THE EFFECT OF ORGANIC BIOSTIMULANTS AND THE MODE OF APPLICATION ON THE GROWTH AND BIOCHEMICAL COMPOSITION OF *AMARANTHUS HYBRIDUS* L.

By

NELSON NGOROYEMOTO

Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg, South Africa

September 2020

TABLE OF CONTENTS

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Student Number: **216076243**

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science, Higher Degrees Office for examination by the University appointed Examiners.

SUPERVISOR:

PROFESSOR J. VAN STADEN

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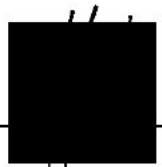


PROFESSOR J.F. FINNIE

CO-SUPERVISOR:

DR M.G. KULKARNI

PUBLICATIONS FROM THIS THESIS

1. **Ngoroyemoto, N.,** Gupta, S., Kulkarni, M.G., Finnie, J.F. and Van Staden, J., 2019. Effect of organic biostimulants on the growth and biochemical composition of *Amaranthus hybridus* L. South African Journal of Botany 124, 87-93.

2. Gupta, S., Hrdlička, J., **Ngoroyemoto, N.,** Nemahunguni, N.K., Gucký, T., Novák, O., Kulkarni, M.G., Doležal, K. and Van Staden, J., 2019. Preparation and standardisation of smoke-water for seed germination and plant growth stimulation. Journal of Plant Growth Regulation 39, 338-345.

3. **Ngoroyemoto, N.,** Gupta, S., Stirk, W.A., Kulkarni, M.G., Finnie, J.F. and Van Staden, J., 2020. Interactions between Microorganisms and a Seaweed-Derived Biostimulant on the Growth and Biochemical Composition of *Amaranthus hybridus* L. Natural Product Communications 15, 1-11.

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

1. **N. Ngoroyemoto**, M.G. Kulkarni, J.F. Finnie, J. Van Staden. A preliminary survey of commonly-consumed traditional fruits and vegetables in four informal/rural settlements in KwaZulu-Natal. South African Association of Botanists (SAAB), 43rd Annual Conference, 9-13 January 2017, Cape Town, Lagoon Beach Hotel. Oral Presentation.

2. **N. Ngoroyemoto**, M.G. Kulkarni, J.F. Finnie, J. Van Staden. Effect of organic biostimulants on the growth and biochemical composition of *Amaranthus hybridus* L. South African Association of Botanists (SAAB), 45th Annual Conference, 8-11 January 2019, University of Johannesburg. Oral Presentation.

3. **N. Ngoroyemoto**, M.G. Kulkarni, S. Gupta, J.F. Finnie, J. Van Staden. The effects of microbes and biostimulants on the growth and biochemical composition of the leafy vegetable *Amaranthus hybridus* L. South African Association of Botanists (SAAB), 46th Annual Conference, 7-10 January 2020, University of Free State, Qwa-Qwa. Oral Presentation.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing for each publication).

Publication 1: Contributions: Experimental work and draft manuscript and literature search were done by NN. MGK and SG assisted with the experimental design. JFF and JVS supervised the whole study and edited the manuscript before submission.

Publication 2: Contributions: Experimental work was done by NN and NKN. MGK, TG and ON assisted with experimental design and draft manuscript was done by SG and JH. MGK and JVS supervised the whole study and edited the manuscript before submission.

Publication 3: Contributions: Experimental work, literature search and draft manuscript were done by NN. SG, MGK and WAS assisted with the experimental design. JFF and JVS supervised the whole study and edited the manuscript before submission.

Authors' abbreviations:

NN	Nelson Ngoroyemoto
MGK	Manoj G. Kulkarni
SG	Shubhpriya Gupta
WAS	Wendy A. Stirk
NKN	Nkhanedzeni K. Nemahunguni
HJ	Jakub Hrdlička
TG	Tomáš Gucký
ON	Ondřej Novák
JFF	Jeffrey F. Finnie
JVS	Johannes Van Staden

Signed…… 

ACKNOWLEDGEMENTS

I would like to express my very sincere appreciation and gratitude to:

- My supervisor, Professor J. Van Staden, for his continuous encouragement and moral support toward the completion of this study.
- My co-supervisor, Professor J.F. Finnie for his patience, unwavering support, words of inspiration and tireless constructive criticism in the writing of the current thesis.
- Drs M.G. Kulkarni and S. Gupta for their advice, valuable assistance and suggestions during experimental work, proof-reading and critical comments during the write-up of this thesis.
- Researchers at the Laboratory of Growth Regulators, Institute of Experimental Botany, and Olomouc, Czech Republic team for assistance with analysing plant samples from some parts of my experimental work.
- The National Research Foundation (NRF) and the University of KwaZulu-Natal for scholarships and financial assistance.
- All the Staff and Students at the Research Centre for Plant Growth and Development, particularly Mrs L.A. Warren, for her priceless assistance, patience, moral support as well as her valuable administrative work.
- Mrs A. Young, Mr G. Carelse and all other Staff members of the UKZN Botanical Garden for their assistance during the greenhouse experiments.
- My family, especially my kids , Celeste, Alcides and Ariel who had to make do without the presence of their dad at home and my ever-loving wife Scholastica for her unwavering support and prayers for the completion of this programme.
- I also dedicate this work to my late mom and aunt, how I wish they could be around to celebrate with me, May Their Souls Rest in Eternal Peace.
- To all colleagues and friends from Lab 102 for quality time shared.
- Daniel said “Let the name of God be blessed forever and ever, for wisdom and power belong to Him” Daniel 2:20. Amen!!

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AGR	Absolute growth rate
AMF	Arbuscular mycorrhiza fungi
ANOVA	Analysis of variance
Al	Aluminium
ASC	Ascorbic acid
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BL	*Bacillus licheniformis*
Ca	Calcium
CC	Cyanidin chloride
CCE	Cyanidin chloride equivalent
Cu	Copper
DPPH	2, 2-diphenyl-1-picryhydrazyl
DW	Dry weight
EBIC	European Biostimulants Industry Council
EC	Electroconductivity
ECK	Eckol
EDTA	Ethylene diamine tetraacetic acid
FAO	Food and Agriculture Organisation
Fe	Iron
FRAP	Ferric cyanide reducing antioxidant power
GA	Gibberellic acid
GAE	Gallic acid equivalents

H_2O	Water
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HNO_3	Nitric acid
HNS	Hoagland’s nutrient solution
HS	Humic substances
K	Potassium
KAR_1	Karrikinolide
KEL	Kelpak®
MeOH	Methanol
Mg	Magnesium
Mn	Manganese
N	Nitrogen
Na	Sodium
NBT	Nitroblue tetrazolium
ORR	Oxygen reduction reaction
P	Phosphorus
PBS	Phosphate-buffered saline
PBs	Plant biostimulants
PF	*Pseudomonas fluorescens*
PGPF	Plant growth promoting fungi
PGPRs	Plant growth promoting rhizobacterias
PGR	Plant growth regulators
PH	Protein hydrolysates
RCPGD	Research Centre for Plant Growth and Development
RGR	Relative growth rate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSA	Radical scavenging activity
SAAB	South African Association of Botanists

SE	Standard error
SOD	Superoxide dismutase
SW	Smoke-water
SWE	Seaweed extracts
TFC	Total flavonoid content
TLVs	Traditional leafy vegetables
TPC	Total phenolic content
UKZN	University of KwaZulu-Natal
VCL	Vermicompost leachate
WHO	World Health Organisation
Zn	Zinc

ABSTRACT

Amaranthus hybridus L. (Amaranthaceae family) commonly known as Pigweed, Imbuya, Vowa, Umfino or Isheke, is a species originating from Central America and Mexico which has become a delicacy as a vegetable in many countries in southern Africa including South Africa. Even though the nutritional and nutraceutical properties of *A. hybridus* have been documented and that it has been consumed for many decades by the locals in South Africa, the plant is not usually planted. It occurs as a volunteer crop after first rains and is often harvested from the wild, therefore its production levels are not known. There is limited information on the cultivation of *A. hybridus* or its improvement as compared to the commercial crops like spinach, rice, wheat, etc. For this plant to be introduced in commercial agriculture and developed as a domesticated crop, it is pertinent to find ways of improving the crop in terms of quality and extent of production. To this end, the current study first evaluated the optimal growth conditions for *A. hybridus*. The effect of nitrogen (N), phosphorus (P) and potassium deficiency, nutrient strength using Hoagland's nutrient solution (HNS), watering frequency, and light intensity on growth of the crop were investigated. A further experiment was carried out to investigate the effect of the absence of N and substituting N with organic biostimulants, Smoke-water (SW) 1:500 v/v, Karrikinolide (KAR_1) 10^{-6} M, Vermicompost leachate (VCL) 1:5 v/v, Kelpak® (KEL) 10^{-8} M and Eckol (ECK) 10^{-8} M, on the growth of *A. hybridus*. The second stage of the research was to evaluate the effect of organic biostimulants (SW, KAR_1, VCL, KEL and ECK) on the growth and biochemical composition of *A. hybridus*, with water (H_2O) and gibberellic acid (GA) being used as the negative and positive controls respectively. The third stage of the research was to investigate the effect of the interaction of microorganisms, *Bacillus licheniformis* (BL) and *Pseudomonas fluorescens* (PF) and a biostimulant (KEL) on the growth and chemical composition of *A. hybridus*. The final stage was to investigate the effect of organic biostimulants on the growth of two other amaranth species, *Amaranthus caudatus* and *Amaranthus retroflexus*.

In terms of the general requirements for the successful establishment of *A. hybridus*, it was observed that nitrogen, phosphorus and potassium (NPK) play a very critical role. The plant can grow to some extent in the presence of small amounts of P and K but cannot survive in the absence of N. In terms of nutrient strength, the crop achieved

significant growth at 50% HNS to indicate the plant grows well with the availability of nutrients. With regard to the experiment on water requirements, it was concluded that watering frequency has a strong influence on the growth of *A. hybridus* as most vegetative growth of the plant was achieved when water was applied to the plant three times per week. On the effect of light intensity on growth and chemical composition of *A. hybridus*, it was concluded from the results of the experiment that the crop is strongly light- dependent since the most significant growth was achieved at the highest light intensity of 600 µmol m^{-2} s^{-1}. This could be due to the fact that large amounts of photosynthetic products are synthesised at high light intensity. This explains the increased growth of the crop.

A. hybridus could not grow in the absence of N and in another investigation to try to replace the lack of N in the soil with biostimulants, the plant was only able to grow with KEL supplementing for N, i.e. in –N (HNS) + KEL. The conclusion from the experiment was that KEL can be used as a substitute for N when growing *A. hybridus* in a soil where the element is lacking.

The treatment of *A. hybridus* with 50% HNS + biostimulants (SW, KAR_1, VCL, KEL and ECK) at different irrigation frequencies (once, twice and thrice) resulted in significant effects when the plant was irrigated more than once a week. When the application was done twice a week, 50% HNS + SW had a significant effect on AGR and RGR (height) and RGR (leaf number) whilst 50% + KAR_1 had a significant effect on both AGR and RGR (height) and RGR (leaf number). Application of treatments thrice a week resulted in KAR_1 having a significant effect on both AGR and RGR for height and number of leaves. 50% HNS + KAR_1 is the best treatment that can be applied twice or thrice a week to increase the AGR and RGR for both the height and leaf number since there is bound to be more branching and more leaf formation as the plant grows taller. This increases the number of tender leaves being formed as they are suitable for human consumption as green vegetables.

In the investigation of the effect of organic biostimulants and mode of application on growth and biochemical composition of *A. hybridus*, KAR_1 significantly improved most growth parameters of the plant when applied via soil drenching. VCL was observed to enhance growth in *A. hybridus* as a foliar spray as it had a significant effect on most growth parameters of the crop. The same could be said of KEL when the biostimulants

were applied via a combination of soil drench and foliar spray. In terms of the biochemical composition of *A. hybridus*, KAR_1, VCL and KEL significantly influenced the biochemical composition when applied as a foliar spray. KAR_1 and VCL enhanced the protein content in the plant while KEL significantly increased the protein content and photosynthetic pigment content of *A. hybridus*. In terms of the mineral composition of *A. hybridus*, KEL, ECK and VCL had notable effects on nutrient levels of the crop, with KEL significantly improving N, Ca, Mg, K, Na, Zn, Cu and P levels. It was concluded that KEL is the ideal biostimulant for enhancing the mineral content of *A. hybridus*. This biostimulant boosts levels of Zn which is normally lacking in the diet and this is important for nutrition security.

In the experiment to investigate the effect of organic biostimulants and mode of application on the antioxidant activity and phytochemical composition of *A. hybridus,* it was concluded from the results that, generally, the treatments and mode of application had little or no influence at all. SW applied via drenching was the only treatment which significantly enhanced the amount of condensed tannins in the green leafy vegetable.

In the experiment to investigate the effect of the interaction of microbes and biostimulants on the growth and biochemical composition of *A. hybridus*, it was observed that microbes alone had no effect. Microbes were only effective in combination with KEL. KEL improved the growth and photosynthetic pigment content and PF generally enhanced the mineral content of *A. hybridus*. It can be concluded that microorganisms and KEL could work in a complementary or interactive manner to improve growth, biochemical composition and mineral content of *A. hybridus*.

The other two species of amaranth, *A. caudatus* and *A. retroflexus* responded in a similar manner to *A. hybridus* in terms of general growth when treated with organic biostimulants using different application methods. They could also be harnessed to increase amaranth vegetable production.

CHAPTER 1: General Introduction

1.1 Introduction

An estimated 9 billion people are expected to inhabit planet Earth midway through this century **(ABRAHAM et al., 2014; KHOURY et al., 2014)**, feeding this huge population is a major global challenge. This has been aggravated by climate change, which is often linked with increases in both biotic and abiotic stresses, culminating in crop failure in many affected regions **(ALEMAYEHU et al., 2015; CHENG et al., 2017)**. Many countries, particularly those in moist, hot, dry and arid regions of the world, face the brunt of climate change **(ABRAHAM et al., 2014; GORNALL et al., 2010; MASSAWE et al., 2015)**. Besides climate change, other factors limiting crop production include natural resources which have been abused, increased soil erosion and accelerated land degradation **(ARAUS et al., 2008; ASHRAF et al., 2016; CHENG et al., 2017; DELGADO et al., 2011)**. Statistical results show that nearly 795 million humans do not have sufficient food **(BURCHI et al., 2011; OGUNTOYINBO et al., 2016)**, with an estimated 2 billion people worldwide suffering from "hidden hunger" i.e. micronutrient deficiencies **(BURCHI et al., 2011)**. The majority of those affected are found on the African continent (with the highest occurrence of "undernutrition" in sub-Saharan Africa) and the Indian subcontinent **(MUTHAYYA et al., 2013; OGUNTOYINBO et al., 2016)**. There is a need to increase food production to meet the demands of the growing world population and address climate change challenges **(ALEMAYEHU et al., 2015; MASSAWE et al., 2015)**. The provision of a diversity of food sources and agricultural systems remain the only critical premise for assuring future food security **(FAO, 2015; MASSAWE et al., 2015)**. There has been a huge loss of genetic diversity and this also retards crop improvements and negatively affects sustainable agriculture **(FU, 2015)**. About 2.7 million people worldwide die every year due to insufficient consumption of vegetables and fruits **(KEDING, 2010)** and this is one of the top 10 mortality risk factors **(EZZATI et al., 2002)**. These deaths are more common in low and middle income countries **(KEDING, 2010)**. A solution to this current epidemiological scenario calls for foods rich in nutrients which are easy to obtain **(MAURYA and ARYA, 2018)**. Rampant micronutrient deficiencies, especially vitamin A, iron, iodine, magnesium, selenium and zinc, are responsible for diseases

that include cardiovascular diseases, cancer, chronic respiratory diseases and diabetes **(KEDING, 2010)**, all of which are on the increase according to the **World Health Organisation (2007)**. There is also a high incidence of non-communicable diseases globally, such as stunted growth, goitre, blindness, kwashiorkor and marasmus, due to the preference for simple diets with high energy content but low in micronutrients **(MEDOUA and OLDEWAGE-THERON, 2014; NNAMANI et al., 2015)**. The International Food Policy Research Institute (IFPRI) predicted an 18% rise in the number of children suffering from malnutrition in sub-Saharan Africa by the year 2020 **(ROSEGRANT et al., 2001)**, with many countries facing difficulties in solving under-nutrition and deficiencies of micronutrients **(LOPRIORE and MUEHLHOFF, 2003)**. To ensure a healthy and high-quality diet, it is imperative to consume the right qualities of a wide range of food categories **(SCHREINEMACHERS et al., 2018)**. There is a need to promote the increased consumption of fruits and vegetables, since they are known to contain micronutrients which are beneficial for health. Many contain other non-nutrient phytochemicals linked to health maintenance and prevention of chronic diseases **(STEINMETZ and POTTER, 1996; UUSIKU et al., 2010)**. This makes fruits and vegetables essential sources of micronutrients required for healthy diets **(BALDERMANN et al., 2016; SCHREINEMACHERS et al., 2018)**. Vegetables contain K, which aids, in the maintenance of a healthy blood pressure. Cholesterol levels are controlled by fibre content of the vegetables thereby lowering the risk of heart diseases. The risks of birth defect are reduced by folate (folic acid), whilst skin and eye health is maintained by vitamin A. The health of teeth and gums and the absorption of iron is partially the responsibility of vitamin C **(NESAMVUNI et al., 2001; SCHREINEMACHERS et al., 2018; YANG and KEDING, 2009)**. In this era of the AIDs pandemic, micronutrient and antioxidant rich diets are now highly recommended to complement HIV/AIDS therapies **(ELISHA et al., 2016; YANG and KEDING, 2009)**. The WHO recommends a minimum intake of 400 g per day of fruits and vegetables to minimise the risks of chronic diseases **(SMITH and EYZAGUIRRE, 2007)**.

From a total of 10 000 edible plant species used by mankind, only a paltry 150 species are commercialised on a global scale. Only 12 provide 80 percent of dietary energy which is derived from plants. Only four species provide approximately 60 percent of the global protein requirement. Many plant species and varieties are classified as underutilised or neglected crops because of their marginalisation by both agriculture

and nutrition researchers **(VENSKUTONIS and KRAUJALIS, 2013)**. One way of achieving sustainable agriculture is by unlocking the genetic potential of these underutilised crops **(CHENG et al., 2017)**. Of concern is the regular and constant improvement and transformation of highly productive crops, mainly exotic crops, with little or nothing being done on the numerous underutilised traditional crops (also known as "neglected or orphan plants") **(DANSI et al., 2012; JACOBSEN et al., 2013)**. Therefore, it is imperative to value neglected and underutilised crops by placing them at the core of scientific research so that they can also be improved and transformed into highly productive crops to feed and meet the demands of humankind **(DOEBLEY et al., 2006)**. Some of these crops demonstrate notable tolerance to different types of stress factors which include drought and heat **(CHENG et al., 2017; NNAMANI et al., 2015)**. The value of these underutilised and neglected crops goes far beyond being climate-resilient as many of them are packed with superior nutritional and nutraceutical attributes **(CHENG et al., 2017; MOYO et al., 2013; RASTOGI and SHUKLA, 2013)**. Diversification of crops and consumption patterns to include underutilised traditional leafy vegetables is one of the most sustainable ways to reduce and control micronutrient deficiency disorders **(NNAMANI et al., 2015; SALVI and KATEWA, 2016)**. Neglected and underutilised crops have the potential to improve food and nutrition security, increasing agricultural diversification and reducing environmental land degradation **(ALEMAYEHU et al., 2015; FOMSGAARD et al., 2011; MASSAWE et al., 2016)**.

1.2 Rationale for the research

One of the recommended strategies for climate change mitigation and adaptation is the incorporation of drought and heat stress tolerant crops into agriculture so as to establish different cropping systems **(DELGADO et al., 2011; MORTON, 2007; SCHREINEMACHERS et al., 2018)**. KwaZulu-Natal is rich in traditional leafy vegetables (TLVs), which are diverse, have short growing cycles and use irrigation water efficiently, thereby reducing a farmer's vulnerability to climate change effects **(SCHREINEMACHERS et al., 2018)**. Despite the importance and high nutritional value of TLVs in household food security in rural and peri-urban KwaZulu-Natal **(MODI et al., 2006)**, there is no information about the improvement and production potential

of TLVs in the province **(LEWU and MAVENGAHAMA, 2010)**. There is an urgent need to carry out more empirical investigative research on improving the productivity, nutritional value, domestication and promotion of TLVs for their adoption and cultivation in mainstream commercial agriculture. This could go a long way in alleviating nutritional deficiencies among rural and peri-urban communities in KwaZulu-Natal **(DOVIE et al., 2007; MHLONTLO et al., 2007)**. A number of wild plant species are utilised as vegetables in KwaZulu-Natal by rural and peri-urban communities. According to **ODHAV et al. (2007)**, 12 out of 20 wild vegetable species have a mineral concentration above one percent of plant dry weight and this is much higher than that in exotic vegetables like cabbage and spinach. Among such wild traditional vegetables of superior nutritional content are the amaranths, which are one of the most popular TLVs which have been recommended for cultivation **(ODHAV et al., 2007)**. Exploitation of local biodiversity with regards to vegetables is a practical and sustainable way of addressing the burden of malnutrition in rural and peri-urban communities **(NEUGART et al., 2017)**. Studies have shown that TLVs have high energy and protein contents, are good sources of fat and also have a high fibre content. They also have high amounts of micronutrients such as zinc. There is minimal cultivation of these plants despite their vital importance, as more attention is being directed towards exotic species **(ODHAV et al., 2007)**. TLVs are suitable for growing in areas with unfavourable weather conditions, hence the need for them to be explored, developed and exploited more. TLVs could be a solution to the critical state of food security and for meeting the nutritional and medicinal demands of an ever increasing human population **(CHENG et al., 2017)**.

Amaranths are an underutilised crop and are a cheap source of proteins, minerals, vitamins A and C, making them a promising crop for the future. These plants are currently in demand due to a change in consumer demands which now favours organically grown crops which are more nutritionally balanced **(ALEMAYEHU et al., 2015)**. The plant is normally harvested from the wild as a weed on a seasonal basis, since it only grows in summer **(TALENI and GODUKA, 2013)**.

The amaranth plant has unique nutrient composition, medicinal qualities **(LAKSHMI and VIMALA, 2000; SHUKLA et al., 2006)** and a high tolerance of drought and other stress factors, including diseases **(BARRIO and AÑÓN, 2010)**. This makes it an ideal candidate for climate change mitigation strategies and extended use in marginal

agricultural land to improve agricultural systems in rural and semi-urban areas of KwaZulu-Natal. *Amaranthus* can grow on different soils and under different agro-climatic conditions **(KATIYAR et al., 2000; SUDHIR and SINGH, 2000)**. All these positive attributes substantiate the use of amaranth as a vegetable which could serve as a cheap, alternative source of protein and other nutrients for rural and peri-urban people in KwaZulu-Natal. *Amaranthus* is now perceived as a promising food crop due to its resistance to stresses like heat, drought, diseases and pests in addition to the high nutritional value of both its leaves and seeds **(WU et al., 2000)**. Improvement of *Amaranthus* through research and development could go a long way in malnutrition elimination, health promotion and ensuring food security **(ACHIGAN-DAKO et al., 2014)** for subsistence and small scale farmers in of KwaZulu-Natal and other provinces of South Africa. The main focus of this research was to investigate the effects of organic biostimulants on the growth of *Amaranthus hybridus* and to evaluate other methods of enhancing its growth and productivity.

1.3 Research aims and objectives

- To develop strategies to improve the nutritional value of *A. hybridus* through the use of organic biostimulants in terms of growth rate, leaf size, stem length and diameter;
- To assess the effects of biostimulants on *A. hybridus* seed germination, (germination rate, seedling length and seedling weight);
- To calculate absolute growth and relative growth rate of *A. hybridus*;
- To evaluate the effects of organic biostimulants on the biochemical composition of *A. hybridus* (protein content, chlorophyll content, carotenoids and total carbohydrate content);
- To evaluate the effects of organic biostimulants on pharmacological properties of the traditional leafy vegetable (*A. hybridus*) i.e. antioxidants, total phenolic content, vitamins and tannins;
- To assess the effect of mode of application of biostimulants on the growth of *A. hybridus* (drenching, foliar application and drenching/foliar application);
- To investigate the effect of nutrient strength, (NPK) and –N + organic biostimulants on *A. hybridus* growth and,

- To assess both the effects of organic biostimulants and mode of application on two other species of *Amaranthus*; *A. caudatus* and *A. retroflexus*.

CHAPTER 2: Literature Review

2.1 Background

Since the shift of humans from hunting and gathering some many years ago and the introduction of the Green Revolution, agriculture has become the mainstay of food supply systems for mankind **(HUESTON and MCLEOD, 2012)** and has been mainly responsible for emerging civilisations and their increasing populations **(MABHAUDHI et al., 2018a)**. Agriculture has evolved over time with humans gaining more knowledge and coming up with inventions, with the Industrial Revolution in Europe being one of the most notable events, which saw an advancement of farming practices and inventions of new farming technologies **(HUESTON and MCLEOD, 2012)**. These successful scientific advancements, because of demand shaped by social and economic forces, caused a shrinkage of the list of crops feeding the world **(HUESTON and MCLEOD, 2012),** especially as a result of the Green Revolution. Though successful in most industrialised countries (such as parts of Asia, North America and many parts of Europe), the Green Revolution was less successful in most parts of Latin America and a large chunk of Asia and Africa, which are still languishing with chronic hunger **(PINGALI, 2012)**. Noteworthy products of the Green Revolution are high-yielding crop varieties with a greater dependency on high fertilizer and water inputs **(GOLLIN and EVENSON, 2003)**. The Green Revolution, though successful with regards to increased production, also gave rise to new challenges for mankind such as environmental degradation and pollution **(KERR, 2012; PINGALI, 2012)** and biodiversity loss as more forests had to be cleared to expand agricultural land **(DUDLEY and ALEXANDER, 2017; KERR, 2012)**. This resulted in an increase in malnutrition due to a lack of diversity in the diet **(GÓMEZ et al., 2013)**, as it brought homogeneity to global food supplies, with only three crops being cultivated to supply the world's food energy requirements. Only a paltry 20 plant species comprise 90% of the world's calories, making human diets around the world very similar **(KHOURY et al., 2014; MASSAWE et al., 2016)** with the "big three cereals", maize (*Zea mays*), wheat (*Triticum aestivum)* and rice (*Oryza sativum*), dominating the diet **(ABRAHAM et al., 2014; KHOURY et al., 2014)**. Most other crops are losing ground both

agriculturally and commercially **(ABRAHAM et al., 2014)**. This is a very sad scenario since some of these crops favoured for intensive agriculture may not be the most suitable species to grow in marginal areas and may also not be able to cope with the current prevailing adverse weather conditions in many parts of the world caused by climate change **(MIJATOVIĆ et al., 2013)**. Currently dominating the global Sustainable Development Agenda is the failure of the global food system to provide the basic food needs of the citizens of the world in an equitable manner **(MABHAUDHI et al., 2018a; MABHAUDHI et al., 2018b)**. The lack of diversity in modern food supply systems makes them more vulnerable to shocks of both an economic and climatic nature due to a lack of a buffering effect and non-resilience against these risks **(GREENBERG, 2017)** and exposure to pests and diseases **(PINGALI, 2012)**. The post-colonial replacement and eventual relegation of underutilised and traditional crops by the introduction of high yielding but input intensive exotic crops, has to some extent contributed to the limited success of the global food supply system **(SEBURANGA, 2013; SHELEF et al., 2017)**. These crops, which used to form the basis of local food systems, are now in neglect, especially in sub-Saharan Africa, including South Africa **(MABHAUDHI et al., 2018a)**. It is evident that conventional agriculture is encountering very serious limitations in the form of a decline in natural resources and environmental damage inflicted by current agricultural practices **(LE MIRE et al., 2016)**. Urbanization and multinational food industries are further aggravating the situation since the majority of humans now consume processed and fast foods **(CORDAIN et al., 2005; SANZ-CAÑADA AND MUCHNIK, 2016; TILMAN and CLARK, 2014)**. This exacerbates the rise of excessive calorie intake and poor nutrition induced non-communicable diseases such as diabetes, strokes, heart attack, and some types of cancer **(DWIVEDI et al., 2017; GREENBERG, 2017; SANZ-CAÑADA AND MUCHNIK, 2016)**. Another risk posed by the current food production systems is that any slight “glitch” would have far-reaching consequences like serious famine and even civil unrest, particularly in developing countries **(DUDLEY and ALEXANDER, 2017)**. Increased crop diversification can go a long way to alleviating such global problems of hunger and civil unrest **(BUA and ONANG, 2017)**. The cultivation of a wide variety of crops is not only a strategy to ensure sustained global food supplies but a tool for fighting famine, hidden hunger and “over nutrition” **(MASSAWE et al., 2016; SANZ-CAÑADA AND MUCHNIK, 2016)**. The main challenges facing agriculture today are; the high demand for food which is safe, rich

in nutrients and healthy and also the obligation to safeguard biodiversity and other natural resources in addition to the challenges being caused by climate change **(DWIVEDI et al., 2017)**. There are mounting calls of late for diets which are diverse and healthy and are generally plant-based, to curb or minimise illnesses which are linked to poor diet **(DWIVEDI et al., 2017)**. A diet is considered to be healthy and sustainable when it provides all essential nutrients, minerals and vitamins with minimal impact on the environment **(PERIGNON et al., 2017)**. Out of the approximately 50 000 edible plant species, only a mere 300 species are consumed **(JACQUES and JACQUES, 2012)**. This global food consumption pattern is worrisome and authorities implore humans to utilise a wide range of food sources so as to fight against the ever growing global challenge of malnutrition and food insecurity **(MASSAWE et al., 2016)**. The world should consume more of the so called underutilised or orphan crops (also classified as 'minor crops') **(MASSAWE et al., 2016; MASSAWE et al., 2015)**. These so called underutilised crop plants possess resilient traits and are capable of resisting stresses such as drought, flooding, extreme temperatures, as well as pests and diseases unlike the major staples **(MAYES et al., 2011)**. The incorporation of such crops into global food systems would go a long way towards addressing climate change challenges, in addition to tackling the problem of malnutrition facing the world at the moment **(CHENG et al., 2017)**.

It is only prudent that food systems and diets be diversified since it improves human health, besides having other multiple benefits such as healthy ecosystems. Much has been documented about the numerous benefits derived from biodiversity, such as how critical it is for the well-being of humans, and how adopting a food-based dietary diversity strategy has social, cultural economic and environmental benefits **(DWIVEDI et al., 2017)**. There is a need for researchers to come up with innovative methods to produce more food and improve its nutritional quality but at the same time reduce the negative impacts of agriculture on the environment.

2.1.1 Agroecology

According to **LE MIRE et al. (2016),** agroecology involves the application of ecological principles to agricultural systems in the context of sustainable production. The aim is to optimize both the economic and environmental performances of beneficial

ecosystem services so as to enhance productivity and resilience of the cultivated ecosystems. This also goes a long way in the preservation of natural ecosystems.

Performance maintenance can only be achieved through the development of new technologies that increase tolerance of plants to various abiotic and biotic stresses. Agroecology offers an important scientific strategy which takes cognisance of concerns of society concerning agriculture, the economy and particularly the environment **(LE MIRE et al., 2016)**. The main aim of agroecology is making use of ecological principles to study and design agricultural systems reliant on interactions of their biophysical, technical and socioeconomic components. To this end, a lot of research is focussing on agroecological principles to reduce chemical inputs which can be harmful, and managing ecological relationships and agrobiodiversity **(LE MIRE et al., 2016)**. A lot of technological tools have been developed in the past decades which promote sustainable agroecosystems. One such innovation is the use of biostimulant products as substitutes for the use of chemical fertilisers **(LE MIRE et al., 2016)**. This also saves energy and provide farmers with new opportunities for sustainable fertilization and control of pests and diseases **(CALVO et al., 2014; MEJÍA-TENIENTE et al., 2010)**.

2.1.2 Plant biostimulants (PBs)

The world is faced with a very serious dilemma of the ever-rising human population which has in turn imposed pressure on agricultural land. This has led to an increased demand for production on a unit area and has also resulted in a shift of cultivation to extreme marginal areas and types of soils. High yields could be achieved by farmers when they also use high nutrient fertilisers but the heavy chemical fertiliser requirements have also brought about costs for the farmers. One of the most important restrictions of agricultural production worldwide is the immoderate use of fertilisers under non-biological stress conditions **(JEWELL et al., 2010)**. The situation is being further aggravated by climate change and global warming. These have worsened the occurrence and severity of many stresses of a non-biological nature, such as high temperatures and drought, and have caused notable yield reductions in food crops. As scientists are looking for more advances to produce more crops for food to feed the ever-rising human population and benefits of harvesting from the agricultural economy, the agro-ecosystem and socio-economic challenges get more severe

(ZHANG et al., 2018). Unpredictable climate, shrinking of agricultural lands, depleting of natural resources, poor soil nutrition and reduced crop responses to agrochemicals are the major problems now being faced by farmers. This has caused an increase in concerns for agricultural sustainability and improving the health of plants.

Feeding an ever increasing world population in a proper manner without upsetting the delicate balance of nature is the major challenge of agriculture. There is a need to achieve the second goal of the United Nations, the Sustainable Development goal of "ending hunger, achieving food security and improved nutrition and promoting sustainable agriculture". Climate change and global warming effects will result in more environmental stress being imposed on crop plants globally **(PACHAURI et al., 2014)**. Rising seas, soil erosion, salinization and desertification will consume a large chunk of high quality agricultural lands as climate change progresses into the 21st century. This calls for the need to maintain crop yields against a backdrop of reduced agriculturally productive land and more adverse climatic conditions since agricultural production has to be sustained so as to meet consumer demands. This can only be attained through the efficient utilization of the available resources for making and providing products which are healthy **(COLLA and ROUPHAEL, 2015)**.

The current scenario of climate change, natural resource depletion and increase in hunger and malnutrition, calls for sustainable agricultural production and utilisation of resources **(SHUBHA et al., 2017)**. Today's agriculture is faced with a plethora of challenges like a decline of natural resources and damage inflicted on the environment by current agricultural practices **(LE MIRE et al., 2016)**, hence the need for the adoption of scientific approaches which are sustainable. Food is one of the basic needs of mankind and is crucial in health and development of human societies, so there are undesirable and disastrous outcomes caused by the extensive increases in both environmental degradation and continued population increase as it is inevitable that the current food supplies will not be able to meet world food demand in the long run **(GAVELIENĖ et al., 2018)**. Scientists are looking for food production strategies which are sustainable and environmentally friendly because of the increased demand for better yields and quality of food crops **(XU and GEELEN, 2018)** as prompted by the ever increasing world population. An estimated 9.7 billion people are expected to inhabit planet Earth by 2050 and this compels modern agriculture to become more efficient in producing more food in an eco-friendly and sustainable manner

(ROUPHAEL et al., 2018). Food is one of the basic needs and it plays a key role in health and development of any society **(ETESAMI and MAHESHWARI, 2018)**. A lot of research has been done to discover substances for use in crop production capable of improving growth of plant, productivity and quality as well as assisting plants to overcome stresses imposed by the environment **(PARAĐIKOVIĆ et al., 2019)**. One way of achieving this is the production of fertilisation reagents of biological origin to minimise the use of chemical fertilisers as fertiliser usage has been the most popular strategy for enhancing agricultural production. With degradation of agricultural land and uncertainty of climate change, biostimulants promise to be a most viable and sustainable option **(PARAĐIKOVIĆ et al., 2019)**.

Biostimulant is the name given to substances and microorganisms which act on plants' metabolic and enzyme processes, thereby increasing plant growth, nutrition efficiency, crop quality traits and environmental stress tolerance **(SHUBHA et al., 2017; WOO and PEPE, 2018; XU and GEELEN, 2018)**. The European Biostimulants Industry Council (EBIC) defines biostimulants as "substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere, is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop productivity **(DU JARDIN, 2015; XU and GEELEN, 2018)**. Besides anti-stress support, growth stimulation, increasing nutrient absorption and crop productivity enhancement, biostimulants also assist in breaking dormancy, increase the size of fruits, increase root development and photosynthetic and vegetative tissue activities. They also increase plant vigour and uniformity, control flowering and aid fruit setting and ripening. All these attributes of biostimulants result in crop improvement, growth, development and production **(BULGARI et al., 2019; PARAĐIKOVIĆ et al., 2019)**, hence the move for their inclusion in agricultural practices with the aim of reducing chemical inputs, enhancing productivity and reinstating the natural balance of agro-ecosystems **(WOO and PEPE, 2018)**. The mode of application of biostimulants depends on their composition and expected outcomes so they can be applied to the soil (drenching) or on leaf surfaces (foliar application) **(KUNICKI et al., 2010)**.

Plant biostimulants are normally incorporated into agricultural practices with the aim of reducing the use of chemical inputs, to increase production, and to re-establish the natural equilibrium in agro-ecosystems. The substances are also applied to enhance crop productivity and nutritional quality of agricultural foods. Biostimulant formulations

can be mixtures of different substances such as, humic and fulvic acids, protein hydrolysates, extracts of plants or seaweeds, silicon, chitosan, inorganic compounds, beneficial fungi (i.e. arbuscular mycorrhizal fungi; AMF and *Trichoderma* spp.) and plant growth-promoting bacteria **(CANELLAS et al., 2015; COLLA and ROUPHAEL, 2015; ROUPHAEL et al., 2015; YAKHIN et al., 2017)**. The term biostimulants is a bit ambiguous because not all elements mentioned in the description of biostimulant formulations are biological and "bio" prefix could be because of the living organism component and substances of biological origin. Non-organic components are also believed to stimulate biological processes, regulating the physiology of the plant metabolic processes instead, and the non-organic factors can be considered as positive effectors of the "biological" processes that regulate plant physiology, metabolism, morphology and relationships in the agro-ecosystem **(PARAĐIKOVIĆ et al., 2019; YAKHIN et al., 2017)**.

2.1.3 Biostimulants and vegetable production

Currently, consumers and scientists the world over prefer vegetables grown organically because of the growing awareness for healthy and safer foodstuffs **(DORAIS and ALSANIUS, 2015)** and this has seen global organic agriculture doubling since 2008. By 2014, a staggering 3.5 million hectares was under organic cultivation and organic farming was being practised in over 87 countries **(WILLER and LERNOUD, 2016)**. Reports on organic horticulture confirm that it is a system which is environmentally friendly and enables food production without being detrimental to the environment, resulting in conservation of both water and the environment with minimal chemical inputs **(DORAIS, 2007)**. The major limitation of this strategy is the reduced yield when compared to conventional agriculture **(DORAIS and ALSANIUS, 2015; SEUFERT et al., 2012)**, so more land is required to get the same amount of food as that produced by conventional agriculture. This encourages deforestation, as there is a need for more land so as to generate the same amount of food, and this compromises the benefits to the environment derived from organic farming **(TREWAVAS, 2001)**. Therefore a better approach would be the use of biostimulants, which seem to be a potential and environmentally friendly strategy to increase food production without compromising the environment **(ROUPHAEL et al., 2017b)**. According to greenhouse studies, nutrient uptake and assimilation are promoted in

those plants grown under biostimulant application **(COLLA et al., 2015a)**. Factors which are believed to be responsible for the increase of uptake of nutrients by plants are; an increase in soil enzymatic and microbial activities, changes/alteration of root architecture as well as an enhancement in micronutrient mobility and solubility **(COLLA et al., 2015b; ERTANI et al., 2009; LUCINI et al., 2015)**.

Biostimulants influence development in all the different phases of the crop's life cycle, starting from seed germination up to maturity. They improve the efficiency of the plant's metabolism to induce yield increases and enhanced crop quality **(COLLA et al., 2017a, CALVO et al., 2014)**. They are also known to enhance plant tolerance to both abiotic and biotic stress **(COLLA et al., 2017a, CALVO et al., 2014)**. Biostimulants enhance plant nutrient assimilation and also increase the quality of agro-products like sugar content, colour, fruiting, seeding, etc. They improve water use efficiency of plants and also foster the development of complimentary soil microorganisms which improves soil fertility **(COLLA et al., 2017a; ROUPHAEL et al., 2015**).

Application of biostimulants can be done directly onto the plant itself, to seeds, the soil or in any growing media capable of enhancing the ability of the plant to assimilate nutrients. Plant growth promotion resulting from better nutrient uptake caused by biostimulants of microbial origin are linked to several mechanisms such as supplying N through biological N_2 fixation **(COLLA et al., 2017a)**. They make more soil nutrients available for uptake by plants via the solubilisation of mineral phosphates and other nutrients through the production of small metal-binding molecules such as organic acids and siderophores and also through the release of specific enzymes like phosphatases **(COLLA et al., 2017a; CALVO et al., 2014)**. Plant access to soil nutrients is enhanced by the increase in volume of soil accessed by the root system **(HAYAT et al., 2010; ROUPHAEL et al., 2015)**.

Biostimulants are very different from crop inputs that have been traditionally used in terms of operation mechanisms as they are quite distinct from those of fertilisers. Also, biostimulants are very different from products used in crop protection, in that they only influence the plant vigour, with no direct action on either pests or diseases **(CALVO et al., 2014)**.

2.1.4 Classification of plant biostimulants (PBs)

Plant biostimulants (PBs) fall under different categories according to their influence and/or role in influencing plant growth. Plant biostimulants have been defined as a variety of substances and microorganisms which are administered to plants in order to increase their nutrition efficiency, abiotic stress tolerance and/or crop quality traits regardless of their nutrient content **(DU JARDIN, 2015)**.

Plant biostimulants can be used to enhance nutrient availability to those plants exposed to nutrient deficiency caused by lack of nutrients in the soil or poor solubility of the nutrients in the soil solution. Plant biostimulants can increase nutrient availability through increasing soil cation exchange capacity by providing N to crops and/or increasing the solubility of nutrients in soil **(DE PASCALE et al., 2017)**. Humic substances (HS) are obtained from the chemical and biological transformation of dead organic matter as well as microbial metabolism **(CANELLAS et al., 2015; DU JARDIN, 2015)**. HS are known to have a key role on physico-chemical parameters of soils and are responsible for root-growth stimulation and plant nutrition improvement because of soil nutrients **(CANELLAS et al., 2015; DU JARDIN, 2015)**. Humic substances make nutrients available for plants by increasing the cation exchange capacity and buffering (neutralise) soil pH **(CANELLAS et al., 2015; DU JARDIN, 2015)**. Humic substances also form complexes with micronutrients (this prevents leaching, thereby making micronutrients more available for plants) **(CHEN et al., 2004b; GARCÍA-GIL et al., 2004)**. Humic substances stimulate plasma membrane ATPase activity, thereby increasing H^+ extrusion from roots and this lowers the root surface pH, which triggers soil nutrient availability for improved uptake and translocation **(CANELLAS et al., 2015)**. It is also believed that HS could have notable effects on secondary metabolism and stress alleviation **(DE PASCALE et al., 2017)**. Humic substances have been shown to be effective in plant tolerance to salinity in tomato and okra according to **TÜRKMEN et al. (2004)** and **PAKSOY et al. (2010)** respectively.

The protein hydrolysates (PH) are another group of important PBs and are actually mixtures of polypeptides, oligopeptides and amino acids made from partial hydrolysis of protein sources **(SCHAAFSMA, 2009)**. According to **COLLA et al. (2017a)**, they are found in granular or powder form as well as liquid extracts and can be applied on

leaves as foliar sprays or dosed near the root system. Protein hydrolysates act on plants by improving soil respiration, microbial biomass and activity, so that microorganisms can easily use amino acids and peptides as a C and N source **(FARRELL et al., 2014)**. They also enhance plant nutrition by forming complexes and chelates between peptides/amino acids and soil micronutrients (Cu, Fe, Mn and Zn) making nutrients more available and easily acquired by the root system **(COLLA et al., 2015a; DU JARDIN, 2015)**. Protein hydrolysates are involved in chelate formation with macro- (K, Ca and Mg) and micronutrients (Cu, Fe, Mn and Zn) which are used by industries to develop fertilisers with high nutrient use efficiency **(DE PASCALE et al., 2017)**.

Plant growth promoting fungi (PGPF) like arbuscular mycorrhiza fungi (AMF) and *Trichoderma* spp. can promote plant growth by making nutrients like N, P, and Fe more available **(DORAIS, 2007)**. For this reason, different symbionts and non-symbionts are now being used in enhancing nutrient availability to improve plant productivity in both conventional and organic farming **(CALVO et al., 2014; HAYAT et al., 2010)**. The mechanism of how PGPF-mediated improvement to crop productivity still needs to be explained **(DEY et al., 2004)**. Many PGPF can produce plant hormones such as auxins, cytokinins, gibberellins, ethylene and abscisic acid **(HAYAT et al., 2010)**. This may explain their role in stimulating plant growth and development by stimulating root growth and nutrient and water uptake (auxins). Cytokinins promote mitotic cell division in both shoots and roots and also delay senescence. Gibberellins play an important role in fruit and flower formation and fruit improvement. They also break dormancy of vegetative organs and promote seed germination **(MÉTRAUX, 1987)**. Abscisic acid is responsible for plant adaptive responses to environmental stresses like drought, high salinity and plant development (seed maturation and dormancy) **(MIYAKAWA et al., 2013)**.

Many PGPF increase phosphate solubility in the soil, making phosphate readily available for uptake by plants **(CANBOLAT et al., 2006)**. Arbuscular mycorrhizal fungi (AMF) act in the same manner as PGPF by enhancing the availability of phosphorus **(ROUPHAEL et al., 2015)**.

There are several factors at play when plants absorb nutrients, some of which include conditions of the environment, the microorganisms related to the roots of the plant and

lastly the species of plant. According to many studies, plant biostimulants such as humic substances (HS), protein hydrolysates (PHs) and seaweed extracts (SWE) are able to enhance root growth and development due to auxins found in most PBs. According to other reports, the recommended rate of PBs when applied is below threshold in terms of causing root stimulation by auxins **(WALLY et al., 2013)**, thus attributing the root stimulation effect by biostimulants to the interaction of organic molecules from biostimulants with receptors on the cell membrane causing a signal transduction pathway through modulation of hormones within cells **(BATTACHARYYA et al., 2015)**. Protein hydrolysates as plant biostimulants have been reported to have root stimulation effects in tomato, lettuce and corn **(COLLA et al., 2014; COLLA et al., 2015b)**.

