Impacts of climate change on cowpea (*Vigna unguiculata* L. Walp) treated with biostimulants

By

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Master of Science

Research Centre for Plant Growth and Development
School of Life Sciences

College of Agriculture, Engineering and Science

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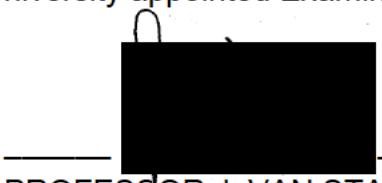

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List of abbreviations

ANOVA	Analysis of variance
C	Carbon
CO_2	Carbon dioxide
CAM	Crassulacean acid metabolism
CH_4	Methane
d	Day(s)
DAS	Days after sowing
dH_2O	Distilled water
DMRT	Duncan's Multiple Range Test
FW	Fresh weight
GHGs	Greenhouse gases
h	Hour(s)
IPCC	Intergovernmental Panel on Climate Change
KAR_1	Karrikinolide
KEL	Kelpak®
MeOH	Methanol
min	Minute(s)
N_2O	Nitric oxide
N	Nitrogen
NSC	Non-structural carbohydrates
O_2	Oxygen
PPM	Parts per million
PPF	Photosynthetic photon flux
P	Phosphorus
pH	Potential of hydrogen
ROS	Reactive oxygen species
SWC	Seaweed concentrates
SW	Smoke-water
TMB	Trimethylbutenolide
VCL	Vermicompost leachate

Abstract

Vigna unguiculata L. Walp, commonly known as cowpea, is a warm-season herbaceous legume considered native in Africa and Asia. The crop is traditionally consumed as both a leafy vegetable and staple pulse. Although the growth behaviour and nutritional composition of *V. unguiculata* have been explored by the research community, information regarding the plant's response to biostimulants under abiotic stresses remain limited. Therefore, studies of this nature are pertinent, especially in the presence of climate change which manifests into global warming, drought episodes and dissipating of natural resources. Hence, a better understanding of the effects of temperature and drought stress on *V. unguiculata* physiology, morphology, nutrition and phytochemistry are important to ensure high yields which is important for meeting the goals of global food security. Firstly, this study investigated the effects of seed priming with biostimulants [vermicompost leachate, VCL (1:20 v/v), commercial seaweed extract Kelpak®, KEL (0.6%) and smoke-water, SW (1:1000 v/v)] and distilled water (dH_2O) on *V. unguiculata* germination and seedling parameters under constant day/night temperatures of 30/30, 35/35 and 40/40 ˚C in Conviron® plant chambers. In addition, hydroponic experiments were set-up to evaluate biostimulant efficacy on rooting. Secondly, post-germination effects of VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) were evaluated under similar temperature conditions to ascertain the influence on morphological parameters 28 days after sowing (DAS). Thirdly, post-germination effects of VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) were investigated under different watering regimes using greenhouse protocols to ascertain biostimulation influence on growth variables and flowering after 13 weeks. Finally, the effects of VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) were tested on *V. unguiculata*'s photosynthetic pigments [chlorophyll *a*, *b*, (*a* + *b*) and carotenoids], carbohydrates, proteins and phytochemicals (total phenolics and flavonoids) grown for 11 weeks under different watering regimes in the greenhouse.

Despite the biostimulants not differing significantly with the corresponding controls, KEL and SW induced marked germination at 30, 35 and 40 ˚C while VCL being potent under 30 and 40 ˚C. Seed priming (i.e. biostimulant and hydropriming) significantly improved shoot length and root length over non-priming, with biostimulant-priming being more effective under 40 ˚C by also inducing significantly higher leaf number,

fresh weights and seedling areas compared to non-primed controls. At 30 ˚C, priming with the three biostimulants improved peduncle diameter, fresh weight and established a significant increase on root length and dry biomass over hydropriming. VCL was most effective at promoting shoot length, root elongation and dry weight under 30 ˚C while KEL was most effective in increasing seedling leaves, shoot length, root number, fresh and dry biomass of plants exposed to 35 ˚C.

Although overall, biostimulant-non-priming was second best after biostimulant-priming, non-priming with biostimulants was able to promote key variables compared to both controls (i.e. non-primed and hydroprimed controls). Hydroponic results revealed that non-priming with VCL and SW increased root number by 3 and 4-fold, respectively, at 40 ˚C. SW also stands out at enhancing a significant increase in leaf number and seedling area whereas KEL was the most significant solution promoting shoot length and seedling area. At 30 ˚C, SW-non-priming promoted significant root elongation, improved fresh and dry weights while VCL was best at promoting root number and root length. KEL and SW also exhibited post-germination effects over the control at 40 ˚C by improving leaf number on a weekly basis. Fresh and dry weight were improved similarly with significant improvements at 30 ˚C by KEL and SW.

