The role of biostimulants on the physiology, nutrition, phytochemistry and endogenous phytohormone content in *Ceratotheca triloba* under abiotic stress conditions

By

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Research Centre for Plant Growth and Development

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1. **Nqobile A. Masondo**, Manoj G. Kulkarni, Jeffrey F. Finnie, Johannes Van Staden 2018. Influence of biostimulants-seed-priming on *Ceratotheca triloba* germination and seedling growth under low temperatures, low osmotic potential and salinity stress. Ecotoxicology and Environmental Safety147: 43 – 4.

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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 of each publication)

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

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Publication 3 Contributions: NAM performed the literature search and drafted the manuscript under guidance and supervision of JVS and JFF.

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SCP Srinivas C. Pendota
JFF Jeffrey F. Finnie
JVS Johannes Van Staden

Signed

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List of Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
BR	Brassinosteroids
Ca	Calcium
CH_3CN	Acetonitrile
*cis*OPDA	cis-(+)-12-oxo-phytodienoic acid
CKs	Cytokinins
DMRT	Duncan's Multiple Range Test
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
Eckol	4-(3,5-dihydroxyphenoxy) dibenzo-*p*-dioxin-1,3,6,8-tetrol
ESI	Electrospray ionisation
ET	Ethylene
ETR	Relative electron transport rate
Fe	Iron
Fm	Maximum fluorescence
Fo	Minimal fluorescence
FW	Fresh weight
Fv/*Fm*	Maximum quantum efficiency of PSII
GA	Gibberellic acid
HCOOH	Formic acid
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HNO_3	Nitric acid
HS	Hoagland' solution
IAA	Indole-3-acetic acid
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
JA	Jasmonic acid
JA-Ile	Jasmonoyl isoleucine
K	Potassium
KAR_1	Karrikinolide
LAE	Late embryogenesis abundant
LOX	Lipoxygenases
MeOH	Methanol
MIC	Minimum inhibitory concentration
Mg	Magnesium
Mn	Manganese
MRM	Multiple ion monitoring mode
N	Nitrogen
Na	Sodium
NaCl	Sodium chloride

NPQ	Non-photochemical quenching
P	Phosphorus
PAL	Phenylalanine ammonia lyase
PEG	Polyethylene glycol 6000
PG	Phloroglucinol
PGRs	Plant growth regulators
PPF	Photosynthetic photon flux
ΦPSII	Actual quantum yield of photosystem II
qP	Photochemical quenching
RDA	Recommended daily allowance
ROS	Reactive oxygen species
SA	Salicylic acid
SL	Strigolactones
SW	Smoke water
UHPLC	Ultra High Performance Liquid Chromatography
VCL	Vermicompost leachate
Zn	Zinc

Abstract

Ceratotheca triloba (Bernh.) Hook.f. (Pedaliaceae), commonly known as African foxglove is a southern African indigenous species. The species is commonly consumed as a traditional green leafy vegetable and possesses some medicinal properties. Even though the nutritional content and pharmacological properties of *C*. *triloba* have been documented, information relating to critical aspect of the plant's cultivation remain inadequate. A better understanding of the effect of abiotic stress on plant physiology, endogenous phytohormones, phytochemicals and nutritional content of *C*. *triloba* is pertinent if the plant is to be developed as a domesticated vegetable crop. Firstly, the current study evaluated the role of biostimulants [smoke-water (SW 1:500 v/v), synthesized smoke derived compound karrikinolide (KAR_1 10^{-6} M), a commercial seaweed extract Kelpak® (0.4 %), compounds isolated from Kelpak® phloroglucinol (PG 10^{-6} M) and eckol (10^{-6} M)] on seed germination and early seedling growth of *C*. *triloba* at different temperature regimes. In addition, the effect of biostimulant-seed-priming on germination and seedling growth under low temperatures, low osmotic potential and different sodium chloride (NaCl) concentrations was determined. Secondly, the study evaluated the effect of different watering regimes on growth and development of *C*. *triloba* in order to understand water requirements under greenhouse conditions. Thirdly, the study examined the role of commonly used biostimulants [Kelpak® 0.4 % and vermicompost leachate (VCL 1:10 v/v)] on *C*. *triloba* seedling growth under salinity and nutrient-deficient stresses in the greenhouse. Lastly, the effect of different harvesting stages (2 and 4 months) on the growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content were assessed in *C*. *triloba* seedlings grown at different watering regimes, salinity and nutrient-deficient stress conditions.

Temperature played an important role in *C*. *triloba* seed germination. Low temperatures (10 and 15 ºC) completely inhibited seed germination, 25 ºC was the optimum temperature with the highest percentage germination and 35 ºC slightly decreased seed germination. Smoke-water and KAR_1 were found to significantly improved seed germination at 25 °C compared to the control. Furthermore, Kelpak®, PG and eckol also stimulated seed germination at 25 °C relative to the control treatment. In addition to the inhibitory effects of low temperatures; low osmotic potential (PEG) and different NaCl concentrations completely inhibited *C*. *triloba* seed germination even with the application of biostimulants. Therefore, seed-priming (biostimulants or distilled water) proved to be an indispensable technique in the germination of *C*. *triloba* during these aforementioned stresses. Priming seeds with SW, KAR_1 and Kelpak® ameliorated the detrimental effects of low temperature (15 °C), low osmotic potential (-0.05 and -0.15 MPa) and NaCl concentrations (5 – 50 mM). Furthermore, SW and PG treatments improved seedling growth at low temperature (15 °C) and low osmotic potential (-0.05 MPa), whereas hydroprimed seeds produced longer shoots and roots with a higher vigour index at different NaCl (5 – 25 mM) concentrations.

During greenhouse cultivation, different watering regimes [7 (daily); 3 (thrice); 2 (twice); 1 (once) day(s) per week] significantly influenced *C*. *triloba* seedling growth. In comparison to plants watered daily, watering plants once a week reduced (c.a. 1.4-fold) plant growth and chlorophyll fluorescence parameters [maximum quantum efficiency of PSII (*Fv*/*Fm*), quantum yield of photosystem II (ΦPSII), phytochemical quenching (qP), relative electron transfer rate (ETR)] while increasing non-photochemical quenching (NPQ) values. From the quantified endogenous phytohormones, plants watered once a week had significantly lower jasmonoyl

isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), salicylic acid (SA), jasmonic acid (JA) and indole-3-acetic acid (IAA) content with an increase in abscisic acid (ABA) concentrations. On the contrary, watering plants daily enhanced the aforementioned phytohormone concentration while reducing ABA production in leaves and roots of 2 and 4-month-old plants. Phenolic acid quantification in *C*. *triloba* yielded a total of six (4-hydroxybenzoic, protocatechuic, vanillic, caffeic, ferulic and 4-coumaric acid) metabolites which were mostly concentrated in 4-month-old leaves watered once a week. The reduction in water supply (1 d) caused a decline in mineral and carbohydrate content in 2-month-old leaves, while plants watered twice a week had increased mineral and total carbohydrate content after 4-months of growth. Overall, prolonged duration (4 months) of growth enhanced endogenous phytohormones, phenolic acids and mineral content in *C*. *triloba* plants.

The deleterious effects of NaCl-stress (75; 150 mM) on the growth of *C*. *triloba* were to some extent alleviated with the application of Kelpak® and VCL. The promotive effects of biostimulants were observed in the chlorophyll fluorescence (*Fv*/*Fm*, ΦPSII, qP, NPQ, relative ETR) of leaves especially after 2 months, with prolonged growth (4 months) significantly reducing the photosynthetic activity of plants. The applied biostimulants also decreased endogenous phytohormone content (JA-Ile, *cis*OPDA, ABA, SA, JA and IAA) in 2-month-old leaves at different NaCl concentrations. Furthermore, Kelpak® induced the synthesis of bioactive compounds (4-hydroxybenzoic, protocatechuic, vanillic, caffeic, ferulic and 4-coumaric acid) at 150 mM NaCl concentration in 2-month-old plants, while both biostimulants reduced phenolic acid content at different NaCl conditions, particularly in 4-month-old plants. Application of Kelpak® and VCL generally enhanced the mineral and carbohydrate

content in *C*. *triloba* leaves at different NaCl concentrations. The stimulatory effect of biostimulants on the mineral content was more pronounced in plants grown for 4 months, except in Kelpak®-treated plants with 75 mM NaCl. Duration of *C*. *triloba* growth influenced the evaluated parameters, in which 2-month-old plants had higher chlorophyll fluorescence, endogenous phytohormones and phenolic acid content.

Nutrient-deficient [nitrogen (-N), phosphorus (-P) and potassium (-K)] soils significantly reduced the leaf weight, root length, root weight and plant height in *C*. *triloba*. This was marked by a 7-fold reduction in leaf weight of N and P-deficient plants after 4 months of growth. Detrimental effects of insufficient nutrient supply were ameliorated with the application of Kelpak® and VCL treatments. Even though nutrient-deficiency had minimal effect on the chlorophyll fluorescence parameters (*Fv*/*Fm*, ΦPSII, qP, NPQ, relative ETR) after 2 and 4 months of growth, application of biostimulants improved the photosynthetic activity of plants especially after 4 months. Nutrient-deficiency together with biostimulant application enhanced phytohormone content (JA-Ile, *cis*OPDA, ABA, SA, JA and IAA) in *C*. *triloba* leaves and roots after 2 and 4 months of growth. However, phenolic acids in *C*. *triloba* leaves were better regulated by nutrient-deprivation than by supplementation with Kelpak® and VCL. Despite the inhibitory effects of N and P-deprivation in plants, biostimulant application increased mineral content in these treatments. Prolonged (4 months) duration of growth significantly enhanced endogenous phytohormones, phenolic acid and nutritional content relative to 2-month-old plants. The current findings demonstrates the deleterious effect of abiotic stresses during growth of *C*. *triloba* plants. However, *C*. *triloba* plants were able to acclimatize and adapt to the different stresses with the application of biostimulants possibly through the mediation of growth and development, ion exclusion (Na^+ and Cl^-) and nutrient allocation. Most importantly, the ability of Kelpak® and VCL to

efficiently aid in nutrient assimilation and translocation during different stresses remarkably improved plant adaptation thus enhancing plant growth, survival and mineral content in *C*. *triloba*.

Chapter 1: General introduction

1.1. Introduction

In Africa, the number of hungry people is continuously increasing with the current estimate placed at 233.5 million **(FAO et al. 2015)**. About 23 million people in 11 African countries are affected by acute food shortage. An average of one in four individuals on the Continent are undernourished **(FAO 2012)**. In addition, an estimated 3.7 million deaths in children is attributed to malnutrition while 750 000 - 850 000 deaths are caused by iron, zinc or vitamin A deficiency **(FAO 2004)**. According to **Okigbo (1990)**, deficiency in vitamin A is the leading cause of blindness and even death amongst children in semi-arid and arid regions of Africa. Despite the relative abundance of vegetables and fruits in Africa, their low intake remains one of the top 10 risk factors contributing to mortality on the Continent **(Ezzati et al. 2002)**. According to the World Health Organization **(WHO 2003a)**, humans require a daily intake of more than 400 g of vegetables and fruits to prevent diet-related chronic diseases. As a supplement to carbohydrates (which forms the bulk of the daily diet among Africans), vegetables are the major sources of nutrients such as vitamins, essential amino acids, minerals and antioxidants **(Uusiku et al. 2010)**.

African indigenous traditional foods are categorized into grains, vegetables and fruits. In South Africa, traditional vegetables are collectively called *morogo* (Sesotho and Sepedi) or *imifino* (IsiZulu and IsiXhosa). Consumption of these traditional vegetables in the country is dependent on various factors including poverty status, degree of urbanization and plant seasonality **(Vorster et al. 2002)**. Unlike in South Africa; production, trade and consumption rate of indigenous traditional vegetables in other African countries is well-developed **(Schippers 2000)**. Thus, scientists and policymakers have increased the levels of attention devoted to traditional vegetable

research including both indigenous and indigenized species **(Department of Agriculture 2004)**.

1.2. Status and value of traditional vegetables in South Africa

In the past two decades, documentation of traditional vegetable consumption among South Africans has increased with several studies conducted in KwaZulu-Natal and Limpopo Provinces **(Dovie et al. 2007; Ntuli et al. 2012)**. In addition, the availability, nutritional value, pharmacological potential, conservation status and cultivation of these vegetables has been highlighted **(Bvenura and Afolayan 2015; Odhav et al. 2007; Uusiku et al. 2010)**. This is due to their health benefits which extend beyond basic nutrition **(Van Wyk and Gericke 2000)**. Most importantly, these vegetables can be harvested from the wild because they are known to grow in the field without the need for cultivation. Indigenous vegetables are also known to be resistant, adaptable and tolerant to different harsh climatic conditions when compared to exotic species. Even though they can be easily cultivated, they still remain under-utilized due to inadequate knowledge on their value and lack of cultivation practices **(Dweba and Mearns 2011)**. Thus, more stringent studies are necessary to evaluate the unexplored potential of indigenous traditional vegetables. Furthermore, there is a need to determine the effect of diverse environmental conditions on their growth, development and survival.

1.3. Effect of abiotic stress on plant growth and development

Environmental factors such as extreme temperatures, drought, salinity and nutrient-deficiency are the major causes of reduced seed germination, plant growth, survival rate and overall crop productivity. These factors have been estimated to cause more than a 50% decline in crop yields **(Bray et al. 2000)**. Furthermore, the impact of

environmental stresses is dependent on the developmental stage, plant tissues or organs affected and severity of stress. The ability of plants to respond to abiotic stress is activated by molecular networks that are involved in stress perception, signal transduction and expression of stress-related genes and metabolites **(Vinocur and Altman 2005)**. Furthermore, endogenous phytohormones play a crucial role in the plant's ability to adapt/acclimatize to different stress conditions, through nutrient distribution and source/sink transitions **(Peleg and Blumwald 2011)**. Therefore, biotechnological approaches have focused on enhancing the plant's endogenous defense mechanism in order to counteract the effect of abiotic stress. However, such approaches might have dire consequences on plant growth due to the over-expression of regulatory elements that are triggered by crosstalk between developmental and stress-responsive pathways **(Cabello et al. 2014)**. Thus, studies have been aimed at understanding the synergistic or antagonistic cross-talk of phytohormones in order to regulate their synthesis in response to different stress factors **(Peleg and Blumwald 2011)**. Furthermore, more research has focused on the search for new and alternative technologies for the improvement of plant survival under abiotic stress conditions. Therefore, finding inexpensive and accessible organic fertilizers (e.g. biostimulants) with the ability to ameliorate the effect of abiotic stress via the regulation of endogenous phytohormones during seed germination, early seedling growth, plant survival and crop productivity is of outmost importance.

1.4. Effect of biostimulants on plant growth under abiotic stress conditions

Biostimulants improve the growth and development of plants through nutrient uptake, nutrient efficiency and abiotic stress tolerance **(European Biostimulants Industry Council 2012)**. They encompass diverse compounds, microorganisms, plant growth regulators (PGRs) and seaweed extracts **(Hamza and Suggars 2001)**. A number of

biostimulants have been extensively evaluated for their role during plant growth and development in different plant species. Smoke-water (SW) is one of the well-recognized and most commonly used biostimulant especially during seed germination **(Brown and Van Staden 1997; Van Staden et al. 2000)**. The discovery of SW lead to the isolation of a biologically active compound known as karrikinolide [(3-methyl-2*H*-furo[2,3-*c*]pyran-2-one; KAR_1] **(Flematti et al. 2004; Van Staden et al. 2004)**. Seaweed extracts is a well-known biostimulant which has been extensively reviewed for its efficiency in agriculture **(Calvo et al. 2014; Craigie 2011)**. In South Africa, Kelpak® is a commercially manufactured biostimulant from a brown seaweed *Ecklonia maxima* (Osbeck) Papenfuss (Phaeophyceae) **(Troell et al. 2006)**. Recently, two compounds phloroglucinol [benzene-1,3,5-triol; PG] and eckol [4-(3,5-dihydroxyphenoxy) dibenzo-*p*-dioxin-1,3,6,8-tetrol] were isolated from *Ecklonia maxima* **(Kannan et al. 2013)**. Vermicompost leachate (VCL) produced through the activity of earthworms from organic residues has been widely utilized in the improvement of growth and development during crop production **(Ievinsh 2011)**.

1.5. *Ceratotheca triloba*

Ceratotheca triloba (Bernh.) Hook.f. (Pedaliaceae) is an indigenous leafy vegetable commonly known as a wild foxglove. The genus is relatively small, consisting of five species that are widely distributed in some parts of Africa **(Duncan 2011)**. The species serves as a supplement to the starch-based diets of under-developed communities in southern Africa. Although not well-researched, *C. triloba* has been reported to have relatively high nutritional content **(Odhav et al. 2007)**. In South Africa, the species has been widely documented for its medicinal and pharmacological potential **(Masondo et al. 2016a)**. For conservation purposes, the species is propagated through seed germination during spring or early summer in rich well-drained soils. However, there

is still limited literature on the cultivation practices of *C*. *triloba* particularly under abiotic stress conditions.

1.6. Aims and objectives

The aims and objectives of the study were to:

- Determine the effects of biostimulants (SW, KAR_1, Kelpak®, eckol and PG) on seed germination of *C*. *triloba* under different temperature regimes, low osmotic potential and varying sodium chloride (NaCl) concentrations;
- Investigate the role of different watering regimes on plant growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content in *C*. *triloba*;
- Evaluate the effect of biostimulant (Kelpak® and VCL) application on *C*. *triloba* seedling growth under salinity stress and nutrient-deficiency. Additionally, plant growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional contents were evaluated;
- Determine the effect of different harvesting stages (2 and 4 months) on growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content on seedlings cultivated at different watering regimes, salinity and nutrient-deficient stress.

1.7. General overview of the thesis

Chapter 2 highlights the unexplored nutritional and pharmacological potential of the *Ceratotheca* genus, an African indigenous leafy vegetable. The review documents and discusses the limited studies conducted on the utilization, safety and conservation of *Ceratotheca* species.

Chapter 3 evaluates seed germination of *C*. *triloba* under different temperature regimes when supplemented with biostimulants such as SW, KAR_1, Kelpak®, PG and eckol. Furthermore, the influence of biostimulant-seed-priming under low temperature, low osmotic pressure and NaCl concentrations were investigated.

Chapter 4 determines the effect of different watering regimes on *C*. *triloba* seedling growth under greenhouse conditions.

Chapter 5 evaluates the effect of biostimulants (Kelpak® and VCL) on the growth of *C*. *triloba* under salinity stress under greenhouse conditions.

Chapter 6 determines the effect of biostimulants (Kelpak® and VCL) on the growth of *C*. *triloba* under nutrient-deficient conditions (nitrogen, phosphorus and potassium) in the greenhouse.

Chapter 7 presents a summary of the main findings of the study.

The section '**References**' is a list of all the literature cited in this thesis.

Chapter 2: Nutritional and pharmacological potential of the genus *Ceratotheca*: An underutilized leafy vegetable of Africa

2.1 Introduction

The genus *Ceratotheca* is a member of the Pedaliaceae, comprising of five species endemic to Africa. The generic name for *Ceratotheca* is derived from the Greek names *kerato* (horned) and *theke* (a case) describing the plant's discrete sharp horned capsule **(Duncan 2011)**. Similar to other members of the family, the genus is characterised by distinct type of mucilaginous glands, covering most parts of the plant including the leaves. These glands secretes mucilage once in contact with water **(Ihlenfeldt 2004)**. The genus closely resembles the genus *Sesamum* which produces sesame seeds and oil **(Bedigian 2004)**.

Among the five species, *C*. *sesamoides* Endl. and *C*. *triloba* (Bernh.) Hook.f. are the only two species documented for their nutritional and pharmaceutical importance. Even though the species have an acrid smell and hairs on their leaves, they are still included in diets (commonly classified as traditional leafy vegetables) in some parts of Africa. These species are good sources of proteins, sugars, and calcium **(Fasakin 2004; Grubben and Denton 2004; Yadang et al. 2009)** and contain high energy (kJ), lipids and magnesium levels **(Odhav et al. 2007)**. Apart from their nutritional value, the species are also widely utilized against various ailments. In some parts of Nigeria, *C*. *sesamoides* is used in the treatment of diarrhoea, conjunctivitis, snakebites, skin diseases and to facilitate child birth in both humans and animals **(Grubben and Denton 2004)**. In South Africa, *C*. *triloba* leaves are extensively used by the Zulu people for the relief of painful menstruation, stomach cramps and diarrhoea **(Pooley 1998; Watt and Breyer-Brandwijk 1962)**. Hence, the species have been studied in both *in vitro* and *in vivo* bioassays and reported to possess antimicrobial,

antidiarrhoeal, anti-inflammatory and antidiabetic properties **(Mohanlall and Odhav 2013; Toyin et al. 2012)**. Thus, the species can be classified as functional foods due to their health benefits extending beyond basic nutritional value **(Van Wyk and Gericke 2000)**. Although the importance of these plants in Africa has been over-looked and poorly documented, the available literature supports their high and valuable nutritional status as well as their pharmacological potential. Therefore, this review aims to document the unexplored potential and significance of the African endemic species namely *C*. *sesamoides* and *C*. *triloba*, and their nutritional and pharmacological properties.

2.2. Distribution and general morphology of *Ceratotheca* species

The five species of *Ceratotheca* include the annual *C*. *integribracteata* Engl. distributed in Angola and Namibia; the annual *C*. *saxicola* E.A.Bruce endemic to Soutpansberg Centre, Limpopo Province, South Africa; the annual *C*. *sesamoides* Endl. endemic to the northern parts of West Africa; the annual and biennial *C*. *triloba* (Bernh.) Hook.f. distributed in southern Africa and the perennial *C*. *reniformis* Abels found in Angola **(Duncan 2011)**.

The genus consists of erect plants which grow to a height ranging from 0.6 - 2.0 m. The leaves are arranged in opposites with lower leaves larger and the uppermost leaves narrowly to broadly lanceolate. The flowers have a foxglove-like shape, produced in pairs that vary from white, pink or mauve (**Fig. 2.1**). Flowering season commences from late January (summer) until May (autumn), depending on the species. The plant produces distinctive horn-shaped seeds that are dark brown or black in colour **(Duncan 2011)**. The species are covered with mucilage producing

glands, that allow plants to withstand severe dehydration without any tissue damage, which makes them drought-resistant **(Bedigian and Adetula 2004)**.

Fig. 2.1: *Ceratotheca triloba* morphology A-whole plant; B-flowers; C-seed capsule.

2.2.1. Ceratotheca sesamoides

Ceratotheca sesamoides commonly known as 'false sesame' was first described by Professor Stephan Endlicher (1804 - 1849), the Director of the Botanical Garden of Vienna in *Linnaea* **(Grubben and Denton 2004)**. The species has two synonyms *C. melanosperma* Hochst. ex Bernh. (1842) and *Sesamum heudelotii* Stapf (1906). The plant is also known by various names such as Eku and Bungu (Nigeria), Tchaba-laba (Guinea Bissau) and Lalu-caminho (Senegal). The species is found in West Africa and has extended its distribution from Senegal to Tanzania, Democratic Republic of

Congo, southwards to Botswana, Mozambique, Zimbabwe and Zambia. Even though the species grows as a weed, it has become one of the most frequently cultivated leafy vegetables in most parts of sub-Saharan Africa. The plant is easily adapted to a wider range of environments, including open grasslands and tree savanna with well-drained sandy soils **(Grubben and Denton 2004)**. It grows well in sunny habitats making it a heat and drought-tolerant species.

2.2.2. Ceratotheca triloba

Ceratotheca triloba commonly known as African foxglove, was first documented in southern Botswana (1812) by William Burchell at Chue Spring (now Heuningvlei). It was initially recorded as *Sporledera triloba* by Johann Bernhardi a German botanist (1774–1850), then changed to *Ceratotheca* by J.D. Hooker in *Curtis Botanical Magazine* **(Duncan 2011)**. Its synonyms include *C*. *lamiifolia* (Engl.) Engl., *S*. *lamiifolium* Engl., *S*. *kraussiana* Bernh., and *S*. *triloba* Bernh. **(Raimondo et al. 2009)**. The species is commonly referred to as Wild foxglove (English); Vingerhoedblombossie (Afrikaans); Udoncalwabathwa, Udonqabathwa (IsiZulu); Mudyangaringa, Nyamanhuwe (Shona). *Ceratotheca triloba* is widely distributed in southern African countries including South Africa, Mozambique, Zimbabwe, Zambia and Angola **(Pickering and Roe 2009)**. The species either grows as a weed or cultivated in some regions. In South Africa, the plant grows best in summer rainfall grasslands. Seeds germinate during spring or early summer, in rich and well-hydrated soils and plants start flowering before the onset of winter.

2.3. Consumption of *Ceratotheca* species

In the last two decades, there has been growing interest in the potential of traditional vegetables in overcoming the nutritional gap experienced by the population in

developing countries. However, several leafy vegetable species are yet to be recognized and documented for their important role in human health. In addition, data collection on leafy vegetables has been poorly characterized within international genebanks **(Maggioni 2002)**. Due to this, knowledge on the use (nutritional and medicinal) of neglected or underutilised crop species is crucial for conservation purposes.

Ceratotheca sesamoides is amongst the important African indigenous vegetables ans is widely utilized, especially for the oil it produces. Although the plant has been reported to be widely consumed in most parts of West Africa **(Bedigian and Adetula 2004; Ruffo et al. 2002)**, literature on its consumption in other regions is still limited (**Table 2.1**). Furthermore, the available literature has described the consumption of the species as being dependent on its distribution and scarcity of other preferred food sources. For instance, **Mertz et al. (2001)** reported on the plant as being the least consumed leafy vegetable in Burkina Faso even though it was described as having a pleasant taste. **Dansi et al. (2012)** found that the species was abundant in Benin yet its consumption was inconsequential compared to the most consumed leafy vegetables. Even when cultivated, *C*. *sesamoides* plants have a low consumption rate in some regions **(Lykke et al. 2002; N'Danikou et al. 2011)**. Nevertheless, there are regions where the importance of *C*. *sesamoides* has been widely documented in the diet of the population. In Benin (Sudano-Guinean and Sudanian regions), the plant is amongst the most commonly consumed non-cultivated leafy vegetable **(Achigan-Dako et al. 2011)**. The species is also reported as one of the most frequently consumed vegetable with millet in south-western Niger **(Ayantunde et al. 2009)**. The plant serves as a significant wild crop in Zambia, with 37% of households reporting its consumption **(Bedigian 2004)**. Unlike *C*. *sesamoides*, literature on the consumption

of *C*. *triloba* in southern Africa is scarce. The species has thus far been recorded to be consumed in drier areas of Zimbabwe and the leafy vegetable was classified as a 'poor man's food' **(Maroyi 2011)**. The infrequent consumption of *C*. *sesamoides* and *C*. *triloba* in some areas might be due to several factors such as preference for other vegetables, availability/seasonality of the species and the status of the leafy vegetable as a "poor man's food". Nonetheless, these vegetables may serve to supplement the mainly starch-based diets of many under-developed communities in Africa because they can grow in the wild and be easily cultivated.

Table 2.1: Documented surveys on the consumption of *Ceratotheca sesamoides* and *Ceratotheca triloba* in African countries.

Scientific name	Countries	Status of distribution	Result highlights	Reference
C. *sesamoides* Endl.	Benin (Sudano– Guinean and Sudanian region)	Wild	Among the 245 species surveyed in Benin, *C*. *sesamoides* was the most frequently consumed non-cultivated vegetable in the two regions.	**Achigan-Dako et al. (2011)**
C. *sesamoides* Endl.	Benin	Wild	The species is recognized as one of the most widely distributed and uncultivated leafy vegetables indigenous to the country.	**Adéoti et al. (2009)**
C. *sesamoides* Endl.	Benin	Wild	In some parts of Benin, the availability of *C*. *sesamoides* is abundant, however its consumption is negligible.	**Dansi et al. (2012)**
C. *sesamoides* Endl.	Benin (North)	Cultivated	The species was ranked at number 13 in order of its importance in the market trade.	**Dansi et al. (2008)**
C. *sesamoides* Endl.	Bénin (Dan forest)	Wild	The species occurs in different habitats and its nutritive value has been ranked at number 27 in the southern region of Benin.	**N'Danikou et al. (2011)**
C. *sesamoides* Endl.	Burkina Faso	Wild	Even though *C*. *sesamoides* is the least consumed vegetable in Silmiogou and Ningaré regions, it is still considered the tastiest vegetable.	**Mertz et al. (2001)**
C. *sesamoides* Endl.	Burkina Faso	Cultivated	Consumption of *C*. *sesamoides* accounted for less than 1% of the meals in Silmiogou households.	**Lykke et al. (2002)**

C. *sesamoides* Endl.	Niger (South-western)	Wild	The species was reported as one the four most recognized plants consumed with solid millet. The plant was also found to be consumed by livestock.	**Ayantunde et al. (2009)**
C. *triloba* (Bernh.) Hook.f.	Zimbabwe	Wild	Of the 32 edible traditional vegetables identified in Zimbabwe, *C*. *triloba* was found to be consumed in Beitbridge, Binga, Tanganda Halt and Umguza Districts. It has been classified as a “poor man’s food” therefore not traded in the markets.	**Maroyi (2011)**

2.4. Nutritional value of *Ceratotheca* species

Nutrient-rich foods which are a good source of proteins, vitamins, minerals, fibers and amino acids are vital for growth and development in children and adults. The recommended dietary allowance (RDA) for minerals in adults per day has been estimated as follows: calcium 1000 mg; phosphorus 800 mg; copper 900 mg; zinc 10 mg; magnesium 400 mg; manganese 7 mg and iron 8 mg **(Odhav et al. 2007)**. Even though the importance of nutrients and minerals is well-established, the majority of the population in developing countries still lack the daily required amounts in their diets. Hence, several studies have reported on the value of vegetables as major constituents of micro/macronutrient as well as dietary energy **(Aletor et al. 2002; Barminas et al. 1998; Uusiku et al. 2010)**. Therefore, supplementing poor diets with vegetables can contribute greatly to the reduction of malnutrition in sub-Saharan Africa **(Bedigian 2004; Van Wyk and Gericke 2000)**.

During the preparation of *C*. *sesamoides* and *C*. *triloba*, most parts of the plant are consumed including leaves, flowers and seeds. *Ceratotheca sesamoides* is prepared by grinding dry leaves into fine powder and mixing them with groundnut flour, salt and hot water then cooking for a few minutes. The mixture is eaten as a sauce with porridge. Onion and tomatoes can be added in order to soften and reduce the bitterness of the leaves **(Grubben and Denton 2004)**. Furthermore, crushed seeds of *C*. *sesamoides* can also be eaten with beans or cassava and the plant seed oil added in salads **(Grubben and Denton 2004)**. The leaves of *C*. *sesamoides* are frequently served with other leafy vegetables, possibly due to their laxative effect **(Bedigian 2004)**. *Ceratotheca triloba* is cooked as a spinach. The unpleasant smell of leaves disappears once boiled. The vegetable has a sweet taste and is also included as a relish in some dishes **(Tredgold 1986)**.

The nutritional composition of *C*. *sesamoides* and *C*. *triloba* is presented in **Table 2.2**. Leaf extracts of *C*. *sesamoides* contain high protein, carbohydrate, minerals and energy (kJ) levels **(Bedigian and Adetula 2004)**. The authors found that the seeds had even higher energy, protein, fat, carbohydrate and calcium contents compared to the leaves. Seed oil composition when extracted with petroleum ether was 35% **(Bolton 1919)**. According to the authors, extraction of *C*. *sesamoides* seeds tested negative for the presence of sesame oil when tested with the Baudouin reaction colour test. In addition, results from other tests (e.g. free fatty acids) showed similarities in oil seed constituents with a slightly lower specific gravity in *C*. *sesamoides*. The potentially good nutritional content of wild and cultivated *C*. *sesamoides* (leaves and seeds) has been highlighted in a number of studies **(Fasakin 2004; Fasola and Ogunsola 2014; Mitchikpe et al. 2008; Yadang et al. 2009)**. The species had high concentration of mineral and provitamin-A [3681 retinol equivalents (RE)/100 g] **(Bedigian 2004)**. The high nutritional value of C. *sesamoides* shows the plant's importance in supplementing most diets. However, no studies have evaluated the nutritional content of *C*. *sesamoides* flowers even though they are included in some dishes. Nutritional composition of *C*. *triloba* was evaluated by **Odhav et al. (2007)**. The plants comprises of high energy levels (kJ) as well as high fat content, however the mineral content (calcium, phosphorus, sodium, manganese, copper, zinc and iron) was relatively low **(Odhav et al. 2007)**.

Table 2.2: Documented studies on the nutrient content of *Ceratotheca sesamoides* and *Ceratotheca triloba*.

Scientific name	Plant part tested	Finding(s)	Reference
C. *sesamoides* Endl.	Leaves	Two cultivars (leaf material) of *C*. *sesamoides* contain high levels of protein (29.85%), calcium (2.62%) and phosphorus (0.27%). Even though seeds had high levels of oil (21.00 %), crude protein (22.15%), crude fiber (29.75 %), calcium (3.15%) and phosphorus (0.54%).	**Fasakin (2004)**
C. *sesamoides* Endl.	Leaves	At three different growth stages (6, 8 and 10 weeks); moisture and carbohydrate content in leaves was highest on the 6th week 9.30% and 59.90%, respectively. Even though the highest protein (12.85%), ash (8.55%) and crude fibre content (8.60%) was recorded in the 8th growth week.	**Fasola and Ogunsola (2014)**
C. *sesamoides* Endl.	Leaves	The nutritional content of *C*. *sesamoides* was 93.39 dry matter (%), 15.11 protein, 0.09 lipid, 75.43 total sugar, 8.20 reductors sugar per 100 g/l DM and 1515.75 energy (kJ).	**Yadang et al. (2009)**
C. *sesamoides* Endl.	Leaves	Leaf extracts of *C*. *sesamoides* contained: 81 g water, 226 kJ energy (54 kcal), 4.2 g protein, 0.5 g fat, 11.0 g carbohydrate, 300 mg calcium, 86 mg phosphorus, 3.2 mg iron, 28 mg ascorbic acid per 100 g. However the seeds contained: 7.0 g water, 2303 kJ energy (550 kcal), 14.2 g protein, 46.5 g fat, 27.5 g carbohydrate, 887 mg calcium, 38 mg iron, 0.75 mg thiamin, 0.3 mg riboflavin and 4.4 mg niacin.	**Grubben and Denton (2004)**
C. *sesamoides* Endl.	Leaves	The leaf extracts contained: 84.6% dry matter, 25 protein, 4.3 fat, 51.1 fibre, 6.1 carbohydrates, 13.6 g ash per 100 g dry matter and energy 673 (kJ)/161 (kcal). Inorganic constituents: 146.2 iron, 5.0 zinc, 1207 calcium, 377 phosphorus, 2125 potassium, 2.6 copper, 20.7 manganese, 592 magnesium mg/100 g dry matter.	**Mitchikpe et al. (2008)**

C. *triloba* (Bernh.) Hook.f.	Leaves	*C*. *triloba* extracts contained: energy 259 kJ (62 kcal), 85 g moisture, 2 g protein, 2.1 g fat, 2.07 g fibre, 2.27 g ash, 8.28 g carbohydrates. Mineral content: 705 calcium, 223 phosphorus, 115 sodium, 8 manganese, 3 copper, 3 zinc, 428 magnesium, 19 iron mg/100 g dry weight.	**Odhav et al. (2007)**

Trypsin inhibitors are compounds that interfere with protein digestion causing pancreatic enlargement and enhancement of chemically-induced pancreatic tumors **(Venter and Van Eyssen 2001)**. A study by **Vanderjagt et al. (2000)** evaluated the antinutrient properties (compounds that interfere with the absorption of nutrients) in *C. sesamoides* and reported that the plant had weak trypsin inhibitory activity (0.21 – 0.45 µg/mg dry weight). Leaves of *C. sesamoides* contained minor traces of trypsin-neutralizing activity when compared to other leafy vegetables and commercial species [e.g spinach (*Amaranthus spinosus*) 3.45 µg/mg dry weight)] found in Niger Republic. The trypsin activity observed in the leaf extracts showed resistance to boiling with 100% heat resistance. The ability of the plants to resist changes in trypsin activity is of interest because the co-presence of protease inhibitors in ingested plants might reach the small intestine and block the trypsin activity which catalyses the hydrolysis of dietary proteins. This is an important process for protein digestion and absorption especially for *C. sesamoides* which remains a poor source of protein **(Yadang et al. 2009)**. *Ceratotheca triloba* leaf extracts contained approximately 913 (TAU) trypsin inhibition with small traces of phytic acid (0.04 mg/ml). The presence of phytic acid in vegetables leads to the inhibition of iron inhibition in both infants and adults **(Hurrell 2003)**. It has been estimated that exclusion of phytic acid to ≤ 0.01 mg/g enhances iron absorption (4 - 5 fold) in comparison to its accumulation which results in decreased iron absorption **(Hurrell et al. 1992)**. Presence of phytic acid in such low quantities will allow for absorption of important minerals e.g. iron in humans. The slightly higher levels of phytic acid levels in *C. triloba* might at times hinder the absorption of nutritionally essential minerals, consequently having a negative effect on body functions. Therefore, further tests are of importance to validate the phytic acid content in the plant species.

2.5. Medicinal properties of *Ceratotheca* species

Globally, approximately 80% of the population continues to utilize traditional medicine for several ailments **(WHO 2003b)**. Thus, extensive research focusing on scientific evaluation of traditional medicine from plant origin has been on-going. Apart from their nutritional value, leafy vegetables have long been known to possess medicinal properties **(Tredgold 1986; Van Wyk and Gericke 2000; Watt and Breyer-Brandwijk 1962)**. In this respect, *C*. *sesamoides* and *C*. *triloba* have been widely documented for their utilization in traditional medicine.

In Nigeria, *C*. *sesamoides* are traditionally used in the treatment of diarrhoea. The leaves are soaked in water and drops of the slimy residue is used to cure conjunctivitis. The mucilage is occasionally used as an emollient and lubricant **(Grubben and Denton 2004)**. A mixture of warmed, crushed leaves with ash aids in relieving pressure on inflamed cervical lymph nodes. The infusion of *C*. *sesamoides* leaves with rhizomes of *Anchomanes difformis* (Blume) Engl. can be applied topically for the treatment of leprosy. The plant is used as an aphrodisiac, treatment for jaundice, snakebites and skin ailments. *Ceratotheca sesamoides* leaf infusions are used to facilitate delivery in both humans and animals **(Grubben and Denton 2004)**. In northern Nigeria, *C*. *sesamoides* seeds are used to relieve circumcision pain in males **(Abubakar et al. 2007)**. Leaf decoctions of *C*. *sesamoides* can either be administrated orally or as a bath for the treatment of malaria in Mali **(Diarra et al. 2015)**. In Baskoure, Kourittenga Province, Burkina Faso, the whole plant is used as a decoction to cure stomach ache **(Nadembega et al. 2011)**.

In South Africa, *C*. *triloba* is traditionally used to relieve painful menstruation, stomach cramps, nausea, fever and diarrhoea **(Pooley 1998)**. In Zimbabwe, the leaves of the

species are administered in order to induce abortion **(Hutchings et al. 1996)**. Leaf infusions of the plant aids in the relief of gastro-intestinal cramps and flatulence **(Roberts 1990; Watt and Breyer-Brandwijk 1962)**. *Ceratotheca triloba* leaves are used as an abortifacient and for dysmenorrhoea **(Van Wyk and Gericke 2000; Watt and Breyer-Brandwijk 1962)**. Root infusions are utilized in the treatment of sore eyes and ears **(Hutchings et al. 1996)**. When soaked, the whole plant can be used as soap or shampoo. Due to the unpleasant odour the plant produces, its leaves have been utilized as insect-repellent sprays **(Roberts 1990)**.

2.6. Phytochemistry of *Ceratotheca* species

The phytochemicals from *C*. *sesamoides* and *C*. *triloba* are summarised in **Table 2.3**. Phytochemical analysis of *C*. *sesamoides* leaves yielded flavonoids, saponins, alkaloids, tannins and phenols **(Fasola and Ogunsola 2014)**. According to **Fasola and Ogunsola (2014)**, different stages of growth had a significant impact on the phytochemical content of *C*. *sesamoides*, with a decline in phytochemicals observed when the plants approached their reproductive stages. Crude extracts of *C*. *triloba* contained phenolics, phlobatannins, saponins, steroids, alkaloids and terpenoids, with the absence of flavonoids, tannins, cardiac glycosides and cynogenic glycosides **(Akula and Odhav, 2008; Mohanlall et al., 2011; Mudzwiri, 2007)**. Presently, few studies have investigated the phytochemical content of these plant species. Thus, more studies need to be conducted in order to critically evaluate and document secondary metabolites synthesized by *Ceratotheca* species in order to fully explore their benefits in pharmacology.

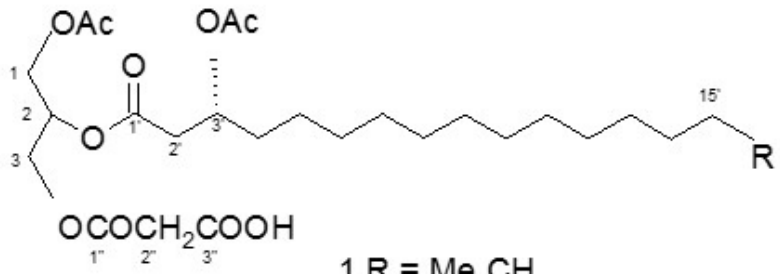
Table 2.3: Phytochemical content of *Ceratotheca sesamoides* and *Ceratotheca triloba*.

Scientific name	Plant part tested	Extracting solvent(s)	Phytochemical bioassay(s)	Finding(s)	Reference
C. sesamoides Endl.	Leaves	ND	Flavonoids	Leaf extracts showed higher flavonoid content after 8 weeks (1400 mg/100 g) of growth in comparison to 6 and 10 weeks.	**Fasola and Ogunsola (2014)**
C. sesamoides Endl.	Leaves	ND	Saponins	Leaves accumulated more saponins as growth (6 – 10 weeks) progressed.	**Fasola and Ogunsola (2014)**
C. sesamoides Endl.	Leaves	ND	Alkaloids	Alkaloid content ranged from 825 – 855 mg/100 g with the highest concentration recorded after 10 weeks.	**Fasola and Ogunsola (2014)**
C. sesamoides Endl.	Leaves	ND	Tannins	There was a decrease in tannin content (130 – 125 mg/100 g) in leaf extracts after 10 weeks.	**Fasola and Ogunsola (2014)**
C. sesamoides Endl.	Leaves	ND	Phenols	Leaf extracts have the highest phenol content (48 mg/100 g) after 8 weeks	**Fasola and Ogunsola (2014)**
C. sesamoides Endl.	Leaves	Methanol	Phenolics	Approximately 186 mg GAE 100 g/l of phenolic content was measured in leaf extracts of *C. sesamoides*.	**Konan et al. (2014)**

C. sesamoides Endl.	Leaves	Water	Alkaloids, saponins flavonoids, tannins phenolics, cardiac glycosides, steroids, cardenolides, dienolides	Extracts contain alkaloids, saponins, flavonoids and phenolics with no traces of tannins, cardiac glycosides, steroids, cardenolides and dienolides.	**Toyin et al. (2012)**
C. *triloba* (Bernh.) Hook.f.	Leaves	Methanol, Water	Phenolics	Methanol extracts had high phenolic content (35.2 mg/g) when compared to aqueous extracts (14.6 mg/g).	**Akula and Odhav (2008)**
C. *triloba* (Bernh.) Hook.f.	Roots	Water	Phlobatannins, saponins, steroids, terpenoids, flavonoids, tannins and cardiac glycosides	Major phytochemical compounds including phlobatannins, saponins, steroids and terpenoids were detected from crude extracts of *C*. *triloba* with no detection of flavonoids, tannins and cardiac glycosides.	**Mohanlall et al. (2011)**
C. *triloba* (Bernh.) Hook.f.	Leaves	80% Methanol, 10% Acetic acid in ethanol, Water, Hydrochloric acid	Saponins, alkaloids, oxalic acid and cyanogenic glycosides	Leaf extracts contained saponins (0.58 mg/ml), alkaloids (0.6 g/5g) and oxalic acid (0.1275 % w/w) with no detection of cyanogenic glycosides.	**Mudzwiri (2007)**

ND – Not defined

Plant extraction in *C*. *sesamoides* and *C*. *triloba* resulted in the isolation and identification of a number of compounds with significant importance in nutrition and pharmacology. Sesamin and sesamolin were extracted from the seeds of *C*. *sesamoides* **(Bedigian 2003)**. These compounds are usually isolated from the *Sesamum* genus and possess pharmacological properties **(Jeng and Hou 2005)**. In glandular trichomes of *C*. *triloba* extracts, **Ohkawa et al. (2012)** isolated nine malonylated glycerolipids (**Fig. 2.2**).

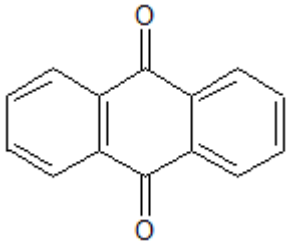


1 R = Me_2CH
2 R = $MeCH_2CH_2$
3 R = Me_2CHCH_2
4 R = MeEtCH
5 R = $MeCH_2CH_2CH_2$
6 R = $Me_2CHCH_2CH_2$
7 R = $MeCH_2CH_2CH_2CH_2$
8 R = $Me_2CHCH_2CH_2CH_2$
9 R = $MeEtCHCH_2CH_2$

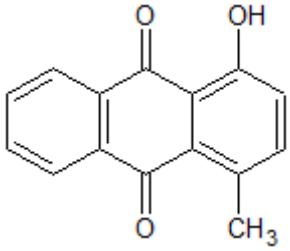
Fig. 2.2: Structures of 1-O-acetyl-2-O-(3-acetyloxy-fatty acyl)-3-O-malonylglycerols (1–9). The methyl esters of 1–9 are referred to as 1A–9A, whereas methyl esters of 3-hydroxy-fatty acids obtained by hydrolysis of 1–9 are designated as 1a –9a.

Ohkawa et al. (2012) found that malonylated glycerolipids represented approximately 90% of the glandular trichome cell content in *C*. *triloba*, with 1-*O*-acetyl-2-*O*-[(R)-3-acetyloxyicosa-noyl]-3-*O*-malonylglycerol being the most abundant constituent (41%)

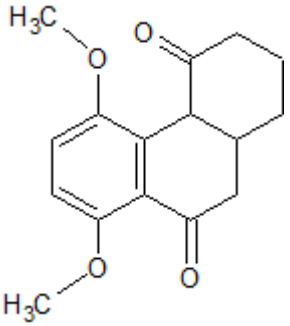
followed by 1-*O*-acetyl-2-*O*-[(R)-(3-acetyloxyoctadecanoyl)-3-*O*-malonylglycerol (21%). Glandular trichomes contained minor compounds with iso- and anteiso-type structures in the 3-acetyloxy-fatty acyl groups in the fatty acyl moiety. Three new anthraquinones and steroid androgen, androst-5-ene-3, 17, 19-triol were isolated from the root extracts of *C*. *triloba* **(Mohanlall et al. 2011)**. These included 9,10-anthracenedione, 1-hydroxy-4-methylanthraquinone, 5,8-dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione; a steroid androst-5-ene-3,17,19-triol as well as other two compounds 1,2 benzenedicarboxylic acid, mono(2-ethylhexyl)ester and octadecanoic acid (**Fig. 2.3**). Amongst the isolated compounds, the authors reported on the high antioxidant and antibacterial activities displayed by 9,10-anthracenedione while 1-hydroxy-4-methylanthraquinone had poor anti-inflammatory activity (**Table 2.4**). The compounds showed potency towards the inhibitors of the human topoisomerase II enzyme. Anthraquinones structures have common characteristics with that of mitoxanthrone used in the treatment of related ailments such as prostate cancer, acute myelogenous leukemia (AML) and breast cancer. In addition, anthraquinones (natural and synthetic) have been recognized by pharmaceutical industries to have a crucial role in antibacterial, antitrypanosomal and antineoplastic activities **(Heyman et al. 2009; Tarus et al. 2002)**.