According to **PACHOLCZAK et al. (2016)** and **VERNIERI et al. (2006)**, SWE stimulate rhizogenesis and root growth when applied to plants or cuttings. In essence SWE are mixtures of bioactive compounds such as polysaccharides, fatty acids, phytohormones, vitamins and mineral nutrients **(BATTACHARYYA et al., 2015)**. **HERNÁNDEZ-HERRERA et al. (2014)** discovered a vigorous root growth-promoting effect from polysaccharide-enriched extracts.

Anecdotes strongly support the idea that PBs are able to enhance uptake of nutrients through improvement of nutrient uptake activity by the root system, and applications of HS, PHs or SWE are reported to up-regulate genes encoding for nutrient transport **(DE PASCALE et al., 2017)**. A good observation was made with *Brassica napus* when it was treated with humic acids and there was stimulation of root growth and increased uptake of N and sulfate due to upgrading of genes in roots which encode for nitrate transporters (BnNRT1.1 and BnNRT2.1) and transporters for sulfate (BnSultr1.1 and BnSultr1.2) **(JANNIN et al., 2012)**.

Many technological ideas have been brought to the fore with the aim of improving sustainable agriculture by drastically minimizing the amount of fertilisers and other chemicals used. One of the most exciting strategies for reducing the use of chemicals and enhancing crop resistance to stress of an abiotic nature such as nutrient deficiency is the employment of biostimulants such as SWE. The incorporation of biostimulants in agricultural production can drastically minimise the use of synthetic

fertilisers since they can increase yields and enhance crop production with no harm to the environment **(KHAN et al., 2009; SHARMA et al., 2014)**.

There has been wide-spread acceptance of the application of SWE in horticulture **(BATTACHARYYA et al., 2015)**. Special attention should be given to SWE such as those made from brown macroalgae especially; *Ascophyllum nodosum*, *Ecklonia maxima*, *Durvillea potatorum* and *Macrocystis pyrifera*. These brown macroalgae species are known to be vital sources of polysaccharides, phenolic compounds, osmolytes like mannitol and phytohormones, which include abscisic acid, auxins, brassinosteroids, cytokinins and gibberellins **(BATTACHARYYA et al., 2015; PAPENFUS et al., 2013; STIRK et al., 2014)**. Scientists have been able to identify and quantify many of these growth regulators from *E. maxima* (Kelpak®) as the chemicals responsible for stimulating growth in different agricultural crops **(PAPENFUS et al., 2013; STIRK et al., 2004)**. KEL has been documented to have beneficial effects in many horticultural crops, ornamentals, trees and monocotyledonous crops and also cuttings. A number of researchers have recorded that microalgae, when applied either via foliar spray or through drenching, can stimulate both physiological and biochemical responses in plants. Plant responses include enhanced root and shoot growth, influence on flowering time, increase plant productivity and nutritional quality as well as mobilization of both macro- and micronutrients **(BATTACHARYYA et al., 2015; CRAIGIE, 2011)**. According to **CRAIGIE (2011)**, **ROUPHAEL et al. (2017a)** and **ROUPHAEL et al. (2015)**, there was improved tolerance to adverse conditions such as heat and drought stress by plants to which *A. nodosum* and *E. maxima* had been applied. Phlorotannins like phloroglucinol and its derivative Eckol, isolated from *E. maxima*, are major compounds found in SWE which also stimulate growth. Eckol has been reported to improve growth of maize in terms of elongation of both shoots and roots and seminal roots. Eckol was also reported to act like auxins in mung beans where it increased root number, elongation of shoots and weight of seedlings. From these reports it is clear that Eckol can play a key role in enhancing agricultural productivity **(RENGASAMY et al., 2015b)**. SWE enhance root and shoot growth, improve nutrient uptake, enhance flower formation and fruit setting, and this translates into increased yields, delay of senescence and confers longer shelf life to fruits **(CROUCH and VAN STADEN, 1994; KHAN et al., 2009)**. In addition, plants also have improved resistance to attacks by

insects and pathogens and to drought and frost stress **(CRAIGIE, 2011; CROUCH and VAN STADEN, 1994; KHAN et al., 2009)**. According to **CRAIGIE (2011), CROUCH and VAN STADEN (1994)** and **KHAN et al. (2009)**, the application of SWE is usually done at low application rates through drenching or foliar spray, so the many benefits derived are not as a result of an increase in macro- and micronutrients found in the extract. The physiological responses are caused by plant growth regulators (PGRs) and other active compounds such as oligomers and polysaccharides which work at very low concentrations **(CROUCH and VAN STADEN, 1994; KHAN et al., 2009)**. A liquid extract marketed as Kelpak® is prepared by a cell burst method from the kelp *Ecklonia maxima* (Osbeck) Papenfus, harvested on the west coast of South Africa.

Biostimulants from algal biomass contain a wide range of different molecules such as phytohormones (cytokinin, auxins, gibberellins, brassinosteroides, ethylene, and abscisic acids) **(LANGE and LANGE, 2006; WERNER and SCHMÜLLING, 2009; ZHAO et al., 2010)** amino acids **(COLLA et al., 2017b; HOQUE et al., 2007)** and polyamines. Phytohormones were reported as the putative ingredients present in SWE **(FUELL et al., 2010; KHAN et al., 2009; STIRK and VAN STADEN, 2014)**. Besides these hormones, there are also carbohydrates like alginate, fucoidan, betaines and proteins and minerals present in algal extracts, all of which support plant growth **(SHARMA et al., 2014)**.

Biostimulants can also be made from wastes from food and agricultural industries **(XU and GEELEN, 2018)** like vermicompost. Vermicompost, by definition, is organic matter produced by worms **(SHARMA et al., 2014)**. This technique of vermicomposting has been extensively utilised to alleviate accumulation of wastes from plants, food wastes, as well as sludge from sewage **(ALLARDICE et al., 2015; DOMÍNGUEZ et al., 2010)**. The technique also gets rid of disease-causing organisms found in manure such as faecal coliforms, species of the *Salmonella* genus, enteric viruses, as well as helminthes **(EDWARDS et al., 2010)**, making it a more sustainable waste management strategy which reduces environmental contamination. Biostimulants can be obtained from vermicompost, which can be used in plant growth media, an amendment of soil, to replenish nutrients and improve resistance against abiotic stress **(AREMU et al., 2012; AREMU et al., 2014; CHINSAMY et al., 2013)**. **AREMU et al. (2015)** demonstrated the presence of the PGRs, cytokinins, auxins,

abscisic acid, gibberellins and brassinosteroids in leachate of garden waste vermicomposted commercially. This may explain the biostimulatory activity of vermicompost, because of these substances which possess phytohormonal activity.

Vermicomposting is vital in sustainable agriculture and recycling of nutrients since it is a cheap method of managing wastes which can be scaled up **(YADAV and GARG, 2013)**. The strategy enables farmers to recycle wastes from their activities, making use of both plant materials and animal manure. In addition, wastes which are suitable are converted into organic fertilisers for crop improvement to enhance yields as well as amending soils **(LAOSSI et al., 2010; VAN GROENIGEN et al., 2014)**.

From all the attributes discussed above on the importance, benefits and roles of biostimulants, they could be harnessed in the improvement and commercial production of underutilised and often neglected crop plants like traditional leafy vegetables (TLVs) so as to ameliorate problems of hunger and malnutrition bedevilling the African continent, particularly sub-Saharan Africa.

2.2 Global hunger and malnutrition challenges

The major challenges faced by the world today are malnutrition, poor health, hunger and in some cases starvation **(BALDERMANN et al., 2016)**. According to the Food and Agriculture Organisation (FAO), an estimated 800 million people are currently afflicted with food and nutrition insecurity **(DA SILVA, 2014)**. Over a third of the world's population is estimated to experience micronutrient deficiency (hidden hunger) **(TONTISIRIN et al., 2002)** a scenario that has been overlooked by most policy makers **(VINCETI et al., 2013)**. The well-being of over 2 billion people worldwide is threatened by malnutrition, with pregnant women and children being the most affected resulting in failure to resist infections, impairments of foetal and child growth and acute cerebral development **(ASARE-MARFO et al., 2013)**. This scenario is more prevalent among low income earners, food insecure and vulnerable households in those countries which are still developing due to lack of access to a variety of foods or ignorance of a suitable diet. This gives rise to numerous incidences of infectious diseases within the population, since there is a very close association between malnutrition and disease **(BALDERMANN et al., 2016)**. The vicious cycle of underdevelopment is as a result

of dietary deficiency because of its impediments on health, ability to learn as well as productivity particularly on the most vulnerable groups **(WHO, 2006)**. The lack of micronutrients and poor nutrition is responsible for nearly half of the annual deaths of children under the age of five, which is about 3.1 million mortalities. According to **KENNEDY et al. (2003)**, the issue often persists without being noticed in communities, despite having far reaching impacts on human growth, functioning of the immune system and brain development.

Nutritional insecurity is prevalent amongst the majority of Africans because of a deficiency of essential vitamins and minerals in the diet, caused by the insufficient consumption of fruits and vegetables **(AFARI-SEFA et al., 2012)**, despite the continent being endowed with a high diversity of underutilised vegetables and fruits known to be rich sources of nutrients **(BUA and ONANG, 2017)**. This contributes immensely to the high figures for malnutrition which increases non-communicable diseases globally **(HALL et al., 2009; MOKDAD et al., 2018)**. Scientists and policy makers are now seeking alternative strategies to enhance agricultural production and increase biodiversity ecosystem services. Such strategies could enhance crop yields and profits **(GARIBALDI et al., 2017)**. There is not a lot of documentation on the link between nutrition and developments in agriculture which are responsible for diet inadequacies being experienced at the moment **(KADIYALA et al., 2014; PINGALI, 2012)**. There have been great losses of biodiversity in agricultural food systems because of the focus on increasing yields of some staple cereals by the Green Revolution **(MELDRUM et al., 2018)**. It is unsustainable in the long term to rely on a few major crops, since this comes with agronomic, ecological, nutritional and economic risks **(EBERT, 2014)**.

Neglected and underutilised vegetables and fruits have the potential to play a pivotal role in food security by providing a diet rich in the essential nutrients. The high levels of micronutrients in TLVs could make a significant contribution to nutritional security if they are incorporated in the diet **(KEATINGE et al., 2011)** since they can contribute both micronutrients and bioactive compounds **(SMITH and EYZAGUIRRE, 2007)**. Many countries on the African continent are failing to address issues to do with under-nutrition and shortages of micronutrients in the diet **(LOPRIORE and MUEHLHOFF, 2003)**. Empirical evidence has shown the many benefits associated with TLVs, which include their superior micronutrient content, useful medicinal properties as well as

agronomic advantages like short growth periods (ready to harvest 3-4 weeks after planting for some crops), as well as low input requirements. This could make TLVs crucial to food and nutrition security in many parts of Africa, especially in times of famine and natural disasters, two scourges haunting sub-Saharan Africa **(HUGHES, 2008)**. The African continent is rich in edible traditional vegetables, amongst which, the amaranth is known to be one of the most nutritious, and this has been attributed to its high protein and lysine content, an amino acid not found within most of the cereals **(MAUNDU et al., 2009; MLAKAR et al., 2009a)**. TLVs have been neglected in terms of research and investment, despite the numerous attributes they possess. The main constraints of production cited include; poor seed quality, absence of production technologies and poor strategies for marketing and processing **(DINSSA et al., 2013)**.

Much attention is now being given to micronutrient malnutrition due to its recognition as a global disease threat. Several non-specific physiological disorders which include non-resistance to infections, problems in metabolism and slow and retarded physical and mental development are caused by micronutrient deficiencies. The severity and frequency of micronutrient deficiencies is more pronounced in poor communities, even though they can also be a menace to public health in some industrialised countries **(FLYMAN and AFOLAYAN, 2006; GLETSU-MILLER and WRIGHT, 2013; VENSKUTONIS and KRAUJALIS, 2013)**. The lack of iron, iodine, vitamin A and zinc was acknowledged as the world's most serious risk factors with regards to health in the 2000 World Health Report. Problems associated with deficiencies in the diet are actually immense when it comes to designing control strategies and prevention of diseases like malaria, HIV/AIDS, tuberculosis and certain chronic diseases resulting from diets which are not balanced **(WHO, 2000)**. According to **SOETAN et al. (2010)** mineral nutrients present in body tissues and fluids are key in maintaining certain physicochemical processes which are vital for life. With regards to the human body's mineral nutrient requirements, there are major secondary micro or trace minerals, nutrient minerals and organic nutrients deemed essential and they are all present in foods derived from plants. Although most foods have mineral nutrients, they are found in different amounts, with some having higher amounts of certain minerals and for this reason recommended doses vary **(JIMOH et al., 2018)**. By definition, a dietary requirement is the minimum continuing intake of a particular nutrient required to

maintain a defined level of the nutritive need of an individual **(SUTHERLAND et al., 1998)**. The daily nutritional requirements for humans are dependent on sex, size, age and occupation. Vegetables are a vital component of any balanced diet **(AGTE et al., 2000)** and their nutritional value varies according to the plant part consumed. Health promoting ingredients in vegetables such as vitamins, amino acids, as well as sugars, have been acknowledged for their benefits to man as far as health is concerned, with other substances in vegetables previously ignored, now being recognised and given due attention **(RAI et al., 2012)**. Plants such as TLVs now offer the potential hope of reclaiming "lost" dietary requirements to fill the nutrient vacuum afflicting many vulnerable populations globally **(BUA and ONANG, 2017; HAWKESWORTH et al., 2010)**.

Many people rely on staple crops, mainly rich in carbohydrates, most of which are often micronutrient deficient and lacking nutrients which are vital for human health. Essential nutrients required by humans in the diet include vitamins A, C and E and iron, zinc and iodine **(AFARI-SEFA et al., 2012)**, hence the increase in malnutrition in parts of sub-Saharan Africa, where it is causing ill health in children and contributing to stunting and mortality **(AFARI-SEFA et al., 2012)**. This sad scenario can only be addressed by increased consumption of fruits and vegetables to attain a balanced diet. According to **KEATINGE et al. (2011)** the continued lack of vegetable and fruit consumption the world over has very serious effects on health of humans and this adversely affects the attainment of a number of the Millennium Development Goals (MDGs). Ironically, African food systems make up the richest sources of biodiversity and are also good sources of beta-carotene **(CHWEYA and EYZANGUIRE, 1999)**. It is imperative to diversify diets with vegetables since they assist with biofortification by making available diets which are not only nutritious but are also balanced **(TOLEDO and BURLINGAME, 2006; UUSIKU et al., 2010; VAN RENSBURG et al., 2014)**.

2.3 Traditional Leafy Vegetables (TLVs)

2.3.1 Definition for Traditional Leafy Vegetables (TLVs)

VAN RENSBURG et al. (2007a) define traditional leafy vegetables (TLVs) as plant species whose leafy parts include young succulent stems, flowers and young fruit and

are utilised as a vegetable. Other authors define TLVs as plants that have been cultivated or grown within communities for many years, whose leaves, immature green pods, stems, roots, seeds, flowers, fruits or even bark are accepted by the society to be consumed as vegetables **(KIMIYWE et al., 2007; NGUNI and MWILA, 2007; SEEISO and MATERECHERA, 2014; TALENI and GODUKA, 2013)**. Some authors consider them as plants whose parts such as leaves, fruits as well as roots are deemed acceptable and utilised as vegetables by rural, peri-urban and urban communities by virtue of custom, habit or tradition **(CHWEYA and EYZAGUIRRE, 1999; LEWU and MAVENGAHAMA, 2010; MUHANJI et al., 2011)**. The presence of several classes of active compounds like carotenoids, and polyphenols in TLVs means that they can be further defined as food-medicine **(GUARRERA and SAVO, 2013)**. **GOCKOWSKI et al. (2003)**, define TLVs as those that have been domesticated or cultivated in Africa for several centuries. According to **ETÈKA et al. (2010)**, TLVs are those cultivated in specific regions or continents and include those indigenous to Africa and introduced species which now form part of the local diet. These vegetables are adapted to specific locations geographically, becoming part of the local culture **(MABHAUDHI et al., 2017)**. TLV consumption has been practised for many centuries by many rural communities on the African continent, so it is a tradition. There are approximately 1 000 different indigenous and naturalised vegetable species that can be used in dietary diversification, attainment of food security and subsequent improvement in livelihoods of people in sub-Saharan Africa **(TOWNS and SHACKLETON, 2018)**.

Malnutrition, also known as “hidden hunger”, is responsible for health problems such as high mortality and low economic productivity in tropical Africa **(BIESALSKI, 2013)**. Emphasis is now being given by WHO-FAO nutritionists that a portion of different vegetables has to be consumed on a daily basis to achieve a balanced diet, especially for children, and women who are pregnant **(GRUBBEN et al., 2014)**. In comparison with the recommended intake, vegetable consumption, which is a cheap and available source of micronutrients, is low and it is imperative that projects on food and security in Africa focus more on promoting the cultivation of vegetables for health purposes **(GRUBBEN et al., 2014)**. The scourge of “hidden hunger" is quite alarming in sub-Saharan Africa, according to a report by a team of hunger experts who embarked on a global investigation into how incidences of malnutrition had implications on health and economic growth. Stunting of growth and anaemia caused by a deficiency of iron

and vitamin A were used as indices in the research **(MUTHAYYA et al., 2013)**. Signs of malnutrition and hunger were not clearly visible, hence the term “hidden hunger”. “Hidden hunger” usually has lifelong and debilitating effects on health, productivity and mental development and mainly affects women of reproductive age as well as children. Globally, the most common micronutrient and vitamin deficiencies are those of iron, zinc, vitamin A, iodine and folate, but deficiencies of vitamin B12 and other B vitamins are also common **(GRUBBEN et al., 2014)**.

Among the top ten factors which contribute to mortality on a global scale is the low intake of vegetables and fruits **(EZZATI et al., 2002)**. Cereal-based and nutrient-poor diets are the order of the day for the poor who have little or no access to animal foods, fruits and vegetables in most developing countries **(HOTZ and GIBSON, 2007)**.

Many TLVs found on the African continent are high sources of important nutrients such as folate, iron, zinc, proteins and dietary fibre. They also demonstrate a better water use efficiency in comparison with exotic vegetable species **(MASEKO et al., 2017)**. The main aim of dietary diversification, together with nutrition education, is to improve production, availability, access to affordable foods and the use of food with a nutrient diversity and which is available throughout the year **(AFARI-SEFA et al., 2012)**. Another positive characteristic of vegetables is the high diversity among TLVs. In addition to nutrition improvement they also add colour, flavour and texture to meals and this makes food more attractive and palatable **(AFARI-SEFA et al., 2012; MELDRUM et al., 2018)**. Of great importance would be the exploitation of the agronomic and yield potential of TLVs since this could go a long way in food and nutrition security in addition to livelihood strategies for communities under threat from migration, civil disorder as well as diseases like HIV-AIDS **(BUA and ONANG, 2017)**. It has also been observed that TLVs require less labour and economic inputs compared to other global vegetables. Based on their nutritional value and adaptation to local conditions, there is now literature supporting the notion that the increased production and consumption of TLVs could go a long way towards supporting nutritional security and income **(LEGWAILA et al., 2011; VAN JAARSVELD et al., 2014)**. Few TLVs have been domesticated or semi-domesticated and the majority grow as weeds **(MOLINA et al., 2014; RUBATZKY and YAMAGUCHI, 2012)**. To this end, more research should be done to explain and support the roles and potential of particular species since a lot of information on relationships between nutritional yields,

water availability and soil quality still needs to be explored. Additionally, information on consumer preferences and incorporation into value chains needs to be researched and explored **(MELDRUM et al., 2018)**. The promotion of TLVs is generally hindered by the lack of information because just like many other neglected and underutilised crops, there is limited research and breeding efforts on TLVs. In addition, germplasm characterisation and species distribution is not known for TLVs **(GALLUZZI and LÓPEZ NORIEGA, 2014)**. Useful species may be overlooked due to lack of information, and poor awareness, through neglect and underutilisation **(MELDRUM et al., 2018)**. On a global scale the trend is a general decline in the use of TLVs and this jeopardises their future and also minimises the delivery of benefits to society **(MELDRUM and PADULOSI, 2017)**. Most TLVs are neglected and underutilised as there is no comprehensive information on cultivation practises, since most are regarded as weeds **(BUA and ONANG, 2017; MELDRUM et al., 2018)**.

2.3.2 The history of the use of Traditional Leafy Vegetables (TLVs) in South Africa

The consumption of TLVs in South Africa is as old as history, because the Khoisanoid people who inhabited southern Africa about 120 000 years ago depended mostly on gathering wild plants for their livelihoods **(FOX and NORWOOD YOUNG, 1982; PARSONS, 1993)**. When the Bantu-speaking tribes settled in South Africa some 2 000 years ago they also relied on harvesting wild leafy vegetables, making hunting and edible plant collection vital activities in their food system, especially in times of crop failure or death of livestock herds due to natural disasters **(BUNDY, 1988; PEIRES, 1982)**. This made wild plants and animals the only components of the diet for the hunter-gatherer cultures **(BHARUCHA and PRETTY, 2010)**. The knowledge and practice of TLV collection has always been a female domain, both historically and in contemporary South Africa **(MBHENYANE, 2017; VAN RENSBURG et al., 2004)**. Evidence shows that men only get involved when a particular plant species becomes domesticated and starts to be grown as a crop, particularly when it is produced commercially **(VAN AVERBEKE and JUMA, 2006)**. According to **MODI et al. (2006)** and **VAN RENSBURG et al. (2004)**, there is still continued collecting and cultivation of leafy vegetables by African people in sub-Saharan Africa, including South Africa, despite the modification of food consumption patterns of locals by western influences **(VAN RENSBURG et al., 2007b)**. The pattern of consumption is variable and

dependent on poverty status, degree of urbanization, distance to markets of fresh produce and season **(MBHENYANE, 2017; VORSTER et al., 2002)**. According to **VORSTER et al. (2002)**, the consumption of vegetables collected from the wild as weeds is inversely proportional to the income of the household, with poor households using them more than rich ones due to lack of finance to buy conventional vegetables from markets. The consumption of food from the wild is part of the safety net used by rural people in coping with poverty, disaster and other life stresses **(RUBAIHAYO, 1997; SHACKLETON et al., 2000)**. The majority of these people consume traditional foods as they are believed to reduce the risk of certain ailments **(MAKUSE and MBHENYANE, 2011)**. The increased use of wild food like TLVs can be a result of several factors, such as drought, loss of employment or demise of the breadwinner, as well as social strife **(DOVIE et al., 2002; SHACKLETON, 2003; VORSTER and JANSEN VAN RENSBURG, 2005)**.

In contemporary South Africa, TLVs have been labelled as weeds since the 1960s by both research and extension workers, with households being encouraged to produce food similar to those sold in the shops. It is because of this negative perception that people are not willing to use or conserve foods they label “poverty foods”, a term coined to describe TLVs by many communities **(BUA and ONANG, 2017; MASEKO et al., 2017)**.

For many years, agricultural research in South Africa concentrated mainly on commercial large scale agriculture and mono-culture systems, but there has been a shift of focus of late towards small-scale and subsistence farmers but still focussing on the common commercial crops **(VORSTER, 2007)**. The Brundtland Commission identified three types of agriculture based on how agro-ecological and socioeconomic factors interact, namely; industrial, green revolution and resource-poor agriculture **(WCED, 1987)**. Unfortunately most countries in sub-Saharan Africa are found in the resource-poor category, relying mainly on rain-fed marginal soils for growth **(VORSTER, 2007)**. Associated with resource-poor agriculture are farming systems which are complex and exposed to risk, thus farmers use different methods to survive **(VORSTER, 2007)**.

Resource-poor farmers in South Africa practise subsistence farming using both traditional and conventional methods **(VORSTER et al., 2007)**. It is only recently that

the value of indigenous knowledge of traditional crops as a survival strategy for rural people has been acknowledged by research. Extension personnel still treat TLVs as weeds and criticise farmers for failing to keep weed populations under control **(VORSTER et al., 2007)**, which they label not worthy of the space they occupy **(BUA and ONANG, 2017)**. From the wild, different plant parts are harvested as food and these include, leaves, stems, tubers, rhizomes, roots, flowers, fruits, gums, nuts, cereals, berries and legumes.

South Africa is a country endowed with a high biodiversity of both plants and animals, with some of these plants being used for many purposes by locals. Some of these uses include; food, shelter, fuel, medicine and tools **(VAN WYK and GERICKE, 2000)**. The wild vegetables in South Africa possess remarkable nutritious qualities in the form of macro- and micronutrients, minerals and vitamins **(LEWU and MAVENGAHAMA, 2010; VAN DEN HEEVER, 1995)**. TLVs not only play an important role in the diet but also support the local economy and provide excellent environmental services such as an increase in biodiversity, reduced pollution, pest management **(FRISON, 2016)** and are an integral part of African traditional medicine **(PADULOSI et al., 2013)**. Of great concern is the underutilization of TLVs in South Africa **(VORSTER and JANSEN VAN RENSBURG, 2005)**. The average South African consumes about 200 g of fruits and vegetables on a daily basis, which falls far short of the WHO recommended daily intake of 400 g per day. Traditional leafy vegetables have a long utilisation history by local communities across Africa **(MOYO et al., 2013)** but there has been a marked decline in their consumption and utilisation of late by the locals, due to the emergence of exotic vegetables. Traditional leafy vegetables are commonly known as ‘imfino’ in isiZulu / isiXhosa, ‘morogo’ in seSotho / sePedi and ‘muhuro’ in Tshivenda **(VAN DER WALT et al., 2009; VAN RENSBURG et al., 2007b)**. In South Africa, just like in many rural communities in Africa, vegetables are mainly used to supplement the diet usually consisting of staples like cassava, maize, millet, sorghum and wheat **(MAROYI, 2013)**. It is known that plant material consumption is a fundamental requirement for the well-being of mankind **(MOYO et al., 2013; SALVI and KATEWA, 2016; SCHREINEMACHERS et al., 2018)** since the dietary requirements for bio-available micronutrients and phytochemicals are obtained via consumption of leafy vegetables **(MOYO et al., 2013; SALVI and KATEWA, 2016; UUSIKU et al., 2010)**. Most of these vegetables are seasonal and usually in abundance during the rainy season. Their

consumption is vital in achieving a balanced diet, thereby preventing the chronic effects of “hidden hunger”. According to **SALVI and KATEWA (2016)** and **YAHIA (2010)**, results from epidemiological research show that a high intake of plant-derived products in the diet results in a reduced risk of various chronic conditions such as cancer, neurodegenerative and cardiovascular ailments **(BUA and ONANG, 2017; WHO, 2015; SCHREINEMACHERS et al., 2018)**.

In the past few years there has been a recognition of the nutritional and cultural importance of TLVs in South Africa. This has resulted in their being incorporated into the core business of the Agricultural Research Centre (ARC), a national centre for research in South Africa. The Agricultural Research Council Roodeplaat, together with the Vegetable and Ornamental Plant Institute, are now concentrating on improvement, distribution and the conservation status of TLVs. There is a need for active promotion, use and conservation of TLVs so as to increase production in order to tap into their potential contribution towards food security in South Africa.

More than 100 different types of TLV species are known in South Africa but only a handful of these are being utilised **(VAN RENSBURG et al., 2007a)**. The commonly consumed ones include *Corchorus olitorius* (jute mallow), *Amaranthus cruentas* (pigweed), *Citrullas lanatus* (bitter melon), *Vigna unguiculata* (cowpea), *Cleome gynandra* (spider plant), *Cucurbita* spp. (pumpkin) and *Brassica rapa* subsp. *chinensis* (non-heading Chinese cabbage). These TLVs are known by different vernacular names in their cultural areas and they are utilised differently according to different authors **(MAVENGAHAMA, 2013; VAN RENSBURG et al., 2007b)**.

One of the most commonly consumed TLV in South Africa is the amaranth from the Amaranthaceae family, which consists of a number of species found growing in many different parts of the country **(MAVENGAHAMA et al., 2013; OELOFSE and VAN AVERBEKE, 2012)**. The main amaranth species found growing in South Africa include *A. thunbergii*, *A. greazicans*, *A. spinosas, A. deflexus*, *A. hypochondriacus*, *A. viridus* and *A. hybridus* **(MAVENGAHAMA et al., 2013; OELOFSE and VAN AVERBEKE, 2012)**. These amaranth groups thrive well in harsh climatic conditions although flowering can be induced by long dry spells, thereby reducing plant yield **(MAVENGAHAMA et al., 2013; OELOFSE and VAN AVERBEKE, 2012)**.

2.3.3 Utilization of Traditional Leafy Vegetables (TLVs)

There has been a marked reduction in the use of TLVs in South Africa **(NESAMVUNI et al., 2001; VAN WYK, 2005)** despite their high nutritive value and high potential as cash crops **(MASEKO et al., 2017)**. The reason for the decline in utilisation of TLVs is consumer preferences, with locals preferring exotic vegetables **(VAN RENSBURG et al., 2004)**, so there is variable use of these vegetables currently. There are a number of factors contributing to this state of affairs with regards to adoption of TLVs by locals. People do not cultivate TLVs since most of them are gathered from the wild, cultivated fields and from land left fallow **(MAVENGAHAMA, 2013; VENTER et al., 2007)** and harvesting of TLVs mainly involves women. Furthermore, the youth associate the consumption of TLVs with poverty, and have coined TLVs as food for the poor during hard times **(TALENI and GODUKA, 2013; VORSTER et al., 2002)**. The other reason for the non-cultivation of TLVs is the loss of local knowledge about them **(MODI et al., 2006)**. Some of them are unpalatable due to poor preparation methods, so they are shunned by the young. Also youths are not knowledgeable about which species to collect and sometimes they confuse them with poisonous species **(MAVENGAHAMA et al., 2013)**.

Current trends of harvesting TLVs are not sustainable since there is no control over their availability. The increase in promotion and use needs to match with propagation or cultivation, otherwise there would be a problem of overharvesting from the wild which could lead to species extinction **(LEWU et al., 2007; MAVENGAHAMA, 2013)**. The best way forward is to try and incorporate TLVs in the current cropping systems, hence the need to carry out more research in order to come up with appropriate methods for the production of these crops so as to meet supply and demand **(MASEKO et al., 2017)**.

The ability of TLVs to thrive in adverse conditions makes them potential candidates for use in enhancing world food production **(SWART et al., 2005)**. The other positive attributes of TLVs is their low input requirements for their production compared with exotic crops and in addition to this, they are generally very resistant to pathogens. Therefore they are cost effective in terms of chemicals and pesticides. These crops are also more tolerant to both abiotic and biotic stresses than other crops **(ADEBOOYE and OPABODE, 2004; OKENO and CHEBET, 2003)**. They could be a

worthwhile and profitable substitution of other crops in the fight against malnutrition, since they can increase nutrient uptake **(TESFAYE et al., 2016)**. TLVs have a great potential to be utilised for enhancing food and nutrition security since they do not need a great deal of water **(MASEKO et al., 2017)**.

An estimated 11.1 million males and 12.5 million females of 15 years or older had a below average intake of vegetables and fruits in the year 2000 in South Africa **(ROSE and CHARLTON, 2002; SCHNEIDER et al., 2007)**. The fruit intake for these groups was half the World Health Organisation's recommended daily intake of 400 g of vegetables and fruits **(WHO, 2003)**, which can prevent non-communicable diseases caused by malnutrition or hidden hunger **(LOCK et al., 2005; VAN JAARSVELD et al., 2014)**. According to **LABADARIOS et al. (2011)**, the least consumed foods by adults in South Africa are the vitamin A-rich vegetables and fruits, eggs and legumes and this reduces variety in the diet resulting in wide spread micronutrient deficiencies of vitamin A, iron and zinc.

Despite their acclaimed importance with regards to nutrient value, there is very little domestication and no large-scale commercial cultivation of TLVs in South Africa **(DEPARTMENT OF AGRICULTURE, 2013)**. There are several reports of the cultivation of TLVs on a subsistence scale by rural communities in South Africa, mostly in the Limpopo and KwaZulu-Natal Provinces **(UUSIKU et al., 2010; VAN RENSBURG et al., 2007a)**. According to **VAN DER HOEVEN et al. (2013)**, TLVs provide essential nutrients required for human health in the same manner as conventional vegetables with some of the TLVs having superior amounts of the essential nutrients than the conventional vegetables **(RAMOS et al., 2013)**.

TLVs have been part of the daily livelihoods of the African people for many centuries and they were mainly harvested from the wild and at little cost, which made people assume that they would always remain part of their lives. The trend was that the responsibility and knowledge would be passed on to younger generations by aged ladies and in this manner, seeds of certain species were conserved. Sadly, such systems are currently non-existent due to social, political and economic factors **(VAN RENSBURG et al., 2007a)**. In terms of occurrence, TLVs can either be cultivated, semi-cultivated, weedy or wild crops with ecological, social and cultural values **(OLASANTAN, 2007; THAMAGA-CHITJA et al., 2011)**.

Of global interest is food production, utilising fewer resources, so as to ensure food security for the ever rising world population. Concerns about food security were raised at the Hot Springs Conference convened by the Food and Agriculture Organisation in 1943 from which the definition evolved. The World Food Summit of 2006 came up with the most recent definition of food security as: 'a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life' **(FAO, 2006)**. In order to come up with a proper definition of food security there are four aspects which are taken into account viz, food availability, access, utilisation and stability **(FAO, 2006)**. There is a close link between food security and agriculture. According to **HART (2009)**, South Africa as a country is food secure at national level, but not secure at the level of households. Incorporation of TLVs in the diet could go a long way in alleviating problems of micronutrient deficiency.

Investment has been centred mainly on cereal crops with very negligible investment on TLV crop development **(SCHREINEMACHERS et al., 2018)**. The current production of vegetables runs short in meeting the needs of the human population **(SIEGEL et al., 2014)** with poorly developed vegetable value chains thereby limiting accessibility to consumers **(BANDULA et al., 2016; CHAGOMOKA et al., 2014)**. A major priority challenge to policy makers is to facilitate access to vegetables at an affordable price, since populations are more reliant on exotic foods bought from the shops **(HAWKES and FANZO, 2017; MILLER et al., 2016)**. There is now recognition for the need for nutrition-sensitive agriculture and food with calls for increased vegetable production through new approaches such as horticulture, home gardens, urban and peri-urban agriculture, agroforestry and school-feeding programmes **(SINGH and DWIVEDI, 2017)**.

There is perpetual pressure on agricultural production as the world population increases in size and affluence and demand keeps on outpacing supply **(AFARI-SEFA et al., 2012; EBERT, 2014)**. The impact on agriculture is wide-spread because of intensive agriculture, hence there is an urgent need for a global change to alternative farming approaches to guarantee food security and nutrition, as well as provision of social and economic equity and also conserve and maintain ecosystem services which support agriculture.

A lot of attention is now being given to traditional vegetables as important components of African diets due to the recognition of the medicinal properties of their non-bioactive compounds **(AYODELE, 2005; LIU, 2013; OKENO and CHEBET, 2003)**. In sub-Saharan Africa TLVs have been used as ingredients in traditional soups taken with carbohydrate staple foods **(CHWEYA and EYZAGUIRRE, 1999)**.

According to Plant Resources of Tropical Africa (PROTA), there are approximately 6376 indigenous African plants considered useful, of which 397 comprise vegetables **(GRUBBEN and DENTON, 2004)**. There is an estimated 20 leafy vegetables of nutritional importance found in Africa which are usually used in daily diets **(GUARINO, 1997)**. Research has demonstrated the synergistic effect played by TLVs as nutraceuticals since they possess both nutritional and medicinal attributes **(SMITH and EYZAGUIRRE, 2007)**.

Evidence of a comparison of starchy staple crop production and vegetable production shows that it is more profitable to cultivate vegetables since they provide more opportunities for employment and income-generation, in addition to enhancing the commercialization of the rural sector **(WEINBERGER and LUMPKIN, 2007)**. It is now well documented that TLVs such as amaranth (*Amaranthus* spp.), cow pea leaves and pods (*Vigna unguiculata*), African nightshade (*Solanum scabrum* and *S. villosum*) and spider plant (*Cleome* spp.) have a superior micronutrient content as well as being rich in antioxidants **(YANG and KEDING, 2009)** and health promoting phytochemicals. Vegetables are also known to restore the balance of beneficial bacteria responsible for decomposition found in the human gut because of their antibiotic and prebiotic properties **(AFARI-SEFA et al., 2012; ERASTO et al., 2004; VELURI et al., 2004)**. Increased consumption of vegetables and other different food stuffs can also reduce chronic diseases, hence the repeated calls for increased vegetable and fruit consumption because they contain non-nutrient phytochemicals known to prevent chronic diseases **(AFARI-SEFA et al., 2012; STEINMETZ and POTTER, 1996)**. It is for the same reason that recommended micronutrient and antioxidant rich diets work hand in hand with medicinal therapies in the fight against HIV-AIDS **(FRIIS, 2006)**.

2.3.4 Current status, utilisation and production of Traditional Leafy Vegetables (TLVs) in South Africa

According to **FRD (1992)** South Africa is a country of national food sufficiency and can export food but ironically hunger and malnutrition are still rife in both rural and urban areas. The solution to this dilemma lies in the incorporation of traditional vegetables and fruits in daily diets because of their potential to make significant contributions to both calorie and nutrient content of diets **(KUCICH and WICHT, 2016)**.

Fruit and vegetable scarcity in the diet is responsible for vitamin A deficiency responsible for blindness and even death in young children in many parts of the African continent **(OKIGBO, 1990)**. Taking in an excess of at least seven portions per day of fruits and vegetables greatly lowers the risk of mortality, yet many poor South Africans have very low or zero intake of fruits and vegetables, running the risk of "hidden hunger" **(KUCICH and WICHT, 2016)**. Colonisation worsened the dietary patterns of local South Africans with nutritionally superior traditional crops being slowly displaced by cash crops. This is very unfortunate for rural communities and places poor rural children at a greater risk of malnutrition **(CHIVENGE et al., 2015; FRISON et al., 2005)**.

South Africa as a nation has always been faced with the problem of food insecurity at household levels rather than chronic malnutrition **(FABER and WENHOLD, 2007)**, with 21.5% of its population living under the poverty datum line (PDL). The problem of food insecurity is further compounded by water scarcity and population growth, which are also rife in South Africa **(ODHAV et al., 2007)**. Food insecurity is a result of the lack of a variety of food, lacking fruits and vegetables in particular, leading to a plethora of chronic diseases such as high incidents of lower respiratory infections and acute chronic diarrhoea. This affects children in the long run, resulting in poor mental development, leading to poor academic ability and also stunted growth **(VAN RENSBURG et al., 2004)**. This problem can be alleviated by the consumption of TLVs since they offer an affordable and nutrient rich alternative **(MASEKO et al., 2017)**. Among TLVs are some that are known to be rich sources of vitamins, minerals and antioxidants as well as anticancer factors for health maintenance and disease prevention **(ABUKUTSA-ONYANGO, 2003)**. They could make a difference in poor rural communities who cannot afford to buy vegetables from the local markets

(MASEKO et al., 2017). Most of these communities are found living in marginal areas with critical water challenges with high crop failure due to drought and heat stress. TLVs are ideal crops for cultivation in marginal areas **(VAN AVERBEKE et al., 2012)** since they are known to be tolerant to stresses like drought, pests and diseases. Other additional attributes of TLVs are their adaptation to low-input agriculture compared to their exotic counterparts like Swiss chard. This makes them a potential food source for under privileged inhabitants of marginal areas who practice low-input agriculture **(MASEKO et al., 2017; MAUNDER and MEAKER, 2007; VAN RENSBURG et al., 2004)**.

Local people in South Africa used to eat diets of meat, milk, wild cereals and wild plants, but the reality of late, for most South Africans, is what the Pedi proverb states "Meat is a visitor, but 'morogo' a daily food", meaning that meat is only eaten when there is a visitor in the home **(WALDMANN, 1980)**. The use of traditional leafy vegetables in communities has been noted in several studies of foods from the wild carried out from 1936 to 1982.

Research is required to ascertain the extent of the use and conservation status of TLVs in South Africa. Research is needed to establish the extent of the use, conservation status and awareness of the plants. The main aim is promotion of the use of TLVs via better preparation, enhanced consumption, processing, landrace improvement and genetic diversity control, adding science technologies on indigenous knowledge where required.

According to **CHENG et al. (2017)**, traditional vegetables are increasingly becoming more relevant in relation to global health and diet trends which clamour for 'gluten-free' and 'super foods' diets. The introduction of an uncommon food crop to local people is not easy since the people have the tendency of choosing what they know rather than what could add more value. This is the challenge with the introduction and adoption of TLVs in diets because of their limited documentation and non-consideration in mainstream research **(JAENICKE and VIRCHOW, 2013; NAYLOR et al., 2004)**.

One of the most important TLVs in South Africa is the amaranth and despite its high nutritious and nutraceutical properties very little if nothing has been done in terms of its improvement and commercial cultivation, with only a few pockets of the population

carrying out subsistence cultivation of this TLV. Most of it is harvested from the wild as a weed and sold on the local markets and on road sides. The amaranth is a crop with features which allows it to acclimatise and thrive in unfavourable and rapidly changing climatic conditions **(ABRAHAM et al., 2014; MASSAWE et al., 2007)**. The crop's adaptation to the socio-economics of the region could make it a favourite with both farmers and locals **(JAENICKE, 2011)**.

2.4 The amaranth

The Mayan civilisation of South America was recorded to be the first to domesticate *Amaranthus*, according to historical evidence. Another piece of evidence suggests that the Aztec civilisation in Mexico used amaranths as a staple food they called 'huahtli' **(LEHMANN, 2018; SAUER, 1950)**. The belief of the Aztecs was that the plant possessed magical attributes giving it strength and as such the plant grain was used in religious practices. The practice was ended in the 1500s by the Spanish conquistadors who banned the cultivation of *Amaranthus* as a way to suppress the culture and religion of the Aztecs **(RASTOGI and SHUKLA, 2013)**.

One of the oldest known edible vegetables (dating back to a Tehuacan puebla Mexico in about 4000 BC) is the amaranth according to the earliest archaeological records **(SINGHAL and KULKARNI, 1988)**. Historically, amaranth (*Amaranthus* spp.) has been consumed by many civilizations such as the Incas, Mayas and Aztecs who used it as a staple food. Amaranth is the collective name given to about 60 members of the genus *Amaranthus* of the Amaranthaceae, whose members are mainly used as grains and leafy vegetables **(BRENNER et al., 2000; RAY and ROY, 2008)**. The amaranths are found in the tropics, subtropical and temperate regions of the world **(SAUER, 1976)**. Amaranth is native to Central and South America and is believed to have been first domesticated by the Aztecs as a crop 8000 years ago. It has been extensively used as a green vegetable in tropical regions **(BRENNER et al., 2000)**. Amaranth is a crop which grows fast with low production costs, thus making it an inexpensive leafy vegetable to produce. The vegetable is often linked with the term 'poor man's food' and is mainly cultivated in summer, unlike most green vegetables, and was often the only available vegetable on the market **(SINGH and WHITEHEAD, 1996)**. Amaranths are C4 plants and grows well under stresses of heat and drought and is also known to

be tolerant to stresses such as high salinity, acidity and alkalinity, making it unique and suitable for subsistence agriculture **(ACHIGAN-DAKO et al., 2014)**. They have the potential to improve health and curb malnutrition **(MAUGHAN et al., 2011)**. To optimally grow the plant, it requires fertile and well drained soils of a loose nature with a pH > 6 **(GRUBBEN and DENTON, 2004)**. The vegetable amaranth grows well at temperatures above 25 °C.

Even though the amaranths have been neglected for many years, they have recently been rediscovered as a promising food crop, mainly due to their resistance to heat, drought, diseases and pests. The nutritional value of both the seeds and leaves is excellent and superior compared to most plants under commercial cultivation **(HAUPTLI and JAIN, 1977; RASTOGI and SHUKLA, 2013; VENSKUTONIS and KRAUJALIS, 2013)**.

Although the value of amaranth is both as a grain and vegetable, it is generally classified as a pseudo-cereal since it is not a 'true cereal' like maize or wheat. Caryophyllales is the order to which amaranth belongs and the family is Amaranthaceae, with *Amaranthus* being the genus. The botanical genus name, *Amaranthus* is derived from a Greek word "amarantos", which means "unfading" because it has long lasting flowers. About 400 amaranth species are known worldwide with the majority being classified as weedy species **(SUMA et al., 2002)**. Of special mention is *Amaranthus retroflexus*, known to be one of the worst weeds in the world **(BRESSANI et al., 1993)**. The amaranth is a multipurpose crop since it can be used as a source of grain and tasty leafy vegetable of superior nutritional value and can also be used ornamentally because of the attractive colour of its inflorescence **(BREENE, 1991; MLAKAR et al., 2009b)**. Hence it is used as food for both human and animals as a leafy vegetable, for ornamental purposes and forage.

As a C4 crop, the amaranth grows fast and is well adapted to different soil types and climates and has a higher atmospheric carbon conversion rate to plant sugars than C3 plants. The crop can achieve optimal growth in warm conditions making it a summer growing plant **(MASEKO et al., 2017)**. Its good performance under adverse conditions makes it one of the few major crops which are resilient to climate change **(RASTOGI and SHUKLA, 2013)**. The amaranth is mostly cultivated for its seeds/grain in India and as a green leafy vegetable in Africa **(RASTOGI and SHUKLA, 2013)**.

2.4.1 Taxonomic classification of amaranths

Kingdom – Plantae

Division – Magnoliophyta

Class – Magnoliopsida

Order – Caryophyllales

Family – Amaranthaceae

Genus – *Amaranthus*

Fig. 2.1: *Amaranthus hybridus* grown in the greenhouse at UKZN Botanical Gardens.

2.4.2 Distribution of Amaranthus

Amaranthus is distributed worldwide and is found in temperate, subtropical and tropical climates with a total of about 400 species in existence **(SUMA et al., 2002)**. In India there are about 20 cultivated/wild species. Native to south and Central America are some grain species of *Amaranthus* **(GRUBBEN and VAN SLOTEN, 1981)**, and some are native to the European, Asian, African and Australian continents **(BECKER et al., 1981; TEUTONICO and KNORR, 1985)**.

2.4.3 Nutritional value of amaranths

The lysine content of amaranth is twice that found in wheat and thrice that in maize. A lot of attention is being paid to the genus *Amaranthus* in many countries because of the superior nutritional value of some species, which makes them an important source of food as either a vegetable or grain. Leaves of amaranths have a protein content of 17.5 to 38.3% dry matter with lysine making up 5% **(AYODELE and SHITTU, 2013)**. Significant levels of vitamin A and C are present in the leaves and amaranths contain a vitamin C, calcium and niacin content which is three times that found in spinach. There is 18 times more vitamin A, 13 times more vitamin C, 20 times more calcium and 7 times more iron **(GUILLET, 2004)** in *Amaranthus* compared with lettuce. The crop is highly nutritious and can grow well on marginal lands, since it can withstand hot and dry conditions. Amaranth species, besides having nutritional properties, are also known to have medicinal properties and some species are known to be good sources of flavonoids which have antioxidant properties. Just like most TLVs, amaranth cultivation in South Africa is very rare since locals have the belief that the plant grows naturally **(MAVENGAHAMA et al., 2013)**.