Increasing watering regimes from once to thrice a week significantly increased the number of leaves, root length and flower number. The number of nodules, however, did not differ significantly. Restricting watering frequency to once a week significantly increased shoot length, root length and leaf area in SW-treated plants compared to the control. Shoot length and root length of KEL-treated plants were also increased similarly. Remarkably, VCL increased the number of nodules and shoot length by 4 and 3-fold, respectively. Relative leaf weekly growth in SW, VCL and KEL was higher by 1, 4 and 5 leave(s), respectively, after 11 weeks under high water deficits. This foliage increase remained high by more than 4 leaves in KEL and SW-treated plants watered twice and thrice a week. Accompanying increase in number of flowers was only established in SW water-stressed plants. However, raising watering frequency to twice a week increased flower number in SW, VCL and KEL-treated plants by 2, 4 and 7-fold, respectively, compared to the control. This floral increase was still comparatively high by 2, 4 and 2-fold, respectively, in plants watered thrice a week with biostimulants. VCL also induced a marked significant increase on root length, peduncle diameter and dry weights of plants watered thrice a week.

Decreasing substrate water availability from thrice to once a week induced a general increase in leaf soluble proteins, total phenolics and flavonoids. This watering transition significantly enhanced root soluble carbohydrates and proteins while root phenolics and flavonoids markedly declined. VCL, KEL and SW promoted leaf carbohydrates coupled with significant increases in those of roots of SW plants compared to the corresponding controls. Remarkably, leaf soluble proteins of biostimulant plants significantly declined to within the ranges of the plants watered twice and thrice a week. Root proteins were significantly greater to those of leaves in high water-stressed plants and statistically the same to those of roots of plants watered twice and thrice a week. Total phenolics and flavonoids of foliage of the biostimulant plants were lowered and relatively the same in the different watering regimes. Root total phenolics were highly inhibited in less watered plants and gradually increased with an increase in watering regimes. Similar trends were established in flavonoids although they were greater than in the corresponding controls.

Biostimulant photosynthetic pigments [i.e. chlorophyll *a*, *b*, (*a* + *b*) and carotenoid contents] did not differ significantly with those of the control in plants watered once a week. However, the three biostimulants were able to improve chlorophyll *a* and *a* + *b*. KEL and SW induced higher increase in chlorophyll *b* and carotenoid concentrations. The biostimulants increased chlorophyll *a* in 3-day-watered plants by more than 2-fold. These biostimulants also improved the chlorophyll *a* + *b* and carotenoid contents. Both increasing and decreasing trends in compatible solutes (i.e. soluble sugars and proteins), photosynthetic pigments and phytochemicals under water deficits indicated biostimulant-induced capacity in cowpea for osmotic adjustment, drought tolerance or adaptive mechanisms to water stress. These findings demonstrated the biological potential of VCL, KEL and SW to improve germination, seedling/plant growth and yield in legumes even under temperature stress and drought stress. Thus, establishing their stress amelioration properties to offset negative impacts of climate change on plants and yield.

Chapter 1: General introduction

1.1 Introduction

Although cowpea (*Vigna unguiculata* L.) can thrive in hostile ecological niches, growth remains susceptible to excessively wet conditions, poorly drained soils **(Gómez, 2004)**, extreme temperatures (i.e. too hot or cold) and prolonged drought events **(Masenya, 2016)**. This places major limitations on the productivity of cowpea. These conditions, particularly reduced weather precipitation, elevated temperature regimes and prolonged drought incidences, are modern and prevalent consequences of climate change and are detrimental to autotrophic plants **(IPCC, 2014)**. The Intergovernmental Panel on Climate Change (IPCC) described this change in climate as a variation in the state of the atmosphere that can be measured using barometrical tools and statistical tests to obtain variations in the mean and/or variability of its properties, for an extended time-frame, usually decades or centuries **(UNFCCC, 2011)**. These variations are a result of both natural variability and anthropogenic emissions such as the release of ozone-depleting substances known as greenhouse gases (GHGs) such as carbon dioxide (CO_2), methane (CH_4), nitric oxide (N_2O) and fluorinated gases **(IPCC, 2014)**. The build-up of GHGs in the atmosphere triggers temperature variations, causing the global warming phenomenon and subsequently, unprecedented weather patterns with catastrophic natural disasters for humans, flora and fauna **(Dusenge et al., 2019; OECD, 2008)**. The impact of climate change, especially those that are caused by increasing temperatures on natural systems, are considered unequivocal and present a huge cost to the world economy and societies at large **(OECD, 2008)**.