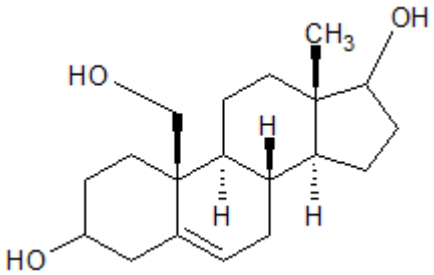
9,10 anthracenedione



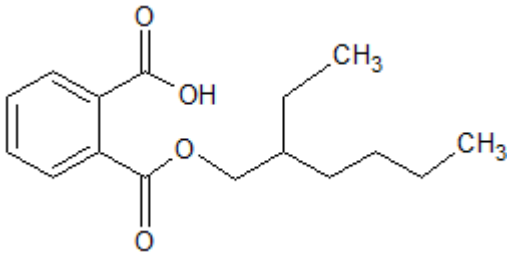
1-hydroxy-4-methylanthraquinone



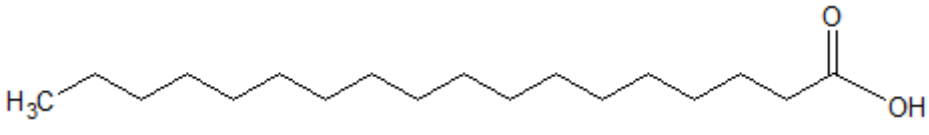
5,8-dimethoxy-2,3,10,10a-tetrahydro-1H, 4aH-phenanthrene-4,9-dione



androst-5-ene-3,17,19-triol



1,2 benzenedicarboxylic acid, mono(2-ethylhexyl)ester



octadecanoic acid

Fig. 2.3: Compounds isolated from the roots of *Ceratotheca triloba*.

2.7. Pharmacological properties of *Ceratotheca* species

The increasing resistance of microorganisms to available antibiotics is a major concern to researchers and clinicians globally. Existing drugs are becoming ineffective against viruses, bacteria, fungi and protozoa which is a problem in the fight against microbial-related ailments. In addition to the search for new drugs from medicinal plants, leafy vegetables might be a complementary option because of their health benefits which encompass both nutritional and medicinal properties. Furthermore, *in vitro* and epidemiologic studies have shown the benefits of foods rich in phenolic compounds in reducing the risk of health problems due to their antioxidant, anti-mutagenic, anti-inflammatory and antibacterial properties **(Gülçin 2012; Surh 2002)**. The antioxidant, antimicrobial, antidiarrhoeal, anti-inflammatory, antidiabetic, antiplasmodial, antiviral and antivenom activities of *C*. *sesamoides* and *C*. *triloba* are presented in **Table 2.4**. Different plant parts and extracting solvents have been assessed using *in vitro* and *in vivo* screening for *Ceratotheca* species.

2.7.1 Antioxidant activity

Antioxidant activity was moderate in methanol leaf extracts of *C*. *sesamoides* when evaluated against DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) free radicals **(Konan et al. 2014)**. The authors found that there was a high correlation coefficient (0.93) between extract concentration and free radical scavenging activity. Leaf extracts of *C*. *triloba* showed noteworthy antioxidant activity when compared to the positive control (rutin, 100% activity) **(Odhav et al. 2007)**. Similar antioxidant activity (> 80% DPPH) was reported by **Akula and Odhav (2008)**. However, the authors reported on the low DPPH radical activity in leaf aqueous extracts.

Table 2.4: Pharmacological properties of *Ceratotheca sesamoides* and *Ceratotheca triloba*.

Scientific name	Plant part(s) tested	Extracting solvent (s)	Test system and positive control (concentration)	Bioactivity	Report on the activity	Reference
C. *sesamoides* Endl.	Leaves	Methanol	*In vitro* Ascorbic acid, Gallic acid (ND)	Antioxidant DPPH activity	The extracts were effective (57%) against DPPH radical scavengers with an IC_{50} of 7.5 µg/ml.	**Konan et al. (2014)**
C. *triloba* (Bernh.) Hook.f.	Leaves	Methanol, Water	*In vitro* Rutin (1 mM)	Antioxidant DPPH activity	Methanol extracts recorded high (84.9%) activity while the water extracts showed low (36.7%) activity against DPPH radical scavengers.	**Akula and Odhav (2008)**
C. *triloba* (Bernh.) Hook.f.	Roots	Methanol	*In vitro* Quercetin-3-rutinoside (1 mM)	Antioxidant DPPH activity	Approximately 50% scavenging activity was observed from purified root extracts of *C*. *triloba*, with *CTRE 02 extract recorded to have the highest activity (IC_{50} - 1 mg/ml).	**Mohanlall and Odhav (2013)**
C. *triloba* (Bernh.) Hook.f.	Leaves	Methanol	*In vitro* Rutin (ND)	Antioxidant DPPH activity	Leaf extracts demonstrated high (84%) antioxidant activity.	**Odhav et al. (2007)**
C. *triloba* (Bernh.) Hook.f.	Leaves, Roots	Methanol, Water	*In vitro* Gentamycin (100 µg/ml) Ampicillin (100 µg/ml)	Antibacterial agar disk diffusion activity	Leaf extract exhibited inhibitory activity against *Bacillus cereus*, *Enterobacter aerogenes* and *Micrococcus luteus*. The root extract showed inhibitory activity against *B*. *cereus* and *M*. *luteus*.	**Mohanlall and Odhav (2013)**

C. *triloba* (Bernh.) Hook.f.	Leaves, Roots	Acetone, Water	*In vitro* Gentamycin (100 µg/ml) Ampicillin (100 µg/ml)	Antibacterial agar disk diffusion activity	*The six isolated compounds showed good activity against the Gram-positive bacteria; *M*. *luteus, B*. *cereus* and *Staphylococcus aureus*. Although the purified extracts exhibited moderate activity against Gram-negative bacteria; *E*. *coli* and *Salmonella typhimurium*.	**Mohanlall and Odhav (2013)**
C. *triloba* (Bernh.) Hook.f.	Leaves, Roots	Methanol, Water	*In vitro* Amphotericin B (5 µg/ml)	Antifungal activity	There was minimal antifungal activity observed against the two fungal strains (*Aspergillus flavus* and *Fusarium verticilloides*).	**Mohanlall and Odhav (2013)**
C. *sesamoides* Endl.	Plant	Saline	*In vitro* Human epidermoid carcinoma HEP-2 cell line (ND)	Antiviral activity	Extracts exhibited a dose dependent activity against measles virus (HEP-2). Virus + cell + extract showed antiviral activity at 10 and 15 mg/ml extract concentration. Although the cells treated with an extract and virus exerted a mild antiviral activity at 15 mg/ml extract concentration.	**Obi et al. (2006)**
C. *sesamoides* Endl.	Leaves	Water	*Ex vitro* Loperamide hydrochloride (1 ml)	Antidiarrhoeal activity	Extracts at 25 mg/kg body weight (194.50 min) prolonged the onset time of diarrhea in rats. Even though, there were no signs of diarrhoea in rats with 50 and 100 mg/kg body weight.	**Toyin et al. (2012)**

C. *triloba* (Bernh.) Hook.f.	Leaves	Methanol	*In vitro* Nordihydroguaiaretic acid (4.1 µg/ml) Rutin (7.3 µg/ml)	Anti-inflammatory activity	Plant extracts exhibited minimal inhibitory activity (IC_{50} - 56.1 µg/ml) towards the 5-LOX enzyme.	**Akula and Odhav (2008)**
C. *triloba* (Bernh.) Hook.f.	Leaves Roots	Methanol Water	*In vitro* Nordihydroguaretic acid (ND)	Anti-inflammatory activity	*C*. *triloba* leaves and roots showed poor anti-inflammatory activity (IC_{50} 300 µg/ml) against the 5-LOX enzyme.	**Mohanlall and Odhav (2013)**
C. *triloba* (Bernh.) Hook.f.	Leaves	Water	*In vitro* Acarbose (1 mg/ml)	Anti-diabetic activity	Extract had a dose dependent response in the inhibition of α-amylase (1 mg/ml: 43.23 %; 3 mg/ml: 99%; 5 mg/ml: 99%) enzyme.	**Odhav et al. (2010)**
C. *sesamoides* Endl.	Whole plant	Petroleum ether, Acetone, Ethanol	*In vitro* Chloroquine (400 ng/ml)	Antiplasmodial activity	Plant extracts had an IC_{50} ranging from 20 to >50 µg/ml in both fresh and stored material against *Plasmodium falciparum* (FcM29-Cameroon strain).	**Benoit-Vical et al. (2008)**
C. *sesamoides* Endl.	Whole plant	Petroleum ether, Acetone, Ethanol	*In vitro* Chloroquine (60 ng/ml)	Antiplasmodial activity	Plant extracts had an IC_{50} ranging from 4 to >50 µg/ml against *Plasmodium falciparum* FcB1-Colombia strains, with the best activity observed from stored material extracted with ethanol.	**Benoit-Vical et al. (2008)**
C. *triloba* (Bernh.) Hook.f.	Leaves	Water, Dichloromethane/Methanol (1:1)	*In vivo* N,N-diethyl-*meta*-toluamide	Antiplasmodial activity	Dichloromethane:Methanol extracts were 50% effective against *Anopheles arabiensis* in rats, while water extracts had 30% *A*. *arabiensis* inhibition.	**Maharaj et al. (2010)**

C. *triloba* (Bernh.) Hook.f.	Twigs	Water, Dichloromethane/Methanol	*In vivo* N,N-diethyl-*meta*-toluamide	Antiplasmodial activity	Dichloromethane:Methanol and water extracts had a repellent effect of 40 and 31%, respectively against *A*. *arabiensis* in rats.	**Maharaj et al. (2010)**
C. *triloba* (Bernh.)	Fruits	Water	*In vivo* N,N-diethyl-*meta*-toluamide	Antiplasmodial activity	Water extracts recorded a 26% *A*. *arabiensis* inhibition in rats.	**Maharaj et al. (2010)**
C. *sesamoides* Endl.	Herb	Water, Ethanol	*In vitro* Aristolochic acid (ND)	Hyaluronidase activity	Water extracts had higher hyaluronidase inhibition (14 %) than ethanol extracts (1%) for *Bitis arietans* venom. However, better hyaluronidase inhibition was observed from both extracts against *Naja nigricollis* venom with 50% and 27%, respectively.	**Molander et al. (2014)**
C. *sesamoides* Endl.	Herb	Water, Ethanol	*In vitro* EDTA (ND)	Phospholipase A_2 activity	Aqueous extracts had 27% phospholipase A_2 inhibition and the ethanol extracts had 10% when tested against *Bitis arietans* venom.	**Molander et al. (2014)**
C. *sesamoides* Endl.	Herb	Water, Ethanol	*In vitro* 4-(2-Aminoethyl)benzenesulfonyl fluoride, EDTA (ND)	Proteolytic activity	Both solvent extracts showed low (≤ 4) proteolytic inhibition against *B*. *arietans* venom.	**Molander et al. (2014)**

DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) bioassay, ND – not defined; * (i) 9,10 anthracenedione, (ii) 1-hydroxy-4-methylanthraquinone, (iii) 5,8-dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, androst-5-ene-3,17,19-triol, (iv) 1,2 benzenedicarboxylic acid, (v) mono (2-ethylhexyl) ester (vi) octadecanoic; CTRE 01: 9,10 anthracenedione + 1-hydroxy-4-methylanthraquinone; CTREh02 and CTREh03; HEP-2 cell line - Human epidermoid carcinoma HEP-2 cell line.

9,10-Anthracenedione and 1 hydroxy-4-methyl anthraquinone compounds isolated from *C*. *triloba* root extracts had approximately 50% antioxidant activity in the DPPH assay **(Mohanlall and Odhav 2013)**. From the documented studies, *C*. *sesamoides* and *C*. *triloba* showed moderate – good activity against the DPPH free radicals, however, more studies need to be conducted in order to determine the *β*-carotene activity of both species, as choosing at least two antioxidant bioassays (DPPH and *β*-carotene) is necessary due to their complex processes **(Moon and Shibamoto 2009)**.

2.7.2. Antimicrobial and antiviral activity

In the *in vitro* agar disk diffusion bioassay, leaves of *C*. *triloba* exhibited inhibitory activity against Gram-positive bacteria (*B*. *cereus* and *M*. *luteus*) and a Gram-negative bacterium (*E*. *aerogenes*) **(Mohanlall and Odhav 2013)**. However, root extracts were more effective against *B*. *cereus* and *M*. *luteus*. 9,10-Anthracenedione had an inhibitory effect against *E*. *coli* and *S*. *typhimurium*, while 1-hydroxy-4-methylanthraquinone inhibited *S*. *aureus* and *M*. *luteus* **(Mohanlall and Odhav 2013)**. Nevertheless, **McGaw et al. (2000)** reported on the poor antibacterial activity of *C*. *triloba* extract when tested against Gram-positive bacteria (*B*. *subtilis* and *S*. *aureus*) and Gram-negative bacteria (*E*. *coli* and *K*. *pneumoniae*). Antiviral activity of *C*. *sesamoides* plant extracts was evaluated using an *in vitro* assay **(Obi et al. 2006)**. The extracts were effective against the measles virus on human epidermoid carcinoma HEP-2 cell line when tested at high concentrations (10 and 15 mg/ml). The authors suggested on the use of *C*. *sesamoides* against the measles virus because in Nigeria it is widely utilized in the management of conjunctivitis, a viral infection. Thus far, studies on the antimicrobial and antiviral activity of *Ceratotheca* species are still inadequate. Especially since *C*. *sesamoides* is traditionally used for microbial-related ailments such as conjunctivitis and leprosy. Nonetheless, the antiviral activity of *C*.

sesamoides could be a stepping stone in the search for antiviral drugs as well as other viral related diseases that are a major concern worldwide.

2.7.3. Antidiarrhoeal activity

Castor oil induces problems such as water and electrolyte permeability, resulting in a change in the intestinal mucosal membrane that lead to fluids and watery luminal content flowing rapidly through the small and large intestines causing diarrhoea. When the antidiarrhoeal properties of *C*. *sesamoides* were evaluated *in vivo* using castor oil, aqueous leaf extracts inhibited the onset of diarrhoea at concentrations of 50 and 100 mg/kg plant extract per body weight in rats **(Toyin et al. 2012)**. At these concentrations, the typical diarrhoeal symptoms did not manifest in the experimental rats. Most importantly, the extracts showed almost similar potency (194.50 min) as that of the positive control (Loperamide hydrochloride, 233 min) when tested at the lowest concentration (25 mg/kg body weight). *Ceratotheca sesamoides* showed potential in the inhibition (55% defecation inhibition) of diarrhoea at the lowest extract concentration, similar to the positive control, with a 100% defecation inhibition observed in plant extract at 50 and 100 mg/kg body weight in rats. The authors suggested that diarrhoea may have been prevented by the activity of *C*. *sesamoides* on prostanglandin synthesis, nitric oxide and the production of platelet activating factor, since prostaglandin inhibitors and nitric oxide synthases have been attributed to the delay of diarrhoea induced by castor oil in rats (**Toyin et al. 2012**).

2.7.4. Anti-inflammatory activity

Inflammation results from excess generation of reactive oxygen species (ROS) stimulated by the production of cytokines and enzyme activation including

lipoxygenases (LOXs) from inflammatory cells. The LOX enzyme is responsible for several inflammation-related diseases **(Dobrian et al. 2011)**. Lipoxygenases are involved in the biosynthesis of leukotrienes and prostagladins which if inhibited, results in the prevention of several diseases triggered by oxidative stress **(Rådmark and Samuelsson 2007)**. Regulation of ROS production is an important step in the down-regulation of the immune response thus preventing chronic inflammation **(Seifried et al. 2007)**.

Table 2.4 summarises the *in vitro* analysis of 5-LOX enzyme activity in *C*. *triloba* leaf and root extracts. Methanol leaf extracts exhibited minimal inhibitory effects against the 5-LOX enzyme with an IC_{50} of 56.1 µg/ml **(Akula and Odhav 2008)**. Similarly, **Mohanlall and Odhav (2013)** reported on the poor anti-inflammatory activity of leaves and root extracts (IC_{50} of 300 µg/ml) when tested against the 5-LOX enzyme. The poor activity of *C*. *triloba* is somehow contradictory to its traditional use in pain-related ailments such as stomach cramps and painful menstruation. Such insignificant/poor anti-inflammatory activity of plant extracts against the 5-LOX enzyme might be due to several factors including the tested enzyme. According to **Jäger et al. (1996)**, active compounds of the extracts might be more effective when tested at other sites in the complex process of inflammation. Therefore, more studies examining the activity of *C*. *triloba* against LOX enzymes and other enzymes such as cyclooxygenase-1 and 2 are required.

2.7.5. Antidiabetic activity

Traditionally, there is no record on the use of *C*. *triloba* in the treatment of diabetes. Thus, the inhibitory activity of leaf extracts against the α-amylase enzyme at the lowest (1 mg/ml) and highest (5 mg/ml) concentrations recorded to be 43.3% and 99.7%,

respectively is noteworthy **(Odhav et al. 2010)**. The inhibitory activity of *C*. *triloba* extracts at 3 and 5 mg/ml was equivalent to that of the positive control with a 99.2% inhibitory activity (acarbose at 1 mg/ml). Therefore, findings from **Odhav et al. (2010)** might contribute to the search for plants with antidiabetic properties. Furthermore, these findings are of interest given that the plant species will not only provide nutritional benefits but also play a vital role in inhibiting the α-amylase enzyme, thereby reducing blood glucose levels in diabetic people.

2.7.6. Antiplasmodial activity

The *in vitro* and *in vivo* antiplasmodial properties of *C*. *sesamoides* and *C*. *triloba* are presented in **Table 2.4**. Plant extracts of *C*. *sesamoides* were more effective after storage then fresh material against the *P. falciparum* (FcB1-Colombia) strain **(Benoit-Vical et al. 2008)**. Amongst the solvents used, ethanol extracts gave good activity (IC_{50} - 4 µg/ml) compared to petroleum ether (IC_{50} - 15 µg/ml) and acetone (IC_{50} - 33 µg/ml). Generally, plant extracts (fresh or stored) were more effective against the FcB1-Colombia strain in comparison to the FcM29-Cameroon strain, with dichloromethane:methanol leaf extracts effective (50%) against *A*. *arabiensis* in rats after 2 min during *in vivo* studies **(Maharaj et al. 2010)**. Even though the antiplasmodial inhibitory activity of other plant parts such as twigs and leaves ranged from 26 – 40% when extracted with either dichloromethane:methanol or water. These results revealed the difference in the plant extracts ability to inhibit malarial strains, thus more strains need to be investigated. These results also justify the traditional use of *C*. *sesamoides* in the treatment of malaria.

2.7.7. Hyaluronidase, phospholipase and proteolytic activity

Given that *C*. *sesamoides* is traditionally used against snakebites, it was evaluated for plant compounds effective against the necrosis-inducing enzymes of snake venom **(Molander et al. 2014)**. Plant extracts (5 mg/ml) showed ≤ 50% activity against the tested enzymes of *Bitis arietans* (5 mg/ml) and *Naja nigricollis* (5 mg/ml). In most cases, water extracts were more potent in inhibiting hyaluronidase, phospholipase and proteolytic enzymes from *B*. *arietans* and *N*. *nigricollis* venom compared to ethanol extracts. From the *N*. *nigricollis* venom, water extracts exhibited high (50%) hyaluronidase enzyme inhibition in comparison to the ethanol extracts. The ability of *C*. *sesamoides* to inhibit some enzymes found in snake venom might be due to polyphenols such as tannins (**Molander et al. 2014**). Aqueous extracts inhibitory activity (50%) towards hyaluronidase enzymes from *N*. *nigricollis* venom is remarkable. However, in order to fully elucidate the effect of *C*. *sesamoides* against necrosis-inducing enzymes of snake venom, more studies need to be conducted on the different plant parts, extraction solvents and other snake venom enzymes.

2.7.8. Other documented pharmacological properties

Table 2.5 represents additional biological activities of *C*. *sesamoides*. *In vivo* insecticidal activity was investigated with *C*. *sesamoides* seeds **(Uddin II and Abdulazeez 2013)**. Seed extracts (powdered and aqueous treatments) exhibited high mortality rates (3.00) against *Callosobruchus maculatus* at the lowest tested concentration (1.5%) after 72 h in infested cowpea (*Vigna unguiculata*) seeds. In comparison to the negative control (water), seed extracts prevented more oviposition of *C*. *maculatus* even at the lowest extract concentration. *Ceratotheca sesamoides* is reported to be pest tolerant **(Grubben and Denton 2004)** and has been found to be visited by 149 insects with minimal damage observed on the plants **(Uddin II and Adesiyun 2011)**. The potential organic “pesticide” of the plant against cowpea seeds

needs to be further evaluated. Especially some of the bioactive compounds synthesized by the plant which account for its pest tolerance. Such findings may give an understanding on the insecticidal activity of *C*. *sesamoides* since more studies are now focused on ‘green farming’ with organic fertilizers becoming more popular **(Aguilera et al. 2013)**. Furthermore, studies evaluating volatile compounds in *C*. *sesamoides* are pertinent because the plant has an acrid smell which might be the reason for its insecticidal activity.

Table 2.5: Other documented pharmacological activities of *Ceratotheca sesamoides* and *Ceratotheca triloba*.

Species	Plant part(s) tested	Extracting solvent (s)	Test system and positive control (concentration)	Bioactivity	Report on the activity	References
C. sesamoides Endl.	Seeds	Water	*In vivo* Pirimiphos-methyl (0.5% w/w)	Insecticidal activity	In extracts, *Callosobruchus maculatus* mortality was highest at 1.5% concentration after 72 h of cowpea infestation. Aqueous extracts prevented oviposition and F_1 progeny emergence of *C. maculatus* at a lower concentration compared to powder extracts.	**Uddin II and Abdulazeez (2013)**
C. sesamoides Endl.	Whole plant	Petroleum ether, Acetone, Ethanol	*In vitro* Etoposide (2×10−5 M)	Cytotoxicity activity	Plant extracts showed slight toxicity at high concentrations.	**Benoit-Vical et al. (2008)**
C. triloba (Bernh.) Hook.f.	Leaves	Water, 80% Methanol	*In vitro* ND	Cytotoxicity activity	Methanol extracts had minimal cytotoxicity, the aqueous extracts exhibiting no cytotoxic effect.	**Mudzwiri (2007)**
C. triloba (Bernh.) Hook.f.	Leaves, Roots	Methanol, Water	*In vitro* ND	Cytotoxicity activity	Purified extracts of *C. triloba* had 41.4 – 73.2% $HepG_2$ cell death at 12.5 µg/25 µl, low concentrations (1.56 µg/ 25 µl) exhibited 1 – 14.4% $HepG_2$ cell death.	**Mohanlall (2010)**

ND – Not defined

In the current review, the phytochemistry section shows few studies devoted to the isolation and identification of different compounds (**Fig. 2.2** and **2.3**) in *Ceratotheca* plant species **(Bedigian 2003; Mohanlall and Odhav 2013; Ohkawa et al. 2012)**. From the available studies, the plant species yielded a wide range of compounds that can be useful in nutritional and pharmacological sectors. These compounds are of value, especially the anthraquinones isolated from *C*. *triloba* which are recognized for their similarities with mitoxanthrone compounds used for cancer-related diseases. Nevertheless, more studies directed towards the isolation and identification of novel compounds are needed.

Ceratotheca species showed potential antimicrobial, antiviral, antidiarrhoeal, anti-diabetic and antiplasmodial properties. Although *C*. *triloba* has been documented in the treatment of a wide range of microbial ailments in traditional medicine, their antimicrobial properties for crude plant extracts were very poor **(McGaw et al. 2000)** compared to isolated compounds **(Mohanlall and Odhav 2013)**. Diarrhoea is caused by a wide range of entero-pathogenic microorganisms such as *Shigella flexneri, Staphylococcus aureus, Escherichia coli, Salmonella typhi and Candida albicans*. The antidiarrhoeal activity demonstrated by *C*. *sesamoides* was noteworthy **(Toyin et al. 2012)**. These findings serve to confirm the claims made in traditional medicine about the plants usefulness against diarrhoea. Furthermore, *C*. *sesamoides* plant extracts were reported to inhibit the measles virus on the human epidermoid carcinoma HEP-2 cell line **(Obi et al. 2006)**. The ability of *Ceratotheca* species to inhibit microbial-related and viral infections is of interest due to the increasing resistance of microorganisms to available antibiotics worldwide. Even though *C*. *triloba* showed minimal inhibitory effects against the 5-LOX enzyme, the plant extracts were effective against the α-amylase enzyme. However, further isolation and identification of the

active hypoglycemic and hypolipidemic principles in the plants are needed in order to understand their mechanisms of action. *Ceratotheca sesamoides* extracts were found to have antiplasmodial and insecticidal activities. The high potency of dried material against *Plasmodium* strains is of importance because most plants in traditional medicine are dried, ground and stored before usage.

Besides the inadequate literature on the biological activities of *Ceratotheca* species, few studies have focused on investigating the effect of plant parts (crude or isolated compounds), solvent extracts and extract dose/concentration. From the available studies, leaves were the most commonly used plant part followed by the roots, seeds and the whole plant from both species. The potency of leaf extracts in the evaluated biological activities is important for conservational purposes of *Ceratotheca* species. Generally, water extracts are known to exhibit lower activity when compared to non-polar solvent extracts in various pharmacological studies. Nevertheless, aqueous extracts of *Ceratotheca* species showed good antidiarrhoeal, anti-diabetic, hyaluronidase enzyme and insecticidal activities. Furthermore, the species showed diverse bioassay activities including *in vitro* and *in vivo*. From these studies, it is evident that there is still a dire need for more research especially for both species because they might have similar potency against various diseases. Once the pharmacological potential of plants has been established in *in vitro* and *in vivo* bioassays, isolation and investigation of the compounds will be necessary.

2.8. Safety and toxicity of *Ceratotheca* species

Even though *Ceratotheca* species play a major role as food supplement in most diets (**Table 2.2**), the species are also widely used for their medicinal properties in some African countries (**Table 2.4**). Therefore, it is crucial to determine their safety and

toxicity especially because the plants are consumed in large quantities (**Table 2.5**). In a test to determine the toxicity of *C*. *sesamoides*, the extracts showed minimal toxicity against the vero cell line with acetone extracts having no cytotoxic effect even at the highest concentration tested **(Benoit-Vical et al. 2008)**. Purified extracts of *C*. *triloba* exhibited cytotoxic activity (41.4 – 73.2%) against $HepG_2$ cells at high concentrations **(Mohanlall 2010; Mohanlall and Odhav 2013)**. Nevertheless, low purified extract concentration (1.56 µg/ 25 µl) showed slight cytotoxicity against $HepG_2$ cells. At 1000 µg/ml, organic extracts of *C*. *triloba* demonstrated low levels of cytotoxicity with ≤ 5% cell death in $HepG_2$ cells while aqueous extracts were not at all cytotoxic **(Mudzwiri 2007)**. Toxicity analysis of *C*. *triloba* showed that plant extracts did not contain cyanogenic glycosides **(Mudzwiri 2007)**. However, aqueous extracts were considered toxic when consumed at levels above 100 and 1000 µg/ml **(Mudzwiri 2007)**. *Ceratotheca triloba* extracts showed no mutagenic activity when evaluated in the Ames test assay **(Mohanlall and Odhav 2013; Mudzwiri 2007)**. Both species (crude and purified extracts) had inhibitory activity against the topoisomerase II enzyme. Overall, *C*. *sesamoides* and *C*. *triloba* were reported to be potentially toxic when tested at higher concentrations. Thus, more stringent studies need to be conducted in order to understand the toxicity levels of these leafy vegetables since they are consumed as starch supplements at large quantities.

2.9. Propagation strategies for *Ceratotheca* species

Leafy vegetables are known to form a substantial part of diets and are often considered the cheapest and most readily available source of food. Nevertheless, their conservation has become a concern in international agricultural research sectors. Thus, strategies combining the standard *ex situ* and *in situ* conservation for indigenous vegetables need to be implemented in order to promote and improve their utilization,

particularly, since these vegetables are known to be resistant, adaptable and tolerant to different harsh climatic conditions compared to exotic species **(Raghuvanshi and Singh 2001)**.

To date, few studies have documented propagation strategies for *C*. *sesamoides* and *C*. *triloba*. However, *C*. *sesamoides* and *C*. *triloba* are easily cultivated from seeds. The seeds are sown at the onset of the rainy season during early summer. Seedlings grow best in rich well-drained sandy soils with full sun exposure or semi-shade **(Duncan 2011; Grubben and Denton 2004)**. Seedlings of *C*. *triloba* are often slow to grow initially but the use of Seagro or Humac fertilizers improves their growth. *Ceratotheca sesamoides* seeds do not show dormancy and can be intercropped with okra (*Abelmoschus esculentus*), eggplant (*Solanum melongena*), cowpea (*V*. *unguiculata*), amaranth (*Amaranthus*), sorghum (*Sorghum bicolor*), sweet potato (*Ipomoea batatas*) and sesame (*S*. *indicum*) **(Grubben and Denton 2004)**. The species are considered to be drought, disease and pest tolerant. Frequent pruning of shoots permits sustained vegetative growth and flowering, prolonging the growth cycle. In an effort to improve the mutagenesis of *C*. *sesamoides*, **Nura et al. (2014)** found that 0.1 mM colchicine was effective in the genetic improvement of different growth parameters. Improved growth parameters were as a result of the plant's ability to respond well to the mutagens (colchicine), inducing favourable mutants. Similarly, 0.1 mM colchicine concentration increased the overall yields of cultivated *C*. *sesamoides* plants **(Nura et al. 2012)**. **Fasakin and Olofintoye (2005)** established that for different cultivars, row spacing as well as seeding rate had a positive effect on plant population density in *C*. *sesamoides* while the overall yields were improved by different cultivar and seeding rates.

2.10. Conclusions

To date, studies have evaluated the nutritional and medicinal role of the under-utilized leafy vegetables, *C*. *sesamoides* and *C*. *triloba*. The current review has revealed the nutritional potential (relatively high energy levels, carbohydrates, protein, fat content and inorganic constituents) of both species. Furthermore, these results shows that utilization of these leafy vegetables could play a crucial role in dietary diversification, improve nutrition and human health. However, more work is still needed to further validate the vegetable's nutritional role if they are to supplement the starch-based diets of African communities. Research also needs to highlight the potential variations in the chemical compositions of *Ceratotheca* leafy vegetables especially the change in nutritional content caused by environmental/growth conditions, harvest stages, their preparation and preservation.

Ceratotheca sesamoides and *C*. *triloba* showed potentially good antibacterial, antidiarrhoeal, antidiabetic, antiplasmodial and antiviral properties against various tested microorganisms. These properties might be due to the different secondary metabolites (flavonoids, alkaloids, saponins, tannins, phenolics and steroids) synthesized by the species. Most importantly, *C*. *sesamoides* and *C*. *triloba* were reported to yield various compounds including sesamin, sesamolin, anthraquinones similar to mitoxanthrone (for cancer treatment) and malonylated glycerolipids. From the available information on the pharmacological role of *Ceratotheca* species, there is still a dire need for studies to evaluate the plant's biological activities in *in vitro* and *in vivo* bioassays, different plant parts, solvent extracts and extract dose/concentrations. Furthermore, more work is still required in order to elucidate the plants phytochemistry, their relation to the plant's traditional use and their significance in human health especially with the compounds they produce. In order to introduce the species as a

conventional vegetable crop as well as medicinal plants, renewed efforts aimed at cultivation practices are pertinent because information regarding propagation strategies of species remains insufficient. Safety and toxicity analysis of *C*. *sesamoides* and *C*. *triloba* remains a major concern and needs to be extensively examined given that the plants are consumed in high quantities.

Chapter 3: Influence of biostimulants on *Ceratotheca triloba* germination under different abiotic stress conditions

3.1. Introduction

Seed germination is a process that commences with the absorption of water and is completed with the protrusion of the embryonic axis **(Bewley 1997)**. Germination is considered complete when the radicle penetrates structures surrounding the embryo (emergence of the radicle), defined as visible germination. For germination to be successful, seeds have to transition through an "environmental sieve" (environmental impediments) in order to reach a "safe site" (ideal environmental conditions), triggered by appropriate stimuli and resources **(Kaldy et al. 2015)**. Seed dormancy is then an adaptation which results from unfavourable environmental conditions that seeds go through before germination can take place **(Bewley 1997; Finch-Savage and Leubner-Metzger 2006)**. Generally, environmental factors such as extreme temperatures, drought and salinity stress induce physiological "damage" that affects seed germination and seedling growth.

Temperature is one of the most crucial climatic factors influencing seed germination. Changes in temperature significantly affect seed germination through the inhibition of radicle emergence and post-germination growth in seedlings **(Probert 2000)**. Successful seed germination and seedling establishment is dependent on surrounding temperatures with each species having a particular set of requirements. Outside these, seed germination declines gradually. Furthermore, drought and salinity stress severely affects seed germination by preventing water uptake and through the toxic effect of sodium (Na^+) and chloride ions (Cl^-). These factors result in inhibited or delayed seed germination and seedling growth **(Ashraf and Foolad 2005)**. In order to improve seed germination under extreme temperatures, drought and salinity stress conditions, seed-

priming has proven to be an indispensable technique in the production of stress tolerant plants **(Jisha et al. 2012; Paparella et al. 2015)**.

Priming is a process by which seeds are hydrated in different solutions for certain metabolic processes (e.g. protein synthesis using mRNA and DNA as well as repairing or synthesizing new mitochondria), which permits preliminary germination but not the final stage **(Jisha et al. 2012; Paparella et al. 2015)**. The technique also improves seedling shoot and rooting frequency, vigour index and ultimately crop yields. There are several priming approaches currently applied in seeds of various species including hydropriming, osmopriming, chemical priming, hormonal priming, biological priming, redox priming and solid matrix priming. Nonetheless, seed germination after priming is mostly dependent on the priming agent, severity of stress and crop species **(Jisha et al. 2012)**. The use of biostimulants in order to counteract the effect of abiotic stress is well-established **(Bulgari et al. 2014; du Jardin 2015; Sharma et al. 2014)**. In addition, the promotory effects of biostimulants such as smoke-water (SW), synthesized smoke-derived compound karrikinolide (KAR_1) **(Kulkarni et al. 2011; Light et al. 2009)**; Kelpak® (commercial seaweed extract) **(Stirk and Van Staden 2006)**; phloroglucinol (PG) and eckol isolated from Kelpak® **(Aremu et al. 2015a; Rengasamy et al. 2015a)** have been documented. Therefore, the current study aimed to determine the effect of biostimulants on *Ceratotheca triloba* seed germination and seedling growth at different temperature regimes. Furthermore, the role of biostimulant-seed-priming during seed germination and early seedling growth in *C. triloba* under low temperature, low osmotic potential and varying concentrations of sodium chloride (NaCl) was evaluated.

3.2. Materials and methods

3.2.1. Biostimulants and chemicals

Smoke-water and KAR_1 were prepared according to previously described methods **(Baxter et al. 1994; Flematti et al. 2004; Van Staden et al. 2004)**. Kelpak® [Kelp Products (Pty) Ltd, Simon's Town, South Africa] solution was prepared as indicated on the product label (0.4%). Eckol was isolated from *Ecklonia maxima* as described by **Kannan et al. (2013)**. Phloroglucinol (PG), polyethylene glycol 6000 (PEG) solution (Merck, Darmstadt, Germany) and NaCl (ACE Chemicals, Johannesburg, South Africa) were of analytical grade.

3.2.2. Seed germination using biostimulants

Ceratotheca triloba seeds were purchased from Silverhill Seeds Nursery, Cape Town, South Africa. During seed germination, 25 seeds were placed in 90 mm Petri dishes (5 seeds per Petri dish) lined with two layers of Whatman No. 1 filter paper. Seeds were treated with different biostimulants at varying concentrations [(SW 1:1500; 1:1000; 1:500; v/v), KAR_1 (10^{-6}; 10^{-7}; 10^{-8} M), Kelpak® solution (0.4%), eckol (10^{-6}; 10^{-7}; 10^{-8} M), PG (10^{-6}; 10^{-7}; 10^{-8} M)]. Distilled water was used as a control. Seeds were incubated at 25 °C in a 16/8 h light and dark regime with a photosynthetic photon flux (PPF) of 45 µmol m^{-2} s^{-1} for 20 days. Seed germination was recorded daily for the duration of the experiment. Seed germination was considered successful when the radicle had protruded 2 mm.

Based on the preliminary results above, seeds were germinated using the most effective biostimulant concentration [SW 1:500 v/v, KAR_1 10^{-6} M, Kelpak® 0.4 %, PG 10^{-6} M, eckol 10^{-6} M] and distilled water (control). During seed germination and seedling growth experiments, biostimulant treatments were separated as either smoke- (SW, KAR_1) or seaweed- (Kelpak®, PG, eckol) derived with their respective

control treatment (water). To determine the effect of temperature, 25 seeds were germinated in 90 mm Petri dishes (5 seeds per Petri dish) lined with two layers of Whatman No. 1 filter paper. Seeds were incubated at 10; 15; 25 and 35 °C in a 16/8 h light and dark regime with a PPF of 45 µmol m^{-2} s^{-1} for 25 days and germination was recorded daily.

In order to determine the effect of drought and salinity stress, different osmotic solutions were used to create low water potential during seed germination (e.g. PEG and NaCl) due to the chemicals non-destructive nature towards the seeds. Twenty-five seeds were germinated with different concentrations of (0; -0.05; -0.15; -0.30; -0.49 MPa) PEG 6000 for low osmotic potential as developed by **Michel and Kaufmann (1973)** and NaCl (0; 5; 15; 25; 50 mM) for NaCl stress in 90 mm Petri dishes (5 seeds per Petri dish) lined with two layers of Whatman No.1 filter paper. Polyethylene glycol 6000 and NaCl treatment solutions were prepared with respective biostimulants (SW, KAR_1, Kelpak®, eckol, PG). Control treatments contained different concentrations of PEG or NaCl concentrations prepared with distilled water. Solutions of PEG and NaCl were replaced on a 3 day interval for the duration of the experiment. Seeds were incubated at 25 ºC in a 16/8 h light and dark regime; PPF of 45 µmol m^{-2} s^{-1} for 25 days with germination recorded daily.

3.2.3. Seed imbibition, priming and germination using biostimulants

For imbibition tests, 90 seeds were placed in 90 mm Petri dishes (30 seeds per Petri dish) with two layers of filter paper (Whatman No.1) moistened with 3 ml distilled water and allowed to imbibe for 6, 12, 24, 48 and 72 h at 25 °C. Thereafter, percentage seed imbibition was calculated as: Imbibition (%) = (weight of seeds after imbibition - initial weight of seeds)/initial weight of seeds **(Govender et al. 2008)**.

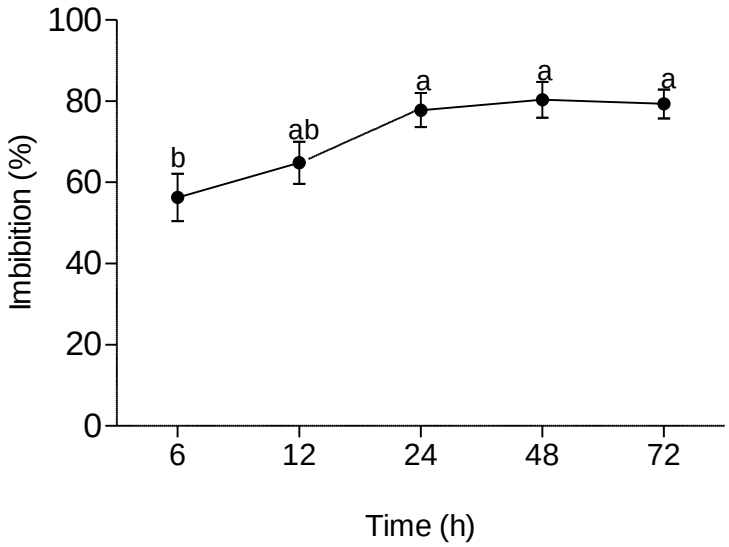


Fig. 3.1: Imbibition curve for *Ceratotheca triloba* seeds incubated at 25 ºC. The letter(s) (± SE; *n* = 90) are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT).

Due to the lack of seed germination under relatively low temperatures, low water potential and NaCl conditions, seeds were primed with different biostimulants (SW 1:500 v/v, KAR_1 10^{-6} M, Kelpak® 0.4%, PG 10^{-6} M, eckol 10^{-6} M) as well as distilled water (control) for 48 h at 25 °C based on an imbibition curve in **Fig. 3.1**. To determine the effect of temperature, 25 biostimulant-primed seeds were germinated in 90 mm Petri dishes (5 seeds per Petri dishes) lined with two layers Whatman No. 1 filter paper and incubated at 10, 10/15 and 15 °C. During a temperature-shift experiment, seeds were initially incubated at 10 °C for 15 days followed by another 10 days at 15 ºC which is presented as 10/15 ºC above. Germination in biostimulant-primed seeds was conducted as described in **Section 3.2.2** for different PEG and NaCl concentrations. Seeds germination was recorded daily and all germination experiments were repeated twice. After 25 days of seed germination and seedling growth, survival rate, shoot

length and root length were measured. Seedling vigour index was calculated as VI = seedling length (mm) × percentage germination **(Dhindwal et al. 1991)**.

3.3. Data analysis

Data were subjected to analysis of variance (ANOVA) using SPSS for Windows (SPSS®, Version 23.0. Armonk, New York, USA). For statistical significance ($P \leq 0.05$), the mean values were further separated using Duncan's Multiple Range Test (DMRT).

3.4. Results

3.4.1. Effect of biostimulants on seed germination

Based on preliminary studies, germination of *C*. *triloba* seeds was best stimulated with the application of biostimulants, particularly at the highest tested concentration (SW 1:500; KAR_1 10^{-6} M, eckol 10^{-6} M and PG 10^{-6} M) (**data not shown**). *Ceratotheca triloba* seed germination was significantly influenced by different temperature regimes (**Figs. 3.2** and **3.3**). For instance, low temperatures (10 and 15 ºC) completely inhibited germination, while 25 ºC had the highest seed germination percentage, with 35 ºC slightly decreasing percentage germination. Relative to the control treatment, SW and KAR_1 significantly improved seed germination at 25 °C, whereas KAR_1 greatly stimulated germination at 35 °C (**Fig. 3.2**). Biostimulants (Kelpak®, PG, eckol) increased seed germination at 25 °C compared to the control treatment (**Fig. 3.3**). However, percentage germination was enhanced in seeds treated with Kelpak® at 35 °C.

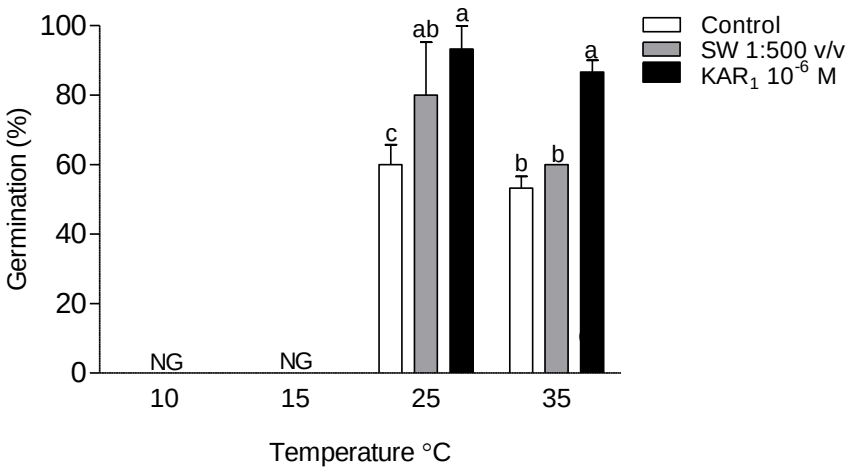


Fig. 3.2: Effect of distilled water (control), smoke-water (SW) and karrikinolide (KAR_1) on seed germination of *Ceratotheca triloba* incubated at different temperatures for 25 days. For each temperature, bars (± SE; *n* = 25) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range (DMRT). No germination (NG).

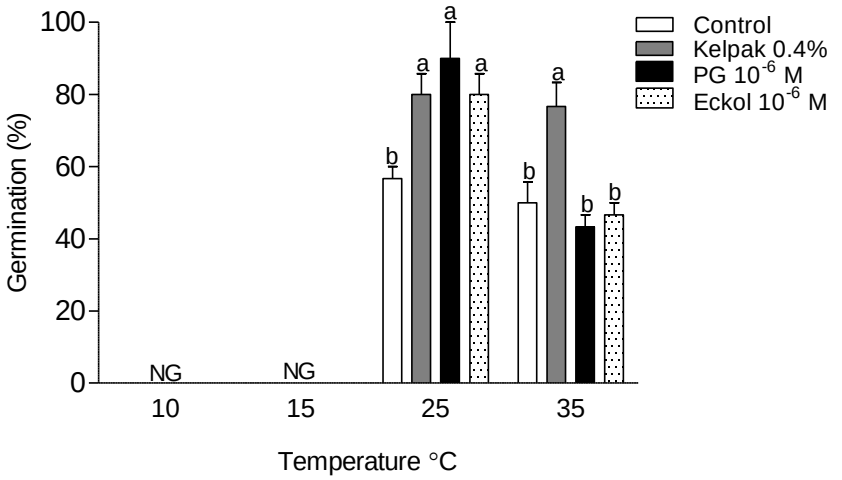


Fig. 3.3: Effect of distilled water (control), Kelpak®, phloroglucinol (PG) and eckol on seed germination of *Ceratotheca triloba* incubated at different temperatures for 25 days. For each temperature, bars (± SE; *n* = 25) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range (DMRT). No germination (NG).

3.4.2. Effect of biostimulant-priming on seed germination and seedling growth under low temperature conditions

In order to stimulate germination under low temperature, *C*. *triloba* seeds were primed with biostimulants as well as distilled water (control) (**Figs. 3.4** and **3.5**). Regardless of priming with different agents, there was still no germination observed at 10 °C. Nevertheless, temperature shifts from 10 to 15 °C (10/15 °C) considerably improved germination. Generally, germination was higher in seeds incubated at 15 °C than at 10/15 °C, with some exceptions. In **Fig. 3.4**, SW and KAR_1-primed seeds had significantly higher germination percentages than hydroprimed seeds. Likewise, Kelpak® and eckol treatments significantly improved seed germination at 10/15 °C than in the control treatment (**Fig. 3.5**). Eckol-primed seed had less than 20% germination at 15 °C while Kelpak® and hydroprimed seeds had significantly higher germination percentages.

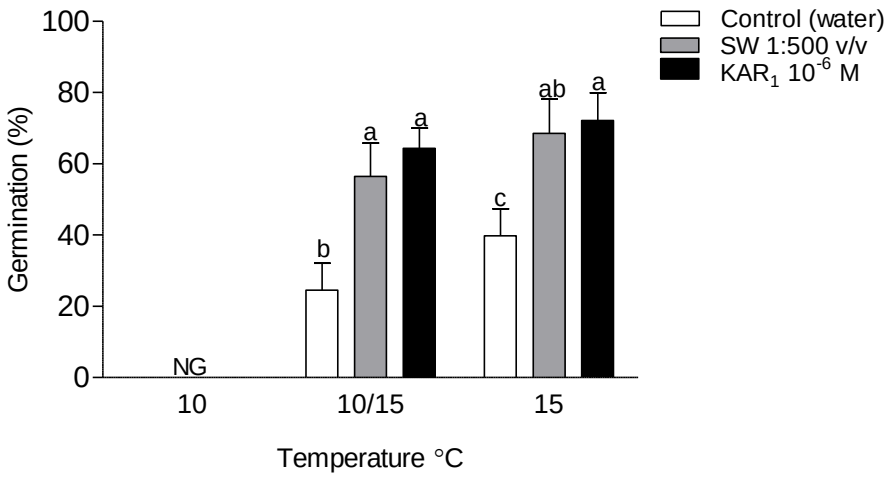


Fig. 3.4: Germination (%) of *Ceratotheca triloba* seeds primed with distilled water (control), smoke-water (SW) and karrikinolide (KAR_1) under low temperatures for 25 days. For each temperature, bars (± SE; n = 25) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). No germination (NG).