Below is a summary of why the amaranth should be seriously considered for improvement, adoption and cultivation by people in KwaZulu-Natal and South Africa as a whole:

- It is cheap to grow by resource poor locals in rural areas.
- It is easy to establish and harvest.
- Takes a short period to mature, producing many seeds.

- Has a superior nutritive value consisting of proteins of high quality and contains lysine and methionine; minerals and vitamins, and fibres needed in the diet.
- Has good taste and is capable of being used uncooked, cooked or ground as flour.
- Has multiple uses in the diet as green vegetables, oil, flour and food for animals.

CHAPTER 3: Effects of nutrient strength, nitrogen, phosphorus and potassium (NPK) deficiency, irrigation frequency and light intensity on the growth and biochemical composition of *Amaranthus hybridus* L.

3.1 Introduction

One vital component of food security is the ability of any food to supply almost all the essential nutrients **(MAVENGAHAMA et al., 2013)**. This makes wild plants such as the amaranths important, hence, their consideration as 'safety nets' or food sources in emergencies **(RAMDWAR et al., 2017; SHACKLETON et al., 2006)**. Climate change effects are exerting pressure on the production of exotic crops currently under commercial agriculture. There is, therefore, an urgent need to incorporate wild and neglected plant species like the amaranths into mainstream agriculture **(RASTOGI and SHUKLA, 2013)**. Wild edible plants are known for their resilience to stresses and plant diseases, unlike exotic crops, making them better adapted to climate change effects **(FEYSSA et al., 2011; VORSTER et al, 2007)**. There is also a need to preserve edible plants like the wild amaranths to prevent them from going extinct as a result of human activities **(RAMDWAR and SIEW, 2017)**.

The utilization of chemically reactive nitrogen fertilisers has resulted in immense benefits as far as agricultural productivity and food security is concerned **(FOWLER et al., 2013)**. Despite these benefits, this form of food production is a typical case of 'the tragedy of commons' as described by the late Garrett Hardin in his 1968 seminal paper. Individual farmers act in rational pursuit of fulfilling their self-interest but at the same time sacrificing the long-term viability of a shared resource, which in this case is the environment, for short-term gain. The subsequent consequence is a disaster in a society which operates on the notion of freedom of the 'commons' because environmental pollution affects everyone **(HARDIN, 1968)**. Any individual farmer aims to maximise short-term economic yields through the over-application of N fertilisers and this can cause long-term damage to the environment. Global climate change is a consequence of such irresponsible actions. Excess N is known to pollute the air, soil and water, and also increases emissions of greenhouse gases with adverse effects

on the biodiversity and functioning of ecosystems. Of all the reactive N in fertilisers applied to increase crop yields, only a fraction is consumed as food with the rest remaining in the environment.

The situation is quite dire in sub-Saharan African countries where the soils are characterised by reduced organic matter content and low fertility, coupled with a deficiency of essential macro and micro-elements **(TILMAN et al., 2002)**. On the other hand, the use of organic manure has many shortcomings which include low nutrient content, slow decomposition, and different nutrient compositions depending on the organic materials, compared to chemical fertilisers. Despite these disadvantages, organic manure has multiple benefits such as balanced nutrient supply, including micronutrients, increased soil nutrient availability due to increased soil microbial activity, decomposition of harmful elements, improvements of both soil structure and root development and increased soil water availability **(MALERBA and CERANA, 2018)**. Organic manure derived from animal by-products has been utilised to alleviate problems of environmental contamination and reduction of plant productivity as a result of the excessive and constant use of chemical fertilisers **(HAN et al., 2016)**.

Among the limiting nutrients to plant production, N and phosphorus (P) are the most prominent. Smallholder farmers cannot use fertilisers to their maximum potential because of the exorbitant costs. Nutrients such as N and P become limiting for efficient food production **(SARWAR et al., 2012)**. The race is now on for modern agriculture to search for new strategies that could enable a reduction in the utilization of chemical inputs without compromising crop yield or farmers' income **(MAFAKHERI and ASGHARI, 2018)**. Nitrogen is a vital nutrient in agriculture, and life in general, in that it is a key limiting nutrient for many crops as well as in numerous aquatic and terrestrial ecosystems.

Fertilizer usage has continually increased globally due to declining arable land and loss of fertility **(OUYANG et al., 2018)** with farmers applying it via soil (for plant roots uptake) or leaves (foliar uptake) as well as in aquatic environments to promote both plant and fruit growth. Of particular concern in environmental quality management are N and P because they are lost via several pathways including surface runoff, subsurface flow of water and wind erosion. Also gaseous emissions of N can be deposited by atmospheric precipitation **(PIETRZAK, 2013)**. A healthy environment can

only be achieved by reducing the accumulation of pollutants in agroecosystems and refraining from the use of toxic chemicals, particularly synthetic fertilisers and chemical pesticides.

Water is another crucial requirement for plant growth with different plant species having specific water requirements. There is a need for studies to investigate the minimum water requirements for neglected and underutilised plants like the amaranths. In this way, water could be saved in the advent of drought which happens to be a critical threat in this era of climatic change **(GODFRAY et al., 2010b; WANG and FREI, 2011)**. Adequate and relevant water-saving strategies are required to curb the effects of water shortages and severe drought on food security in a world faced with an ever-increasing population **(WEI et al., 2016)**, particularly in arid and semi-arid areas. One primary environmental concern in agriculture is the use of fresh water for irrigation **(POSTEL et al., 1996)** since water resources are threatened by the increased water demand in agriculture, with climate change worsening, and the gap between water availability and demand **(AFZAL et al., 2016)**.

The major consumer of available freshwater globally is irrigated agriculture, “gobbling” an estimated 70% of available freshwater supplies **(EVANS and SADLER, 2008)**. There is a general opinion that there is usually a wastage of water in agriculture and that its use is extremely inefficient **(HSIAO et al., 2007)**. Crop yields in semi-arid environments are very low with only a small percentage of available water being utilised. Normally, rain fed crops use 15% to 30% of precipitation **(WALLACE, 2000)**, compared to the 13% to 18% used by irrigated crops in similar environments **(WALLACE and GREGORY, 2002)**, with low values of 5% of available water-use being reported in western Africa **(ROCKSTRÖM and FALKENMARK, 2000)**. Hence, the calls for agricultural efficiency in water-use which has been defined as the ability to produce the desired effect with minimal effort, expense and minimal water wastage **(JENSEN, 2007; PEREIRA et al., 2012)**.

Farmers are now improving irrigation strategies so that they provide crops with exact water requirements **(MORILLE et al., 2013)**. Many studies show short interval irrigation events improve both crop growth and development **(MEKONNEN et al., 2012)**. Optimal water management and increased crop yield in areas with scarce water resources can only be achieved with control or timing of irrigation scheduling.

The outcome of the worldwide climate change has brought about the expansion of extreme meteorological events such as dry seasons **(PENALBA and RIVERA, 2013)**. These are adversely affecting agricultural systems. Drought happens to be the most dynamic and worst abiotic stress affecting plant growth and development. It limits the productivity of crop plants depending on duration and intensity of the drought stress and stage of development of the plant as well as its genotype **(DRESSELHAUS and HÜCKELHOVEN, 2018; IRMAK et al., 2000; PATTERSON, 1995; SHAO et al., 2009)**. If there is a lack of water in any crop's growing environment, there is the potential for a decrease in the yield and profitability of the crop. It causes detrimental effects on both the physiological and biochemical processes of the crop plant **(ANJUM et al., 2017; RICCARDI et al., 2016; YANG et al., 2019)**. According to **ARBEX DE CASTRO VILAS BOAS et al. (2017)**, much interest has been generated of late to improve fruit and vegetable quality sustainably to meet future food requirements and tackle environmental stress caused by climate change.

Other critical environmental factors affecting crop physiology and biochemistry are light intensity and quality **(YANG et al., 2018)**. A slight increase or decrease in light intensity can have a significant influence on leaf morphology and structure **(WU et al., 2017)**. According to comparative studies done earlier, low light conditions detrimentally impact on plant growth since they cause a decrease in root, stem, leaf and whole plant dry matter. They also affect the rate of photosynthesis, transpiration and stomatal conductance and stem diameter **(MIELKE and SCHAFFER, 2010; WANG et al., 2009; YANG et al., 2017)**. Crops have also been observed to produce smaller and thinner leaves in low light conditions than in full sunlight **(WU et al., 2017)**. Temperature and light intensity are climatic conditions with a strong influence on growth, yield and nutritional quality of vegetables **(SAVVAS and PASSAM, 2002)**. According to **HUNTER and BURRITT (2004)**, the growth-promoting effect of light only operates within a specific range of light intensities. According to **HANGARTER (1997)**, light plays a critical role in the growth and development of a plant. Plant growth and photosynthetic efficiency are regulated by quality, quantity and direction **(SYSOEVA et al., 2010)**.

Several studies have associated the accumulation of phytochemicals in plants with genotype, light conditions and environmental temperature as well as irrigation and fertilisation **(KOPSELL and KOPSELL, 2008; MOU, 2009; PÉREZ-BALIBREA et al.,**

2008). Photosynthetic energy is solely derived from light and this makes it a vital signal from the environment responsible for photosynthetic biosynthesis and photo-morphogenesis **(CHEN et al., 2004a)**. Light triggers a wide range of signals and information for morphogenesis and several physiological processes in plants **(CHEN et al., 2004a)**. Light characteristics such as the composition of the spectrum (wavelengths), intensity, duration and direction all have significant effects on plant growth and development including the process of photosynthesis **(KOZAI, 2016)**. A correlation between phytochemical biosynthesis and accumulation and the amount of photosynthates has been reported in plants, and this makes light conditions of vital importance for optimising the accumulation of phytochemicals **(BIAN et al., 2015)**. According to **KOZAI (2016)** plants respond differently according to the lighting environment, season, genotype, cultivation practices and many other factors. All plants have their optimal light intensity ranges for growth, hence too high or too low light intensities affect morphology, photosynthetic physiology, and subsequently secondary metabolite production of plants **(PAN and GUO, 2016)**. It is essential to cultivate crops under optimal light intensity to maximise growth, development and yield **(PAN and GUO, 2016)**.

In a bid to promote the cultivation of *Amaranthus hybridus*, experiments were carried out to investigate the effects of nitrogen, phosphorus and potassium (NPK) deficiencies, nutrient strength, irrigation frequency and light intensity on the growth of *A. hybridus*. A further experiment was carried out to investigate the effects of N deficiency plus organic biostimulants on the growth of *A. hybridus*.

3.2 Materials and methods

3.2.1 Site of experiments

Pot experiments were conducted in the 2018 growing season in a greenhouse at the University of KwaZulu-Natal (UKZN) Botanical Garden (290 37.55' S; 300 24.13' E), Pietermaritzburg Campus, KwaZulu-Natal Province, South Africa.

3.2.2 Plant material

Amaranthus hybridus seeds were obtained from McDonalds Seed Company in Pietermaritzburg, South Africa. All the treatments in this investigation were arranged in a randomized complete block design.

3.2.3 Effect of nutrient strength on Amaranthus hybridus *growth under greenhouse conditions*

The main aim of this experiment was to investigate and establish the optimal nutrient requirement for the growth and development of *A. hybridus*. To this end, the Hoagland's nutrient solution (HNS) of different strengths was tested to establish the optimal concentration at which the vegetable could thrive.

3.2.3.1 Seed germination and transplantation

See11ds of *A. hybridus* were sown in brown polyvinyl chloride pots of 10 cm diameter filled with acid-washed, white, sterilised sand. Seedlings were treated with three different concentrations (50, 25 and 12.5%) of HNS **(ARNON and HOAGLAND, 1952)**. The respective treatments of 50 mL were applied once a week per pot as a soil drench for three weeks. The sand was hydrated regularly to prevent water stress. Leaf number and plant height were recorded after first, second and third weekly applications for each plant. The plants from the three treatments were harvested at the termination of the experiment after four weeks. The plants were uprooted, excess soil and water were removed from the roots using paper towels. Subsequently, different growth parameters (leaf number, shoot length) were recorded. After recording these parameters, the leaves and roots were separately placed in brown paper bags and oven-dried at 70°C for 72 h, after which, plant material was weighed to determine dry weight. Absolute Growth Rate (AGR) and Relative Growth Rate (RGR) were calculated using the collected data based on the following formulae:

$AGR = n_2 – n_1 / t_2 – t_1$ [yields (n = number of leaves or plant height) average slope over that time (t) interval] and $RGR = \ln n_2 – \ln n_1 / t_2 – t_1$ [yields (n = number of leaves or plant height) constant slope (t = time interval) during logarithmic (In) phase].

3.2.4 Effect of NPK deficiency on Amaranthus hybridus *growth under greenhouse conditions*

3.2.4.1 Preparation of Hoagland's nutrient solution

Hoagland's nutrient solution was prepared to mimic nutrient deficiencies as follows; half-strength solution with N, P and K served as the control **(ARNON and HOAGLAND, 1952)** and half-strength solution without N, P or K served as the nutrient deficiency treatments.

3.2.4.2 Treatments

Fifteen pots were arranged in three rows (5 pots in each row) and being tiny seeds, an undefined number of *A. hybridus* seeds were sown into the pots. The pots were left in the greenhouse for seeds to germinate. After five days, the resulting seedlings were thinned out to three seedlings per pot and the remainder were transplanted into another 50 PVC pots of the same size (10 mm diameter) filled with sterile white sand. All the pots were irrigated manually with 100 mL of half-strength HNS and left for a week for the transplanted seedlings to establish, before commencement of different drenching treatments of NPK, –N, –P, and –K as described in Section 3.3.1. Each treatment had five replicates with three plants in each pot. The treatments were applied once a week for three weeks from the time of seedling establishment. Data were collected weekly after the first application.

Plants were harvested after four weeks and the following parameters were measured; shoot length (mm) and number of leaves. Subsequently, the plant material was dried at 70°C for a week in an incubator for dry weight measurements.

3.2.5 Effect of different watering frequencies on growth of Amaranthus hybridus

Optimal water management and increased crop yield in areas of limited water resources can only be achieved through accurate control of irrigation scheduling. Hence, the main objective of this study was to investigate the effects of different

watering frequencies on the growth, physiology and biochemical composition of *A. hybridus*.

3.2.5.1 Seed germination and transplantation

The seeds used for this experiment were sown in germination trays. Seedlings were allowed to grow for two weeks, and at the two-leaf stage, seedlings were transplanted to experimental pots (15 cm diameter) filled with the soil of the following composition [bark, compost, limestone, ammonium nitrate and NPK (2:3:2) and sand)] **(KULKARNI et al., 2006)**. There were four replicates for each treatment. Seedlings were given two weeks to establish before irrigation frequency treatments were applied and commencement of growth assessed.

3.2.5.2 Treatments and plant maintenance

For the determination of the effects of watering frequency on growth of *A. hybridus*, pots were arranged on a bench in a greenhouse at 24 ± 2 °C. On a weekly basis, three levels of watering frequency of once, twice and thrice were applied with pots receiving 100 mL at each watering level.

3.2.5.3 Determination of plant growth traits

Plant growth traits were determined using methods described in Section 3.2.3.1.

3.2.6 Effect of light intensity on growth, chlorophyll and biochemical content of Amaranthus hybridus

The current study was aimed at investigating the effects of light intensity on the growth, chlorophyll and nutritional content of *A. hybridus*.

3.2.6.1 Seedling establishment

Seeds of *Amaranthus* were first germinated in 10 cm diameter PVC pots containing garden soil under greenhouse conditions. Upon germination, the seedlings from the pots were thinned by transplanting them in other pots full of soil, leaving three plants

in every pot, resulting in 40 pots having approximately 120 seedlings of *A. hybridus*. The seedlings were watered and allowed to establish under greenhouse conditions. Upon establishment, the seedlings were exposed in the garden to midday light intensities of 600, 450, 300 and 150 µmol m^{-2} s^{-1}. The first set of 30 seedlings was covered by a single layer of 1 mm green mesh net which represented a light intensity of 600 µmol m^{-2} s^{-1}. The next set was covered by two layers of green mesh net representing 450 µmol m^{-2} s^{-1} .The third set of seedlings was covered with three layers and the fourth set with 4 layers representing 300 and 150 µmol m^{-2} s^{-1} respectively. The plants were allowed to grow under these conditions following all the cultural practices. They were watered twice a week throughout the experiment. The following parameters; the number of leaves and plant height, were measured and recorded at different light intensities every two weeks for six weeks. The freshly harvested material was analysed for total chlorophyll, carotenoid, protein and starch content.

3.2.6.2 Chlorophyll content evaluation

The photosynthetic pigments [Total chlorophyll (a + b) and carotenoids] were estimated by the methods of **LICHTENTHALER (1987)** as described by **AMOO et al. (2014)**. One-hundred milligrams of fresh leaf material was ground with 5 mL ice-cold acetone with a pinch of sand washed with acid (BDH Chemicals Ltd, England). The resultant solution was filtered using Whatman No. 1 filter paper and centrifuged (Hettich Universal, Tuttlingen, Germany) at 3000 g for 10 min at ambient room temperature. The absorbance of the solution was read at 470, 645 and 662 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). Chlorophyll and carotenoid content were calculated as;

Chlorophyll a = 11.23A662 – 2.04A645
Chlorophyll b = 20.13A645 – 4.19A662
Chlorophyll a + b = 7.05A662 + 18.09A645
Total carotenoids = (1000A470 –1.90Chla–63.14Chlb)/214

3.2.6.3 Protein content evaluation

Total protein was estimated using bovine serum albumin (BSA) as a standard **(BRADFORD, 1976)**. Two-hundred milligrams of the sample was taken and

homogenized in an ice-chilled mortar and pestle with 6 mL ice-cold phosphate-buffer saline (PBS) [8 g NaCl (137 mM), 0.2 g KCL (2.7 mM), 1.44 g NA_2HPO_4 (10 mM), 0.24 g KH_2PO_4 (1.8 mM) in 1 l of dH_2O(pH 7.2)]. The homogenate was centrifuged at 15000 RCF(g) for 15 min at 4 °C. One-hundred microliter samples were pipetted out into test tubes and the volume made up to 1 mL in all test tubes with PBS. One millilitre Bradford dye was added to all the test tubes. The contents of the test tubes were mixed by vortexing and allowed to stand for 5 min. The red dye turns blue as it binds protein. Absorbance was recorded at 595 nm against a control.

3.2.6.4 Carbohydrate content evaluation

Total carbohydrate was estimated according to the method of **SADASIVAM and MANICKAM (2008)** with minor modifications. Two-hundred milligrams of plant material (leaf) were weighed and hydrolysed by keeping in a boiling water bath for 3 h with 5 mL 2.5 N hydrochloric acid (HCl) and then cooled to room temperature. The hydrolysed plant material was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 5 mL by adding distilled water and centrifuged at 10000 RCF(g). One-hundred microlitre of the supernatant was taken and the volume was made up to 1 mL with distilled water before adding 4 mL of anthrone reagent. The test tubes were heated in a boiling water bath for 8 min and were cooled rapidly in running tap water. The absorbance was read at 630 nm as the colour changed from green to dark green. A standard curve was prepared using 0-100 µg glucose.

3.3. Statistical analysis

The data obtained for different parameters were statistically analysed using one-way analysis of variance (ANOVA) to observe significant differences. The significance of the differences among the treatment means was evaluated by the Duncan's Multiple Range Test (DMRT) at 5% level of probability according to **GOMEZ and GOMEZ (1984)**.

3.4 Results

3.4.1 Effect of nutrient strength on the growth of Amaranthus hybridus

Nutrient strength had a notable influence on the general growth of *A. hybridus*. Nutrient strength had a significant influence on AGR and RGR of both leaf number and plant height at the highest tested concentration of 50% compared to the lowest concentration of 12.5% (**Fig. 3.1 A** and **B**). The nutrient strength did not show any notable influence on RGR for height of the plants. The notable influence of nutrient strength has been visually depicted in **Fig. 3.2 A** and **B**. In these figures, the plants treated with 50% HNS showed more prolific growth followed by those grown at 25% HNS with the least growth being observed at 12.5% HNS (**Fig. 3.2 A** and **B**). These results clearly show that the availability of nutrients has a substantial influence on the growth of *A. hybridus*.

3.4.2 Effect of NPK deficiency on the growth of Amaranthus hybridus

The results show notable influences on both AGR and RGR for both number of leaves and height of plants. NPK and –N had an influence on AGR and RGR in terms of leaf number of *A. hybridus* with the other treatments (–P and –K) showing significantly lower values (**Fig. 3.1 C**) to mean that lack of these nutrients have a negative effect in terms of AGR and RGR of *A. hybridus*. In terms of the AGR for the height of the plant, none of the treatments had an influence with the exception of –K, (**Fig. 3.1 D**). All the treatments had no significant effects on the RGR for the height of plant (**Fig. 3.1 D**). In the absence of K, the height of plant (AGR) increased significantly compared to the other treatments (**Fig. 3.1 D**). **Fig. 3.2 C** and **D** shows the effect of NPK deficiency on *A. hybridus* plants grown under greenhouse conditions.

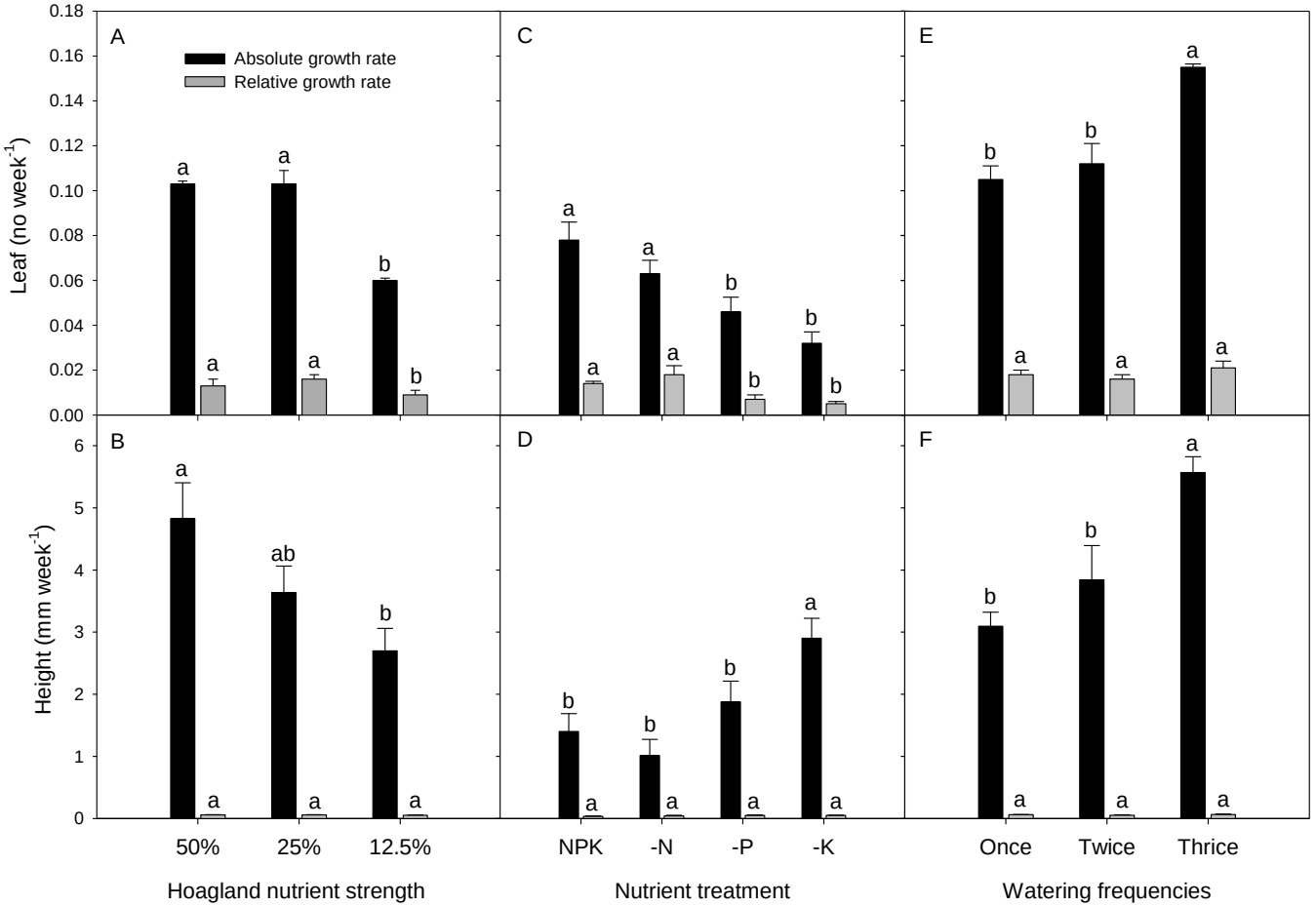


Fig. 3.1: Effect of nutrient strength, nutrient deficiency and watering frequency on absolute growth rate (AGR) and relative growth rate (RGR) of *Amaranthus hybridus*. Bars of absolute growth rate and relative growth rate in each graph with different letter(s) are significantly different according to Duncan's multiple range test ($P < 0.05$).

Fig. 3.2: Effect of nutrient strength (**A** and **B**), NPK deficiency (**C** and **D**) and watering frequency (**E** and **F**) on the growth of *Amaranthus hybridus*.

3.4.3 Effect of watering frequency on the growth of Amaranthus hybridus

From the results shown in **Fig. 3.1 E** and **F**, it is evident that watering frequency had a notable influence on the vegetative growth of *A. hybridus*. The results show a gradual increase in the growth of *A. hybridus* as watering frequency increases from once, to twice and thrice a week. The highest and significant improved growth was recorded at a watering frequency of thrice a week for AGR for both leaf number and height (**Fig. 3.1 E** and **F**). Watering frequency did not affect the RGR for both leaf number and height (**Fig. 3.1 E** and **F**). Plants irrigated three times a week exhibited maximum growth in terms of the number of leaves and height. There was a gradual increase in plant size as irrigation frequencies increased (**Fig 3.2 E** and **F**).

3.4.4 Effect of light intensity on growth of Amaranthus hybridus

The growth of *A. hybridus* was significantly influenced by light intensity (**Fig. 3.3**). Both the AGR and RGR were significantly improved for both leaf number and plant height at a light intensity of 600 µmol m^{-2} s^{-1} (**Fig. 3.3 A-D**). At 450 µmol m^{-2} s^{-1} light intensity, AGR and RGR were significantly greater than at 300 and 150 µmol m^{-2} s^{-1} light intensities for plant height (**Fig. 3.3 B** and **D**). **Fig. 3.4** shows *A. hybridus* plants grown under four light intensities.

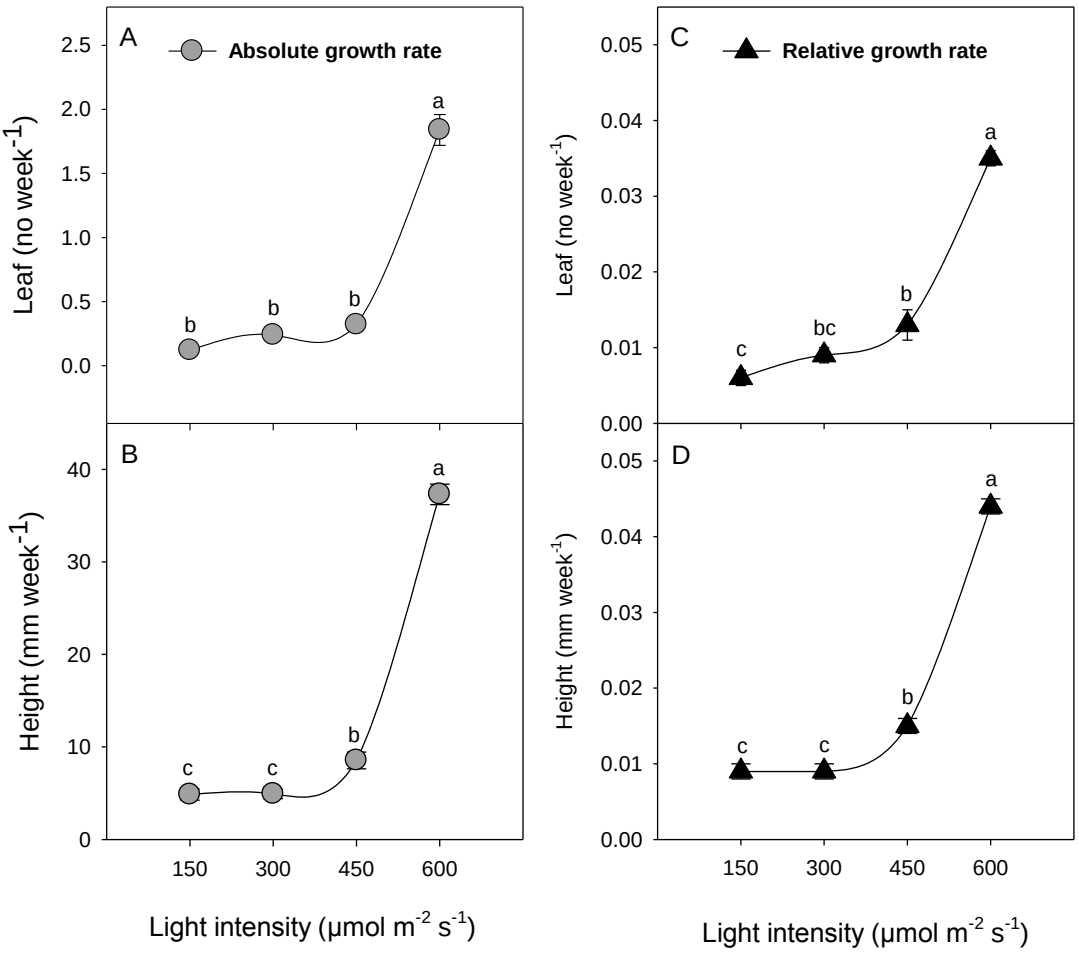


Fig. 3.3: Effect of light intensity on the absolute growth rate (AGR) and relative growth rate (RGR) of *Amaranthus hybridus*. Symbols in each graph with different letter(s) are significantly different according to Duncan's multiple range test ($P < 0.05$).

Fig. 3.4: *Amaranthus hybridus* plants grown at different light intensities. Light intensity values displayed on the pots are in µmol m^{-2} s^{-1}.

3.4.5 Effect of light intensity on the photosynthetic pigments and biochemical composition of Amaranthus hybridus

The effect of varying light intensities on the biochemical composition of *A. hybridus* is shown in **Table 3.1**. Results indicate that the different light intensities did not seem to have any notable influence on the amount of Chlorophyll a since the values obtained were statistically not significantly different when compared to each other. Light intensity of 300 µmol m^{-2} s^{-1} improved the content of Chlorophyll b as well as Chlorophyll a + b (**Table 3.1**). Other light intensities generally showed increasing levels but were not significantly different to the lowest light intensity of 150 µmol m^{-2} s^{-1} in terms of chlorophylls. Light intensities of 450 and 600 µmol m^{-2} s^{-1} enhanced the content of carotenoid, protein and carbohydrate compared to the lowest light intensity tested in *A. hybridus* (**Table 3.1**). Although there was an increment in the carotenoid, protein and carbohydrate contents with light intensity of 300 µmol m^{-2} s^{-1} and above, only the protein content was significantly lower at the lowest light intensity. These results indicate that light intensity has an influence on both photosynthetic pigments and biochemical composition of *A. hybridus*.

Table 3.1: Effect of light intensity on the biochemical composition of *Amaranthus hybridus*.

Light intensity µmol m^{-2} s^{-1}	**Chlorophyll a µg g^{-l} FW**	**Chlorophyll b µg g^{-l} FW**	**Chlorophyll a + b µg g^{-l} FW**	**Carotenoids µg g^{-l} FW**	**Proteins µg g^{-l} FW**	**Carbohydrates µg g^{-l} FW**
150	770 ± 25 a	73 ± 20 b	844 ± 47 b	207 ± 30 b	7.1 ± 0.31 b	25.8 ± 1.77 c
300	855 ± 106 a	338 ± 30 a	1194 ± 137 a	239 ± 8 b	12.5 ± 0.18 a	42.5 ± 10.6 bc
450	915 ± 44 a	189 ± 42 b	1104 ± 83 ab	301 ± 14 a	11.0 ± 1.39 a	57.2 ± 11.9 ab
600	868 ± 34 a	160 ± 42 b	1029 ± 73 ab	334 ± 8 a	12.2 ± 0.64 a	77.3 ± 8.46 a

Mean values (± SE) in a column with different letter(s) are significantly different according to Duncan's multiple range test ($P < 0.05$).

3.5 Discussion

3.5.1 Effect of nutrient strength on the growth of Amaranthus hybridus

The growth of *A. hybridus* was influenced by the amount of nutrients applied, as evidenced by the plants grown under the three different nutrient strengths used for the investigation (**Fig. 3.1 A** and **B**). Maximum growth was observed when the plants were treated with 50% HNS. The results from this experiment concur with those obtained by **KANG and IERSEL (2004)** when they worked with sub-irrigated *Salvia splendens,* where shoot and total dry weight and leaf area increased significantly when HNS concentration was increased from 0.125 to 1.0_X HNS concentration. The possible explanation raised is that the increase in these parameters could have been attributed to carbon allocation within the plant. This could be the same scenario operating in the current investigation with *A. hybridus*. Also, these results are in agreement with those obtained in lettuce plants with increasing electroconductivity (EC) levels by **SUBLETT et al. (2018)** and those of **ABOU-HADID et al. (1995)** and **KANE et al. (2006)** on *Allium cepa* where 50% HNS produced higher total biomass and edible biomass than plants grown in other solutions. **AYI et al. (2019)** obtained similar results with *Alternanthera philoxeroides* where the growth of the plants were affected by the lack of nutrients. This resulted in the relative growth rates of plant fresh weight (RGR_{FW}) and plant stem height (RGR_H) of plants growing in 5% strength standard Hoagland's solution being lower than those of plants growing in 50% and 100% strength solutions. According to **CHAPIN (1991)** there are notable examples of plant responses to deficiencies of different resources and the responses could be morphological or physiological. The most important responses are those which support an increase in resource uptake and efficient use of limited resources **(HERMANS et al., 2006; POORTER et al., 2009)**. Normally plants tend to improve root production and enlarging the root environment interface when there is limited nutrient availability to allow for maximum uptake of nutrients **(HERMANS et al., 2006)**.

3.5.2 Effect of nutrient treatment (NPK) deficiency on the growth of Amaranthus hybridus

It was evident that *A. hybridus* cannot grow well in the absence of P and K since it only showed some growth in plant height but not in terms of number of leaves. The most significant growth for leaf number was achieved when all three mineral elements (NPK) were present (**Fig. 3.1 C**). NPK and –N enhanced the AGR for leaf number but all treatments had no influence on RGR of the height of the plant. These results confirm that all three mineral elements (NPK) are critical for the growth and development of plants. The results of this investigation show that *A. hybridus* generally cannot grow well in the absence of P and K (**Fig. 3.1 C** and **D** and **Fig. 3.2 C** and **D**). The limited growth of *A. hybridus* in this research could be explained in the following manner; although HNS lacking P and K were applied to the plant, the plants managed to grow making use of the scant minerals found in the sand in which the seedlings where grown. According to **CARSTENSEN et al. (2018)** and **MALHOTRA et al. (2018)**, P is an essential macronutrient whose deficiency limits plant growth and productivity. Phosphorus is known to be a structural component of biochemicals such as nucleic acids, sugars and lipids, and also plays a vital role in the developmental processes of plants at both cellular and whole plant level. These processes include seed germination, seedling establishment, root, shoot, flower and seed development, photosynthesis, respiration and N fixation **(MALHOTRA et al., 2018)**. Plants are believed to undergo different adaptations in terms of morphology, physiology and biochemistry in response to P deficiency **(MALHOTRA et al., 2018)**. According to **FRYDENVANG et al. (2015)**, research has shown effects of P deficiency on electron transport to photosystem I (PSI), although the underlying mechanisms are not known. P is a key element found in compounds such as ATP, NADPH, nucleic acids, sugar phosphates and phospholipids, all of which are involved in photosynthesis. This goes a long way in confirming that any marginal shortage of P can have serious consequences on the growth and development of a plant **(WHITE and HAMMOND, 2008)**. Consequently, even marginal P deficiency has a major impact on plant growth and development **(CARSTENSEN et al., 2018)**. An estimated 30% of the global arable soils lack P and require artificial fertilisers to enhance crop yields **(MACDONALD et al., 2011)**, so the research provides evidence that *A. hybridus* has limited growth in

soils with a low P content since they could not increase in both height and number of leaves as demonstrated in this investigation (**Fig. 3.1 C** and **D**).

A. hybridus was seen to increase in height in treated sandy soil treated with K-deficient ½ strength HNS but not for number of leaves and this again shows the vital role played by K in plant growth. Potassium happens to be the second most abundant element absorbed by plant roots, after N **(SINGH et al., 2018)**. Potassium is one of the key elements required for plant growth **(CAKMAK, 2005; WANG et al., 2013)** since it snot only forms a constituent of the plant structure but also has a regulatory function in many biochemical processes involved with protein synthesis, carbohydrate metabolism, and activation of enzymes **(HASANUZZAMAN et al., 2018)**. Several physiological processes depend on K, such as stomatal regulation and photosynthesis, so its shortage can lead to malfunctions of many physiological and biochemical processes **(TU et al., 2017)**. Any deficiency in K results in limitation of growth and yield in crop plants due to the adverse impairment of key processes in plants such as cell turgidity, cell-elongation, transport of assimilates and activation of enzymes, all which has to do with water relations **(PETTIGREW, 2008; RÖMHELD and KIRKBY, 2010)**. In the current investigation, *A. hybridus* was unable to grow well in K-deficient ½ strength HNS- treated soils although it still managed to increase in height. This goes a long way to show the resilience of the plant under different abiotic stresses. **SINGH et al. (2018)** investigated the effects of K on soybean growth and they reported that K-deficiency limited soybean growth traits more than photosynthesis. In this research, *A. hybridus* showed variable growth responses in soil HNS lacking K.

Nitrogen is the most abundant mineral element absorbed by plant roots from the soil and its balance in the environment is vital for the maintenance of life **(WEATHERS et al., 2016)**. It is not surprising that *A. hybridus* could not achieve much growth in terms of plant height in the absence of N since it is a vital element in nucleic acids such as DNA and RNA, the two most important of all biological molecules, vital for all living organisms. The lack of N in plants causes a failure to produce amino acids required for protein synthesis and plant growth. **BRADY et al. (2010)** and **RAZAQ et al. (2017)** found that *Acer mono* plant seedlings treated with N fertilizer exhibited significantly greater levels of Chlorophylls a and b and carotene as well as greater height of plants and diameter of root ($P < 0.05$) than the untreated ones. **VAN AVERBEKE et al.**

(2007) investigated the growth and yield response of *Solanum retroflexum* Dun. (nightshade) and *Brassica rapa* L. subsp*. chinensis* (non-heading Chinese cabbage) to N, P and K availability in the soil. *S. retroflexum* was found to be more sensitive to N availability in the soil and required sufficient N to attain optimal growth. In addition to N, the production of the crop also required adequate supplies of P and K. An optimum availability range for N and K was identified for *B. rapa*, including a critical level of availability for P. **CHEN et al. (2018)** obtained similar results when they measured growth and photosynthetic parameters in *Eustoma grandiflorum* (Raf.) Shinn where the height of plants, number of nodes and leaf area were all reduced under NPK deficiencies. All the above reports demonstrate how critical the elements NPK are, with regards to plant growth and production. These reports concur with findings of this investigation. *A. hybridus* could not achieve optimal growth for both height and growth in the absence of N, P and K.

3.5.3 Effect of watering frequency on the growth of Amaranthus hybridus

Generally, water management is key to obtaining high yields and conservation of irrigation water within the different growth stages of crop plants **(WANG et al., 2017)**. Little water or excess water can affect plant growth negatively since plants require the proper moisture content in the soil **(BERTOLINO et al., 2019; CHILUNDO et al., 2016)**. The results of the investigation showed that watering frequency had a marked influence on the AGR of *A. hybridus*. There was a gradual increase in the AGR even though not significant for once and twice a week frequencies. The watering frequency was highly significant on the AGR of *A. hybridus* for both leaf number and height when applied thrice a week, as can be seen in **Fig. 3.1 E** and **F**, and **Fig. 3.2 E** and **F**. The results of this experiment are in agreement with the results obtained with mini Chinese cabbage (*Brassica pekinensis* cv. “Lvguan F1”) when an investigation was carried out on the effect of irrigation level and irrigation frequency by **XIANG et al. (2019)**. In this investigation, both irrigation frequency and level had a significant effect on the growth and yield of the mini Chinese cabbage. Similar results were obtained by **ISLAM et al. (2018)** on wheat (*Triticum aestivum* L.) when they investigated the effect of irrigation levels on the crop, where they established that the irrigation level and time had a strong influence on morphology, growth and yield. The results also coincide with those of **KHOKHAR et al. (2010)** who reported a higher spike length in wheat irrigated five

times per week. Also, findings of **NGWAKO and MASHIQA (2013)** indicated that continuous irrigation of wheat during the growth stages has a positive effect on the yield of grain. This indicated that when irrigation water increased, yield also increased to a certain point. **SENYIGIT and KAPLAN (2013)** also concluded that when irrigation water increased, so does the yield up to a certain extent, and when the amount of irrigation water is more than that required by the plant, the yield of lettuce decreased. All these findings support the results of the current investigation in which increased watering frequency was observed to improve the vegetative growth of *A. hybridus*.

3.5.4 Effect of light intensity on growth and biochemical composition of Amaranthus hybridus

The results of the above investigation show the significant effects of light intensity on the growth and biochemical composition of *A. hybridus,* as can be seen for light intensities 600 and 450 µmol m^{-2} s^{-1} ($P < 0.05$) and then at 300 and 150 µmol m^{-2} s^{-1}. The results are in agreement with those of **PAN and GUO (2016)** where *Epimedium pseudowushanense* B.L.Guo, a medicinal plant, was exposed to five different levels of light intensity and leaf dry biomass was highest under Level 4 (90.9 ± 2.5 µmol $m^{-2}s^{-1}$) associated with the highest net photosynthetic rate. Leaf areas were higher under low light intensities to maximise light absorption. Light as the original source of energy for plant photosynthesis and growth signals a wide range of signals and growth and information for morphogenesis and many other physiological processes **(CHEN ET AL. 2004)**. Different characteristics of light such as spectral composition (wavelengths), intensity, duration and direction can influence plant growth and development **(NAOYA ET AL., 2008)**. Higher light intensities resulted in more branches than low light intensities and the explanation could be that with more light, more photosynthesis occurred, thereby stimulating the plant to grow more branches and more leaves. Light intensity has a big influence on many different characteristics of plants which include leaf area, number of branches and water content **(DAI et al., 2009)**. Differences in plant morphology for different species are a function of adaptations to varied light environments **(ALERIC and KIRKMAN, 2005)**. **FAN et al. (2013b)** and **TANG et al. (2015)** studied the growth and leaf development in tomato plants under different light intensities and the results showed that fresh weight, dry weight, stem diameter and health index were higher in plants grown under 450 and

550 µmol m^{-2} s^{-1} than those grown under 300 µmol m^{-2} s^{-1}. This again demonstrates the effect of light intensity on growth of plants and concurs with the trend obtained in the current investigation even though plants for the current investigation did not grow well at 300 µmol m^{-2} s^{-1}. A further investigation was carried out by **NGUYEN et al. (2019)** on *Spinacia oleracea* L. to investigate the effect of four different light intensities (90, 140, 190 and 240 µmol m^{-2} s^{-1}) on the growth, photosynthesis and leaf microstructure of spinach. Plant height, leaf number, leaf area, Chlorophyll a, Chlorophyll a + b and photosynthetic capacity were observed to increase with increasing light intensity.

Results from this investigation (**Table 3.1**) show that light intensity influences the amounts of chlorophyll, carotenoids, proteins and carbohydrates in *A. hybridus* when exposed to different light intensities. Findings from this investigation support those obtained by **FENG et al. (2018)** on soybean where different light intensity treatments affected the chlorophyll and carotenoid content of the crop. The amount of pigments increased as light intensity increased. According to the same authors, there is a direct relationship between chlorophyll and carotenoids and alterations in light intensity and this concurs with results of the present investigation. Results from this investigation are similar to those reported by **WITTMANN et al. (2001)** in *Fagus sylvatica* and aspen (*Populus tremula*), and **FAN et al. (2018)** in soybean intercropped with maize, in which there was an increase in chlorophyll content as light intensity was increased. The current results differ from results reported by **LI et al. (2014)** in sorghum. **LI et al. (2014)** claimed that a reduction in light intensity resulted in an increase in chlorophyll, which is opposite to results from the current investigation.

Increase in light intensity enhances the rate of photosynthesis, stomatal conductance, levels of intercellular carbon dioxide and the rate of transpiration. The promotion of growth in the crop could be a result of the increased gain of carbon due to the improvement of photosynthetic parameters **(LIAO et al., 2006)**. The increase in carbohydrates as light intensity increases is due to an improvement of the assimilation rate of carbon dioxide (sucrose and starch production) in response to the improvement of morphological parameters and enzymatic activities involved in the process **(FENG et al., 2018)**. Also, plant responses to changes in light intensity vary from one variety to another and between crop species **(FENG et al., 2018)**. Similar results were obtained by **POORTER et al. (2019)** where they determined how 70 traits related to

plant anatomy, morphology, chemistry, physiology, growth and reproduction are affected by daily light integral (DLI; mol photons m^{-2} d^{-1}). **POORTER et al. (2019)** also observed that the carbohydrate content increased with an increase in DLI. From a database of 500 experiments involving 760 plant species, they were able to determine dose-response curves and discovered that most of the traits increased with DLI in a saturating way with some increasing by ten-fold over the DLI range of 1-50 mol m^{-2} d^{-1}. These results are further supported by **MONOSTORI et al. (2018)** in hexaploid wheat (*T. aestivum* ssp. *aestivum* cv. 'Mv Kikelet') where elevated light intensities achieved using LEDs enhanced the rate of photosynthesis, the number of tillers, biomass and yield. According to **FAN et al. (2013b)**, blue and red irradiations play a vital role in photosynthesis and also stimulate the biosynthesis of chlorophyll and carotenoids. This could be another explanation of the results obtained in this investigation. Results demonstrate the dynamic nature of light intensity to which plant species respond and change at differing time scales, and from season to season **(ASSMANN and WANG, 2001)**. All these illumination dynamics have also been observed in major crop species such as soybean (*Glycine max*; **PEARCY et al. (1997)**, rice (*Oryza sativa*; **NISHIMURA et al. (2000)** and maize (*Zea mays*; **WANG et al. (2008)** through experiments and modelling approaches **(SLATTERY et al., 2018)**.