In recent years, biological treatments such as biofertilizers, biopesticides as well as biostimulants, have received more research attention than synthetic agricultural inputs **(Poberežny et al., 2020)**. This has changed the way farmers cultivate their crops. A farmers' incentives to adopt bio-derived products to improve productivity may be driven by several key constraints. These constraints include an elevation in plant stresses as a result of recent environmental changes; increasing governmental support for environmental-friendly agri-inputs; public demand for high-value crops as well as the biological capabilities of biostimulants to improve crop growth, yield and

quality under constraint conditions **(Poberężny et al., 2020)**. Biostimulants such as vermicompost leachate (VCL) **(Agrawal et al., 2018; Kaur et al., 2018; Morales-Corts et al., 2018)**, commercial seaweed extract Kelpak® (KEL) and smoke-water (SW) exhibit potential for use in agriculture and horticulture **(Aremu et al., 2012; Gutiérrez-Miceli et al., 2017; Kocira et al., 2013; Kulkarni et al., 2011; Light et al., 2009)**. These biostimulants are reported to improve seed germination, growth and yield in many plant species **(Carlos et al., 2008; Kocira et al., 2020; Kulkarni et al., 2011)**. Literature reports that their active ingredients can protect plants against abiotic stresses, improve micro- and macronutrient up-take efficiency without compromising plant nutrition, environmental integrity and are cost-effective **(Battacharyya et al., 2015; Esakkiammal et al., 2015; Kulkarni et al., 2011; Sharma and Garg, 2018)**. Therefore, with rising poverty and malnutrition there is a need to explore every viable option in agriculture with a potential to improve plant yield and alleviate poverty. This includes increasing the use of biostimulants on staple crops as an additional avenue of ensuring food security. This is crucial for food-insecure countries like South Africa, where the production and productivity of staple crops such as *V. unguiculata* still remain relatively low, due to climate constraints, scanty breeding research, lack of stress resistant varieties and poor adoption of improved varieties **(Asiwe, 2009)**.

1.2 Rationale/problem statement of the study

From an agricultural point of view, the current increase in temperatures, drought episodes and reduced precipitation triggers a negative response on the growth and productivity of many crops including *V. unguiculata* resulting in reduced yields. A significant reduction in *V. unguiculata* yield and that of other staple crops is anticipated to increase in the near future, given a predicted rise of 2-4 °C in global temperature and a carbon dioxide (CO_2) accumulation rate of one part per million (ppm) per annum in the atmosphere, based on the current rate of emissions **(Kiprotich et al., 2015; Vadez et al., 2012)**. Climate change also presents an additional challenge to both food security and food safety as it contributes to the elevation of hunger and malnutrition **(FAO, 2008)**. Malnutrition is responsible for over one-third of all child deaths around the world with an alarming 54% in low-income countries **(Bain et al., 2013; Jayathilake et al., 2018)**.

Another concern is the significant variation in yield response of *V. unguiculata* to climate change across agro-ecological zones **(Kiprotich et al., 2015)**. Adaptation of crop varieties to local conditions can partially mitigate the negative impacts of climate change. However, the adoption of comprehensive agricultural practices that can immediately offset negative impacts of climate change and global warming, could allow for the cultivation of crop varieties in any given region with relatively similar abiotic and biotic stresses. The use of biostimulants could provide a viable research avenue to resolve agricultural challenges faced by *V. unguiculata* farmers in different agro-ecological zones as a result of climate change.

1.3 Aim and objectives

1.3.1 Aim

To investigate the effects of VCL, KEL and SW on growth, photosynthesis, phytochemicals, biochemicals and flowering of *V. unguiculata* cultivated under temperature stress and drought stress.

1.3.2 Main objectives

In pursuit of the above main aim, experimental trials were designed in order to achieve the following objectives:

i. To determine effects of priming and non-priming with VCL, KEL and SW on seed germination and seedling growth of *V. unguiculata* 7 and 28 days after seed sowing under elevated temperatures of 30, 35 and 40 °C;

ii. To determine effects of VCL, KEL and SW on growth and flowering of *V. unguiculata* planted under different watering regimes using greenhouse protocols and;

iii. To investigate effects of VCL, KEL and SW on photosynthetic pigments, phenolics, flavonoids, total carbohydrates and proteins of *V. unguiculata* after 13 weeks of growth under different watering regimes in the greenhouse.

Chapter 2: Literature review

2.1 Introduction

In order to meet the food demand for a surging world population of over 7.7 billion people, agricultural output needs to double by 2050 **(UN, 2019)**. This increase in productivity must encompass sustainable ways of land-use, more efficient irrigation and eco-friendly practices. The increased food supply will also reduce hunger and malnutrition in 1 billion people from sub-Saharan Africa, Asia and elsewhere **(FAO, 2011)**. Yet, this must be done in the presence of climate change, dissipating natural resources and under the manifestation of intense temperatures and drought episodes unprecedented in the history of agriculture. Crop production already utilizes 11% of available arable land on earth **(FAO, 2011)** influenced by high intake of crops in the human diet **(Bonhommeau et al., 2013)**. This shows the significance of plants to humans, particularly angiosperms which accounts for 50-90% of our plant-derived diet and total energy intake **(Procheş et al., 2008)**. Therefore, it is evident that the required increase in food supply should come from crops.