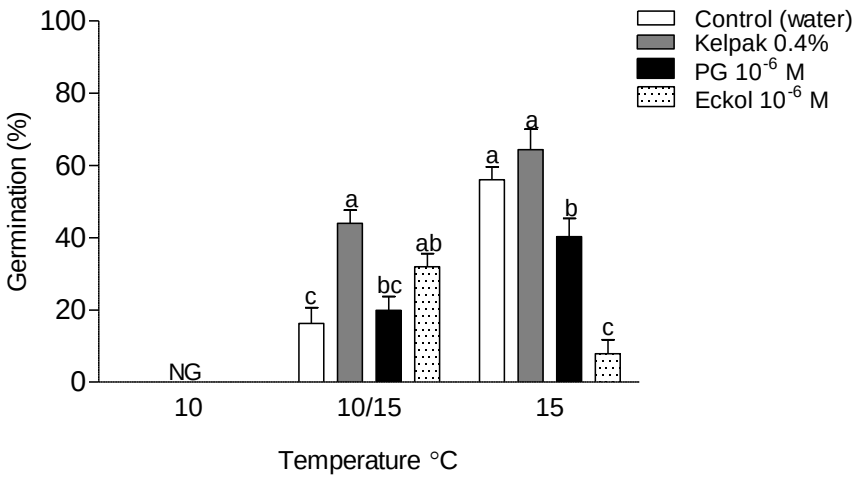


Fig. 3.5: Germination (%) of *Ceratotheca triloba* seeds primed with distilled water (control), Kelpak®, phloroglucinol (PG) and eckol under low temperatures for 25 days. For each temperature, bars (± SE; *n* = 25) with different letter(s) are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT). No germination (NG).

As shown in **Table 3.1**, SW improved shoot and root length as well as vigour index in *C*. *triloba* seedlings. Even though Kelpak® stimulated seed germination, continuous application of the biostimulant was detrimental on seedling growth and survival rate (**Table 3.2**). Relative to the control, PG and eckol treatments significantly improved shoot and root length.

Table 3.1: Effect of distilled water (control), smoke-water (SW) and karrikinolide (KAR_1) on seedling growth of *Ceratotheca triloba* seeds at 15 °C after 25 days.

Treatment	Survival rate	Shoot length		Root length		Vigour Index	
	(%)	(mm)		(mm)			
Control (water)	16	5.4 ± 2.3	b	8.5 ± 4.3	b	215 ± 91.4	c
SW 1:500 v/v	20	12.0 ± 0.7	a	28.8 ± 2.7	a	822 ± 50.0	a
KAR_1 10^{-6} M	52	7.9 ± 0.5	ab	20.8 ± 2.5	a	571 ± 34.8	b

Mean values ± standard error (*n* = 10) in each column with different letter(s) are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT).

Table 3.2: Effect of distilled water (control), Kelpak®, phloroglucinol (PG) and eckol on seedling growth of *Ceratotheca triloba* seeds at 15 °C after 25 days.

Treatment	Survival rate	Shoot length		Root length		Vigour Index	
	(%)	(mm)		(mm)			
Control (water)	20	6.8 ± 2.3	c	13.2 ± 4.5	b	381 ± 128.5	b
Kelpak 0.4%	4	1.5 ± 1.5	d	2.1 ± 2.1	c	97 ± 96.5	c
PG 10^{-6} M	60	17.7 ± 1.6	a	29.3 ± 3.1	a	713 ± 65.5	a
Eckol 10^{-6} M	40	12.6 ± 1.4	b	29.3 ± 3.7	a	99 ± 10.8	c

Mean values ± standard error (n = 10) in each column with a different letter is significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.4.3. Effect of biostimulant-priming on seed germination and seedling growth under low osmotic potential

Smoke-water and KAR_1-primed seeds significantly increased germination percentage at 0 MPa PEG concentration (**Fig. 3.6**). Likewise, the biostimulants stimulated seed germination at -0.05 and -0.15 MPa low osmotic potential, even though -0.15 MPa concentration was higly detrimental to the seeds. Kelpak® and PG-primed seeds had significantly higher germination percentage at low osmotic potential (-0.05 and -0.15 MPa) than hydroprimed seeds (**Fig. 3.7**). As shown in **Figs. 3.6** and **3.7**, hydroprimed seeds were completely inhibited by low osmotic potential (-0.15 MPa). The sensitivity of *C*. *triloba* seeds towards PEG 6000 solutions was evident especially at -0.30 and -0.49 MPa concentrations, with complete inhibition even after seeds were primed with different biostimulants (**Data not shown**).

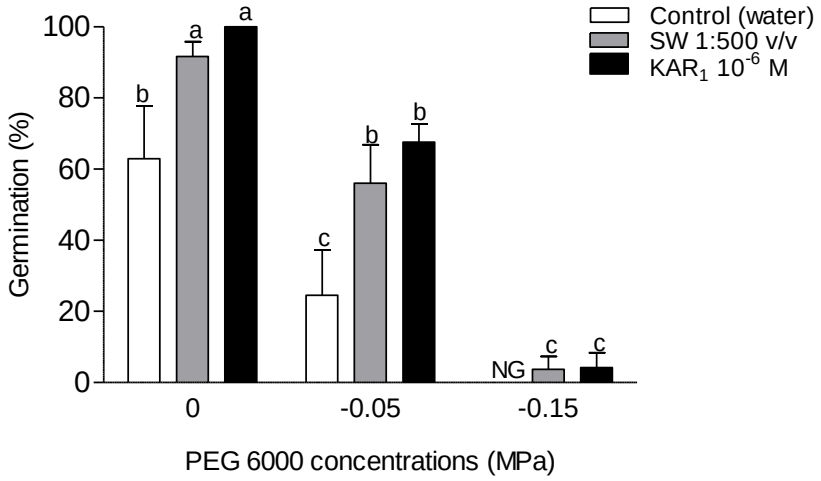


Fig. 3.6: Germination (%) of *Ceratotheca triloba* seeds primed with distilled water (control), smoke-water (SW) and karrikinolide (KAR_1) under low osmotic potential for 25 days. Bars (± SE; *n* = 25) with different letter (s) are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT). No germination (NG), polyethylene glycol 6000 (PEG).

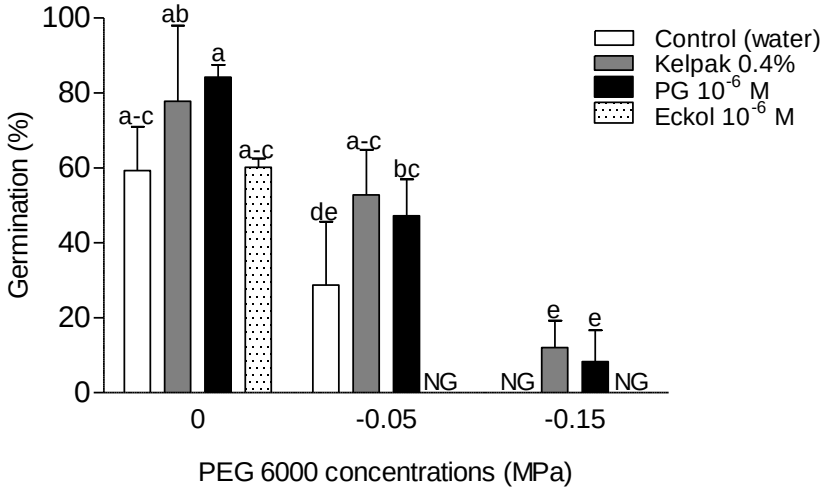


Fig. 3.7: Germination (%) of *Ceratotheca triloba* seeds primed with distilled water (control), Kelpak®, phloroglucinol (PG) and eckol under low osmotic potential for 25 days. Bars (±SE; *n* = 25) with different letter(s) are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT). No germination (NG), polyethylene glycol 6000 (PEG).

Despite the fact that seeds germinated at -0.15 MPa PEG concentration (**Figs. 3.6** and **3.7**), no seedling growth was observed at this concentration (**Tables 3.3** and **3.4**). Under normal conditions (0 MPa), the control treatment had significantly longer shoots than SW and KAR_1-treated seedlings (**Table 3.3**). However, there was a drastic decline in shoot length in the control treatment after exposure to PEG (-0.05 MPa) solution, with SW-treated seedlings producing the longest shoots at -0.05 MPa concentration when compared to the control and KAR_1 treatment. Smoke-water and KAR_1 treatments significantly improved root length in seedlings with PEG. Irrespective of the fact that eckol-treated seedlings had longer roots (37 mm) at 0 MPa PEG concentration, PG treatment maintained greater shoot and root length as well as a higher vigour index at -0.05 MPa osmotic potential (**Table 3.4**).

Table 3.3: Effect of water (control), smoke-water (SW) and karrikinolide (KAR_1) on seedling growth of *Ceratotheca triloba* seeds after incubating at different osmotic potentials for 25 days.

Treatment	Osmotic potential	Survival rate	Shoot length		Root length		Vigour Index	
Smoke	(MPa)	(%)	(mm)		(mm)			
Control (water)	0	84	18.1 ± 0.8	a	28.7 ± 2.5	a	1140 ± 51.8	a
	-0.05	16	7.6 ± 3.3	c	11.5 ± 4.8	b	186 ± 81.8	d
	-0.15	NG	NG		NG		NG	
SW 1:1500 v/v	0	84	13.6 ± 1.1	b	33.8 ± 2.2	a	1247 ± 100.6	a
	-0.05	48	12.9 ± 0.9	b	32.5 ± 3.5	a	723 ± 51.8	b
	-0.15	NG	NG		NG		NG	
KAR_1 10^{-6} M	0	96	7.6 ± 0.6	c	34.2 ± 3.9	a	760 ± 58.1	b
	-0.05	60	6.1 ± 0.6	c	26.6 ± 2.5	a	412 ± 43.3	c
	-0.15	NG	NG		NG		NG	

Mean values ± standard error (n = 10) in each column with a different letter is significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). No growth (NG).

Table 3.4: Effect of water (control), Kelpak®, phloroglucinol (PG) and eckol on seedling growth of *Ceratotheca triloba* seeds after incubating at different osmotic potentials for 25 days.

Treatment	Osmotic potential (Mpa)	Survival rate (%)	Shoot length (mm)		Root length (mm)		Vigour Index	
Control (water)	0	72	16.2 ± 1.14	a	30.4 ± 2.98	ab	960.0 ± 67.74	b
	-0.05	16	6.7 ± 3.33	b	13.1 ± 4.77	c	218.1 ± 95.70	d
	-0.15	NG	NG		NG		NG	
Kelpak 0.4%	0	44	13.2 ± 1.17	a	15.1 ± 1.30	c	1026.7 ± 91.15	b
	-0.05	NG	NG		NG		NG	
	-0.15	NG	NG		NG		NG	
PG 10^{-6} M	0	88	17.5 ± 1.31	a	29.9 ± 2.65	ab	1474.5 ± 110.40	a
	-0.05	40	13.6 ± 2.56	a	26.1 ± 4.00	b	642.2 ± 120.95	c
	-0.15	NG	NG		NG		NG	
Eckol 10^{-6} M	0	64	16.6 ± 1.06	a	37.0 ± 2.42	a	999.1 ± 63.57	b
	-0.05	NG	NG		NG		NG	
	-0.15	NG	NG		NG		NG	

Mean values ± standard error (n = 10) in each column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). No growth (NG).

3.4.4. Effect of biostimulant-priming on seed germination and seedling growth under salinity stress conditions

Priming with KAR_1 and Kelpak® slightly improved seed germination at different NaCl concentrations (except 50 mM) relative to hydroprimed seeds (**Figs. 3.8** and **3.9**). In most cases, an increase in NaCl concentration decreased *C*. *triloba* germination in biostimulant treated or hydroprimed seeds. The severity of salinity stress in *C*. *triloba* seeds was most evident at 25 and 50 mM NaCl concentrations.

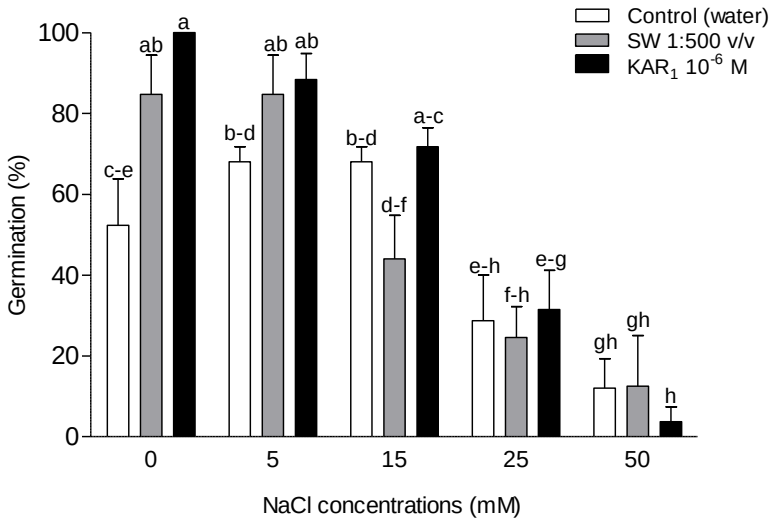


Fig. 3.8: Germination (%) of *Ceratotheca triloba* seeds primed with distilled water (control), smoke-water (SW) and karrikinolide (KAR_1) under salinity conditions for 25 days. Bars (± SE; *n* = 25) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Sodium chloride (NaCl).

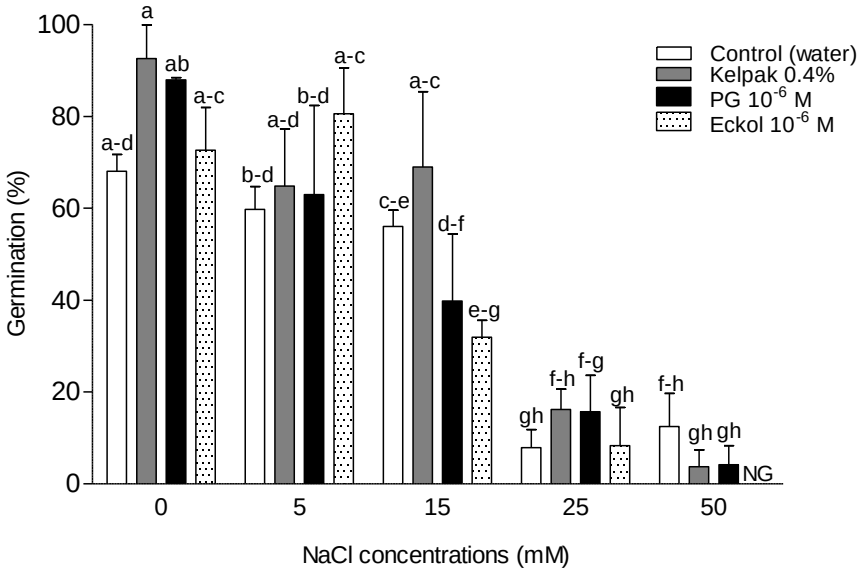


Fig. 3.9: Germination (%) of *Ceratotheca triloba* seeds primed with distilled water (control), Kelpak®, phloroglucinol (PG) and eckol under salinity conditions for 25 days. Bars (± SE; *n* = 25) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). No germination (NG), sodium chloride (NaCl).

During seedling growth, control treatment produced longer shoots with a higher vigour index than SW and KAR_1-stimulated seedlings, particularly at different NaCl concentrations (**Table 3.5**). Nevertheless, KAR_1 treatment improved seedling root length, except at 50 mM NaCl where no growth was observed. In a similar manner, hydroprimed seedlings improved shoot and root length as well as vigour index compared to biostimulant-treated seedlings under different NaCl concentrations (**Table 3.6**). However, Kelpak-treated seedlings showed improved seedling growth when tested at 15 mM NaCl compared to the control treatement. Overall, increase in NaCl concentrations caused a severe decline in seedling growth and survival rate.

Table 3.5: Effect of distilled water (control), smoke-water (SW) and karrikinolide (KAR_1) on seedling growth of *Ceratotheca triloba* seeds after incubating at different sodium chloride (NaCl) concentrations for 25 days.

Treatment	NaCl	Survival rate	Shoot length		Root length		Vigour Index	
Smoke	(mM)	(%)	(mm)		(mm)			
Control (water)	0	84	17.9 ± 0.7	a	28.3 ± 2.1	ab	938 ± 36.6	b
	5	56	11.6 ± 1.4	c	27.5 ± 3.0	ab	789 ± 95.0	c
	15	44	4.0 ± 0.8	e	16.4 ± 3.3	d	272 ± 51.4	e
	25	12	0.7 ± 0.4	f	0.5 ± 0.4	e	19 ± 10.4	f
	50	NG	NG		NG		NG	
SW 1:500 v/v	0	84	14.0 ± 1.0	b	32.9 ± 1.9	a	1186 ± 87.5	a
	5	76	4.1 ± 0.2	e	22.6 ± 2.1	bc	345 ± 19.3	de
	15	24	1.1 ± 0.4	f	4.9 ± 1.7	e	50 ± 17.1	f
	25	8	0.4 ± 0.3	f	0.7 ± 0.5	e	10 ± 6.7	f
	50	NG	NG		NG		NG	
KAR_1 10^{-6} M	0	96	8.3 ± 0.5	d	32.4 ± 2.6	a	833 ± 54.0	bc
	5	76	4.8 ± 0.3	e	28.7 ± 2.0	a	424 ± 27.6	d
	15	56	3.3 ± 0.5	e	19.1 ± 3.1	cd	239 ± 32.6	e
	25	20	1.4 ± 0.5	f	3.5 ± 1.5	e	44 ± 17.1	f
	50	NG	NG		NG		NG	

Mean values ± standard error (n = 10) in each column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). No growth (NG).

Table 3.6: Effect of distilled water (control), Kelpak®, phloroglucinol (PG) and eckol on seedling growth of *Ceratotheca triloba* seeds after incubating at different sodium chloride (NaCl) concentrations for 25 days.

Treatment	NaCl (mM)	Survival rate (%)	Shoot length (mm)		Root length (mm)		Vigour Index	
Control (water)	0	64	14.5 ± 1.37	b	26.3 ± 2.99	bc	989.07 ± 92.95	c
	5	56	11.6 ± 1.40	c	27.5 ± 3.03	b	692.78 ± 83.41	e
	15	44	4.0 ± 0.76	ef	16.4 ± 3.28	de	224.07 ± 42.35	gh
	25	12	1.1 ± 0.61	gh	1.1 ± 0.62	h	8.40 ± 4.76	i
	50	NG	NG		NG		NG	
Kelpak 0.4%	0	44	9.8 ± 1.76	c	11.4 ± 2.05	ef	907.41 ± 162.95	cd
	5	36	6.9 ± 1.57	d	10.2 ± 2.39	e-g	445.06 ± 101.77	f
	15	60	11.1 ± 0.81	c	20.1 ± 1.52	cd	763.40 ± 55.70	de
	25	NG	NG		NG		NG	
	50	NG	NG		NG		NG	
PG 10^{-6} M	0	88	18.0 ± 1.02	a	30.1 ± 2.64	ab	1556.67 ± 97.02	a
	5	28	3.5 ± 1.04	e-g	18.6 ± 5.54	d	218.27 ± 65.28	gh
	15	16	1.5 ± 0.70	f-h	4.2 ± 2.46	gh	61.05 ± 27.98	hi
	25	8	0.5 ± 0.32	h	1.9 ± 1.36	h	7.35 ± 5.06	i
	50	NG	NG		NG		NG	
Eckol 10^{-6} M	0	64	16.7 ± 1.12	ab	34.5 ± 2.25	a	1211.42 ± 81.39	b
	5	52	4.6 ± 0.58	de	26.6 ± 3.38	bc	370.56 ± 46.38	fg
	15	28	2.0 ± 0.59	e-h	6.9 ± 2.25	fg	63.89 ± 18.70	hi
	25	4	0.3 ± 0.33	h	1.6 ± 1.60	h	2.78 ± 2.78	i
	50	NG	NG		NG		NG	

Mean values ± standard error (n = 10) in each column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test. No growth (NG).

3.5. Discussion

Biostimulants are diverse substances and microorganisms which improve growth and development of plants through nutrient uptake and efficiency, water use and tolerance to abiotic stresses **(European Biostimulants Industry Council 2012)**. These biostimulants enhance plant development from seed germination up to maturity and improve plant adaptability to diverse environments. In the current study, the role of biostimulants during seed germination and seedling growth under temperatures, low osmotic potential and NaCl concentrations varied greatly based on the stress and biostimulant used as well as the evaluated physiological growth processes. The

valuable role of biostimulants was more noticeable during seed priming, where they significantly improved seed germination and seedling growth under harsh environmental conditions. Efficiency of priming agents is dependent on various factors such as plant species, seed morphology and physiology as well as environmental stress. Priming is known to activate cellular and molecular changes through several pathways involved in different metabolic processes in order to alleviate the deleterious effects of stress **(Jisha et al. 2012; Paparella et al. 2015)**. Nevertheless, the detrimental effects of low temperature, low osmotic potential and high NaCl concentrations was evident during seed germination and early seedling growth of *C. triloba*.

3.5.1. Effect of biostimulant-priming on seed germination and seedling growth under low temperature

Changes in ambient temperature occur more frequent than other stress factors, thus exacerbating drought and salinity stress problems **(Ashraf and Foolad 2007)**. Extreme temperatures are a significant challenge during crop growth and have been predicted to increase due to current climatic changes. This poses serious threats for plant survival because temperature controls germination which affects final seed germination rate **(Dürr et al. 2015)**. In this case, temperature played a significant role during *C. triloba* seed germination. A low temperature of 10 °C completely inhibited germination, indicating that seeds might have experience metabolic arrest at such low temperature. However, detrimental effect of low temperature after 15 days was overcome with a shift in temperature (10/15 °C), thus stimulating seed germination. On the other hand, high temperatures might have induced conformational changes in proteins **(Jain et al. 2006)** thereby reducing seed germination at 35 ºC. Optimum germination temperature in *C. triloba* was 25 °C, comparable to its ambient

temperature in the natural environment **(Duncan 2011)**. Seed germination and seedling growth was also influenced by the different biostimulant-priming agents. Smoke-water, KAR_1 and Kelpak® treatments enhanced seed germination, whereas seedling growth was improved with SW and PG treatments. The promotive role of SW and KAR_1 during germination and seedling growth of different species under normal or abiotic stress conditions has been reviewed **(Kulkarni et al. 2011; Light et al. 2004)**. Furthermore, SW and KAR_1 have been shown to improve seed germination and seedling growth under extreme temperatures in tomato seeds **(Jain et al. 2006; Jain and Van Staden 2007)**. Therefore, the current results further support the promotive effect of biostimulants under low temperature, indicating their impact in overcoming the deleterious effects of temperature fluctuations on seed germination and seedling growth.

3.5.2. Effect of biostimulant-priming on seed germination and seedling growth under low osmotic potential and salinity stress conditions

Drought and salinity stress are responsible for both inhibition or delayed seed germination and seedling establishment **(Almansouri et al. 2001; Farooq et al. 2009)**. These stresses share similar physiological mechanistic responses during plant growth. Once seeds have been exposed to these conditions, they develop an osmotically enforced “dormancy”, triggered by insufficient water uptake. This adaptive response prevents seed germination under unfavourable conditions in order to ensure proper seedling establishment. In most cases, low osmotic potential and NaCl concentrations reduced/inhibited seed germination and seedling growth in *C*. *triloba*. The severity of stress was most prominent at -0.15 MPa osmotic potential and 50 mM NaCl concentration during both germination and seedling growth. Deleterious effects of PEG and NaCl application during germination could have been attributed to limited

seed water uptake during germination **(Almansouri et al. 2001)**. Nevertheless, seed priming with SW and KAR_1 stimulated seed germination and seedling growth under low osmotic potential (-0.05 and -0.15 MPa). In similar studies, **Ghebrehiwot et al. (2008)** found that *Eragrostis tef* seeds treated with SW and butenolide (KAR_1) had a higher germination percentage with decreasing osmotic potential. Furthermore, the promotive role of SW and KAR_1 during seed germination and seedling growth under diverse environmental conditions has been reported in tomato **(Jain and Van Staden 2007)** and rice **(Jamil et al. 2014)**.

The stimulatory effect of Kelpak® has been documented for a number of plant species under favourable and unfavourable conditions during seed germination and seedling growth **(Khan et al. 2009; Stirk and Van Staden 2006)**. The biostimulant enhanced seed germination in *C*. *triloba* under low osmotic potential and NaCl concentrations. However, its continuous application had an inhibitory effect on seedling growth and survival rate. Therefore, seedling growth was improved in PG-primed and hydroprimed seeds at low osmotic potential and NaCl concentrations, respectively. The stimulatory effect of PG might be due to its cytokinin and auxin-like activity **(Teixeira da Silva et al. 2013)**. Furthermore, PG application has been shown to enhance seedling and root growth in *Zea mays* **(Rengasamy et al. 2015b**, **c)**. Although the exact molecular mechanism of seed priming is not completely understood, it is assumed that sensitization associated with inactive signaling proteins in primed cells could be hyperactived under abiotic stress conditions, thus enhancing signal transduction in plants which trigger defense responses **(Conrath et al. 2006)**. Different seed priming agents are able to regulate signaling pathways during early developmental stages leading to a faster defense response by plants **(Jisha et al. 2012)**. Therefore, biostimulants might have induced various biochemical changes such as hydrolysis,

activation of enzymes and breaking seed dormancy in order for *C*. *triloba* seeds to germinate and grow at such conditions **(Jisha et al. 2012; Paparella et al. 2015)**. According to **Bedigian (2004)**, species in the Pedaliaceae have various adaptation strategies including enforced dormancy, which in turn delays germination until the commencement of the rainy season. Induced dormancy in *C*. *triloba* seeds was noticeable during seed germination under low osmotic potential (-0.30 and -0.49 MPa). However, suppressed growth in seedlings incubated at PEG -0.15 MPa and 50 mM NaCl concentrations might suggest that although the seeds germinated they still did not enter phase III which is the embryonic axes elongation stage **(Bewley 1997)**. Nevertheless, at certain osmotic potential and NaCl concentrations biostimulants must have provided adequate moisture content for metabolic activities such as DNA synthesis, transcription, translation, cell elongation and cell division to be activated and seed germination to commence **(Bewley 1997)**. These metabolic activities were stimulated with very low concentrations of plant growth regulators (PGRs) contained in biostimulants. In addition, SW and KAR_1 have been documented to have PGR like properties. These PGRs include cytokinins (CKs), auxins, abscisic acid (ABA), gibberellins (GAs), brassinosteroids (BRs), strigolactones (SLs) and polyamines **(Light et al. 2009; Papenfus et al. 2012; Stirk et al. 2004; Stirk et al. 2014)**. The remarkable effect of these biostimulants was possibly prompted by the combination of diverse PGRs which may act individually or synergistically, in turn alleviating the detrimental effects of low osmotic potential and NaCl stress during seed germination and seedling growth in *C*. *triloba* species.

3.6. Concluding remarks

Existing knowledge on seed germination and seedling growth of traditional vegetables including *C*. *triloba* under various environmental conditions remains inadequate. Therefore, understanding factors that contribute towards low seed germination during abiotic stress is crucial, especially with the current climatic conditions. Furthermore, finding inexpensive and accessible biostimulants which can counteract the effect of harsh environmental conditions during seed germination and seedling growth is of utmost importance. As in most plant species, temperature played a crucial role in *C*. *triloba* seed germination and seedling establishment, with 25 °C being the optimum temperature. Biostimulant-seed priming as well as hydropriming proved to be an efficient technique in alleviating the detrimental effects of low temperature, low osmotic potential and NaCl concentrations in *C*. *triloba*. During seed germination and seedling growth, SW, KAR_1 and Kelpak® were the most effective biostimulants in stimulating germination under abiotic stress conditions. Seedling growth was greatly improved with SW, PG and control treatments in these aforementioned stresses. The deleterious effect of PEG was more intense than the toxic effect of NaCl during germination and seedling establishment. The current findings demonstrate the potential of seed priming with biostimulants in ameliorating the diverse effects of abiotic stresses during *C*. *triloba* seed germination and seedling growth. Nevertheless, more studies are needed in order to better understand the molecular role of biostimulants during seed germination and seedling growth in *C*. *triloba* under abiotic stress conditions.

Chapter 4: Effect of different watering regimes on *Ceratotheca triloba* seedling growth

4.1. Introduction

Environmental stresses such as drought, high salinity and nutrient deficiency are well-recognized for their deleterious effects during plant growth and survival. These stresses usually occur concurrently or at different times throughout the plants developmental life. Most environmental factors encountered by plants during growth are directly or indirectly linked to water status **(Verslues et al. 2006)**. Plants response towards water stress is very diverse, thus altering the plants metabolism, growth and development. The decrease in water supply causes a reduction in water flow conductivity along the soil-plant-atmosphere pathway **(Bacelar et al. 2007)**. This process occurs via stomatal closure and root conductivity in order to permit sufficient water flow throughout the entire plant. Furthermore, water stress is regulated by various mechanisms such as stress sensors, signalling pathways (protein-protein reactions), transcription factors and promoters as well as secondary metabolites. The complexity of these processes causes an increase in phytohormones such as abscisic acid (ABA), compatible solutes and protective proteins, antioxidants as well as expression or induction of genes **(Bartels and Sunkar 2005)**.

Phytohormones are compounds derived from plant biosynthetic pathways and function at the site of synthesis or are transported in order to facilitate growth and development under diverse environmental conditions **(Peleg and Blumwald 2011)**. These include ABA, auxins, gibberellins (GAs), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JAs), brassinosteroids (BRs), strigolactones (SL) and peptides. Abscisic acid is the key phytohormone induced during water stress. Accumulation of ABA regulates the expression of stress-responsive genes such as

compatible osmolytes and late embryogenesis abundant (LAE) protein synthesis, as well as dehydrins and stress-induced proteins **(Verslues et al. 2006)**. However, over-expression of ABA consequently leads to retarded growth and development through stomatal closure in plant species **(Sreenivasulu et al. 2012)**. Cytokinin, SA, ET and JA are amongst the major phytohormones directly or indirectly involved in plant stress responses. For instance, CK is an ABA antagonist which results in reduced CK concentrations when plants are exposed to limited water resources **(Peleg and Blumwald 2011)**. Therefore, the maintenance of precise dose/response ratio in phytohormones is crucial for plant adaptation/tolerance towards water stress.

Plant secondary metabolites also play a fundamental role in the defence against unfavourable environmental conditions. These secondary metabolites are divided into three chemically distinct groups namely: terpenes, phenolics and nitrogen-containing compounds **(Taiz and Zeiger 2002)**. The bioactive compounds are known to modulate various physiological processes such as transcriptional regulation, membrane permeability, respiration and photosynthetic rate **(Cheynier et al. 2013)**. Furthermore, they are considered to be essential compounds in signalling molecules, although their involvement in signal transduction remains to be fully elucidated **(Edreva et al. 2008)**. Unlike primary metabolites, secondary metabolites are usually present in very low quantities. Their production is also reported to be site specific and are now understood to play an important role during plant growth and development during abiotic stress.

Ceratotheca triloba (Bernh.) Hook.f. either grows as a weed along roadsides or is cultivated in some regions. Plant species in the family Pedaliaceae are covered with mucilage glands enabling the plants to withstand severe dehydration without tissue damage, making them drought resistant **(Ihlenfeldt 2004)**. Therefore, the current

study aimed to determine the effect of different watering regimes on growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content in *C. triloba* cultivated under greenhouse conditions. In addition, the study also evaluated the influence of 2 and 4 month harvesting stages on these aforementioned parameters.

4.2. Materials and methods

4.2.1. Seed source, germination and transplantation

Ceratotheca triloba seeds were purchased from Silverhill Seeds Nursery, Cape Town, South Africa. The experiment commenced in October 2015 and was conducted in a greenhouse at the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa. Based on results obtained from **Section 3.2.2**, seeds were primed with SW 1:500 (v/v dilution). Using seed trays, seeds were sown at 1 cm depth in soil (prepared through the combination of refuse and compost tea for 18 months, sieved then poisoned with methyl bromide for 48h in order to kill off weed seeds, fungus and nematodes). For one month in the mist house, seedlings were grown with day and night temperatures of 30/15 °C, relative humidity of 80 - 90% and 10 s misting at 15 min intervals. Thereafter, seedlings were transplanted into 7.5 cm diameter pots with soil for 3 weeks in the greenhouse with 30 - 40% relative humidity, day and night temperatures of 30/15 °C and an average photon flux density (PPF) of 450 µmol m^{-2} s^{-1}. Established seedlings were completely randomized into four treatments. The treatments were made up of plants watered daily (7 d), thrice (3 d), twice (2 d) and once (1 d) per week and grown for 2 and 4 months. For each treatment, 50 ml of water was applied per pot. After 2-months of growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content were evaluated. At the termination of the experiment (4-month growth), plant fresh weight (leaf weight, root

length, root weight, plant height) as well as the abovementioned parameters were recorded.

4.2.2. Chlorophyll fluorescence evaluation

After 2 and 4 months of growth in the greenhouse, chlorophyll fluorescence of *C. triloba* leaves cultivated at different watering regimes was evaluated. Chlorophyll fluorescence was measured with a pulse modulated fluorometer (Model FMS-2, Hansatech Instruments, King's Lynn, UK). Intact fully developed leaves were covered for 10 min with a black ring (10 mm diameter) to exclude light. Minimal fluorescence (Fo) (representing the basal level of fluorescence emitted without exerting any photochemistry) was measured on the dark-adapted leaves. Maximum fluorescence (Fm) in dark-adapted leaves was measured after a brief saturating flash of approximately 8 s. The following parameters were derived from the final measurements: maximum quantum efficiency of PSII (*Fv/Fm*), actual quantum yield of photosystem II (ΦPSII), photochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transport rate (ETR) **(Beckett et al. 2005; Maxwell and Johnson 2000)**. The above mentioned parameters were measured at four different actinic light intensities: 56, 134, 850 and 1279 µmol PAR m^{-2} s^{-1} at a regular interval (5 min). Data was downloaded to a computer, recorded automatically and subjected to analysis of variance (ANOVA).

4.2.3. Quantification of stress-related phytohormones using Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC–MS/MS)

Lyophilized 2 and 4-month-old *C. triloba* leaves and roots (2.5 – 3 mg) were transferred into Eppendorf tubes containing 2 mm ceria-stabilized zirconium oxide beads (Retsch

GmbH & Co. KG, Haan, Germany). Extraction of jasmonoyl isoleucine (JA-Ile), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA), SA, ABA, jasmonic acid (JA) and indole-3-acetic acid (IAA) was quantified as described by **Floková et al. (2014)**. Analyte recovery during purification process was determined with the addition of stable isotopically labelled internal standards (20 pmol) in each sample. Extracts were homogenized in 1 ml of ice cold 10% methanol (MeOH) solution for 5 min using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) at 27 Hz frequency. The samples were sonicated at 4 °C for 3 min with an ultrasonicator in an ice block-filled bathtub (Transsonic T310, Elma GmbH & Co KG, Singen, Germany) and then extracted using a benchtop laboratory rotator (Stuart SB3 BibbyScientific Ltd., Staffordshire, UK) at 4 °C for 30 min. Samples were centrifuged (fixed angle rotor) for 10 min at 20 000 rpm, 4 °C (BeckmanAvanti™ 30) and the supernatants were transferred into microtubes. The pellet was re-extracted with 1 ml of ice cold 10% MeOH using a vortex (Velp Scintifica, Usmate, Italy), benchtop laboratory rotator and centrifuged for 10 min at 20 000 rpm, 4 °C. Thereafter, eluates were evaporated until they were dry.

Stress-related phytohormones were analyzed using an Acquity UHPLC® system (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer Xevo™ TQ MS (Waters MS Technologies, Manchester, UK). Samples (10 µl) were injected into a RP column (Acquity UHPLC® CSH™ C18; 2.1 x 100 mm; 1.7 µm; Waters, Ireland) at a flow rate of 0.4 ml/min and the column was incubated at 36 °C. The extracted compounds were separated by gradient elution using 10 mM formic acid (HCOOH) (A) and acetonitrile (CH_3CN) (B) over 35 min. Thereafter, 100% acetonitrile was used to wash the column and equilibrated to the initial condition of 15% A, v/v for 5 min. The eluate was placed into the electrospray ion source of a tandem MS analyzer

and analyzed with MS/MS conditions including source/desolvation temperature, 120/550 °C; cone/desolvation gas flow, 70/650 l/h; capillary voltage, 3 kV; cone voltage, 23 - 30 V; collision energy, 12 - 23 eV; collision gas flow (argon), 0.21 ml/min. Analyzed compounds and internal standards were quantified in multiple ion monitoring mode (MRM) using optimized MS conditions and continuous polarity-switching data. The MRM transitions were recorded in a chromatographic run in 10 targeted scan windows to obtain the highest possible MS signal intensity for each compound. MassLynx™ software package (version 4.1, Waters, Milford, MA, USA) was used to control the instrument and to obtain and process the MS data. Analysis was done in triplicate through technical replication.

4.2.4. Phenolic acid quantification using Ultra High performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC–MS/MS)

Lyophilized 2 and 4-month-old *C*. *triloba* leaves (30 mg) were transferred to an Eppendorf tube containing 2-mm ceria-stabilized zirconium oxide beads (Retsch GmbH & Co. KG, Haan, Germany). The material was extracted with 80% MeOH and a 20 µl internal standard [10^{-4} mol/l; solution of salycilic acid (3,4,5,6-$^{2}H_4$) and 4-hydroxybenzoic acid (2,3,5,6-$^{2}H_4$)]. The mixture was homogenized for 3 min in the oscillatory ball mill homogenizer at 27 Hz frequency and extracted for 15 min in an ultrasonicator and centrifuged (fixed angle rotor) for 10 min (17 000 rpm) at 4 °C. Supernatants were transferred into Eppendorf tubes and the pellet was re-extracted. Both extracts were combined and evaporated to dryness in an oil evaporator for 6 – 8 h. The resultant supernatants were filtered through 0.45-lm nylon membrane filters (Alltech, Breda, Netherlands) and then analysed using a UHPLC™ system (Waters, Milford, MA, USA) connected simultaneously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro micro™ API benchtop

triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating in a negative mode as described by **Gruz et al. (2008)**. The extracts were injected into a reversed phase column (BEH C_8, 1.7 µm, 2.1 × 150 mm, Waters, Milford, MA) and incubated at 30 °C. Linear gradients and isocratic flows of the mobile phase had a sequencing of 9.5-min with solvent B (acetonitrile) balanced with solvent A (aqueous 7.5 mM HCOOH) at a flow rate of 250 µl/min. After sequencing, the column was equilibrated for 2.5 min under initial conditions with pressure from 4000 to 8000 psi during the chromatographic run. Eluent was added into a PDA detector (scanning range 210 – 600 nm, resolution 1.2 nm) then into an electrospray source (source block temperature 100 °C, desolvation temperature 350 °C, capillary voltage 2.5 kV, cone voltage 25 V). Argon was applied as a collision gas (collision energy 16 eV) and nitrogen as a desolvation gas (500 l/h). Different retention windows were used for quantification. Analysis was done in triplicates through technical replication.

4.2.5. Total carbohydrate and elemental analysis

Total carbohydrates were estimated as described by **Sadasivam and Manickam (1996)**. Briefly, 0.1 g of dried leaf material (2 and 4 months) was hydrolysed using 5 ml of 2.5 N hydrochloric acid (HCl) and cooled at room temperature. Hydrolysed leaf material was neutralized with sodium carbonate until effervescence ceased. Thereafter, a neutralized solution was made up to 10 ml with distilled water and the mixture was centrifuged at 3000 rpm for 15 min. In triplicate test tubes, 1 ml of the supernatant was added to 1 ml of 5% phenol and 5 ml 96% sulfuric acid. The mixture was shaken well for 10 min and placed in a water bath at 30 °C for 20 min. After cooling, the absorbance was measured at 490 nm in a UV-visible spectrophotometer (Varian Cary 50, Australia) against a blank. A standard graph of carbohydrate was

prepared using glucose to calculate the carbohydrate content (% DW). Samples were analyzed in triplicate through technical replication.

Dry leaf material of *C*. *triloba* was digested using an aluminium heating block. In Teflon vessels, 8 ml nitric acid (HNO_3) was added to 0.1 g DW of plant material and heated in an aluminium heating block for 30 min at 90 °C, and again at 120 °C for another 30 min. Thereafter, 1 ml hydrogen peroxide (H_2O_2) was added to the mixture and heated at 150 °C for 60 min. After allowing the mixture to cool for 5 min, 1 ml HCl was added to the vessels and heated at 150 °C for 20 min. Completely digested solution and a blank was transferred to 10 ml volumetric flasks made up to volume with distilled water. Samples were kept in pill vials at 10 °C until analyzed.

An Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Varian 720-ES, Varian Inc, Palo Alto, CA, USA) instrument was used to determine the element concentration in samples **(Hou and Jones 2000)**. The instrument was operated as follows: RF power 1.0 kW; viewing geometry axial; Argon gas used as plasma gas flow at the rate of 15.0 l/min; auxiliary gas flow rate 1.50 l/min; nebulizer gas flow rate 0.75 l/min; and replicate reading time 9.0 s.

4.3. Data analysis

Data was subjected to analysis of variance (ANOVA) and Student's *t* test using SPSS for Windows (SPSS®, Version 22.0. Armonk, New York, USA). For statistical significance ($P \leq 0.05$), the mean values were further separated using the Duncan's Multiple Range Test (DMRT). Statistical differences between the mean values of different growth duration were further analysed using a Student's *t* test package. For phenolic acids and endogenous phytohormones, data processing was performed with MassLynx™ software (version 4.0, Waters, Milford, MA, USA).

4.4. Results

4.4.1. Effect of different watering regimes on growth and chlorophyll fluorescence

The growth of *C*. *triloba* was considerably influenced by different watering regimes (**Table 4.1**). For instance, there was a significant improvement in the growth of plants watered daily compared to plants watered once a week. Limited water supply resulted in a more than 1.4-fold decrease in leaf weight, root length and weight as well as plant height in plants watered once a week. The severity of watering once a week was more noticeable in the root weight which was 1.7 g relative to 11.1 g in plants watered daily (**Fig. 4.1**). Overall, there was a significant decline in growth with decreasing watering regimes in 4-month-old *C*. *triloba* plants.

Table 4.1: Effect of different watering regimes per week on the growth of *Ceratotheca triloba* after 4 months in the greenhouse.

Watering regimes per week	Leaf weight (g)		Root length (mm)		Root weight (g)		Plant height (mm)	
7 (daily)	5.5 ± 0.40	a	92.5 ± 10.82	a	11.1 ± 1.06	a	1211.4 ± 61.21	a
3 (thrice)	5.0 ± 0.56	ab	82.1 ± 5.10	ab	4.4 ± 0.65	b	805.0 ± 49.21	b
2 (twice)	4.8 ± 0.43	ab	65.8 ± 6.38	bc	3.6 ± 0.30	b	724.3 ± 31.56	b
1 (once)	3.9 ± 0.42	b	56.7 ± 2.47	c	1.7 ± 0.24	c	577.9 ± 19.21	c

Mean values ± standard error (n = 8) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

Fig. 4.1: *Ceratotheca triloba* roots grown under different watering regimes per week after 4 months under greenhouse conditions. Watering regimes per week: daily (7 d), thrice (3 d), twice (2 d) and once (1 d).

As shown in **Table 4.2**, there was minimal/no change in the *Fv/Fm* value of plants watered at different regimes in *C*. *triloba* leaves. However, watering once a week negatively affected chlorophyll fluorescence in 4-month-old plants, with an *Fv*/*Fm* value of 0.80. Even though, leaves of plants watered daily, thrice and twice a week showed no signs of stress (0.83 – 0.85) during the course of growth.

Table 4.2: Effect of different watering regimes per week on chlorophyll fluorescence (*Fv/Fm*) of *Ceratotheca triloba* after 2 and 4 months in the greenhouse.

Watering regimes	*Fv/Fm*			
per week	2 Months		4 Months	
7 (daily)	0.85 ± 0.00	a	0.84 ± 0.01	a
3 (thrice)	0.84 ± 0.00	b	0.83 ± 0.01	ab
2 (twice)	0.85 ± 0.00	a	0.85 ± 0.00	a
1 (once)	0.84 ± 0.00	b	0.80 ± 0.01	b

Mean values ± standard error (n = 8) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Maximum quantum efficiency of photosystem II (*Fv/Fm*).

There was a gradual decline in ΦPSII and qP levels at actinic light intensities of 56 - 1279 µmol PAR m^{-2} s^{-1}, whereas NPQ and ETR levels increased as light intensities increased in 2 and 4-month-old plants (**Figs. 4.2** and **4.3**). Generally, chlorophyll fluorescence (ΦPSII, qP and relative ETR) in leaves significantly decreased when plants were watered once a week. In a similar manner, ΦPSII and relative ETR levels in plants watered daily declined after 4 months of growth. Non-photochemical quenching was higher in plants watered once a week after 4 months of growth (**Fig**

4.3 a-b). The duration of growth had minimal effect on chlorophyll fluorescence parameters in *C*. *triloba* plants (**Appendix A, Table 1**).