It can be concluded that light intensity influences the amount of chlorophyll and carotenoids in *A. hybridus,* as shown by the results of the current investigation. There was a substantial increase in all the photosynthetic pigments assessed to show that as light intensity was increased it also enhanced the rate of photosynthesis, resulting in increased amounts of photosynthates.

CHAPTER 4. Effects of organic biostimulants on the growth and biochemical composition of *Amaranthus hybridus* L.

4.1 Introduction

By definition, biostimulants are substances and microorganisms that have been discovered to regulate plant growth in a number of ways **(TARANTINO et al., 2018)**. Research has shown their ability to enhance the following parameters in plants; plant growth, nutrition efficiency, abiotic stress tolerance and crop quality traits **(VAN OOSTEN et al., 2017)**. These positive attributes of biostimulants could be used to promote the commercial cultivation of neglected and underutilised traditional leafy vegetables such as *Amaranthus hybridus*. Many neglected and underutilized vegetables have the potential to bring diversity in human diets and increase levels of food production thereby bringing about agro- and horti-food systems, which are more sustainable and resilient **(BALDERMANN et al., 2016)**. Traditional vegetables are cheaper and rich sources of vitamins and other health-promoting nutrients **(BUA and ONANG, 2017)**. Vegetable consumption confers palatability and taste to diet, thereby enhancing appetite. Vegetables also provide fibre which is an important component of a balanced diet. Vegetables have many health benefits since most of them are known to be rich sources of secondary metabolites, which play a crucial role in the prevention of diseases. It is for this reason that plants (fruits and vegetables) are also valued for their medicinal attributes in many cultures where they play a pivotal role in folklore medicine **(BUA and ONANG, 2017)**.

The main cause of the development and progression of a number of diseases is oxidative stress **(KASOTE et al., 2015)**. Several metabolic reactions in the human body produce free radicals such as reactive oxygen species (ROS) as by-products with undesirable effects **(RAVIMANNAN and NISANSALA, 2017)**. Plants are able to produce a variety of secondary metabolites classified as antioxidants that can alleviate oxidative damage caused by ROS. Antioxidants attenuate ROS-induced oxidative damage by inhibiting the oxidation of oxidizable substrates found at concentrations much lower than the substrate **(RAVIMANNAN and NISANSALA, 2017)**. Plants are rich sources of antioxidants and two-thirds of global plant species are believed to have

medicinal value, with most having excellent antioxidant attributes **(KRISHNAIAH et al., 2011)**. An antioxidant is defined as a substance that protects the oxidation of substrates which can be oxidised when its concentration is lower than that of an oxidizable substrate. Antioxidants work individually or in synergy in enhancing cellular defences against oxidative stress caused by various ROS and reactive nitrogen species (RNS) **(LÜ et al., 2010; YOUNG and WOODSIDE, 2001)**. There are two main categories of antioxidants; natural enzymatic and non-enzymatic. Natural enzymatic antioxidants include enzymes such as superoxide dismutase (SOD), (located in chloroplasts, mitochondria, peroxisomes and/or the cell wall) and catalases (located in peroxisomes). Vitamin E, Vitamin C, butylated hydroxytoluene (BHT), BHA butylated hydroxyanisole (BHA), carotenoids, glutathione and its derivatives, phenolic compounds, flavonoids and alkaloids fall under non-enzymatic and/or synthetic antioxidants **(ALSCHER et al., 2002; MOUSSA et al., 2019)**. Superoxide dismutases (SOD) constitute the first line of defence against ROS in plant cells **(ALSCHER et al., 2002)**. They are responsible for the removal of charged oxygen molecules (O_2^-) from mitochondrial compartments where they are produced. Charged oxygen molecules (O_2^-) are not able to pass through the phospholipid membranes **(TAKAHASHI and ASADA, 1983)**.

Antioxidants have been discovered to reduce chronic health disorders such as cardiac problems, aging process and also cancers associated with the respiratory tract, alimentary canal, lungs, bladder and breast **(HAJHASHEMI et al., 2010; HARASYM and OLEDZKI, 2014)**. The main focus of workers in the fields of functional foods and nutraceuticals is now centred on the detection of secondary metabolites which are beneficial to health and which act as antioxidants **(LUIS et al., 2006)**.

1. The objective of this study was to investigate the effects of various biostimulants, [smoke-water (SW), smoke-isolated karrikinolide (KAR_1), seaweed-based Kelpak® (KEL), earthworm-derived vermicompost leachate (VCL) and a seaweed-isolated bioactive compound eckol (ECK)] and the method of application on growth and biochemical composition of *A. hybridus* in order to promote its adoption and cultivation in mainstream crop production. As part of this investigation, the effect of biostimulants + nutrients on the growth of the plant was also investigated. Also in the same investigation, the effect of organic biostimulants and the method of application on antioxidant activity was

assessed. Antioxidants which were targeted in this research were; total phenolics, condensed tannins, flavonoids and beta-carotene oxidation.

2. 4.2 Materials and methods

3. *4.2.1 Site of experiment*

4. The seed germination experiments were carried out under laboratory conditions using an incubator in the Research Centre for Plant Growth and Development and the pot experiment was carried out in a greenhouse at the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg Campus (290 37.55' S; 300 24.13' E).

4.2.2 Plant material

Amaranthus hydridus seeds were obtained from McDonalds Seed Company in Pietermaritzburg, South Africa.

4.2.3 Seed germination experiments

Seeds were first subjected to different temperatures and light conditions and treated with different biostimulants to determine optimal germination. Twenty-five seeds were placed in 65 mm plastic Petri dishes lined with two sheets of Whatman No. 1 paper and moistened with 3 ml of different concentrations of biostimulants; SW 1:500 v/v , KAR_1 10^{-6} M, VCL 1:5 v/v, KEL 0.8% and ECK 10^{-8} M, GA 10^{-8} M and water were used as the positive and negative controls respectively and incubated in plant growth chambers at 25 ± 2 °C under 16:8 fluorescent tube light (80 µmol m^{-2} s^{-1}) and dark conditions. Another set was subjected to 24 h dark. Seeds treated with biostimulants were placed in light-proof wooden boxes and inspected daily under green “safe light” (0.3 µmol m^{-2} s^{-1}). This experiment was carried out for 14 days with seed germination being recorded daily. The seed was considered germinated when radicles had protruded 2 mm.

4.2.4 Biostimulants and chemicals

Smoke-water and KAR_1 solutions were prepared according to previously described methods **(BAXTER et al., 1994; FLEMATTI et al., 2004; VAN STADEN et al., 2004; GUPTA et al., 2020)**. Kelpak® [Kelp Products International (Pvt) Ltd, Simon's Town, South Africa] was supplied by the company and was prepared as prescribed on the product label. Vermicompost leachate was purchased from Wizzard Worms (a commercial supplier), Greytown, South Africa and ECK was extracted, isolated and identified from the ethyl acetate fraction of the seaweed *Ecklonia maxima* **(RENGASAMY et al., 2016a)**. Gibberellic acid (GA) and water (C) were used as the positive and negative controls respectively. Fifty percent Hoagland Nutrient solution was prepared using the method described by **ARNON and HOAGLAND (1952)**. Folin and Ciocalteu phenol reagent, gallic acid (3,4,5-trihydroxibenzoic acid), vanillin (4-hydroxyl-3 methoxybenzaldehyde), catechin, DPPH (2,2-diphenyl-1-picryl hydrazyl), rhodanine, β-carotene, and diosgenin were obtained from Sigma-Aldrich Co. (Steinheim, Germany); ferric ammonium sulfate, sodium nitrite, sodium hydroxide, aluminium chloride, sodium hydrogen carbonate, BHT, and potassium ferricyanide from BDH Chemicals Ltd (Poole, England); harpagoside from Extrasynthèse (Genay, France); trichloroacetic acid, ascorbic acid (ASC), Tween 20 (polyoxyethylene sorbitan monolaurate, surfactant), ferric chloride, chloroform, n-butanol and methanol from Merck KGaA (Darmstadt, Germany). All chemicals used in the assays were of analytical grade.

4.2.5 Experimental design and greenhouse conditions

Amaranthus seeds were sown directly in 15 cm diameter pots containing potting soil mixture (described in Chapter 3, Section 3.5.1). Upon germination, the seedlings were thinned leaving a single seedling in each experimental pot and were grown in a completely randomised design with 15 replicates per treatment. After 14 days of seedling growth, the biostimulants were applied once a week with three different modes of application viz. drenching (50 ml), foliar spray [with a handheld spray bottle to the solution (2 to 3 drops of Tween 20 were added) runoff point] and a combination of drenching and foliar spray. The pots were placed on a metal bench in a greenhouse with an average midday photosynthetic photon flux density ranging from 550-600 µmol

m^{-2} s^{-1} at 24 ± 2 °C with 60 ± 5% relative humidity. All experimental pots were monitored on a daily basis. The seedlings were irrigated twice weekly with water except on the day of biostimulant treatment. The number of leaves and plant height were recorded on the seventh, fourteenth and twenty-first day. The plants were harvested after six weeks and the number of leaves and roots were counted, shoot and root length were measured, fresh/dry weight was recorded for both shoot and root. The leaf area was measured using a leaf area meter (Li-3100, LI-COR Inc., Lincoln, NE, USA) and stem thickness with Vernier callipers. Photosynthetic pigments (chlorophylls and carotenoids), proteins and carbohydrates were determined at harvest as described in the previous chapter. Plants were dried in an oven (Memmert, UF55plus, Germany) at 50°C for determining shoot and root dry weight. Absolute growth rate, relative growth rate and leaf area ratio was calculated **(WAREING and PHILIPS, 1981)** as follows:

Absolute Growth Rate (AGR)

AGR = dn/dt

$= n_2 - n_1/t_2 - t_1$

Relative Growth Rate (RGR)

RGR= $dn/dt \times 1/n$

$= \ln n_2 - \ln n_1/t_2-t_1$

Where n_1 and n_2 = number, size (height, leaf area) at time t_1 and t_2

ln = natural logarithm

Leaf area ratio (LAR)

Over any time interval, LAR = $LA_2 – LA_1/ W_2 – W_1$

Where LA = Leaf area and W = plant dry weight

4.3 Antioxidant activity assays

4.3.1 Ferric cyanide (Fe^{3+}) Reducing Antioxidant Power (FRAP)

The FRAP assay described by **PULIDO et al. (2000)** was used to determine the antioxidant potential of *A. hybridus* extracts. A potential antioxidant reduces ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}). The ferric ion reagent consists of 20 mmol.L^{-1} TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol.L^{-1} HCl, 20 mmol.L^{-1} $FeCl_3 \cdot 6H_2O$ and 300 mmol.L^{-1} acetate buffer pH 3.6 in the ratio of 1:1:10. 900 µL of FRAP reagent was added to 30 µL of sample extract and the mixture was made up to 1 mL with distilled water. The reaction mixture solution was vigorously shaken and incubated for 30 min before reading the absorbance at 517 nm. Methanol solutions of $FeSO_4 \cdot 7H_2O$ were prepared as a standard curve between 10 to 100 µmol.L^{-1}. Results were expressed as mmol Fe^{2+} equivalents per gram of dry extract.

4.3.2 DPPH (1-1-diphenyl-1picryhydrazyl) radical scavenging activity

The ability of both water and methanolic extracts of *A. hybridus* treated with different biostimulants to scavenge the DPPH radical was estimated using the method described by **KARIOTI et al. (2004)** with some modifications. Sample extracts or standard antioxidants were made by suspending in 50% methanol to known concentrations starting with highest concentration at 50 mg/mL. Each sample was diluted with 735 µL of 50% methanol and then added to 750 µL (50 µM in methanol) of freshly prepared methanolic DPPH solution (0.1 mM) in a brown Schott bottle, making a final volume of 1.5 mL. The reaction was performed under dim light and incubated in a dark room for 30 min. The absorbance of the mixture was recorded at 517 nm with absolute methanol as the blank using a Varian Cary 50 UV-visible spectrophotometer (Varian, Australia) so as to measure the discolouration of the purple colour of DPPH. Ascorbic acid and BHT, which are standard antioxidants, were used as the positive controls. The negative control was prepared by replacing the sample extract with 50% methanol. To complete background correction of sample absorbance (DPPH absent), absorbance readings for methanol only were subtracted from corresponding readings obtained with DPPH present. Assays were performed in

triplicate. The following formula was used to calculate the free radical scavenging activity (RSA):

$$\%RSA = 100 \times 1 - A_E/A_D$$

In which A_E is the absorbance of the reaction mixture containing the sample extract or standard antioxidant and A_D is the absorbance of the negative control. The normalised logarithmic regression curve derived from the plot data was used to calculate the IC_{50} values. Using the formula by **SCHERER and GODOY (2009)**, the DPPH antioxidant activity was further expressed as the antioxidant activity index (AAI).

$$AAI = \text{Final DPPH concentration}/EC_{50}$$

4.3.3 β-carotene-linoleic acid (BCA) model system

Methods described by **AMAROWICZ et al. (2004)** were used to determine the coupled inhibition of β-carotene and linoleic acid oxidation. The procedure involved the dissolving of dried *A. hybridus* extracts and BHT (positive control) in 50% aqueous methanol to a known concentration of 7 mg/mL. β-carotene was dissolved in 10 mL of chloroform in a brown Schott bottle with the excess chloroform being allowed to evaporate under vacuum. This resulted in a thin layer of β-carotene being formed. Linoleic acid (200 µL) and Tween 20 (2 mL) were immediately added to the thin layer of β-carotene and mixed with aerated distilled water (497.8 mL) to give a final concentration of 20 µg/mL of βeta-carotene. Vigorous agitation of the reaction mixture produced an orange-coloured emulsion caused by saturated oxygen. The emulsion (4.8 mL) was dispensed in a test tube then samples of *A. hybridus* extracts or BHT (200 µL, 7 mg/mL) were added giving a final concentration of 280 µg/mL of reaction mixture. Absorbance at 470 nm of reaction mixture was immediately measured at time, t = 0 with Tween 20 as the blank. At 30 min intervals, subsequent absorbance readings were taken for 180 min with samples being incubated at 50°C in a water bath. The negative control was prepare replacing sample extract with 50% methanol. The rate of β-carotene bleaching was calculated using the equation:

$$\text{Rate of β-carotene bleaching} = [\ln (A_{t\,=\,0}/A_{t\,=\,t}] \times 1/t$$

Where $A_{t\,=\,0}$ is emulsion absorbance at 0 min; $A_{t=t}$ is absorbance at time, t (30, 60, 90, 120 min). Calculated average rates of β-carotene bleaching was based on rates at 30,

60 and 90 min. The antioxidant activity (ANT) of the sample was then determined using the calculated average rate and expressed as a percentage using the formula:

$$\%ANT = (R_{control} - R_{sample} / R_{control}) \times 100$$

Where $R_{control}$ and R_{sample} are the respective average β-carotene bleaching rates of negative control and plant extracts. To further express the antioxidant activity of sample extracts, the oxidation rate ratio (ORR) was calculated using the formula:

$$ORR = R_{sample} / R_{control}$$

The antioxidant activity (AA) was calculated according to **BRACA et al. (2003)** based on the coupled inhibition of β-carotene and linoleic acid oxidation against a negative control at t= 60 min and t = 120 min, using the following formula:

$$\%AA = [1- (A_0 - A_t) / (A_{00}- A_{0t})] \times 100$$

Where A_0 is the initial absorbance of sample before incubation; A_t is absorbance at time t = 60 and 120 min for sample extract; A_{00} and A_{0t} represent the absorbance of the negative control at start of incubation and at time t = 60 and 120 min respectively.

%ANT, ORR and % AA were then calculated

4.4 Quantification of polyphenolic compounds

4.4.1 Total flavonoid content determination

The aluminium chloride colorimetric assay of **MAKKAR et al. (2000)** was used to measure the total flavonoid content. The different *A. hybridus* filtrates of 250 µL were individually diluted with distilled water in a test tube to attain a 1 mL volume. To the mixture, 5% (w/v) sodium nitrate (75 µL), 10% (w/v) aluminium chloride (75 µL), 1 M sodium hydroxide (500 µL) and 0.6 mL of distilled water were also added. The reference compound used was Catechin (CA). The absorbance of the reaction mixture was measured immediately at 510 nm using a Cary UV-visible spectrophotometer (Varian, Australia) with 50% aqueous methanol as the blank. A standard calibration curve was obtained using the same procedure described above for the *A. hybridus* filtrates, to the standard solution of CA. Samples were done in triplicate. The total flavonoid content was expressed as mg/mL Catechin equivalent (CAE) per mg/g DW

of crude extracts of the different treatments of *A. hybridus*. CAE values were expressed as X ± SE of the performed triplicate reactions.

4.4.2 Total phenolic content determination

The Folin-Ciocalteu (Folin-C) colorimetric method of **SINGLETON and ROSSI (1965)** was used to determine the total phenolic contents (TPC) spectrophotometrically. Choice of method was due to its high sensitivity and that it is reproducible **(MAKKAR et al., 2007)**. A reaction mixture containing 50 µL of sample filtrates, 950 µL of distilled water, 500 µL of 1N Folin-C phenol reagent and 2.5 mL of 2% (w/v) sodium carbonate was made. Reaction mixture was incubated at room temperature for 40 min. The standard for this reaction was (0.1 mg/mL) gallic acid, using 50% aqueous methanol as the blank. The absorbance of the reaction mixture was measured at 725 nm using a Cary UV-visible spectrophotometer (Varian, Australia). The assay was done in triplicate. Applying the same procedure to the standard solution of gallic acid a standard curve was obtained. The total phenolic content was expressed as mg/mL gallic acid equivalents (GAE) with the GAE values being given as X ± SE of the three replicates done.

4.4.3 Determination of condensed tannins

Condensed tannins were quantified using the method described by **(MAKKAR et al., 2000)** with minor modifications. To 500 µL of each sample, 3 mL of butanol-HCl reagent (95:5 v/v) were added followed by the addition of 0.1 mL of ferric reagent (0.2% [w/v] ferric ammonium sulfate in 2N HCl). The mixture was further vortexed and incubated in a boiling water bath for 1 h. The standard for this method was cyanidin chloride (CC). The absorbance of the mixture after incubation was measured at 550 nm using a Cary UV-visible spectrophotometer (Varian, Australia) with an unheated mixture of 500 µL, butanol-HCl reagent (3 mL) and ferric reagent (100 µL) as the blank. The assay was done in triplicate with condensed tannins being expressed as cyanidin chloride equivalent mg/mL (CCE) per mg/g DW of crude extracts.

4.5 Statistical analysis

The quantification of all parametric data was done in replicate and results presented as mean ± standard error. Mean value comparison was computed using one-way analysis of variance (ANOVA) using SPSS for Windows (SPSS, Version 24.0. Armonk, New York, USA). Duncan's multiple range test was utilised for statistical significance ($P \leq 0.05$) to separate the mean values**.** General analysis of variance was computed for main effects and their interactions.

4.6 Results

4.6.1 Effect of biostimulants on Amaranthus hybridus *growth*

Different organic biostimulants and different methods of application of the biostimulants influence the growth of *A. hybridus* in many different ways as shown in **Table 4.1**. Application of the different organic biostimulants via drenching resulted in only two organic biostimulants enhancing the growth in some parameters of *A. hybridus.* KAR_1 significantly improved the shoot length, shoot fresh weight and the dry shoot weight of the plant (390 ± 26 mm, 6.723 ± 0.03 and 0.878 ± 0.006 g) respectively. KEL notably improved the number of leaves (20.7 ± 2.2). Most of the treatments had a negative influence on growth parameters and yielded lower values compared to the control. Foliar application of VCL on *A. hybridus* resulted in a significant increase in most of the growth parameters. VCL increased leaf area, fresh shoot and root weights and dry shoot weight. Most of the biostimulants affected the growth of *A. hybridus* in a negative manner for some parameters with some having no or minimal improvement for other parameters. The third method of application was a combination of both drenching and foliar application and again for this treatment, only two biostimulants yielded positive results by improving some of the growth parameters. VCL increased the root length (47.1 ± 3.4 mm), leaf area (84.2 ± 7.7), both shoot and root fresh weight (4.865 ± 0.015 and 0.880 ± 0.016) g respectively as well as shoot dry weight (1.338 ± 0.022 g). On the other hand, KEL significantly improved all the parameters under observation apart from shoot length, yielding; 22.2 ± 3.0 for leaf number, 6.0 ± 1.7 for root number, 45.3 ± 6.2 mm for root length, 3.9 ± 0.04 mm for stem thickness, 86.9 ±

15.0 cm^2 for leaf area, 4.893 ± 0.009 g for shoot fresh weight, 1.052 ± 0.010 g for root fresh weight, 0.882 ± 0.011 g for shoot dry weight and 0.320 ± 0.013 g for root dry weight. The effects of the other treatments were generally either neutral and did not differ from the control or yielding negative values which were lower than the control.

Table 4.1: Effect of different applications of biostimulants on the growth of *Amaranthus hybridus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3). [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Leaves (no.)	Root (no.)	Shoot length (mm)	Root length (mm)	Stem thickness (mm)	Leaf area (cm^2)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Drenching										
Control	17.4 ± 1.7 ab	4.5 ± 0.7 a	301 ± 24.4 b	51.0 ± 3.2 a	4.4 ± 0.3 a	61.9 ± 11.4 a	5.066 ± 0.041 c	0.895 ± 0.014 a	0.466 ± 0.013 b	0.318 ± 0.015 a
SW 1:500 (v/v)	13.9 ± 1.4 b	3.7 ± 0.6 a	312 ± 34.9 ab	39.6 ± 4.1 b	4.2 ± 0.1 a	61.6 ± 6.2 a	4.467 ± 0.020 e	0.488 ± 0.007 d	0.524 ± 0.006 c	0.194 ± 0.001 c
KAR_1 (10^{-6} M)	16.6 ± 1.0 ab	4.4 ± 0.4 a	390 ± 26.4 a	51.0 ± 3.4 ab	4.2 ± 0.1 a	83.3 ± 10.0 a	6.723 ± 0.031 a	0.871 ± 0.008 a	0.878 ± 0.006 a	0.265 ± 0.003 b
VCL 1:5 (v/v)	15.7 ± 0.8 b	4.9 ± 0.6 a	282 ± 25.6 b	47.1 ± 2.3 ab	3.8 ± 0.1 a	68.7 ± 6.2 a	4.847 ± 0.024 d	0.631 ± 0.017 c	0.636 ± 0.010 b	0.205 ± 0.002 c
KEL (0.8%)	20.7 ± 2.2 a	4.7 ± 0.8 a	313 ± 33.9 ab	43.5 ± 3.2 ab	4.3 ± 0.1 a	65.2 ± 9.1 a	5.446 ± 0.028 b	0.785 ± 0.010 b	0.620 ± 0.004 b	0.240± 0.006 bc
ECK (10^{-8} M)	15.1 ± 1.5 b	3.8 ± 0.5 a	272 ± 24.2 b	43.3 ± 2.7 ab	3.6 ± 0.1 a	65.8 ± 9.9 a	4.553 ± 0.022 e	0.539 ± 0.008 d	0.625 ± 0.006 b	0.211 ± 0.005 c
GA (10^{-6} M)	15.7 ± 1.2 b	3.2 ± 0.5 a	288 ± 25.9 b	44.0 ± 3.1 ab	3.5 ± 0.1 a	54.6 ± 11.3 a	4.593 ± 0.029 e	0.622 ± 0.012 c	0.591 ± 0.002 b	0.225 ± 0.003 bc
Foliar										
Control	26.0 ± 2.3 ab	8.4 ± 1.2 ab	386 ± 30.0 ab	46.8 ± 2.6 bc	4.9 ± 0.01 a	78.3 ± 10.6 b	9.388 ± 0.030 b	0.945 ± 0.010 d	1.184 ± 0.004 b	0.297 ± 0.001 ab
SW 1:500 (v/v)	23.1 ± 2.4 abc	6.4 ± 1.0 ab	401 ± 38.4 ab	50.6 ± 4.0 ab	5.1 ± 0.01 a	92.1 ± 14.5 ab	9.528 ± 0.021 b	1.409 ± 0.017 c	1.259 ± 0.011 ab	0.316 ± 0.006 ab
KAR_1 (10^{-6} M)	19.2 ± 1.2 c	5.3 ± 0.8 b	320 ± 31.2 b	46.6 ± 2.7 bc	4.1 ± 0.02 a	67.9 ± 10.0 b	6.403 ± 0.017 c	0.940 ± 0.006 d	0.830 ± 0.011 c	0.225 ± 0.001 c
VCL 1:5 (v/v)	27.8 ± 2.3 a	8.9 ± 1.6 a	478 ± 24.5 a	56.2 ± 1.9 a	5.0 ± 0.03 a	116 ± 16.5 a	10.647 ± 0.026 a	1.687 ± 0.020 a	1.338 ± 0.022 a	0.337 ± 0.009 a
KEL (0.8%)	22.2 ± 1.6 abc	5.6 ± 0.8 ab	322 ± 33.6 b	39.0 ± 3.0 c	4.4 ± 0.03 a	61.5 ± 8.1 b	6.251 ± 0.030 c	0.942 ± 0.010 d	0.630 ± 0.009 d	0.237 ± 0.009 c
ECK (10^{-8} M)	20.8 ± 2.1 bc	7.3 ± 1.1 ab	395 ± 34.3 ab	45.2 ± 2.7 bc	5.2 ± 0.04 a	96.8 ± 13.0 ab	9.594 ± 0.051 b	1.531 ± 0.018 b	1.169 ± 0.014 b	0.306 ± 0.006 ab
GA (10^{-6} M)	19.4 ± 1.6 c	5.0 ± 0.7 b	322 ± 30.4 b	45.1 ± 3.3 bc	4.4 ± 0.03 a	62.3 ± 9.6 b	5.651 ± 0.023 d	0.943 ± 0.003 d	0.706 ± 0.011 d	0.272 ± 0.008 bc
Drenching and Foliar										
Control	13.3 ± 1.2 b	2.7 ± 0.4 b	227 ± 22.7 ab	32.9 ± 3.7 b	2.9 ± 0.01 b	52.3 ± 6.7 c	3.514 ± 0.045 c	0.514 ± 0.010 d	0.445 ± 0.009 c	0.193 ± 0.006 bc
SW 1:500 (v/v)	13.6 ± 1.2 b	2.4 ± 0.5 b	222 ± 27.0 ab	29.3 ± 3.3 b	3.0 ± 0.02 b	45.5 ± 7.4 c	3.242 ± 0.023 d	0.486 ± 0.006 d	0.386 ± 0.007 c	0.190 ± 0.002 bc
KAR_1 (10^{-6} M)	16.2 ± 1.4 b	2.3 ± 0.4 b	261 ± 16.8 ab	38.0 ± 2.3 ab	3.0 ± 0.02 b	59.1 ± 7.4 bc	4.262 ± 0.018 b	0.740 ± 0.020 c	0.436 ± 0.006 c	0.232 ± 0.009 bc
VCL 1:5 (v/v)	14.4 ± 0.6 b	2.2 ± 0.3 b	297 ± 18.7 a	47.1 ± 3.4 a	3.4 ± 0.01 ab	84.2 ± 7.7 ab	4.865 ± 0.015 a	0.880 ± 0.016 b	0.668 ± 0.016 b	0.242 ± 0.005 b
KEL (0.8%)	22.2 ± 3.0 a	6.0 ± 1.7 a	296 ± 31.2 a	45.3 ± 6.2 a	3.9 ± 0.04 a	86.9 ± 15.0 a	4.893 ± 0.009 a	1.052 ± 0.010 a	0.882 ± 0.011 a	0.320 ± 0.013 a
ECK(10^{-8} M)	14.6 ± 1.1 b	2.1 ± 0.4 b	197 ± 27.0 b	32.0 ± 2.5 b	2.7 ± 0.02 b	51.9 ± 11.7 c	3.368 ± 0.023 cd	0.465 ± 0.009 d	0.384 ± 0.004 c	0.202 ± 0.002 bc
GA (10^{-6} M)	15.7 ± 1.1 b	1.9 ± 0.3 b	288 ± 25.9 a	29.0 ± 2.7 b	1.9 ± 0.01 c	33.5 ± 5.0 c	2.238 ± 0.021 e	0.314 ± 0.015 e	0.242 ± 0.006 d	0.180 ± 0.010 c

Mean values (± SE) in a column for each application with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

4.6.2 Effect of different application methods of biostimulants on the growth of Amaranthus hybridus

The statistical and graphical analysis of combined results of plant dry and fresh weight and plant height and of leaf number, plant height and leaf area ratio are shown in **Fig. 4.1** and **4.2** respectively. The KAR_1 drenching significantly increased plant height, plant fresh and dry weight compared to both negative (water) and positive (GA) control (**Fig. 4.1**). Similar results were obtained when VCL was applied via the leaves (foliar application) which significantly improved the height of the plant and plant fresh weight and dry weight. Results of a combination of drenching and foliar application show the significant influence of VCL and KEL on plant height and plant fresh weight (**Fig. 4.1**) as well as the significant improvement on plant height, plant fresh weight and plant dry weight by KEL (**Fig. 4.1**) compared to the control. The following parameters, leaf number, plant height and leaf area ratios were used to calculate AGR and RGR and from the results obtained in **Fig. 4.2**, KEL applied via both drenching and combined drenching and foliar application had a significant influence on the AGR as shown in **Fig. 4.2**. Plant height per week was increased by KAR_1 application via drenching.

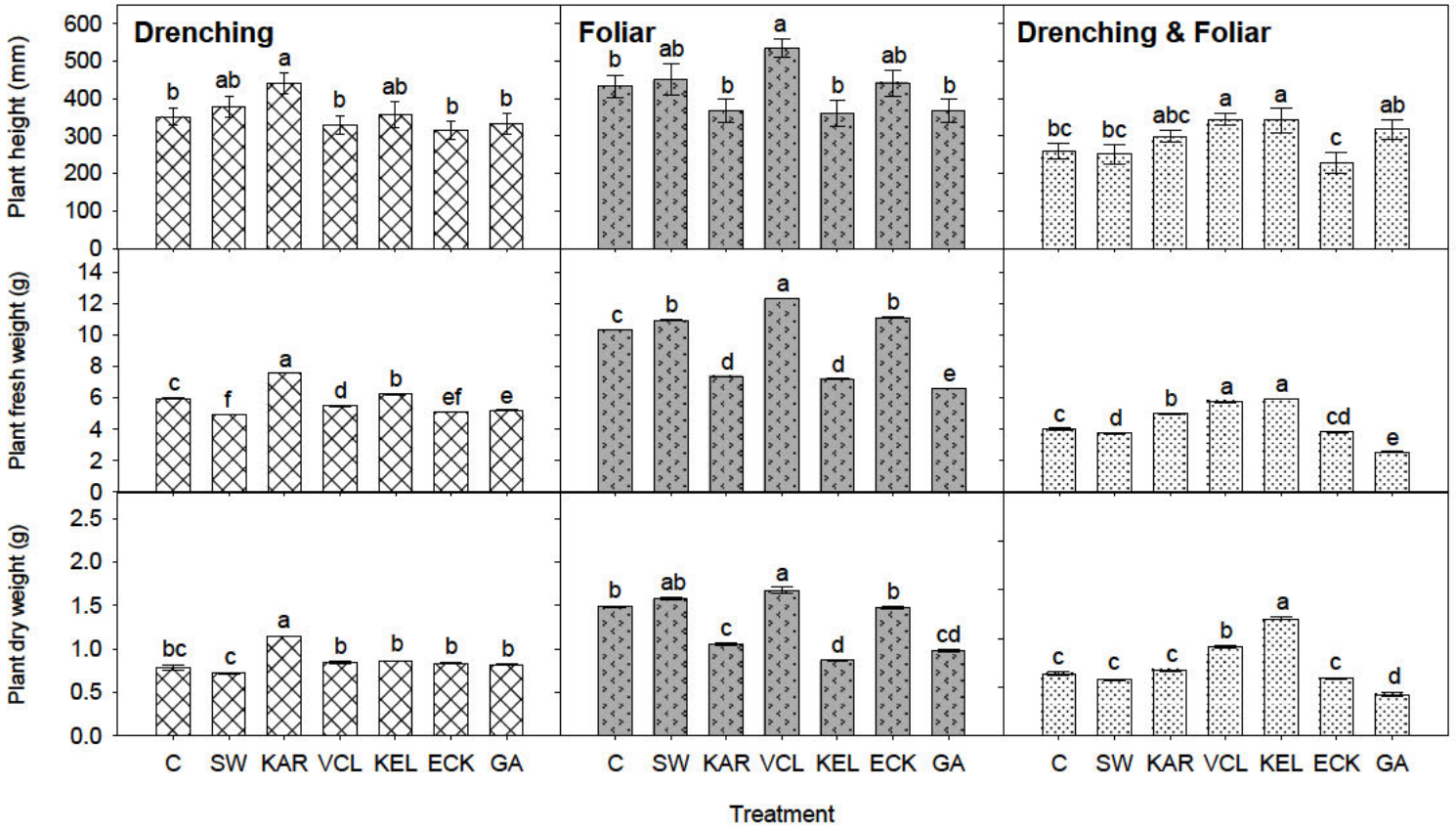


Fig. 4.1: Effect of different application methods of biostimulants on the growth of *Amaranthus hybridus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after six weeks (n = 5; rep = 3). [Control (water), C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

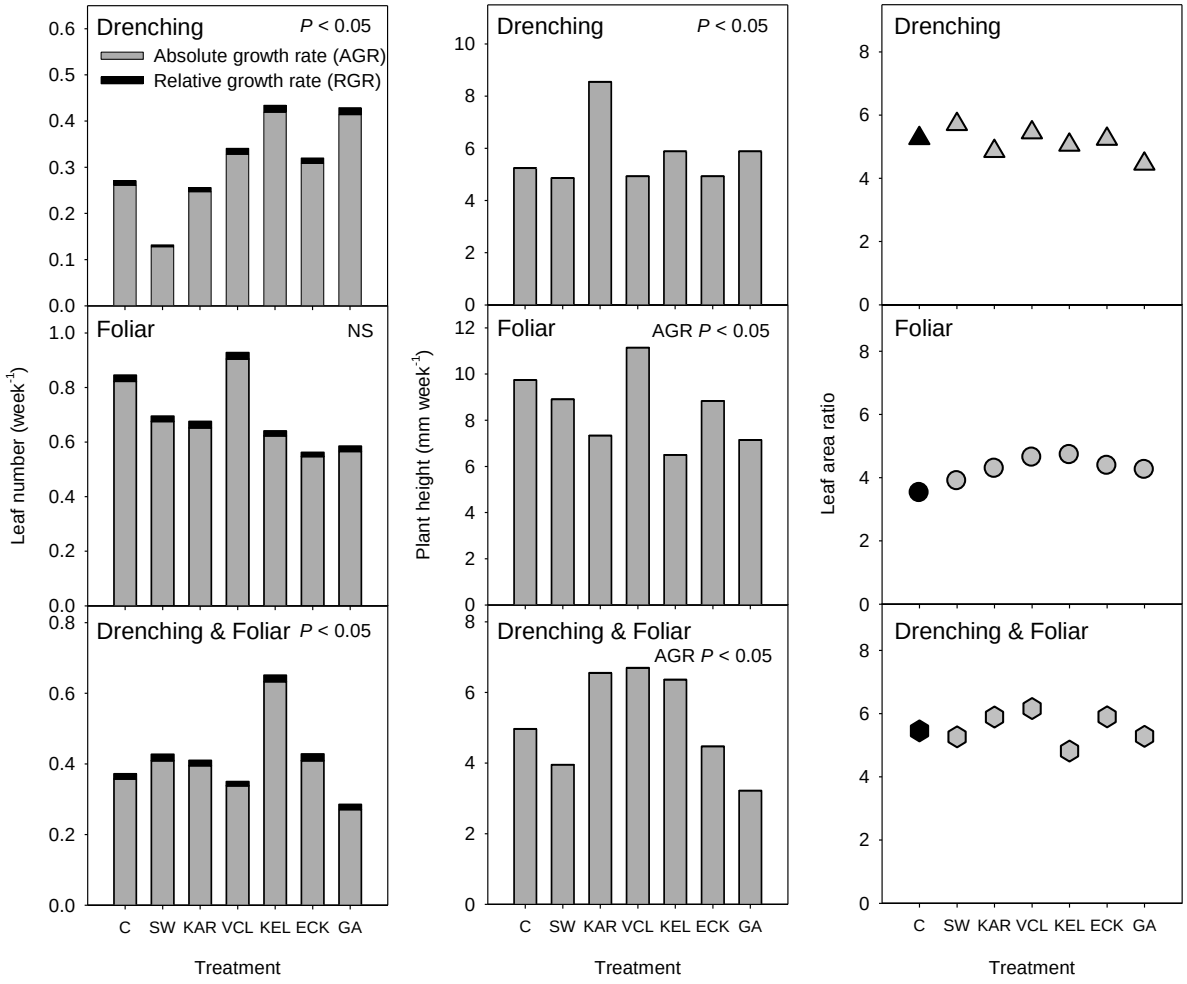


Fig. 4.2: Effect of different organic biostimulants on the absolute and relative growth rate (leaf number and plant height) and leaf area ratio of *Amaranthus hybridus* at 24 ± 2°C under greenhouse conditions. The plants were harvested after six weeks (n = 5; rep = 3). [Control (water), C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

4.6.3 Effect of different organic biostimulants and method of application on the biochemical (pigments, proteins and carbohydrates) composition of Amaranthus hybridus

Foliar application generally showed better results compared to the other methods of application (**Fig. 4.1**), when considering the enhancement of morphological parameters, hence the estimation of biochemicals was done for foliar application only.

Chlorophyll, carotenoid, protein and carbohydrate contents were assessed. Results of biochemical estimations are summarised in **Table 4.2**.

Table 4.2: Effect of foliar application of biostimulants on the chlorophyll, carotenoid, protein and carbohydrate content of *Amaranthus hybridus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3). [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Chlorophyll a µg/g FW	Chlorophyll b µg/g FW	Chlorophyll a + b µg/g FW	Carotenoids µg/g FW	Proteins µg/g FW	Carbohydrates µg/g FW
Control	444 ± 0.09 f	129 ± 0.36 f	573 ± 0.43 f	135 ± 0.04 f	18.4 ± 1.37 c	465.6 ± 9.24 a
SW 1:500 (v/v)	515 ± 0.22 d	131 ± 0.32 e	647 ± 0.51 d	139 ± 0.04 e	22.7 ± 1.26 bc	170.1 ± 0.78 d
KAR_1 (10^{-6} M)	427 ± 0.31 g	117 ± 0.70 g	545 ± 1.00 g	139 ± 0.19 e	34.8 ± 4.13 a	102.4 ± 0.85 f
VCL 1:5 (v/v)	636 ± 0.30 b	168 ± 0.28 b	804 ± 0.58 b	177 ± 0.04 c	39.6 ± 1.85 a	157.1 ± 1.10 e
KEL (0.8%)	641 ± 0.71 a	172 ± 0.22 a	813 ± 0.61 a	197 ± 0.10 a	33.3 ± 0.92 a	208.0 ± 1.38 b
ECK (10^{-8} M)	487 ± 0.88 e	147 ± 0.17 d	634 ± 0.76 e	181 ± 0.29 b	26.7 ± 1.66 b	203.8 ± 0.94 b
GA (10^{-6} M)	584 ± 1.49 c	157 ± 0.42 c	742 ± 1.11 c	164 ± 0.28 d	21.7 ± 1.20 bc	186.2 ± 1.27 c

Mean values (± SE) in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

Table 4.3: Analysis of variance for comparing different application methods for the growth of *Amaranthus hybridus* with different biostimulants ($P < 0.05$).

Treatment comparison	Plant height (mm)			Plant fresh weight (g)			Plant dry weight (g)		
	difference	*t*-value	significant	difference	*t*-value	significant	difference	*t*-value	significant
Foliar vs Drenching	1.234	0.650	no	3.619	7.733	yes	0.444	5.908	yes
Foliar vs Drenching and Foliar	10.635	5.641	yes	5.004	10.691	yes	0.586	7.796	yes
Drenching vs Drenching and Foliar	9.400	4.950	yes	1.385	2.958	yes	0.142	1.887	no

From the results in **Table 4.2**, it can be observed that treating plants with KEL and VCL significantly augmented Chlorophyll (a, b and a + b) content as well as carotenoids and proteins in comparison to water control and the positive control, GA_3. KAR_1 also increased the protein content (34.8 ± 4.13 µg/g) in *A. hybridus* with KEL on the other hand, significantly improving the carotenoid content of the plant (197 ± 0.10 a). The carbohydrate content was, however, significantly decreased in all the tested biostimulants when compared with the control.

With reference to **Table 4.3**, it can be concluded that *A. hybridus* growth was significantly enhanced when the different biostimulants used in this study were applied via foliar application. The fresh weight and dry weights of the plant were more improved in foliar application compared with drenching, and drenching and foliar treatment.

4.6.4 Effect of biostimulants on the mineral composition of Amaranthus hybridus

The effects of organic biostimulants on the mineral composition of *A. hybridus* was also assessed in this study. **Table 4.4** is a summary of the results of the investigation. Most of the treatments did not have any notable positive effects on the mineral composition of *A. hybridus*. KEL had the most significant effect on a number of mineral elements when compared with the negative control C (water). It significantly improved the content of the following mineral elements, N, calcium (Ca), magnesium (Mg), K, sodium (Na), zinc (Zn), copper (Cu) and P (**Table 4.4**). The effect of KEL on N, Na, and Cu was the same as that of GA, the positive control. VCL significantly improved the Zn and Mn content of *A. hybridus* compared with both controls and ECK significantly increased the aluminium content of the plant but slightly increased the Na and Cu content even though the increase was not significant. Other treatments increased the content of some minerals but the increment was not significant. KAR_1 increased the levels of Ca, Mg, Na, Cu and Al. SW slightly increased the Na and Al content (**Table 4.4**).

Table 4.4: Effect of biostimulants on mineral element composition *Amaranthus hybridus.* [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Nitrogen (%)	Calcium (%)	Magnesium (%)	Potassium (%)	Sodium (mg kg^{-1})	Zinc (mg kg^{-1})	Copper (mg kg^{-1})	Manganese (mg kg^{-1})	Iron (mg kg^{-1})	Phosphorus (%)	Aluminium (mg kg^{-1})
Control	2.55 ± 0.02 b	1.17 ± 0.01 e	0.52 ± 0.01 c	6.33 ± 0.04 d	604 ± 97 bc	56.5 ± 0.70 d	4.80 ± 1.30 b	153 ± 0.78 d	252 ± 7.14 a	0.94 ± 0.02 bc	103 ± 7.2 b
SW	2.28 ± 0.04 e	1.23 ± 0.01 d	0.52 ± 0.00 c	6.80 ± 0.06 c	842 ± 61 abc	55.9 ± 0.79 d	4.56 ± 1.10 b	163 ± 0.69 c	172 ± 2.55 b	0.87 ± 0.03 bc	112 ± 4.9 ab
KAR_1	2.38 ± 0.01 d	1.37 ± 0.01 b	0.58 ± 0.01 b	7.14 ± 0.07 b	837 ± 128 abc	62.2 ± 0.00 c	6.32 ± 1.39 ab	147 ± 0.14 e	182 ± 5.02 b	0.97 ± 0.03 b	117 ± 12.1 ab
VCL	2.40 ± 0.04 cd	1.20 ± 0.01 de	0.53 ± 0.00 c	5.65 ± 0.02 e	558 ± 58 c	69.8 ± 0.05 b	5.63 ± 1.01 ab	223 ± 0.15 a	146 ± 1.43 cd	0.87 ± 0.04 bc	60 ± 1.51 c
KEL	2.79 ± 0.03 a	1.47 ± 0.01 a	0.64 ± 0.01 a	8.38 ± 0.08 a	989 ± 66 a	71.6 ± 0.69 a	9.06 ± 1.32 a	179 ± 1.26 b	157 ± 2.96 c	1.09 ± 0.04 a	98 ± 3.6 b
ECK	2.48 ± 0.02 bc	1.29 ± 0.01 c	0.53 ± 0.00 c	7.24 ± 0.06 b	879 ± 91 ab	56.1 ± 0.15 d	6.41 ± 0.71 ab	148 ± 0.69 e	139 ± 3.66 d	0.91 ± 0.03 bc	127 ± 7.3 a
GA	2.81 ± 0.02 a	1.36 ± 0.02 b	0.58 ± 0.01 b	7.24 ± 0.05 b	898 ± 104 ab	56.8 ± 0.41 d	7.50 ± 0.76 ab	144 ± 1.20 f	117 ± 2.57 e	0.84 ± 0.04 c	50 ± 0.6 c

4.6.5 Effect of application frequency of biostimulants + 50% HNS (nutrients) on growth of Amaranthus hybridus

A. hybridus was exposed to biostimulants + 50% HNS irrigation regime of once, twice and thrice a week. Results of this experiment are summarised in **Table 4.5**. Irrigation of treatments of the plant once a week had no effect on the leaf number of *A. hybridus* with all treatment having the same effect as the control. KEL had a significant effect on both shoot and root length (71.9 ± 5.9 and 35 ± 4.1 mm) respectively, and also on both shoot and root fresh weights, (648 ± 73 and 79 ± 12 mg) respectively. KAR_1 significantly improved root length and root fresh weight (34 ± 4.1 mm and 89 ± 11 mg) respectively and ECK only significantly improved the root fresh weight (83 ± 6 mg) compared with the control. The remaining treatments had little effect on the growth parameters. Irrigation of the plant twice a week resulted in SW significantly increasing all the growth parameters; leaf number, shoot length, root length, shoot fresh and root fresh weight (7.0 ± 0.4, 118 ± 11, 81.2 ± 9 mm, 1.067 ± 99 and 428 ± 89 mg) respectively. KAR_1 significantly increased shoot length (119 ± 7.3 mm) and this was much higher than the control. ECK significantly enhanced the root length of the plant (78.6 ± 4.8 mm). When plants were irrigated thrice a week, SW and VCL only significantly improved the shoot length whilst KAR_1 had a significant effect on all the growth parameters except leaf number (**Table 4.5**).