2.2 Importance of legumes

Legumes form a unique taxonomic group of angiosperm plants that produce an average of 1-12 seeds per pod **(Morel et al., 2012)**. They belong to the Fabaceae family, which has approximately 770 genera and more than 19 500 species. The Fabaceae is the third largest angiosperm family after the Asteraceae and Orchidaceae **(Menéndez et al., 2019)**. Among important members of this family are legumes such as cowpea (*V. unguiculata*), soybean (*Glycine max* L.), peas (*Pisum sativum*), mungbean (*Vigna radiata*), common bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris*), alfalfa (*Medicago sativa*) and chickpea (*Cicer arietinum*). Legumes play a crucial role as forage crop to rear livestock, in soil nutrient-replenishment and as a cost-effective food source with rich plant-based proteins. Their specialised root organs called nodules are inhabited by bacteria containing an enzyme known as nitrogenase that can fix up to 350 kg of N_2/ha/year **(Foyer et al., 2019)**. This gives legumes a competitive advantage to thrive in habitats with less soil fertility and low levels of N. Moreover, this also makes N_2-fixing legumes suitable to grow with “N-hungry” crops

such maize (*Zea mays*), millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*) using an intercropping agricultural system **(FAO 2016a)**. Globally, legumes are harvested as seeds called a pulse, green food (usually pods), or as biomass for oil extraction as well as to extract biochemicals for pharmaceutical purposes **(Lewis et al., 2005)**. Their multipurpose use coupled with broad genetic diversity in species (which are adapted to flourish in various climates) has led crop breeders to deduce that legumes can allow for the development of high-yielding and climate resilient varieties **(Calles, 2016; FAO 2016a)**.

2.3 An overview of *Vigna unguiculata*

V. unguiculata is an annual, warm-season and herbaceous crop considered native in Africa and Asia. It is globally known as cowpea, frijol or lubia, depending on the country and the language. Its common names in South Africa, varying by language include in English: cowpea, china pea or cowgram; in Afrikaans: akkerboon, koertjie or swartbekboon; in isiZulu: isihlumaya or indumba; in Shangaan: tinyawa, munaoa, dinaba; in Venda: munawa, indumba and; in Sepedi: dinawa. In 1647, an Italian botanist called Domenico Vigna was honoured by the naming of the cowpea, *Vigna unguiculata* L. Walp after him **(Pooley, 1998)**. Globally, *V. unguiculata* is an important food and fodder crop. It is cultivated as a highly palatable and nutritious legume in Asia, Africa, the Middle East, United States of America and tropical regions **(Sheahan, 2012)**. It is also a staple crop that can be consumed at any growth stage **(Masenya, 2016)** by subsistence farmers in semi-arid areas **(Kiprotich et al., 2015)**. Edible parameters include fresh pods, seeds, flowers and foliage **(Jayathilake et al., 2018)**. As a fodder crop, it can either be used as forage, silage or hay. When used as forage, it must be lightly grazed after flowering. When used as silage, it should be mixed with maize, sorghum, or molasses to give fermented sugar **(FAO, 2012)**.

2.3.1 Morphology

V. unguiculata has complex taxonomic varieties identified by shape, colour, pulse size, taste, yield, stress tolerance and life cycle. Varieties include brown-eyed peas, black-eyed peas, pink-eyed peas, purple hull peas, white acre type, crowder peas, cream peas and clay type pulse. Seeds can readily germinate, producing seedlings with vigorous growth. Plants can either be tall and vine-like, prostrate or short and bushy

with trifoliate, egg-shaped and hairless leaves. It has branchless inflorescences with white or purple flowers along the main axis. All varieties flower in spring and summer to produce cylindrical straight or curved pods that contain approximately 6-13 seeds **(Sheahan, 2012)**. The seeds have distinctive colours, namely, red, yellow-brown and black-brown, depending on the variety **(Xaba, 2007)**. The seeds are also either round or kidney shaped and weigh between 8-32 mg **(Sheahan, 2012)**. The long peduncles are the distinguishable feature of *V. unguiculata* to other legumes **(Davis et al., 1991)**. A single peduncle can bear 2-3 pods, or even 4 or more pods under favourable conditions **(Masenya, 2016)**. *V. unguiculata* seeds are rich in proteins, starch, carbohydrates and fat content. An average single pulse contains 23-32% crude protein **(Cruz et al., 2014)**, 50-65% carbohydrates, and 1-2% fat **(FAO, 2012; Kirse and Karklina, 2015)**. The pulse also has 0.00074% thiamine, 0.00281% niacin and 0.00042% riboflavin **(Davis et al., 1991)**.

V. unguiculata exhibits optimal performance in well-drained sandy or sandy-loam soils where growing seasons have 670-1520 mm rainfall **(Masenya, 2016)** under long and dry summer conditions **(Sheahan, 2012)**. It also shows considerable adaptation to warm and hostile ecological niches due to persistent morpho-physiological capabilities **(Masenya, 2016)**. These existing capabilities include low stomatal water-loss, a vine-like system which flourishes with less nutrients and water and a deep taproot system with long adventitious roots for prolific water up-take, nutrient absorption and effective N_2-fixation **(Cruz et al., 2014; Masenya, 2016)**. Its deep root system and wide vegetative spread also helps in weed suppression and prevention of soil erosion. Allelopathic compounds present in *V. unguiculata* give additional capacity to suppress noxious weeds **(Clark, 2007).** This benefits other intercropped plants by making N available, weed suppression and preventing soil degradation. However, *V. unguiculata* struggles to thrive under frequent and long wet conditions, drought and extreme temperatures **(Gómez, 2004)**. These abiotic conditions affect its seed germination, growth and yield.