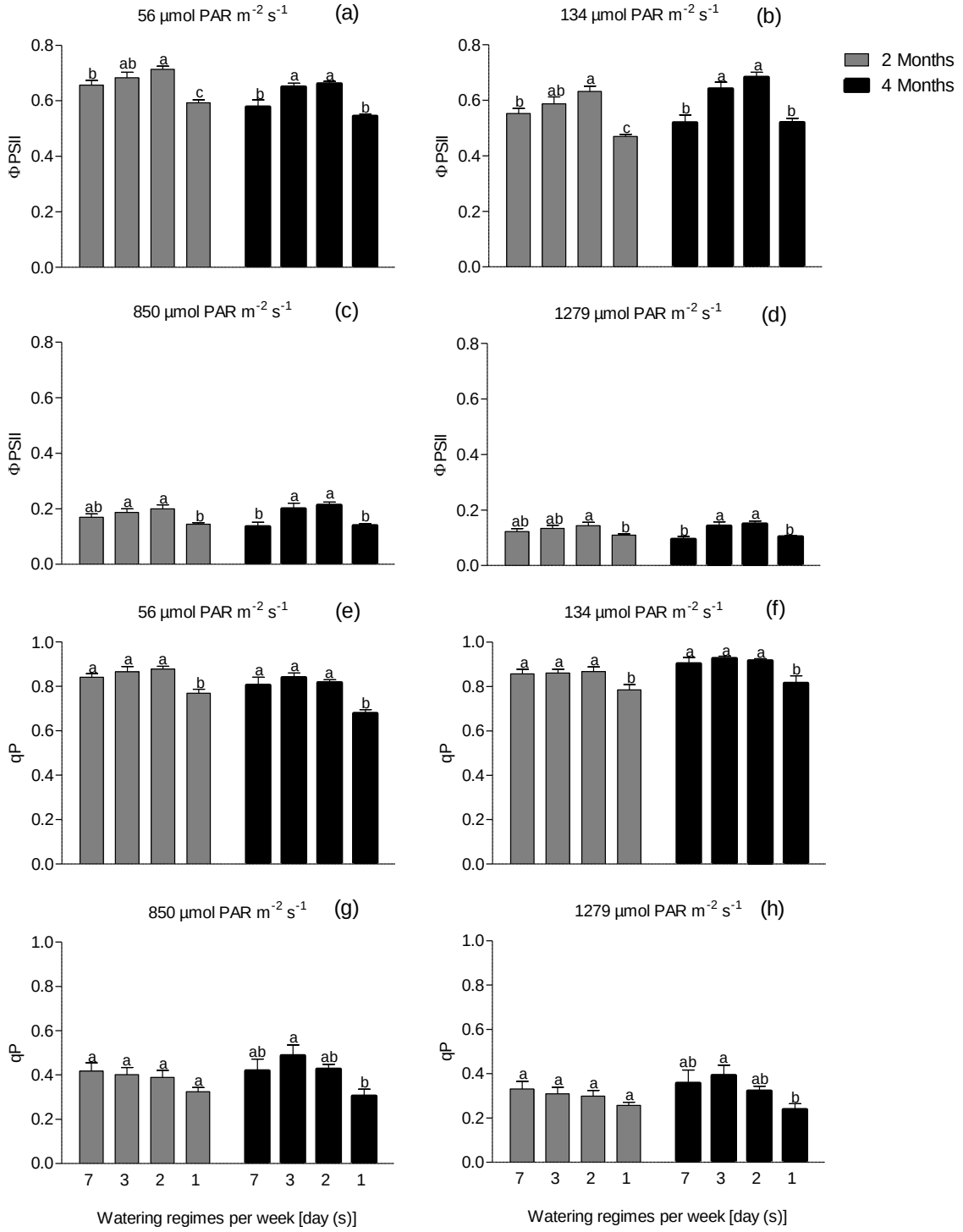


Fig. 4.2: Effect of different watering regimes per week on the quantum yield of photosystem II (ΦPSII, a-d) and phytochemical quenching (qP, e-h) in *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse. In each harvested growth period graph, bars represent mean values ± standard error (n = 8) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

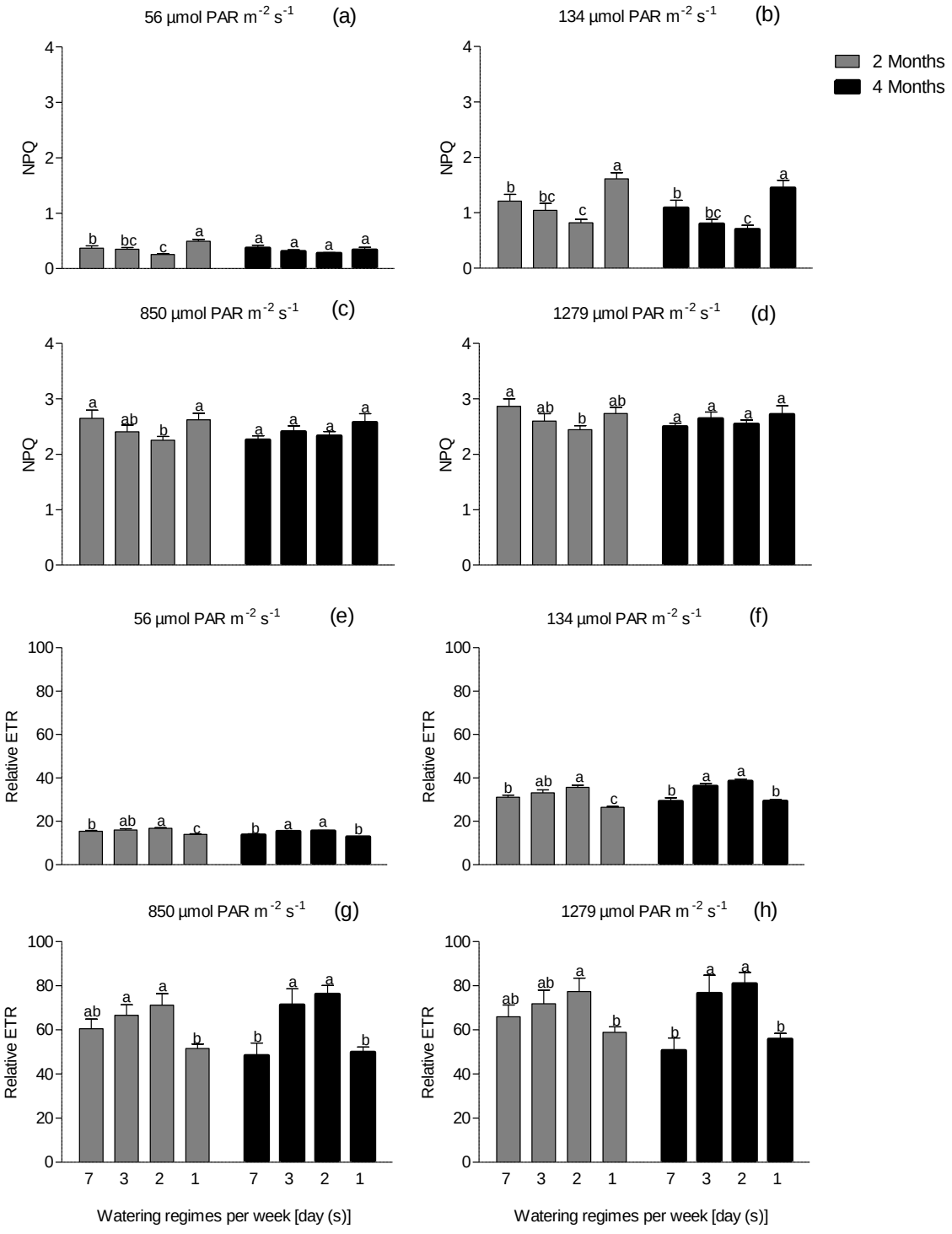
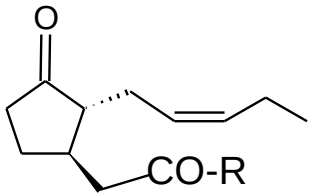


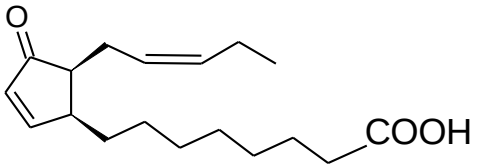
Fig. 4.3: Effect of different watering regimes per week on the non-photochemical quenching (NPQ, a-d) and relative electron transfer rate (relative ETR, e-h) in *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse. In each harvested growth period graph, bars represent mean values ± standard error (n = 8) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

4.4.2. Effect of different watering regimes on stress phytohormone content

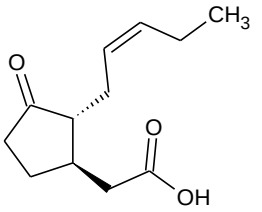
A total of six stress-related phytohormones were quantified using a UHPLC in *C. triloba* plants (leaves and roots) cultivated in the greenhouse for a duration of 2 and 4 months (**Fig. 4.4**). These included: JA-Ile (active form of JAs), *cis*OPDA (JAs precursor), ABA, SA, JA and IAA. As shown in **Fig. 4.5**, plants watered daily produced high concentrations of JA-Ile (238 pmol/g DW), *cis*OPDA (0.5 nmol/g DW) and SA (11.8 nmol/g DW) in 2-month-old leaves. However, plants watered daily had reduced ABA levels (266.9 pmol/g DW) while plants watered once a week accumulated high concentrations of ABA (2048.5 pmol/g DW). Unlike plants watered daily, plants watered once a week had significantly low levels of JA-Ile, *cis*OPDA and SA. Essentially, there was a notable variation in endogenous phytohormone production in 4-month-old plants (**Fig. 4.6**). This was observed in roots which accumulated high levels of JA-Ile, *cis*OPDA, JA and IAA, whereas leaves had increased concentrations of ABA and SA. In most cases, phytohormone synthesis was significantly induced in roots of plants watered daily. Abscisic acid and SA were more concentrated in leaves of plants watered once a week compared to plants watered daily. Furthermore, *C. triloba* leaves accumulated high levels of SA (8 – 98 nmol/g DW) relative to other quantified phytohormones. In general, duration of growth significantly influenced JA-Ile and SA production in leaves, with 4-month-old plants accumulating more phytohormones than 2-month-old plants (**Appendix A, Table 2**).



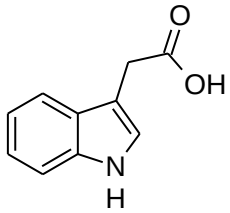
Jasmonoly isoleucine (JA-Ile)



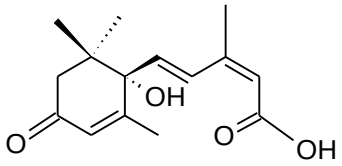
cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA)



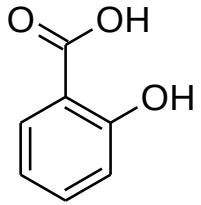
Jasmonic acid (JA)



Indole-3-acetic acid (IAA)



Abscisic acid (ABA)



Salicylic acid (SA)

Fig. 4.4: Chemical structures of analysed endogenous phytohormones in greenhouse cultivated *Ceratotheca triloba* (leaves and roots) after 2 and 4 months of growth.

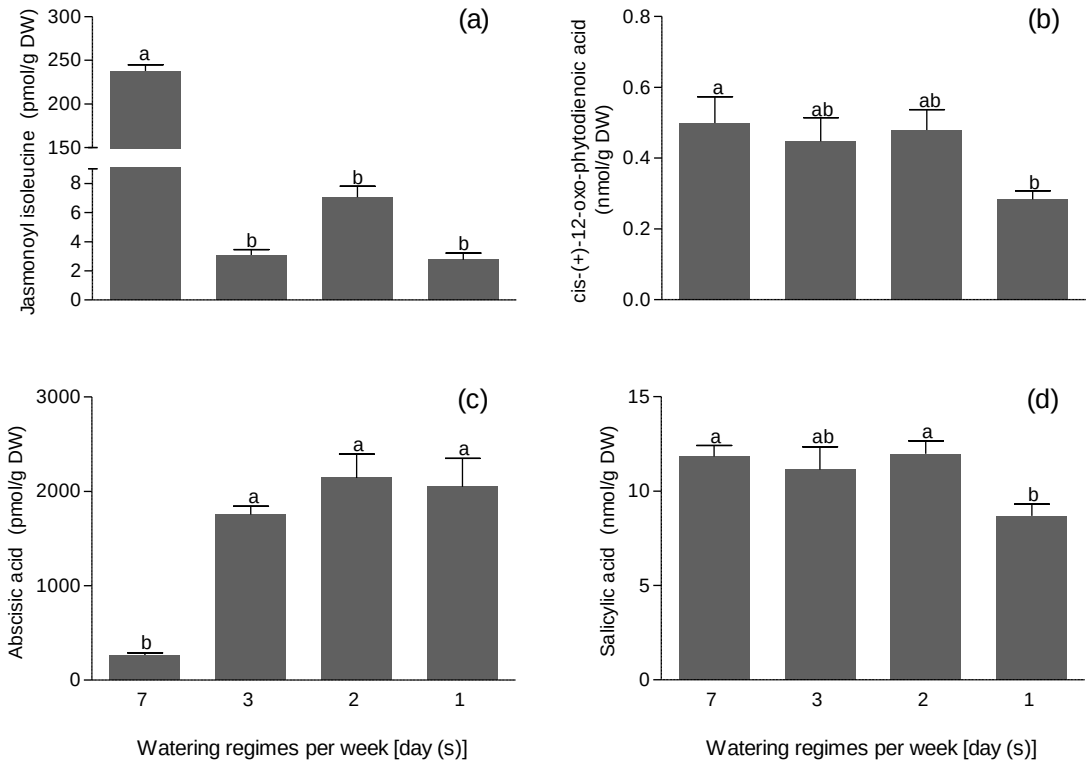


Fig. 4.5: Effect of watering regimes per week on endogenous phytohormones: jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), abscisic acid (ABA) and salicylic acid (SA) in *Ceratotheca triloba* after 2 months of growth in the greenhouse. In each graph, bars represent mean values ± standard error (n = 3) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

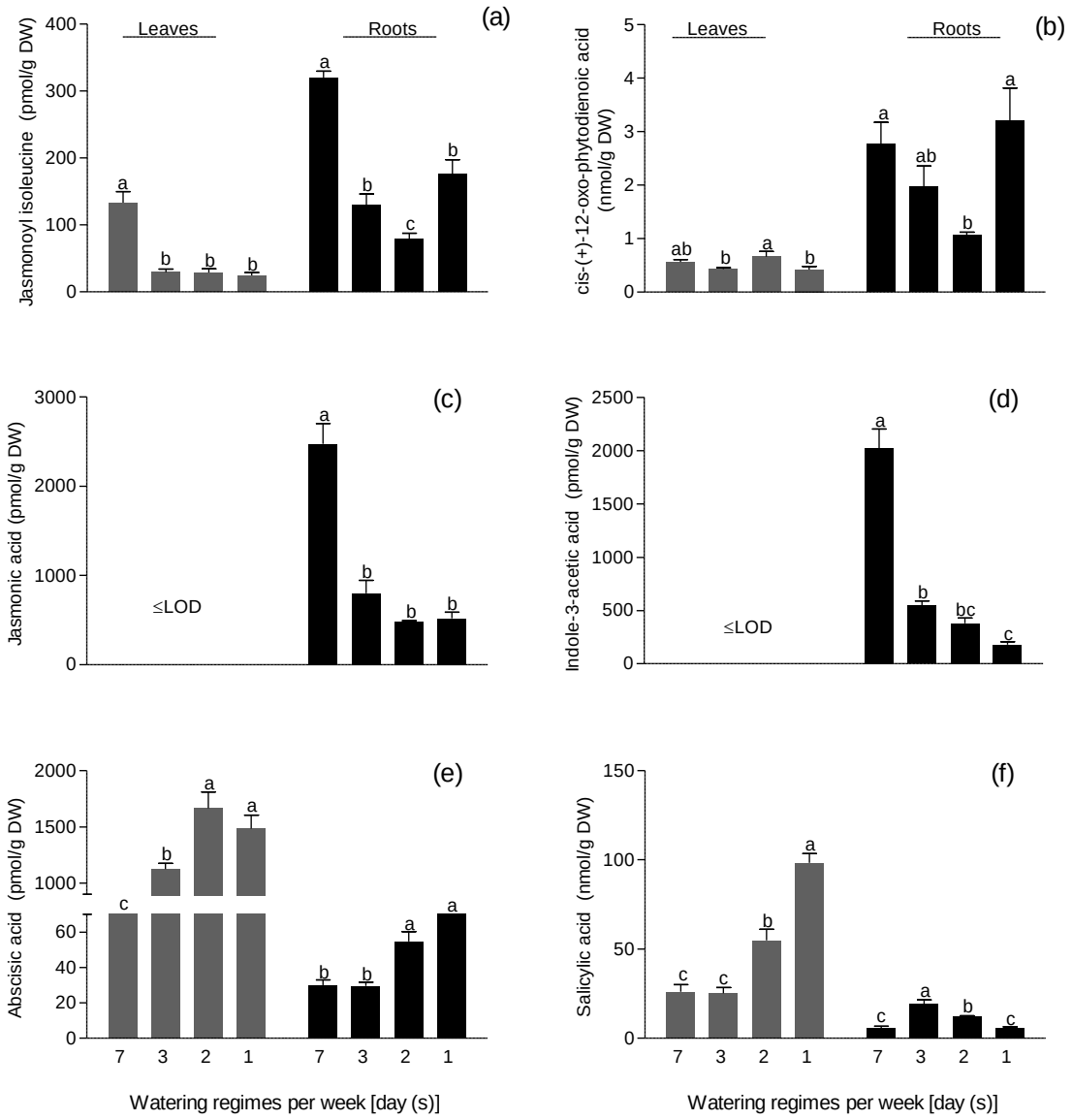
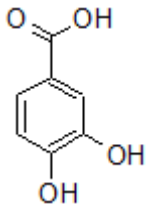


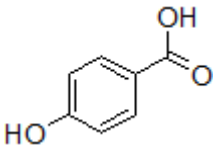
Fig. 4.6: Effect of watering regimes per week on endogenous phytohormones: jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), jasmonic acid (JA), indole-3-acetic acid (IAA), abscisic acid (ABA) and salicylic acid (SA) in *Ceratotheca triloba* after 4 months of growth in the greenhouse. In each harvested part graph, bars represent mean values ± standard error (n = 3) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Under detection limit of the LC-MS/MS method (<LOD).

4.4.3. Effect of different watering regimes on phenolic acid content

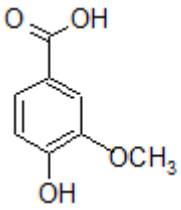
The UHPLC analyses showed the presence of three hydroxybenzoic and three hydroxycinnamic acids in greenhouse cultivated *C*. *triloba* (**Fig. 4.7**). These included protocatechuic, 4-hydroxybenzoic, vanillic, ferulic, caffeic and 4-coumaric acids. 4-Hydroxybenzoic and vanillic acids were detected in relatively low concentrations, whereas 4-coumaric acid was the highest detected phenolic acid in leaves of *C*. *triloba* (**Fig. 4.8**). Phenolic acid production was significantly higher in plants watered once a week particularly in 4-month-old plants, except for protocatechuic acid. Basically, phenolic acid concentrations gradual increased in 2 and 4-month-old plants with a reduction in water supply in plants. However, plants watered twice a week had the highest protocatechuic acid content after 2 and 4 months. The time of harvest significantly influenced phenolic acid concentration especially in 4-month-old plants compared to 2-month-old plants (**Appendix A, Table 3**).



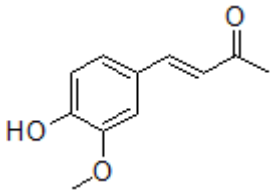
Protocatechuic acid



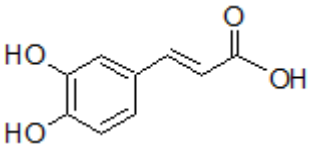
4-Hydroxybenzoic acid



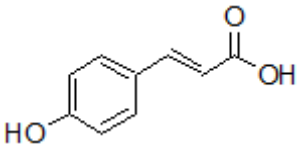
Vanillic acid



Ferulic acid



Caffeic acid



4-Coumaric acid

Fig. 4.7: Chemical structures of phenolic acids quantified in the leaves of greenhouse cultivated *Ceratotheca triloba* after 2 and 4 months of growth.

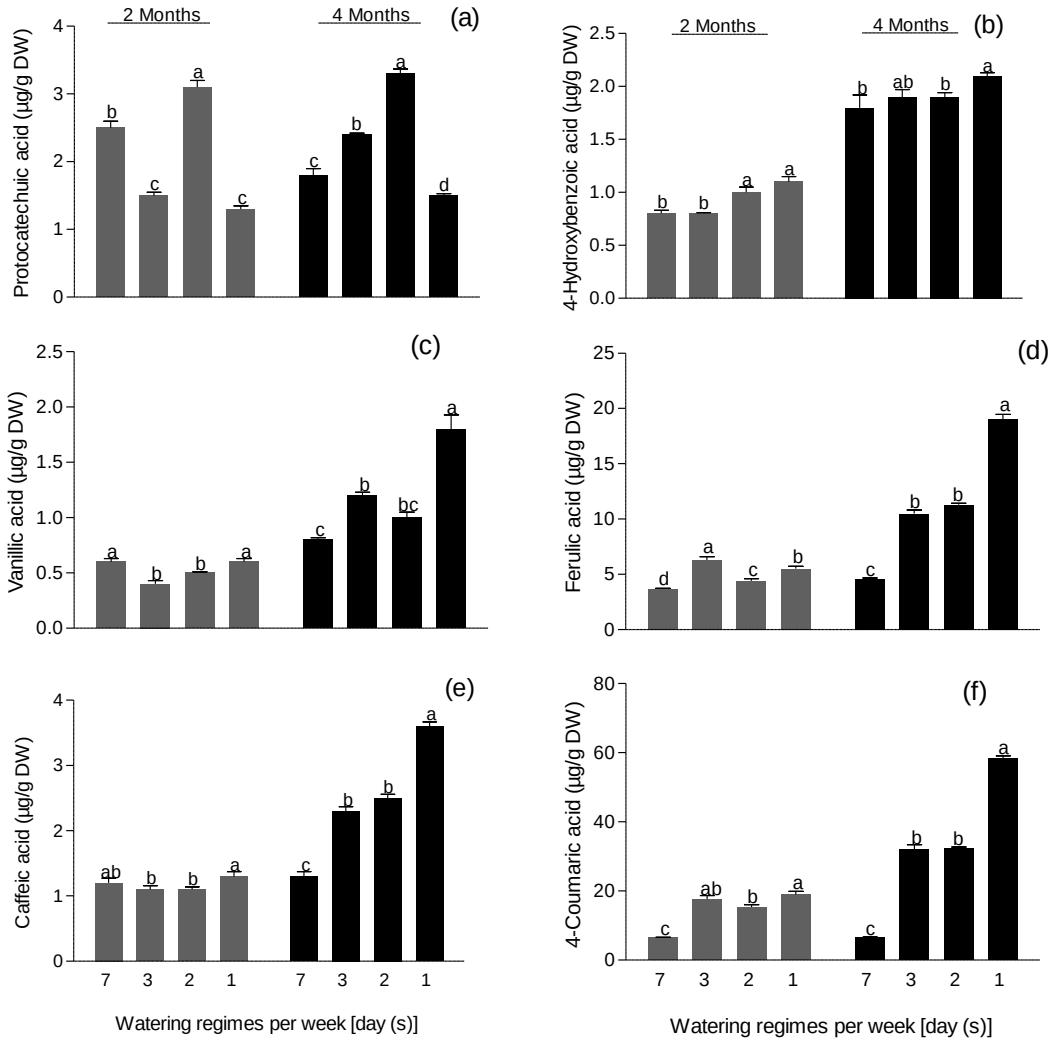


Fig. 4.8: Effect of different watering regimes per week on phenolic acid content in the leaves of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse. In each harvested growth period graph, bars represent mean values ± standard error (n = 3) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

4.4.4. Effect of different watering regimes on mineral and total carbohydrates content

Mineral content and total carbohydrate percentage in *C*. *triloba* after 2 and 4 months of growth are presented in **Table 4.3**. Leaves contained relatively high levels of calcium (Ca) and potassium (K) >10000 mg/kg (DW), with some exceptions. There was significant variation in mineral and total carbohydrate content in plants watered at

different regimes. After 2 months of growth, plants watered daily had the highest Ca and Mn content, plants watered thrice had high levels of sodium (Na), zinc (Zn) and total carbohydrate, whereas plants watered once a week accumulated more iron (Fe), K and magnesium (Mg). Plants watered twice a week produced high Ca, Zn and total carbohydrate levels after a 4-month growth period. In most cases, mineral content and total carbohydrate content decreased in plants watered daily (after 4-months) while plants watered twice a week had enhanced nutritional content in 4-month-old plants.

Table 4.3: Effect of different watering regimes per week on mineral content and total carbohydrates (%) in *Ceratotheca triloba* after 2 and 4 months of growth in a greenhouse.

Harvest	Watering regimes per week	Ca	Fe	K	Mg	Mn	Na	Zn	% Carbohydrates
		mg/kg (DW)							
2-Months	7 (daily)	18103	520	26521	3856	79	228	104	22
	3 (thrice)	13876	364	25880	2966	57	282	123	24
	2 (twice)	737	48	1054	187	5	16	30	14
	1 (once)	13852	564	26647	3963	5	14	8	17
4-Months	7 (daily)	18698	396	20508	2698	4	226	18	11
	3 (thrice)	15786	556	25680	2127	62	200	52	8
	2 (twice)	24377	548	27152	3637	58	209	117	15
	1 (once)	18580	532	28441	3766	8	12	36	13

Calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), zinc (Zn).

4.5. Discussion

Plants have developed several complex mechanisms in order to cope with water stress. These strategies include six aspects which are: (1) drought escape, (2) drought avoidance, (3) drought tolerance, (4) drought resistance, (5) drought abandonment and (6) drought-prone biochemical-physiological traits e.g. genetic mutation and genetic modification **(Xu et al. 2010)**. The multi-aspect processes may occur

synchronously in a plant's developmental life when subjected to water stress and rewatering. Once plants have been exposed to water stress, their growth is restricted due to the disturbance of photosynthesis, respiration, translocation, ion uptake, carbohydrate production/utilization, nutrient metabolism and growth promoters **(Jaleel et al. 2009)**. Nonetheless, a plants reaction towards water stress differs at various organizational levels and is dependent on a number of factors such as the intensity and duration of stress, plant species and developmental stages **(Chaves et al. 2002)**.

4.5.1. Effect of different watering regimes on growth and chlorophyll fluorescence

The growth of *C*. *triloba* was predominantly dependent on water availability in order for plants to maximize their photosynthetic potential, thus improving growth and development. Hence, limited water supply caused an excessive decline in growth, especially in plants watered once a week. Similarly, the deleterious effects of limited water supply during the irrigation of *Solanum scabrum* **(Muthomi and Musyimi 2009)**, *Olea europaea* **(Bacelar et al. 2007)**, *Amaranthus cruentus*, *A*. *hypochondriacus*, *Brassica carinata*, *S*. *scabrum* and *S*. *villosum* **(Luoh et al. 2014)** has been reported. According to **Anjum et al. (2011)**, the relationship between source and sink limitations of shoot and root systems must have a well-established equilibrium for successful plant growth. This in turn improves the growth and development of plants under various stress conditions. In *C*. *triloba*, limited water caused an excessive decline in plant leaf weight due to leaf shedding, a common adaptation trend employed by plants in order to minimize water loss through transpiration and decreased soil water potential **(Chaves et al. 2009)**. Unlike shoot growth, root development is usually less inhibited by water stress and in some cases even improved **(Sharp et al. 2004)**. However, this was not the case with *C*. *triloba*, whereby root weight was severely reduced by limited water supply which eventually caused excessive reduction in leaf weight. Extensive

rooting systems improves plant growth especially when plants are grown in water deficit soils **(Bengough et al. 2011)**. Factors that restrict root growth include the reduction in water potential, hypoxia or anoxia (limited oxygen) and mechanical impedance (hardened soil) **(Bengough et al. 2011)**. Nevertheless, low water potential causes the morphology of the roots to change and become thinner, an adaptive response for plants to be able to grow under such conditions **(Sharp et al. 2004)**. Root length in *C*. *triloba* was not severely impaired by limited water. This must have enabled plants to absorb more water and improve growth and survival rate after a period of 4 months. Despite the fact that *Ceratotheca* is presumed to be a drought-resistant genus **(Bedigian and Adetula 2004)**, *C*. *triloba* plants still need to be grown in well-drained soil for improved growth and development **(Duncan 2011)**. Nevertheless, the plants are still capable of surviving/adapting to soils with limited water supply even though their growth is impaired.

Photosynthetic response of plants towards diverse environmental conditions including water stress is frequently assessed using chlorophyll fluorescence analysis. In leaves, light energy absorbed by chlorophyll molecules is either used during photosynthesis (photochemistry), dissipated as heat or re-emitted as light (chlorophyll fluorescence) energy **(Maxwell and Johnson 2000)**. These processes occur in a competitive manner. Therefore, chlorophyll fluorescence emission provides essential information regarding the quantum efficiency of photochemistry necessary for photosynthesis and heat dissipation in plants. Water stress can impair chlorophyll molecules through photo-inhibition which causes photo-damage to the photosynthetic apparatus thus resulting in irreversible inactivation of PSII. In addition, water stress causes a reduction in chlorophyll concentration of plants, a common symptom of oxidative stress caused by pigment photo-oxidation and chlorophyll degradation **(Anjum et al. 2011)**.

Therefore, regulation of light harvesting in plants is an important step towards absorption and utilization of light energy which reduces photo-oxidative damages under diverse environmental conditions **(Müller et al. 2001)**. The photosynthetic potential of *C*. *triloba* plants was assessed under different watering regimes. Chlorophyll fluorescence had minimal effect on the *Fv*/*Fm* ratio at different watering regimes after 2 and 4 months of growth. The optimum value for unstressed plants has been documented to be approximately 0.83 **(Johnson et al. 1993)**. Therefore, the values expressed in plants with reduced water supply are consistent with those of unstressed leaves, suggesting that the quantum efficiency of PSII was not considerably affected by reduction in water supply. However, plants watered once a week showed some signs of stress with an *Fv/Fm* value of 0.80 after 4 months of growth. Such a decline in photosynthetic activity indicates the occurrence of photo-damage to PSII in plants watered once a week compared to plants watered daily. The maximum quantum yield of PSII in *C*. *triloba* can be related to the severe reduction in growth after 4 months, which was almost half of that in plants watered daily. The reduction in photosynthetic activity of plants is caused by various factors including stomatal closure, inefficient electron transport and a decrease in photosynthetic enzymes **(Bartwal et al. 2013)**.

Chlorophyll fluorescence in *C*. *triloba* leaves was significantly reduced in terms of ΦPSII, qP and relative ETR in plants watered once a week for a duration of 2 and 4 months (**Figs. 4.2** and **4.3**). Interestingly, plants watered daily also showed a reduction in ΦPSII and relative ETR compared to plants watered thrice and twice a week particlularly after 4 months. Quantum yield of PSII measures the proportion of energy absorbed and utilized for photochemistry, thus determining the photosynthetic capacity of plants **(Maxwell and Johnson 2000)**. The ΦPSII parameter is the main

target damaged during photo-inhibition. The decrease in ΦPSII of *C*. *triloba* leaves might have caused PSII photochemical changes which reduced the overall photosynthetic capacity of the plants. **Lu and Zhang (1999)** suggested that reduction in ΦPSII and increase in NPQ in water stressed plants is a mechanism to down-regulate photosynthetic electron transport to match the decline in CO_2 assimilation. Therefore, a decrease in ΦPSII and an increase in NPQ rate in *C*. *triloba* must have been an adaptation strategy for the plants to reduce their photosynthetic activity while preventing leaf cell death. Similar to the findings by **Liberato et al. (2006)**, reduction in ΦPSII and relative ETR possibly had detrimental effects on the capacity of the plastoquinone complexes which are responsible for the photochemical transport of electrons amongst photosystems I and II for oxi-reduction reactions in plants watered once a week. However, it is not very clear what might have caused the reduction of ΦPSII in plants watered daily since the *Fv*/*Fm* value in leaves showed no signs of stress. The significant increase in NPQ particularly in 4-month-old plants watered once a week triggered leaf protection as a response to light-induction damage **(Maxwell and Johnson 2000)**. Non-photochemical quenching regulates and protects photosynthetic processes in environments which have light energy absorption exceeding light utilization. Nonetheless, there was minimal change in NPQ levels at high actinic light intensities suggesting that plants could not efficiently regulate photosynthetic processes and therefore induced photo-inhibition in leaves.

4.5.2. Effect of different watering regimes on endogenous phytohormones

Biosynthesis, distribution and signal transduction of phytohormones can be modified by water stress, thus stimulating induction of specific protective mechanisms. In the current study, greenhouse cultivation of *C*. *triloba* lead to the accumulation of six stress-related phytohormones (four after 2 months and six after 4 months).

Biosynthesis of JA-Ile, *cis*OPDA and SA compounds was significantly reduced in 2-month-old plants watered once a week (**Fig. 4.5**). Even though, the reduction in water supply enhanced the accumulation of ABA in these plants. Biosynthesis of ABA during drought stress in *Zea mays* **(Du et al. 2010; Wang et al. 2008)** and *Phaseolus vulgaris* **(Pospisilova et al. 2005)** has been documented. Abscisic acid has been recognized in the induction of a number of stress-response genes that are expressed individually or synergistically. These include activation of stress-associated genes which results in high production of osmoprotectants, LEA proteins, signalling and transcriptional regulation **(Bartels and Sunkar 2005)**. The expressed genes maintain cellular water status in plants while protecting the protein/enzyme molecules together with cellular organelles from injuries caused by water stress. Activated signalling pathways can result in stomatal closure and reduction in leaf expansion, thus reducing plant growth. Accumulation of ABA with a decline in some of the quantified phytohormones played an important role in the survival of *C*. *triloba* watered once a week. It is likely that the phytohormone triggered the activation of various stress response mechanisms resulting in the reduction of chlorophyll fluorescence inevitably reducing plant growth.

Stress-related phytohormones in 4-month-old plants were largely dependent on the evaluated plant parts. For instance, JA-Ile and *cis*OPDA (**Fig. 4.5 a** and **b**) production was higher in roots. Jasmonic acid and IAA (**Fig. 4.5 c** and **d**) were only detected in roots. Abscisic acid and SA (**Fig. 4.5 e** and **f**) were higher in leaves. Jasmonic acid and IAA were detected in low concentrations in plants watered once a week relative to plants watered daily. Likewise, induction of drought stress via reduction in soil moisture content caused a decline in IAA concentration and an increase in ABA levels in wheat **(Bano and Yasmeen 2010)**. **Du et al. (2013)** also found a significant decline in IAA levels with increased JA concentration in *Zea mays* after being exposed to

severe drought stress. The authors suggested that JA and IAA biosynthesis/signalling can be regulated by drought stress, and precise hormonal balance is essential for plant growth and development. Jasmonic acid together with its biosynthetic precursor (*cis*OPDA) and its active form (JA-Ile) are known to be regulated during osmotic stress and wounding **(Floková et al. 2014; Grebner et al. 2013)**. These JAs drastically increase in leaves and roots of *Arabidopsis* during osmotic stress **(Grebner et al. 2013)**. However, in the present study, lower concentrations of JA-Ile and *cis*OPDA were observed in leaves of plants watered once a week. These JAs were accumulated in much smaller quantities than in roots. Therefore, high accumulation of JA-Ile and *cis*OPDA in roots rather than in leaves of *C*. *triloba* suggest the involvement of jasmonates in root development. The phytohormones (JA-Ile and *cis*OPDA) accumulation in 2 and 4-month-old leaves might have prevented leaf senescence triggered by low water potential in plants. Abscisic acid and SA content was significantly higher in leaves of plants watered once a week (**Fig. 4.6**). Abscisic acid plays a significant role in signifying osmotic stress and activation of signal pathways which convey the message to shoots and roots, eventually resulting in water-saving anti-transpiration mechanisms **(Wilkinson et al. 2012)**. However, excessive production of ABA and reduced SA levels in roots and SA increase in 4-month-old leaves might have been a stress response triggered by reduced water availability in *C*. *triloba*. In *Arabidopsis* mutants, production of SA increased accumulation of reactive oxygen species (ROS) thereby inducing stomatal closure and drought tolerance mechanisms **(Miura et al. 2013)**. *Phillyrea angustifolia* shrubs activated various photo- and antioxidative protection mechanisms through the upregulation of SA levels under drought conditions **(Munné-Bosch and Peñuelas 2003)**. Therefore, higher levels of SA in 4-month-old plants compared to 2-month-old plants is indicative of the

phytohormone induced defensive mechanisms after prolonged growth with limited water supply. Phytohormone fluctuation alters cellular dynamics in plants under stress conditions, thereby regulating plant growth and survival **(Kohli et al. 2013)**. Activation of signal pathways by phytohormones is dependent on the intensity, nature and period of stress in plants **(Khan and Khan 2013)**. Therefore, these factors contributed greatly to the biosynthesis of stress responsive phytohormones particularly in plants with limited water supply.

4.5.3. Effect of different watering regimes on phenolic acid content

Phenolic acid production is triggered by various environmental stress conditions and biosynthesized via the shikimic and malonic acid pathways. Most phenolics are derived from phenylalanine catalysed by phenylalanine ammonia lyase (PAL) **(Taiz and Zeiger 2002)**. However, the activity of PAL is complicated by its presence in multiple PAL-encoding genes, activated in specific tissues and upregulated by certain environmental stresses in different species **(Logemann et al. 1995)**. Water availability and soil composition are well-known to have a marked effect on the biosynthesis of phenolic acids. Water availability in the soil significantly influenced phenolic acid (hydroxybenzoic and hydroxycinnamic acid) accumulation in *C*. *triloba* after 2 and 4 months of growth. Amongst the quantified compounds, ferulic and *p*-coumaric acids were the most abundant derivatives detected in leaves. Their production was mostly enhanced in plants watered once a week particularly after 4-months of growth than in plants watered daily. Ferulic and *p*-coumaric acid compounds are produced in plant cell walls and can be esterified to pectins and arabinoxylans or cross-linked to cell wall polysaccharides in a form of dimers such as dehydroferulates and truxillic acid **(Naczk and Shahidi 2006)**. The cross-links improve cell-cell bonds, serves as a site for lignin formation and adds to the thermal stability of plant texture. Furthermore, these

hydroxycinnamates are synthesized in response to wounding and defence against pathogens **(Kroon and Williamson 1999)**. Therefore, high accumulation of total phenolics in *C*. *triloba* might be due to the increased production of ferulic and *p*-coumaric acids **(Masondo et al. 2016a; Masondo et al. 2016b)**. Besides the aforementioned compounds, watering once a week significantly induced bioactive compounds especially in 4-month-old plants. Similarly, shoots and roots of *Hypericum brasiliense* accumulated more phenolic acids after being exposed to drought conditions **(Nacif de Abreu and Mazzafera 2005)**. Drought stress induces phenolic acid synthesis through the phenylpropanoid pathway **(Dixon and Paiva 1995)**. Therefore, limited water supply and an extended periods of exposure (4-months) might have triggered the expression of PAL genes thus enhancing phenolic acid levels in *C*. *triloba*. Based on these results, there was a trade-off between growth and phenolic acid production in plants watered once a week. The trade-off might have resulted from fewer resources allocated for primary metabolism (e.g. growth), thus bringing about protective secondary metabolites under water stress conditions **(Cheynier et al. 2013)**. Furthermore, phenolic acids are regulated by agronomic conditions and harvesting period similar to most vegetable species **(Tiwari and Cummins 2013; Tomás-Barberán and Espin 2001)**.

4.5.4. Effect of different watering regimes on mineral and total carbohydrate content

Conventional edible leafy vegetables with mineral composition exceeding 1% per plant dry weight are considered to have relatively high mineral content. The Food and Nutrition Board, Institute of Medicine, National Academies has estimated the daily recommended dietary allowance (RDA) of minerals in adults as Ca (1000 mg), phosphorous (P; 800 mg), copper (Cu; 900 mg), Zn (10 mg), Mg (400 mg), Mn (7 mg) and Fe (8 mg) as reported by **Odhav et al. (2007)**. Mineral content in traditional

vegetables has been estimated to contain about 10% of the RDA. Moreover, *C*. *triloba* is amongst the traditional vegetables with mineral concentrations exceeding 1% of plant dry weight **(Odhav et al. 2007)**. Based on the RDA values, the estimated mineral content in *C*. *triloba* was remarkable (**Table 4.6**). Estimation of mineral content in *C*. *triloba* showed a remarkable increase in Ca and K, which were >10000 mg/kg (DW). High contents of Ca have been reported in *C*. *sesamoides* leaves **(Fasakin 2004)**. Similar to *C*. *triloba*, Ca levels in *C*. *sesamoides* increased with extended periods of growth. On the contrary, carbohydrate content in *C*. *triloba* was higher in 2-month-old plants relative to 4-month-old plants. This was also the case in *C*. *sesamoides* which accumulated high levels of carbohydrates after 6 weeks of growth with a slight decrease in 10-week-old plants **(Fasola and Ogunsola 2014)**. Essentially, the nutritive value of plants was mostly influenced by the different watering regimes as well as the duration of growth. This was observed in the high levels of mineral and carbohydrate content in plants watered daily and thrice a week for a period of 2 months. On the other hand, plants watered twice and once a week accumulated high mineral and carbohydrate content after 4-months of growth. The high mineral content in plants watered once a week (4-months) was remarkable suggesting that the plants have the ability to maintain relatively good mineral value even after prolonged exposure to limited water supply. Likewise, drought stress had no effect on the nutritional value (Ca, Fe and Zn) in *Amaranthus hypochondriacus* and *Solanum scabrum* **(Luoh et al. 2014)**. Watering plants once a week reduced Mn, Na and Zn content in 2 and 4-month-old *C*. *triloba*. The decline in Zn levels in *C*. *triloba* is of concern because the mineral accounts for micronutrient deficiency in humans. A number of factors directly or indirectly affect the nutritional value in crops including organic matter content and soil-water relationships, plant species/cultivar and cultural

practices **(Hornick 2009)**. According to **Murphy et al. (2008)**, there has been a significant decline in mineral content (except Ca) with an increase in wheat grain yields over the past decades. The authors suggested that the variation in mineral content of different cultivars of wheat might be due to the selection of low ash content in soft white wheat cultivars, consequently reducing mineral nutrition in modern wheat cultivars. According to a review by **Frossard et al. (2000)**, the consistent decline in essential minerals is due to breeding techniques that merely focus on increasing crop yields and disease resistance in wheat and rice grains. Therefore, these shortcomings can be counteracted by application of fertilizers and good plant breeding techniques.

4.6. Concluding remarks

In an attempt to improve cultivation of *C*. *triloba*, the effects of different watering regimes on growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acid content and nutritional value were assessed. Generally, the growth of *C*. *triloba* was significantly reduced in plants watered once a week relative to plants watered daily. The deleterious effect of limited water supply was evident in the photosynthetic potential of the plants, with *Fv*/*Fm* values consistent with those of stressed plants. Furthermore, watering plants once a week significantly reduced the levels of ΦPSII, qP and relative ETR while enhancing NPQ rate in 4-month-old plants. Increase in NPQ levels together with a decline in ΦPSII values brought about the survival of the plants via the reduction of photosynthetic activity, thus preventing leaf cell death. This can then be related with the excessive production of ABA and SA in plants watered once a week relative to plants watered daily. These results therefore showed that plants grown in a limited water supply had enhanced ABA content, which activated stress response signalling pathways, in turn resulting in stomatal closure and reduction in leaf expansion, ultimately leading to plant survival. Therefore, precise regulation of

phytohormone content during the cultivation of *C*. *triloba* must have resulted in hormonal cross-talk, thus improving plant growth. Furthermore, there was also a trade-off between primary and secondary metabolic pathways which resulted in less resources assigned for plant growth and high production of phenolic acids for plant defence. On the contrary, primary metabolic pathways involving mineral and carbohydrate accumulation in leaves of *C*. *triloba* were induced with remarkably high amounts of Ca, K and Mg in plants. These mineral nutrients were also enhanced in plants watered once a week especially after 4-months of growth even though the plants had reduced growth due to limited resource allocated for primary metabolic pathways.

Chapter 5: Effect of Kelpak® and vermicompost leachate (VCL) on *Ceratotheca triloba* seedling growth under salinity stress condition

5.1. Introduction

Globally, salinity stress is one of the major environmental factors limiting plant growth, crop productivity and survival rate. An estimated 800 million ha of arable land is salt-affected, equating to more than 6% of the world's total land area **(FAO 2008)**. Based on the FAO/UNESCO Soil Map of the World, of the available 230 million ha of arable land utilized for irrigation, 45 million ha (19.5%) has been estimated to be salt-affected, while of 1500 million ha of agricultural land, 32 million ha (2.1%) is salt-affected. This continuous increase in salinization is estimated to decrease arable land productivity by up to 30% in the next 25 years, and 50% by the year 2050 **(Wang et al. 2003)**. A number of factors have been accounted to contribute to salinized agricultural soil including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water and poor cultivation practices **(Ashraf and Foolad 2007)**.

The deleterious effects of salinity stress on plant growth and development are associated with ion toxicity (Na^+ and Cl^-), low osmotic potential (water stress), nutritional imbalance or a combination of these factors **(Ashraf and Harris 2004)**. Ion toxicity in the soil interferes with various processes in plants including enzyme structure and certain macromolecules, cell organelle damage, reduction in photosynthesis and respiration, inhibition of protein synthesis and ion deficiencies **(Porcel et al. 2011)**. The toxicity levels of Na^+ towards important enzymes has been estimated to be above 100 mM, equitably similar to Cl^- levels **(Munns 2002)**. In order to prevent damage caused by Na^+, plants restrict ion uptake at the plasma membrane and promote the extrusion of ions from the cytoplasm **(Hasegawa et al. 2000)**. Plasma membrane H^+-ATPases are responsible for the transport of solutes such as Na^+ and

K^+ in plants. An increase in ATPase-mediates H^+ translocation in the plasma membrane, thus preventing a build-up of NaCl concentration in plant cells **(Watad et al. 1991)**. Another major problem associated with salinity stress is the loss of intracellular water availability (low water potential). Generally, water transpiration in plant's is approximately 30 - 70% higher compared to the water utilized for cell expansion especially under different weather conditions **(Munns 2002)**. Therefore, accumulation of solutes in the soil have a 30 – 70% chance of being taken up by the plant if not excluded by the roots. Plants then avoid such stress through the production of compatible solutes such as sugars (e.g. fructose, sucrose, trehalose and fructans), glycine, betaine, proline and ectoine **(Mahajan and Tuteja 2005)**. Biosynthesis of these osmolytes prevent intracellular water loss in the cells through the reduction of cell water potential. In addition, their synthesis under low osmotic potential does not affect the plants normal metabolic reaction as their function aids in plant cell osmotic adjustment. Lastly, the uptake of excessive soluble salts by the roots can potentially cause osmotic stress in plants subsequently impeding sufficient nutrient uptake. Accumulation of Na^+ and Cl^- disturbs and subsequently decreases the uptake of K^+, Ca^{2+} and Mn^{2+} **(Karimi et al. 2005)**. The toxic effect of Na^+ in the cytosol is caused by the disturbance of K^+ homeostasis (an essential element required for growth and development) with K^+ competing for the binding sites with Na^+ **(Bartels and Sunkar 2005)**. Excessive Na^+ accumulation inhibits nutrient uptake through root plasma membrane transport interference and root growth hindrance. Toxic ions restricts water and nutrient (e.g. P, Fe or Zn) uptake as well as growth promoting microorganisms such as mycorrhizal fungi **(Parvaiz and Satyawati 2008)**. The combination of these factors not only impairs plant growth but also affects soil fertility, therefore more

research is focusing on finding methods that can alleviate the effect of salinity stress on plant growth and development.

To date, research has been geared towards the use of biostimulants in order to improve plant growth and development as well as stress tolerance under salinity stress conditions **(Chinsamy et al. 2013; Çimrin et al. 2010; Ertani et al. 2013)**. However, the mode of action of these biostimulants in improving stress tolerance remains poorly understood and could be attributed to the presence of a number of plant growth regulators (PGRs) and their role in enhancing endogenous concentrations of stress-related molecules such as cytokinins, proline, antioxidants and antioxidant enzymes **(Calvo et al. 2014)**. Information regarding mechanisms activated by biostimulants is very complex because of the number of naturally occurring or commercially applied micronutrients, sugars and amino acids that could potentially have complementary or no effects on plant growth **(Saa et al. 2015)**. Therefore, isolating the effect of a single active ingredient present in a biostimulant solution is likely to give unreliable results due to the synergistic effects of the different compounds. For these reasons, biostimulants should be characterised based on their action in plants or the plants physiological response towards the biostimulants rather than on their composition **(Bulgari et al. 2014)**. The current study was therefore aimed at determining the effect of biostimulants [Kelpak® and vermicompost leachate (VCL)] on *C*. *triloba* growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional contents under salinity stress conditions. In addition, the study evaluated the influence of 2 and 4 month harvesting stages on the aforementioned parameters.

5.2. Materials and methods

5.2.1. Biostimulant source and preparation

Kelpak® was obtained from Kelp Products (Pty) Ltd, in Simon's Town, South Africa and diluted as described on the manufacture's label (0.4%). Vermicompost leachate (VCL 1:10 v/v) was purchased from Wizzard Worms Commercial Company Ltd, Greytown, South Africa. According to the manufacturer, VCL (pH 7.82) is derived from garden waste using the red earthworm *Eisenia fetida* and contains nitrogen (2.26%), phosphorus (0.99%), potassium (0.64%), calcium (2.52%) and sodium (631.03 mg/kg). Details on VCL are available from the company (http://www.wizzardworms.co.za).

5.2.2. Seed source, germination and transplantation

Ceratotheca triloba seeds were purchased from Silverhill Seeds Nursery, Cape Town, South Africa. The experiment was conducted in the greenhouse at the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa. Seed germination and seedling growth of *C*. *triloba* was performed as described in **Section 4.2.1**. Established seedlings were randomized into six treatments with their respective controls (untreated). Seedlings were treated with varying concentrations of NaCl (0, 75, 150 mM) concentrations. For each NaCl concentration, 20 ml was applied three times a week per pot (soil drench) for the duration of the experiment. The different treatments (0, 75, 150 mM) were treated with 50 ml of Kelpak® or VCL which was soil drenched five times (per pot) for the duration of the experiment. Essentially, Kelpak® or VCL were applied twice in the first 2 months and again three-times within 4 months. The soil was kept hydrated daily to prevent water stress reported in **Chapter 4**. After 2-months; chlorophyll fluorescence, endogenous phytohormone, phenolic acids and nutritional content were measured. At the termination of the experiment (4 months);

plant fresh weight (leaf weight, root length, root weight, plant height) as well as the aforementioned parameters were recorded.

5.2.3. Chlorophyll fluorescence determination

During the 2 and 4-month growth periods, intact leaves of *C*. *triloba* grown under salinity conditions were measured for chlorophyll fluorescence using a pulse modulated fluorometer (Model FMS-2, Hansatech Instruments, King's Lynn, UK) as described in **Section 4.2.2**. Parameters evaluated included the maximum quantum efficiency of PSII (*Fv*/*Fm*), actual quantum yield of photosystem II (ΦPSII), photochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transport rate (ETR). Chlorophyll fluorescence was measured at different actinic light intensities: 56, 134, 850 and 1279 µmol PAR m^{-2} s^{-1} switched on at a regular (5 min) intervals. Data was downloaded to a computer, recorded and subjected to analysis of variance (ANOVA).

5.2.4. Quantification of stress-related phytohormones using Ultra High Performance Liquid Chromatography-tandem mass spectrometry (UHPLC–MS/MS)

Lyophilized 2 and 4-month-old *C*. *triloba* leaves and roots (2.5 – 3 mg) were transferred into Eppendorf tubes containing 2 mm ceria-stabilized zirconium oxide beads (Retsch GmbH & Co. KG, Haan, Germany). Extraction of jasmonoyl isoleucine (JA-Ile), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA), jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and indole-3-acetic acid (IAA) was conducted as described in **Section 4.2.3**. The phytohormones were analyzed using an Acquity UHPLC® system (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer Xevo™ TQ MS (Waters MS Technologies, Manchester, UK) **(Floková et al. 2014)**. The analyzed compounds and appropriate internal standards were quantified in multiple

ion monitoring mode (MRM) using optimized MS conditions and continuous polarity-switching data measurements. The MRM transitions were recorded in a chromatographic run in ten targeted scan windows to get the highest possible MS signal intensity for each compound. MassLynx™ software package (version 4.1, Waters, Milford, MA, USA) was used to control the instrument, obtain and process the MS data. Analysis was done in triplicate.