Table 4.5: Effect of application frequency of biostimulants + 50% HNS on growth of *Amaranthus hybridus* [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK].

Treatment	Leaf (no.)	Shoot length (mm)	Root length (mm)	Shoot fresh weight (mg)	Root fresh weight (mg)
Once a week					
Control	4.8 ± 0.6 a	58.1 ± 7.2 b	25 ± 4.0 c	306 ± 53 bc	40 ± 5 b
SW 1:500 v/v	4.3 ± 0.3 a	46.9 ± 4.0 b	25 ± 3.2 bc	224 ± 10 bc	34 ± 3 b
KAR_1 (10^{-6} M)	4.5 ± 0.4 a	57.7 ± 3.8 b	34 ± 4.1 ab	294 ± 30 bc	89 ± 11 a
VCL 1:5 v/v	4.5 ± 0.3 a	51.9 ± 2.9 b	27 ± 3.5 abc	211 ± 21 c	40 ± 3 b
KEL 0.8%	5.3 ± 0.7 a	71.9 ± 5.9 a	35 ± 4.1 a	648 ± 73 a	79 ± 12 a
ECK (10^{-8} M)	5.0 ± 0.3 a	54.0 ± 3.4 b	34 ± 3.7 abc	325 ± 28 b	83 ± 6 a
Twice a week					
Control	5.7 ± 0.3 bcd	79.5 ± 5.9 b	53.2 ± 6.5 c	408 ± 43 c	188 ± 13 b
SW 1:500 v/v	7.0 ± 0.4 a	118 ± 11 a	81.2 ± 9.8 a	1.067 ± 99 a	428 ± 89 a
KAR_1 (10^{-6} M)	6.3 ± 0.3 ab	119 ± 7.3 a	73.6 ± 6.1 ab	686 ± 60 b	261 ± 21 b
VCL 1:5 v/v	5.0 ± 0.4 d	93.2 ± 9.2 b	57.3 ± 8.1 bc	479 ± 46 c	161 ± 17 b
KEL 0.8%	5.4 ± 0.3 cd	89.2 ± 6.5 b	73.8 ± 7.4 ab	490 ± 47 c	225 ± 28 b
ECK (10^{-8} M)	6.0 ± 0.1 bc	86.4 ± 5.1 b	78.6 ± 4.8 a	501 ± 41 c	143 ± 43 b
Thrice a week					
Control	6.1 ± 0.5 a	99.5 ± 12.9 b	67.5 ± 9.1 bc	726 ± 121 b	242 ± 45 b
SW 1:500 v/v	6.4 ± 0.5 a	142.9 ± 11.6 a	85.4 ± 9.6 b	803 ± 50 b	255 ± 46 b
KAR_1 (10^{-6} M)	6.6 ± 0.6 a	164.5 ± 14.8 a	129.5 ± 13.2 a	1.191 ± 117 a	467 ± 103 a
VCL 1:5 v/v	7.1 ± 0.7 a	147.5 ± 14.2 a	83.1 ± 8.9 b	797 ± 108 b	204 ± 34 b
KEL 0.8%	6.0 ± 0.5 a	83.6 ± 9.7 b	43.1 ± 7.4 c	667 ± 70 b	116 ± 10 b
ECK (10^{-8} M)	6.5 ± 0.6 a	99.5 ± 12.0 b	65.9 ± 11.2 bc	272 ± 40 c	256 ± 35 b

Main effects and their interactions for the growth of *A. hybridus* are summarised in the ANOVA **Table 4.6**.

Table 4.6: General analysis of variance with main effects and their interactions for the growth of *Amaranthus hybridus* with different biostimulants [Control; Smoke-water 1:500 (v/v); Karrikinolide (10^{-6} M); Vermicompost leachate 1:5 (v/v); Kelpak® (0.8%); Eckol (10^{-8} M); Gibberellic acid (10^{-6} M)].

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance	*F*-probability
Leaf (no.)					
Treatment (T)	5	4.809	0.962	0.41	0.840
Frequency (F)	2	104.080	52.040	22.32	<.001
T X F	10	43.551	4.355	1.87	0.052
Residual	182 (16)	424.251	2.331		
Total	199 (16)	564.000			
Plant height (mm)					
Treatment (T)	5	103104	20621	10.73	<.001
Frequency (F)	2	497329	248665	129.35	<.001
T X F	10	157775	15778	8.21	<.001
Residual	182 (16)	349885	1922		
Total	199 (16)	029063			
Plant fresh weight (mg)					
Treatment (T)	5	5.41581	1.08316	12.44	<.001
Frequency (F)	2	12.56909	6.28455	72.17	<.001
T X F	10	10.70491	1.07049	12.29	<.001
Residual	113 (85)	9.83963	0.08708		
Total	130 (85)	27.94062			

From the ANOVA **Table 4.6**, it is noted that for leaf number, treatment (T) had no significant effect but frequency had a significant effect ($P < 0.01$) on leaf number. The interaction of treatment (T) x frequency (F) did not have a significant effect on the number of leaves of the plant. Considering plant height, treatment alone, frequency alone and the interaction of the two, treatment and frequency (T x F) were all significant ($P < 0.01$) on the height of the plant. A similar trend was observed on plant fresh weight. All the three treatments, T, F and F x T had a significant influence ($P < 0.01$) on plant fresh weight.

The relative and absolute growth of the plants were further calculated and the yield is summarised in **Fig. 4.3**.

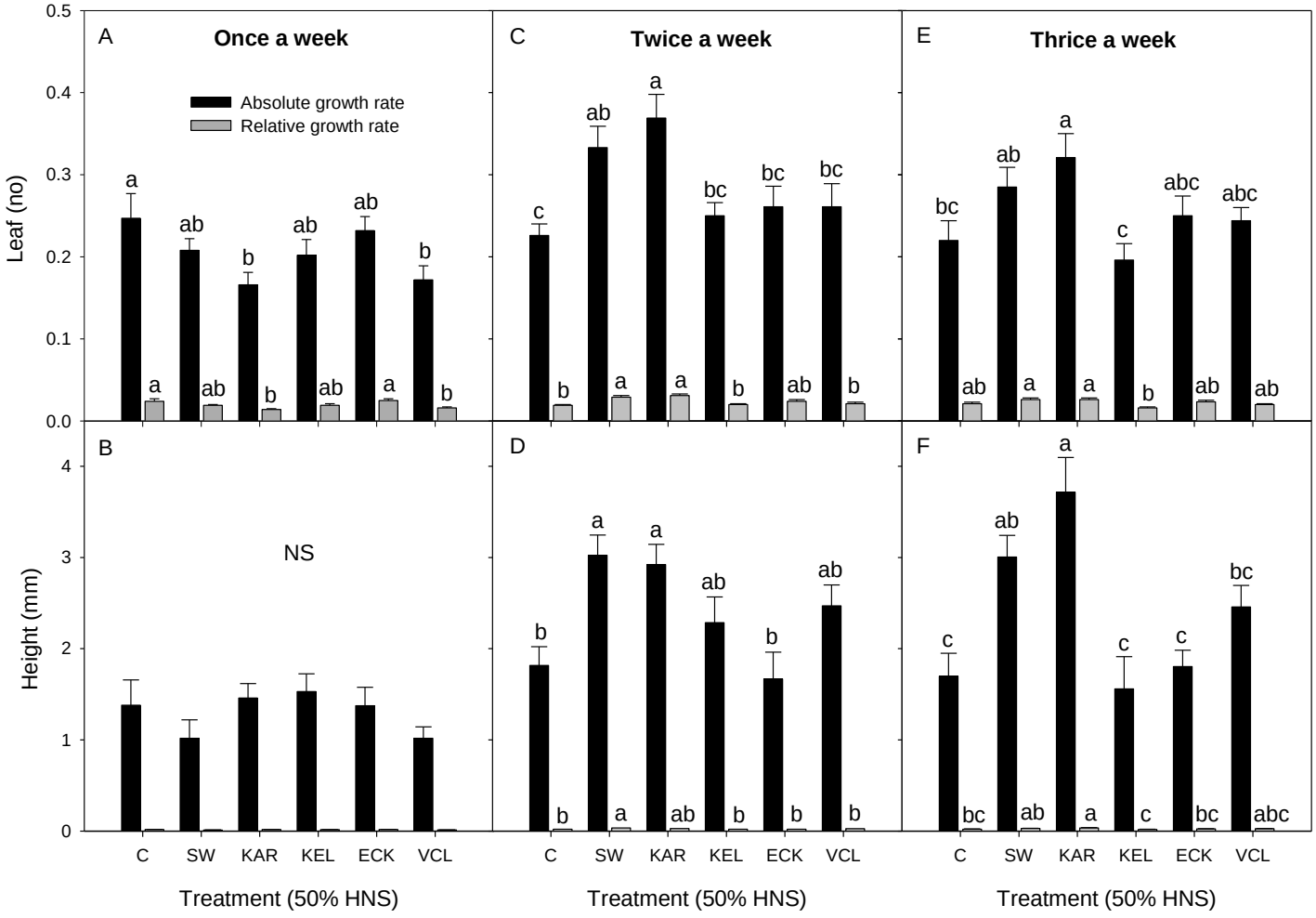


Fig. 4.3: Effect of irrigation frequency of biostimulants + 50% HNS on the absolute and relative growth rate (leaf number and plant height) and leaf area ratio of *Amaranthus hybridus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after six weeks (n = 5; rep = 3). [Control, C; Smoke-water, SW 1:500 (v/v); Karrikinolide, KAR_1 (10-6 M); Vermicompost leachate, VCL 1:5 (v/v); Kelpak®, KEL (0.8%); Eckol, ECK (10-8 M)].

From **Fig. 4.3**, it can be noted that for plants irrigated once per week with biostimulants + 50% HNS, all treatments did not have an increased effect on AGR for leaf number with the control yielding a higher AGR than all other treatments. All treatments had no significant influence on both AGR and RGR for leaf number and height compared to the control (**Fig. 4.3 A** and **B**). SW and KAR_1 were the only treatments with a significant

effect on AGR for leaf number of plants irrigated twice a week compared to the control (**Fig. 4.3 C**). In terms of the RGR for leaf number, SW and KAR_1 significantly increased the RGR for leaf number. SW and KAR_1 also significantly improved the AGR for height when plants were irrigated twice a week and only SW significantly enhanced the RGR for height (**Fig 4.3 D**). The AGR for leaf number and height was significantly increased by KAR_1 when plants were irrigated thrice a week compared to the control (**Fig. 4.3 E** and **F**).

4.6.6 Effect of –N + biostimulants on the absolute growth rate (AGR) of Amaranthus hybridus

In another investigation, the effect of applying HNS lacking N + biostimulants was also assessed and the results of this investigation are shown in **Fig. 4.4**. From the results it is evident that all treatments did not have a significant improved effect on AGR for both leaf number and height of the plant but it can also be noted that all the treatments had a negative effect on AGR of leaf number although there was a slight increase in –N + KEL treated plants. The increase was closer to the positive control but not significantly (**Fig. 4.4 a**). **Fig. 4.4 b** illustrates the amaranths plants growing under different –N + biostimulant concentration regimes.

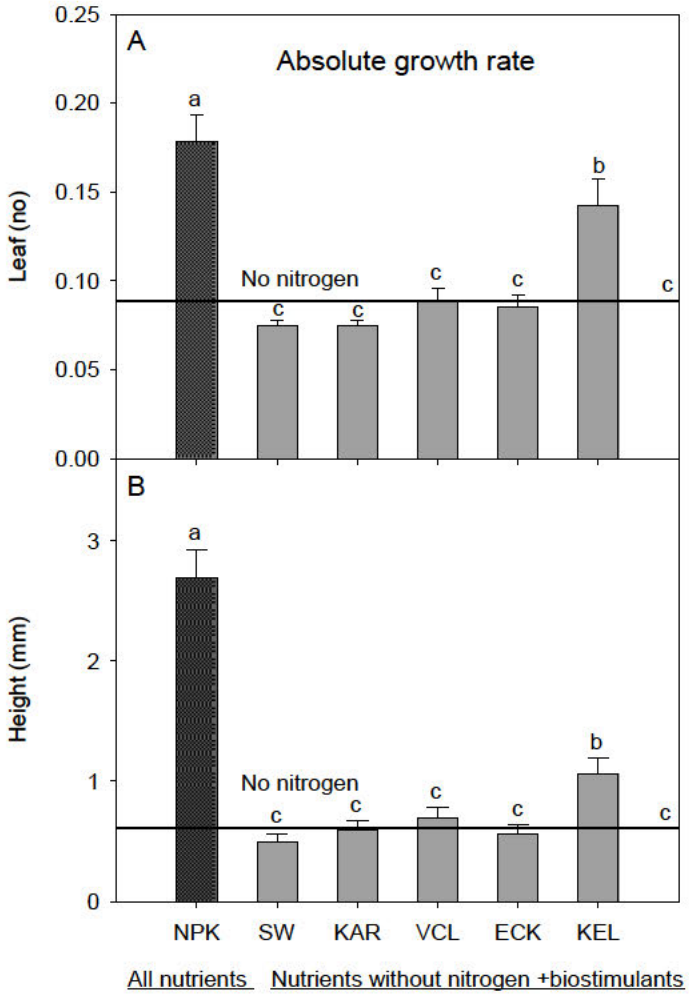


Fig. 4.4 A: Effect of –N +biostimulants on the absolute growth rate of *Amaranthus hybridus.*

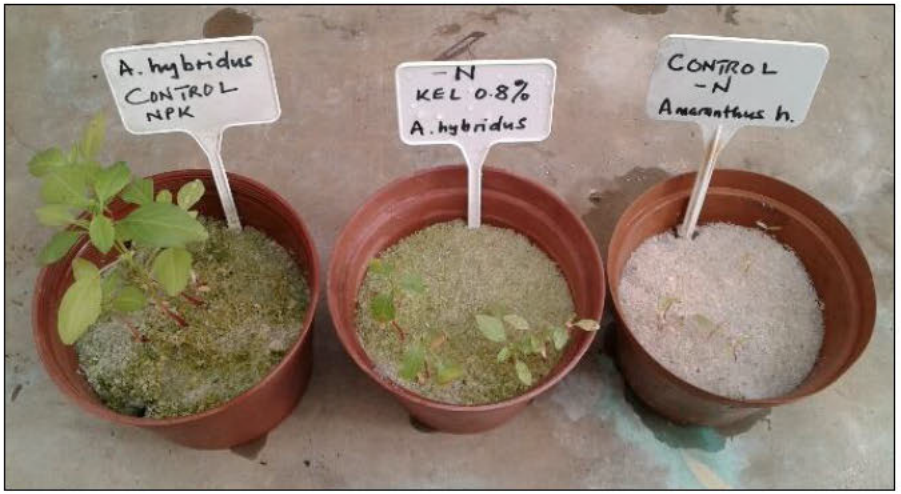


Fig. 4.4 B: *Amaranthus hydridus* showing the effect of –N + KEL result compared with positive and negative control.

4.7 Effect of organic biostimulants on antioxidant activity of *Amaranthus hybridus*

The effect of different biostimulants and methods of application on the antioxidant activity was also evaluated in this research. In this regard, several assays were carried out with harvested plant material to assess the antioxidant activity of *A. hybridus* exposed to different biostimulants and different methods of application.

4.7.1 β-Carotene/linoleic acid oxidation of crude methanolic extracts of Amaranthus hybridus

The β-carotene oxidation was assessed on both water and crude methanolic extracts of *A. hybridus* for the three methods of application to establish their ability in preventing or delaying the coupled oxidation of β-carotene and linoleic acid. Results of this investigation are shown in **Table 4.7**. The average rate of β-carotene bleaching, %ANT was significantly high in VCL, KAR_1 and SW (94 ± 4.0, 84 ± 3.9 and 80 ± 2.1%) respectively under the drenching method. These values are comparable with the value obtained for the standard control, BHT (82.6 ± 1.14%) (**Table 4.7**). The corresponding ORR associated with antioxidant capacity for the same mode of application (drenching) ranged from 0.06 ± 0.04 - 0.39 ± 0.07 making VCL and KAR_1 the most active since they had the lowest significant ORR values (0.06 ± 0.04 and 0.16 ± 0.04) respectively (**Table 4.7**). Furthermore, the antioxidant activity (AA) based on the inhibition of β-carotene oxidation was determined at t = 60 and t = 120 min and this ranged from 20. ± 2.54 to 100 ± 8.39 and from 23 ± 3.02 to 90 ± 12.9 respectively under drenching (**Table 4.7**). From the results of the investigation, most treatments showed a high β-carotene oxidation with the lowest value being 61 ± 6.8 and 61 ± 5.2 for the control (C) and ECK respectively.

When the treatments were applied via foliar application, the average rate of antioxidant activity (%ANT) was significantly high for KEL , 97 ± 2.9 with SW, ECK, and KAR_1, (87 ± 2.2, 85 ± 1.7 and 79 ± 0.6%) respectively having values which were also comparable with BHT (80.16 - 0.19%) but not significantly different statistically when compared with the control (C). The ORR ranged from 0.05 ± 0.02 to 0.32 ± 0.09. The highest activity was found in KEL, SW and ECK which had low values of ORR (0.05 ± 0.02,

0.13 ± 0.02 and 0.15 ± 0.02) respectively. The determination of AA based on β-carotene oxidation inhibition was also calculated at t = 60 and t = 120 min and was seen to vary from 62 ± 4.64 to 99 ± 0.84 for t = 60 min and from 33 ± 2.14 to 73 ± 5.79. KEL had the highest AA60 and AA120, (99 ± 0.84 and 73 ± 5.79). KAR_1 had a high AA60 (95 ± 3.47) whilst and ECK had 79 ± 5.55, but all these elevated values were not statistically different from the controls. The same parameters were established for drenching and foliar application where the highest %ANT was obtained with SW, ECK and KEL (86 ± 4.80, 85 ± 3.3 and 81.23 ± 6.0) respectively and again the values were comparable with BHT (80.16 ± 0.19). The values for AA60 were not significantly different from the positive control but were significantly higher than the negative control (**Table 4.7**). Antioxidant activity was also high for the combination of drenching and foliar spray. The value for ORR for drenching and foliar application was determined and it ranged from 0.14 ± 0.07 to 0.42 ± 0.03 making SW and ECK the most active at ORR (0.14 ± 0.07 and 0.15 ± 0.04) respectively. The AA at t = 60 min ranged from 53 ± 2.13 to 88 ± 3.56 and for t = 120 min it ranged from 44 ± 4.01 to 82 ± 7.52.

Table 4.7: Effect of organic biostimulants and mode of application on β-carotene oxidation of water extracts of *Amaranthus hybridus*.

Mode of application	Treatment	β-carotene Oxidation			
		%ANT	ORR	AA60	AA120
Drenching	Control	61 ± 6.8 fg	0.39 ± 0.07 ab	43 ± 5.15 g	29 ± 3.82 ef
	SW	80 ± 2.1 bcde	0.20 ± 0.02 cdefg	88 ± 3.31 abc	55 ± 2.94 bcdef
	KAR_1	84 ± 3.9 abcd	0.16 ± 0.04 defgh	89 ± 1.84 abc	68 ± 1.36 abc
	VCL	94 ± 4.0 ab	0.06 ± 0.04 gh	100 ± 8.39 a	75 ± 9.59 abc
	KEL	75 ± 6.0 cdef	0.25 ± 0.06 bcde	62 ± 21.3 defg	90 ± 12.9 a
	ECK	61 ± 5.2 fg	0.39 ± 0.1 ab	20 ± 2.54 h	23 ± 3.02 f
	GA	63 ± 0.1 fg	0.38 ± 0.00 ab	50 ± 2.15 fg	65 ± 3.50 abcd
Foliar Spray	Control	80 ± 3.3 bcde	0.20 ± 0.03 cdefg	79 ± 1.55 abcd	61 ± 10.53 abcde
	SW	87 ± 2.2 abc	0.13 ± 0.02 efg	92 ± 3.85 ab	61 ± 5.57 abcde
	KAR_1	79 ±0.6 bcde	0.21 ± 0.02 cdef	95 ± 3.47 a	33 ± 2.14 def
	VCL	71 ± 7.0 defg	0.29 ± 0.07 abcd	71 ± 7.63 bcde	51 ± 14.69 bcdef
	KEL	97 ± 2.9 a	0.05 ± 0.02 h	99 ± 0.84 a	73 ± 5.79 abc
	ECK	85 ± 1.7 abcd	0.15 ± 0.02 defg	79 ± 5.55 abcd	64 ± 5.57 abcd
	GA	68 ± 8.9 efg	0.32 ± 0.09 abc	62 ± 4.64 defg	69 ± 19.69 abc
	Control	58 ± 0.9 g	0.42 ± 0.01 a	58 ± 3.06 efg	44 ± 4.01 cdef
	SW	86 ± 4.8 abcd	0.14 ± 0.02 defg	88 ± 3.56 abc	59 ± 8.05 abcde
	KAR_1	69 ± 2.6 efg	0.31 ± 0,14 abc	53 ± 2.13 efg	82 ± 7.52 ab

Drench/Foliar	VCL	71 ± 5.2 defg	0.29 ± 0.32 abcd	69 ± 9.80 cdef	69 ± 10.86 abc
	KEL	81 ± 6.0 bcde	0.19 ± 0.06 cdefg	85 ± 3.28 abc	60 ± 23.69 abcde
	ECK	85 ± 3.3 abcd	0.15 ± 1.20 defg	81 ± 3.89 abcd	78 ± 12.44 abc
	GA	75 ± 0.8 cdef	0.25 ± 0.22 bcde	68 ± 1.67 cdef	61 ± 3.56 abcde
	BHT	81.60 ± 1.84 bcde	0.170 ± 0.01 cdef	69.± 5.43 cdef	56.80 ± 2.31 bcdef

Values represent mean ± of three replicates. Different letters in same column indicate significant differences at 5% level of significance. Control= Water, SW= Smoke-water, KAR_1= Karrikinolide, VCL= Vermicompost leachate, KEL= Kelpak®, ECK= Eckol, GA = Gibberellic acid and BHT= butylated hydroxyl-toluene. %ANT= Antioxidant activity was calculated using rate of β-carotene bleaching at t= 60 and 120 min, ORR= oxidation rate ratio with stronger activity being associated with low value of ORR. AA60 and AA120 = % antioxidant activity of extracts or BHT at t= 60 or 120 min.

4.7.2 DPPH activity of methanolic and water extracts of Amaranthus hybridus

The antioxidant activity of both methanolic and water extracts of *A. hybridus* treated with different organic stimulants applied via three different methods of application was determined by the DPPH radical scavenging activity assay. These results are recorded in **Table 4.8**. There was a wide variation of antioxidant activity as depicted by the results in **Table 4.8**, with RSA values differing according to extracts used, with methanolic extracts generally having low values and water extracts showing more potent radical scavenging activity (**Table 4.8**). Methanolic extracts under drenching had RSA activity ranging from 42.71 ± 1.47 to 64.18 ± 2.02 and IC_{50} values ranging from 0.1967 ± 0.01 to 0.3327 ± 0.02. VCL had the least significant RSA value which fell below those of the controls. On the other hand, water extracts generally exhibited high values of radical scavenging activity across all three modes of application ranging from 45.85 ± 0.71 to 94.69 ± 0.18 (**Table 4.8**). The highest values of radical scavenging activity were shown by the control and SW under a combination of drenching and foliar spray (94.69 ± 0.18 and 90.43 ± 0.15) respectively. The high values of RSA of water extracts were not statistically significant but were comparable with the standards ASC and BHT (90.65 ± 0.43 and 97.08 ± 2.65) respectively. IC_{50} values of water extracts across modes of applications and treatments ranged from 0.1648 ± 0.01 to 0.4044 ± 0.01 (**Table 4.8**). Generally, there were no statistically significant differences in IC_{50} values between treatments and across modes of applications except for KAR_1 (foliar) methanolic extracts, which had significantly lower values of IC_{50} compared with the control (0.1967 ± 0.01 and 0.1932 ± 0.00) respectively (**Table 4.8**).

Table 4.8: DPPH (1-1-diphenyl-1-picryhdrazyl) radical scavenging activity (RSA) and IC_{50} (half maximal inhibitory concentration) values for methanolic and water extracts of *Amaranthus hybridus*.

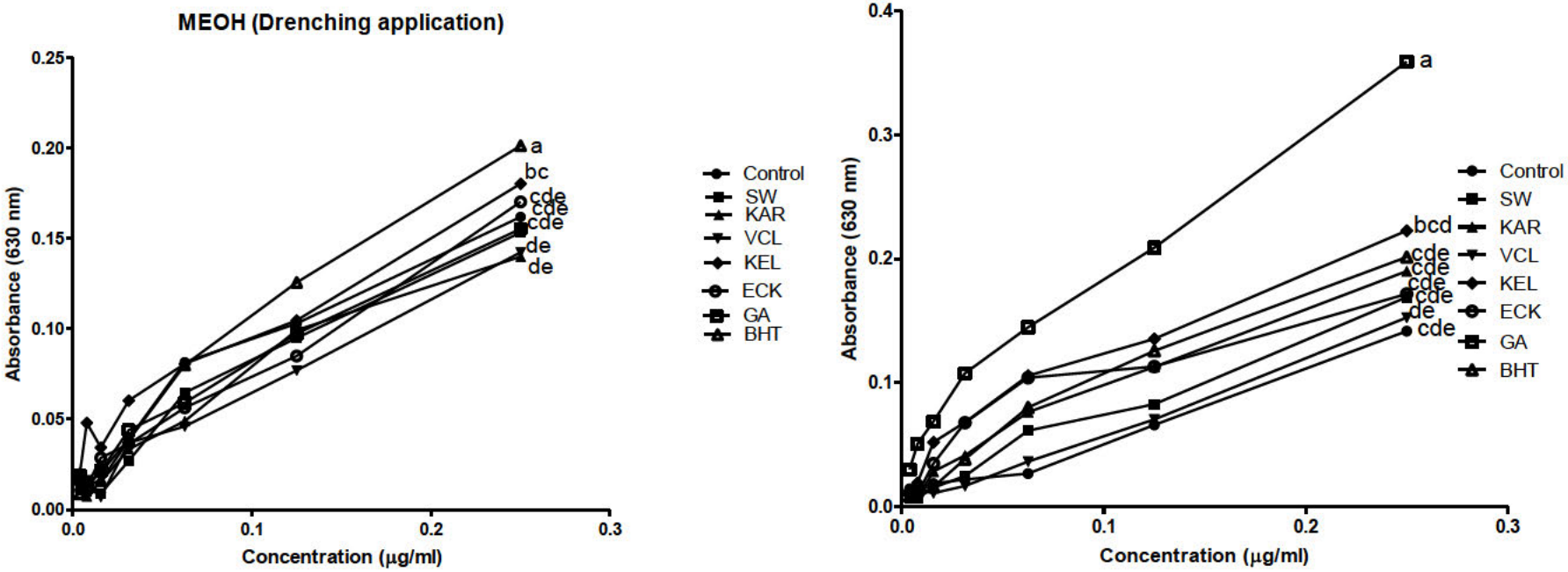
Mode of application	Treatment	DPPH			
		%RSA (MeOH)	**IC_{50}**	**%RSA (H_2O)**	**IC_{50}**
Drenching	Control	55.03 ±1.64 cdefg	0,2525 ± 0.01 cdef	83.56 ±2.11 bc	0,1648 ± 0.01 f
	SW	64.18 ± 2.02 bc	0,2488 ± 0.01 cdefg	86.56± 1.44 b	0,1904 ± 0.01 ef
	KAR_1	46.43 ± 2.20 ghij	0,2852 ± 0.03 abcd	86.02 ± 6.24 b	0,4044 ± 0.01 a
	VCL	42.71 ± 1.47 ij	0,1967 ± 0.01 fghijk	86.60± 1.59 b	0,2118 ± 0.03 de
	KEL	48.30± 1.02 fghij	0,3327 ± 0.02 a	86.67± 1.59 b	0,2491 ± 0.01 bcd
	ECK	51.02± 0.34 defghi	0,2113 ± 0.01 efghi	86.95 ± 1.56 b	0,2675 ± 0.02 bc
	GA	51.06 ± 2.21defghi	0,2279 ± 0.01 defgh	87.31± 2.20 b	0,2690 ± 0.01 bc
Foliar	Control	39.12 ± 0.82 j	0,2658 ± 0.02 bcde	87.31 ± 1.33 b	0,2653 ± 0.01 bc
	SW	50.31 ± 2.32 efghi	0,2859 ± 0.02 abc	86.39 ± 0.49 b	0,2527 ± 0.01 bcd
	KAR_1	57.84 ± 3.36 cde	0,1932 ± 0.00 ghijk	86.05 ± 0.50 b	0,2687 ± 0.01 bc
	VCL	61.20 ± 8.59 bcde	0,2778 ± 0.02 abcd	79.47± 0.17 c	0,2478 ± 0.02 bcd
	KEL	50.29± 3.69 efghi	0,2889 ± 0.03 abc	86.40 ± 0.17 b	0,2658 ± 0.03 bc
	ECK	50.20 ± 3.61 efghi	0,2471 ± 0.03 cdefg	86.05 ± 0.30 b	0,2736 ± 0.02 bc
	GA	47.56± 1.73 fghij	0,2741 ± 0.03 bcd	86.57± 0.69 b	0,2659 ± 0.01 bc

	Control	61.93± 0.49 bcd	0,1893 ± 0.01 hijk	94.69 ± 0.18 a	0,2255 ± 0.02 cde
	SW	77.27 ± 0.99 ab	0,3151 ± 0.02 ab	90.43 ± 0.15 ab	0,2501 ± 0.01 bcd
	KAR_1	58.14 ± 2.50 cdef	0,2770 ± 0.01 abcd	87.74± 2.59 b	0,3737 ± 0.01 a
Drenching and foliar	VCL	58.05 ± 0.89 cdef	0,2164 ± 0.01 efghi	58.05 ± 0.29 e	0,2642 ± 0.01 bc
	KEL	45.85 ± 2.72 hij	0,2953 ± 0.01 abc	45.85 ± 0.71 f	0,2399 ± 0.01 bcd
	ECK	69.04± 6.27 ab	0,1588 ± 0.01 jk	69.04 ± 0.97 d	0,2843 ± 0.01 b
	GA	57.08 ± 4.21 cdefg	0,1664 ± 0.03 ijk	57.08± 0.93 e	0,2691 ± 0.01 bc
	ASC	89.65 ± 0.30 a	0.08± 0.148 l	90.65 ± 0.43 ab	0. 032 ±0.02
	BHT	85.41 ± 0.99 ab	0.15± 0.08 jk	97.08 ± 2.65 a	0. 031 ± 0.01

Values represent mean ± of three replicates. Different letters in same column indicate significant differences at 5% level of significance. Control= Water, SW= Smoke-water, KAR_1= Karrikinolide, VCL= Vermicompost leachate, KEL= Kelpak®, ECK= Eckol, GA = Gibberellic acid, ASC= Ascorbic acid and BHT= butylated hydroxytoluene.

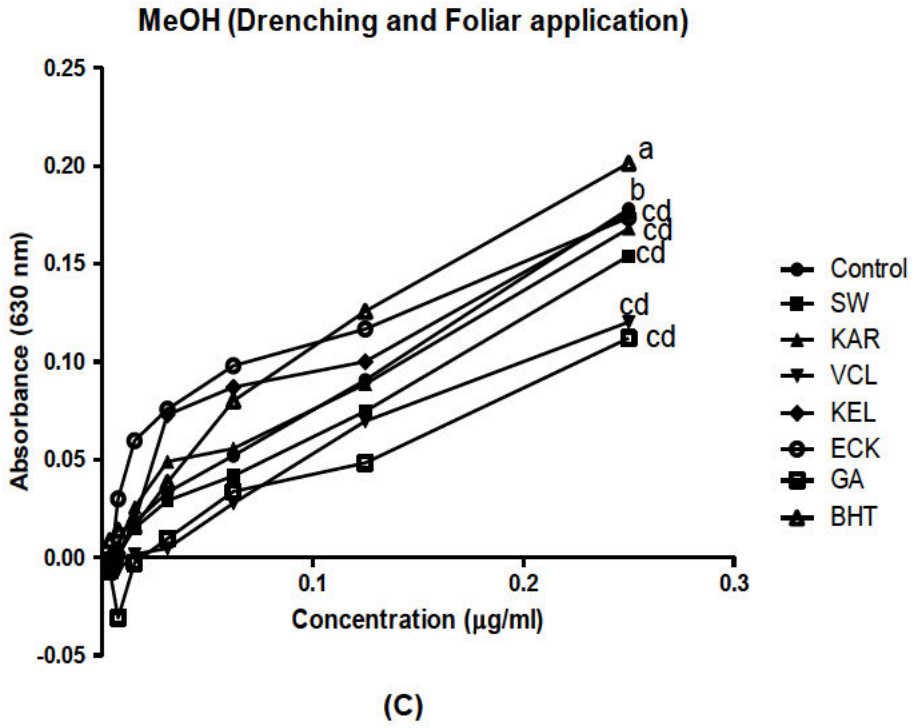
4.7.3 Ferric-cyanide (Fe3+) reducing antioxidant power (FRAP) results

To evaluate the antioxidant potential of both water and aqueous crude methanolic extracts of *A. hybridus* on their ability to reduce ferricyanide (Fe^{3+}) complexes in solution to the ferrous form (Fe^{2+}), the ferric-reducing power assay was employed. According to **NDHLALA et al. (2014)**, a strong antioxidant reduces Fe^{3+} complexes resulting in Perl's Prussian blue colour change that is detected by a spectrophotometer at absorbance 630 nm. The dose-dependent ferric-reducing powers of both methanolic and water extract samples and the positive control (BHT) are presented in **Fig. 4.5 A, B and C** and **Fig. 4.6 A, B and C** respectively. In this study, antioxidant activity varied depending on treatment and method of application. Methanolic extracts from the drenching method had a significant lower antioxidant activity which was far below that of the standard solution, BHT. Antioxidant activity of methanolic extracts among treatments were not significantly different from each other for the drenching method (**Fig. 4.5 A**). Methanolic extracts from foliar application method had an antioxidant activity significantly lower than that of the positive control (GA). There were no significant differences in antioxidant activity among treatments under foliar application (**Fig. 4.5 B**). There was no notable antioxidant activity in methanolic extracts from drenching and foliar applications. The highest antioxidant activity was observed for BHT (**Fig. 4.5 C**). A similar trend was also observed on water extracts of *A. hybridus*, with all the treatments showing a significantly low antioxidant activity compared with BHT, the standard solution (**Fig. 4.6 A, B and C**). SW and VCL, under drenching application, had the lowest antioxidant activity (**Fig. 4.6 A**). A similar trend was observed in foliar spray although KAR_1 had a high but not significant antioxidant activity. KEL demonstrated the least significant antioxidant activity (**Fig. 4.6 B**). The differences in antioxidant activity for most of the other remaining treatments were not statistically significant (**Fig. 4.6 B**). Antioxidant activity among treatments under combined drenching and foliar application was significantly different with ECK exhibiting an elevated antioxidant activity (**Fig. 4.6 C**). Consequently, both methanolic and water extracts of *A. hybridus* did not have a major influence on the antioxidant activity of the leafy vegetable according to the FRAP assay.


MEOH (Drenching application)
Absorbance (630 nm)
Concentration (µg/ml)
0.00
0.05
0.10
0.15
0.20
0.25
0.0
0.1
0.2
0.3
a
bc
cde
cde
cde
de
de
Control
SW
KAR
VCL
KEL
ECK
GA
BHT
MeOH (Foliar application)
Absorbance (630 nm)
Concentration (µg/ml)
0.0
0.1
0.2
0.3
0.4
a
bcd
cde
cde
cde
cde
de
cde
Control
SW
KAR
VCL
KEL
ECK
GA
BHT

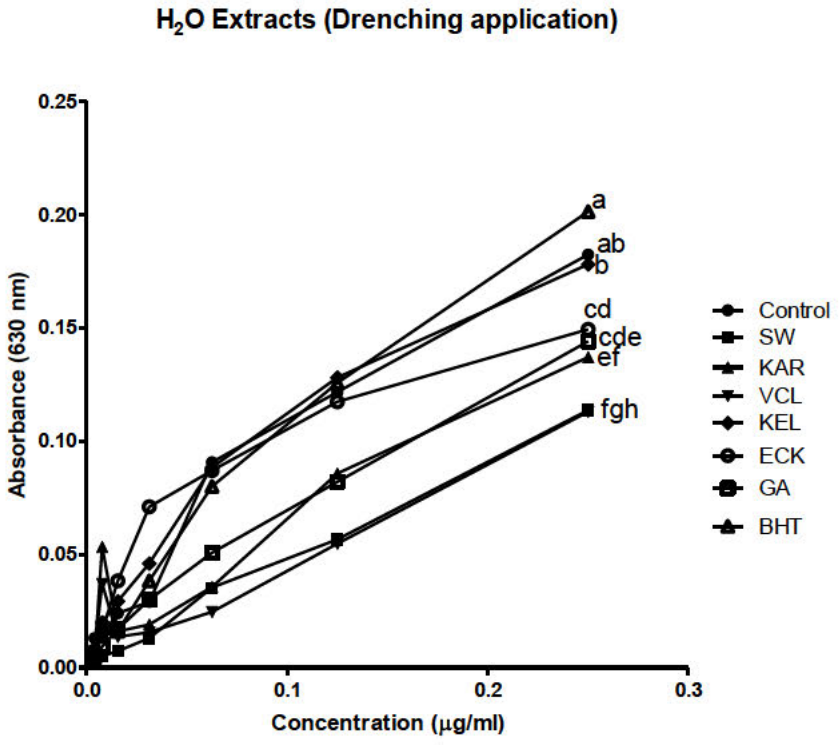
(A)

(B)

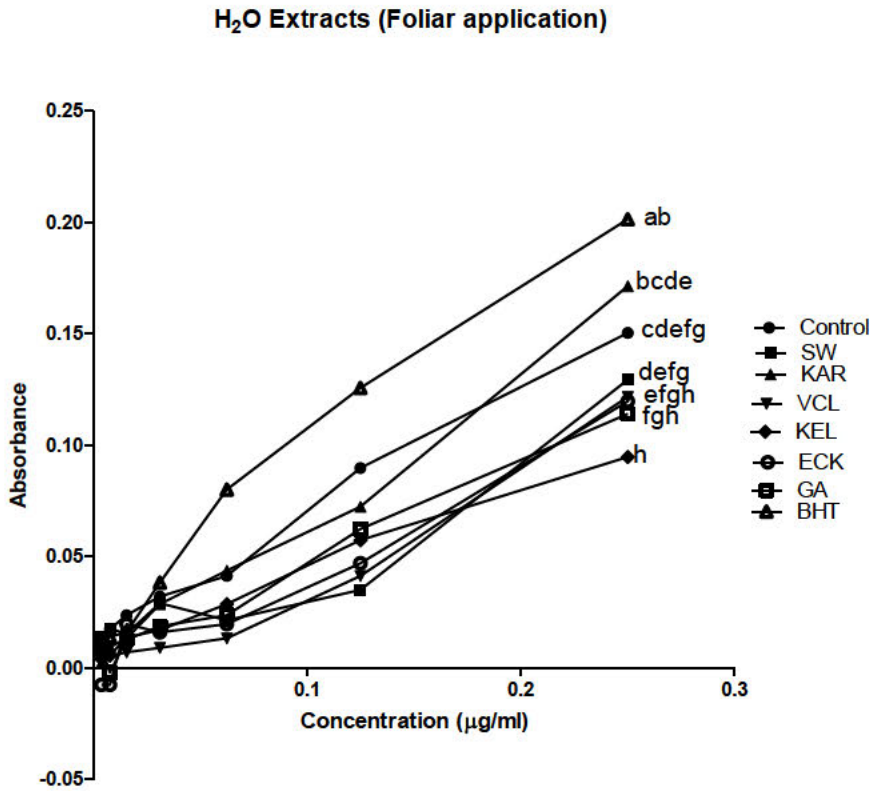


(C)

Fig. 4.5 A, B, C: Dose dependent ferric reducing antioxidant power of methanolic extracts of *Amaranthus hybridus*. BHT= butylated hydroxytoluene. Different treatments are represented in the figure above. C= Water, SW= Smoke water, KAR_1= Karrikinolide, VCL= Vermicompost leachate, KEL= Kelpak®, ECK= Eckol and GA= Gibberellic acid. Values indicate mean ± of three replicates. Different letters in same column indicate significant differences at 5% level of significance.


H2O Extracts (Drenching application)
Absorbance (630 nm)
0.25
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
Concentration (µg/ml)
a
ab
b
cd
bcde
ef
fgh
Control
SW
KAR
VCL
KEL
ECK
GA
BHT

(A)


H2O Extracts (Foliar application)
Absorbance
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.1
0.2
0.3
Concentration (µg/ml)
ab
bcde
cdefg
defg
efgh
fgh
h
Control
SW
KAR
VCL
KEL
ECK
GA
BHT

(B)

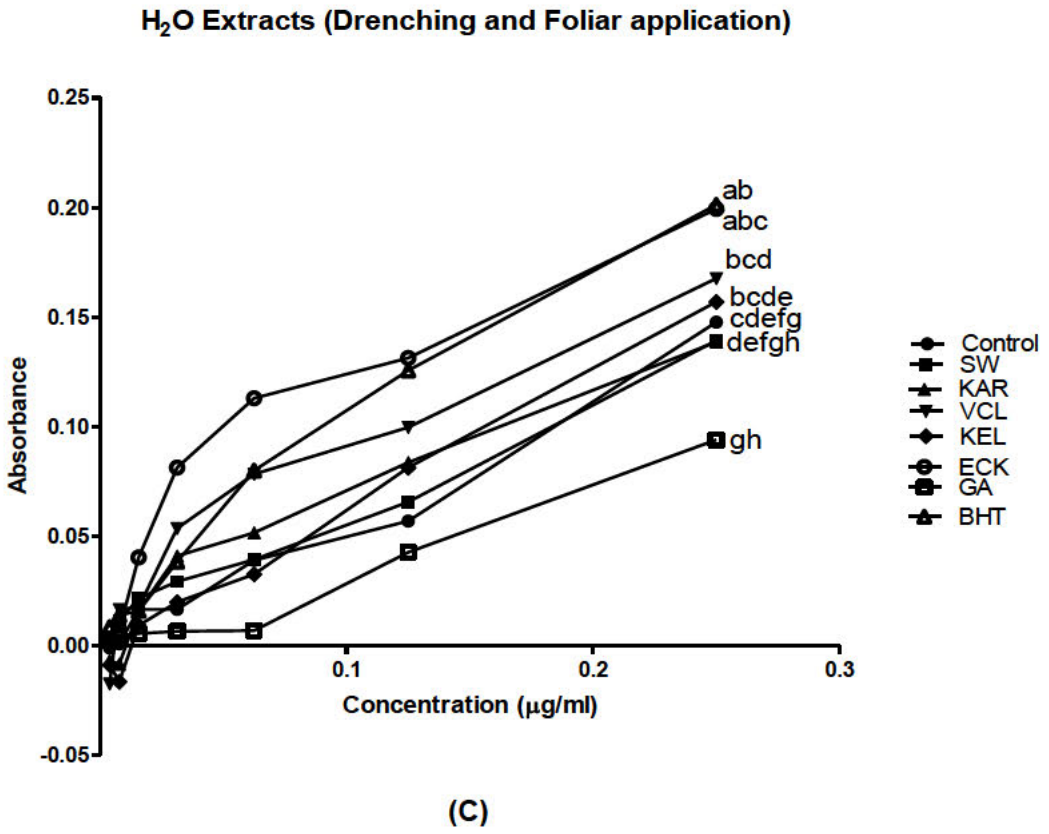


Fig. 4.6 A, B, C: Dose dependent ferric reducing antioxidant power of water extracts of *Amaranthus hybridus*. BHT= butylated hydroxytoluene. Different treatments are represented in the figure above; C= Water, SW= Smoke water, KAR_1= Karrikinolide, VCL= Vermicompost leachate, KEL= Kelpak®, ECK= Eckol and GA= Gibberellic acid. Values indicate mean ± of three replicates. Different letters in same column indicate significant differences at 5% level of significance.

4.7.4 Effect of organic biostimulants and mode of application on total phenolic content (TPC) in Amaranthus hybridus

The Folin-Ciocalteu reagent was used to determine the phenolic content in methanolic extracts of *A. hybridus* with the results being derived from a calibration curve (y = 9.53x - 0.13, R^2 = 0.996) of gallic acid (0 - 250 ug/mL). The resultant phenolic content amount was expressed as gallic acid equivalents (GAE) per gram of dry extract weight (GAE /g DW). **Fig. 4.7** shows the total phenolic content in the extracts. Among the different treatments, the highest phenolic content was observed in the control under drenching (1.409 ± 0.298). VCL under foliar application had the least statistically significant

amount of total phenolic content (0.536 ± 0.017) (**Fig. 4.7**). Total phenolic content was slightly elevated in the controls under drenching and drenching combined with foliar application (**Fig. 4.7**). Differences in total phenolic content between treatments and mode of application were found to be not statistically different for most of the treatments.