2.3.2 Domestication

The lack of archaeological records about *V. unguiculata*'s place of origin has resulted in different opinions. Many people support the idea that it originated from South

America, Asia and Africa. Till today, its place of origin still remains debatable. However, the most popular claim is that the crop originated in southern and west Africa **(DAFF, 2013, 2014)**. The reason for such a claim is the way in which its wild species are distributed across the sub-Saharan African region, particularly in west Africa **(Ngalamu et al., 2015)**. Another fact in favour of the African theory comes from the great genetic diversity of *V. unguiculata* found in almost the entire continent with southern, central and west African countries having the largest variation in their savannah biomes **(Agbogibi and Egho, 2012; Ngalamu et al., 2015)**.

2.3.3 Global and local production status

Despite extensive global cultivation of *V. unguiculata*, it is often difficult to accurately quantify and access reliable data on cowpea. This is due to patchy production among subsistence farms in villages, limited research funding **(Asiwe, 2009)** and substantial use as a N_2-fixer for major cereals and cash crops including cotton (*Gossypium*) and sugarcane (*Saccharum officinarum*). Nevertheless, it is known that the global output of *V. unguiculata* is approximately 3 million metric tons per annum **(Frota et al., 2017)**. In 2008-2010, this total output increased to 5.3 million metric tons and most of the contributions came from Africa **(Nedumaranaa et al., 2015; Frota et al., 2017)** since it is the world's largest producing continent (**Fig. 2.1**) **(Gómez, 2004)**. Under cultivation, western and central Africa produce 64% of the 68% (**Fig. 2.1**) total output from 8 million ha of the global 12.5 million ha **(Ngalamu et al., 2015; Frota et al., 2017)**. In west Africa, Nigeria is the largest producer of *V. unguiculata* with an average production of 2.58 ± 0.31 million metric tons from 2001-2010 **(Adeola et al., 2011; AATF, 2012)**. However, due to high demand the country still imports *V. unguiculata* from countries such as Burkina Faso, Mali, Niger and Ghana. Other important emerging producers include United States of America and countries in Asia and South America **(Ngalamu et al., 2015)**.

For many years in South Africa, *V. unguiculata* has not received enough farming and research attention. Factors such as lack of proper agronomic practices, scarcity of improved varieties, unavailability of good seeds and insignificant marginal returns exacerbated its negligence as a crop **(Asiwe, 2009)**. Its limited production is mainly prevalent among subsistence farmers inhabiting areas prone to drought in Gauteng,

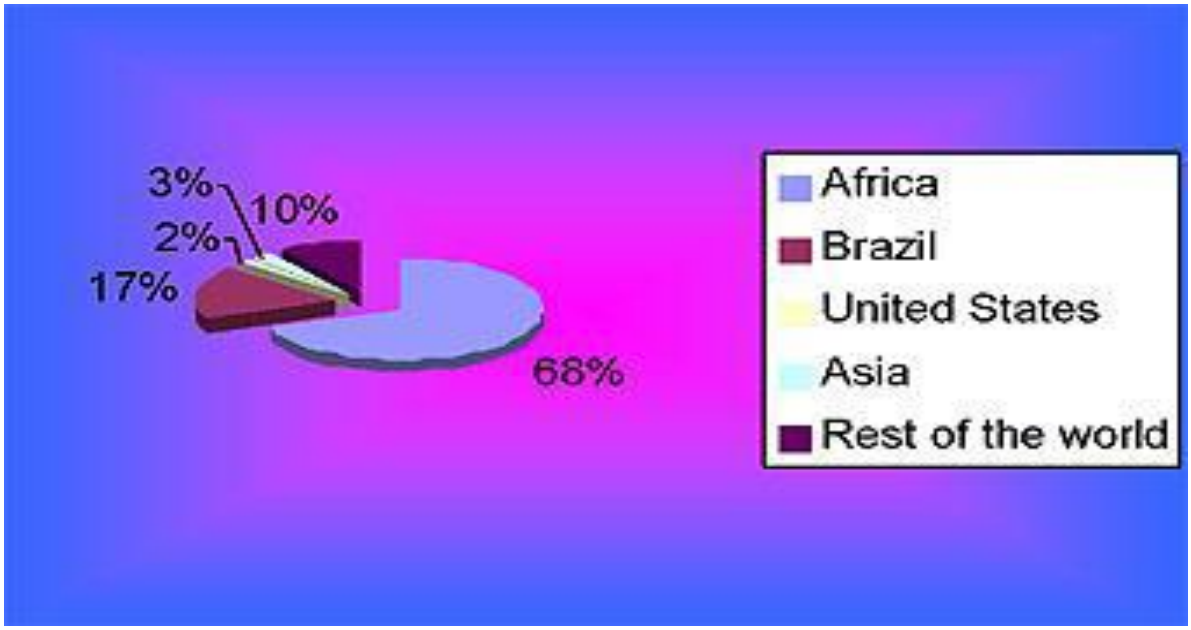

Fig. 2.1: Cowpea production throughout the world in dry grain states **(Gómez, 2004)**.