5.2.5. Phenolic acid quantification using Ultra High Performance Liquid Chromatography-tandem mass spectrometry (UHPLC–MS/MS)

Lyophilized 2 and 4-month-old leaf material (30 mg) was transferred into Eppendorf tubes. Phenolic acid analysis of *C*. *triloba* material was conducted as described in **Section 4.2.4**. The UHPLC-MS/MS quantification was evaluated using UHPLC™ system (Waters, Milford, MA, USA) connected synchronously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating in a negative mode **(Gruz et al. 2008)**. Analysis was done in triplicate.

5.2.6. Total carbohydrate and elemental analysis

Total carbohydrates were estimated using 0.1 g of 2 and 4-month-old leaf material as outline in **Section 4.2.5**. Absorbance was measured at 490 nm against a blank in a UV-visible spectrophotometer (Varian Cary 50, Australia). A standard graph of carbohydrate was prepared using glucose to calculate the carbohydrate content (% Dry Weight). Analysis was done in triplicate.

Dry leaf material (0.1 g) was digested using an aluminium heating block. Elemental analysis of *C*. *triloba* was performed as described in **Section 4.2.5**. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Varian 720-ES, Varian Inc, Palo Alto, CA, USA) instrument was used to evaluate the elemental concentration in samples **(Hou and Jones 2000)**. The ICP-OES was operated as follows: RF power 1.0 kW; viewing geometry axial; Argon gas used as plasma gas flow at the rate of 15.0 l/min; auxiliary gas flow rate 1.50 l/min; nebulizer gas flow rate 0.75 l/min; and replicate reading time 9.0 s.

5.3. Data analysis

Statistical differences between the mean values of treated and untreated plants was analysed using a Student's *t* test package. Data was further subjected to ANOVA using SPSS for Windows (SPSS®, Version 22.0. Armonk, New York, USA). The significant levels were determined at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). For endogenous phytohormones and phenolic acids, data processing was carried out with MassLynx™ software (version 4.0, Waters, Milford, MA, USA).

5.4. Results

5.4.1. Effect of Kelpak® and vermicompost leachate (VCL) on growth and chlorophyll fluorescence

Salinity stress caused a severe decline in the overall growth of *C*. *triloba* grown for 4 months (**Tables 5.1** and **5.2**). The deleterious effects of NaCl were evident at the highest concentration (150 mM; **Data not shown**), with no survival observed after 4 months of growth. To some degree, application of Kelpak® improved the leaf weight (7.4 g) and root length (97.1 mm) in plants treated with 75 mM NaCl.

Table 5.1: Effect of Kelpak® on the growth of *Ceratotheca triloba* under salinity stress after 4 months in the greenhouse.

Concentration	Leaf weight (g)			Root length (mm)			Root weight (g)			Plant height (mm)		
of NaCl (mM)	Untreated	Kelpak-treated		Untreated	Kelpak-treated		Untreated	Kelpak-treated		Untreated	Kelpak-treated	
Control	9.3 ± 0.74	9.7 ± 0.60	ns	98.7 ± 12.31	133.7 ± 13.25	ns	17.0 ± 1.55	14.7 ± 1.58	ns	1725.7 ± 59.20	1732.9 ± 45.54	ns
75	4.6 ± 0.38	7.4 ± 1.09	*	66.3 ± 7.62	97.1 ± 10.05	*	6.5 ± 1.07	6.9 ± 0.97	ns	1171.7 ± 78.38	1310.0 ± 72.24	ns

For each parameter, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

Table 5.2: Effect of vermicompost leachate (VCL) on the growth of *Ceratotheca triloba* under salinity stress after 4 months in the greenhouse.

Concentration	Leaf weight (g)			Root length (mm)			Root weight (g)			Plant height (mm)		
of NaCl (mM)	Untreated	VCL-treated		Untreated	VCL-treated		Untreated	VCL-treated		Untreated	VCL-treated	
Control	16.2 ± 1.41	8.7 ± 0.37	***	107.8 ± 12.00	91.6 ± 15.39	ns	12.5 ± 1.63	11.3 ± 2.59	ns	1210.0 ± 42.65	1299.3 ± 94.93	ns
75	3.2 ± 1.25	5.2 ± 1.71	ns	30.0 ± 10.00	52.5 ± 7.50	ns	2.4 ± 0.73	2.5 ± 0.16	ns	425.0 ± 125.00	620.0 ± 20.00	ns

For each parameter, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

The growth of *C*. *triloba* was also slightly improved with the application of VCL at 75 mM NaCl concentration. However, VCL proved to be more detrimental to plants grown without NaCl (control). The damaging effects of prolonged exposure to salinity stress in leaves was apparent in the measured *Fv*/*Fm* values, particularly after 4 months (**Tables 5.3** and **5.4**). Maximum quantum yield of PSII ranged from 0.71 – 0.82 in 4-month-old plants treated with 75 mM NaCl (with/without biostimulants). In 2-month-old plants, VCL showed inhibitory effect on the *Fv*/*Fm* value (0.79) in plants grown at 150 mM NaCl concentration. Kelpak® and VCL slightly increased *Fv/Fm* levels in 4-month-old NaCl-treated (75 mM) plants.

Table 5.3: Effect of Kelpak® on the chlorophyll fluorescence (*Fv/Fm*) of *Ceratotheca triloba* under salinity stress after 4 months of growth in the greenhouse.

Concentration of NaCl (mM)	*Fv*/*Fm* 4 months	
	Untreated	Kelpak-treated
Control	0.82 ± 0.00	0.82 ± 0.00 ns
75	0.80 ± 0.02	0.82 ± 0.01 ns

For each parameter, data is represented as mean value ± standard error ($n = 7$) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl), maximum quantum efficiency of photosystem II (*Fv/Fm*).

Table 5.4: Effect of vermicompost leachate (VCL) on the chlorophyll fluorescence (*Fv/Fm*) of *Ceratotheca triloba* under salinity stress after 2 and 4 months of growth in the greenhouse.

Concentration of NaCl (mM)	*Fv*/*Fm* 2 months		4 months	
	Untreated	VCL-treated	Untreated	VCL-treated
Control	0.85 ± 0.00	0.85 ± 0.00 ns	0.83 ± 0.00	0.84 ± 0.00 ns
75	0.85 ± 0.00	0.85 ± 0.00 ns	0.71 ± 0.01	0.75 ± 0.03 ns
150	0.82 ± 0.00	0.79 ± 0.01 *	NG	NG

For each parameter, data is represented as mean value ± standard error ($n = 7$) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl), maximum quantum efficiency of photosystem II (*Fv/Fm*), no growth (NG).

There was a significant decline in ΦPSII and qP levels with an increase in actinic light intensity (1279 µmol PAR m^{-2} s^{-1}), while NPQ and ETR levels were enhanced with increasing levels of actinic light intensities after 2 and 4 months of plant growth. Chlorophyll fluorescence levels (ΦPSII, qP, NPQ, relative ETR) in leaves of *C*. *triloba* were not considerably improved with the application of Kelpak® in 4-month-old plants, with some exceptions (**Figs. 5.1** and **5.2**). There was a severe decline in ΦPSII, qP and relative ETR in VCL-treated plants grown at 150 mM NaCl compared to the control treatment after 2 months of growth (**Figs. 5.3** and **5.4**). Even so, VCL improved the levels of ΦPSII, qP and relative ETR in plants exposed to 150 mM NaCl. Severity of NaCl on the evaluated chlorophyll fluorescence parameters was more noticeable after 4 months of growth. Despite the application of biostimulants, there was minimal/insignificant improvement in the chlorophyll fluorescence of *C*. *triloba* leaves under NaCl conditions. In general, there was a significant decline in the chlorophyll parameters (ΦPSII, qP, NPQ, relative ETR) evaluated particularly after 4 months of growth (**Appendix B, Table 1**).

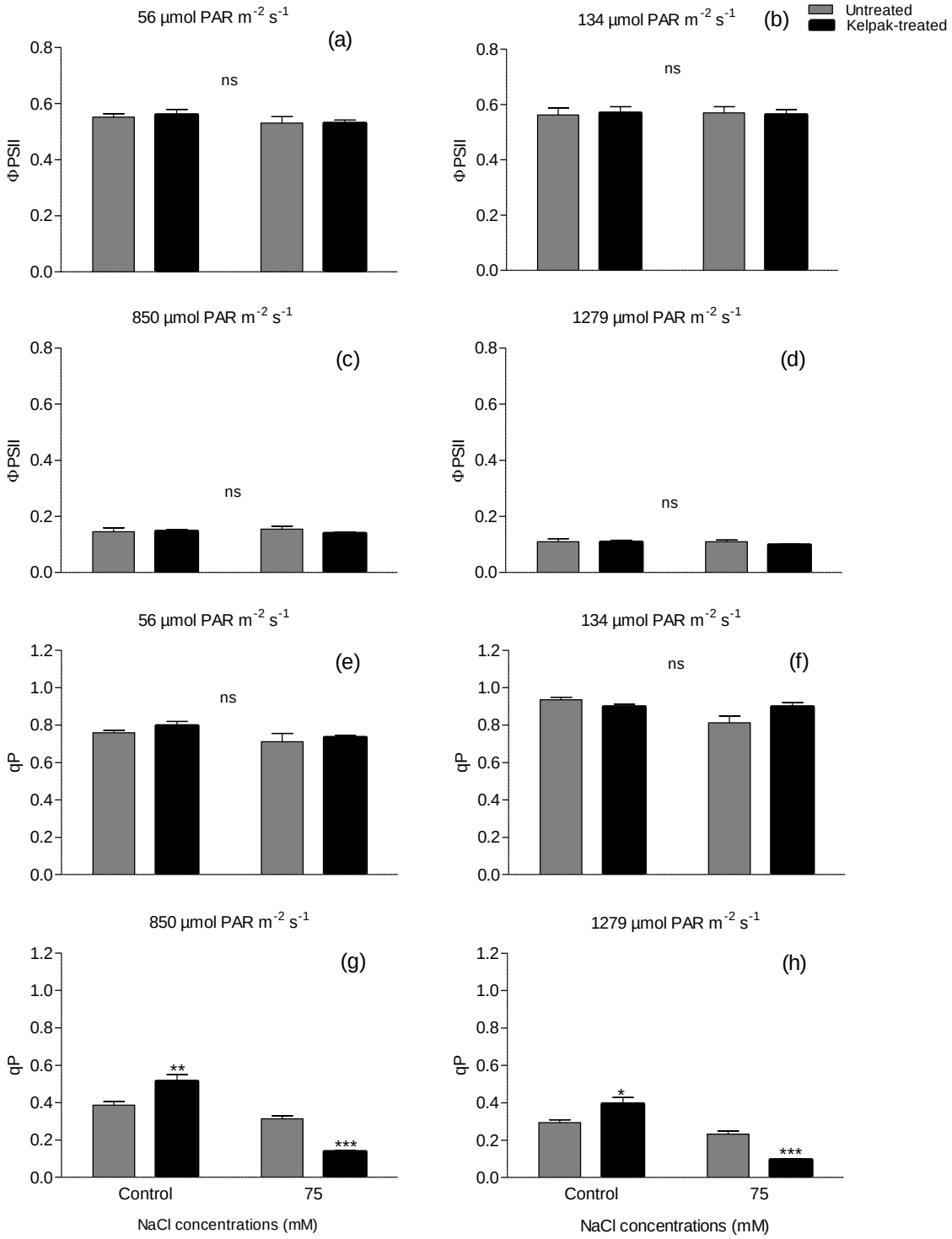


Fig. 5.1: Effect of Kelpak® on quantum yield of photosystem II (ΦPSII, a-d) and phytochemical quenching (qP, e-h) in *Ceratotheca triloba* under salinity stress after 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

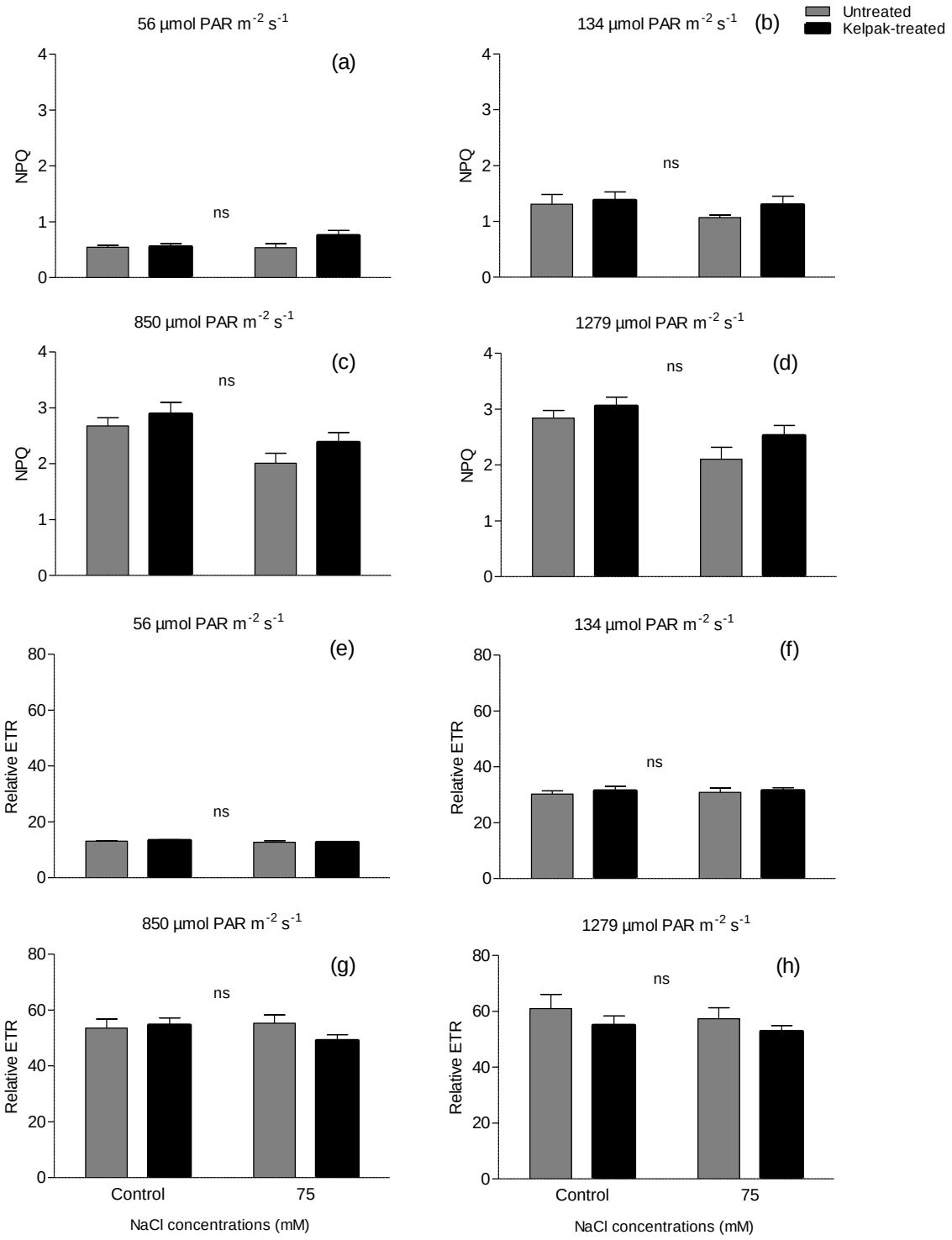


Fig. 5.2: Effect of Kelpak® on non-photochemical quenching (NPQ, a-d) and relative electron transfer rate (ETR, e-h) in *Ceratotheca triloba* under salinity stress after 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

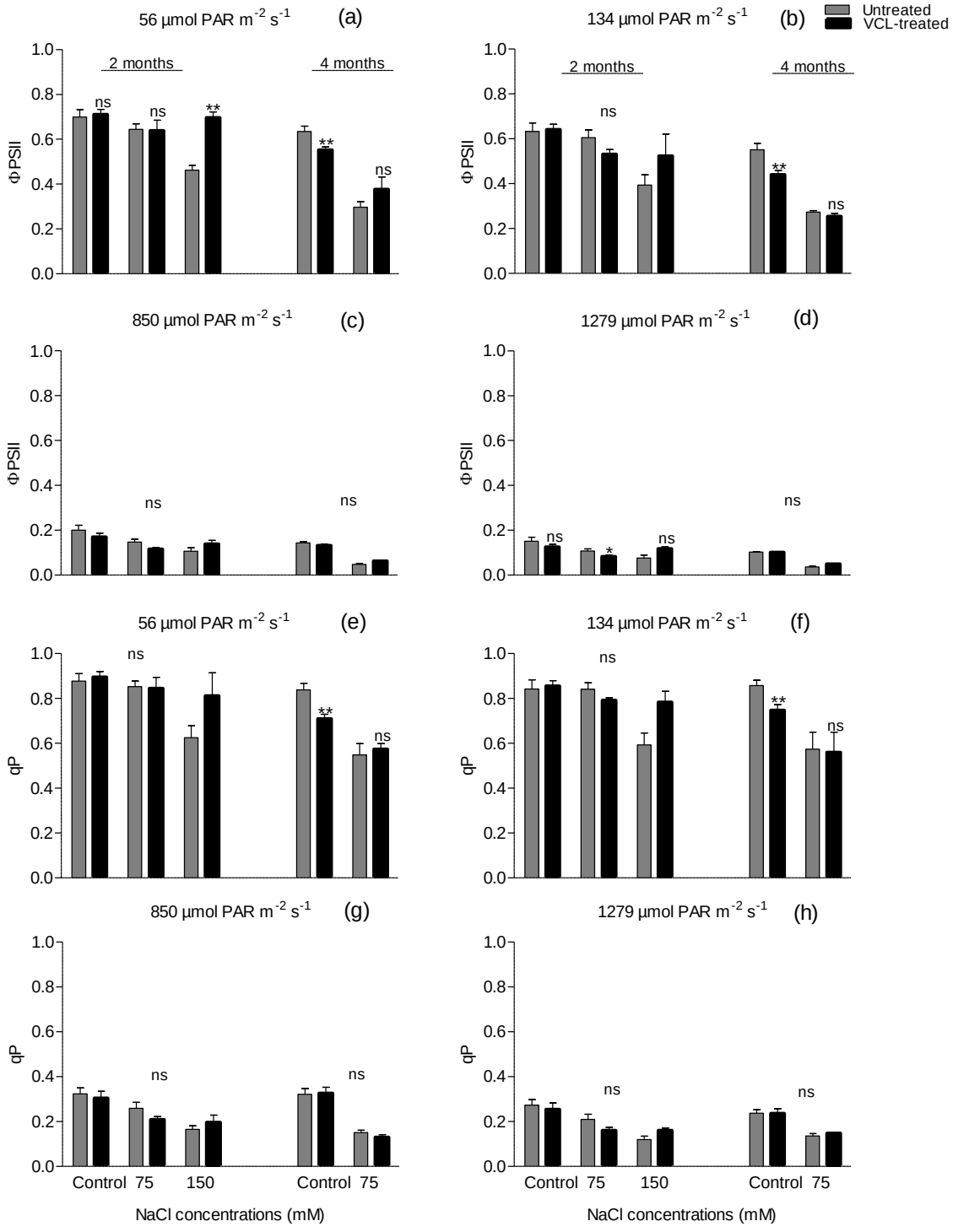


Fig. 5.3: Effect of vermicompost leachate (VCL) on quantum yield of photosystem II (ΦPSII, a-d) and phytochemical quenching (qP, e-h) in *Ceratotheca triloba* under salinity stress after 2 and 4 months of growth in greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl).

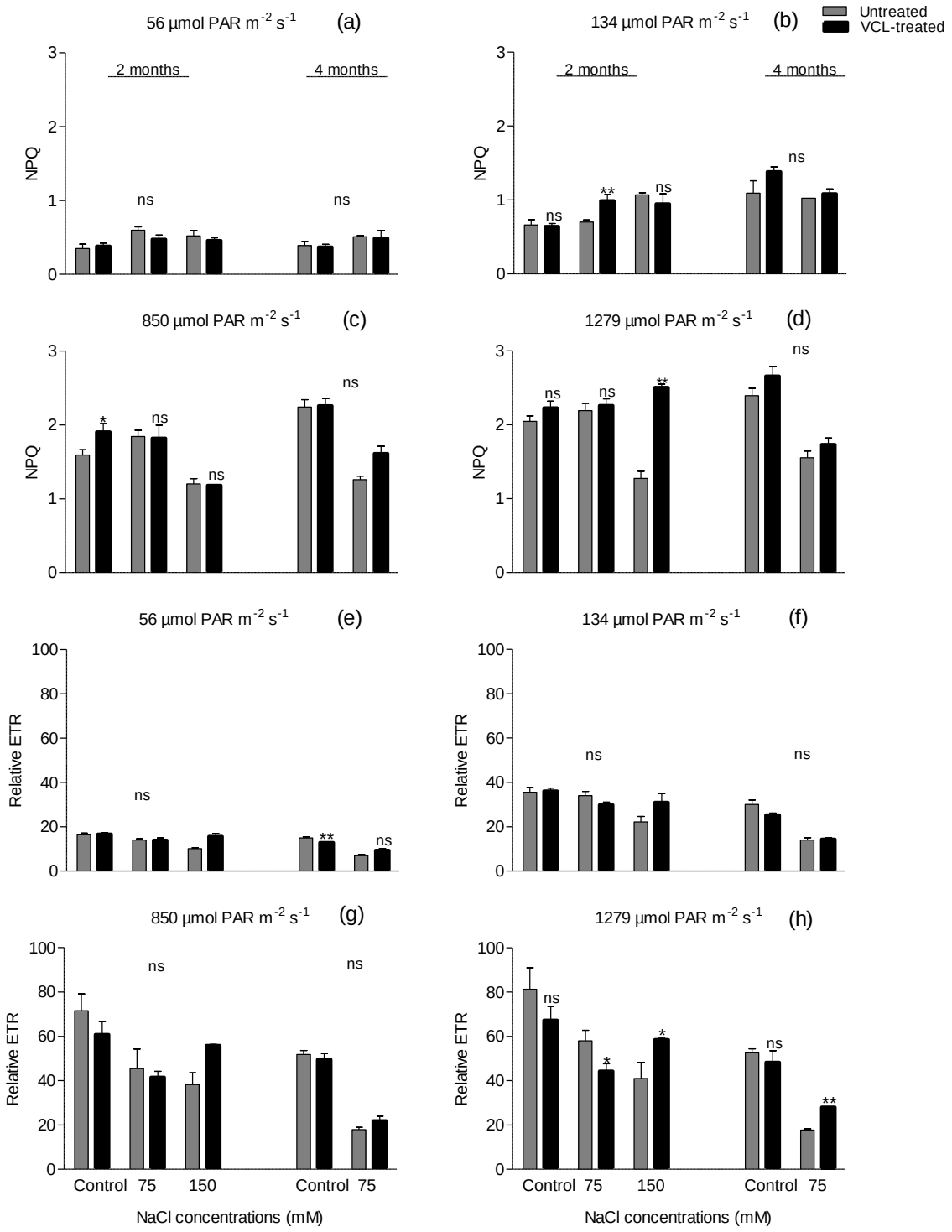


Fig. 5.4: Effect of vermicompost leachate (VCL) on non-photochemical quenching (NPQ, a-d) and relative electron transfer rate (ETR, e-h) in *Ceratotheca triloba* under salinity stress after 2 and 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. P ≤0.05 (*), P ≤0.01 (**), P ≤0.001 (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

5.4.2. Effect of Kelpak® and vermicompost leachate (VCL) on stress-related endogenous phytohormones

The UHPLC analysis resulted in the identification of a total of six endogenous phytohormones; with only five phytohormones identified in 2-month-old leaves, four identified in 4-month-old leaves and all six phytohormones identified in 4-month-old roots (**Figs. 5.5** - **5.10**). These included JA-Ile (active form of JAs), *cis*OPDA (JAs precursor), ABA, SA, JA and IAA. Amongst these, SA was detected in higher quantities ranging from 5 – 50 nmol/g DW. Furthermore, JA was only detected in 2-month-old leaves (**Fig. 5.7**) as well as 2 month-old leaves and 4 month-old roots (**Fig**. **5.10)**, while IAA was only detected in roots. In most cases, phytohormone levels were reduced in NaCl-stressed plants treated with Kelpak® and VCL. For instance, JA-Ile and *cis*OPDA content significantly declined in leaves treated with Kelpak® at 75 (2 and 4 months) and 150 mM (4 months) NaCl concentrations (**Fig. 5.5**). However, the biostimulant enhanced the production of JA-Ile and *cis*OPDA in roots at 75 mM NaCl concentration. Abscisic acid concentration decreased in 2-month-old NaCl-stressed (75 and 150 mM) plants treated with Kelpak®, while the biostimulant enhanced ABA levels in 4-month-old roots (**Fig. 5.6**). Likewise, Kelpak® increased the concentration of SA in 2-month-old leaves under 75 and 150 mM NaCl. In 4-month-old plants, Kelpak® reduced SA in leaves while increasing phytohormone levels in roots at 75 mM NaCl.

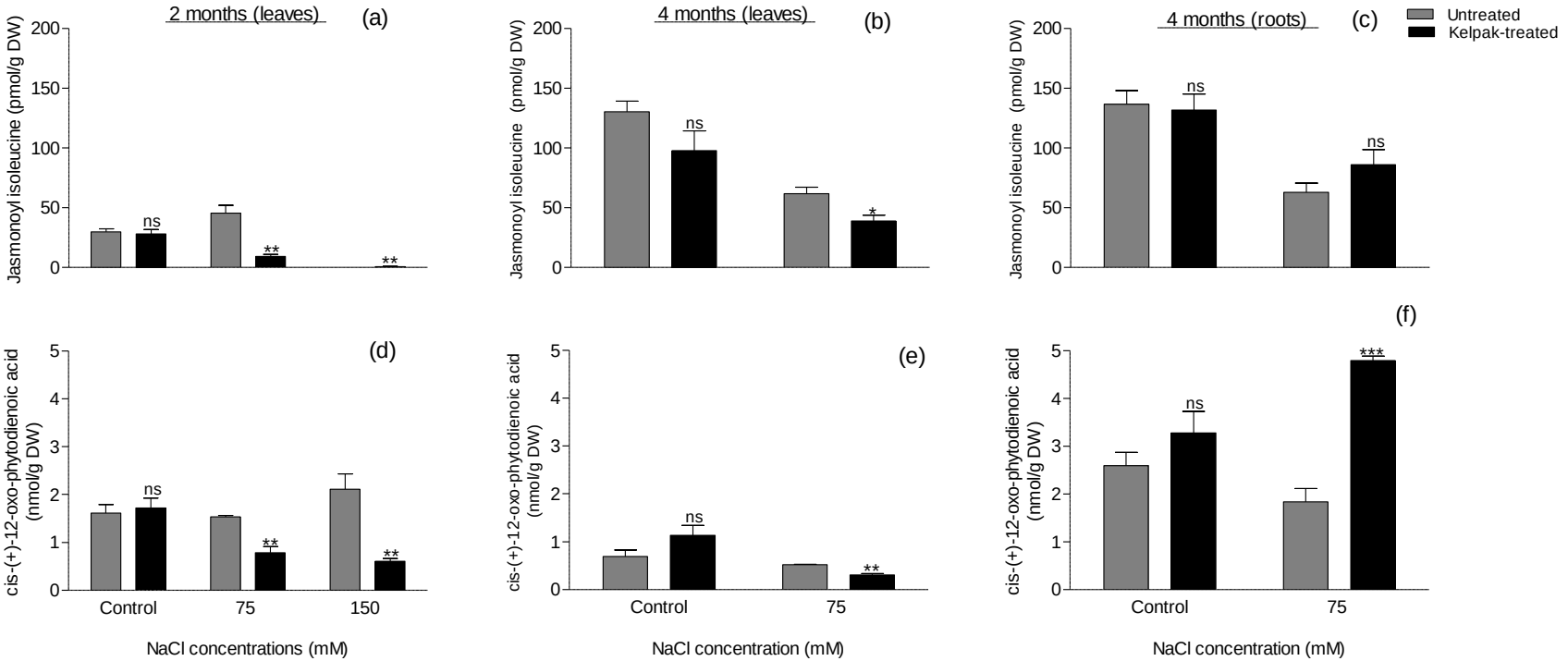


Fig. 5.5: Effect of Kelpak® on endogenous phytohormones: jasmonoyl isoleucine (JA-Ile; active form of JAs) and cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

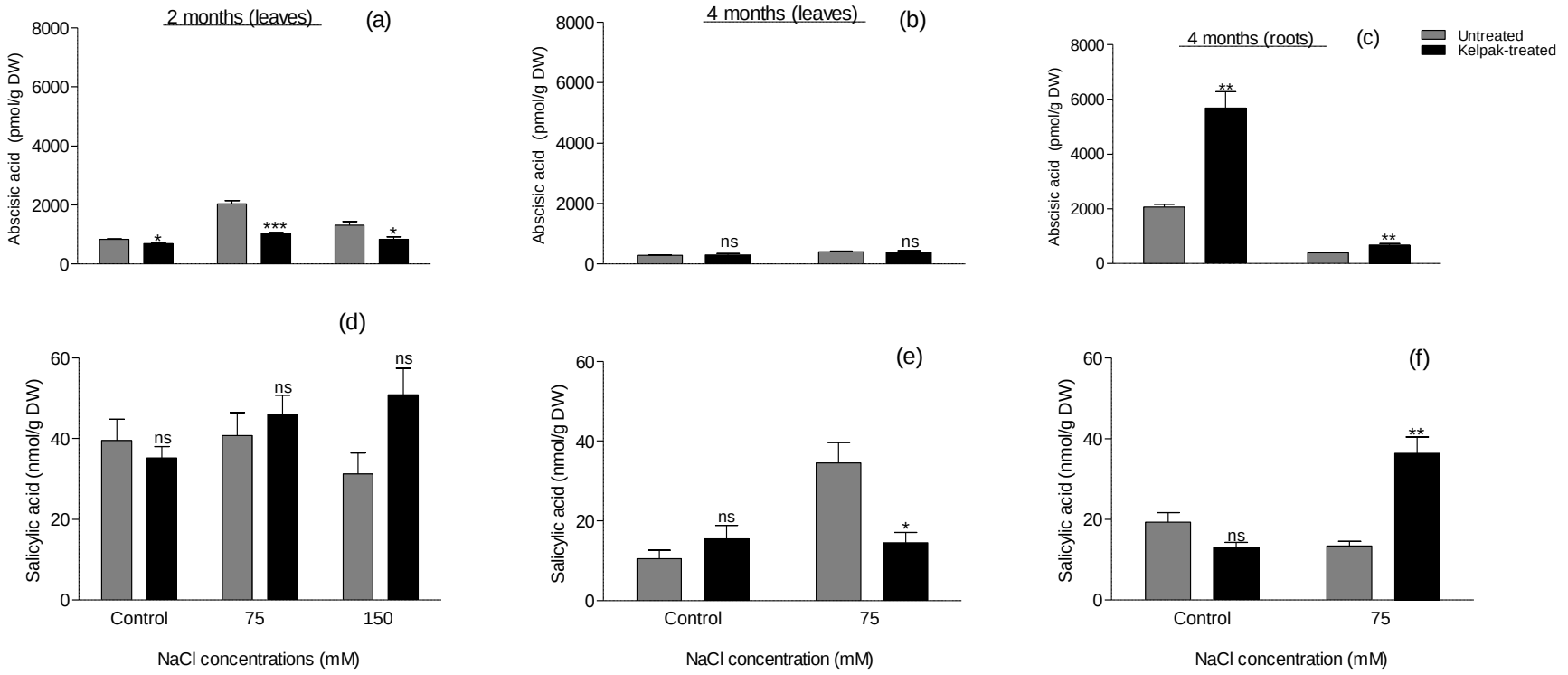


Fig. 5.6: Effect of Kelpak® on endogenous phytohormones: abscisic acid (ABA) and salicylic acid (SA) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

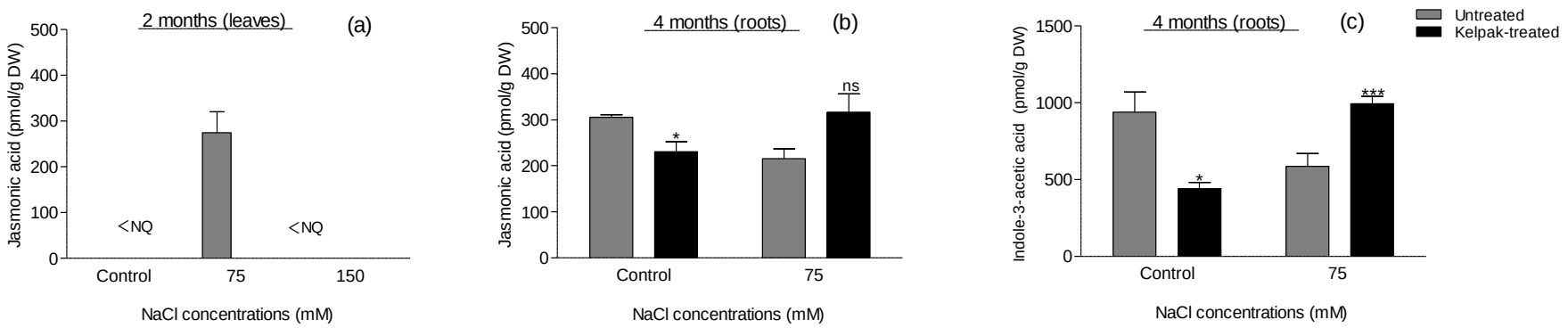


Fig. 5.7: Effect of Kelpak® on endogenous phytohormones: jasmonic acid (JA) and indole-3-acetic acid (IAA) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl), not quantified (Internal standard was not detected; NQ).

Vermicompost leachate application significantly inhibited JA-Ile production in 2-month-old leaves at 75 and 150 mM NaCl (**Fig. 5.8**). In 75 mM NaCl-treated plants, VCL application induced JA-Ile production (25 pmol/g DW) in 4-month-old leaves while reducing JA-Ile content (23 pmol/g DW) in roots. Biosynthesis of *cis*OPDA was higher in 2-month-old leaves than in 4-month-old leaves and roots with/without VCL. A gradual decline was observed in the biosynthesis of ABA and SA content with prolonged exposure to NaCl in untreated leaves and roots of *C*. *triloba* plants, with some exceptions (**Fig. 5.9**). In most cases, VCL reduced ABA and SA content in the control treatment after 2 and 4 months of growth, while increasing phytohormone content in NaCl-stressed (75 mM) leaves. Abscisic acid and SA significantly declined in roots treated with VCL at 75 mM NaCl. Jasmonic levels were higher in roots than in 4-month-old leaves (**Fig. 5.10**). Addition of VCL was inhibitory towards the production of JA in the control treatment and at 75 mM NaCl in leaves and roots. Although VCL decreased IAA (211 pmol/g DW) concentration in the control treatment, the biostimulant significantly enhanced the levels of IAA (585 pmol/g DW) at 75 mM NaCl. Overall, the leaves of *C*. *triloba* accumulated high levels of *cis*OPDA, ABA and SA after 2 months of growths, and JA-Ile was better stimulated in 4-month-old plants (**Appendix B, Table 2** and **3**).

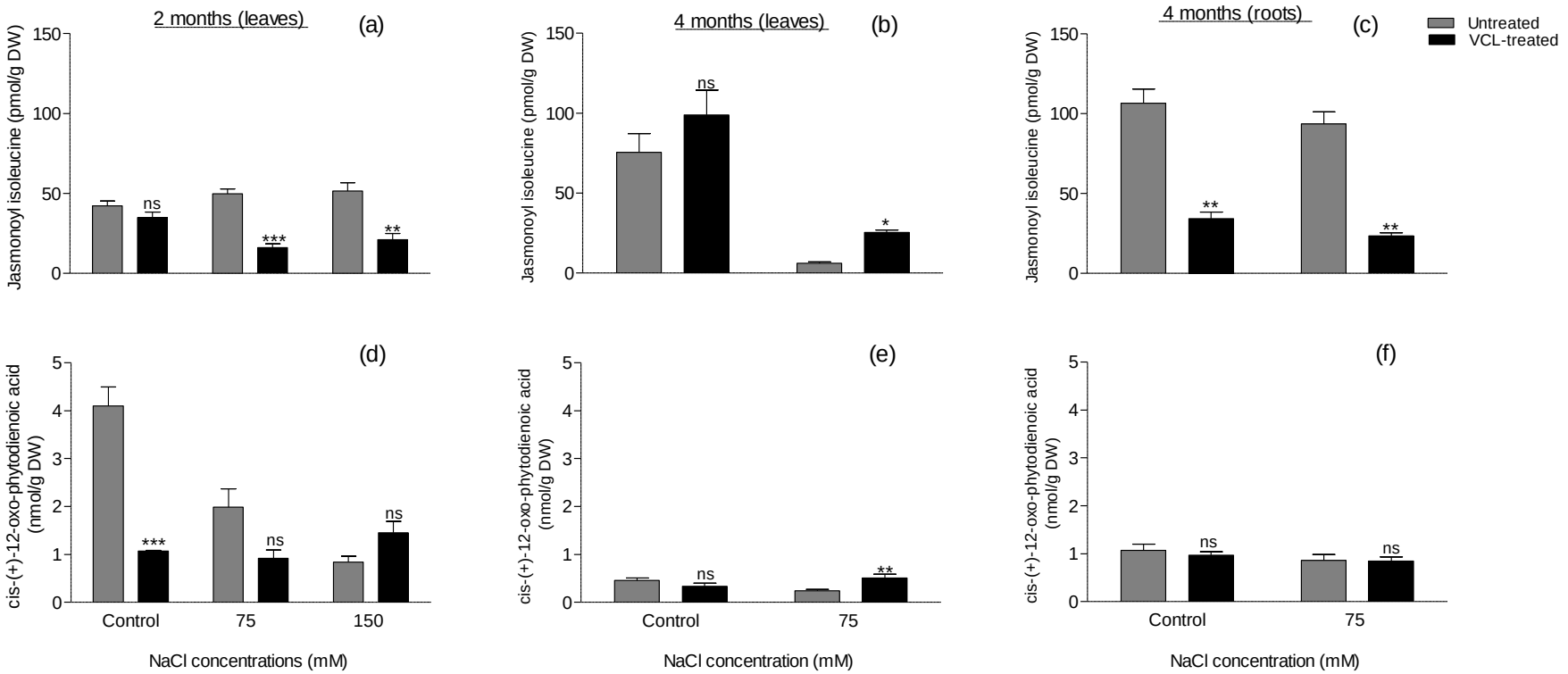


Fig. 5.8: Effect of vermicompost leachate (VCL) on endogenous phytohormones: jasmonoyl isoleucine (JA-Ile; active form of JAs) and cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

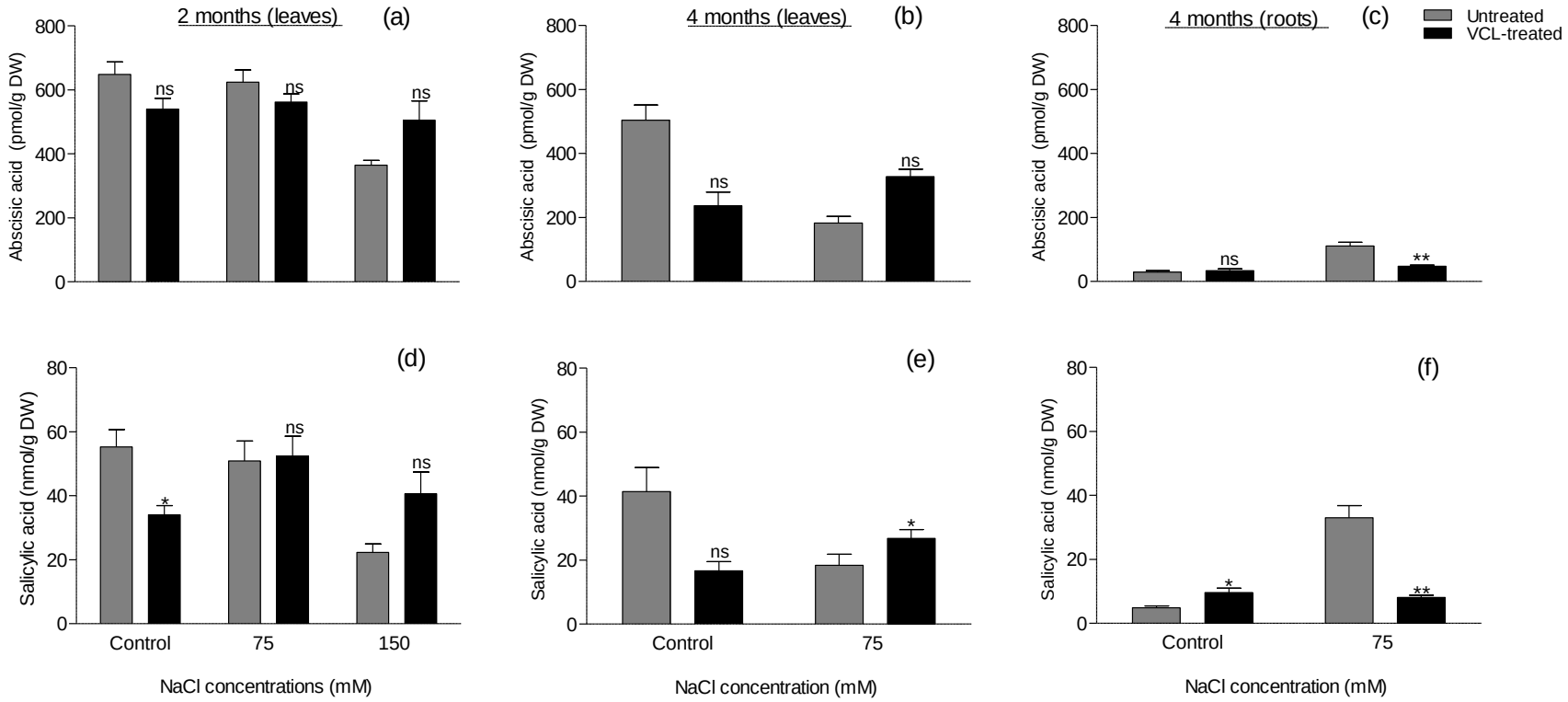


Fig. 5.9: Effect of vermicompost leachate (VCL) on endogenous phytohormones: abscisic acid (ABA) and salicylic acid (SA) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

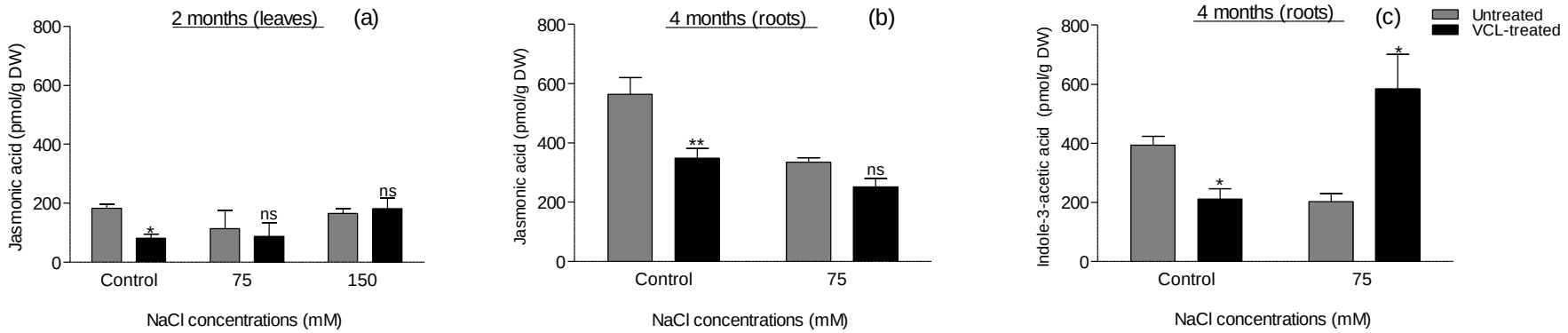


Fig. 5.10: Effect of vermicompost leachate (VCL) on endogenous phytohormones: jasmonic acid (JA) and indole-3-acetic acid (IAA) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

5.4.3. Effect of Kelpak® and vermicompost leachate (VCL) on phenolic acid content

As shown in **Figs. 5.11** and **5.12**, three hydroxybenzoic and three hydroxycinnamic acids were detected in greenhouse-cultivated *C*. *triloba*. From these, 4-coumaric acid (c.a. 70 µg/g DW) and ferulic acid (c.a. 15 µg/g DW) were the most abundant phenolics in the plants. In most cases, 2-month-old NaCl-stressed (75 and 150 mM) plants had increased phenolic acid content relative to the control treatment (**Fig. 5.11**). Nonetheless, application of Kelpak® enhanced phenolic acid content in 150 mM NaCl-stressed plants, with exception to ferulic and 4-coumaric acids. In comparison to 2-month-old plants, accumulation of bioactive compounds was significantly lower in 4-month-old plants with/without Kelpak®, especially under salinity stress conditions (**Appendix B, Table 4**). The synthesis of phenolic acids in 2 and 4-month-old *C*. *triloba* was largely reduced with the application of VCL (**Fig. 5.12**). This was more evident in the control treatment after 4 months of growth. Except for the untreated control treatment, phenolic acid concentration was more abundant in 2-month-old plants than in 4-month-old plants (**Appendix B, Table 5**).