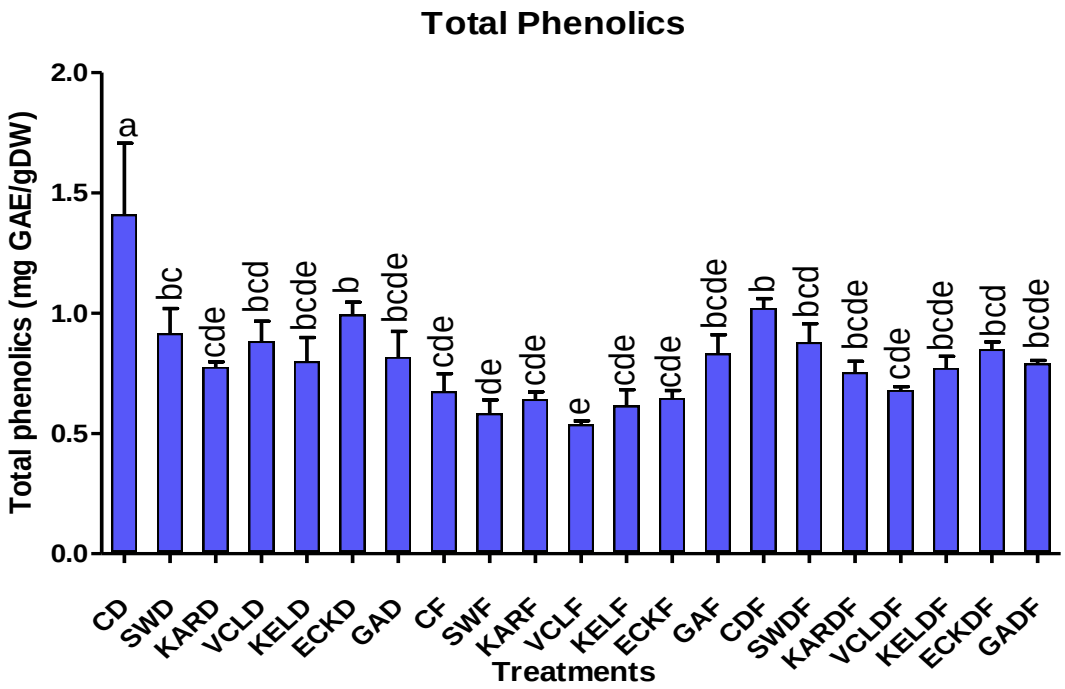


Fig. 4.7: Total phenolic content of methanolic extracts of *Amaranthus hydridus*. Values indicate mean ± SE of three replicates and different letters between treatments indicate significant differences at 5% level of significance. CD= Control (drenching), SWD = Smoke Water (drenching), KARD = Karrikinolide (drenching), VCLD= Vermicompost leachate (drenching), KELD= Kelpak® (drenching), ECKD= Eckol (drenching) and GAD= Gibberellic acid (drenching). Same treatments with F= Foliar spray. Same treatments with DF= Drenching and foliar spray.

4.7.5 Effect of organic biostimulants and mode of application on total flavonoids content (TFC) in Amaranthus hybridus

Fig. 4.8 shows the total flavonoid content (TFC) of methanolic extracts of *A. hybridus*. Among all treatments and mode of application, the highest amount of flavonoid content was found in the control (10.752 ± 1.024) under drenching and foliar application. This was followed by ECK (9.210 ± 0.824) under drenching which was also statistically significant compared to the other treatments. The organic biostimulants and their mode of application seem to have had little effect on the total flavonoid content of *A. hybridus* (**Fig. 4.8**). The lowest statistically significant amounts of flavonoids were found in the control (foliar application) (4.423 ± 0.293), VCL, KEL and ECK all under a combination of drenching and foliar application (4.666 ± 0.146, 4.788 ± 0.040 and 4.666 ± 0.107) respectively compared to the other treatments (**Fig. 4.8**). Generally, differences in flavonoid content among treatments (organic biostimulants) and mode of application in terms of total flavonoid content were found to be not statistically significant.

4.7.6 Effect of biostimulants and mode of application on condensed tannins in Amaranthus hybridus

The butanol-HCL assay was employed in the determination of quantities of condensed tannins in methanolic extracts of *A. hybridus* treated with different organic biostimulants. Results of this investigation are shown in **Fig. 4.9**. The highest amounts of condensed tannins were obtained in SW (2.745 ± 0.630) under drenching application and this was statistically significant compared with all the other treatments. Also, elevated amounts of condensed tannins were observed in ECK (1.690 ± 0.243) under drenching application although not statistically significant overall (**Fig. 4.9**). The general trend showed no significant differences in amounts of condensed tannins due to treatment and mode of application (**Fig. 4.9**).

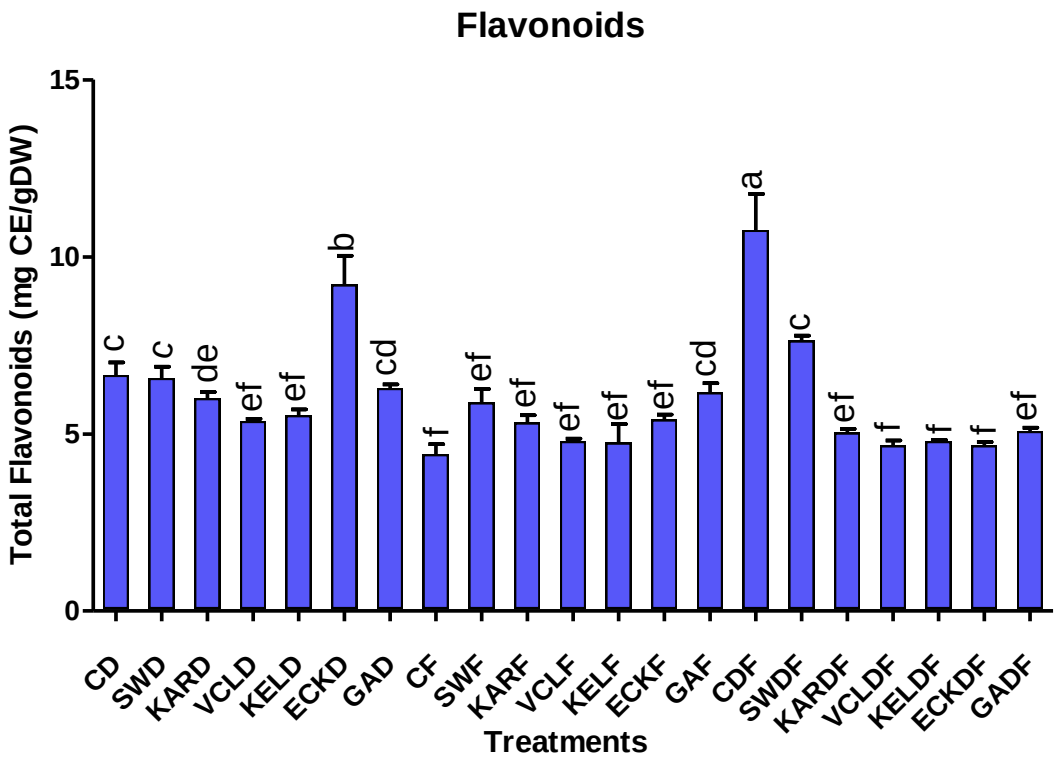


Fig. 4.8: Total flavonoid content of methanolic extracts of *Amaranthus hydridus*. Values indicate mean ± SE of three replicates and different letters between treatments indicate significant differences at 5% level of significance. CD= Control (drenching), SWD= Smoke water (drenching), KARD= Karrikinolide (drenching), VCLD= Vermicompost leachate (drenching), KELD= Kelpak® (drenching), ECKD= Eckol (drenching) and GAD= Gibberellic acid (drenching). Same treatments with F= Foliar spray. Same treatments with DF= Drenching and foliar spray.

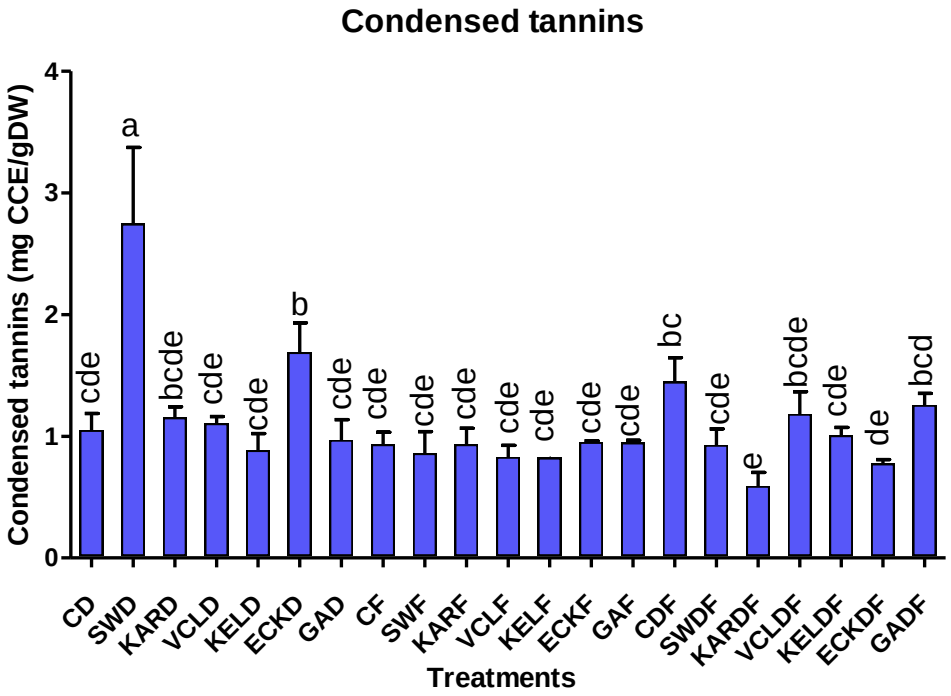


Fig. 4.9: Condensed tannins of methanolic extracts of *Amaranthus hybridus*. Values indicate mean ± SE of three replicates and different letters between treatments indicate significant differences at 5% level of significance. CD= control (drenching), SWD= Smoke water (drenching), KARD = Karrikinolide (drenching), VCLD= Vermicompost leachate (drenching), KELD= Kelpak® (drenching), ECKD= Eckol (drenching) and GAD= Gibberellic acid (drenching). Same treatments with F= Foliar spray. Same treatments with DF= Drenching and foliar spray.

4.8. Discussion

4.8.1 Effect of biostimulants on Amaranthus hybridus *growth*

The best concentration of different biostimulants for growth study was selected based on promotory effects on seed germination and seedling growth of *A. hybridus* from preliminary experiments carried out prior to this study. Under greenhouse conditions, the foliar application of VCL generally showed an increase in several growth parameters of *A. hybridus* seedlings compared to other biostimulants. In combined drenching and foliar treatment, most of the growth parameters with KEL treatments were significantly higher compared to the control, GA and other tested biostimulants (**Table 4.1**). KAR_1 drenching treatment significantly increased plant height and fresh/dry weight compared to the water control, positive GA control and other tested biostimulants when results of shoot and root were combined and analysed (**Fig. 4.1; Table 4.1**). These results were similar for foliar VCL and combined drenching and foliar KEL treatments (**Fig. 4.1**). General analysis of variance shows that different biostimulant concentrations and mode of applications used in this experiment had a significant effect on *A. hybridus* plants (**Table 4.2**). However, the only exception was for plant height where treatments did not have a significant effect. KEL treatment improved absolute and relative growth rates of leaf number in drenching and combined drenching and foliar application (**Fig. 4.2**). For plant height, KAR_1 drenching and combined drenching and foliar treatment were significantly effective. VCL treatment with foliar and combined drenching and foliar treatment showed an increment in absolute growth rate. However, no significant difference with relative growth rate was observed (**Fig. 4.2**). Foliar application of biostimulants was effective in increasing the leaf area ratio.

Plants have been discovered to exhibit a wide range of responses when exposed to different organic biostimulants. VCL, KEL and KAR_1 were effective in improving some of the growth parameters of *A. hybridus* plants. It has been shown that Chinese cabbage (*Brassica rapa* cv. Bonsai) treated with VCL resulted in increased fresh/dry weight and leaf area **(PANT et al., 2009)**. Foliar application of VCL improved quality and yield of strawberry (*Fragaria* × *ananassa*) fruit **(SINGH et al., 2010)**. *A. hybridus* plants, when treated with KEL, both foliar as well as drenching, showed improved

growth. A study done *in vitro* has reported that the root-growth-promoting activity of KEL was effective when applied on cultured tomato roots **(FINNIE and VAN STADEN, 1985)**. In another study, wheat plants treated with KEL showed an increase in root: shoot dry mass **(NELSON and VAN STADEN, 1986)**. Vermicompost leachates and KEL have been shown to enhance growth and yield and alleviate different biotic and abiotic stresses **(AREMU et al., 2015)**. This has been attributed to the presence of plant growth regulators (PGRs) such as cytokinins, polyamines, abscisic acid, indole acetic acid, brassinosteriods and gibberellins **(AREMU et al., 2015; PAPENFUS et al., 2013; STIRK et al., 2004; STIRK et al., 2014)**. Treatment of tomato (*Solanum lycopersicum*), okra (*Abelmoschus esculentus*) and bean (*Phaseolus vulgaris*) seeds with KAR_1 exhibited a significant improvement in seedling length and growth compared to water control **(VAN STADEN et al., 2006)**. Foliar application of KAR_1 to the seedlings of okra and tomato significantly improved their growth **(KULKARNI et al., 2006; KULKARNI et al., 2007)**. Number of leaves and shoot height were promoted in KAR_1-treated maize (*Zea mays*) seedlings **(VAN STADEN et al., 2006)**. It has been speculated that there may be interactions between KAR_1 and gibberellins, auxin and strigolactones, which results in stimulating plant growth **(LIGHT et al., 2009)**. These interactions between PGRs and KAR_1 are being extensively researched.

4.8.2 Effect of biostimulants on the biochemical composition of Amaranthus hybridus

Chlorophyll, carotenoid, protein and carbohydrate contents of *A. hybridus* were calculated for foliar application, as it showed better results than the other two applications in enhancing morphological parameters (**Table 5.3**). KEL- and VCL-treatment significantly augmented the levels of chlorophylls (a, b and a + b), carotenoids and proteins in comparison to the water control, positive control GA and other tested biostimulants. However, carbohydrate content was significantly decreased in all tested biostimulants compared to the control. These results are in agreement with those obtained for spinach (*Spinacia oleracea*), where all the tested plant growth biostimulants significantly increased total chlorophyll, carotenoids and protein content **(KULKARNI et al., 2019)**. Similarly, biostimulants enhanced the efficiency of photosynthesis and other physiological processes, which resulted in improved growth of tomato and snap bean **(HERNÁNDEZ-HERRERA et al., 2014; SEIF et al., 2016)**. Chlorophyll increase in treated plants could be attributed to

nutrients such as N and Mg present in biostimulants, which are essential for chlorophyll synthesis **(YAKHIN et al., 2017)**. Another explanation for increased chlorophyll content would be the ability of biostimulants to efficiently translocate water and mineral supply to plants **(ABBAS, 2013)**. Carotenoid content was significantly enhanced in all tested biostimulants in comparison to water control and the positive control GA. Carotenoids are an important component of the plants as they have the capacity to augment antioxidant activity **(HAN and XU, 2014)**. Higher carotenoid content recorded with biostimulants indicates that *A. hybridus* plants may have better nutritive value with greater antioxidant capacity. Similarly, increased protein content indicates that the rate of protein synthesis was higher in *A. hybridus* plants. This could be due to the ability of the biostimulants to efficiently mobilize the uptake of N, which is correlated with protein synthesis **(ABBAS, 2013)**. The negative effects of GA could be explained by the fact that the analysis was done at the end of the experiment when plants were harvested, six weeks after planting. The plant could be using more carbohydrates resulting in some imbalance and maybe if the analysis was done between stages of data collection, i.e. after every 2 weeks, a different result could have been obtained. One other explanation could be the soil nutrients, the crop variety used or the different watering regimes could have affected carbohydrate production in the plant. We feel more work needs to be done to ascertain what could be the cause of the reduction in carbohydrates in the crop. Maybe the GA could have caused some changes in the biochemical activity of crop which negatively affected carbohydrate production, but this still need to be investigated.

Biostimulants used in the present study stimulated the growth of *A. hybridus* when applied as a foliar treatment. The use of biostimulants in agriculture is attributed to macro- and micronutrients as well as amino acids, vitamins, cytokinins, auxins and abscisic acid **(HERNÁNDEZ-HERRERA et al., 2014)**. This influences plant cell metabolism resulting in better growth and yield **(CROUCH and VAN STADEN, 1993a; STIRK et al., 2004)**. According to **STEPHENSON (1974)**, some of the biostimulants may contain a precursor of elicitor compounds that stimulate germination, growth and plant health **(HERNÁNDEZ-HERRERA et al., 2014)**. The synergistic interactions of these components from biostimulants illustrate their beneficial properties in crop production. The results of this experiment provide vital information on the utilization of biostimulants for commercial cultivation of *A. hybridus*.

4.8.3 Effect of organic biostimulants and mode of application on antioxidant activity and phytochemical composition of Amaranthus hybridus

The total phenolic, flavonoid and condensed tannin content in methanolic extracts of *A. hybridus* treated with different biostimulants using three methods of application were determined using various methods. For total phenolic content, it was observed that the controls had the highest amounts of total phenolics. Organic biostimulants such as SW and ECK under drenching application had enhanced amounts of total phenolics although the amounts were not statistically significant as they were lower than the control. Foliar application of VCL yielded significant lower amounts of total phenolic content. The differences in total phenolic content between treatments and mode of application of treatments were not statistically significant. A similar result was observed in the total flavonoid content within treatments and mode of applications and again the control had the highest amount of flavonoid content. The general trend was a decrease in flavonoid contents, with all treatments having values lower than that of the control (C) when VCL, KEL and ECK were applied via a combination of drenching and foliar application. For condensed tannins, there was also generally no significant differences in the amount of condensed tannins with the exception of SW applied via drenching which significantly enhanced the content of condensed tannins in *A. hybridus.* The results of the investigation are supported by those obtained by **RENGASAMY et al. (2016)** in cabbage (*Brassica oleracea* var. *capitata*) in which the total phenolics, total flavonoids and condensed tannins were found to be lower in plants treated with ECK at time of harvesting. In another study on maize treated with ECK, there was a significant increase in the amount of total phenolics but the amount of total flavonoids and condensed tannins did not change **(RENGASAMY et al., 2015b)**. Results from this investigation concur with those of **PEREIRA et al. (2019)** on spinach on effects of biostimulants application on nutritional quality and bioactive properties of the plant. High amounts of total phenolic compounds were found in the control. In the same investigation, β-carotene was also enhanced in tomato plants with low concentrations of saffron extract. In another study on tomato by **COLLA et al. (2017a)**, neither total phenols nor total ascorbic acid levels were influenced by the application of biostimulants and this concurs with the results from the current investigation. Similar results were also obtained when differences in tomato quality

were tested with or without treatment using Stimplex® (a liquid seaweed extract), a biostimulant. The results showed that there was no significant difference in the content of total phenolics among cultivars treated with Stimplex®. Control plants had significantly higher DPPH scavenging activity than that in the Stimplex®-treated tomatoes. There was also no significant difference in reducing power among different treatment groups **(SIDHU et al., 2017)**. The foliar application of legume-derived protein hydrolysate (LDPH) to baby lettuce resulted in enhanced antioxidant activity in baby lettuce (*Lactuca sativa*) leaves **(DI MOLA et al., 2019)**. This could be a result of the stimulation of key enzymes involved in antioxidant homeostasis in cells coupled with assimilation of macro and micronutrients in biostimulant-treated plants. This could have resulted in the synthesis of amino acids, phenylalanine and tyrosine **(COLLA et al., 2017a; COLLA et al., 2015b)**. **KHOULATI et al. (2019)** had different results from those of this investigation when tomato plants treated with a biostimulant via foliar application resulted in significant improvements ($P < 0.05$) in amounts of polyphenols, flavonoids and condensed tannins. Only SW increased the amount of condensed tannins in this investigation. *Brassica oleraceae* cultivars treated with *Ascophyllum nodosum* extracts, resulted in enhanced amounts of total phenolics and total flavonoids **(LOLA-LUZ et al., 2013)**, and this does not concur with response from *A. hybridus* in the current investigation.

4.9. Conclusions

The mode of application of liquid supplements to the crops is crucial for economic returns. Three modes of application of biostimulants at very low concentrations were studied on *A. hybridus,* a leafy vegetable. KAR_1, VCL and KEL showed better growth performance with drenching, foliar and combined drenching and foliar applications respectively. However, the best results were achieved with foliar application of VCL, showing a significant effect on both the growth and biochemical parameters of *A. hybridus*. Major biochemical components such as carotenoid and protein were improved with all tested biostimulants. This suggests that not only will some biostimulants have an effect on the growth but they may also influence the nutritional components of a crop plant. These biostimulants are eco-friendly and can be used in low concentrations and in combination with inorganic fertilisers. These biostimulants

should be seriously considered as a worthwhile production strategy for traditional leafy vegetables such as *A. hybridus* for increasing the yield and nutritive value of the crop.

Chapter 5: Effects of microorganisms and biostimulants on the growth and biochemical composition of *Amaranthus hybridus* L.

5.1 Introduction

Modern agriculture is facing emerging threats such as a rapid population increase, global warming and environmental pollution. All these threats have impacted negatively on food production worldwide **(JI et al., 2019)**. Sustainable and eco-friendly approaches are now required to address challenges of increased global food demand, decrease in arable lands and resources and several environmental pressures caused by climate change **(BARGAZ et al., 2018; TILMAN et al., 2017)**. Even though macronutrients such as N, P, K and sulfur (S) found in mineral fertilisers are important in agriculture, microorganisms play a vital role in crop production through N_2 fixation, P solubilization and production of phytohormones. Agriculturally-beneficial microorganisms also play an indirect role through the production of antimicrobial compounds and elicitation of induced systemic resistance **(BARGAZ et al., 2018; SINGH, 2016)**.

Plants interact with a wide variety of soil-inhabiting organisms in different ways, either competitive, exploitative, neutral, commensal or mutualistic **(BONKOWSKI et al., 2009; JACOBY et al., 2017)**. Both plants and microorganisms benefit in such a symbiotic relationship.There are an estimated 20 000 plant species that cannot survive without symbiotic associations with microorganisms **(VAN DER HEIJDEN et al., 2008)**. These associations have been in existence for the past 450 million years, since the ancestral plant lineages first colonized land **(HASSANI et al., 2018)**. The association involves plants being a source of nutrition and a habitat for the microorganisms and in return, the plants get numerous benefits from the microbes. Some of these benefits include growth promotion and stress reduction **(HARDOIM et al., 2008)**. Plant-microbe interactions are of vital importance for the growth of plants under adverse climatic conditions. This should be taken into consideration when designing novel strategies targeting yield improvement and stress resistance in crop plants **(KORENBLUM and AHARONI, 2019)**.

Soil microbiomes have been manipulated to optimize crop productivity since 300 BC **(FINKEL et al., 2017)**. Deposition of organic litter by plants and their metabolic activities alters both the physical and chemical properties of the soil and the plants get nutrients from the soil. Direct benefits plants derive from microorganisms include controlling hormone signalling and resistance to pathogens, with the plants communicating with the microorganisms via metabolites exuded by the roots **(JACOBY et al., 2017)**. This relationship is illustrated in **Fig. 5.1** below.

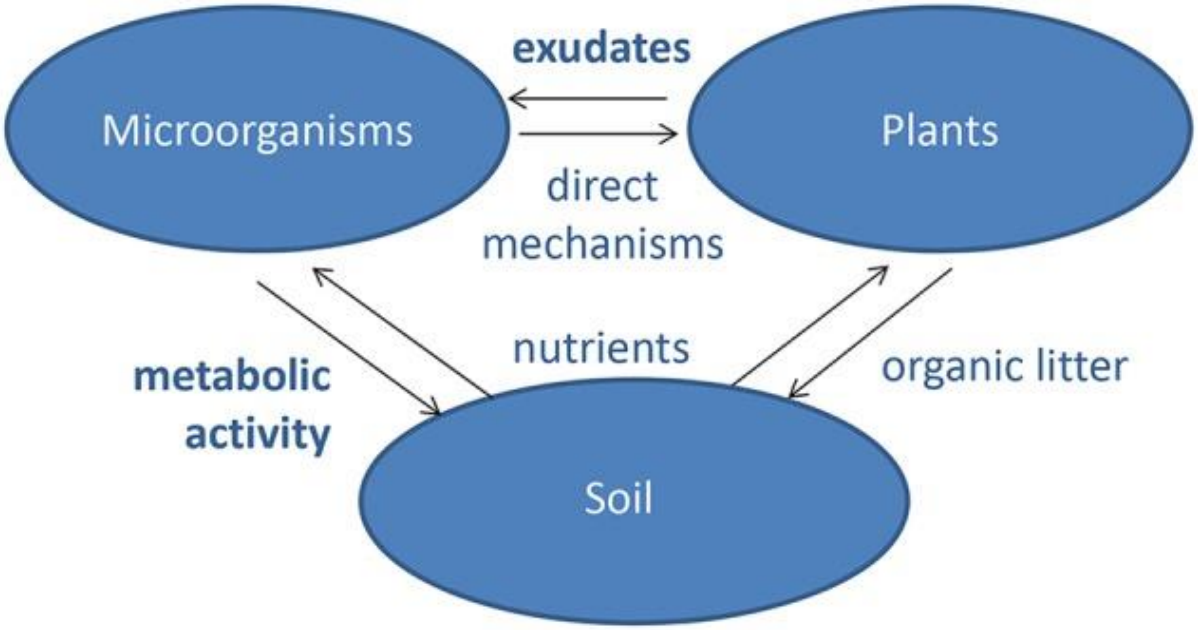


Fig. 5.1: An illustration of how plants interact with microorganisms and the soil. Source: **JACOBY et al. (2017)**.

There is a wide variety of microorganisms which interact in a beneficial way with plants and these include arbuscular mycorrhizal fungi (AMF) or plant growth-promoting rhizobacteria (PGPR) **(TODESCHINI et al., 2018)**.Some of these microorganisms could be exploited in the production of efficient biofertilisers. The plant growth-promoting traits are due to the presence of phytohormones, siderophores, amino acids and polysaccharides, all of which enhance plant growth **(TODESCHINI et al., 2018)**. There are a wide range of benefits to plant health that are derived from microorganisms. Some of these include suppression of diseases **(RITPITAKPHONG et al., 2016)**, priming of plant immune systems **(VAN DER ENT et al., 2009)**, inducing

systemic resistance **(ZAMIOUDIS et al., 2015)**, increasing the acquisition of nutrients **(VAN DER HEIJDEN and HARTMANN, 2016)**, tolerance of abiotic stresses **(ROLLI et al., 2015)**, coping with variations in environmental conditions **(HANEY et al., 2015)** and establishment of mycorrhizal associations **(GARBAYE, 1994)**.

There is a great need for the production of microbial-based bio-formulations that work in a complementary and synergistic manner with mineral fertilisers to increase plant performance **(BARGAZ et al., 2018)**. Nutrients can limit yields of plants, for example, Fe and Zn can be found in abundance in soils but in forms that are not available for crops **(BACKER et al., 2018)**. Several strains of bacteria can enhance the availability of Fe by producing siderophores (organic acids) **(AHMED and HOLMSTRÖM, 2014)**. The same siderophores can also control pathogenic microorganisms by limiting the availability of Fe to the microorganisms **(AHMED and HOLMSTRÖM, 2014; SAHA et al., 2016)**. For example, cotton yield was increased when PGPRs were combined with compost and NPK fertilizer **(KHALIQ et al., 2006)** and long-term PGPR application in combination with compost enhanced straw biomass, grain yields, and grain nutrition of wheat **(HU and QI, 2013)**. PGPR could also contribute to ecosystem recovery when inoculated with plants **(ARMADA et al., 2018; MAJEED et al., 2018)**. The predominant plant growth-promoting bacteria are *Pseudomonas* and *Bacillus* species **(RADHAKRISHNAN et al., 2017)**. *Rhizophagus irregularis* (formerly *Glomus intraradices*), *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* (the most extensively studied species) **(VÁZQUEZ et al., 2000)** are usually used as commercial soil additives because of their ability to promote plant growth **(XIE et al., 2018)**. AgriLife (India) has developed a commercial formulation of *Acidithiobacillus ferroxidans*, a zinc-mobilising bacterium. This has been shown to enhance the uptake of Zn, thereby increasing yield in a number of crops including rice **(SHAKEEL et al., 2015)**, soybean and wheat **(RAMESH et al., 2014)**.

Natural seaweed extracts have been used to partially replace conventional synthetic fertilisers **(SHARMA et al., 2014)**. The seaweed extracts are used as biostimulants in agriculture where they are known to enhance crop productivity. One such seaweed extract is Kelpak® (KEL), derived from *Ecklonia maxima* (Osbeck) Papenfuss, a brown kelp, commonly known as “sea bamboo” that is harvested on the west coast of South Africa **(ANDERSON et al., 2007)**. The growth-promotory effects of KEL on a number of agricultural crops is well documented **(BECKETT et al., 1994; CROUCH and VAN**

STADEN, 1992; PAPENFUS et al., 2013; RENGASAMY et al., 2015a). KEL is effective when applied at low concentrations, suggesting that elicitor compounds such as plant hormones are the active ingredients. Plant hormones such as auxins, cytokinins, gibberellins and brassinosteroids **(STIRK et al., 2004; STIRK and VAN STADEN, 2014)**, polyamines (putrescine and spermine) **(PAPENFUS et al., 2012)** and a phlorotannin (eckol) **(RENGASAMY et al., 2015a)** have been identified in KEL.

Plants often face growing challenges in adverse environmental conditions, such as water deficit or excess, high light intensity, low or high temperature, salinity, heavy metals, UV rays, insect and pest attacks **(BERWAL and RAM, 2018)**. Such stresses induce many metabolic changes, such as the occurrence of an oxidative stress which adversely affects the plant's growth and development **(DÍAZ-VIVANCOS et al., 2008)** resulting in crop failure. Abiotic stresses increase the production of reactive oxygen species (ROS) which has cytotoxic effects **(BERWAL and RAM, 2018)**. Superoxide dismutases (SODs) are ubiquitous metalloenzymes that form the first line of defense against ROS **(TAKAHASHI and ASADA, 1983)**. SOD is one of the most effective components of a plant's cell antioxidant defense system against ROS toxicity **(FRIDOVICH, 1986)**.

With increased interest is organic-based agriculture and the need to minimize fertilizer use, the potential to combine the application of PGPRs and plant-based biostimulants such as KEL to improve the growth, yield and quality of crops needs to be investigated. This study was therefore carried out to determine the effect of PGPRs (*Bacillus licheniformis* and *Pseudomonas fluorescens*) applied in combination with the seaweed-based biostimulant KEL on the growth, biochemical composition and SOD activity of *A. hybridus* L.

5.2 Materials and methods

5.2.1 Site of the experiment

The experiment was carried out in a greenhouse at the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg Campus (290 37.55′ S; 300 24.13′ E), South Africa.

5.2.2 Bacterial inoculum

Bacillus licheniformis (ATCC 12759) and *Pseudomonas fluorescence* (ATCC 13525) were purchased from The American Type Culture Collection (ATCC). Bacterial inoculum was prepared by culturing *B. licheniformis* and *P. fluorescence* in 200 mL Mueller-Hinton (MH) broth on an orbital shaker at 35°C and 27°C respectively for 2 days. The optical density was measured at 660 nm by spectrophotometer (Varian Cary 50 UV-Vis Spectrophotometer, Australia) to achieve uniform populations of bacteria of 10^8 colony-forming units (CFU) per mL. The inoculum was centrifuged at 5 000 g for 10 min (4°C) (Avanti J-E Centrifuge, Beckman Coulter, Inc., California, USA) and the pellet was rinsed with distilled water to remove traces of MH broth. A bacterial suspension was made using distilled water so that the absorbance value was 1.0 when measured at 660 nm.

5.2.3 Pot trial

A. hybridus seeds were purchased from McDonald's Seed Company, Pietermaritzburg, South Africa. New pots (15 cm diameter) were filled with 242 g autoclaved garden soil (described in Chapter 3, Section 3.5.1). Seeds were sown in a nursery to raise seedlings and after seven days (two-leaf stage), three healthy seedlings were transplanted into a pot (15 cm diameter) with five pots per treatment. Pots were arranged on a metal bench in a greenhouse with a daily maximum and minimum temperature of 22 ± 3°C and 15 ± 2°C, respectively, and midday light intensity of 500 - 600 µmol m^{-2} s^{-1}. A randomized pot trial was carried out in which the interactions between *B. licheniformis*, *P. fluorescence* and Kelpak® (KEL) were investigated and compared to a control treatment with/without *B. licheniformis*, *P. fluorescence* and KEL application. Seven days after transplanting, the seedlings were treated with either 10 mL bacterial inoculum per pot or 10 mL KEL (1% v/v) per pot applied to the soil around the plants. For the combination treatments, KEL (1% v/v) was incorporated into the 10 mL bacterial inoculum. The treatments were as follows: control (distilled water), distilled water + *B. licheniformis*, distilled water + *P. fluorescence*, KEL (1% v/v) + *B. licheniformis*, KEL (1% v/v) + *P. fluorescence* and KEL (1% v/v). A second and third application of these solutions was done 2 and 4 weeks later. The seedlings were irrigated twice weekly with water (100 mL) for the

duration of the pot trial except on the day of treatment. Plants were harvested 6 weeks after sowing. Fresh weights of roots and shoots (combined leaf and stem material) and leaf area (measured with a leaf area meter LI-31000, LI-COR Inc., Nebraska, USA) were recorded as a measure of growth. Fresh material (leaf) was randomly harvested from the five pots to make three replicates of each treatment for biochemical analyses.

5.2.4 Determination of total chlorophyll and carotenoid content

This was carried out as described in Chapter 3, Section 3.6.2.

5.2.5 Protein content evaluation

This was carried out as described in Chapter 3, Section 3.6.3.

5.2.6 Carbohydrate content evaluation

This was carried out as described in Chapter 3, Section 3.6.4.

5.2.7 Superoxidase dismutase (SOD) activity

The SOD enzyme activity of leaf protein was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of **GIANNOPOLITIS and RIES (1977)**. The reaction mixture (3 mL) consisted of 50 mmol phosphate buffer (7.8 pH), 13 mmol methionine, 75 µm NBT, 2 µmol riboflavin, 50 µmol EDTA and 100 µL of crude enzyme extract (protein). Tubes were vortexed and were subjected to illumination under 20 W fluorescent tubes. The reaction was allowed to proceed for 15 min and the tubes were kept in the dark. The absorbance of the reaction mixture was taken at 560 nm. One enzyme unit was defined as the amount of enzyme which causes 50% inhibition of NBT photoreduction. The enzyme activity was expressed as Unitsμg^{-1} FW.

5.2.8 Mineral element analysis

The nitrogen (N) content was determined by the method of **(DUMAS, 1831)** using a LECO-Truspec CNS analyser and the other minerals viz. aluminium (Al), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), calcium (Ca), magnesium (Mg), sodium (Na), phosphorus (P) and potassium (K) were determined using inductively coupled plasma - optical emission spectrometry (ICP-OES), VISTA-MPX. For the determination of N, the milled plant material was kept in an oven at 110°C overnight. The following day the samples were cooled in a desiccator for 30 min and then 0.125 g samples were weighed. The samples were introduced and burnt in a module consisting of an electric furnace working at a temperature of 950 °C. For the determination of Al, Fe, Mn, Zn, Cu, Ca, Mg, Na, P and K, 0.5 g milled samples were weighed and placed in crucibles (crucibles were preheated overnight in an oven set at 110°C and were cooled in a desiccator and weighed) in an oven set at 110°C for 2 h. Crucibles with the samples along with blanks were arranged in order and placed into a furnace set at 450°C for 4 h. After 4 h, the furnace was opened and allowed to cool off. Subsequently, crucibles were removed from the furnace and cooled. Samples were then digested where a few drops distilled water was added, followed by addition of 2 mL concentrated HCl to each sample. The samples were slowly evaporated to dryness in a water bath in a fume hood. Then 25 mL of freshly prepared 1:9 HCl solution was added to each sample and stirred with a glass rod. The samples were filtered through Advantec 5B: 90 mm diameter filter papers into clean sample crucibles. The filtrate was diluted with de-ionized water, ratio 5:20 and the diluted solution was analysed for elements in the ICP-OES system. The calibration standards were treated similarly. The raw data from the ICP-OES was taken for further calculations using dry-matter determined earlier as well as the sample weight. All sample vials, sample crucibles, and glassware were cleaned by soaking in 10% (v/v) HNO_3 and rinsed with deionized ultrapure water (Milli-Q, Millipore, Bedford, MA) before use. The appropriate standards were prepared by dilution of each pure element standard within the concentration range of the elements in the samples. The results were obtained from triplicate measurements.

5.3 Statistical analysis

The quantification of all parametric data was done in triplicate and results presented as mean ± standard error. Mean value comparison was computed using one-way analysis of variance (ANOVA) using GenStat 18th Edition. Duncan's multiple range test was used for statistical significance ($P < 0.05$) to separate the mean values. Graphs were plotted using Sigma Plot for Windows Version 11.

5.4 Results

5.4.1 Effect of Bacillus licheniformis*,* Pseudomonas fluorescens *and Kelpak® on the growth of* Amaranthus hybridus

B. licheniformis and *P. fluorescens* caused a major decline in the growth of *A. hybridus* compared to the control plants. The overall application of microorganisms alone on *A. hybridus* had a deleterious effect on the growth and development of the plant. In contrast, Kelpak® had a beneficial effect of plant growth with significantly higher values recorded in these plants compared to the control treatment (**Fig. 5.2 D, E** and **F**). Application of KEL in combination with the rhizobacteria overcame the negative effect of the microorganisms. The combined treatment of KEL + *P. fluorescens* showed a significant positive effect on most of the growth parameters (leaf number, root length, both shoot and root fresh weights and leaf area) compared to *A. hybridus* control plants and to a lesser extent, when combined with *B. licheniformis* (**Fig. 5.2 A, C, D, E** and **F**, respectively). The results of the study are supported by ANOVA where microorganisms alone had no significant influence on the leaf number (**Table 5.1**). Only treatment (T) and the interaction of microbes (M) and T (M x T) had a significant effect on leaf number ($P < 0.05$). Microbes (M) alone, T and the interation of M and T (M x T) had a significant influence on leaf area (**Table 5.1**). Microbes alone showed no significant influence on the height of *A. hybridus* plant while notable significant effects where observed due to T and the interaction of M and T (M x T). The fresh weight of *A. hybridus* was significantly influenced by the microbes, treatment and their interactions (**Table 5.1**).

5.4.2 Effect of Bacillus licheniformis*,* Pseudomonas fluorescens *and Kelpak® on the biochemical composition and antioxidant enzyme superoxide dismutase (SOD) of* Amaranthus hybridus

B. licheniformis and *P. fluorescens* treated plants had a significantly lower Chlorophyll a content compared to the control plants while KEL treated plants yielded significantly higher Chlorophyll a. Although not as high as the KEL treated plants, KEL + *B. licheniformis* and KEL + *P. fluorescens* treated plants showed a significant increase in the amount of Chlorophyll a compared to the plants treated with microbes alone. However, control plants were not significantly different from these treatments (**Table 5.2**). None of the treatments showed any significant influence on Chlorophyll b production apart for the significant decrease in *B. licheniformis* treated plants (**Table 5.2**). For Chlorophyll a + b and carotenoid content, the same trend was apparent as with Chlorophyll a where the KEL treated plants showed the most significant increase, PGPR caused a significant decrease and the combined treatments caused increased levels although not significantly different to the control plant (**Table 5.2**).

B. licheniformis and *P. fluorescens* treated plants had a significant decrease in protein and carbohydrate content while the KEL treatment had no significant effect. Protein and carbohydrate levels were similar to the control plants for the combination treatments (**Table 5.2**).

All the treatments significantly reduced the activity of SOD compared to *A. hybridus* control plants with the combination treatments having significantly low activity (**Table 5.2**).

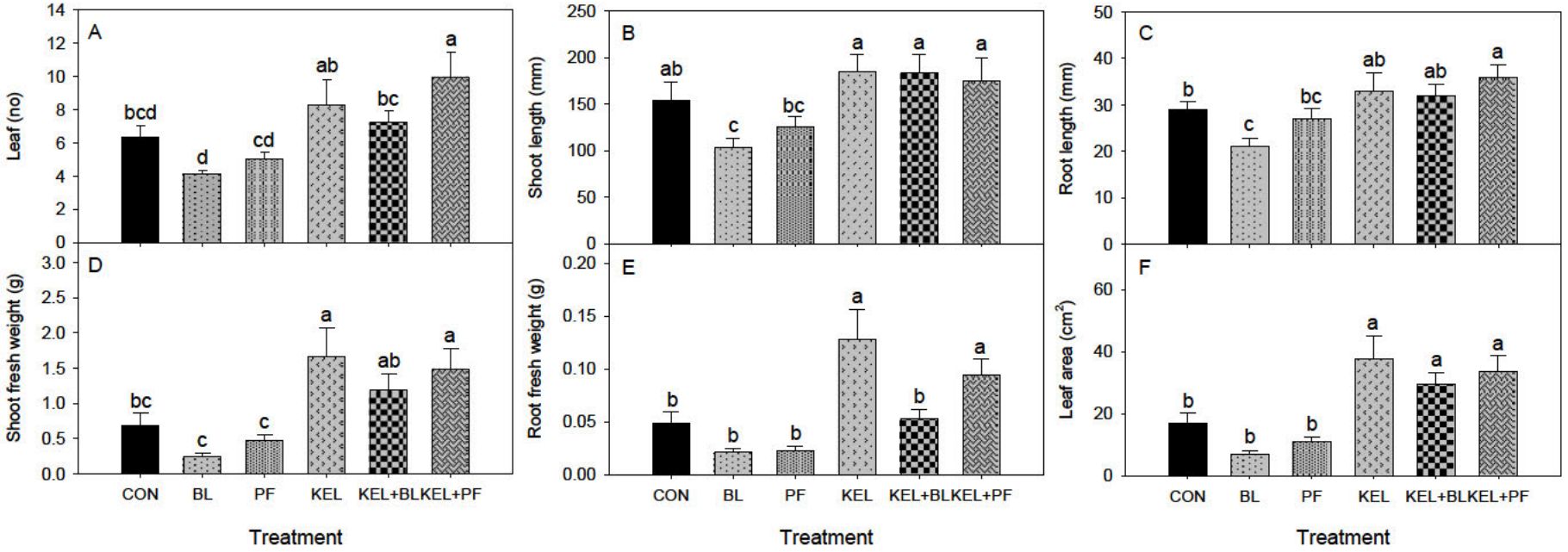


Fig. 5.2: Effect of microorganisms [*Bacillus licheniformis* (BL) and *Pseudomonas fluorescens* (PF) and the biostimulant Kelpak® (KEL)] on the general growth of *Amaranthus hybridus*. The plants were grown under greenhouse conditions at a temperature of 25 ± 2 °C. Bars (± SE) of each growth parameter with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

Table 5.1: Analysis of variance for comparing different treatments and interactions for the growth response of *Amaranthus hybridus* (*P* < 0.05).

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance	*F*-probability
Leaf (no.)					
Microbes (M)	1	14.02	14.02	0.86	0.356
Treatment (T)	5	445.15	89.03	5.45	< 001
M X T	4	445.15	111.29	6.88	< 001
Residual	114	1844.80	16.18		
Total	119	2303.97			
Leaf area (cm^2)					
Microbes (M)	1	1381.5	1381.5	4.65	0.033
Treatment (T)	5	14778.5	2955.7	9.95	< 001
M X T	4	14778.5	3694.6	12.55	< 001
Residual	114	33572.1	294.5		
Total	119	49732.2			
Plant height (mm)					
Microbes (M)	1	16951	16951	2.73	0.101
Treatment (T)	5	131155	26231	4.22	< 001
M X T	4	131155	32789	5.32	< 001
Residual	114	702386	6161		
Total	119	850492			
Plant fresh weight (g)					
Microbes (M)	1	3.6407	3.6407	3.95	0.049
Treatment (T)	5	34.2282	6.8456	7.44	< 001
M X T	4	34.2282	8.5571	9.38	< 001
Residual	114	104.0319	0.9126		
Total	119	141.9008			

Table 5.2: Effect of *Bacillus licheniformis*, *Pseudomonas fluorescens* and Kelpak® on photosynthetic pigments, biochemical and superoxide dismutase enzyme (SOD) content of *Amaranthus hybridus* after 6 weeks.

Treatment	Chlorophyll a (µg g^{-1} FW)	Chlorophyll b (µg g^{-1} FW)	Chlorophyll a + b (µg g^{-1} FW)	Carotenoid (µg g^{-1} FW)	Protein (µg g^{-1} FW)	Carbohydrate (µg g^{-1} FW)	SOD (Unit mg^{-1} FW)
Control	919 ± 90 b	283 ± 38 a	1202 ± 60 b	255 ± 12 b	9.5 ± 0.12 a	210 ± 34 ab	0.041 ± 0.007 a
BL	329 ± 60 d	148 ± 20 b	478 ± 80 c	112 ± 14 d	3.5 ± 0.26 c	131 ± 14 c	0.025 ± 0.001 b
PF	753 ± 21 c	250 ± 6 a	1004 ± 26 b	212 ± 3 c	6.1 ± 1.49 b	154 ± 4 bc	0.012 ± 0.003 cd
KEL	1129 ± 66 a	297 ± 20 a	1426 ± 86 a	298 ± 16 a	9.3 ± 0.37 a	204 ± 26 abc	0.020 ± 0.003 bc
KEL + BL	992 ± 54 ab	256 ± 12 a	1249 ± 67 ab	278 ± 9 ab	10.6 ± 0.75 a	267 ± 30 a	0.007 ± 0.001 d
KEL + PF	925 ± 92 ab	254 ± 28 a	1179 ± 120 b	254 ± 18 ab	8.9 ± 0.95 a	179 ± 10 bc	0.005 ± 0.001 d

Mean values (± SE) in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

5.4.3 Effect of Bacillus licheniformis*,* Pseudomonas fluorescens *and Kelpak® on the mineral composition of* Amaranthus hybridus

The mineral composition in *A. hybridus* varied with different treatments (**Table 5.3**). Plants treated with *P. fluorescens* generally had the highest mineral content with significantly higher Ca, Mg, K, P, Zn and Mn content and significantly lower Fe and Al compared to the control plants while N and Cu were similar to the control plants (**Table 5.3**). In contrast, KEL treated plants had significantly higher K and P and significantly lower N, Ca, Fe and Al compared to the control plants while Mg, Zn, Cu and Mn were similar (**Table 5.3**). The combination treatment of KEL + *B. licheniformis* generally decreased the mineral composition (K, P, Zn, Fe and Al) compared to the KEL treatment with only N and Ca increasing in the combination treated plants and Mg, Cu and Mn being similar in both treatments (**Table 5.3**). For mineral analysis only effective combinations showing good activity were selected for further analysis.

Table 5.3: Effect of *Bacillus licheniformis* (BL), *Pseudomonas fluorescens* (PF) and Kelpak® on the mineral composition of *Amaranthus hybridus*.