Limpopo, KwaZulu-Natal, Mpumalanga and the North West province **(DAFF, 2013, 2014)**. Hence, its local production has not been accurately quantified nor the actual size of its area of cultivation **(Asiwe, 2009; Masenya, 2016)**. Therefore, there is a need for more research on *V. unguiculata* in order to increase its production in South Africa since the country is becoming more food insecure, drought-prone and water-scarce due to climate change and global warming.

2.4 Introduction to climatic natural response

Climate change is the change in atmospheric integrity determined by employing barometric tools and statistical tests to determine variation that has occurred over many years **(UNFCCC, 2011)**. When this change occurs in climate systems as the result of either natural (e.g. volcanic eruption and desert dust) or anthropogenic sources, it causes perturbation on the earth's radiation budget through radiative forcing. Radiative forcing, also called climate forcing, is a degree of the net change in the energy balance as the result of a specific source inducing external perturbation **(Cubasch et al., 2013)**. These sources release greenhouse gases (GHGs) with the capacity to change solar irradiance, atmospheric gas and aerosol concentrations **(Goosse et al., 2010)**. Anthropogenic activities contribute to radiative forcing through emissions and changes in land-use which subsequently cause changes in the climate and weather by warming the earth. These changes eventually and systematically

influence climate and weather in five major components of the climate system, namely atmosphere, cryosphere (solid water), hydrosphere (liquid water), terrestrial surface and the biosphere **(IPCC, 2007)**.

2.4.1 Natural and enhanced greenhouse effect

The earth and atmosphere can be compared to a gigantic greenhouse wherein the biosphere thrives. Atmosphere is 100 km above the earth and has four layers, viz, troposphere, stratosphere, mesosphere and thermosphere or ionosphere. The troposphere and stratosphere are the closest layers to earth with the troposphere being the lowest where changes in weather patterns take place including warming. In the stratosphere, a region called ozone (O_3) absorbs solar energy and re-radiates it to the earth as shortwave radiation (SWR). The dry "air" in the atmosphere is 78.08% N_2, 20.95% oxygen (O_2) and 0.93% Argon (Ar) as non-GHGs **(North et al., 2015)** and less than 1% GHGs that have 3 or more atoms. When GHGs receive radiation, they vibrate and emit infrared radiation (heat) away-and-to the earth as infrared photons **(Brath et al., 2015)**. CO_2 and water vapour are the two major GHGs in the lower atmosphere at 0.04% and 1-5%, respectively **(Goosse et al., 2010)**. Other GHG concentrations making the layer include 0.0002% CH_4 and 0.00003% N_2O as well as varying levels of O_3.

The global energy budget shows that earth absorbs 50% of SWR, 30% remains trapped in atmospheric constituents and 20% is reflected (i.e. albedo) back to space by clouds, GHGs, aerosols and the earth's surface **(Trenberth et al., 2000; Cubasch et al., 2013)**. The earth surface emits longwave radiation (LWR) as an outgoing energy flux which is also largely absorbed and re-emitted by GHGs in all directions, including toward the earth where they make surface temperatures warmer than they should be **(Cubasch et al., 2013)**. In this manner, GHGs act as a blanket that traps heat which subsequently warms the atmosphere and earth surface. This phenomenon is known as natural greenhouse effect and it allows for life on earth, by keeping surface temperatures warm **(Lacis et al., 2013; Cloy and Smith, 2015)**. Without natural GHGs nights on earth will be about -21 °C and days will be too hot with dangerous radiation for life to thrive **(Goosse et al., 2010; Lacis et al., 2010)**.

Nevertheless, the increasing concentrations of non-water vapour and non-condensing GHGs such as CO_2, NH_3, CH_4, N_2O, perfluorocarbons (PFCs), hydrofluorocarbons (HFCs) and sulphur hexafluoride (SF_6) from anthropogenic emissions, elicit extra heating of the atmosphere. The enrichment of these GHGs in the atmosphere have increased the total mean temperature of the earth surface by approximately 15 °C **(Murray and Holbert, 2015)**. This increase, due to enriched GHGs, is called enhanced greenhouse effect or global warming, describing an additional warming of the atmosphere, oceans and land **(Trenberth et al., 2000)**. According to the **IPCC (2007, 2018)**, anthropogenic activities have caused a 1 °C increase in global warming above pre-industrial levels and it is expected to reach 1.5 °C in 2030 if the current rate of 0.2 °C per annum is not reduced. This increase has projected impacts such as rise in sea levels, oceanic and surface temperatures, changes in weather precipitation patterns, ocean salinity, melting of snow and ice sheets and modified wind patterns **(IPCC, 2007).** These changes induce elevated frequency of life-threatening weather events such as heavy rainfalls, heat waves, drought spells and a rising intensity of tropical cyclones **(IPCC, 2007)**.

