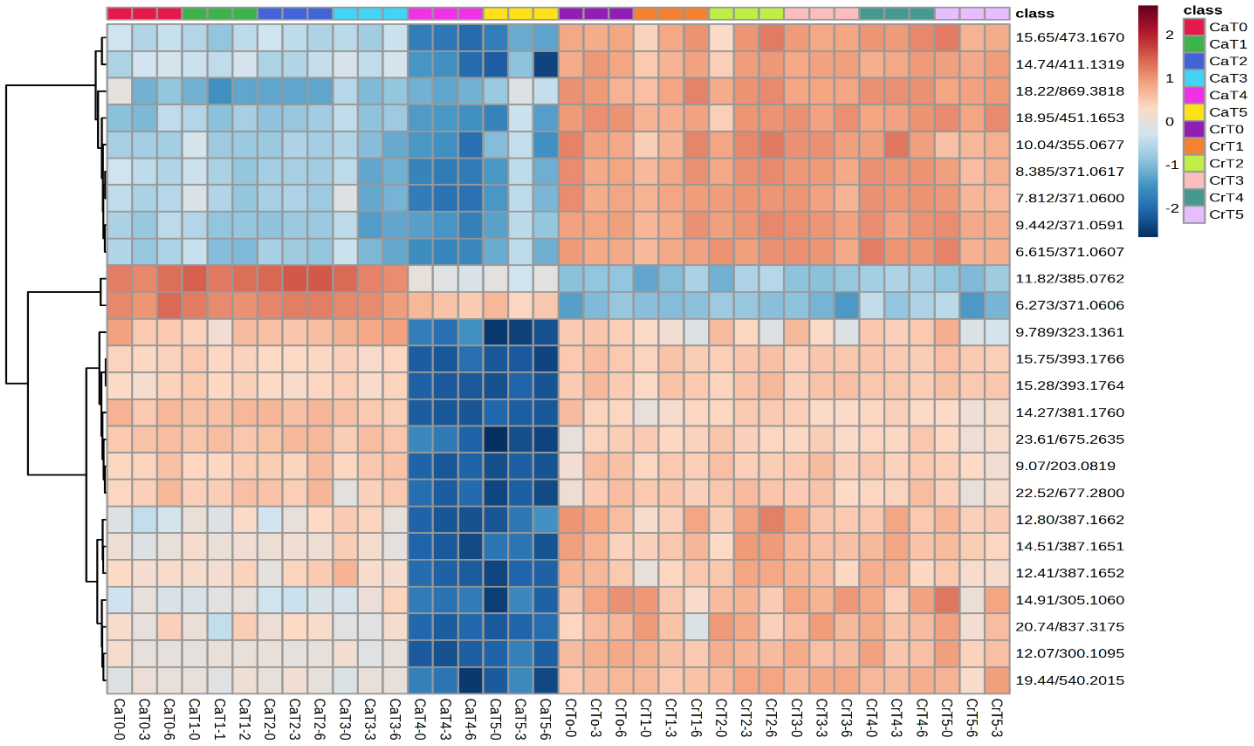


Figure 5.3: Heatmap of untargeted metabolites in hierarchical clustering in the leaves of *Amaranthus species*. Ca= *Amaranthus caudatus*; Cr= *Amaranthus cruentus*; T= Phytostim® biostimulant at T0=0%, T1=0.5%, T2=1%, T3=1.5%, T4=2.5% and T5=3%. e (Q-r-g). The pattern and magnitude relating to the color intensity (hue) from +2 to −2 and 0 as symmetry relating to visualization of response intensities of identified and unidentified compounds present in *Amaranthus species*.

CONCLUSION

The effects of different concentrations of Phytostim® biostimulant on the secondary metabolites showed to vary in phenolic compounds and based on species. Phytostim® biostimulant applied at 3% resulted in an improvement of phenolic compounds until the end of the storage as it preserved more phenolic compounds. Suggesting that Phytostim® biostimulant should be considered more effective for slowing down the degradation of phenolic compounds at 3%. These results can be useful in providing information that 3% of Phytostim® biostimulants preserve the phenolic compounds of *Amaranthus species* which are designated to be therapeutic.

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CHAPTER 6

SUMMARY OF FINDINGS, SIGNIFICANCE, RECOMMENDATIONS AND CONCLUSIONS

6.1 Summary of findings

Leafy vegetables crops contribute immensely to improving the food security. Yet, they are underutilized and neglected. On the other hand, sustained cultivation of modern crops is a major concern. The findings of this study revealed that Phytostim® biostimulant improved the growth and yield attributes, escalated the shelf life of *Amaranthus species*, enhanced the nutritional components and secondary metabolites. The first phase of the investigation was aimed at assessing the effects of Phytostim® biostimulant on growth and yield attributes. The results documented in this study reported that Phytostim® biostimulant improved the plant height (cm), stem diameter (mm), no of branches, chlorophyll index (SPAD), biomass (g), aerial mass (g), root mass (g), leaf length (cm), leaf width (cm), root length (cm) and no of leaves. Thus, it was clearly concluded that the optimum concentrations for improving the growth and yield attributes of *Amaranthus species* was 1.5%. The next stage was aimed at investigating the effects of Phytostim® biostimulant on postharvest quality attributes and nutritional components. From the results of this investigation, it was reported that Phytostim® biostimulant enhanced the postharvest quality attributes, meaning that the shelf life of the studied crop escalated the retailers shelf life of three days and nutritional components were also enhanced. It was clearly concluded that the attributes were improved at 2.5% of Phytostim® biostimulant. A further investigation was carried out to establish if Phytostim® biostimulant also impacted the secondary metabolites of the studied crop. It was observed that Phytostim® biostimulant increased the metabolites at 3%.

6.2 Significance

The information documented in this study will be used in the future to improve the production of underutilized crops like amaranth using the organic or sustainable agriculture practices. The information will also be shared with small- scale farmers and growers of underutilized crop to improve the yield without compromising the nutritional components and secondary metabolites.

6.3 Recommendations

It is recommended to use Phytostim® biostimulant as an alternative to synthetic fertilizer to improve the growth, yield, postharvest attributes, nutritional components, and secondary metabolites. Since it eco-friendly and mitigate the issue of climate change. Moreover, it is recommended to be used with less than 3% dose because according to the results reported in this study it produced good yield. Furthermore, the effect of biostimulant application varied form one plant to the next and from genotypes of the same species. This raises the need for more research focusing on the effects Phytostim® biostimulant on genotypes of *Amaranthus species* and other leafy vegetables. More future studies on the needed to assess antioxidant activity of *Amaranthus species*, in different soils and climatic conditions.

6.4 Conclusion

In conclusion, the current study findings authenticated the potential use of biostimulants (Phytostim®) on plant growth and yield, and nutritional components. The current study further revealed that the efficacy of biostimulants on concentrations dependent. However, a more in-depth assessment of the efficacy of biostimulants on growth, yield, postharvest attributes, nutritional components, and secondary metabolites is encouraged in order to establish methods and doses that will enhance the effect of biostimulants on these parameters. The current study provided insights on the effect of Phystostim® biostimulants on different plant growth, yield, postharvest attributes, nutritional components and secondary metabolites.