Treatment	Nitrogen	Calcium	Magnesium	Potassium	Phosphorus	Zinc	Copper	Manganese	Iron	Aluminium
				(%)					(mg kg^{-1})	
Control	2.06 ± 0.01 a	0.93 ± 0.0 b	0.44 ± 0.01 b	5.06 ± 0.02 d	0.43 ± 0.01 c	40.1 ± 0.81 b	4.53 ± 0.14 a	97 ± 0.95 b	549 ± 13.7 a	172 ± 8.2 a
KEL	1.65 ± 0.02 c	0.89 ± 0.0 c	0.42 ± 0.01 b	5.52 ± 0.02 b	0.66 ± 0.02 a	40.5 ± 0.41 b	4.19 ± 0.70 a	109 ± 0.10 b	264 ± 5.0 b	148 ± 6.8 b
KEL + BL	1.86 ± 0.01 b	0.92 ± 0.0 b	0.43 ± 0.01 b	5.35 ± 0.02 c	0.51 ± 0.02 b	36.4 ± 0.42 c	4.02 ± 0.16 a	105 ± 1.09 b	221 ± 5.3 c	88 ± 4.10 c
PF	1.96 ± 0.06 ab	1.06 ± 0.0 a	0.50 ± 0.01 a	6.26 ± 0.03 a	0.57 ± 0.02 b	43.8 ± 0.69 a	5.03 ± 0.51 a	116 ± 0.67 a	246 ± 5.1 bc	140 ± 6.7 b

Mean values (± SE) in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

5.5 Discussion

There are numerous environmental and public health issues generated by conventional agricultural practices, particularly the use of chemical fertilisers. Hence, there is a need for approaches or strategies which are eco-friendly involving the inclusion of biostimulants and microorganisms in agriculture **(GANGWAR et al., 2017; MISHRA et al., 2017)**. There is a need to create a balance between the needs of the future generations and the currently available natural resources because of the continued exploitation of available resources in response to a general population increase **(MISHRA et al., 2017)**. The most promising eco-friendly approach for sustainable agriculture involves harnessing plant growth-promoting rhizobacteria (PGPRs), endo- and ecto-mycorrhizal fungi, cyanobacteria and numerous other useful microorganisms present in the soil **(GANGWAR et al., 2017)** along with biostimulants such as KEL. Soil fertility is partly a function of the diversity of microorganisms and their functions **(ANSARI and AHMAD, 2019)**. Interactions between the different species of soil microbes are responsible for maintaining the microbial dynamics **(SØRENSEN et al., 2005)**. According to **ANSARI and AHMAD (2019)**, two organisms can interact either positively, negatively or in a neutral manner even though it is believed that PGPRs were correctly named since they are beneficial bacteria with regards to their positive role in plant growth **(SHANK et al., 2011)**. The PGPRs are present in the soil and stimulate plant growth in many ways. They often work in association with roots or leaves or inside tissues of the plant **(GLICK, 2012)**. Among the PGPRs there is a wide variety of microbes belonging to genera *Pseudomonas*, *Rhizobium*, *Azotobacter*, *Klebsiella*, *Enterobacter* and *Bacillus,* to mention only a few **(AHEMAD and KIBRET, 2014)**. According to **DU JARDIN (2015)**, biostimulants are substances or microorganisms which are applied to crop plants to improve nutrition efficiency, tolerance of abiotic stress and/or quality of the crop. The objective of the current research was to investigate the combined effects of some microorganisms (*Bacillus licheniformis* and *Pseudomonas fluorescens*) and the seaweed biostimulant KEL on the growth and biochemical composition of *A. hybridus* grown under greenhouse conditions. The information obtained could be utilised to explain the effects of PGPRs and KEL on this leafy vegetable.

5.5.1 Effect of Bacillus licheniformis*,* Pseudomonas fluorescens *and Kelpak® on the growth of* Amaranthus hybridus

Results from the present investigation showed that KEL, a seaweed-based biostimulant, influenced growth in *A. hybridus* and this supports the reports of **DU JARDIN (2015)** on the role of biostimulants on plant growth. Biostimulants enhance nutrient uptake, support the growth of crop plants and also improve crop tolerance to abiotic stresses **(DROBEK et al., 2019)**. Biostimulants stimulate nutritional processes of the plant, resulting in the efficient use of nutrients by the plant, tolerance to abiotic stresses, improvement of crop quality traits, availability of nutrients present in the soil and rhizosphere, and breakdown of soil organic compounds **(CARADONIA et al., 2019)**. The positive and significant response of *A. hybridus* to the application of KEL could be due to the presence of various phytohormones, polyamines and the phlorotannin eckol that are present in KEL **(PAPENFUSS et al., 2012; RENGASAMY et al., 2015a; STIRK et al., 2004; 2014)**. Similarly, KEL- treated okra seedlings had a significant increase in shoot length, the thickness of stem, leaves and root numbers as well as fresh weight under deficiencies of both P and K **(PAPENFUS et al., 2013)**.

The two rhizobacteria used in the present study had a negative effect on growth of *A. hybridus*. This is contrary to other reports where PGPRs such as *Pseudomonas fluorescens* have potential to be used as an eco-friendly and sustainable tool in modern agriculture. For example, *P. fluorescens* significantly enhanced yields in rice (*Oryza sativa*) and improved resistance against pathogens **(NEHAL, 2015)**. Fluorescent *Pseudomonas*-FAP2 and *Bacillus licheniformis* were able to interact positively in biofilm mode, resulting in an improvement in the growth and photosynthetic attributes of wheat plants **(ANSARI and AHMAD, 2019)**. The positive contribution of *Pseudomonas* in plant growth is attributed to its ability to produce cytokinins and gibberellins (gibberellic acid) **(AMUTHARAJ et al., 2013; NEHAL, 2015)**. The negative effects of the inoculation could be explained by the fact that maybe the bacterial strains used in the researcher are generally not conducive to the development of the crop. It is known that PGPR-activity is species-specific and it could be that the two strains used are not the best species for promoting growth in A. hybridus **(FIGUEIREDO ET AL., 2011; ROSIER, 2018)**. There is need for further

research test more strains of PGPR on the crop maybe we can get the most ideal strain for the growth promoting role.

However, the combination treatment of the PGPR and KEL used in the present study resulted in a significant improvement in the growth of *A. hybridus*, indicating positive interactions between the rhizobacteria and seaweed extract.

5.5.2 Effect of Bacillus licheniformis*,* Pseudomonas fluorescens *and Kelpak® on the biochemical composition of* Amaranthus hybridus

KEL treatments had a positive effect on the chlorophyll and carotenoid content of *A. hybridus*. Plant-based biostimulants and seaweed extracts promote the biosynthesis of chlorophyll or minimise its breakdown, resulting in an increase in the green colour of the leaves **(ABBAS and AKLADIOUS, 2013)**. Leaf colour is an important parameter of quality in leafy vegetables like *A. hybridus* since greenness is more appealing to consumers. Increasing the chlorophyll of a plant is advantageous to the plant since it translates into increased photosynthetic activity within the leaves **(BULGARI et al., 2019)**. Enhanced concentrations of chlorophyll and carotenoids have been observed in rocket (*Eruca sativa*) **(VERNIERI et al., 2005)**, lettuce (*Lactuca sativa*) and endive (*Cichorium endivia*) **(BULGARI et al., 2014)** when treated with biostimulants. An increase in photosynthetic pigment, phytochemicals and myrosinase activity was also observed in cabbage treated with ECK, a phenolic compound isolated from *Ecklonia maxima*, a brown seaweed used in making KEL **(KHAN et al., 2009)**. Contrary to positive reports of increased pigment content in plants whose roots had been inoculated with PGPRs of the genera *Pseudomonas*, *Bacillus* and *Azotobacter* **(BABU et al., 2015)**, chlorophyll and carotenoid content decreased in *A. hybridus* treated with *B. licheniformis* and *P. fluorescens* in the present study. However, the combined treatments with KEL improved the pigment content, indicating mutualistic interactions between the rhizobacteria and seaweed extract.

Both the PGPR and KEL treatments reduced SOD levels in *A. hybridus*, indicating stress reduction in the plants. Results concur with those of wheat inoculated with *Pseudomonas putida*, a thermo-tolerant microbe, resulting in an increased tolerance to heat stress. Inoculated plants had low ROS generation with lower levels of expression of ROS response genes such as SOD, ascorbate peroxidase, and catalase

(ALI et al., 2011). Biostimulants are able to counteract environmental stresses such as water deficit, soil salinization, and exposure to sub-optimal growth temperatures in many ways **(DU JARDIN, 2015; VAN OOSTEN et al., 2017)**. This results in the improvement of plant performance, increases plant growth and productivity **(BULGARI et al., 2019)**. The combination treatments of PGPR + KEL significantly reduced SOD activity compared to the single treatments in *A. hybridus*, indicating a synergistic effect of these treatments.

5.5.3 Effect of Bacillus licheniformis*,* Pseudomonas fluorescens *and Kelpak® on the mineral composition of* Amaranthus hybridus

P. fluorescens treatment enhanced the content of most mineral elements in *A. hybridus* with the exception of N, Cu, Fe and Al. KEL and a combination of KEL + *B. licheniformis* treatments only improved the K and P content in *A. hybridus* but had a significant negative influence on the content of most of the other minerals quantified. These results are not in agreement with those documented for other plant species treated with KEL. Significant increases in yield and concentration as well as amounts of Ca, K and Mg were recorded in leaves of lettuce treated with KEL **(CROUCH et al., 1990)**. Microtubers of potatoes (*Solanum tuberosum*) had high amounts of Mg, Cu, Fe, Zn and Ni when treated with KEL (**WIERZBOWSKA et al., 2015)**.

The increase in mineral content in *A. hybridus* treated with *P. fluorescens* is in agreement with other studies. For example, the seeds of radish (*Raphanus sativus*) inoculated with *Bacillus subtilis* and *P. fluorescens* resulted in significant increases of P, N, K, Mg and Ca in roots and leaves of the plants **(MOHAMED and GOMAA, 2012)**. When broccoli (*Brassica oleracea* L., var. *italica*) roots were inoculated with PGPRs, there was an increase in amounts of chlorophyll along with mineral elements (N, K, Ca, S, P, Mg, Fe, Mn and Zn) content **(YILDIRIM et al., 2011)**. A significant increase in nutrient content of N, P, K, Ca and Mg, occurred in greenhouse-grown tomato plants (*Solanum lycopersicum*) inoculated with different combinations of PGPRs, (*Pseudomonas*, *Azotobacter* and *Azosprillum*) **(SHARAFZADEH, 2012)**. Similar results were obtained in banana plants treated with PGPRs, where there were significant increases in N, P, K, Ca and Mg **(VAYSSIÈRES et al., 2009)**. PGPRs possess mechanisms that are involved in N fixation, solubilizing phosphate and

synthesis of phytohormones, all of which enhance the availability of nutrients and enabling their absorption by plants **(ARORA et al., 2012; BHARDWAJ et al., 2014)**. Microbial enzymes tend to increase the accumulation of bioavailable forms of macronutrients present in the soil and crops **(KUAN et al., 2016)**. All the above reports concur with findings from the current research since *P. fluorescens* individually enhanced the content of most of the mineral elements with the exception of Fe and Al where it had adverse effects.

5.5.4 Benefits of combination treatments

The present study demonstrated the benefit of applying a combination treatment of PGPR and a seaweed extract. KEL improved the growth parameters of the leafy vegetable *A. hybridus* and increased the pigment content, thus improving the greenness and palatability of the leaves. PGPR improved the mineral content of the leaves, thus improving its nutritional value. This is an important consideration when addressing the issues of hidden hunger. In addition, all treatments decreased the SOD levels, indicating reduced stress levels. The lowest SOD levels were measured in the plants treated with the combination treatments, indicating additive effects between the PGPR and seaweed extract.

Several experimental studies have demonstrated similar additive and synergistic effects of different plant biostimulants combined with PGPR to promote growth and productivity of plants **(BETTONI et al., 2014; ROUPHAEL and COLLA, 2018)**. Examples of such beneficial attributes include the significant increases in plant height and branching for humic acid and seaweed extract sprayed on groundnut plants in comparison to the untreated control. The combined applications of both PGPRs together with seaweed extracts and humic acid demonstrated a synergistic interaction on the groundnut plants with a superior increase in general growth **(PRAKASH et al., 2014)**. Plant improvement was associated with the enhancement of N uptake and the subsequent synthesis of chlorophyll which increased the rate of photosynthesis, thereby triggering the movement of the photosynthates to the sinks **(PRAKASH et al., 2014)**. A similar result was observed in onion seedlings treated with humic acid and an inoculum of *Rhizophagus intraradices,* which resulted in a significant increase in root dry weight and carotenoids of 43.9 and 12.1% for humic acid and 29.6 and 57.1%

for mycorrhiza respectively. There was a synergistic effect when both humic acid and mycorrhiza fungi were applied resulting in both parameters measuring 106.7 and 123.6% respectively **(BETTONI et al., 2014)**. The increase in crop performance was attributed to an increase in the availability of nutrients caused by the synergistic action of humic acid and mycorrhizal fungi when they were applied in combination. Mycorrhizal fungi and humic acid had a similar synergistic effect when applied in combination on ryegrass, a perennial plant in which root biomass and chlorophyll biosynthesis were enhanced compared to either application alone **(NIKBAKHT et al., 2014)**. A similar response was also observed in micropropagated pineapple plantlets due to the synergistic action of vermicompost and PGPR, which significantly enhanced both shoot dry weight and leaf area **(ROUPHAEL and COLLA, 2018)**.

5.6 Conclusions

Microorganisms and biostimulants such as Kelpak® can work in a complementary and interactive manner to improve the growth, biochemical composition and mineral content of the highly nutritious but neglected leafy vegetable, *A. hybridus.* Kelpak® overcame the detrimental effects of microorganisms when applied alone by neutralising negative effects of the microorganisms. Hence to improve the general growth performance of *A. hybridus* it is necessary to include both microorganisms and Kelpak®. The total chlorophyll and carotenoid content were enhanced by this synergism, which improves the size and quality of the leaves thereby enhancing production of the crop. Greenness is a parameter of quality and palatability for most leafy vegetables including *A. hybridus*. This is important as it would improve consumer preferences for the vegetable, since it would be more appealing to any would be consumer by its greenness. Also, the mineral content of the vegetable was increased for most minerals and this is of vital importance, as this goes a long way in addressing the issues of hidden hunger. It can be concluded that microbes and biostimulants could be used as an eco-friendly approach to improve the production and mineral content of indigenous leafy vegetables such as *A. hybridus*

CHAPTER 6: Effect of organic biostimulants on growth of *Amaranthus caudatus* L. and *Amaranthus retroflexus* L.

6.1 Introduction

The burden being faced by agriculture today is the intense demand for increased food, feed and biofuel production against a backdrop of a decline in natural resources to meet the needs of the projected nine billion inhabitants expected to be on the planet by the year 2050 **(GODFRAY et al., 2010b)**. It is imperative that agricultural production increase by 70% to cater for the projected 40% increase in global population **(BRUINSMA, 2009; TOPWAL and AGARAWAL, 2018)**. The green revolution has favoured a limited number of crops, ignoring some key crops **(KHOURY et al., 2014)**. The few crops favoured for cultivation are not nutrient sufficient since they lack essential nutrients and vital vitamins to provide a balanced diet. This has resulted in more than two billion people the world over languishing from malnutrition **(CHENG et al., 2017)**. Underutilised crops have the potential to provide food, feed and vitamins to the increasing population **(EBERT, 2010)**. There is a need to incorporate crops with a more balanced nutrient composition in mainstream agriculture to improve diversity in diet and food quality for the fight against starvation and hidden hunger. The amaranth has potential to be used as an alternative food grain in many parts of the world. It happens to be one of the few multipurpose crops underutilised as it produces grain, leafy vegetables, fodder and improved dietary supplements more than the conventional staple crops **(MLAKAR et al., 2009a)**.

The amaranths are a group of plants from the genus *Amaranthus* of the Amaranthaceae family from the order Caryophyllales. The plants are known to be adaptable to different environmental stresses such as extreme temperatures, drought and low input cultivation **(HUERTA-OCAMPO et al., 2009)**. They are also more competitive than many other crops due to their short life-cycle and easy adaptation to new environments **(NORMAN, 1992)**. The genus is native to America and valued for the quality of its leaves and grain, which are much appreciated for their high nutritional attributes and superior mineral content **(SAUER, 1950)**. This has led to the evolution of the amaranth as an essential food crop in South America, Mexico, Africa, parts of

Asia and Europe, as well as in Australia **(STALLKNECHT and SCHULZ-SCHAEFFER, 1993)**.

The amaranth is classified as a pseudo-cereal since it is not a cereal such as wheat, corn, rice or barley. Amaranths leaves have an excellent chemical composition and a mild taste, similar to that of spinach, which makes it a proper leafy vegetable **(AMICARELLI and CAMAGGIO, 2012)**. In terms of its consumption, it may be eaten raw as a salad, cooked and mixed with other vegetables or as a puree base for sauces and as a spice when dried, or as a soup with some greens and cereals. Some combine it with flour cereals to make noodles and pizzas. Amaranths constitute the mainstay of traditional cuisine in the Caribbean. According to the **NATIONAL RESEARCH COUNCIL (1984)**, grain amaranths such as *Amaranthus caudatus*, *A. hypochondriacus* and *A. cruentus* can synthesize two amino acids, methionine and lysine, in high proportions and this gives them a superior nutritional value compared to other cereal grains. All parts of the amaranth plant are edible and in addition they have medicinal properties and are used to treat many nonsurgical diseases. The multiple benefits derived from the amaranth have led to the re-emergence of these plants as crops of immense value as they are also used as sources of energy the world over **(MLAKAR et al., 2010)**. All the above consolidates the vital role played by the amaranths in food and nutrition security **(UUSIKU et al., 2010)**.

There are mainly three types of grain amaranth species, with two of these, *A. cruentas* L. and *A. hypochondriacus* L., originating from Central and North America and the other one, *A. caudatus* L., from South America **(LIGHTFOOT et al., 2017; STETTER et al., 2017)**. In terms of the nutritional content of the amaranth grain, it has a crude protein content of 13.1-21.0%, consisting of the easy to digest albumins and globulins (50-60% of total protein), glutelins (20.8%) and prolamines (12%) **(KONISHI et al., 1985; ZHELEZNOV et al., 1997)**. According to **KAUR et al. (2010)**, there is a variation in the amount of proteins found amongst amaranth species and varieties including the weedy species reportedly having higher amounts of proteins, amino acids and other nutrients when compared with the cultivated species **(ANDINI et al., 2013; SHUKLA et al., 2010)**. The high nutritional and pharmaceutical attributes of amaranth have seen them being preferred as wheat substitutes in diets of celiac disease patients in the USA and Europe **(ANGEL HUERTA-OCAMPO and PAULINA BARBA DE LA ROSA, 2011; TOSI et al., 2001)** since it is a gluten-free ingredient of bread, pasta and other

confectionery products **(ALVAREZ-JUBETE et al., 2009)**. According to **BHAT et al. (2015)** and **SÁNCHEZ-MARROQUÍN et al. (1987)**, treated seeds of amaranths can be taken as instant drinks, with water or milk, or can be added to bread, tortillas, cookies or other preparations. Amaranth seed flour from grain amaranth such as *A. caudatus* is gluten-free and can be utilised in the improvement of both the nutritional value and digestibility of several cereal products to enhance the quality of protein, fat amount and amino acid profile **(BRESSANI et al., 1992)**.

Amaranth seeds are gluten-free and this is the best-known health benefit of the grain, even though there are also several other medically-active compounds present in amaranths that have been reported. The nutraceutical properties of amaranths are attributed to the presence of a mixture of tocopherols, 0.3-0.4% phytosterols and 4-6% squalene in its oil **(KHAMAR and JASRAI, 2014; LOZANO-GRANDE et al., 2018)**. They also contain rich amounts of bioactive flavonoids **(RASTOGI and SHUKLA, 2013)**. Oil from amaranth seed is known to be the best plant-based source of squalene (a triterpene), a very strong antioxidant which prevents premature skin aging by preventing cell-damage **(KHAMAR and JASRAI, 2014; LOZANO-GRANDE et al., 2018)**. Other compounds present in amaranths with anti-inflammatory and anti-cancerous effects include saponins, tannins, phenols, flavonoids, cardiac glycosides, steroids and triterpenoids **(REYAD-UL-FERDOUS et al., 2015)**. Hence, this explains the global use of amaranth in traditional medicines for various ailments **(KUMAR et al., 2012)**.

Amaranthus caudatus is both a vegetable and grain plant that is fast-growing and produces high yields, whose tender leaves are consumed in soups and stews. The young shoots can be dried to be utilised later as fodder **(GRUBBEN and VAN SLOTEN, 1981)**. According to **PETER and GANDHI (2017)**, concentrates of leaf protein can be used to feed children and people in need of high amounts of protein. According to **PACIFICO et al. (2008)**, the leaves and seeds of *Amaranthus retroflexus* L. (redroot pigweed) have been used for many centuries as sources of food by the local people of North and South America, Asia, Africa and Europe.

Both the amaranth species can be consumed as vegetables and grains because of their excellent nutritional and nutraceutical properties. The inclusion and adoption of these two amaranth species in mainstream agriculture in southern Africa, particularly

in South Africa, could go a long way in addressing the challenges of nutrition insecurity and hunger which are rampant in the region. This study aimed to evaluate the effects of organic biostimulants and the mode of application of the biostimulants on the growth and biochemical composition *of A. caudatus* and *A. retroflexus.*

6.2 Materials and methods

6.2.1 Site of experiment

The experiment was carried out in a greenhouse at the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg Campus (290 37.55′ S; 300 24.13′ E), South Africa.

6.2.2 Biostimulants and chemicals

These were prepared as described in Chapter 4, Section 4.2.4. The seeds of one genotype of *A. caudatus* and one genotype of *A. retroflexus* were acquired from Poland.

6.2.3 Seed growth assays

This was carried out as described in Chapter 4, Section 4.2.4.

6.2.4 Experimental design and greenhouse conditions

This was carried out as described in Chapter 4, Section 4.2.5.

6.2.5 Determination of photosynthetic pigments

This was carried out as described in Chapter 3, Section 3.6.2.

6.3 Statistical analysis

The quantification of all parametric data was done in replicates and results presented as mean ± standard error. Mean value comparison was computed using one-way analysis of variance (ANOVA) using SPSS for Windows (SPSS, Version 24.0. Armonk, New York, USA). Duncan's multiple range test was used for statistical significance ($P \leq 0.05$) to separate the mean values. General analysis of variance was computed for the main effects and their interactions.

6.4 Results

6.4.1 Effect of organic biostimulants and mode of application on Amaranthus caudatus

The effects of biostimulants and mode of application were investigated and the results were analysed. **Table 6.1** shows the effects of different organic biostimulants and mode of application on the growth of *A. caudatus*. From the results it can be observed that generally, the application of biostimulants via drenching, foliar and a combination of both drenching and foliar application, did not yield notable effects on some growth parameters of *A. caudatus,* with the exception of a few treatments. When the biostimulants were applied via drenching there were no significant effects on leaf number, shoot and root fresh weight of the plant but SW and KEL had a significant influence on shoot length compared to the control. Significant influences were also recorded for SW and KEL for root length compared to control (**Table 6.1**). Stem thickness was significantly influenced by all the biostimulants compared to the control. However, KAR_1, VCL and ECK reduced the stem thickness compared to GA, the positive control. SW and KEL showed similar effects on stem thickness to GA (**Table 6.1**). SW and GA were the only treatments with a significant influence on leaf area compared to the control. The application of biostimulants via foliar treatment had no notable influence on the growth parameters of *A. caudatus* (**Table 6.1**) except for all the treatments decreasing stem thickness. When the same biostimulants were applied via a combination of drenching and foliar application, there was generally no significant effect on most of the growth parameters with the exception of VCL, which had a

significant influence on root length compared to both controls and other biostimulants (**Table 6.1**).

The ANOVA table for *A. caudatus* (**Table 6.2**), is a summary of the different treatments on the amaranth. The results of the ANOVA table indicated that in terms of leaf number, the interactions of application (A), concentration (C) and treatment (T) (A x C x T) had a significant effect. A similar interaction was obtained for plant height. Only A was significantly effective ($P < 0.001$) for plant fresh weight. Stem thickness of *A. caudatus* was significantly affected by all the variables i.e. A, C, T and their interactions (**Table 6.2**). The same ANOVA table shows that A and T were significantly effective for the leaf area.

Table 6.1: Effect of different applications of biostimulants on the growth of *Amaranthus caudatus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n=5; rep =3. [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Leaf (no.)	Shoot length (mm)	Root length (mm)	Shoot fresh weight (g)	Root fresh weight (g)	Stem thickness (mm)	Leaf area (cm^2)
Drenching							
Control	14 ± 0.5 a	341 ± 20 c	48 ± 4.9 c	5.62 ± 0.65 ab	0.518 ± 0.044 abc	2.8 ± 0.2 c	66 ± 7.6 c
SW 1:500 (v/v)	14 ± 0.7 a	514 ± 53 a	70 ± 10.2 ab	7.02 ± 0.94 a	0.691 ± 0.107 a	4.7 ± 0.2 a	108 ± 6.1 a
KAR_1 (10^{-6} M)	13 ± 0.8 a	345 ± 48 bc	63 ± 8.4 abc	4.65 ± 0.79 b	0.364 ± 0.074 c	3.8 ± 0.1 b	65 ± 8.8 c
VCL 1:5 (v/v)	13 ± 1.5 a	350 ± 44 bc	47 ± 7.9 c	6.30 ± 0.38 ab	0.445 ± 0.049 bc	3.6 ± 0.2 b	65 ± 11.2 c
KEL (0.8%)	12 ± 0.9 a	504 ± 54 a	80 ± 12.7 a	7.30 ± 1.25 a	0.617 ± 0.137 ab	4.3 ± 0.3 a	83 ± 11.4 bc
ECK (10^{-8} M)	13 ± 0.6 a	422 ± 53 abc	61 ± 6.6 bc	5.30 ± 0.88 ab	0.328 ± 0.061 c	3.4 ± 0.2 b	68 ± 11.0 c
GA (10^{-6} M)	14 ± 0.8 a	455 ± 64 ab	61 ± 5.7 bc	6.40 ± 1.54 ab	0.630 ± 0.197 ab	4.7 ± 0.4 a	101 ± 17.3 ab
Foliar							
Control	15 ± 1.5 a	488 ± 77 a	64 ± 9.6 a	8.46 ± 1.82 a	0.765 ± 0.155 a	5.5 ± 0.2 a	90 ± 16.9 a
SW 1:500 (v/v)	13 ± 0.6 a	404 ± 41 a	54 ± 3.6 a	5.70 ± 0.94 a	0.453 ± 0.078 ab	3.1 ± 0.3 c	71 ± 9.5 a
KAR_1 (10^{-6} M)	13 ± 0.6 a	432 ± 50 a	52 ± 7.4 a	5.38 ± 0.83 a	0.353 ± 0.049 b	3.6 ± 0.1 c	81 ± 11.0 a
VCL 1:5 (v/v)	14 ± 0.7 a	450 ± 42 a	55 ± 6.5 a	4.69 ± 0.81 a	0.312 ± 0.045 b	3.9 ± 0.1 bc	72 ± 8.4 a
KEL (0.8%)	15 ± 0.7 a	524 ± 42 a	62 ± 4.2 a	7.41 ± 0.93 a	0.545 ± 0.086 ab	4.2 ± 0.2 bc	104 ± 11.3 a
ECK (10^{-8} M)	14 ± 0.7 a	408 ± 52 a	50 ± 4.7 a	5.11 ± 1.23 a	0.425 ± 0.134 b	3.9 ± 0.3 bc	85 ± 15.6 a
GA (10^{-6} M)	12 ± 0.9 a	436 ± 53 a	65 ± 7.8 a	5.92 ± 1.30 a	0.423 ± 0.124 b	4.5 ± 0.3 b	89 ± 19.8 a
Drenching and Foliar							
Control	13 ± 0.7 ab	442 ± 33 a	53 ± 3.1 bcd	5.53 ± 0.84 a	0.419 ± 0.090 a	3.6 ± 0.2 a	75 ± 9.5 ab
SW 1:500 (v/v)	13 ± 0.6 b	340 ± 37 a	57 ± 3.2 bc	3.19 ± 0.53 b	0.201 ± 0.034 b	2.7 ± 0.1 d	51 ± 8.8 b
KAR_1 (10^{-6} M)	15 ± 0.4 a	419 ± 30 a	44 ± 4.2 cd	4.11 ± 0.47 ab	0.218 ± 0.027 b	2.9 ± 0.1 cd	64 ± 5.8 ab
VCL 1:5 (v/v)	12 ± 0.5 b	415 ± 41 a	85 ± 8.1 a	5.11 ± 0.89 ab	0.405 ± 0.072 a	3.4 ± 0.1 ab	68 ± 10.8 ab
KEL (0.8%)	13 ± 0.5 b	432 ± 36 a	60 ± 4.5 b	4.33 ± 0.86 ab	0.270 ± 0.053 ab	2.9 ± 0.1 cd	63 ± 8.0 ab
ECK (10^{-8} M)	13 ± 0.8 b	371 ± 42 a	42 ± 5.2 d	4.01 ± 0.62 ab	0.240 ± 0.034 b	2.9 ± 0.1 cd	57 ± 7.7 ab
GA (10^{-6} M)	12 ± 0.4 b	415 ± 33 a	54 ± 1.9 bcd	4.97 ± 0.63 ab	0.318 ± 0.038 ab	3.2 ± 0.1 bc	82 ± 6.1 a

Mean value (± SE) of each application in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

Table 6.2: Analysis of variance for comparing different application methods for the growth of *Amaranthus caudatus* with different biostimulants ($P < 0.05$).

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance	*F*-probability
Leaf (no.)					
Application (A)	2	19.480	9.740	1.46	0.235
Concentration (C)	5	53.164	10.633	1.59	0.164
Treatment (T)	1	14.760	14.760	2.21	0.139
A x C x T	12	181.538	15.128	2.26	< 0.010
Residual	224	1496.806	6.682		
Total	244	1765.750	7.237		
Plant height (mm)					
Application (A)	2	93086	46543	2.01	0.136
Concentration (C)	5	230554	46111	1.99	0.081
Treatment (T)	1	26554	26554	1.15	0.285
A x C x T	12	522133	43511	1.88	< 0.038
Residual	224	5187402	23158		
Total	244	6059729	24835		
Plant fresh weight (g)					
Application (A)	2	209.518	104.759	11.06	< 0.001
Concentration (C)	5	97.601	19.520	2.06	0.071
Treatment (T)	1	17.703	17.703	1.87	0.173
A x C x T	12	165.318	13.777	1.46	0.143
Residual	224	2120.858	9.468		
Total	244	2610.998	10.701		
Stem thickness (mm)					
Application (A)	2	0.490685	0.245343	54.94	< 0.001
Concentration (C)	5	0.078299	0.015660	3.51	< 0.005
Treatment (T)	1	0.085810	0.085810	19.21	< 0.001
A x C x T	12	0.678635	0.056553	12.66	< 0.001
Residual	224	1.000336	0.004466		
Total	244	2.333765	0.009565		
Leaf area (cm^2)					
Application (A)	2	16928	8464	6.66	< 0.002
Concentration (C)	5	7665	1533	1.21	0.307
Treatment (T)	1	6329	6329	4.98	< 0.027
A x C x T	12	25642	2137	1.68	0.072
Residual	224	284498	1270		
Total	244	341063	1398		

6.4.2 Effect of organic biostimulants and mode of application on chlorophyll and carotenoid content of Amaranthus caudatus

The general effects of organic biostimulants and mode of application on photosynthetic pigments of *A. caudatus* are summarised in **Table 6.3**.

Table 6.3: Effect of organic biostimulants and mode of application on chlorophyll and carotenoid content of *Amaranthus caudatus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3) [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Chlorophyll a µg g^{-1} FW	Chlorophyll b µg g^{-1} FW	Chlorophyll a + b µg g^{-1} FW	Carotenoid µg g^{-1} FW
Drenching				
Control	550 ± 0.7 b	143 ± 0.2 b	694 ± 0.4 b	169 ± 0.2 c
SW 1:500 (v/v)	427 ± 0.1 d	101 ± 0.4 c	529 ± 0.3 d	131 ± 0.1 e
KAR_1 (10^{-6} M)	452 ± 6.5 c	124 ± 10.9 b	577 ± 17.4 c	148 ± 0.2 d
KEL 0.8%	553 ± 0.4 b	139 ± 0.3 b	692 ± 0.1 b	182 ± 0.1 b
ECK (10^{-8} M)	344 ± 9.0 e	132 ± 14.7 b	476 ± 24.4 e	101 ± 0.2 f
VCL 1:5 (v/v)	649 ± 0.2 a	258 ± 0.2 a	908 ± 0.2 a	210 ± 0.1 a
GA (10^{-6} M)	238 ± 0.1 f	62 ± 0.1 d	300 ± 0.1 f	77 ± 0.1 g
Foliar				
Control	510 ± 5.0 b	145 ± 8.3 b	656 ± 13.4 b	163 ± 0.2 b
SW 1:500 (v/v)	425 ± 0.1 d	110 ± 0.1 b	535 ± 0.2 c	146 ± 0.2 c
KAR_1 (10^{-6} M)	264 ± 0.1 e	65 ± 0.3 c	330 ± 0.2 d	89 ± 0.1 e
KEL 0.8%	219 ± 0.1 f	58 ± 0.1 c	278 ± 0.2 d	80 ± 0.1 g
ECK (10^{-8} M)	581 ± 18.4 a	260 ± 30.6 a	841 ± 49.1 a	164 ± 0.3 a
VCL 1:5 (v/v)	471 ± 13.3 c	243 ± 20.8 a	714 ± 34.2 b	122 ± 0.2 d
GA (10^{-6} M)	237 ± 0.1 f	63 ± 0.2 c	301 ± 0.3 d	87 ± 0.2 f
Drenching and Foliar				
Control	285 ± 0.9 c	76 ± 0.1 b	361 ± 0.1 b	92 ± 0.2 c
SW 1:500 (v/v)	177 ± 0.1 f	46 ± 0.3 d	223 ± 0.3 d	55 ± 0.1 g
KAR_1 (10^{-6} M)	394 ± 0.6 a	94 ± 0.4 a	488 ± 0.2 a	132 ± 0.2 a
KEL 0.8%	242 ± 0.1 d	57 ± 0.2 c	300 ± 0.2 c	83 ± 0.1 d
ECK (10^{-8} M)	162 ± 1.9 g	43 ± 4.1 d	205 ± 6.1 e	61 ± 0.5 f
VCL 1:5 (v/v)	234 ± 0.9 e	58 ± 2.0 c	292 ± 2.9 c	81 ± 0.1 e
GA (10^{-6} M)	291 ± 0.2 b	71 ± 0.1 b	362 ± 0.3 b	105 ± 0.1 b

Mean value (± SE) of each application in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

From **Table 6.3** it can be observed that organic biostimulants and their mode of application did not affect yield much in terms of the chlorophyll and carotenoid content of *A. caudatus*. Under the drenching application, only VCL significantly enhanced the amount of Chlorophyll a, Chlorophyll b, Chlorophyll a + b and carotenoids (**Table 6.3**). For foliar application, only ECK significantly influenced the content of chlorophylls and carotenoids. The influence of VCL applied via foliar spray was observed for Chlorophyll b content (243 µg g^{-1} FW) which was significantly enhanced compared with the control. The other treatments applied via foliar spray lowered the content of photosynthetic pigments. Significant increments in both chlorophylls and carotenoid content were observed for KAR_1 applied via a combination of drenching and foliar spray. All other treatments yielded values which were much lower than the control.

6.4.3 Effect of organic biostimulants and mode of application on absolute growth rate (AGR) and relative growth rate (RGR) of Amaranthus caudatus

For this investigation, the absolute and relative growth rates of *A. caudatus* were calculated to establish the effects of organic biostimulants and mode of application on the plant. **Fig. 6.1** shows the effects of organic biostimulants and their mode of application on both the absolute growth rate (AGR) and relative growth rate (RGR) of *A. caudatus*. The effects were quite varied as could be seen from the figure. When the biostimulants were applied via drenching, there was no significant effect on both AGR and RGR in terms of the number of leaves and plant height of *A. caudatus* as shown in **Fig. 6.1 A** and **D**. On application of the same biostimulants via foliar spray their effects were not significant on the leaf number of the plant (**Fig. 6.1 B**) but were significantly effective for the height of the plant **Fig. 6.1 E** ($P < 0.05$).

On application of the biostimulants via a combination of drenching and foliar spray, (**Fig. 6.1 C** and **F**), there was a significant effect on AGR for number of leaves in *A. caudatus* (**Fig. 6.1 E**) ($P < 0.05$) but there was no effect on the RGR. Considering plant height, the biostimulants had a significant effect on both AGR and RGR ($P < 0.05$) (**Fig. 6.1 F**).

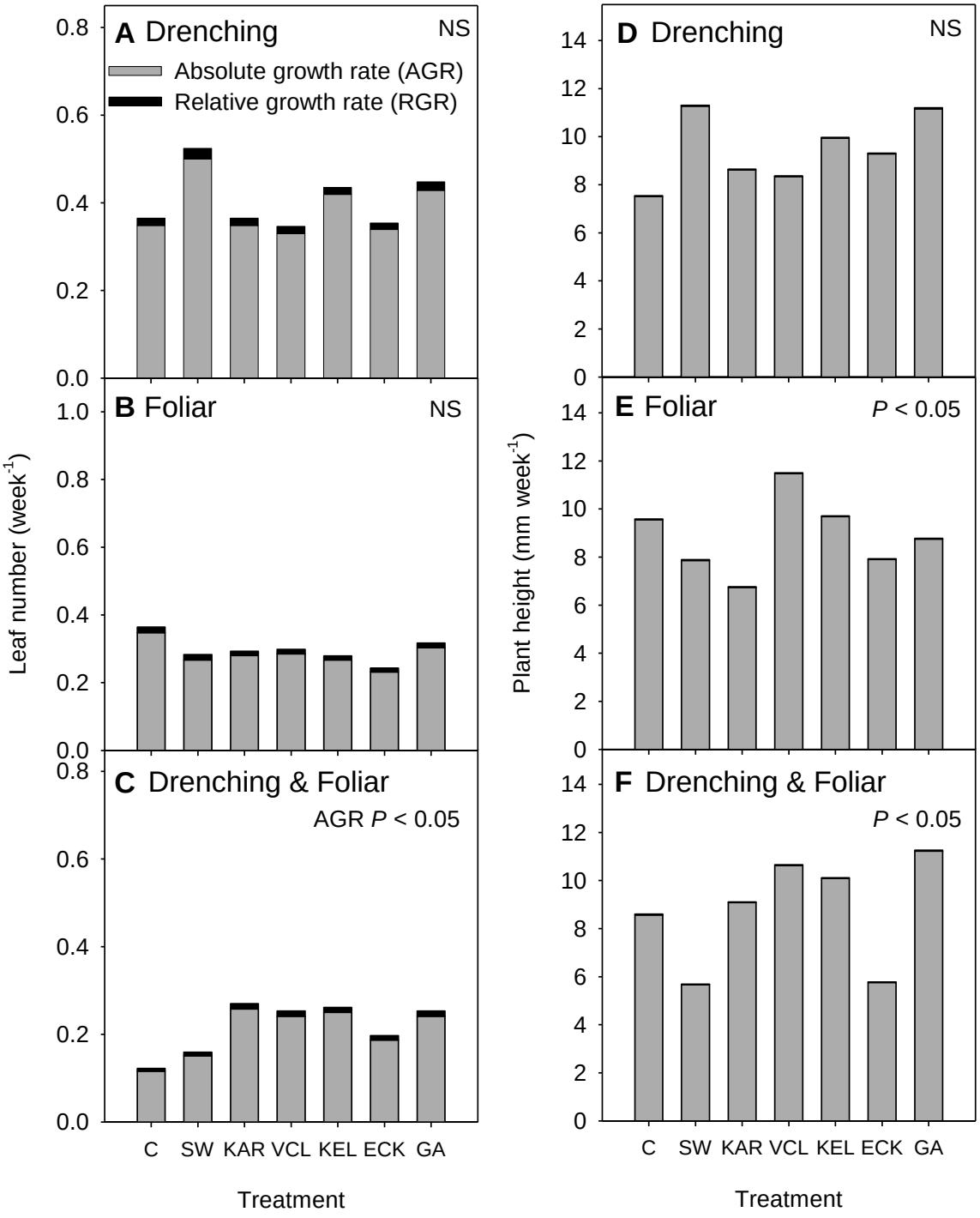


Fig. 6.1: Effect of organic biostimulants using different application methods on the absolute and relative growth rate (leaf number and plant height) of *Amaranthus caudatus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3) [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

6.4.4 Effect of organic biostimulants and mode of application on the growth of Amaranthus retroflexus

The effects of organic biostimulants and the mode of application of these biostimulants were also investigated on another amaranth species which is frequently consumed both as a grain and a leafy vegetable. **Table 6.4** shows a summary of the results of the effect of biostimulants on various growth parameters of *A. retroflexus*. In the drenching treatment, it was observed that all the biostimulants had a significant influence on leaf number compared to the control, with the exception of VCL which increased leaf number but not significantly. All the biostimulants had no significant influence on shoot length even though the longest increment was recorded for ECK (**Table 6.4**). Root length was negatively affected by the treatments. A similar trend was observed for both shoot and root fresh weights with the exception of SW, which showed similar effects to the control for shoot fresh weight. The other biostimulants applied via drenching significantly reduced both growth parameters (**Table 6.4**).

The foliar application of biostimulants on *A. retroflexus* plants did not generally yield notable effects on most growth parameters of the plant, except in a few cases. Most treatments did not have much influence on both leaf number and shoot length of the plant, however SW significantly increased shoot fresh weight compared to the control (**Table 6.4**). SW, KEL and ECK applied via foliar spray significantly enhanced the fresh weight of root compared to the control. Plants under a combination of drenching and foliar application treatments were not influenced much by most of the organic biostimulants in terms of leaf number and root length (**Table 6.4**). All the biostimulants generally lowered the shoot length of the plant compared to the control when applied via a combination of drenching and foliar spray. Similar results were also obtained for root fresh weight with SW, KAR_1, VCL and ECK treatments. KEL significantly influenced the root fresh weight of the plant (**Table 6.4**).

The ANOVA (**Table 6.5**) shows how the different treatments affected growth parameters in *A. retroflexus*. It can be noted that leaf number was significantly influenced by concentration (C) ($P < 0.024$). Plant height was significantly influenced by both mode of application (A), ($P < 0.001$) and concentration (C), ($P < 0.046$). Plant height was also notably influenced by the interaction factors (A x C x T), ($P < 0.001$).

Table 6.4: Effect of different applications of biostimulants on the growth of *Amaranthus retroflexus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3. [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Leaf (no.)	Shoot length (mm)	Root length (mm)	Shoot fresh weight (g)	Root fresh weight (g)
Drenching					
Control	3.1 ± 0.3 b	154 ± 10 ab	50 ± 3.6 a	0.999 ± 0.057 a	0.188 ± 0.040 a
SW 1:500 (v/v)	6.1 ± 0.8 a	161 ± 5 ab	35 ± 2.8 b	0.924 ± 0.112 a	0.064 ± 0.002 b
KAR_1 (10^{-6} M)	6.3 ± 1.0 a	152 ± 10 ab	30 ± 2.1 b	0.588 ± 0.058 b	0.026 ± 0.003 bc
VCL 1:5 (v/v)	5.2 ± 0.6 ab	140 ± 13 b	30 ± 2.9 b	0.398 ± 0.071 bc	0.022 ± 0.001 bc
KEL (0.8%)	6.8 ± 1.1 a	172 ± 9 ab	35 ± 3.1 b	0.263 ± 0.020 c	0.015 ± 0.001 c
ECK (10^{-8} M)	6.5 ± 0.9 a	176 ± 8 a	35 ± 3.3 b	0.562 ± 0.136 b	0.025 ± 0.001 bc
GA (10^{-6} M)	7.0 ± 0.9 a	164 ± 12 ab	48 ± 4.6 a	0.431 ± 0.107 bc	0.031 ± 0.003 bc
Foliar					
Control	5.4 ± 0.5 ab	137 ± 7 ab	41 ± 3.0 a	0.699 ± 0.112 b	0.021 ± 0.002 c
SW 1:500 (v/v)	3.7 ± 0.4 b	110 ± 11 b	34 ± 4.6 a	0.909 ± 0.111 a	0.030 ± 0.004 b
KAR_1 (10^{-6} M)	6.0 ± 0.5 a	133 ± 7 ab	34 ± 3.3 a	0.814 ± 0.054 ab	0.026 ± 0.001 bc
VCL 1:5 (v/v)	5.6 ± 0.6 ab	142 ± 6 a	43 ± 2.6 a	0.359 ± 0.075 c	0.024 ± 0.001 bc
KEL (0.8%)	4.7 ± 0.5 ab	137 ± 9 ab	38 ± 3.6 a	0.535 ± 0.070 bc	0.032 ± 0.002 b
ECK (10^{-8} M)	6.3 ± 0.6 ab	137 ± 5 ab	40 ± 3.5 a	0.694 ± 0.108 b	0.055 ± 0.002 a
GA (10^{-6} M)	5.5 ± 0.6 ab	154 ± 7 a	42 ± 5.0 a	0.581 ± 0.075 bc	0.052 ± 0.006 a
Drenching and Foliar					
Control	5.0 ± 0.3 a	157 ± 10 a	53 ± 3.7 a	0.980 ± 0.098 b	0.040 ± 0.003 c
SW 1:500 (v/v)	5.6 ± 0.6 a	139 ± 7 abc	50 ± 5.0 a	1.177 ± 0.077 ab	0.060 ± 0.006 abc
KAR_1 (10^{-6} M)	5.1 ± 0.7 a	151 ± 7 ab	47 ± 4.8 a	0.930 ± 0.146 b	0.055 ± 0.008 abc
VCL 1:5 (v/v)	4.5 ± 0.4 a	122 ± 6 c	43 ± 5.6 a	0.989 ± 0.137 ab	0.044 ± 0.003 bc
KEL (0.8%)	5.4 ± 05 a	128 ± 8 bc	51 ± 2.5 a	1.431 ± 0.140 ab	0.075 ± 0.005 a
ECK (10^{-8} M)	5.0 ± 0.7 a	118 ± 12 bc	46 ± 5.2 a	0.958 ± 0.135 ab	0.054 ± 0.010 abc
GA (10^{-6} M)	6.3 ± 0.7 a	138 ± 10 abc	49 ± 4.0 a	1.712 ± 0.314 a	0.062 ± 0.011 ab

Mean value (± SE) of each application in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

Table 6.5: Analysis of variance for comparing different application methods for the growth of *Amaranthus retroflexus* with different biostimulants ($P < 0.05$).