2.4.2 Photosynthetic response to CO_2 enrichment

Vascular plants use three different forms of photosynthesises: C_3 photosynthesis, C_4 photosynthesis and Crassulacean acid metabolism (CAM) photosynthesis to produce organic compounds. These plants are known as C_3, C_4 and CAM plants, respectively. The type of photosynthetic pathway in plants determines how much CO_2 will be fixed under prevailing atmospheric CO_2 concentrations, temperatures and illumination **(Allen et al., 1996; Yamori et al., 2014)**. This allows scientists to determine how these plants are or will be affected as GHGs increase by measuring net photosynthesis in relation to climate change conditions. Therefore, these forms of photosynthesis are pertinent to global warming given different responses to changes in CO_2 concentration and temperature **(Ehleringer and Cerling, 2002)**.

Elevated CO_2 concentrations in the atmosphere are expected to cause CO_2 fertilization effect in C_3, C_4 and CAM plants. This phenomenon occurs when CO_2 concentration (as a photosynthesis substrate) is increased which culminates in more CO_2 assimilation in plants **(Farquhar, 1997; Donohue et al., 2013)**. Depending on the

species, high leaf CO_2 assimilation promotes more accumulation of non-structural carbohydrates (NSC) **(Allen et al., 1996)** which can be transformed into soluble carbohydrates, sucrose, fructose, glucose and starch **(Loladze, 2002; Dong et al., 2018)**. NSC transformation requires an adequate supply of ATP and NADPH to the electron transport chain in the thylakoid membranes and stimulates the synthesis of triose phosphate from a series of reactions called the Calvin–Benson cycle **(Yamori et al., 2014)**. Sometimes the propensity of NSC to accumulate in foliage induces photosynthetic feedback inhibition. This can happen when there is an absence of adequate sinks (e.g. stem, reproductive organs, fruits, seeds, root nodules and roots) or the leaf lacks capacity to fully move soluble carbohydrates into the phloem and translocate them to sinks **(Allen et al., 1996; Dong et al., 2018)**.

2.4.2.1 C_3 photosynthesis

C_3 photosynthesis is the most common metabolic pathway in many plant groups. Plants with C_3 photosynthesis are usually dicotyledons that thrive in temperate habitats **(Prasifka and Heinz, 2004)**. These plants include numerous major cereals; wheat (*Triticum*), rice (*Oryza sativa*), all root crops **(Furbank, 2013)**, most trees, vegetables and grain legumes like peanut (*Arachis hypogaea*), *P. vulgaris*, *V. unguiculata* and sunflower (*Helianthus*) **(Ismail and Hall, 1992; Yamori et al., 2014; Storkey et al., 2016)**. Their Calvin-Benson cycles produce 3-phosphoglyceric acid. This metabolic pathway step occurs after drawing in CO_2-enriched air and uses storage energy in the chloroplast. Thereafter, CO_2 is assimilated directly by an enzyme called ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) and the first stable product is an acid compound with 3-carbons, thus, the names C_3 photosynthesis and C_3 plants were derived **(Ehleringer and Cerling, 2002)**. Rubisco catalyses both carboxylation and oxygenation reactions in a 3:1 ratio, respectively **(Gready and Whitney, 2013)**. The latter reaction promotes O_2 production which elevates the photorespiration rate, causing increase in the net energy spent to fix CO_2 molecule **(Long, 1999)** and lowers the efficiency of C_3 photosynthesis **(Ehleringer and Cerling, 2002)**. This can reduce leaf photosynthetic efficiency of Rubisco by 50% **(Gready and Whitney, 2013)** under optimum conditions, temperature stress and low atmospheric CO_2 concentration **(Ehleringer et al., 1997; Cerling**, **1999)**. This can also influence nitrogenase activity in legumes which is extremely volatile and readily denatured under

O_2 exposure **(Sinclair, 2004)**. High photorespiration also reduces quantum yield (i.e. the number of moles in photons absorbed and converted to the moles of CO_2 assimilated, **Bauerle et al., 2020**) of photosynthesis (φCO_2) in C_3 plants to fix CO_2 **(Sommer et al., 2015)** and subsequently affects growth, photosynthate dissolution, plant yield, and N availability **(Singh, 2008)**. Quantum yield of C_3 dicotyledons have no relative discrepancy to that of C_3 monocotyledons in response to CO_2 enrichment and temperature **(Ehleringer and Cerling, 1997)**.