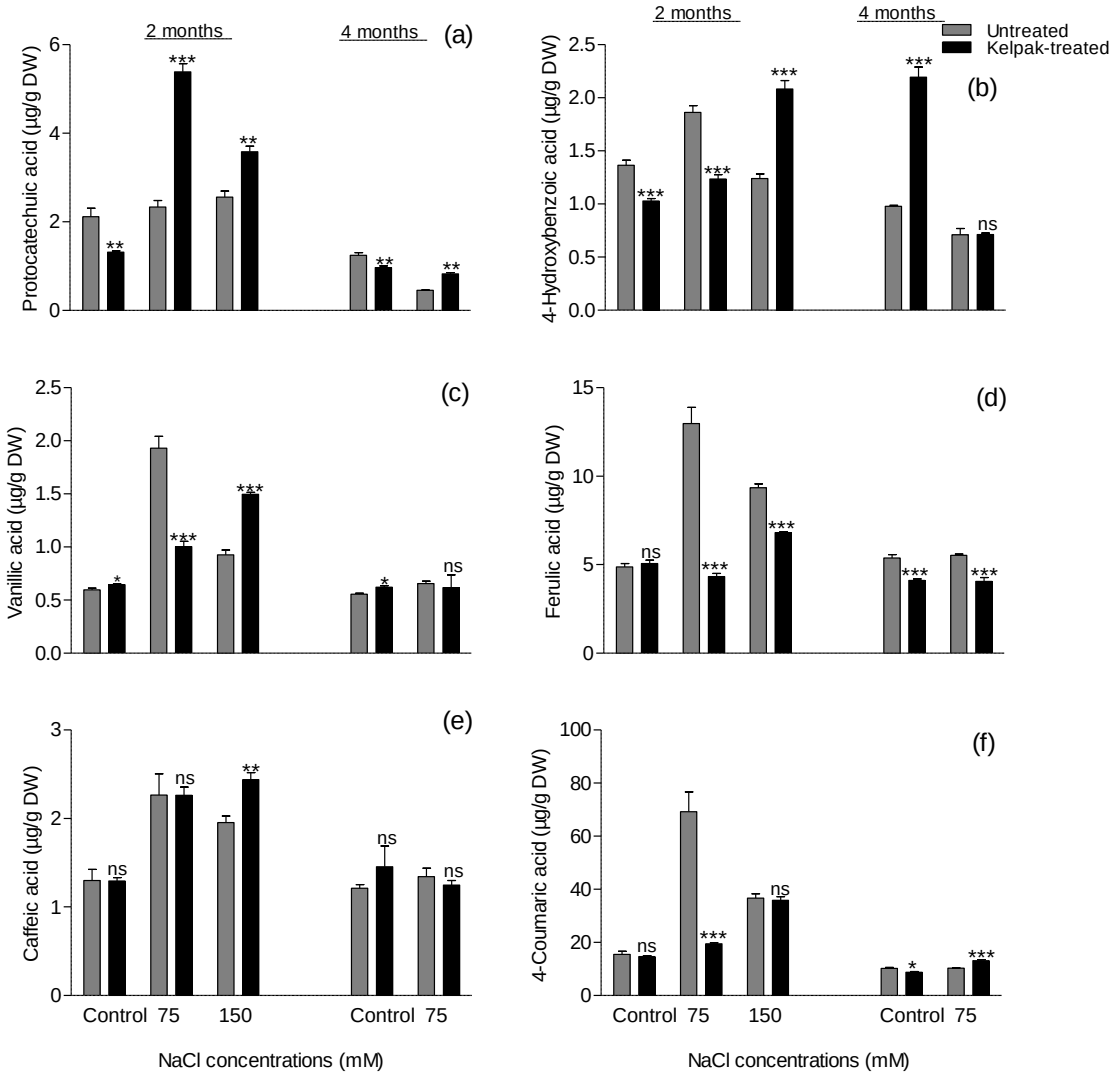


Fig. 5.11: Effect of Kelpak® on phenolic acid content in *Ceratotheca triloba* after 2 and 4 months in the greenhouse under salinity stress conditions. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

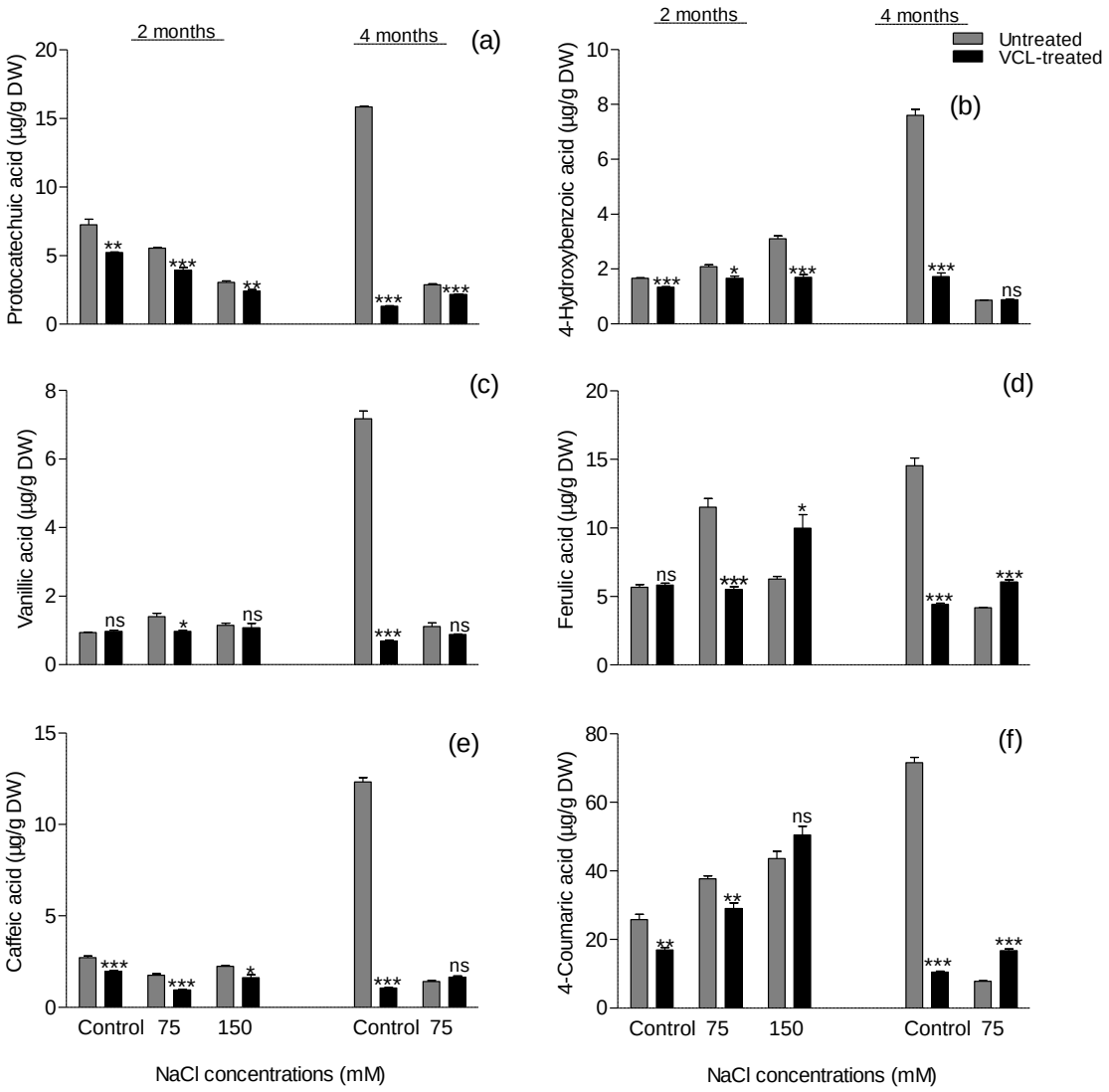


Fig. 5.12: Effect of vermicompost leachate (VCL) on phenolic acid content in *Ceratotheca triloba* after 2 and 4 months in the greenhouse under salinity stress. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Sodium chloride (NaCl).

5.4.4. Effect of Kelpak® and vermicompost leachate (VCL) on mineral and total carbohydrate content

As shown in **Tables 5.5** and **5.6**, nutritional composition of *C*. *triloba* varied greatly with macro elements such as Ca, K, Mg ranging from 303 – 44473 mg/kg; micro elements Fe, Mn, Zn ranging from 1 – 3717 mg/kg and carbohydrate content at 3 – 24%. In a number of cases, application of Kelpak® and VCL increased the mineral and carbohydrate content in NaCl-stressed plants. The promotive role of Kelpak® on the nutritional content of leaves was mostly observed in the control treatment and at 150 mM NaCl after 2 months of growth (**Table 5.5**). However, Kelpak® had an inhibitory effect on the mineral levels of 2 and 4-month-old plants at 75 mM NaCl. In **Table 5.6**, VCL stimulated mineral composition in plants treated with 75 and 150 mM NaCl after 2 and 4 months. Mineral content in these plants was relatively high (almost double) compared to untreated plants. However, VCL application in the control treatment caused a reduction in mineral levels except for the Fe and K elements in 2-month-old plants. Plants grown for 2 months mostly enhanced carbohydrate content (%), whereas 4-month-old plants accumulated higher levels of mineral composition.

Table 5.5: Effect of Kelpak® on the mineral content and total carbohydrates (%) in *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress.

Harvest period	Concentration of NaCl (mM)	Ca		Fe		K		Mg		Mn		Na		Zn		% Carbohydrates	
		mg/kg (DW)															
		Untreated	Kelpak-treated	Untreated	Kelpak-treated	Untreated	Kelpak-treated	Untreated	Kelpak-treated	Untreated	Kelpak-treated	Untreated	Kelpak-treated	Untreated	Kelpak-treated	Untreated	Kelpak-treated
2-Months	Control	1172	14861	93	824	1296	21052	303	4013	3	uv	7	109	14	uv	20	11
	75	15848	13578	334	257	17039	19891	3921	3421	66	1	422	126	372	uv	11	15
	150	14398	15980	440	1466	21616	17431	4033	3805	uv	180	470	2050	35	116	7	7
4-Months	Control	23997	20564	356	405	20101	21085	3411	3251	6	8	78	54	48	22	24	10
	75	27210	18084	427	382	32102	19995	5428	3642	181	9	1177	468	102	53	13	18

Calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), zinc (Zn), below detection limit (uv).

Table 5.6: Effect of vermicompost leachate (VCL) on the mineral content and total carbohydrates (%) in *Ceratotheca triloba* after 2 and 4 months of growth in the greenhouse under salinity stress.

Harvest period	Concentration of NaCl (mM)	Ca		Fe		K		Mg		Mn		Na		Zn		% Carbohydrates	
		mg/kg (DW)															
		Untreated	VCL-treated	Untreated	VCL-treated	Untreated	VCL-treated	Untreated	VCL-treated	Untreated	VCL-treated	Untreated	VCL-treated	Untreated	VCL-treated	Untreated	VCL-treated
2-Months	Control	18681	10684	337	365	21518	22313	4990	3465	101	73	363	362	138	74	8	19
	75	10199	16110	166	209	16196	21889	2364	3855	90	118	1838	2144	100	108	nd	nd
	150	16734	35143	254	505	28421	44473	3285	5639	122	20	3717	1236	96	717	nd	nd
4-Months	Control	21904	20326	409	654	26339	27617	3780	3923	12	9	16	14	9	44	3	10
	75	13072	18807	202	286	11703	33760	2568	4481	7	136	343	2721	46	71	nd	8

Calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), zinc (Zn), not determined (nd).

5.5. Discussion

Salinity stress is amongst the major agricultural constraints limiting crop productivity worldwide. The cause of concern is that several agriculturally important crops have not been bred for such conditions, hence management practices are being challenged beyond their infrastructure capabilities **(Arioli et al. 2015)**. These problems have lead to an urgent need for new and alternative technologies in the improvement of plant productivity under abiotic stresses **(Zalabák et al. 2013)**. Thus, more research has focused on the use of organic fertilizers/products such as seaweed extract and vermicomposting in order to prevent environmental degradation caused by salinity stress while improving crop yields.

5.5.1 Effect of Kelpak® and vermicompost leachate (VCL) on growth and chlorophyll fluorescence

Ceratotheca triloba growth remarkably declined under NaCl stress conditions in 4-month-old plants. Excessive reduction in plant growth was due to the high concentration of salts in the soil which creates a low water potential zone, subsequently reducing the plant's ability to access adequate water **(Mahajan and Tuteja 2005)**. The severe decline in *C*. *triloba* growth as a result of limited water supply and uptake by the roots has been discussed extensively in **Chapter 4**. Furthermore, the harmful effects of high NaCl might have resulted in nutritional imbalance, excessive ion uptake (Na^+ and Cl^-), transport or partitioning within the plants **(Ashraf and Harris 2004)**. According to **Munns (2002)**, plant survival under salinity stress is dependent on the exclusion of salt in older leaves by compartmentalizing it in vacuoles before it reaches toxic levels. Excessive sodium ion levels have a toxic effect in cell metabolism resulting in reduced cell division and expansion, membrane disorganization and osmotic imbalance **(Tuteja 2007)**, premature senescence in older

transpiring leaves and decreased photosynthetic potential **(Munns 2002)**. Therefore, high concentrations of NaCl in *C*. *triloba* exerted similar effects, which caused premature senescence, reduced plant growth and eventually lead to plant death at 150 mM NaCl. Nevertheless, the detrimental effects of NaCl were to some extent ameliorated with the application of Kelpak® or VCL in plants treated with 75 mM NaCl (**Tables 5.1** and **5.2**). Biostimulants have been established to improve plant metabolism for maximum yield production, plant tolerance towards abiotic stresses, efficiently aiding in nutrient assimilation and translocation as well as enhancing crop quality **(Calvo et al. 2014)**. Often, they improve plant growth through effective water usage and promote the growth of beneficial microbes in the soil **(Khan et al. 2009)**. The stimulatory effect of Kelpak® was noticeable in leaf weight and root length of *C*. *triloba* at 75 mM NaCl. This may be partly due to the cocktail of plant growth regulators (PRGs) contained in the Kelpak® extract **(Stirk et al. 2014)**, essential for plant adaptation to salinity stress **(Bulgari et al. 2014)**. On the other hand, VCL had minimal or an even detrimental effect on the growth of *C*. *triloba* in the control treatment. The minimal or detrimental effects of VCL on the growth of *C*. *triloba* has been reported during nutrient stress **(Masondo et al. 2016b)**. Even so, the biostimulant slightly improved plant growth at the 75 mM NaCl treatment. These results indicate the positive role of VCL under NaCl stress conditions relative to its deleterious effect under normal growth conditions (control treatment). According to **Hu and Schmidhalter (2005)**, nutrient application can improve, reduce or have no significant effect on the plant's resistance to saline conditions depending on the severity of the stress. One can then deduce that *C*. *triloba* plants grown without NaCl (control treatment) had sufficient nutrients for the improvement of growth, whereas application of VCL in these plants stimulated high nutrient concentration consequently hindering plant growth.

Salinity stress affects stomatal conductance and photosynthetic gas exchange in plants. These responses are associated with the plants photosynthetic response towards reduced leaf water potential which inevitably leads to stomatal closure thus reducing carbon dioxide uptake. In stressed plants, stomatal closure increases susceptibility to photo-damage, a process which results in increased non-photochemical quenching (NPQ) energy dissipation **(Zribi et al. 2009)**. In the current study, salinity stress caused a reduction in ΦPSII and qP levels while enhancing NPQ and relative ETR levels with an increase in actinic light intensities. Chlorophyll fluorescence levels (ΦPSII, qP, NPQ and relative ETR) in the leaves of *C*. *triloba* were reduced in 4-month-old 75 mM NaCl-stressed plants with/without VCL, suggesting that salinity stress induced the inhibition of PSII electron transport. Although 2-month-old plants grown under NaCl conditions treated with VCL had a slight change in chlorophyll fluorescence relative to plants grown under normal conditions (control). The minimal effect of NaCl in 2-month-old plants might have been due to the increase in NPQ levels whereas, prolonged exposure (4 months) to salinity stress affected leaf photosynthetic activity even though there was an increase in NPQ levels. This was also the case with the leaves of Kelpak®-treated plants even though chlorophyll fluorescence was reduced in plants grown under 75 mM NaCl conditions after 4 months. These results can be related to the decline in the *Fv*/*Fm* values especially after 4-months of growth in NaCl-stressed plants. As reported by **Mehta et al. (2010)**, salinity stress reduces *Fv*/*Fm* values because of electron transport inhibition at the acceptor side of the PSII reaction center. Furthermore, prolonged exposure of plants to salinity stress conditions reduces chlorophyll fluorescence parameters which decreases water potential and stomatal conductance in leaves **(Zribi et al. 2009)**. The severity of salinity stress in plants is mostly noticeable in the decreased efficiency of

excitation energy capture by open photosystem II (PSII) (Fv´/Fm´), ΦPSII and qP parameters **(Zhang et al. 2009)**. Regardless of the minimal stimulatory effect of Kelpak® or VCL in the improvement of chlorophyll fluorescence in the leaves of *C*. *triloba*, salinity stress still reduced the photosynthetic efficiency of plants. The ability of plants in withstanding prolonged NaCl-stress (2 and 4 months) suggests that *C*. *triloba* plants are able to dissipate light energy efficiently in order to reduce photo-damage in leaves while improving plant growth.

5.5.2. Effect of Kelpak® and vermicompost leachate (VCL) on endogenous phytohormone content

Endogenous phytohormones in *C*. *triloba* showed the presence of six stress-related phytohormones under salinity stress conditions. Phytohormone content in *C*. *triloba* was dependent on a number of factors including different NaCl concentrations, type of biostimulants, plant parts evaluated and the duration of growth. In most cases, biostimulants significantly reduced phytohormone content after 2 and 4 months of growth especially under NaCl stress. This was observed in the reduction of ABA levels with the application of Kelpak® and VCL under salinity stress (**Figs. 5.6** and **5.9**). Exposure of plants to salt stress activates several common reactions in plants including endogenous phytohormones. Salinity stress induces ABA levels essential for plant protection and gene expression **(Parida and Das 2005)**. An increase in ABA levels resulting from Ca^{2+} uptake contributes to the maintenance of membrane integrity and regulate the uptake and transport of salt ions when there is excess salt concentration **(Chen et al. 2001)**. However, high levels of ABA in plants results in stomatal closure via the rapid alteration of ion fluxes in guard cells under salinity stress conditions. Furthermore, ABA synthesis has been associated with reduced growth during salinity stress **(Fahad et al. 2015; Javid et al. 2011)** via the regulation and

expression of salt-responsive-genes **(Narusaka et al. 2003)** which usually control plant water status **(Zhu 2002)**. Therefore, the potential effects of Kelpak® and VCL in down-regulating ABA production in *C*. *triloba* might have improved the plants photosynthetic activity, in turn enhancing growth and survival at 75 mM NaCl after 4 months. Biostimulants increase the cell membrane stability index, total free amino acids and IAA levels while reducing lipid peroxidation, electrolyte leakage and ABA levels which alleviates the harmful effects of NaCl stress **(Semida and Rady 2014)**. Regulation of ABA levels with the application of biostimulants in plants ameliorates the negative effects of salinity stress during plant growth and development through the reduction of proline content **(Moghaddam and Soleimani 2012)**. The inhibitory effect of biostimulants during the biosynthesis of ABA induces stress relief in plants via ABA independent pathways **(García et al. 2014)**. Based on established reports, biostimulants ability to repress/suppress ABA synthesis can be associated to the number of PGRs identified in Kelpak® and VCL **(Aremu et al. 2015b; Stirk et al. 2014)**. The PGRs in biostimulants together with endogenous phytohormones resulted in a synergetic or antagonist interaction, subsequently reducing ABA levels while alleviating the detrimental effects of salinity stress.

Salicylic acid is a signal molecule that induces defence mechanisms in plants. The phytohormone improves plant growth via the regulation of ion uptake and transport, photosynthetic rate, stomatal conductance, transpiration and accumulation of proline under salinity stress **(Fahad et al. 2015)**. In *C*. *triloba*, salinity stress increased the accumulation of SA (5 – 50 nmol/g DW) in both leaves and roots. The concentration of SA was also enhanced with the application of Kelpak® and VCL especially in 2-month-old leaves grown under 75 and 150 mM NaCl conditions. However, prolonged exposure of plants to salinity stress reduced the concentration of SA in 4-month-old

plants. Biosynthesis of SA in plants is essential for the development of stress symptoms and hypersensitive responses **(Horváth et al. 2007)**. Salicylic acid reduces the damaging effects of salinity stress by accelerating restoration of growth processes and improving plant tolerance **(Horváth et al. 2007; Sakhabutdinova et al. 2003)**. The phytohormone ameliorates injuries caused by stress through the increase of proline content and leaf electrolyte leakage **(Bastam et al. 2013)**, increase in leaf water relative content, nutrient uptake **(Yildirim et al. 2008)**, suppression of reactive oxygen intermediates (ROI) and lesion formation **(Yang et al. 2004)**. Considering that SA was the most abundant phytohormone detected in *C*. *triloba*, most physiological processes during NaCl stress might have been controlled by this phytohormone. Therefore, accumulation of SA in plants must have altered some stress-related signal pathways, subsequently improving plant tolerance/adaptation to NaCl-stress. These pathways were possibly associated with the regulation of ion uptake and transport as well as nutrient uptake due to the applied biostimulants. In addition, the biostimulants alleviated the harmful effects of NaCl through exogenously applied PGRs e.g. SA contained in seaweed extracts **(Calvo et al. 2014)**. Other phytohormones such as JAs have also been proposed to be signal transducers in responses to abiotic stress. Jasmonates increase rapidly and transiently in response to mechanical stress caused by salt via the up-regulation and down-regulation of specific gene expression **(Dombrowski 2003; Pedranzani et al. 2003)**. In *C*. *triloba*, biosynthesis of JA and its derivatives (*cis*OPDA and JA-Ile) was relatively low with the application of Kelpak® and VCL in 2-month-old plants. Nevertheless, regulation of JA-Ile, *cis*OPDA and JA was more dependent on the applied biostimulants and plant part evaluated in 4-month-old 75 mM NaCl-stressed plants. For instance, Kelpak® reduced JA-Ile and *cis*OPDA content in leaves while increasing the accumulation of JA-Ile, *cis*OPDA and JA in

roots. On the other hand, VCL enhanced JA-Ile and *cis*OPDA in leaves while decreasing JA-Ile and JA production in roots. Likewise, results relating to JA synthesis indicate that phytohormone synthesis is mostly dependent on tissue type, plant cultivar, development, and external stimuli **(Pedranzani et al. 2003; Tani et al. 2007; Wang et al. 2001)**. Furthermore, salinity stress has been shown to regulate endogenous JA levels in plants **(Kramell et al. 1995; Pedranzani et al. 2003)**.

Salinity stress induced the production of *cis*OPDA, ABA and SA with a reduction in JA-Ile, JA and IAA in *C*. *triloba*. Regulation of endogenous phytohormones is crucial in modulating physiological responses and expression of several genes involved in stress tolerance **(Peleg and Blumwald 2011)**. The application of biostimulants played an important role in altering phytohormone content in *C*. *triloba* especially at 75 and 150 mM NaCl. Kelpak® and VCL improved nutrient uptake, efficiency and efficient water use which are some of the major factors contributing to reduced crop yields under saline conditions. Furthermore, Kelpak® and VCL likely prevented or compartmentalized excessive ion uptake while regulating phytohormone production associated with plant growth during salinity stress. Even though biostimulants regulated phytohormone content in plants exposed to NaCl concentrations, 2 and 4 month growth period also influenced phytohormone synthesis. This was observed in the accumulation of *cis*OPDA, ABA and SA in 2-month-old leaves, rather than the production of JA-Ile in 4-month-old plants. These results imply that during the first 2 months of growth, the plants experienced high levels of stress thus accumulating increased levels of stress-responsive phytohormones. However, phytohormone declined after prolonged exposure (4 months) to salinity stress was a result of plant acclimatization and adaptation to salinity stress conditions.

5.5.3. Effect of Kelpak® and vermicompost leachate (VCL) on phenolic acid content

Salinity stress upregulates biosynthetic pathways involved in the production of commercially important bioactive compounds. These compounds are known to protect plants from the damaging effects of stress. In *C*. *triloba*, the leaves yielded a group of hydroxybenzoic and hydroxycinnamic acids under salinity stress conditions. In most cases, NaCl concentrations stimulated the production of phenolic acids in 2 and 4-month-old leaves whereas, Kelpak® and VCL mostly had inhibitory effects on the accumulation of bioactive compounds in plants, with a few exceptions. In those cases where biostimulants were effective, Kelpak® enhanced the synthesis of phenolic acids in 2-month-old 150 mM NaCl-stressed plants. Seaweed extracts are largely known to induce phenolic acid accumulation during stress **(Elansary et al. 2016)**. Kelpak® and eckol regulated hydroxybenzoic and hydroxycinnamic bioactive compounds in *Eucomis autumnalis* **(Aremu et al. 2015a; Aremu et al. 2016)**. The authors proposed that Kelpak® can be a potential elicitor in the enhancement of valuable phytochemicals in medicinal plants. Humic acid (vermicompost) reduces oxidative stress caused by low water potential through the induction of peroxidase enzyme activity which decreases hydrogen peroxide **(García et al. 2012)**. Oxidative stress in plants is prevented by the coordinated activity of antioxidative systems such as secondary metabolites and scavenging enzymes. Accordingly, vermicompost promotes the synthesis of phenolic acids in vegetables **(Theunissen et al. 2010)** under various environmental factors which aid in the improvement of plant quality and stress-tolerance. Vermicompost leachate stimulated the production of ferulic acid (2 and 4 months) and 4-coumaric acid (4-months) in 75 and 150 mM NaCl-stressed plants. Similarly, vermicompost stimulated secondary metabolites such as gallic acid, hydroxycynnamoyl tartaric acids, acylated anthocyanins, flavanols and stilbenes in

Vitis species **(Pardo-García et al. 2014)**; gallic, protocatechuic, syringic, *p*-hydroxybenzoic acid in *Zea mays* **(Ertani et al. 2011)**; phenylalanine ammonia-lyase (PAL) activity in *Zea mays* **(Ertani et al. 2013)**; total phenols and total flavonoids in *Brassica campestris* **(Wang et al. 2010)** under different soil properties. However, VCL was mostly inhibitory towards phenolic acid accumulation with more than a 3-fold reduction when applied to the control treatment after 4 months. The significant decline in phenolic acids was also observed in 75 and 150 mM NaCl-stressed plants after 2 and 4 months of growth. The inhibitory effects of VCL during phytochemical production has been reported in *E*. *autumnalis* and *Tulbaghia ludwigiana* **(Aremu et al. 2014)**. Duration of growth also played an important role in the stimulation of bioactive compounds, with 2-month-old 75 and 150 mM NaCl-stressed plants having higher phenolic acid content. Usually, stress induces accumulation of secondary metabolites in plants, however these bioactive compounds are not very stable over time **(Kim et al. 2007)**. Moreover, secondary metabolite biosynthesis is dependent on the metabolic pathways triggered by the plants growth environment **(Akula and Ravishankar 2011)**. In general, phenolic acid accumulation in leaves of *C*. *triloba* was stimulated with the application of biostimulants, NaCl-stress and the duration of growth. Similarly, several factors including plant cultivar, agronomic conditions, harvesting period, chemical elicitors and abiotic stresses are known to enhance bioactive compounds in fruit and vegetables **(Tiwari and Cummins 2013)**. Therefore, finding suitable means of regulating phytochemicals is a crucial step especially given that their efficacy is directly related to their secondary metabolite efficacy **(Pavarini et al. 2012)**.

5.5.4. Effect of Kelpak® and vermicompost leachate (VCL) on the mineral and total carbohydrate content

Nutritive value in vegetable crops is dependent on a number of factors such as genetics (plant crop/cultivar), environmental conditions (soil type, applied fertilizers, abiotic stress), management practices (irrigation, plant growth regulators, cultivation techniques) and post-harvest practices **(Bourn and Prescott 2002)**. The present study demonstrated the influential effect of biostimulants and salinity stress on mineral and total carbohydrate content in leaves of *C*. *triloba*. The positive impact of biostimulants on the nutritional composition of plants was remarkable especially in plants grown under 75 and 150 mM NaCl conditions, with some exceptions. The stimulatory effect of biostimulants in the accumulation of nutritional content in crop species has been reviewed **(Bulgari et al. 2014; Calvo et al. 2014)**. Furthermore, biostimulants have also been shown to effectively improve nutrient uptake in plants exposed to stress **(Mancuso et al. 2006; Rathore et al. 2009)**. In *Vitis vinifera*, the combination of fertilizers with marine bioactive substances (IPA extracts) increased the accumulation of N, P, K and Mg under stress conditions **(Mancuso et al. 2006)**. Likewise, mineral content in the control treatment and 150 mM NaCl-stressed plants was enhanced with the application of Kelpak®. Nonetheless, Kelpak® was found to reduce mineral and carbohydrate content in 75 mM NaCl-stressed plants after 2 and 4 months of growth. Vermicompost leachate also increased the mineral content in *C*. *triloba* at 75 and 150 mM NaCl while mostly reducing mineral content in plants without NaCl (control). Unlike in the current study, VCL has been shown to increase micro and macronutrients under normal growth conditions **(Peyvast et al. 2008)**. Duration of growth also influenced nutritional content with 4-month-old plants having high composition of minerals. Moreover, *C*. *triloba* remarkably increased mineral composition even after prolonged exposure to salinity stress. Therefore, these results

suggest that *C*. *triloba* can alleviate micronutrient deficiencies in human diet even when grown under salinity stress conditions.

5.6. Concluding remarks

Salinity stress significantly reduced the growth of *C*. *triloba* plants cultivated under greenhouse conditions over a period of 4 months. To some extent, the deleterious effects of NaCl were alleviated with the application of Kelpak® and VCL. Efficacy of these biostimulants was also observed in the chlorophyll fluorescence (e.g. *Fv*/*Fm*) levels of the leaves after prolonged exposure to salinity stress (4-months), even though there was a significant decline in ΦPSII, qP, NPQ and relative ETR in NaCl-stressed plants. The applied biostimulants also regulated endogenous phytohormones, phenolic acids and nutritional content in the plants. This was observed in the reduction of the majority of the quantified phytohormones with the application of biostimulants. Therefore, Kelpak® and VCL have a potential in alleviating the effects of NaCl-stress via the precise regulation of endogenous phytohormones together with phenolic acids. Furthermore, increased accumulation of nutritional content in plants treated with biostimulants under salinity stress is of

utmost importance since the plant is consumed as a leafy vegetable. As in the current study, the effect of biostimulants has been shown to be dependent on several factors including the type and applied concentration, species or cultivar type, time of production and environmental growth factors **(Kunicki et al. 2010)**. Therefore, the current study demonstrated the potential of Kelpak® and VCL in the improvement of growth, regulation of endogenous phytohormones, phenolic acids and nutritive composition in *C*. *triloba* under salinity stress conditions.

Chapter 6: Effect of Kelpak® and vermicompost leachate (VCL) on *Ceratotheca triloba* seedling growth under nutrient deficient (N, P, K) conditions

6.1. Introduction

Globally, the rapidly growing population has lead to increased pressure with regards to food productivity and security. The required increase in food production needs to be achieved in limited cultivated land suitable for agricultural use. However, soil productivity and fertility in the available land has decreased excessively due to extensive use and inappropriate soil-management techniques **(Cakmak 2002; Gruhn et al. 2000)**. According to **Cakmak (2002)**, approximately 60% of arable land has growth-limiting problems due to soil infertility which has been attributed to nutrient depletion, water scarcity, salinization, erosion, acidity, depletion of organic matter and poor drainage. Hence, the use of fertilizers has become the most useful approach for the management of nutrient-deficient soils. However, conventional farming systems are characterised by high quantities of chemical fertilizers which contribute to the reduction of soil, nutrient quality and organic matter **(Liu et al. 2009)**. These conventional farming systems apply pesticides which are known to also reduce soil efficiency, nutrient quality and are hazardous to the environment **(Tilman et al. 2002)**. Nevertheless, cultivation of many important crops is still entirely dependent on the use of high quantities of fertilizers and pesticides. Excessive fertilization has been shown not to always be consistent with increased yields but can also stimulate vegetative growth with high susceptibility to pathogen attack especially with increased levels of nitrogen supply **(Liebman and Davis 2000)**. High fertilizer input is detrimental to the environment, for instance phosphorus impacts on aquatic ecosystems by affecting water quality and aquatic foodweb structure, whereas nitrogen gets converted to ammonia, nitrate and nitrite by bacteria which results in soil leaching **(Tilman 1999)**.

It is understood that the runoffs from these fertilizers negatively affect soil and water resource quality, lifespan and sustainability. Thus, increased attention has focused on organic farming as an alternative agricultural practice for the sustainability of economically viable crop production with minimal environmental problems. Organic fertilizers mitigate nutrient deficiency problems by improving physical and chemical properties of soil including porosity, structure and water-holding capacity **(Zhou et al. 2014)**. Furthermore, they improve root and overall plant growth through the enhancement of nutrient uptake from the soil. Therefore, attention has been directed towards promoting the use of organic fertilizers including biostimulants (seaweed and vermicomposts) in order to prevent environmental degradation while improving crop yields **(Bulgari et al. 2014; Calvo et al. 2014; Craigie 2011; Theunissen et al. 2010)**. Therefore, the current study was aimed at evaluating the effect of biostimulant [Kelpak® and vermicompost leachate (VCL)] application on *Ceratotheca triloba* growth, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content under nutrient deficient [nitrogen (N), phosphorus (P), potassium (K)] conditions. The study also determined the role of 2 and 4 month harvesting stages on the aforementioned parameters.

6.2. Materials and methods

6.2.1. Biostimulant preparation, seed source, germination and transplantation

Kelpak® and VCL were prepared as described in **Section 5.2.1**. Seeds of *C*. *triloba* were purchased from Silverhill Seeds Nursery, Cape Town, South Africa. Experiments were undertaken in the greenhouse at the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa. Seed germination and seedling growth were conducted as described in **Section 4.2.1**. Established seedlings were randomly divided into eight groups. Seedlings treated with 50% Hoagland's (50% HS), N-

deficient, P-deficient and K-deficient nutrient solutions were considered as untreated (controls). The different treatments (HS, -N, -P, -K) were soil drenched with 50 ml of Kelpak® (as per label description, 0.4%) or VCL (1:10 v/v dilution). The different biostimulants were applied five times; two-times in the first 2-months and again three-times within 4 months. The soil was kept hydrated daily to prevent water stress as reported in **Chapter 4**. After 2-months, chlorophyll fluorescence, endogenous phytohormones, phenolic acids and nutritional content were measured. At the termination of the experiment (4-months), plant fresh weight (leaf weight, root length, root weight, plant height) as well as the abovementioned parameters were evaluated.

6.2.2. Chlorophyll fluorescence determination

After 2 and 4 months of growth, intact leaves of *C*. *triloba* grown under nutrient deficient conditions were measured for chlorophyll fluorescence levels using a pulse modulated fluorometer (Model FMS-2, Hansatech Instruments, King's Lynn, UK) as described in **Section 4.2.2**. The leaves were measured for different parameters such as maximum quantum efficiency of photosystem II (*Fv*/*Fm*), quantum yield of photosystem II (ΦPSII), photochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transport rate (ETR). Chlorophyll fluorescence parameters were measured at different actinic light intensities: 56, 134, 850 and 1279 µmol PAR m^{-2} s^{-1} switched on at a regular interval (5 min). Data was downloaded to a computer, recorded automatically and subjected to analysis of variance (ANOVA).

6.2.3. Quantification of stress-related phytohormones using Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC–MS/MS)

Lyophilized leaves and roots of 2 and 4-month-old plants (2.5 – 3 mg) were transferred into Eppendorf tubes containing 2 mm ceria-stabilized zirconium oxide beads (Retsch

GmbH & Co. KG, Haan, Germany). Samples were extracted for jasmonoyl isoleucine (JA-Ile) and cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and indole-3-acetic acid (IAA) as described in **Section 4.2.3**. Phytohormone content was analysed using an Acquity UHPLC® system (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer Xevo™ TQ MS (Waters MS Technologies, Manchester, UK) **(Floková et al. 2014)**. Identified compounds and internal standards were quantified in multiple ion monitoring mode (MRM) using optimized MS conditions and continuous polarity-switching data measurements. The MRM transitions was recorded in a chromatographic run in 10 targeted scan windows to get the highest possible MS signal intensity for each compound. MassLynx™ software package (version 4.1, Waters, Milford, MA, USA) was used to control the instrument and to obtain and process the MS data. Analysis was done in triplicates for each treatment.

6.2.4. Phenolic acid quantification using Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC–MS/MS)

Lyophilized *C*. *triloba* leaves (30 mg) grown for 2 and 4 months under nutrient deficient conditions were transferred into Eppendorf tubes. Phenolic acid quantification was carried out as described in **Section 4.2.4**. The UHPLC-MS/MS quantification was evaluated using an UHPLC™ system (Waters, Milford, MA, USA) connected simultaneously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating in negative mode as described by **Gruz et al. (2008)**. Analysis was done in triplicates.

6.2.5. Total carbohydrate and elemental analysis

Total carbohydrate analysis (0.1 g DW) in 2 and 4-month-old leaves was done as described in **Section 4.2.5**. Extract absorbance was measured at 490 nm against a blank in a UV-visible spectrophotometer (Varian Cary 50, Australia). A standard carbohydrate graph was prepared using glucose to calculate the carbohydrate content (% DW).

Ceratotheca triloba leaf material (0.1 g DW) of 2 and 4 months grown plants was digested using an aluminium heating block. Elemental analysis in plants was performed as described in **Section 4.2.5**. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Varian 720-ES, Varian Inc, Palo Alto, CA, USA) instrument was used to measure element concentration in samples **(Hou and Jones 2000)**. The ICP-OES was operated as follows: RF power 1.0 kW; viewing geometry axial; Argon gas used as plasma gas flow at the rate of 15.0 l/min; auxiliary gas flow rate 1.50 l/min; nebulizer gas flow rate 0.75 l/min; and replicate reading time 9.0 s. Samples were analyzed in triplicate.

6.3. Data analysis

Statistical differences between the mean values of biostimulant-treated and untreated plants was determined using a Student's *t* test. Data was further subjected to analysis of variance (ANOVA) using SPSS for Windows (SPSS®, Version 22.0. Armonk, New York, USA). Significant levels were determined at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). For phenolic acids and endogenous phytohormones, data processing was performed with a MassLynx™ software (version 4.0, Waters, Milford, MA, USA).

6.4. Results

6.4.1. Effect of Kelpak® and vermicompost leachate (VCL) on growth and chlorophyll fluorescence

The effect of Kelpak® and VCL on the growth (4 month) of *C*. *triloba* under nutrient deficient conditions is presented in **Tables 6.1** and **6.2**. Nutrient deficient (-N, -P and -K) soils reduced leaf weight, root length, root weight and plant height relative to plants grown in soil treated with 50% HS. In terms of leaf weight, more than a 7-fold reduction was observed particularly in N and P-deficient plants. Nevertheless, deficiency of these major nutrients was alleviated with the application of Kelpak®. As shown in **Table 6.1**, Kelpak® significantly improved leaf and root weight as well as plant height. The increase was more evident in leaf and root weight, with a 4-fold (-N), 3-fold (-P) and 2-fold (-K) increase in plants treated with Kelpak® than in untreated plants. Kelpak® also improved growth in plants treated with 50% HS. In most cases, application of VCL showed minimal or no observable significant differences when compared to plants grown in nutrient deficient conditions (**Table 6.2**). The biostimulant had a detrimental effect on the root length of P and K-deficient plants. However, application of VCL significantly improved leaf and root weight as well as plant height in P-deficient plants. Likewise, VCL slightly ameliorated the effects of insufficient N during *C*. *triloba* plant growth. Nutrient-deficient conditions exhibited minimal effects on the *Fv*/*Fm* values in 2 and 4-month-old leaves of *C*. *triloba* (**Tables 6.3** and **6.4**). Maximum quantum efficiency of photosystem II (*Fv*/*Fm*) in leaves ranged from 0.81 – 0.86. Reduction of *Fv*/*Fm* values in N, P and K-deficient plants was mostly observed in 2-month-old compared to 4-month-old plants.

Table 6.1: Effect of Kelpak® on *Ceratotheca triloba* growth under nutrient deficient conditions after 4 months in the greenhouse.

Treatment	Leaf weight (g)			Root length (mm)			Root weight (g)			Plant height (mm)		
	Untreated	Kelpak-treated		Untreated	Kelpak-treated		Untreated	Kelpak-treated		Untreated	Kelpak-treated	
HS	3.6 ± 0.30	4.5 ± 0.38	ns	147.1 ± 24.00	161.4 ± 20.69	ns	6.7 ± 0.78	8.4 ± 0.90	ns	733.6 ± 29.03	844.3 ± 39.27	*
-N	0.3 ± 0.12	1.4 ± 0.14	***	51.1 ± 15.91	109.4 ± 9.66	**	0.5 ± 0.18	2.1 ± 0.34	***	148.6 ± 39.79	295.7 ± 32.26	**
-P	0.2 ± 0.05	3.4 ± 0.22	***	91.6 ± 22.17	86.7 ± 7.41	ns	1.6 ± 0.40	5.3 ± 0.56	***	322.9 ± 72.50	524.3 ± 16.60	*
-K	1.7 ± 0.16	4.1 ± 0.28	***	89.4 ± 11.21	98.9 ± 9.37	ns	2.3 ± 0.21	5.6 ± 1.08	***	650.7 ± 62.47	655.0 ± 34.42	ns

For each parameter, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Table 6.2: Effect of vermicompost leachate (VCL) on *Ceratotheca triloba* growth under nutrient deficient conditions after 4 months in the greenhouse.

Treatment	Leaf weight (g)			Root length (mm)			Root weight (g)			Plant height (mm)		
	Untreated	VCL-treated		Untreated	VCL-treated		Untreated	VCL-treated		Untreated	VCL-treated	
HS	2.7 ± 0.22	3.4 ± 0.28	ns	60.7 ± 2.97	66.1 ± 5.91	ns	4.0 ± 0.28	5.1 ± 0.21	**	516.6 ± 36.68	479.3 ± 25.90	ns
-N	0.4 ± 0.12	0.6 ± 0.14	ns	51.3 ± 13.68	54.3 ± 9.29	ns	0.4 ± 0.12	0.6 ± 0.13	ns	117.9 ± 33.25	148.0 ± 11.20	ns
-P	0.1 ± 0.06	1.1 ± 0.10	***	89.2 ± 5.07	69.9 ± 3.31	**	1.1 ± 0.13	3.4 ± 0.23	***	316.3 ± 29.40	484.6 ± 35.31	***
-K	2.9 ± 0.20	3.0 ± 0.13	ns	84.2 ± 4.17	68.6 ± 5.42	*	4.6 ± 0.49	4.0 ± 0.43	ns	385.5 ± 35.53	523.4 ± 24.15	**

For each parameter, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Application of Kelpak® showed an improvement in the *Fv*/*Fm* levels of N-deficient leaves after 2 months whereas *Fv*/*Fm* values were significantly reduced when Kelpak® was applied in 4-month-old leaves with 50% HS and K-deficient plants (**Table 6.3**). In most cases, VCL significantly increased *Fv*/*Fm* levels in P and K-deficient leaves in 2 and 4-month-old plants (**Table 6.4**).

Table 6.3: Effect of Kelpak® on the chlorophyll fluorescence (*Fv/Fm*) of the leaves of *Ceratotheca triloba* under nutrient deficient conditions after 2 and 4 months of growth in the greenhouse.

Treatment	*Fv*/*Fm*					
	2 months			4 months		
	Untreated	Kelpak-treated		Untreated	Kelpak-treated	
HS	0.84 ± 0.00	0.84 ± 0.00	ns	0.85 ± 0.00	0.84 ± 0.00	*
-N	0.80 ± 0.01	0.84 ± 0.00	***	0.82 ± 0.01	0.85 ± 0.00	ns
-P	0.81 ± 0.01	0.81 ± 0.01	ns	0.83 ± 0.01	0.86 ± 0.00	ns
-K	0.82 ± 0.01	0.84 ± 0.00	ns	0.85 ± 0.00	0.84 ± 0.01	*

For each growth stage, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. P ≤0.05 (*), P ≤0.01 (**), P ≤0.001 (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K), maximum quantum efficiency of photosystem II (*Fv/Fm*).

Table 6.4: Effect of vermicompost leachate (VCL) on the chlorophyll fluorescence (*Fv/Fm*) of the leaves of *Ceratotheca triloba* under nutrient deficient conditions after 2 and 4 months of growth in the greenhouse.

Treatment	*Fv*/*Fm*					
	2 months			4 months		
	Untreated	VCL-treated		Untreated	VCL-treated	
HS	0.85 ± 0.00	0.85 ± 0.00	ns	0.85 ± 0.00	0.85 ± 0.01	ns
-N	0.83 ± 0.00	0.83 ± 0.01	ns	0.83 ± 0.01	0.85 ± 0.00	ns
-P	0.81 ± 0.01	0.85 ± 0.00	**	0.82 ± 0.01	0.84 ± 0.00	*
-K	0.83 ± 0.00	0.84 ± 0.00	*	0.86 ± 0.00	0.86 ± 0.00	ns

For each growth stage, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. P ≤0.05 (*), P ≤0.01 (**), P ≤0.001 (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K), maximum quantum efficiency of photosystem II (*Fv/Fm*).

In both 2 and 4-month-old plants, increase in actinic light intensities (56 - 1279 µmol PAR m^{-2} s^{-1}) caused a gradual decline in ΦPSII and qP levels, while NPQ and ETR levels increased with increasing light intensities (**Figs. 6.1** – **6.4**). The minimal change observed in chlorophyll fluorescence values suggest that N, P, K-deficiency in plants was not largely inhibitory towards PSII photochemistry. Even so, Kelpak® and VCL slightly increased ΦPSII, qP, NPQ and relative ETR in plants, with some exceptions. Kelpak® improved NPQ at 56 and 134 µmol PAR $m^{-2}s^{-1}$ actinic light intensities in N-deficient 2-month-old leaves, even though it reduced ΦPSII, qP and relative ETR values, with some exceptions (**Figs. 6.1** and **6.2**). Application of Kelpak® increased ΦPSII, qP and relative ETR in 50% HS-treated and N-deficient plants, with a significant decline in NPQ levels in 50% HS-treated plants after 4 months. In P-deficient plants, Kelpak® improved ΦPSII, qP and relative ETR with a decrease in NPQ levels after 4 months.

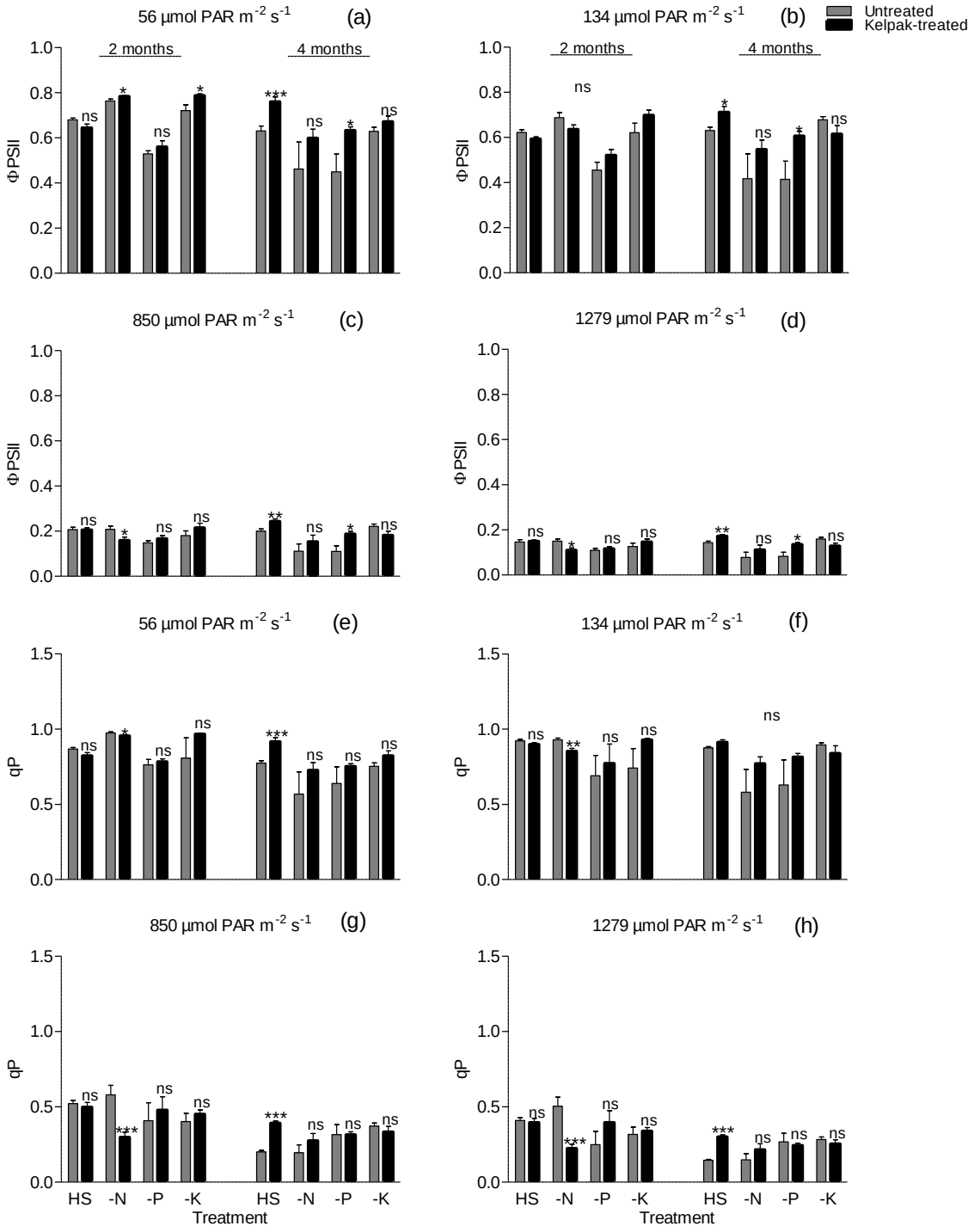


Fig. 6.1: Effect of Kelpak® on quantum yield of photosystem II (ΦPSII, a-d) and phytochemical quenching (qP, e-h) in *Ceratotheca triloba* under nutrient deficient conditions after 2 and 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

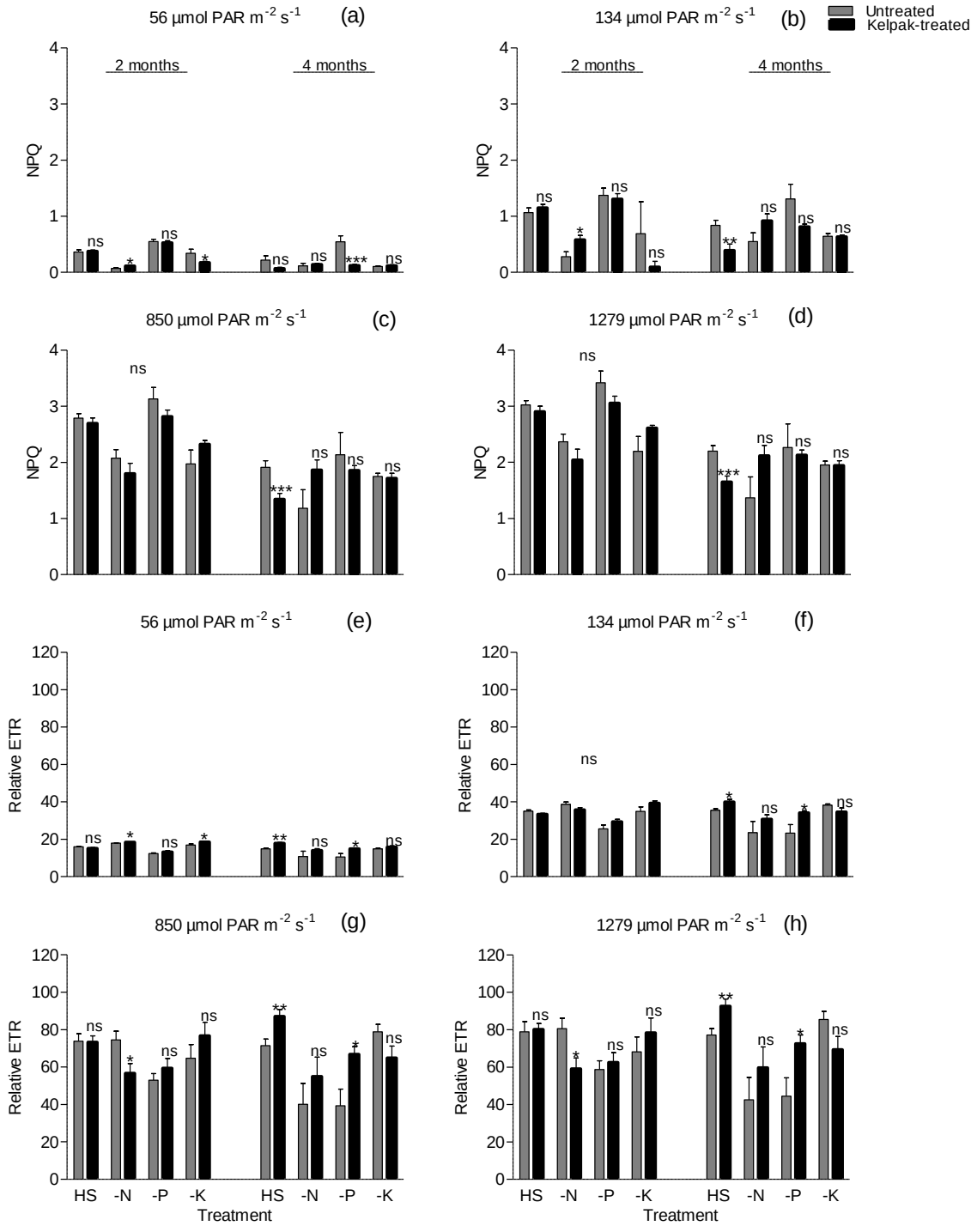


Fig. 6.2: Effect of Kelpak® on non-photochemical quenching (NPQ, a-d) and relative electron transfer rate (ETR, e-h) in *Ceratotheca triloba* under nutrient deficient conditions after 2 and 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Nitrogen-deficient plants treated with VCL had reduced ΦPSII, qP, relative ETR and increased NPQ levels in 2 and 4-month-old plants (**Figs. 6.3** – **6.4**). In most actinic light intensities, VCL application reduced the levels of qP, NPQ and relative ETR in P-deficient plants after 2 months. However, the biostimulant increased NPQ and relative ETR levels in most P-deficient plants after 4 months. Chlorophyll fluorescence levels were enhanced in K-deficient plants treated with VCL after a 4-month growth period. Quantum yields of photosystem II (ΦPSII) and relative ETR levels in 50% HS-treated leaves increased, with a decline in NPQ when VCL treatment was applied in 4-month-old plants. Overall, there was minimal change in chlorophyll fluorescence parameters after 2 and 4 months of growth with/without biostimulants (**Appendix C; Table 1** and **2**). Nonetheless, 2-month-old plant had relatively higher chlorophyll fluorescence content than 4-month-old plants, with some exceptions.