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance	*F*-probability
Leaf (no.)					
Application (A)	2	15.941	7.971	1.41	0.246
Concentration (C)	5	74.843	14.969	2.65	< 0.024
Treatment (T)	1	4.014	4.014	0.71	0.400
A x C x T	12	114.579	9.548	1.69	0.070
Residual	230	1298.750	5.647		
Total	250	1508.127	6.033		
Plant height (mm)					
Application (A)	2	18293	9146	8.30	< 0.001
Concentration (C)	5	12676	2535	2.30	< 0.046
Treatment (T)	1	4247	4247	3.85	0.051
A x C x T	12	27640	2303	2.09	< 0.018
Residual	230	253518	1102		
Total	250	316375	1265		
Plant fresh weight (mg)					
Application (A)	2	17.7342	8.8671	55.63	< 0.001
Concentration (C)	5	4.7008	0.9402	5.90	< 0.001
Treatment (T)	1	0.3709	0.3709	2.33	0.128
A x C x T	12	11.8096	0.9841	6.17	< 0.001
Residual	230	36.6586	0.1594		
Total	250	71.2740	0.2851		

The fresh weight of the plant was significantly influenced by most of the applications except the treatment (T). According to the ANOVA results, the application had a significant influence on the fresh weight of *A. retroflexus* ($P < 0.001$) and so did the concentration (C) ($P < 0.001$). The interaction of A x C x T also had a significant influence on the fresh weight of the plant ($P < 0.001$).

The effect of organic biostimulants and mode of application on photosynthetic pigments was also investigated and the results are shown in **Table 6.6**. VCL applied via drenching had a significant and positive influence on the amount of both chlorophylls and carotenoids in *A. retroflexus* compared to the control (**Table 6.6**).

Table 6.6: Effect of organic biostimulants and mode of application on chlorophyll and carotenoid content of *Amaranthus retroflexus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3) [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

Treatment	Chlorophyll a µg g^{-1} FW	Chlorophyll b µg g^{-1} FW	Chlorophyll a + b µg g^{-1} FW	Carotenoid µg g^{-1} FW
Drenching				
Control	700 ± 11.8 e	233 ± 18.2 c	933 ± 30.0 e	213 ± 1.2 d
SW 1:500 (v/v)	758 ± 10.5 d	267 ± 16.8 bc	1026 ± 27.3 d	213 ± 0.3 d
KAR_1 (10^{-6} M)	1025 ± 9.5 b	357 ± 3.1 a	1383 ± 6.5 b	293 ± 1.0 b
KEL (0.8%)	826 ±15.8 c	300 ± 23.8 b	1126 ± 39.5 c	231 ± 1.6 c
ECK (10^{-8} M)	486 ± 0.4 g	157 ± 0.4 d	643 ± 0.1 g	155 ± 0.1 f
VCL 1:5 (v/v)	1155 ± 0.5 a	394 ± 0.9 a	1549 ± 1.5 a	334 ± 0.1 a
GA (10^{-6} M)	549 ± 0.3 f	173 ± 0.4 d	723 ± 0.2 f	160 ± 0.2 e
Foliar				
Control	928 ± 0.7 f	325 ± 0.1 ab	1254 ± 0.7 c	252 ± 0.2 d
SW 1:500 (v/v)	981 ± 0.9 e	301 ± 0.3 b	1282 ± 0.7 c	269 ± 0.2 c
KAR_1 (10^{-6} M)	1017 ± 0.3 b	307 ± 0.7 b	1324 ± 0.3 b	283 ± 0.2 b
KEL (0.8%)	1128 ± 0.1 a	355 ± 0.5 a	1484 ± 0.6 a	316 ± 0.1 a
ECK (10^{-8} M)	1001 ± 3.6 c	339 ± 30.5 ab	1340 ± 27.1 b	279 ± 9.0 bc
VCL 1:5 (v/v)	994 ± 0.4 d	320 ± 0.2 ab	1315 ± 0.3 b	285 ± 0.1 b
GA (10^{-6} M)	554 ± 0.3 g	190 ± 0.1 c	745 ± 0.2 d	167 ± 0.1 e
Drenching and Foliar				
Control	817 ± 0.2 g	292 ± 0.9 e	1109 ± 0.7 g	239 ± 0.1 g
SW 1:500 (v/v)	1101 ± 0.8 a	343 ± 0.6 a	1445 ± 0.2 a	306 ± 0.2 b
KAR_1 (10^{-6} M)	884 ± 0.5 e	296 ± 0.7 d	1180 ± 0.2 e	268 ± 0.2 c
KEL (0.8%)	947 ± 0.3 c	296 ± 0.4 d	1243 ± 0.1 d	256 ± 0.1 d
ECK (10^{-8} M)	1021 ± 0.9 b	335 ± 0.3 b	1357 ± 0.6 b	309 ± 0.2 a
VCL 1:5 (v/v)	875 ± 0.3 f	288 ± 0.6 f	1164 ± 0.4 f	250 ± 0.1 f
GA (10^{-6} M)	939 ± 0.4 d	313 ± 1.0 c	1252 ± 0.5 c	255 ± 0.3 e

Mean value (± SE) of each application in a column with different letter(s) is significantly different according to Duncan's multiple range test ($P < 0.05$).

The Chlorophyll a content was significantly increased by SW, KAR_1, KEL and VCL treatments compared to both controls, with VCL yielding the highest amount. Three of the biostimulants (KAR_1, KEL, and VCL) also significantly increased the Chlorophyll b content compared with the control (**Table 6.6**). The total Chlorophyll (a + b) content was significantly influenced by SW, KAR_1, KEL and VCL applied via drenching.

The application of biostimulants via foliar spray resulted in all the biostimulants significantly enhancing the Chlorophyll a content in *A. retroflexus*. KEL had the highest yield of Chlorophyll a. The total Chlorophyll (a + b) content was significantly enhanced by most of the biostimulants compared to both controls except for SW, which was not significantly different from the control (**Table 6.6**). All biostimulants significantly influenced the carotenoid content of *A. retroflexus* with KEL yielding the highest amount compared to both controls (**Table 6.6**). Further treatment of *A. retroflexus* with biostimulants via a combination of drenching and foliar application resulted in the biostimulants significantly enhancing the Chlorophyll a content compared to the control. The highest amount of Chlorophyll a was recorded for SW. A similar trend was observed with Chlorophyll b although VCL showed a significantly lower amount of Chlorophyll b compared with the negative control. The total Chlorophyll (a + b) content was significantly enhanced by the biostimulants (SW, KAR_1, KEL, ECK), with the highest amount being recorded for SW, compared to the control (**Table 6.6**). All the biostimulants applied via a combination of drenching and foliar treatment significantly increased the carotenoid content with ECK yielding the highest amount of carotenoids (**Table 6.6**).

6.4.5 Effect of biostimulants and mode of application on the absolute growth rate (AGR) and relative growth rate (RGR) of Amaranthus retroflexus

The effects of different organic biostimulants on the AGR and RGR in terms of the number of leaves and height of *A. retroflexus* were varying as can be observed in **Fig. 6.2**. *Amaranthus retroflexus* treated with biostimulants via drenching significantly increased AGR and RGR for number of leaves ($P < 0.05$) as shown in **Fig. 6.2 A**. However, it was not the same for the height of the plant as shown in **Fig. 6.2 D**, where the biostimulants had no significant effect on both AGR and RGR for height of the plant.

The application of the biostimulants via foliar spray yielded the results as shown in **Fig. 6.2 B** and **E**, where treatments had no significant influence on both the AGR and RGR in terms of the number of leaves and height of *A. retroflexus*. The application of biostimulants via a combination of drenching and foliar application on the plant had significant effects on both AGR and RGR ($P < 0.05$) for leaf number (**Fig. 6.2 C**) but had no significant effects on plant height in terms of AGR and RGR (**Fig. 6.2 F**).

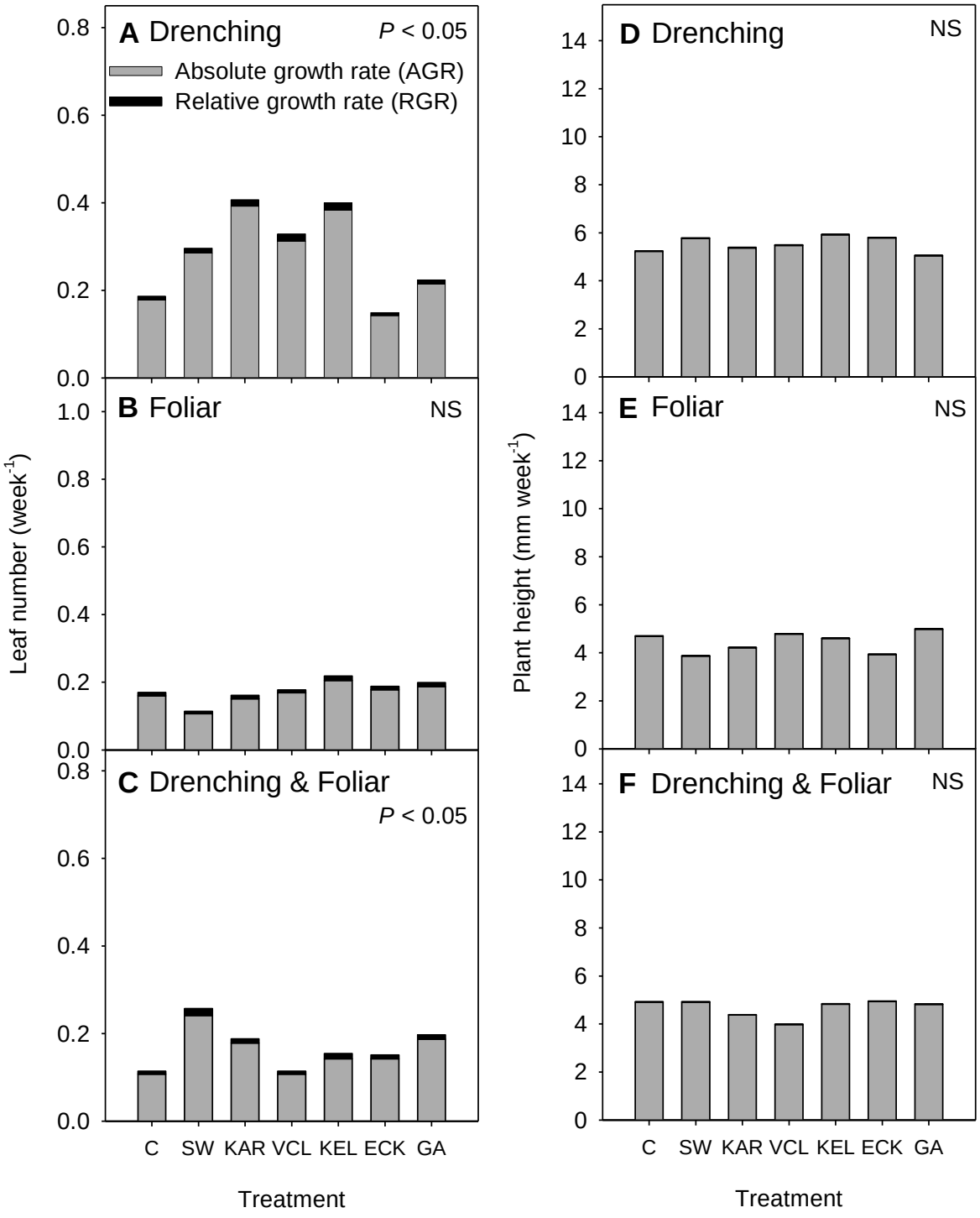


Fig. 6.2: Effect of organic biostimulants and different application methods on the absolute and relative growth rate (leaf number and plant height) of *Amaranthus retroflexus* at 24 ± 2 °C under greenhouse conditions. The plants were harvested after 6 weeks (n = 5; rep = 3) [Control, C; Smoke-water, SW; Karrikinolide, KAR_1; Vermicompost leachate, VCL; Kelpak®, KEL; Eckol, ECK; Gibberellic acid, GA].

6.5 Discussion

Eco-friendly strategies are now being sought in modern agriculture which could be used in the promotion of growth in crops to increase productivity **(XU and GEELEN, 2018)** but at the same time reducing their agricultural carbon footprint **(DIAS et al., 2016; FOLEY et al., 2011)**. For many years, agriculture has relied on chemical fertilisers and pesticides which also pose a threat to the health of mankind and even to the environment **(CHIAIESE et al., 2018)**. In addition to this conundrum, the use of chemical fertilisers has become more expensive due to their continued depletion as the world demand for these resources soars **(CHIAIESE et al., 2018)**. Also, the rising concerns raised by mankind against their use are tilting against their continued use in agriculture as legal frameworks are getting tighter by the day **(COLLA and ROUPHAEL, 2015; YAKHIN et al., 2017)**.

6.5.1 Effect of organic biostimulants and mode of application on growth of Amaranthus caudatus

It can be seen that biostimulants and the mode of application also affects the general growth of the amaranths, *A. caudatus*. It was evident that SW and KEL, applied as soil drenches, had significant effects on some growth parameters of the amaranths. KEL significantly improved shoot and root length (504 ± 54 mm and 80 ± 12.7 mm) respectively, shoot fresh weight (7.30 ± 1.25 g) and stem thickness (4.3 ± 0.3 mm). ECK and KAR_1 enhanced shoot length of *A. caudatus.* These results are similar to those found in ornamental pepper plants (*Capsicum annuum* L.) treated with a biostimulant called Stimplex® as either a soil drench or foliar spray. The application of Stimplex®, a commercial biostimulant product, usually used for the promotion of plant growth, significantly improved the height of the plants, number of leaves, leaf area, shoot fresh and dry weight and root fresh and dry weight when compared with the control plants **(OZBAY and DEMIRKIRAN, 2019)**. Stimplex® is a concentrate extracted from the seaweed, *Ascophyllum nodosum*. Seaweeds and their extracts are known to stimulate growth and development in plants, improve yield and early maturity, improve plant resistance to environmental stresses, improve quality of fruit

and also increase fruit setting in horticultural crops grown conventionally or organically **(CROUCH and VAN STADEN, 1992; NANDWANI et al., 2015)**. KEL, also a concentrate from the seaweed *Ecklonia maxima*, was used in this investigation and produced results similar to those of Stimplex® which emphasizes the notion that seaweed extracts stimulate growth and development, as observed in *A. caudatus* and *A. retroflexus*. Several studies have shown the effects of seaweed extracts on growth and yield of many horticultural plants. This is because they contain traces of some mineral elements, vitamins, amino acids, auxins, cytokinins, as well as natural chelating agents like mannitol, fucoidins and alginic acid. The extracts also stimulate plants to produce their natural growth substances such as auxins and cytokinins which stimulate both cell division and cell elongation, giving rise to larger root systems which improve both plant growth and yield **(CROUCH et al., 1992; CROUCH and VAN STADEN, 1993b; DU JARDIN, 2015; SPANN and LITTLE, 2010; STIRK et al., 2003)**. Seaweed extracts have been reported to have significantly improved growth, enhanced yield and quality in grapes **(NORRIE, 2006)**. Similar findings have been reported in apple **(BASAK, 2008)** and olives **(CHOULIARAS et al., 2009)**. According to **ABDEL-MAWGOUD et al. (2010)** watermelon responded in the same manner when treated with the same seaweed extracts. **SARHAN (2011)** reported the same on cucumber and **MATTNER et al. (2013)** on broccoli, **FAN et al. (2013a)** on spinach and **NANDWANI et al. (2015)** on eggplant. Similar results were reported by **WEBER et al. (2018)** and **ZERMEÑO-GONZÁLEZ et al. (2015b)** on maize and **HERNÁNDEZ-HERRERA et al. (2014)** and **ALI et al. (2016)** on tomato. We still do not have any information from the literature on the effect of biostimulants and application methods on *A. caudatus.*

Just like seaweed extracts, smoke-derived bioactive compounds have also stimulated growth and productivity promotion in many horticultural crops. SW was reported to enhance leaf number in spinach (*Spinacea oleracea* L.) **(KULKARNI et al., 2019)**. The results from this research are in agreement with these results. SW significantly improved shoot length (514 ± 53 mm), shoot and root fresh weight (7.02 ± 0.94 g and 0.691 ± 0.107 g) respectively, stem thickness (4.7 ± 0.2 mm) and leaf area (108 ± 6.1 cm^2). Fire and smoke have been utilised in traditional agricultural systems for many centuries. Smoke and seaweed extracts are organic biostimulants which are being utilised for enhancing growth and productivity in many agricultural crop plants such as

spinach and okra **(KULKARNI et al., 2011; KULKARNI et al., 2019; PAPENFUS et al., 2013)**. Ornamental pepper (*Capsicum annuum* L.) treated with Stimplex® showed the highest increase in stem diameter **(OZBAY and DEMIRKIRAN, 2019)**. The current results on *A. caudatus* concur with these previous studies on the effects of biostimulants on growth of crop plants. In **Table 6.1**, the stem thickness was significantly enhanced by the drenching application of the biostimulants. SW, KAR_1 and KEL increased the shoot length in *A. caudatus* when applied via drenching. This is in agreement with results obtained from ornamental pepper (*Capsicum annuum* L.) treated with Stimplex® **(OZBAY and DEMIRKIRAN, 2019)**. According to **ROUSSOS et al. (2009)** the growth of plants treated with biostimulants, particularly those derived from seaweeds, could be attributed to the significant amount of auxins, cytokinins and betaines, present in seaweeds which influence cell division during the early stages of growth and development in plants.

Vermicompost leachate applied as a drench, ECK applied as a foliar spray and KAR_1 applied as a combination of drenching and foliar spray significantly increased the chlorophyll and carotenoid content of leaves of *A. caudatus* (**Table 6.3**). These results are also in agreement with those reported by **OZBAY and DEMIRKIRAN (2019)** in ornamental pepper where Stimplex®, a seaweed extract applied either as foliar spray or substrate drench was observed to significantly increase the leaf chlorophyll content of *C. annuum*. The above-mentioned biostimulants i.e. VCL, ECK, and KAR_1, applied either by drenching, foliar spray or a combination of both, significantly increased chlorophyll and carotenoid contents in *A. caudatus*. These findings concur with those of previous studies where biostimulants were reported to have significantly enhanced the chlorophyll and carotenoid contents in plants **(BLUNDEN et al., 1996; FAN et al., 2013a; SPINELLI et al., 2010)**. The presence of cytokinin-like effects and betaines found in seaweed could be responsible for this effect **(WHAPHAM et al., 1993)**. Extracts of *A. nodosum* were reported to increase the chlorophyll content in leaves of tomato, bean, wheat, barley and maize in comparison with control plants **(BLUNDEN et al., 1996)**. The increased chlorophyll and carotenoids of the plants could be attributed to the presence of betaines present in seaweed extracts **(MACKINNON et al., 2010)**. Betaines are not included among the known classical plant hormones but are present in seaweeds and their extracts with a higher transcription of betaine aldehyde dehydrogenase being reported in *A. nodosum* **(FAN et al., 2013a)**. Many

studies have shown that biostimulants like seaweed extracts generally enhance vegetative growth in many horticultural crops **(ARTHUR et al., 2003; HERNÁNDEZ-HERRERA et al., 2014; KUMARI et al., 2011; YILDIZTEKIN et al., 2018; ZODAPE et al., 2011)**. An increase in both root and shoot length and fresh weight was also reported by **KUMARI et al. (2011)** in tomato plants treated with liquid seaweed extracts applied via drenching and foliar application. The same was reported for spinach where its fresh weight was significantly increased **(FAN et al., 2013a)**. Seaweed extracts applied to broccoli notably increased the biomass for both shoots, leaf area, and stem diameter of the crop **(MATTNER et al., 2013)**. According to **ZERMEÑO GONZALEZ et al. (2015a)**, the application of organic fertilisers via drenching and foliar application caused a significant increase in plant height, stem diameter and plant dry weight of maize plants. Macro- and microelements, vitamins, amino acids, auxins and cytokinins, which are growth-promoting substances, constituents of seaweed extracts could be responsible for the beneficial attributes of biostimulants, especially those derived from seaweeds like KEL. These substances enhance cell metabolism resulting in increased growth in plants treated with these biostimulants **(CRAIGIE, 2011; CROUCH and VAN STADEN, 1993b; NORRIE and KEATHLEY, 2005)**. According to **KHAN et al. (2011)**, the increase in cytokinin-like responses in *Arabidopsis thaliana* treated with Stimplex® is an indication that seaweed extracts have compounds contributing to the cytokinin-like activity. Cytokinins are responsible for cell division and proliferation of the cells as well as enhancing sink activity of roots culminating in the stimulation of growth **(NELSON and VAN STADEN, 1984)**. Evidence provided by **RAYORATH et al. (2008)** suggests that the enhanced growth in plants treated with extracts from *A. nodosum* is due to the ability of the extracts to regulate the concentration and translocation of auxins.

6.5.2 Effect of organic biostimulants and mode of application on growth of Amaranthus retroflexus

The results from this investigation showed that biostimulants such as SW, KAR_1, KEL and ECK, applied on *A. retroflexus* via drenching significantly stimulated some growth parameters. SW, KAR_1, KEL and ECK enhanced leaf numbers in the plant (**Table 6.4**). When treated with biostimulants via the foliar application, KAR_1, VCL and ECK also had a very significant effect on different growth parameters of *A. retroflexus*. KAR_1

significantly influenced leaf number, (6.0 ± 0.5), VCL increased the length of the shoot, (142 ± 6 mm), whilst SW significantly increased the fresh weight of the shoot, (0.909 ± 0.111 g). The influence of ECK was observed on the fresh weight of roots, (0.075 ± 0.006 g). These results concur with those of **VAN STADEN et al. (2006)** in which SW and KAR_1 significantly ($P < 0.05$) improved seedling growth in maize, okra and bean, as well as increasing both root and shoot lengths of all the seedlings when compared with the control. The roots of KAR_1-treated seedlings of tomato were 10 times longer than control roots, whereas those of okra and bean increased three times compared to the control **(VAN STADEN et al., 2006)**. From these results, it seems the compounds found in smoke could be responsible for the stimulation of either cell elongation and/or division. According to **BROWN (1993)**, enhanced vigorous growth was observed in young seedlings of *Erica* sp., a species from the *Asteraceae* family, treated with smoke. Similarly, the same effect was reported on seeds of the fire-climax grass, *Themeda triandra*. Vigorous and healthy growth was observed, though not statistically significant **(BAXTER and VAN STADEN, 1994)**. Seedling height was also significantly increased in grasses **(BLANK and YOUNG, 1998)** and in indigenous medicinal plants **(SPARG et al., 2005)** treated with both aerosol smoke and smoke water. **THOMAS and VAN STADEN (1995)** and **DREWES et al. (1995)** have investigated the importance and role of SW using crops such as celery and lettuce respectively. **MODI (2002)**, demonstrated that smoke pre-treated seeds gave rise to significantly more vigorous seedlings, which were much heavier and taller compared to untreated ones making use of indigenous methods of storing maize cobs. In another study carried out by **TAYLOR and VAN STADEN (1996)**, it was reported that smoke extracts stimulated the formation of roots in *Vigna radiata* to indicate the significant role played by smoke extracts in root formation. Plants grown via drenching application of SW (maize, okra and been) exhibited significant improvements in shoot fresh and dry weights of these crops compared with the control. KAR_1 was applied via drenching and significantly enhanced ($P < 0.05$) the height of maize shoots. This again demonstrates that SW and KAR_1 promote post-germination plant growth **(VAN STADEN et al., 2006)**. Leaf development in maize plants was also significantly improved by smoke treatments **(VAN STADEN et al., 2006)**. The growth-promoting effects of SW and KAR_1 could be explained by studies of **VAN STADEN et al. (2000)**, which reported the possible interaction of SW with gibberellins, cytokinins, abscisic acid and ethylene in seeds which are both photoblastic and thermo-dormant. Other

researchers, such as **SENARATNA et al. (1999)** and **GARDNER et al. (2001)**, suggested that the active principle(s) of the role of smoke in plant growth is similar to that of other PGRs.

KEL and ECK are both derived from seaweed extracts whose growth-promoting effects have been explained and supported in the previous section on *A. caudatus* **(CRAIGIE, 2011; CROUCH and VAN STADEN, 1993b; NORRIE and KEATHLEY, 2005)** hence they demonstrated similar effects on *A. retroflexus* as shown in our results.

6.5.3 The effect of organic biostimulant and mode of action on chlorophyll and carotenoid contents of Amaranthus caudatus *and* Amaranthus retroflexus

The application of organic biostimulants via drenching resulted in VCL significantly increasing both the chlorophyll and carotenoid content of *A. caudatus* with KEL having a significant influence on the carotenoid content (**Table 6.3**). The foliar application of organic biostimulants resulted in two biostimulants, ECK significantly increasing the chlorophyll and carotenoid content and VCL significantly enhancing the carotenoid content compared to the control in *A. caudatus* (**Table 6.3**). Combining both drenching and foliar application resulted in KAR_1 significantly increasing the chlorophyll and carotenoid content of *A. caudatus*. Organic biostimulants were applied via drenching, foliar spray and a combination of both. KAR_1 and VCL applied via drenching had significant effects on chlorophyll and carotenoid contents. KAR_1 significantly improved the amount of Chlorophyll b, (357 ± 3.1 µg g^{-1} FW) and also increased the total chlorophyll content, Chlorophyll a + b, (1383 ± 6.5 µg g^{-1} FW) but not statistically significantly. VCL improved, in a significant manner, the chlorophyll and carotenoid contents of *A. retroflexus*, Chlorophyll a (1155 ± 0.5 µg g^{-1} FW), Chlorophyll b (394 ± 0.9 µg g^{-1} FW), Chlorophyll a + b (1549 ± 1.5 µg g^{-1} FW) and carotenoid (334 ± 0.1 µg g^{-1} FW) (**Table 6.6**). Results obtained from this investigation are similar to those reported by **AYYOBI et al. (2013)** who obtained tall plants with high amounts of Chlorophyll a, Chlorophyll b, total chlorophyll and carotenoids in peppermint plants (*Mentha piperita* L., Lamiaceae) treated with vermicompost and vermiwash. The same plants also exhibited the highest total plant fresh weight and leaf fresh weight **(AYYOBI et al., 2013)**. Research has shown that the use of vermicompost in agriculture is a

very common and widespread practice with numerous benefits to soil and vegetables **(GUTIÉRREZ-MICELI et al., 2007; OLFATI et al., 2009; SHABANI et al., 2011)**. Several greenhouse and field studies, carried out to investigate the effects of vermicompost on cereals and legumes, vegetables and other field crops have confirmed its beneficial effects on the growth of plants **(AYYOBI et al., 2014; CHAN and GRIFFITHS, 1988; KOCHAKINEZHAD et al., 2012)**. The explanation for these positive attributes of vermicompost can be explained by the way it is formed. There are certain species of earthworms capable of degrading organic materials into fine particles by digesting them in the gizzard **(NDEGWA and THOMPSON, 2001)** and according to evidence by **SUTHAR (2010)**, secretions from earthworms consist of plant hormones such as cytokinins, auxin, amino acid, vitamins, and enzymes which could have been obtained from the interactions between microbes and earthworms. All this information supports the results obtained in this investigation where the organic biostimulant VCL increased the chlorophyll and carotene content of *A. retroflexus* significantly.

In another greenhouse experiment by **FAN et al. (2014)**, humic acid (HA) applied as a foliar application significantly increased the rate of photosynthesis, chlorophyll content, and shoot and root biomass of chrysanthemum (*Chrysanthemum morifolium* R.). Enhancement of the leaf chlorophyll content could be the bioregulators affecting the balance between photorespiration and photosynthesis in plants **(ABOU EL-YAZIED and MADY, 2011; OLAIYA, 2010)**. Dry yeast applied to field bean plants was also reported to significantly increase the amount of Chlorophyll a, b and total chlorophyll **(PRUD'HOMME et al., 1992)**. **CRISTIANO et al. (2018)** also obtained similar results when snapdragon (*Antirrhinum majus* L.) was treated with an animal-derived biostimulant, which caused a significant increase in morphological and qualitative traits of the plant. The physiological parameters enhanced by biostimulant applications include the rate of photosynthesis, rate of transpiration and stomatal conductance. This resulted in an elevated carbon assimilation efficiency **(CRISTIANO et al., 2018)**. The authors concluded that applying small doses of biostimulants to potted snapdragon as a part of a fertilising regime improves the quality of the crop in an agriculturally sustainable manner. In another experiment by **FAROUK et al. (2012)**, all the biostimulants tested, particularly thiamine, increased the following growth parameters in tomato plants; shoot length, number of branches, number of leaves,

both shoot fresh and dry weight and leaf area significantly. The same biostimulants, increased the N, K, P and chlorophyll as well as total carbohydrate content in the shoot. The overall yield of tomato was also higher than that of the control plants **(FAROUK et al., 2012)**.

6.6 Concluding remarks

Both amaranths, *A. caudatus* and *A. retroflexus* also responded to organic biostimulants and mode of application in the same way as *A. hybridus.* SW, KAR_1, KEL and ECK applied via drenching significantly improved some growth parameters in both *A. caudatus* and *A. retroflexus* as could be observed in the results of this investigation. The chlorophyll and carotenoid content of *A. caudatus* was significantly enhanced by the drenching application of VCL and KEL respectively. The application of biostimulants via foliar application resulted in VCL and ECK significantly enhancing the photosynthetic pigments in *A. caudatus*. The application of KAR_1 by a combination of both application methods significantly enhanced the chlorophyll and carotenoid content of *A. caudatus*. KAR_1 and VCL applied via drenching also significantly enhanced the content of photosynthetic pigments in *A. retroflexus*. Results from this investigation validate the potential of organic biostimulants and mode of application for the improvement and promotion of the cultivation of *Amaranthus* species.

Chapter 7: General conclusion

The main challenge being encountered in attaining the United Nations Sustainable Development Goals (UN-SDGs) are; poverty elimination, absence of hunger and good health and well-being and maximisation of food production using scant natural resources in a sustainable manner **(GODFRAY et al., 2010a)**. The major concern is the sustained cultivation of modern crops which require high-inputs against a background of limited resources and diminishing quality and availability of the two key inputs for agricultural productions i.e. water and land **(EBERT, 2014; MLAKAR et al., 2009b)**. Climate change is negatively affecting the quality and availability of these critical resources and thereby impeding food production **(SOARES et al., 2019)**. Future crop productivity is being hampered by climate change effects which include; poor crop yields, desertification, deforestation, erosion, water quality degradation and water resource depletion. All the aforementioned factors go a long way in compromising global food security **(ARAUS et al., 2008; BURRITT, 2019; DELGADO et al., 2011)**. The situation has been further aggravated by increases in energy prices which resulted in the subsequent increases in the cost of agricultural inputs. To meet the global food demand for the ever-rising human population in 2050, food production needs to be increased by 70%. The cultivation of low-resource intensive, resilient and nutritionally rich crops is vital for both the sustainability of the environment and well-being of mankind **(STALLKNECHT and SCHULZ-SCHAEFFER, 1993)**. The only premise to achieve this is the establishment of more diverse cropping systems. There is a need to domesticate the undomesticated wild and neglected crops so as to be able to make use of the natural traits they possess of efficiently using natural resources like N, P, water and land. According to **MASSAWE et al. (2016)**, neglected and underutilised wild plants are a treasure trove for food security. The inclusion of drought and heat stress-tolerant crops as an adaptation to climate variability and pressure from new pests and diseases promises to curb food and nutrition insecurity **(DELGADO et al., 2011; MORTON, 2007)**. These promise the attainment of food security for the future since they are of superior nutritional value and adaptable to adverse environmental conditions **(VAVILOV, 1951)**. There is a big shift in attention towards

underutilised crops as the way forward to increase food production and attain global food security and to mitigate the negative effects of climate change.

Currently there is a shift in consumer demands in favour of organically grown crops which are more nutritiously balanced and thus more interest is now being placed on species like amaranths. In order to mitigate the effects of climate change, the amaranth is a very suitable candidate and needs to be investigated more since they promise to be potential crops to grow in marginal agricultural lands and can improve arid and semi-arid agricultural systems in southern Africa. Both leaf and seed production of *Amaranthus* could be used to address the issue of food security **(FOMSGAARD et al., 2011)**. *Amaranthus* has a unique nutrient composition and inherent tolerance to drought and heat stress, so the promotion of its consumption and cultivation could go a long way in addressing the effects of climate change and assuring food and nutrition security. The nutritional attributes of the amaranth have placed them in high demand by certain groups of consumers like athletes, malnourished children and diabetes and coeliac patients. In terms of antioxidant capacity, amaranth is ranked among the top five leafy vegetables and contains substantial amounts of L-ascorbic acid, β-carotene, polyphenol, anthocyanins and lutein **(WALTER, 2001)**.

The consumption of amaranths such as *A. hybridus* in southern Africa is generally limited by virtue of the fact there is no commercial cultivation of the plant. The leafy vegetable is mainly harvested during the rainy season, from the wild, where it grows like a weed. There is not much literature about the adoption, incorporation and commercial production of the plant in mainstream agriculture.

This research has been motivated by a sudden interest and attention being given to the amaranths globally due to their nutritional and nutraceutical properties. The plant is already consumed as a vegetable (Morogo) but on a small scale in rural and peri-urban areas in several provinces of South Africa.

The first phase of the investigation was aimed at assessing the general requirements for the successful establishment of *A. hybridus*. From the experiments carried out, several conclusions were made with regards to the responses of *A. hybridus* to different factors. On the effects of NPK on *A. hybridus*, it was clearly concluded that the crop can grow well morphologically in the presence of small amounts of P and K

but cannot survive when N is lacking. The next stage was to investigate the effects of nutrient strength where the crop was treated with, 50%, 25% and 12.5% Hoagland's Nutrient Solution (HNS) and from the results of the investigation it was concluded that significant growth was achieved at 50% HNS. A further investigation was carried out to establish if the watering frequency has an influence on the growth of *A. hybridus*. It was observed that the more water that is applied to the plant (3x/week) the greater the vegetative growth though not very different from (2x/week).

The effect of light intensity was also investigated via an experiment in which *A. hybridus* seedlings were exposed to different light intensities of 150, 300, 450 and 600 µmol m^{-2} s^{-1}. From the results of this investigation, it could be concluded that *A. hybridus* growth is light-dependent, with the most significant growth being achieved at 100% light intensity. So when growing *A. hybridus*, it requires maximum exposure to light to achieve optimal vegetative growth and this could be explained by, that at a maximum light intensity, there will be maximum photosynthesis in the plant resulting in large amounts of photosynthates being synthesized, causing better growth.

Since *A. hybridus* could not thrive in the absence of N, a further investigation was carried out by treating *A. hybridus* with –N (HNS) + biostimulants and from the results obtained it was concluded that *A. hybridus* can only grow when treated with –N (HNS) + KEL. In this situation it could be concluded that KEL can be used to supplement *A. hybridus* for N in the event that the element is lacking in the soil.

A. hybridus was treated with 50% HNS + biostimulants at different irrigation frequencies namely (once, twice and thrice) a week. The AGR and RGR were calculated and the effect on growth was further assessed as shown in **Fig. 4.4** and **Fig 4.5** respectively. Irrigation of the plant once a week did not yield any significant effects of 50% HNS + biostimulants in both AGR and RGR on the height of the plants but in terms of leaf number by most treatments. 50% HNS + ECK had a notable improvement on RGR although most of the biostimulants had no effect on both AGR and RGR on the number of leaves of the plant when irrigation was done once a week. From these results it can be concluded that irrigating the plant once a week is not economically viable as it does not result in optimal growth of *A. hybridus.*

When the same treatments were performed twice a week, SW had a significant effect on AGR and RGR (height) and on RGR (leaf number). KAR_1 had a significant effect

on both AGR and RGR (height) and RGR (leaf number). Application of the treatments thrice a week resulted in KAR_1 having a significant effect on both AGR and RGR for both height and number of leaves for *A. hybridus*. From these results, it was revealed that KAR_1 + 50% HNS is the biostimulant which could be applied on *A. hybridus* twice or thrice a week so as to enhance both the AGR and RGR for both plant height and leaf number. The plant grows taller with more branching resulting in more leaves being formed and tender leaves which can harvested for human consumption.

An evaluation of the effect of irrigation frequency on various growth parameters of *A. hybridus* with 50% HNS is summarised in **Table 4.5**. When the plants were irrigated once a week, 50% HNS + KAR_1 had a significantly enhanced fresh weight of roots (89 ± 11 g) and 50% HNS + KEL had significant effects on several growth parameters like, shoot and root length (71.9 ± 5.9 and 35 ± 4.1 mm) respectively and also on both shoot and root fresh weight (648 ± 73 and 79 ± 12 g) respectively. ECK + 50% HNS also had a notable influence on root fresh weight (83 ± 6 g) when irrigated once a week. Irrigation of the crop twice a week resulted in SW having significant effects on several growth parameters of the plant. Effects were observed on leaf number (7.0 ± 0.4), shoot and root length (118 ± 11 and 81.2 ± 9.8 mm) respectively and again on both shoot and root fresh weight (1.067 ± 99 and 428 ± 43 g) respectively. Root length (78.6 ± 4.8 mm) was significantly influenced when ECK + 50% HNS was applied to *A. hybridus* twice a week.

The irrigation of the crop three times a week resulted in SW + 50% HNS influencing only the length of the shoot (142.9 ± 11.6 mm). KAR_1 + 50% HNS had significant effects on both shoot and root length (164.5 ± 14.8 and 129.5 ± 13.2 mm) respectively and on both shoot and root fresh weight (1.191 ± 117 and 467 ± 103 g) respectively. Also observed was the significant effect of VCL + 50% HNS on shoot length (147.5 ± 14.2 mm). The general conclusion made from these results is that the effect of irrigation frequency of 50% HNS + biostimulants on *A. hybridus* is biostimulant dependent with KAR_1, SW and KEL being the main effective biostimulants.

The effects of different biostimulants and mode of application on the growth and biochemical composition of *A. hybridus* under greenhouse conditions was investigated. The results in **Table 4.1**, are a summary of the effect of the organic biostimulants and the mode of application on the growth of *A. hybridus*. Results from

the growth experiments of *A. hybridus* treated with biostimulants via different modes of application show that SW applied via a soil drench had a significant influence on stem thickness and leaf area. Likewise KAR_1 applied via drenching also influenced significantly shoot length (390 ± 26.4 mm), stem thickness (4.2 ± 0.1 mm), leaf area (83.3 ± 10.0 cm^2), both shoot and root fresh weight (6.723 ± 0.0031 g and 0.871 ± 0.008 g) respectively and shoot dry weight (0.878 ± 0.006 g). The application of KEL had a significant influence on the number of leaves (20.7 ± 2.2) in *A. hybridus.* From these results we can conclude that the mode of application influences the growth of the amaranth crop since from all the 5 biostimulants only KAR_1 significantly influenced most growth parameters in *A. hybridus,* making it the biostimulant of choice when treating *A. hybridus* via drenching application.

Results from the foliar application of biostimulants did not yield much and only, VCL had a significant effect on most growth parameters of *A. hybridus.* The influence of VCL was observed for number of leaves (27.8 ± 2.3), root number (8.9 ± 1.6), both shoot and root length (478 ± 24.5 and 56.2 ± 1.9 mm) respectively. It significantly increased, leaf area (116 ± 16.5 cm^2), both shoot and root fresh weight (10.647 ± 0.026 and 1.687 ± 0.020 g) respectively and shoot and dry weight (1.338 ± 0.022 and 0.337 ± 0.009 g) respectively compared to the control (**Table 4.1**). VCL is the only biostimulant which can sustain and enhance the growth of *A. hybridus* when applied as a foliar spray as could be observed from the results presented in **Table 4.1**.

The application of the same biostimulants via a combination of both methods, drenching and foliar spray resulting in VCL having a significant influence on both shoot and root length (297 ± 18.7 and 47.1 ± 3.4 mm) respectively. KEL increased significantly most growth parameters of *A. hybridus*. Significant increases were observed for both leaf and root number (22.2 ± 3.0 and 6.0 ± 1.7) respectively, both shoot and root length (296 ± 31.2 and 45.3 ± 6.2 mm) respectively, stem thickness (3.9 ± 0.04 mm), leaf area (86.9 ± 15.0 cm^2), both shoot and root fresh weight (4.893 ± 0.009 and 1.052 ± 0.010 g) respectively and finally on both shoot dry weight (0.882 ± 0.011 g) and root dry weight (0.320 ± 0.013 g) (**Table 4.1**). It was concluded that KEL applied via a combination drenching/foliar spray had a significant influence on the growth of *A. hybridus*.

In another set of experiments, *A. hybridus* was treated with the same 5 biostimulants via foliar spray and after 6 weeks the plants were harvested and biochemical tests were conducted to establish the chlorophyll, protein and carbohydrate content of the plant. The results are shown in **Table 4.2**. From these results it was observed that KAR_1, VCL and KEL significantly influenced the biochemical composition of *A. hybridus*. KAR_1 significantly increased the protein content (34.8 ± 4.13 μg^{-1} FW) of the crop compared to both the negative and positive controls. The influence of VCL was observed again on the amount of proteins in the amaranth plant (39.6 ± 1.85 μg^{-1} FW). KEL recorded significant influences on Chlorophyll a (641 ± 0.71 μg^{-1} FW), Chlorophyll b (172 ± 0.22 μg^{-1} FW), total Chlorophyll (a + b) (813 ± 0.61 μg^{-1} FW), carotenoid content (197 ± 0.10 μg^{-1} FW) and proteins (33.3 ± 0.92 μg^{-1} FW). In terms of enhancing the biochemical composition of *A. hybridus* via foliar application, KEL is the most appropriate biostimulant to use.

Table 4.4 is a summary of the effects of organic biostimulants on *A. hybridus* mineral composition. From the results it could be observed that some of the biostimulants used in this investigation did not have any significant effects on nutrient levels of the crop except for KEL, ECK and VCL. From these 3 biostimulants the greatest effect on nutrient levels was realised with KEL which significantly improved the levels of N, Ca, Mg, K, Na, Zn, Cu and P. KEL is the ideal biostimulant for enhancing the mineral composition of *A. hybridus* and it is very important especially for nutrition security since it boosts the levels of nutrients like zinc which are normally lacking in the diet.

Another investigation was carried out to evaluate the effect of organic biostimulants and the mode of application on the antioxidant activity and phytochemical composition of *A. hybridus*. From the results of the investigation it was concluded that, generally, treatments and mode of application did not influence the antioxidant activity and phytochemical composition of both methanolic and water extracts of *A. hybridus*. Only SW (**Fig. 4.10**), applied via drenching had a significant influence on the amount of condensed tannins in the leafy vegetable. In some instances, antioxidant activity and phytochemical composition significantly decreased upon application of biostimulants (**Fig. 4.9**).

In another experiment, the effect of the interaction of microbes and biostimulants on growth and biochemical composition of *A. hybridus* was investigated. Effects of KEL

alone, BL and PF alone and combinations of KEL + BL and KEL + PF on the growth and biochemical composition of *A. hybridus* were investigated and **Fig. 5.1** shows the effects of microbes and biostimulants on the crop. The results show that KEL had a significant effect on most growth parameters (**Fig. 5.1 D** and **E**; **Fig. 5.1 B** and **F**). Also, the significant effects were recorded on leaf number (**Fig. 5.1 A**), shoot fresh weight (**Fig. 5.1 D**) both shoot and root length (**Fig. 5.1 B** and **C**) and in *A. hybridus* treated with KEL + PF. In the same manner notable effects were recorded for shoot length (**Fig. 5.1 B**) and leaf area (**Fig. 5.1 F**) when the plant was treated with KEL + BL. These results illustrate the synergistic activities of microbes and biostimulants on *A. hybridus* especially when the microbes alone had no effect but were only effective when they were in combination with the biostimulant KEL. These positive synergistic interactions of microbes and biostimulants could be harnessed so as to improve the growth and productivity of *A. hybridus*.

In terms of the biochemical composition, KEL + BL significantly influenced the carbohydrate (267 ± 30 µg g^{-1} FW) content more than the control, KEL (204 ± 26 µg g^{-1} FW) alone and BL (131 ± 14 µg g^{-1} FW) alone. This again is another demonstration of synergistic interactions between microbe and biostimulant and this could also be utilised to improve productivity in amaranths.

These synergistic and mutualistic interactions between PGPR and biostimulants promise to be a worthwhile strategy in modern agriculture. Microorganisms and biostimulants such as KEL can work in a complementary and interactive manner to improve the growth, biochemical composition and mineral content of the highly nutritious but neglected leafy vegetable, *A. hybridus.* KEL improved the growth and photosynthetic pigment content. The size and greenness of the leaves are important parameters for most leafy vegetables including *A. hybridus*. The mineral content of the vegetable was increased for most minerals when treated with PF and this is of vital importance, as this goes a long way in addressing the issues of hidden hunger. Treatment with the two bacterial strains and KEL caused a decrease in SOD, suggesting stress reduction in *A. hybridus*. This was a synergistic effect with significantly lower SOD levels in the plants where the combination treatments were applied. It can be concluded that microbes and biostimulants could be used in combination as an eco-friendly approach to improve the production and mineral content of traditional leafy vegetables such as *A. hybridus*.

7.1 Concluding remarks

Any successful production and adoption of *A. hybridus* into mainstream agriculture should take into cognisance most of the aspects investigated in this research. Factors such as the type of biostimulant being used and how it is applied on the plant are important since it was observed from the experiments that the plant responds in different ways depending on the nature of the biostimulant and part of the plant to which it is applied. The plant responded in terms of the general growth parameters, photosynthetic pigments (chlorophylls and carotenoids) and carbohydrates. Also the other two species of amaranths, *A. caudatus* and *A. retroflexus* responded in the same manner in terms of the general growth parameters, when treated with biostimulants applied via the three modes of applications. The drenching application of KAR_1 and the foliar application of VCL on *A. hybridus* significantly influenced most of the growth parameters in the plant. The application of biostimulants on *A. hybridus* via a combination of drenching and foliar spray resulted in KEL significantly increasing the most growth parameters of the plant. In terms of light requirements, results from the same research show that *A. hybridus* grows well at maximum light intensity since that is where there is maximum photosynthesis leading to the production of more photosynthates for the enhanced plant growth. Results from this investigation show that generally biostimulant treatments and mode of application did not have any positive influence on antioxidant activity and phytochemical composition of *A. hybridus* with some of the treatments decreasing both antioxidant activity and phytochemical composition. It was also observed in this research that combining microorganisms and biostimulants is an eco-friendly approach in improving the growth and mineral content of *A. hybridus*. From these results it can be concluded that the promotion, improvement and subsequent adoption of *A. hybridus* into the mainstream or commercial agriculture can be achieved via the use of organic biostimulants taking cognisance of the role they play in the plant and how best the biostimulant can be applied.

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