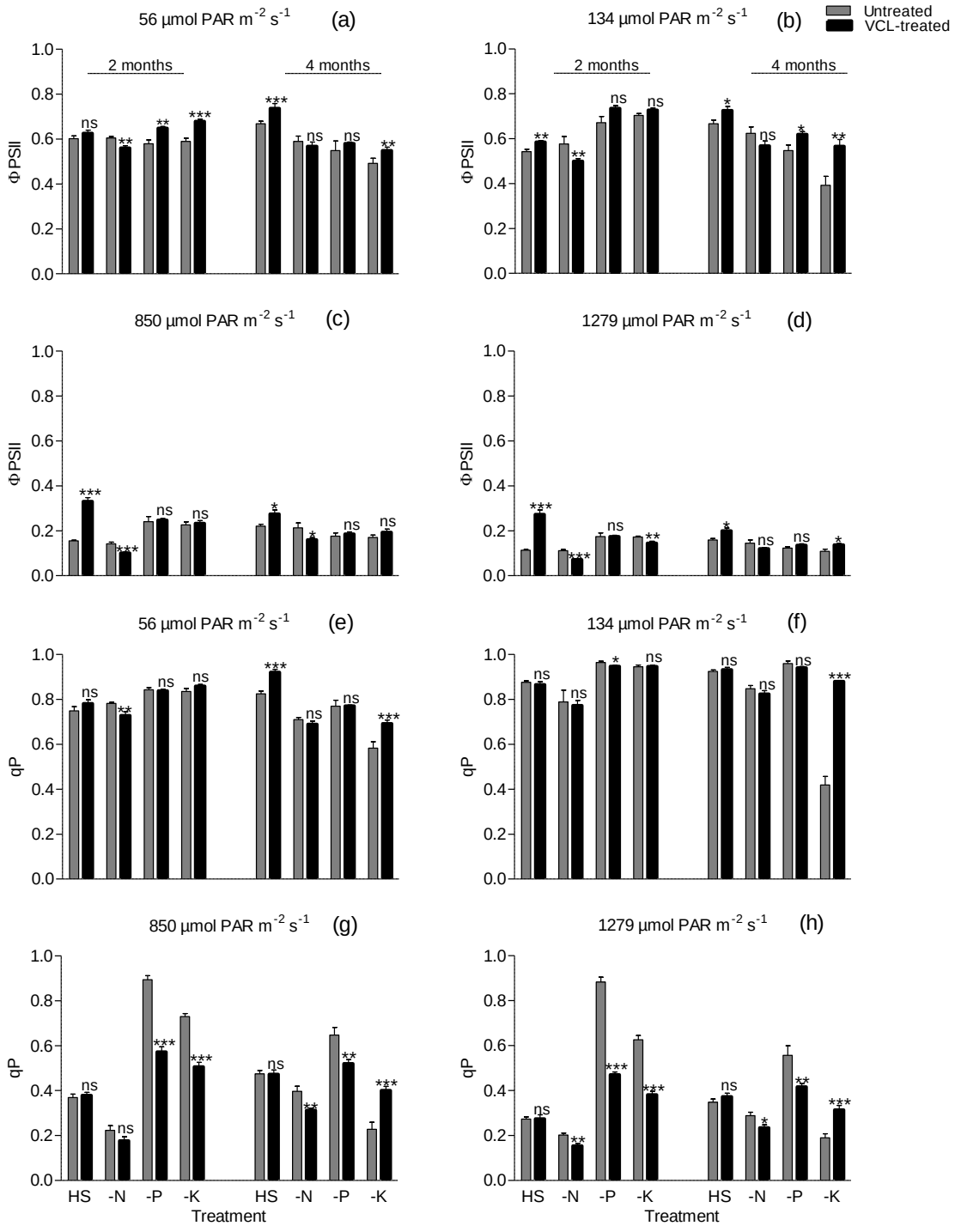


Fig. 6.3: Effect of vermicompost leachate (VCL) on quantum yield of photosystem II (ΦPSII, a-d) and phytochemical quenching (qP, e-h) in *Ceratotheca triloba* under nutrient deficient conditions after 2 and 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

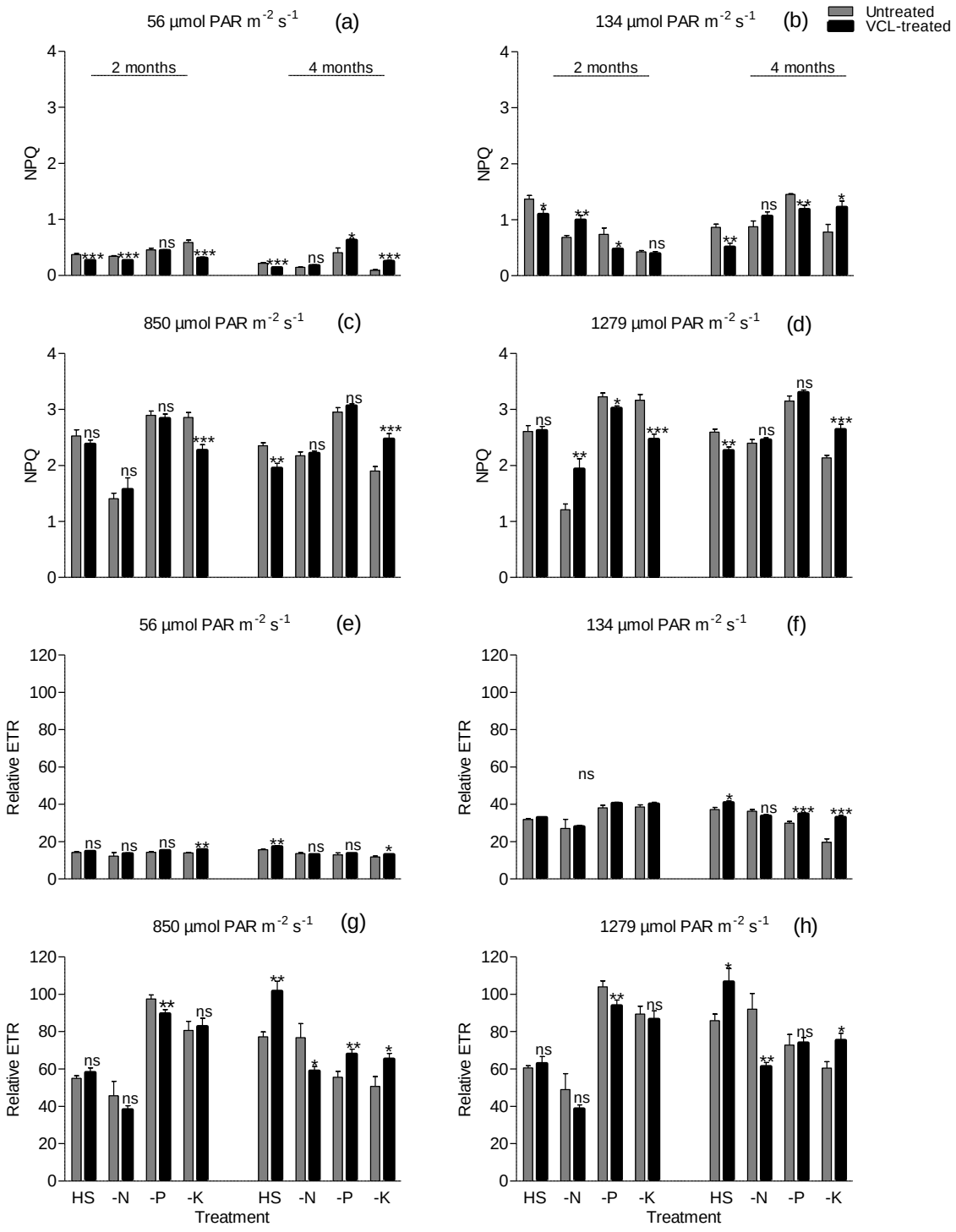


Fig. 6.4: Effect of vermicompost leachate (VCL) on non-photochemical quenching (NPQ, a-d) and relative electron transfer rate (ETR, e-h) in *Ceratotheca triloba* under nutrient deficient conditions after 2 and 4 months of growth in the greenhouse. Bars represent mean value ± standard error (n = 7) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

6.4.2. Effect of Kelpak® and vermicompost leachate (VCL) on endogenous phytohormone content

Endogenous phytohormone quantification in greenhouse-cultivated *C*. *triloba* yielded a total of six stress-related signal molecules in 2 and 4-month-old leaves and roots (**Figs. 6.5 – 6.10**). These consisted of JA-Ile (active form of JAs), *cis*OPDA (JAs precursor), ABA, SA, JA and IAA. Salicylic acid was the most abundant phytohormone, with values ranging from 3 – 70 nmol/g DW. Jasmonic acid and IAA were only detected in roots of 4-month-old plants. In most cases, nutrient-deficient plants (N, P, K) had more endogenous phytohormone content compared to 50% HS-treated plants. Application of Kelpak® and VCL further enhanced the accumulation of endogenous phytohormones in nutrient-deficient plants. Kelpak® enhanced the accumulation of JA-Ile, *cis*OPDA, ABA and SA concentrations in 4-month-old leaves compared to plants grown for 2 (leaves) and 4 (roots) months (**Figs. 6.5** and **6.6**). Jasmonoyl isoleucine (297 pmol/g DW) and *cis*OPDA (2 nmol/g DW) levels significantly increased in 50% HS-treated leaves with Kelpak® after 4 months, while JA-Ile (66 pmol/g DW) and *cis*OPDA (0.6 nmol/g DW) concentration declined in the roots (**Fig. 6.5**). Kelpak® increased the concentration of *cis*OPDA (3.8 nmol/g DW) in N-deficient roots. The biostimulant also stimulated the production of ABA in 4-month-old leaves and roots under N, P and K-deficient conditions (**Fig. 6.6**). Even though, SA content declined with the application of Kelpak® in 4-month-old leaves under P and K-deficient conditions.

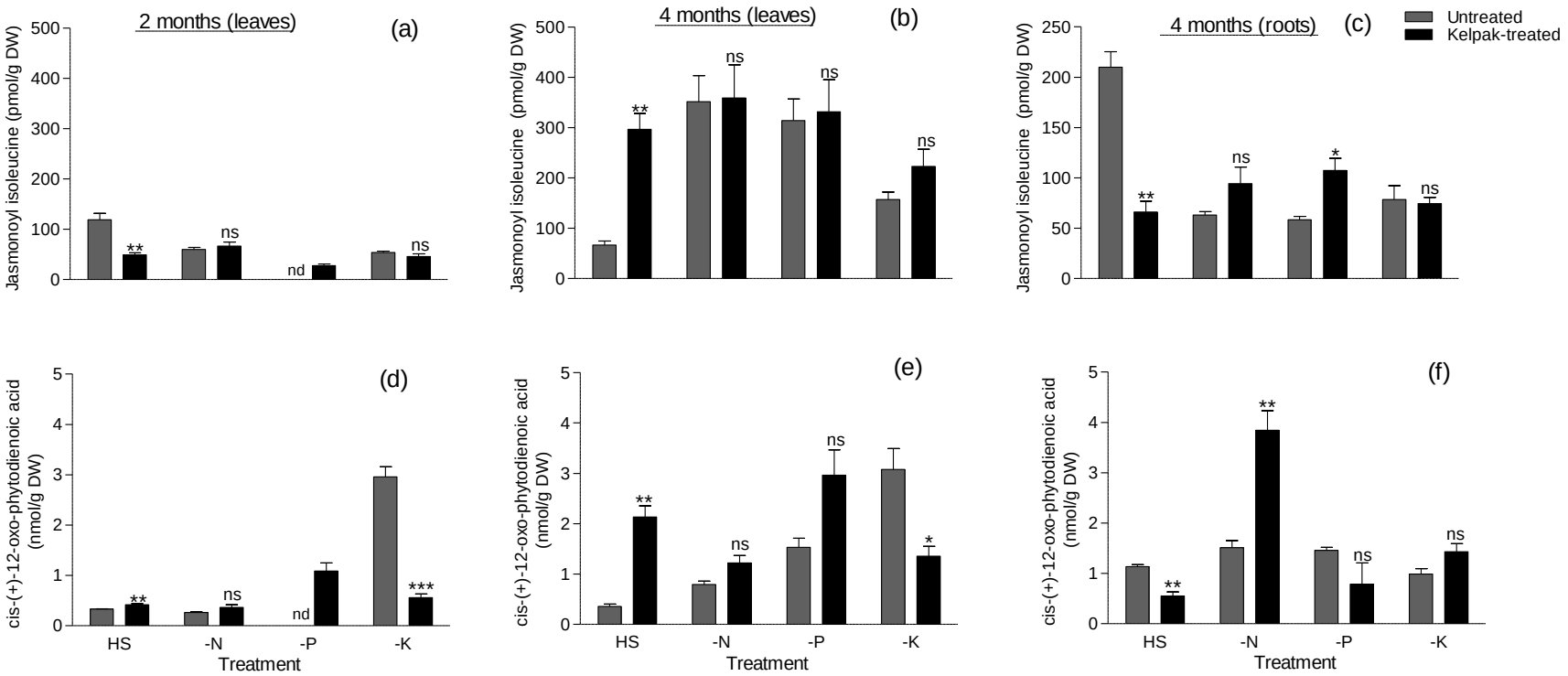


Fig. 6.5: Effect of Kelpak® on endogenous phytohormones: jasmonoyl isoleucine (JA-Ile; active form of JAs) and cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

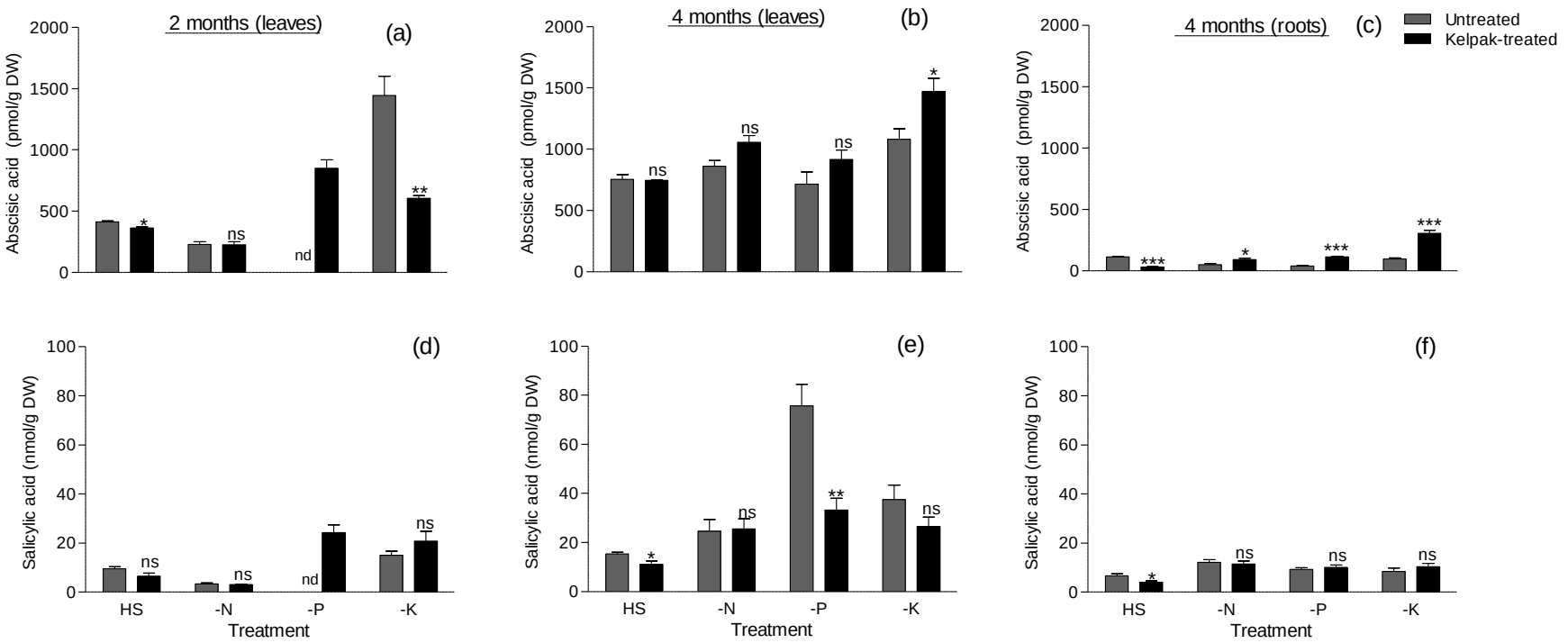


Fig. 6.6: Effect of Kelpak® on endogenous phytohormones: abscisic acid (ABA) and salicylic acid (SA) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Jasmonic acid and IAA concentration were enhanced with Kelpak® in N, P and K-deficient plants, while the biostimulant decreased phytohormone (JA and IAA) content in 50% HS-treated plants (**Fig. 6.7**). Besides nutrient shortage and biostimulants application, duration of growth significantly influenced phytohormone synthesis in leaves of *C*. *triloba*. For instance, prolonged period of growth (4 months) stimulated phytohormone production in leaves relative to plants grown for only 2 months (**Appendix C; Table 3**).

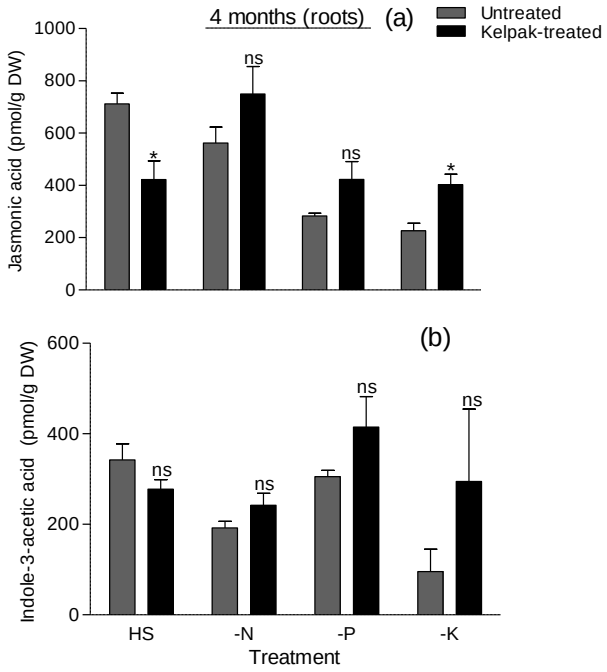


Fig. 6.7: Effect of Kelpak® on endogenous phytohormones: jasmonic acid (JA) and indole-3-acetic acid (IAA) in roots of *Ceratotheca triloba* after 4 months of growth under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (*n* = 3) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. *P* ≤0.05 (*), *P* ≤0.01 (**), *P* ≤0.001 (***); ns (not significant) based on the Student's *t* test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Vermicompost leachate significantly reduced JA-Ile and *cis*OPDA synthesis in 50% HS, P and K-deficient leaves after 2 months (**Fig. 6.8**). In most cases, the concentration of JA-Ile and *cis*OPDA increased in VCL-treated leaves and roots after 4 months of growth in N, P and K-deficient plants. Concentration of ABA significantly decreased in P and K-deficient leaves with the application of VCL in 4-month-old plants (**Fig. 6.9**). Similarly, VCL reduced SA content in N (27 nmol/g DW) and P (23 nmol/g DW) deficient leaves after 4 months whereas, K-deficient plants accumulated high levels of SA with VCL. Jasmonic acid and IAA concentrations were also enhanced with VCL especially in N, P and K-deficient plants (**Fig. 6.10**). Vermicompost leachate in nutrient-deficient plants (N, P and K) increased the concentration of JA by almost 2-fold compared to untreated plants. Overall, *C*. *triloba* plants accumulated high levels of JA-Ile and *cis*OPDA in 2-month-old leaves with an increase in ABA and SA content in 4-month-old leaves (**Appendix C, Table 4**).

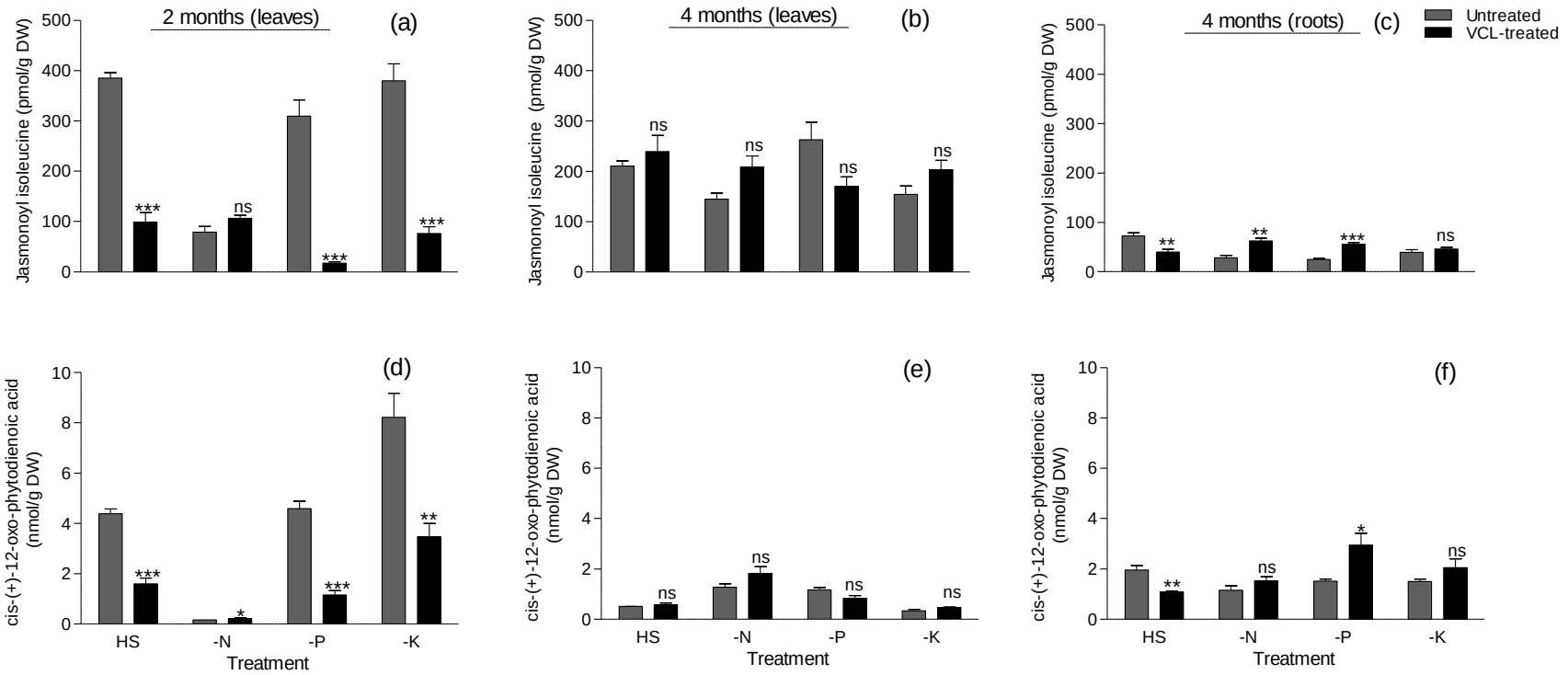


Fig. 6.8: Effect of vermicompost leachate (VCL) on endogenous phytohormones: jasmonoyl isoleucine (JA-Ile; active form of JAs) and cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

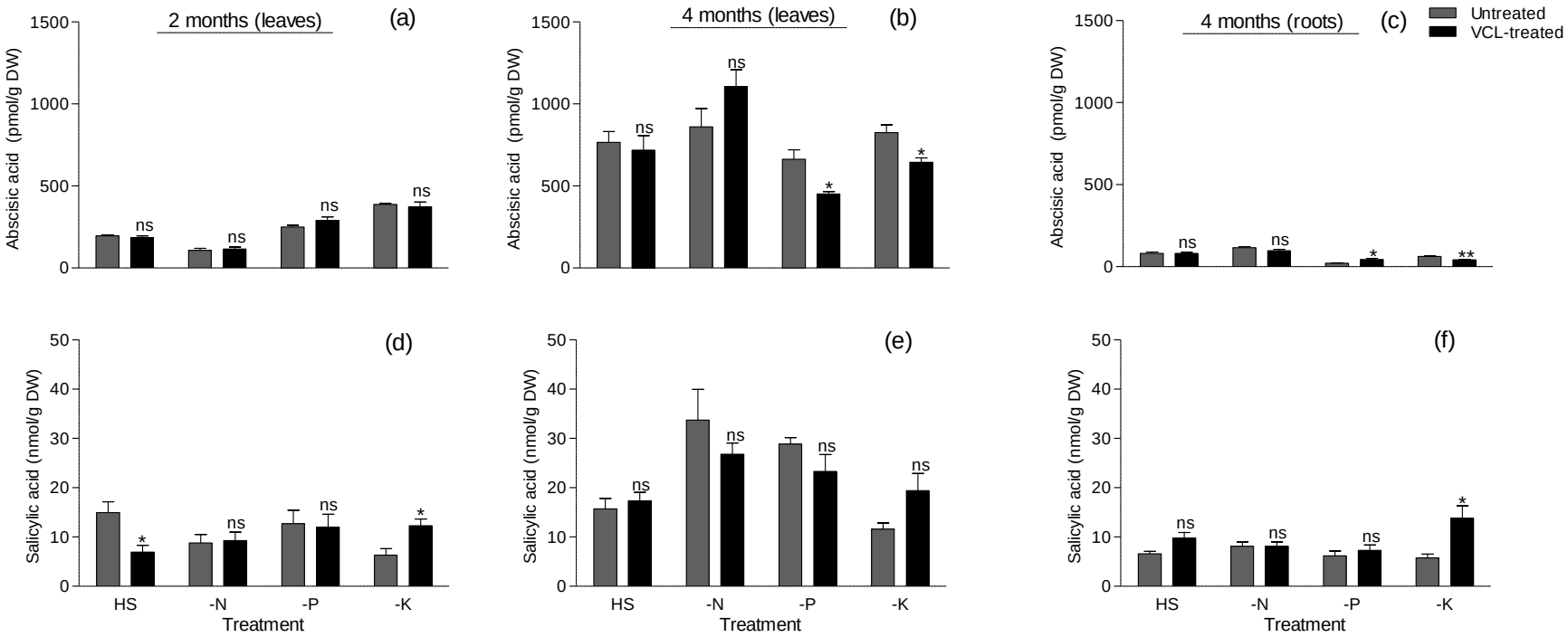


Fig. 6.9: Effect of vermicompost leachate (VCL) on endogenous phytohormones: abscisic acid (ABA) and salicylic acid (SA) in leaves and roots of *Ceratotheca triloba* after 2 and 4 months of growth under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

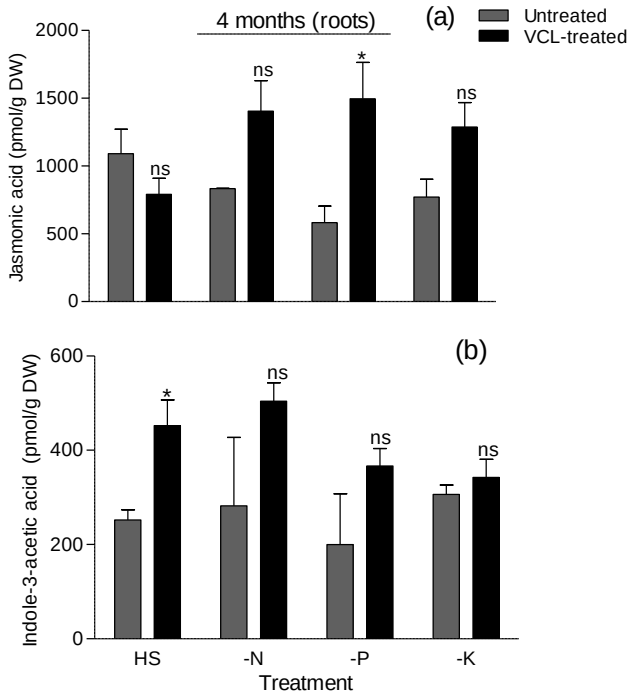


Fig. 6.10: Effect of vermicompost leachate (VCL) on endogenous phytohormones: jasmonic acid (JA) and indole-3-acetic acid (IAA) in roots of *Ceratotheca triloba* after 4 months of growth under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (*n* = 3) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. *P* ≤0.05 (*), *P* ≤0.01 (**), *P* ≤0.001 (***); ns (not significant) based on the Student's *t* test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

6.4.3. Effect of Kelpak® and vermicompost leachate (VCL) on phenolic quantification

Phenolic acid quantification in greenhouse-cultivated leaves of *C*. *triloba* showed the presence of three hydroxybenzoic (protocatechuic, ferulic, 4-hydroxybenzoic) and three hydroxycinnamic (caffeic, vanillic, 4-coumaric) acid compounds in nutrient deficient conditions (**Figs. 6.11** and **6.12**). Amongst the detected phenolics, 4-coumaric acid was the most abundant bioactive compound (c.a. 10 - 60 µg/g DW). Nutrient deficiency largely stimulated phenolic acid production in *C*. *triloba* more than the applied biostimulants. In fact, Kelpak® and VCL induced inhibitory effects when applied to the different treatments (50% HS, -N, -P and -K) in 2 and 4-month-old plants.

As seen in **Fig. 6.11**, application of Kelpak® in K-deficient plants reduced 4-hydroxybenzoic, vanillic, ferulic, caffeic, and 4-coumaric acids after 2 months of growth. The inhibitory effect of Kelpak® treatment was also prominent in 4-month-old plants grown in 50% HS, N and K-deficient soils. Nonetheless, the biostimulant had some stimulatory effects in 50% HS-treated and P-deficient plants after a 2 and 4 months growth period, respectively. Prolonged growth (4 months) of *C*. *triloba* in nutrient deficient soils stimulated high production of phenolic acids in comparison to plants grown for 2 months (**Appendix C; Table 5**).

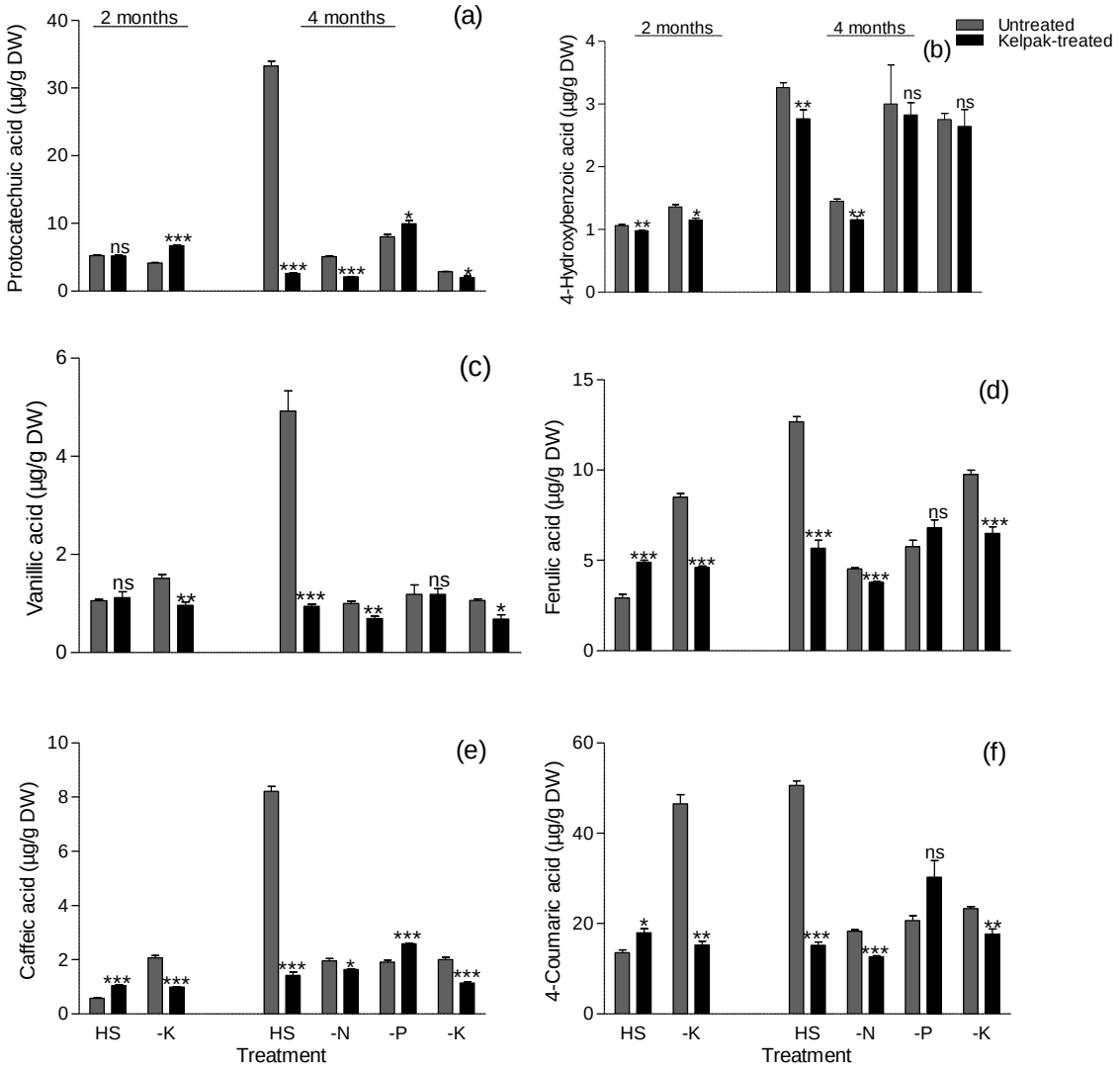


Fig. 6.11: Effect of Kelpak® on phenolic acid content in leaves of *Ceratotheca triloba* after 2 and 4 months under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant differences between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Application of VCL significantly reduced phenolic acid production in 50% HS-treated plants after 2-months of growth (**Fig. 6.12**). A significant decline in protocatechuic and caffeic acids was observed in P and K-deficient plants with VCL after 2 months, while ferulic and 4-coumaric acids were enhanced. Although VCL stimulated the production

of 4-hydroxybenzoic and vanillic acids in 50% HS-treated plants, its application reduced the concentration of protocatechuic, ferulic, caffeic and 4-coumaric acids in 4-month-old leaves. Phenolic acid content in 4-month-old leaves was significantly enhanced in P-deficient soil (untreated), except for 4-hydroxybenzoic acid. Application of VCL in K-deficient plants slightly increased phenolic acid content in 4-month-old leaves, with some exceptions. The duration of growth significantly affected the quantified bioactive compounds, with 4-month-old leaves accumulating high levels of phenolic acids (**Appendix C; Table 6**). However, this was more pronounced in some treatments than others.

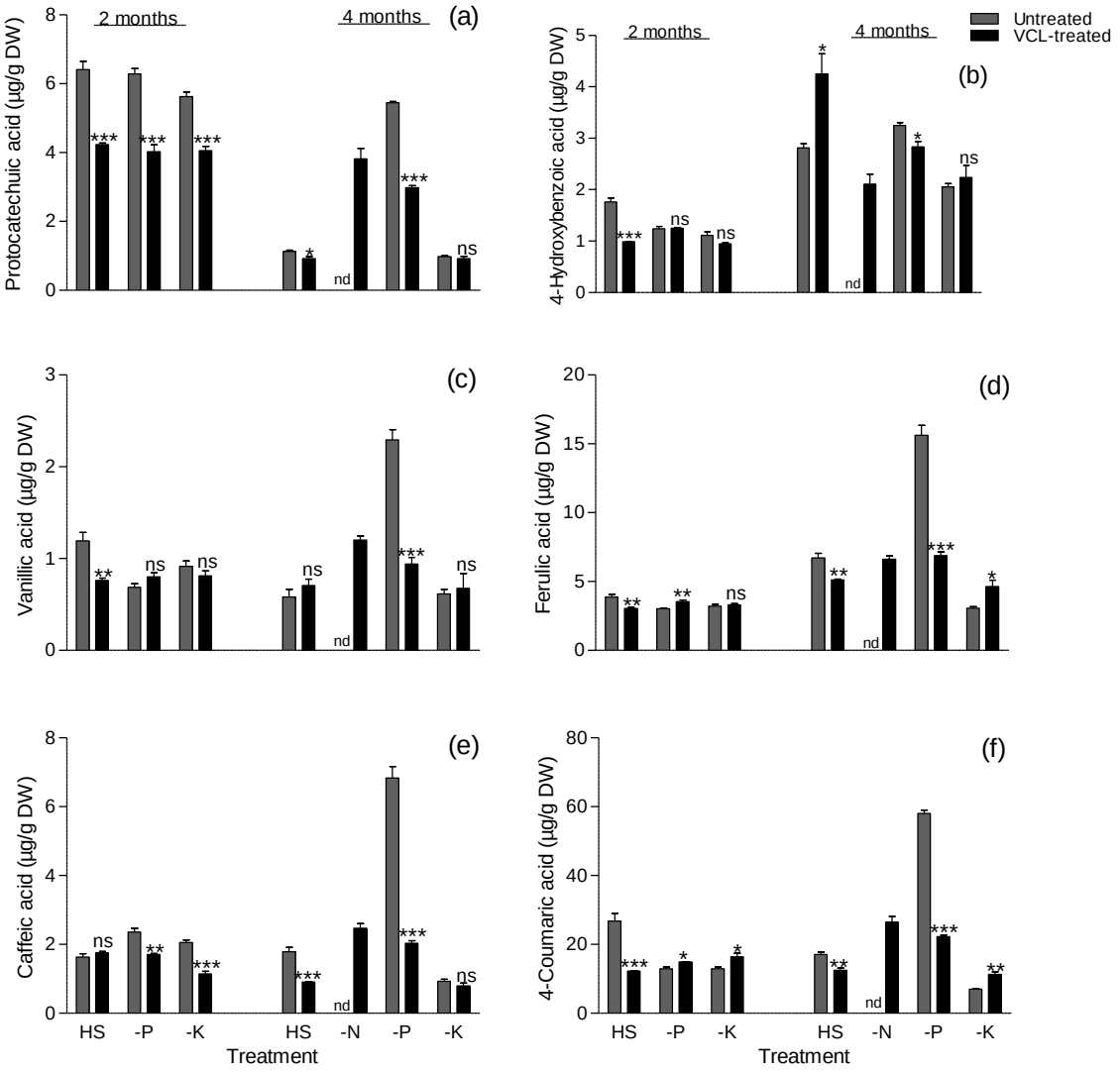


Fig. 6.12: Effect of vermicompost leachate (VCL) on phenolic acid content in leaves of *Ceratotheca triloba* after 2 and 4 months under nutrient deficient conditions in the greenhouse. Bars represent mean value ± standard error (n = 3) and asterisk(s) indicate significant differences between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's t test. Hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

6.4.4. Effect of Kelpak® and vermicompost leachate (VCL) on mineral content and total carbohydrate percentage

Tables 6.5 and **6.6** represent the mineral and total carbohydrate content of leaves of *C*. *triloba* leaves grown in nutrient deficient conditions. Among the analysed minerals,

Ca and K were the most abundant macroelements while microelements Mn and Zn were also present in high concentrations. Total carbohydrates (%) in *C*. *triloba* were relatively low. Application of Kelpak® reduced the mineral content in 50% HS-treated plants after 2 months of growth while increasing Ca, Fe, K, Mg and total carbohydrate content in 4-month-old plants (**Table 6.5**). Generally, Kelpak® decreased the mineral content in K-deficient leaves after 2 and 4 months. Both N and P-deficient treatments had a detrimental effect on some of the estimated mineral content in 4-month-old plants (untreated) compared to K-deficient plants (**Tables 6.5** and **6.6**). The deleterious effect of N-deprivation on the mineral composition was counteracted with Kelpak® or VCL treatments. Although VCL application to 50% HS-treated plants increased Ca, Mg and Na composition in 2 and 4-month-old leaves, the biostimulant reduced Fe, K and Mn content. Phosphorus-deficient leaves accumulated high Ca, Fe, K, Mg and Mn levels in VCL-treated leaves after 2 months. Vermicompost leachate also increased Ca, K and Mg levels in K-deficient plants after 2 months, and only increased Fe and K levels in 4-month-old leaves. Mineral content in leaves of *C*. *triloba* was relatively higher in 4-month-old plants compared to 2-month-old plants.

Table 6.5: Effect of Kelpak® on the mineral content and total carbohydrates (%) in *Ceratotheca triloba* after 2 and 4 months of growth under nutrient deficient conditions in the greenhouse.

Harvest period	Treatment	Ca		Fe		K		Mg		Mn		Na		Zn		% Carbohydrates	
		mg/kg DW															
		Untreated	Kelpak treated	Untreated	Kelpak treated	Untreated	Kelpak treated	Untreated	Kelpak treated	Untreated	Kelpak treated	Untreated	Kelpak treated	Untreated	Kelpak treated	Untreated	Kelpak treated
2-Months	HS	14325	12743	369	338	21830	16711	2938	3188	40	35	372	303	114	85	14	14
	-K	751	584	357	291	194	302	232	191	31	28	1285	551	91	113	12	nd
4-Months	HS	29409	29886	367	417	20993	21417	2874	3113	45	44	329	328	71	42	3	17
	-N	12422	23562	225	312	12674	24017	1362	3287	34	96	277	281	31	68	nd	9
	-P	14762	21794	318	324	18322	26478	2876	2995	38	45	286	309	84	95	nd	9
	-K	33060	31941	394	343	6357	10177	4929	5263	30	31	1628	1010	125	92	5	11

Calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), zinc (Zn), not determined (nd), hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

Table 6.6: Effect of vermicompost leachate (VCL) on the mineral content and total carbohydrates (%) in *Ceratotheca triloba* after 2 and 4 months of growth under nutrient deficient conditions in the greenhouse.

Harvest period	Treatment	Ca		Fe		K		Mg		Mn		Na		Zn		% Carbohydrates	
		mg/kg DW															
		Untreated	VCL treated	Untreated	VCL treated	Untreated	VCL treated	Untreated	VCL treated	Untreated	VCL treated	Untreated	VCL treated	Untreated	VCL treated	Untreated	VCL treated
2-Months	HS	18771	29660	351	294	22110	18981	2857	3300	57	33	264	277	133	39	9	10
	-P	9789	22193	264	302	10497	19839	1685	3008	29	35	300	297	41	41	nd	10
	-K	20551	33755	348	331	4293	4568	4536	5339	27	5	395	231	45	uv	nd	26
4-Months	HS	30846	30897	356	286	23021	22962	3106	3296	45	35	260	307	46	60	16	9
	-N	16169	26571	346	301	19983	26701	1935	3073	29	63	228	304	35	80	nd	9
	-K	34411	29628	384	386	6238	12331	5366	4076	58	41	1175	846	63	40	11	15

Calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), zinc (Zn), below detection limit (uv), not determined (nd), hoagland's (HS), nitrogen (N), phosphorus (P), potassium (K).

6.5. Discussion

Soil nutrient management is amongst the major challenges limiting plant growth and survival rate. In agriculture, crop productivity is greatly dependent on the application of external nutrient supply, particularly N, P and K. During nutrient deficient conditions, plants increase nutrient uptake, remobilisation and redistribution of stored minerals which facilitates the growth of active tissues **(Amtmann et al. 2005)**. Nutrient constituents in biostimulants are known to activate several physiological processes which enhance nutrient uptake and efficiency, subsequently ameliorating the deleterious effects of nutrient stress **(Bulgari et al. 2014; Calvo et al. 2014)**. Biostimulants improve microflora and soil fertility by acting on physical, physico-chemical, chemical and biological properties **(Bulgari et al. 2014; du Jardin 2015)**. Most importantly, biostimulants are able to improve crop quality while keeping nitrate quantities under the limits imposed by European Union (EU) regulations **(Bulgari et al. 2014)**.

6.5.1 Effect of Kelpak® and vermicompost leachate (VCL) on growth and chlorophyll fluorescence

Nutrient-deficiency (N, P, K) caused a severe reduction in the growth of *C*. *triloba* after 4 months. To some extent, the detrimental effects of insufficient nutrients in the soil were mitigated with the use of Kelpak® and VCL. The efficacy of biostimulants as growth promoting substances under N, P and K-deprivation has been documented in a number of plant species **(Arthur et al. 2012; Papenfus et al. 2013; Theunissen et al. 2010)**. In the same way, Kelpak® and VCL were beneficial in alleviating the deleterious effects of N, P and K deficiencies during the growth of *C*. *triloba*. The promotive effect of Kelpak® and VCL were mostly observed with the improvement of leaf weight, root weight and plant height in N and P-deficient plants. The growth yields

in N-deficient plants with Kelpak® were nearly half of those treated with 50% HS (without biostimulants). Kelpak® and VCL applied in P-deficient plants yielded leaf weight, root weight and plant height almost similar to that of plants treated with 50% HS (without biostimulants). Vermicompost leachate improved plant growth in K-deficient plants even more than that of 50% HS-treated plants with/without biostimulants. Interestingly, K-deficient plants with VCL significantly improved root length and plant height when compared to 50% HS-treated plants with VCL. The marked growth responses in *C*. *triloba* are possibly due to the biostimulant's ability to enhance soil organic carbon content and the release of valuable soil microorganisms **(Gopal et al. 2010)**. In addition, biostimulants improve soil properties through increased pH, oxidizable organic carbon, soil microbial activities of dehydrogenase, *β*-glucosidase, urease and phosphatase with a decrease in soil bulk density **(Gopinath et al. 2008)**. The stimulatory effect of biostimulant application on plant growth and development are also associated with the number of identified plant growth regulators (PRGs), mineral elements, humic substances, manure and/or sea kelp extracts **(Calvo et al. 2014; Hamza and Suggars 2001; Theunissen et al. 2010)**. Therefore, Kelpak® and VCL played an important role in the improvement of soil properties and plant growth, although the biostimulants were not able to facilitate recovery in N and P-deficient plants similar to that of plants treated with 50% HS with biostimulants.

Photosystem II efficiency showed minimal changes in N, P and K- deficient plants after 2 and 4 months of growth when compared to 50% HS-treated plants. Nutrient-stressed plants had an *Fv*/*Fm* value consistent with those of unstressed (c.a. 0.83) plants as stipulated by **Johnson et al. (1993)**. Nutrient stress levels that do not reduce photosystem II yields in plants are mostly detected at very low levels or once the plant reaches nutrient starvation levels. This was also the case with *C*. *triloba*, in which

plants showed a slight change in photosystem II after 2 and 4 months of growth under nutrient-deficient conditions. Despite the slight reduction in chlorophyll fluorescence (*Fv*/*Fm*), biostimulants still improved the plants photosynthetic capacity under N, P and K-deficient conditions. This was more noticeable in N-deficient leaves in which Kelpak® significantly increased *Fv*/*Fm* values after 2 months, while VCL improved *Fv*/*Fm* levels in K-deficient (2 months) and P-deficient plants (4 months). The protective role of biostimulants on photosystem II in nutrient-deficient leaves can be related to their efficiency in improving *C*. *triloba* plant growth (**Tables 6.1** and **6.2**). Furthermore, their defined role in enhancing nutrient uptake and efficiency resulted in an increase in *Fv*/*Fm* values in plants deprived of N, P and K nutrients.

Chlorophyll fluorescence (ΦPSII, qP and relative ETR) in 50% HS-treated leaves increased with the application of Kelpak® in 4-month-old plants. Likewise, VCL improved ΦPSII and relative ETR levels in 50% HS-treated 4-month-old leaves. However, both biostimulants inhibited NPQ levels in plants. Activation of excess excitation energy dissipation (NPQ) systems in plants maintains the balance between the photosynthetic energy absorption and utilization. An increase in NPQ levels reduces the efficiency of excitation energy and transfer of PSII reaction centers F'v F'm **(Weng et al. 2007)**. It is clear then that plants grown in 50% HS-treated soil (with/without biostimulant) did not induce an increased susceptibility to photo-inhibition in plants. On the other hand, deficiency of N increased NPQ levels in 2-month-old leaves with a reduction in ΦPSII, qP and relative ETR when plants were treated with Kelpak® and VCL. An increase in NPQ levels of N-deficient plants decreases the excitation pressures of PSII, inducing plant protection against electron transport and/or triplet chlorophyll dependent photo-damage **(Vass 2012)**. Low levels of N reduces quantum efficiency of photosystem II in leaves due to the increase in NPQ together

with increased closure of PSII reaction centre under high photon flux density **(Chen and Cheng 2003)**. Furthermore, N-deficiency in plants reduces phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase and ribulose bisphosphate carboxylase/oxygenase (Rubisco) activity, some of the major carbon-assimilation enzymes involved in the plant's photosynthetic activity **(Sugiharto et al. 1990)**. Nonetheless, Kelpak® improved chlorophyll fluorescence (ΦPSII, qP, NPQ and relative ETR) in N-deficient leaves after 4 months of growth. The increase in chlorophyll fluorescence due to Kelpak® application may be attributed to the plant's ability to efficiently transport nutrients in order to improve the photosynthetic potential of *C*. *triloba*, thereby improving plant growth under N-deficient conditions. However, this was not the case with VCL, given the fact that the biostimulant reduced ΦPSII, qP and relative ETR in 4-month-old plants. Chlorophyll fluorescence reduction in N-deficient plants with VCL can be related to the minimal effect of the biostimulant during plant growth. The deficiency of P in leaves of *C*. *triloba* decreased NPQ levels in 2 and 4-month-old plants when Kelpak® and VCL were applied (with some exceptions). Furthermore, P-deficient plants treated with VCL had a decline in qP and relative ETR after 2 months. The decline in photochemical efficiency of photosystems and stomatal conductance causes inhibition of photosynthesis in plants with low P content **(Starck et al. 2000)**. Phosphorus deficiency reduces chlorophyll fluorescence parameters especially the plants performance index (PI_{ABS}), impairs electron transport activity and the use of absorbed and trapped photosynthetic energy **(Ripley et al. 2004)**. Thus, supplementation of P improves PSII performance through alteration of CO_2 assimilation and fluorescence emissions. This was also the case with the application of Kelpak® and VCL (with some constituents of P) in the improvement of some of the analysed chlorophyll parameters (ΦPSII, qP and relative ETR). Furthermore, Kelpak®

and VCL improved photosystem II efficiency (*Fv*/*Fm*) of 2 and 4-month-old plants even though the biostimulants reduced NPQ levels. Potassium-deficient leaves use a smaller proportion of absorbed photosynthetic photon flux density during photosynthetic carbon reduction due to low activity of Rubisco **(Weng et al. 2007)**. The excess energy is usually higher in K-deficient plants and becomes harmful to the plants photosynthetic apparatus. Therefore, the increase in NPQ levels because of VCL application in K-deficient plant's after 4 months reduced the damaging effects of excess light energy, thus preventing leaf photo-inhibition. The changes in plant photosynthetic activity are dependent on CO_2 diffusion and photosystem activity, associated with nutrient supply. Nutrient deficiency in *C*. *triloba* showed minimal effect on the chlorophyll fluorescence activity of plants, yet application of biostimulants slightly improved some of the measured chlorophyll fluorescence parameters especially in N and P-deficient leaves. These findings show the remarkable effect of biostimulants on the photosynthetic activity of *C*. *triloba* especially in 50% HS-treated plants as well as nutrient deficient plants.

6.5.2. Effect of Kelpak® and vermicompost leachate (VCL) on endogenous phytohormone content

Generally, nutrient-stress (N, P, K) induced endogenous phytohormone production in untreated *C*. *triloba* more than in 50% HS-treated plants. Furthermore, nutrient stress together with the applied biostimulants played an important role in the biosynthesis of endogenous phytohormones. Nutrient stress influences phytohormone signalling pathways which in turn regulates nutrient demand, uptake and utilization. Changes in mineral nutrient concentration in plants acts as signals involved in phytohormone balance **(Amzallag et al. 1992)**. Transcriptome assessment in plants shows a complex interrelationship between nutrient starvation signalling molecules and

changes in phytohormone signalling/metabolism genes **(Rubio et al. 2008)**. These endogenous phytohormones either have a synergistic or antagonistic cross-talk effect in plants during nutrient stress, thus regulating phytohormone biosynthesis or responses. Nitrogen-deficiency in *C*. *triloba* reduced *cis*OPDA, ABA and SA production in 2-month-old plants in comparison to untreated P and K-deficient plants (**Figs. 6.5** and **6.6**). However, application of Kelpak® and VCL in N-deficient plants stimulated the production of endogenous phytohormones after 2 and 4 months of growth. Nitrogen signaling has been associated with the regulation of phytohormones such as auxin, ABA and cytokinins **(Kiba et al. 2011)**. The induction of ABA synthesis in plants coordinate the demands and acquisition of N **(Brewitz et al. 1995; Peuke et al. 1994)**. In addition, N-starvation induces/inhibits auxin translocation from shoots to roots in *Arabidopsis thaliana* **(Zhang et al. 2007)** and increases wound- and volicitin-induced JA levels in *Zea mays* **(Schmelz et al. 2003)**. Therefore, N-deficiency in *C*. *triloba* treated with Kelpak® or VCL brought about a synergistic interaction between ABA, SA, IAA and JA including its active form and precursor. The stimulatory effects of biostimulants on phytohormone content in N-deficient plants might have triggered stress-protective genes while improving nutrient assimilation and efficiency. Biostimulants such as plant-derived protein hydrolysate have been shown to elicit an auxin and gibberellin (GA)-like activity, which induces nitrogen uptake, and improves crop performance **(Colla et al. 2014)**. Furthermore, nitrogen-fixing bacteria contained in biostimulants have the ability to release phytohormones like GA and IAA which stimulate plant growth, absorption of nutrients and photosynthesis **(Rafiee et al. 2016)**. Taken together, increase in phytohormone content due to biostimulant application and N deprivation resulted in increased chlorophyll fluorescence (4 months), contributing greatly towards the increase in the growth of *C*. *triloba*.

Phytohormone content in P-deficient plants varied based on the applied biostimulants in 2 and 4-month-old plants. For instance, Kelpak® induced endogenous phytohormone accumulation, whereas VCL reduced the majority of the quantified phytohormones in P-deficient plants. Both biostimulants increased IAA production in P-deficient roots after 4 months. Auxin acts as an important signalling intermediates in multiple pathways related to P-starvation in plant roots **(Niu et al. 2013)**. This is due to the involvement of auxin as an antagonist in response to P-starvation **(Wittenmayer and Merbach 2005)**. Phosphorus-deficiency induces IAA synthesis which activates several transcriptional factors and protein synthesis while altering the activity of plasma membrane H^+-ATPase enzyme fundamental for P uptake **(Shen et al. 2006)**. Evidence has shown that auxin-dependent and independent pathways implicated in auxin transport may co-exist and aid in the regulation of P-starvation-induced root architectural changes **(Niu et al. 2013)**. Furthermore, IAA synthesis in response to P-starvation shows an increase in lateral root, primary root and lateral primordia **(Nacry et al. 2005)**, enhances root exudation for direct and indirect P mobilization in soil **(Wittenmayer and Merbach 2005)**. Therefore, increased rooting in P-deficient plants (with/without biostimulants) compared to N and K treatments improved nutrient uptake, adaptability and growth of 4 month-old plants.

Potassium-deficiency (untreated) increased phytohormone levels in *C*. *triloba*, particularly in 4-month-old leaves compared to 4-month-old roots. The 4-month-old leaves had increased JA-Ile and ABA production with Kelpak® application. Although, phytohormone content in 4-month-old roots was mostly stimulated with Kelpak® treatment. Application of VCL-treated plants had lower ABA levels in 4-month-old leaves and roots, with increased production of JA-Ile, *cis*OPDA, SA, JA and IAA.

Potassium deficiency stimulates the production of ABA formation in roots that is released to the xylem, then transported to the leaves **(Davies et al. 2005)**. Evidence on increased production of ABA in roots and xylem due to K-deficiency has been reported **(Peuke et al. 2002; Schraut et al. 2005; Wang et al. 2012)**. Increase in ABA levels induces K^+ translocation and accumulation in roots which is mediated by K^+ channels. Hence, a decline in K^+ efflux in the xylem vessels in *Hordeum vulgare* and *Zea mays* has been attributed to the reduction in ABA concentrations **(Roberts and Snowman 2000)**. The modification of ABA levels is due to several factors including plant nutrient status in roots, temperature, availability of extracellular glucose and plant species. Therefore, changes in ABA content between Kelpak® and VCL can be related to the type of biostimulant used. Even though both biostimulants improved the growth of *C*. *triloba* in K-deficient conditions, VCL-treated plants resulted in comparable or even better growth than 50% HS-treated plants. Therefore, the inhibitory effect of VCL on the production of ABA might have reduced stomatal closure, thus ensuring improved growth.

6.5.3. Effect of Kelpak® and vermicompost leachate (VCL) on phenolic acid quantification

Hydroxybenzoic and hydroxycinnamic acids are predominant phenolic derivatives found in plants, with hydroxycinnamic acids detected in high quantities **(Mattila and Hellström 2007)**. Phenolic acids are highly regulated by nutrient deprivation in plant tissues. Nutrient stress induces *de novo* synthesis and triggers metabolic pathways responsible for phenolic acid production **(Akula and Ravishankar 2011)**. These pathways are involved in complex mechanisms regulating the synthesis of phenolic acids and are produced at a specific site during stress conditions **(Cheynier et al. 2013)**. However, mechanisms involved in signal pathways increase bioactive

compounds at the expense of the plants physiological functions. Likewise, nutrient-deficient plants significantly increased the production of hydroxybenzoic and hydroxycinnamic acids while severely hindering the growth of *C*. *triloba*. Several studies have shown the trade-off between plant growth and secondary metabolites in nutrient-deficient plants **(Aremu et al. 2014; Jiang et al. 2007; Trull et al. 1997)**. However, nutrient deficiency can also have inhibitory effects towards the production of bioactive compounds in some species **(Colling et al. 2010; Gremigni et al. 2003)**. External signals including biostimulants activate specific biochemical pathways in plants thus enhancing the production of useful compounds **(Bulgari et al. 2014; Ertani et al. 2011)**. In *C*. *triloba*, phenolic acids were higher in 50% HS-treated plants (2 months) and P-deficient plants (4 months) treated with Kelpak®, whereas VCL enhanced the synthesis of bioactive compounds in K-deficient plants (4 months). The efficacy of biostimulants in the production of secondary metabolites is associated with the different plant growth regulators found in the products **(Aremu et al. 2015b; Stirk et al. 2014)**. Nonetheless, Kelpak® and VCL mostly inhibited bioactive compound production in 2 and 4-month-old plants. Accumulation of phytochemical content in plants is also dependent on the different stages of growth **(Juszczuk et al. 2004; Sarepoua et al. 2015)**. In *C*. *triloba*, phenolic acid accumulation was significantly enhanced with prolonged growth (4 months) compared to plants grown for shorted periods of time (2 months).

6.5.4. Effect of Kelpak® and vermicompost leachate (VCL) on mineral content and total carbohydrates

Nutritional content in *C*. *triloba* was mostly influenced by nutrient stress, biostimulant application and duration of growth. Generally, mineral content was reduced in nutrient deficient plants after 2 months of growth, with 50% HS-treated plants accumulating

high mineral composition (**Tables 6.5** and **6.6**). However, high mineral content was detected in K-deficient plants (untreated) grown for 4 months. Nitrogen and P-deficient plants had relatively low mineral concentration which were considerably stimulated with the application of Kelpak® and VCL. On the contrary, Kelpak® and VCL application mostly reduced mineral content in 50% HS-treated and K-deficient plants, with an increase in total carbohydrate content. Due to the number of plant growth regulators, biostimulants have been shown to enhance mineral content in different plant species **(Mancuso et al. 2006; Padmavathiamma et al. 2008; Peyvast et al. 2008)**. The stimulatory effect of Kelpak® and VCL on the nutritional content of *C*. *triloba* is possibly associated with the ability of the biostimulants to improve nutrient uptake in plants **(Calvo et al. 2014; Rathore et al. 2009; Theunissen et al. 2010)**. Furthermore, prolonged growth increased nutritional value in nutrient-deficient plants grown for 4 months when compared to 2-month-old plants, especially with the application of biostimulants. Increased mineral content in 4-month-old plants might possibly be due to the slow-release and uptake of nutrients from the biostimulants over time **(Chaoui et al. 2003)**.

6.6. Concluding remarks

Nitrogen, P and K are some of the major minerals essential for plant growth and development. Nutrient deprivation in *C*. *triloba* decreased growth, chlorophyll fluorescence and nutritional value, while enhancing endogenous phytohormones. The detrimental effect of nutrient shortage was overcome with the application of Kelpak® and VCL. This was evident in the improved shoot and root weight as well as plant height after 4 months of growth in nutrient deficient soils. Even though minimal changes were observed in chlorophyll fluorescence, biostimulants managed to slightly

improve ΦPSII, qP and relative ETR in nutrient-deficient plants particularly in plants treated with 50% HS after 4 months of growth. Furthermore, Kelpak® and VCL improved photosynthetic activity in plants by increasing NPQ levels which prevented the damaging effects of excess light energy, thus preventing photo-inhibition in plants. Endogenous phytohormones and phenolic acid modification played an important role during the growth of *C*. *triloba*. Nutrient stress together with Kelpak® and VCL contributed to the induction of phytohormone synthesis and the adaptability of plants to nutrient-deficient conditions. Furthermore, application of biostimulants inhibited phenolic acid production which might have alleviated the detrimental pressures resulting from a trade-off between plant growth and secondary metabolites. Kelpak® and VCL mostly increased the nutritional composition in nutrient-deficient plants. Duration of growth also plays a crucial role in the evaluated parameters. Chlorophyll fluorescence was higher in 2-month-old plants, while phytohormone, phenolic acid and nutritional content were significantly enhanced in 4-month-old plants.

Chapter 7: General conclusion

Ceratotheca triloba is an indigenous leafy vegetable that supplements starch-based diets among under-developed communities in southern Africa. The plant is widely used in traditional medicine, and scientific evidence to support some of the traditional claims have been documented. Literature on *C*. *triloba* propagation remains generally insufficient, the plant can be cultivated through seeds germinated in rich well-drained soil. In addition, application of Seagro or Humac fertilizers during the cultivation of *C*. *triloba* contributes to better yields. Therefore, stringent studies evaluating efficient cultivation practices that can be implemented during *C*. *triloba* seed germination, early seedling establishment and plant growth especially under different abiotic stress conditions are needed.

Temperature and water are among the most crucial factors controlling seed germination and seedling growth. During seed germination, *C*. *triloba* germination occurred at temperatures ranging from 15 – 35 °C, with 25 °C being the optimum temperature. However, seed germination at 15 °C was only stimulated with biostimulant-priming or hydropriming techniques. Furthermore, the stimulatory effects of seed priming were observed at low osmotic pressure and high NaCl concentrations. Essentially, seeds primed with smoke-water (SW), karrikinolide (KAR_1) and Kelpak® enhanced germination percentage in *C*. *triloba* under low temperature, low osmotic potential and NaCl concentrations. During seedling growth, SW and phloroglucinol (PG) treatments improved seedling growth at low temperature (15 °C) and low osmotic potential (-0.05 MPa). However, the detrimental effects of NaCl (5 – 25 mM) concentrations in seedlings was alleviated in hydroprimed seeds. Nonetheless, biostimulant-seed-priming and hydropriming techniques also had their limitations on germination and seedling growth at low temperature (10 °C), low osmotic potential (-

0.15 MPa) and NaCl concentration (50 mM). The current findings demonstrate the importance of priming (especially using biostimulants) on the improvement of seed germination, shoot length, root length, vigour index and survival rate in the alleviation of abiotic stresses in *C*. *triloba*.

Similar to other members of the Pedaliaceae, the genus *Ceratotheca* is characterised by a distinct type of mucilaginous glands covering most parts of the plant including the leaves **(Ihlenfeldt 2004)**. These glands allow the plants to withstand severe dehydration without tissue damage, making them drought resistant. The different mechanisms triggered during water stress in *C*. *triloba* in order to prevent severe dehydration and tissue damage remains undocumented. In the current study, the growth of *C*. *triloba* was dependent on water availability to maximize the plant's photosynthetic potential. This was mostly observed in the reduction of chlorophyll fluorescence parameters (*Fv*/*Fm*, ΦPSII, qP, relative ETR) which contributed greatly in the decline of growth in plants watered once a week. The decline in growth and chlorophyll fluorescence can be attributed to a number of factors including the regulation of endogenous phytohormones and phenolic acids. For instance, watering *C*. *triloba* plants once a week caused a significant decline in endogenous phytohormones (JA-Ile, *cis*OPDA, SA, JA and IAA) with an increase in ABA. Accumulation of ABA in plants might have arisen as a result of induced stress-response genes responsible for stomatal closure and plant growth reduction. Furthermore, reduction in water supply significantly induced the production of phenolic acids (4-hydroxybenzoic, protocatechuic, vanillic, caffeic, ferulic and 4-coumaric acid). Based on these results, there was a trade-off between the growth and bioactive compounds thus minimizing tissue damage during limited water supply in *C*. *triloba*. Mineral concentration in *C*. *triloba* watered once a week were also remarkably

enhanced (especially after 4 months of growth) although plants had reduced growth due to limited resources allocated for primary metabolic pathways.

The mode of action of Kelpak® and VCL in the improvement of stress tolerance is partly unknown and might be associated with the presence of stress-related molecules (e.g. plant growth regulators). In the current study, Kelpak® and VCL were effective soil amendment alternatives for the improvement of plant growth and chlorophyll fluorescence under high concentrations of NaCl and nutrient-deficient conditions. To some extent, biostimulants improved photosystem II efficiency (*Fv*/*Fm*) and NPQ levels which contributed greatly to the plants photosynthetic activity and prevented plant susceptibility to photo-damage. Nevertheless, the detrimental effects of NaCl (75 and 150 mM) and nutrient-deficiency (N, P and K) were evident during plant growth. Both stresses had a distinct effect on the regulation of endogenous phytohormones and phenolic acids. Salinity and nutrient-deficiency stresses were regulated by various mechanisms with different pathways. This was observed in the variation of endogenous phytohormones, in which NaCl-stressed (75 and 150 mM) plants had lower phytohormone concentrations relative to the control treatments. Nutrient-deficient plants (N, P and K) had phytohormone content higher than that of plants treated with 50% HS. The synergistic or antagonistic cross-talk associated with either stress modulated the biosynthesis or responses of endogenous phytohormones, thus improving the growth and chlorophyll fluorescence of *C*. *triloba*. The different pathways activated by plants were perhaps linked to the accumulation and toxic effects of NaCl in plant tissues or deprivation of major nutrients for plant growth and development. Therefore, Kelpak® and VCL provided some insights to the regulation of endogenous phytohormones during salinity (decreased phytohormone content) and nutrient-deficiency (increased phytohormone content) stresses in *C*. *triloba* plants. Based on

the differences in phytohormone responses triggered by the stresses and biostimulants, a detailed profiling of transcription factors and promoters, gene expression as well as antioxidant enzymes will be useful in providing further insights on the growth and development of *C*. *triloba* under these conditions. Such results can contribute to understanding the mode of action of biostimulants during abiotic stresses in plants. Furthermore, Kelpak® and VCL mostly inhibited the production of phenolic acids which might have alleviated the detrimental pressures resulting from a trade-off between plant growth and phenolic acids in *C*. *triloba*. In the present studies, there was a remarkable increase in nutritional content of *C*. *triloba* supplemented with biostimulants under salinity stress and nutrient-deficiency. In addition, mineral composition in *C*. *triloba* was enhanced after prolonged periods (4 months) of exposure to the different stress conditions. The enhanced nutritive content of *C*. *triloba* suggests that biostimulants may play a critical role in the alleviation of micronutrient deficiencies in plant grown under salinity and nutrient stresses.

Generally, the current results substantiate the potential use of biostimulants (SW, KAR_1, PG, eckol, Kelpak®, VCL) during seed germination, seedling establishment, plant adaptability and stress tolerance under different environmental factors. Therefore, the efficiency of biostimulants in overcoming some of the detrimental effects of abiotic stress is of utmost importance since *C*. *triloba* is among the commonly consumed vegetable crops in southern Africa. Based on the current findings, use of biostimulants can be possibly adapted to other valuable indigenous crop species especially within the Pedaliaceae. Despite the importance of biostimulants, *C*. *triloba* survival and adaptability strategies associated with prolonged exposure to reduced water supply (1 d), NaCl-stress (75 and 150 mM) and nutrient-deprivation (N, P, K) were mostly regulated by endogenous phytohormones and phytochemicals.

Therefore, a more in-depth assessment of mechanisms and pathways expressed or repressed is pertinent in order to fully understand the plant's metabolic pathways. These studies can also improve our understanding of how *C*. *triloba* is able to maintain/improve its mineral composition under reduced water supply, salinity and nutrient-deficient conditions.

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Appendix A

Table 1: A comparison between 2 and 4 months of growth on the chlorophyll fluorescence parameters under different watering regimes per week in *Ceratotheca triloba* plants analysed using Student's *t* test.

Chlorophyll fluorescence	Watering regime [day(s)] per week	µmol PAR m^{-2} s^{-1}							
		56		134		850		1279	
		F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
ΦPSII	7 (daily)	1.14	0.021 *	1.36	0.325 ns	0.83	0.112 ns	0.06	0.069 ns
	3 (thrice)	1.38	0.199 ns	0.19	0.240 ns	0.33	0.863 ns	0.07	0.959 ns
	2 (twice)	1.56	0.003 **	0.15	0.075 ns	1.90	0.477 ns	1.29	0.672 ns
	1 (once)	0.18	0.003 **	0.97	0.012 ***	0.00	0.609 ns	0.01	0.406 ns
qP	7 (daily)	1.86	0.385 ns	1.001	0.24 ns	0.52	0.988 ns	0.42	0.718 ns
	3 (thrice)	0.00	0.477 ns	1.635	0.01 **	1.35	0.254 ns	1.23	0.242 ns
	2 (twice)	0.36	0.006 **	4.187	0.06 ns	2.87	0.361 ns	1.95	0.496 ns
	1 (once)	0.04	0.003 **	1.048	0.48 ns	0.56	0.597 ns	0.69	0.544 ns
NPQ	7 (daily)	0.29	0.996 ns	0.20	0.496 ns	3.35	0.034 *	3.49	0.031 *
	3 (thrice)	0.17	0.476 ns	1.10	0.206 ns	0.05	0.903 ns	0.01	0.739 ns
	2 (twice)	0.01	0.427 ns	0.26	0.220 ns	0.42	0.467 ns	0.36	0.382 ns
	1 (once)	0.34	0.023 *	2.15	0.351 ns	2.31	0.811 ns	2.56	0.908 ns
Relative ETR	7 (daily)	1.14	0.021 *	1.36	0.325 ns	0.83	0.112 ns	0.06	0.069 ns
	3 (thrice)	1.38	0.199 ns	0.19	0.240 ns	0.33	0.863 ns	0.07	0.959 ns
	2 (twice)	1.56	0.003 **	0.15	0.075 ns	1.90	0.477 ns	1.29	0.672 ns
	1 (once)	0.18	0.003 **	0.97	0.012 **	0.00	0.609 ns	0.01	0.406 ns

Mean values ± standard error (n = 8) in the same column are significantly different, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) based on Duncan's Multiple Range Test (DMRT). Not significant (ns), quantum yield of photosystem II (ΦPSII), phytochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transfer rate (ETR).

Table 2: A comparison between 2 and 4 months of growth on the phytohormone content under different watering regimes per week in *Ceratotheca triloba* plants analysed using Student's *t* test.

Watering regimes	JA-Ile (pmol/g DW)			cisOPDA (nmol/g DW)			ABA (pmol/g DW)			SA (nmol/g DW)		
per week	*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
7 (daily)	1.11	0.004	**	1.11	0.487	ns	1.46	0.068	ns	7.32	0.025	*
3 (thrice)	8.89	0.005	**	1.89	0.833	ns	0.92	0.003	**	3.26	0.016	*
2 (twice)	11.53	0.015	*	1.26	0.179	ns	2.04	0.177	ns	3.84	0.002	**
1 (once)	12.23	0.009	**	2.06	0.100	ns	4.45	0.157	ns	3.52	0.000	***

Mean values ± standard error (n = 3) in the same column are significantly different, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) based on Duncan's Multiple Range Test (DMRT). Not significant (ns), jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), jasmonic acid (JA), indole-3-acetic acid (IAA), abscisic acid (ABA) and salicylic acid (SA).

Table 3: A comparison between 2 and 4 months growth on the phenolic acid content under different watering regimes per week in *Ceratotheca triloba* plants analysed using Student's *t* test.

Watering regimes per week	Protocatechuic acid			4-Hydroxybenzoic acid			Vanillic acid			Ferulic acid			Caffeic acid			4-Coumaric acid		
	µg/g DW																	
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
7 (daily)	0.01	0.010	**	4.54	0.001	***	0.00	0.002	**	2.26	0.000	***	0.07	0.365	ns	1.80	0.801	ns
3 (thrice)	0.80	0.000	***	5.30	0.000	***	0.04	0.000	***	0.04	0.001	***	0.00	0.000	***	0.47	0.001	***
2 (twice)	0.89	0.153	ns	0.01	0.000	***	4.41	0.000	***	0.76	0.000	***	0.48	0.000	***	1.17	0.000	***
1 (once)	3.28	0.075	ns	1.01	0.000	***	6.15	0.001	***	2.82	0.000	***	0.00	0.000	***	0.49	0.000	***

Mean values ± standard error (n = 3) in the same column are significantly different, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) based on Duncan's Multiple Range Test (DMRT). Not significant (ns).

Appendix B

Table 1: A comparison between 2 and 4 months of growth on chlorophyll fluorescence parameters stimulated with vermicompost leachate (VCL) under salinity in *Ceratotheca triloba* plants analysed using Student's *t* test.

Chlorophyll flourescence	Concentration of NaCl (mM)	µmol PAR m^{-2} s^{-1}																					
		56						134				850				1279							
		Untreated			VCL treated			Untreated			VCL treated	Untreated			VCL treated	Untreated			VCL treated				
		F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
ΦPSII	Control	0.22	0.124	ns	1.07	0.000	***	0.68	0.073	ns	0.01	0.000	***	2.90	0.020	*	0.38	0.053	*	7.37	0.013	**	0.50	0.040	*
	75	8.56	0.007	**	2.47	0.029	*	9.16	0.007	**	5.32	0.016	*	9.15	0.006	**	3.92	0.020	*	10.44	0.005	**	3.40	0.022	*
qP	Control	0.25	0.309	ns	0.11	0.000	***	1.30	0.550	ns	3.45	0.016	*	0.08	0.761	ns	0.19	0.796	ns	0.02	0.301	ns	0.14	0.622	ns
	75	4.72	0.013	**	1.61	0.040	*	3.68	0.016	*	2.03	0.035	*	4.27	0.014	*	1.53	0.041	*	3.84	0.017	*	0.78	0.060	ns
NPQ	Control	1.27	0.758	ns	0.38	0.612	ns	1.21	0.059	ns	1.48	0.002	**	2.49	0.002	**	0.57	0.058	ns	1.35	0.064	ns	0.49	0.305	ns
	75	1.70	0.031	*	1.37	0.122	ns	0.56	0.058	ns	0.00	0.138	ns	4.39	0.014	**	0.44	0.080	ns	3.85	0.016	*	0.49	0.078	ns
Relative ETR	Control	0.22	0.124	ns	1.07	0.000	***	3.66	0.068	ns	0.01	0.000	***	2.90	0.020	*	0.38	0.053	*	7.37	0.013	*	0.50	0.040	*
	75	7.67	0.008	**	1.88	0.036	*	9.16	0.007	**	5.32	0.016	*	18.53	0.017	*	3.92	0.020	*	10.44	0.005	**	3.40	0.022	*

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl), quantum yield of photosystem II (ΦPSII), phytochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transfer rate (ETR).

Table 2: A comparison between 2 and 4 months of growth in leaves stimulated with Kelpak® under salinity stress conditions on endogenous phytohormones in *Ceratotheca triloba* plants analysed using Student's *t* test.

Concentration of NaCl (mM)	JA-Ile (pmol/g DW)						cisOPDA (nmol/g DW)						ABA (pmol/g DW)						SA (nmol/g DW)					
	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
Control	6.52	0.000	***	5.77	0.015	*	0.21	0.015	*	0.03	0.116	ns	0.35	0.000	***	0.01	0.003	**	1.90	0.007	**	0.01	0.011	**
75	0.28	0.128	ns	4.55	0.004	**	6.24	0.000	***	4.16	0.022	*	3.94	0.000	***	0.06	0.001	***	0.04	0.472	ns	1.54	0.004	**

For each treatment, data is represented as mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl), jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), abscisic acid (ABA) and salicylic acid (SA).

Table 3: A comparison between 2 and 4 months of growth in leaves stimulated with vermicompost leachate (VCL) under salinity stress conditions on endogenous phytohormones in *Ceratotheca triloba* plants analysed using Student's *t* test.

Concentration of NaCl (mM)	JA-Ile (pmol/g DW)						cisOPDA (nmol/g DW)						ABA (pmol/g DW)						SA (nmol/g DW)					
	Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
Control	7.76	0.050	*	2.26	0.016	*	3.68	0.001	***	7.23	0.000	***	0.09	0.079	ns	0.45	0.005	**	0.75	0.214	ns	0.00	0.012	**
75	8.31	0.000	***	1.01	0.033	*	5.06	0.011	**	0.79	0.093	ns	1.43	0.001	***	0.15	0.002	**	2.22	0.011	**	2.01	0.020	*

For each treatment, data is represented as mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl), jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), abscisic acid (ABA) and salicylic acid (SA).

Table 4: A comparison between 2 and 4 months of growth stimulated with Kelpak® under salinity stress conditions on phenolic acid content in *Ceratotheca triloba* plants analysed using Student's *t* test.

Concentration of NaCl (mM)	Protocatechuic acid						4-Hydroxybenzoic acid						Vanillic acid					
	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
Control	2.64	0.011	**	0.05	0.002	***	9.65	0.001	***	4.88	0.000	***	0.31	0.071	ns	0.58	0.209	ns
75	10.30	0.000	***	10.75	0.000	***	0.03	0.000	***	4.49	0.000	***	6.69	0.000	***	2.55	0.042	*
Concentration of NaCl (mM)	Ferulic acid						Caffeic acid						4-Coumaric acid					
	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
Control	0.00	0.127	ns	1.42	0.014	**	3.99	0.546	ns	9.76	0.536	ns	5.17	0.014	**	0.03	0.000	***
75	6.66	0.001	***	0.01	0.430	ns	1.21	0.025	*	2.63	0.001	***	15.28	0.001	***	0.62	0.000	***

For each treatment, data is represented as mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between Kelpak®-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl).

Table 5: A comparison between 2 and 4 months of growth stimulated with vermicompost leachate (VCL) under salinity stress conditions on phenolic acid content in *Ceratotheca triloba* plants using Student's *t* test.

Concentration of NaCl (mM)	Protocatechuic acid						4-Hydroxybenzoic acid						Vanillic acid					
	Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
Control	9.08	0.000	***	0.85	0.000	***	5.30	0.000	***	2.66	0.032	*	7.83	0.000	***	0.05	0.003	***
75	0.89	0.000	***	8.82	0.001	***	4.46	0.000	***	3.31	0.001	***	0.08	0.124	ns	3.39	0.034	*

Concentration of NaCl (mM)	Ferulic acid						Caffeic acid						4-Coumaric acid					
	Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
Control	4.99	0.000	***	1.72	0.001	***	1.87	0.000	***	5.72	0.000	***	0.09	0.000	***	1.64	0.001	***
75	13.11	0.000	***	0.01	0.086	ns	1.35	0.020	*	2.20	0.001	***	9.91	0.000	***	7.25	0.002	***

For each treatment, data is represented as mean value ± standard error (n = 3) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Sodium chloride (NaCl).

Appendix C

Table 1: A comparison between 2 and 4 months of growth stimulated with Kelpak® in nutrient deficiency conditions on chlorophyll fluorescence in *Ceratotheca triloba* plants using Student's *t* test.

		µmol PAR m^{-2} s^{-1}																							
Chlorophyll		56						134						850						1279					
fluorescence	Treatment	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
		F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
ΦPSII	HS	6.14	0.010	**	6.37	0.000	***	2.57	0.809	ns	0.09	0.000	***	0.12	0.663	ns	8.02	0.028	*	0.08	0.807	ns	10.12	0.060	ns
	-N	0.78	0.000	***	11.82	0.000	***	2.01	0.018	*	0.03	0.000	***	0.27	0.000	***	3.62	0.103	ns	0.11	0.001	***	2.53	0.000	***
	-P	3.62	0.000	***	0.06	0.000	***	7.63	0.002	**	0.84	0.015	*	8.75	0.000	***	0.03	0.306	ns	3.78	0.002	**	8.60	0.428	ns
	-K	3.04	0.001	***	11.62	0.000	***	2.06	0.000	***	1.92	0.000	***	1.92	0.000	***	1.41	0.248	ns	12.36	0.000	***	0.00	0.143	ns
qP	HS	0.88	0.000	***	1.77	0.002	**	0.71	0.002	**	2.22	0.047	*	2.19	0.000	***	0.02	0.000	***	5.07	0.000	***	3.61	0.000	***
	-N	0.37	0.000	***	1.14	0.000	***	0.47	0.000	***	0.44	0.000	***	6.13	0.000	***	1.27	0.335	ns	16.83	0.000	***	4.41	0.445	ns
	-P	1.92	0.000	***	10.60	0.000	***	11.49	0.769	ns	0.24	0.000	***	0.53	0.912	ns	1.99	0.000	***	0.60	0.001	***	1.06	0.000	***
	-K	0.92	0.052	*	0.92	0.056	ns	0.00	0.425	ns	4.38	0.177	ns	1.28	0.000	***	0.20	0.000	***	4.16	0.000	***	0.00	0.000	***
NPQ	HS	2.44	0.000	***	3.95	0.199	ns	6.71	0.000	***	3.51	0.000	***	0.12	0.000	***	0.56	0.000	***	2.31	0.000	***	4.19	0.000	***
	-N	5.50	0.827	ns	29.60	0.987	ns	5.02	0.193	ns	0.72	0.001	***	1.20	0.008	**	10.45	0.009	**	6.94	0.018	*	3.03	0.021	*
	-P	0.05	0.011	**	0.85	0.374	ns	4.44	0.000	***	0.02	0.000	***	1.61	0.002	**	2.80	0.001	***	6.83	0.041	*	1.21	0.000	***
	-K	3.75	0.000	***	5.82	0.000	***	15.76	0.000	***	26.57	0.000	***	12.27	0.000	***	0.22	0.000	***	2.40	0.000	***	0.03	0.000	***
Relative ETR	HS	1.36	0.000	***	10.75	0.000	***	1.99	0.348	ns	0.20	0.000	***	4.05	0.826	ns	16.27	0.000	***	0.15	0.758	ns	11.34	0.002	**
	-N	0.00	0.000	***	1.78	0.000	***	0.31	0.000	***	18.63	0.000	***	3.45	0.000	***	3.97	0.558	ns	6.12	0.000	***	1.07	0.049	*
	-P	3.17	0.000	***	1.01	0.000	***	0.02	0.001	***	0.43	0.013	**	0.94	0.007	**	1.08	0.000	***	0.13	0.010	**	2.45	0.001	***
	-K	9.39	0.000	***	0.71	0.000	***	12.96	0.000	***	2.14	0.000	***	14.03	0.000	***	1.94	0.011	**	21.99	0.000	***	0.24	0.671	ns

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Nitrogen (N), phosphorus (P), potassium (K), quantum yield of photosystem II (ΦPSII), phytochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transfer rate (ETR).

Table 2: A comparison between 2 and 4 months of growth stimulated with vermicompost leachate (VCL) in nutrient deficiency conditions on chlorophyll fluorescence in *Ceratotheca triloba* plants using Student's *t* test.

Chlorophyll fluorescence	Treatment	ΦPSII µmol PAR m^{-2} s^{-1}																					
		56						134						850						1279			
		Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
		F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
ΦPSII	HS	0.93	0.002	**	5.47	0.001	***	0.70	0.000	***	9.09	0.000	***	9.88	0.000	***	0.25	0.044	*	9.05	0.000	***	0.27	0.010	**
	-N	11.41	0.498	ns	4.21	0.738	ns	0.05	0.315	ns	1.71	0.015	*	9.81	0.009	**	1.76	0.000	***	8.77	0.035	*	0.03	0.000	***
	-P	24.21	0.488	ns	0.20	0.000	***	1.17	0.007	**	0.12	0.000	***	2.98	0.038	*	0.41	0.000	***	5.15	0.012	**	0.38	0.001	***
	-K	0.60	0.003	**	1.02	0.000	***	6.65	0.000	***	4.24	0.000	***	0.90	0.010	**	0.15	0.059	ns	2.81	0.000	***	0.04	0.404	ns
qP	HS	0.65	0.004	ns	0.34	0.000	***	0.01	0.000	***	1.59	0.002	**	0.11	0.000	***	1.47	0.002	**	3.18	0.001	***	0.01	0.001	***
	-N	2.27	0.000	***	0.06	0.118	ns	11.43	0.344	ns	1.81	0.085	ns	0.15	0.000	***	7.16	0.000	***	1.96	0.001	***	0.47	0.001	***
	-P	6.51	0.020	*	2.40	0.000	***	12.17	0.702	ns	1.24	0.386	ns	12.05	0.000	***	0.04	0.099	ns	9.21	0.000	***	0.15	0.013	**
	-K	1.99	0.000	***	7.58	0.000	***	16.03	0.000	***	0.70	0.000	***	2.90	0.000	***	0.00	0.002	**	0.01	0.000	***	0.80	0.022	*
NPQ	HS	2.20	0.000	***	0.21	0.000	***	0.58	0.000	***	3.21	0.000	***	2.95	0.166	ns	0.10	0.003	**	2.69	0.913	ns	0.04	0.004	**
	-N	15.70	0.000	***	4.38	0.001	***	29.85	0.081	ns	0.46	0.583	ns	0.71	0.000	***	34.19	0.016	*	2.61	0.000	***	8.16	0.029	*
	-P	4.15	0.564	ns	3.21	0.000	***	44.44	0.000	***	9.50	0.000	***	0.02	0.605	ns	2.10	0.026	*	0.49	0.511	ns	0.05	0.001	***
	-K	9.27	0.000	***	0.43	0.109	ns	7.39	0.020	*	7.13	0.000	***	0.01	0.000	***	0.00	0.180	ns	2.38	0.000	***	0.01	0.228	ns
Relative ETR	HS	1.58	0.007	**	5.54	0.001	***	1.04	0.002	**	9.14	0.000	***	9.47	0.000	***	3.44	0.000	***	11.04	0.000	***	4.14	0.000	***
	-N	5.36	0.227	ns	0.21	0.377	ns	3.66	0.092	ns	0.26	0.000	***	20.73	0.008	**	0.06	0.000	***	10.13	0.002	**	0.06	0.000	***
	-P	26.04	0.201	ns	0.20	0.000	***	6.24	0.000	***	0.41	0.000	***	0.46	0.000	***	0.95	0.000	***	0.68	0.000	***	0.04	0.001	***
	-K	0.01	0.004	**	0.57	0.000	***	4.37	0.000	***	0.78	0.000	***	0.02	0.001	***	0.88	0.007	ns	0.12	0.000	***	0.15	0.084	ns

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Nitrogen (N), phosphorus (P), potassium (K), quantum yield of photosystem II (ΦPSII), phytochemical quenching (qP), non-photochemical quenching (NPQ) and relative electron transfer rate (ETR).

Table 3: A comparison between 2 and 4 months of growth stimulated with Kelpak® in nutrient deficiency conditions on endogenous phytohormones in *Ceratotheca triloba* plants using Student's *t* test.

Treatment	JA-Ile						cisOPDA						ABA						SA					
	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
HS	0.68	0.027	*	8.79	0.001	***	6.86	0.609	ns	7.77	0.002	**	7.31	0.001	***	2.49	0.000	***	0.00	0.007	**	0.01	0.067	ns
-N	4.37	0.005	**	12.06	0.011	**	5.13	0.002	**	1.66	0.005	**	3.63	0.000	***	2.13	0.000	***	12.14	0.013	**	14.57	0.006	**
-K	4.81	0.003	**	11.16	0.006	**	1.59	0.811	ns	2.62	0.019	*	0.50	0.112	ns	4.61	0.001	***	2.54	0.021	*	0.02	0.358	ns

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Nitrogen (N), phosphorus (P), potassium (K), jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), jasmonic acid (JA), abscisic acid (ABA), salicylic acid (SA) and indole-3-acytic acid (IAA).

Table 4: A comparison between 2 and 4 months of growth stimulated with vermicompost leachate (VCL) in nutrient deficiency conditions on endogenous phytohormones in *Ceratotheca triloba* plants using Student's *t* test.

Treatment	JA-Ile						cisOPDA						ABA						SA					
	Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
HS	0.00	0.000	***	1.26	0.019	*	14.11	0.000	***	1.49	0.013	**	5.89	0.001	***	3.34	0.004	**	0.10	0.826	ns	0.28	0.009	**
-N	0.16	0.017	*	2.04	0.011	**	9.55	0.001	***	6.96	0.004	**	12.05	0.002	**	3.46	0.001	***	7.66	0.018	*	0.66	0.004	**
-P	0.01	0.384	ns	9.81	0.001	***	3.69	0.000	***	0.88	0.172	ns	3.48	0.002	**	1.54	0.005	**	3.61	0.006	**	0.08	0.059	ns
-K	2.01	0.004	**	0.25	0.005	**	4.31	0.001	***	11.29	0.004	**	11.01	0.001	***	0.03	0.002	**	0.01	0.043	*	4.56	0.132	ns

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Nitrogen (N), phosphorus (P), potassium (K), jasmonoyl isoleucine (JA-Ile; active form of JAs), cis-(+)-12-oxo-phytodienoic acid (*cis*OPDA; JAs precursor), jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA).

Table 5: A comparison between 2 and 4 months of growth stimulated with Kelpak® in nutrient deficiency conditions on the phenolic acid content in *Ceratotheca triloba* plants using Student's *t* test.

Treatment	Protocatechuic acid						Ferulic acid						4-Hydroxybenzoic acid					
	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
HS	11.6	0.000	***	0.3	0.000	***	1.1	0.000	***	6.3	0.180	ns	3.4	0.000	***	6.8	0.000	***
-K	3.6	0.000	***	3.7	0.000	***	0.0	0.014	**	4.3	0.007	**	3.8	0.000	***	8.7	0.005	**
Treatment	Caffeic acid						Vanillic acid						4-Coumaric acid					
	Untreated			Kelpak treated			Untreated			Kelpak treated			Untreated			Kelpak treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
HS	7.6	0.000	***	9.2	0.037	*	7.7	0.001	***	4.4	0.221	ns	1.5	0.000	***	0.2	0.078	ns
-K	0.1	0.599	ns	4.0	0.050	*	2.4	0.006	**	0.4	0.069	ns	5.8	0.000	***	0.2	0.170	ns

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Nitrogen (N), phosphorus (P), potassium (K).

Table 6: A comparison between 2 and 4 months of growth stimulated with vermicompost leachate (VCL) in nutrient deficiency conditions on the phenolic acid content in *Ceratotheca triloba* plants using Student's *t* test.

Treatment	Protocatechuic acid						Ferulic acid						4-Hydroxybenzoic acid					
	Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
HS	8.2	0.000	***	0.2	0.000	***	2.0	0.002	***	0.0	0.000	***	0.1	0.001	***	15.1	0.001	***
-P	8.0	0.006	**	4.2	0.007	**	9.2	0.000	***	4.0	0.000	***	0.3	0.000	***	7.9	0.000	***
-K	7.3	0.000	***	2.5	0.000	***	0.2	0.393	ns	5.1	0.053	*	0.0	0.001	***	5.2	0.005	**
Treatment	Caffeic acid						Vanillic acid						4-Coumaric acid					
	Untreated			VCL treated			Untreated			VCL treated			Untreated			VCL treated		
	F value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value		*F* value	*P* value	
HS	0.4	0.387	ns	7.6	0.000	***	0.0	0.008	**	4.3	0.495	ns	4.4	0.015	*	2.6	0.746	ns
-P	6.0	0.000	***	3.6	0.015	*	5.4	0.000	***	0.6	0.167	ns	2.5	0.000	***	3.8	0.000	***
-K	0.2	0.000	***	0.0	0.028	*	0.1	0.016	*	5.9	0.469	ns	11.6	0.000	***	0.5	0.015	*

For each treatment, data is represented as mean value ± standard error (n = 7) and asterisk(s) indicate significant difference between VCL-treated and untreated plants. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); ns (not significant) based on the Student's *t* test. Nitrogen (N), phosphorus (P), potassium (K).

Appendix D

Table 1. Composition of Hoagland's nutrient solution (half strength)

Stock	Concentration
$Ca(NO_3)_2.4H_20$	0.75M
KNO_3	0.75M
$MgSO_4.7H_20$	0.30M
KH_2PO_4	0.15M
NaFeEDTA	2.3M
H_3BO_3	7.0mM
$MnCl_24H_20$	1.37mM
$ZnSO_4.7H_20$	0.12mM
$CuSO_4.5H_20$	22µM
$H_2MoO_4.H_20$	16µM
$NaNO_3$	0.75M
$MgCl_2.6H_20$	0.30M
Na_2SO_4	0.30M
NaH_2PO_4	0.15M
$CaCl_2.2H_20$	0.75M
KCl	0.75M