Although CO_2 enrichment is associated with enhanced growth, larger biomass and improved yield in C_3 plants due to greater photosynthetic rates **(Ainsworth et al., 2002)** and carbohydrate accumulation **(Van der Kooi et al., 2018)**, close analysis of C_3 plant's response to long-term CO_2 enrichment, challenges the sustainability and retention of such effects on photosynthesis and plant quality **(Arp, 1991; Niinemets et al., 1999; Lolazde, 2002; Zobayed et al., 2005; Dong et al., 2018)**. These studies suggest that the increased photosynthetic rates under free-air carbon enrichment (FACE) is unsustainable and often culminates in photosynthetic acclimation, a process of down-regulation of net photosynthetic rate in response to long-term exposure to CO_2 enrichment **(Long et al., 2004; Kirschbaum, 2011)**. This shows that doubling of CO_2 concentration can seriously affect quality of C_3 plants **(Dong et al., 2018)**. These studies also report that CO_2 enrichment can cause unpalatable tastes due to abundant cellulose content and increased photosynthates may suppress the N to C ratio and induce an imbalance between source and sinks. Moreover, it has been theorised that given plants will be larger and difficulties in acquiring immobile phosphate in the soil, more foliage will bind up more phosphorus (P) as sugar phosphates, thus increasing P requirements. In *V. unguiculata*, the susceptibility of down-regulation of photosynthesis increases with temperature stress, P stress severity and soil water deficit **(Hall and Schulze, 1980; Ahmed et al., 1993; Sekiya and Yano, 2008)**. Furthermore, effects of CO_2 enrichment on Rubisco activity, protein content and carbohydrates are masked by those of temperature stress and P stress **(Vu et al., 2001; Sekiya and Yano, 2008)**.

2.4.2.2 N_2 fixing of C_3 legumes under CO_2 enrichment

N_2 fixation in legumes by rhizobia bacteria depends on a foliar supply of carbohydrates (i.e. mainly as sorbitol and sucrose) to root nodules which may vary with vegetative

and reproductive phases **(Wang et al., 2019)**. According to **Redden et al. (2013)**, nodules of white lupin (*Lupinus albus*) demand 4.5 gC before anthesis for every 1 gN fixed and translocated to leaves. This accounts for 21.8% of the total amount of C fixed and assimilated in photosynthetic organs. After anthesis and throughout grain-filling, the quantity demand of C increases slightly to 5 g/gN. Conversely, *V. unguiculata* needs 2.3 gC per 1 gN fixed and this accounts for only 10% of its C_3 photosynthesis before anthesis with only a 2.7% in photosynthetic cost during grain-filling **(Redden et al., 2013)**. The relatively high N_2-fixing capability in *V. unguiculata*, *G. max* and *P. vulgaris* is derived from determinate nodules that produce ureides allantoin and allantoic acids with less C while synthesis of amides glutamine and asparagine in indeterminate nodules of *L. albus*, faba bean (*Vicia faba*), *P. sativum*, and *C*. *arietinum* require more C **(Redden et al., 2013)**.

N_2 fixation also depends on the type and solubility of synthesized nitrogenous compounds in nodules. For example, legumes that transport ureides instead of amides show higher N_2 fixation rates, due to efficiency of ureides C:N ratio of 1:1 in transporting N derivatives **(Sinclair, 2004)**. However, because of low solubility of ureides **(Sprent, 1980)**, this demands relatively higher water flux out of the nodules by the xylem and can rapidly inhibit N_2 fixation in the case of hindered waterflow in the phloem **(Serraj et al., 1999)**. This has been proven to be the first and the most sensitive physiological process to occur in ureide-transporting species such as cowpea and soybean under soil water deficit **(Sinclair, 2004)**. Soil water deficit impairs plant growth and N use efficiency by also causing N deficiency through reduced N availability and N up-take **(Nguyen et al., 2017)**. N deficiency should always be avoided in plants by any means necessary due to its adverse effects on physiological processes, morphological growth, biomass accumulation and yield **(Nguyen et al., 2017)**.

2.5 Effects of abiotic stresses on plants

Plant growth and phenology are also prone to effects induced by abiotic factors such as temperature, rainfall and drought **(Allen et al., 1996)**. Any increase or decrease in these environmental factors may induce stress or benefit growth and eventually yield.

2.5.1 Effects of temperature stress on plants

Plant susceptibility to elevated temperatures varies among two types of temperature stresses, viz., moderate temperature stress (MTS) and severe temperature stress (STS). MTS is exhibited 1-10 °C above the optimum temperature and its effects are readily reversible on photosynthesis **(Berry and Björkman, 1980; Weis, 1981)**. Early symptoms to MTS could be limited node development and leaf appearance rate **(Hatfield et al., 2008)**. In contrast, STS occurs at higher temperatures, where the damage on photosynthesis persists even after stress amelioration **(Berry and Björkman, 1980)**. Temperature stress also induces changes in the composition of organic acids, amino acids, and carbohydrates **(Ahmed et al., 1993; Yu et al., 2012)**. These are integral metabolites that serve key metabolic functions in protein synthesis, stress signaling and stress tolerance **(Zobayed et al., 2005; Merewitz et al., 2012)**. Plant response to temperature stress is by activation or enhancing oxygen-scavenging enzymes such as peroxidase, superoxide dismutase, catalase and numerous non-enzymatic phytochemicals **(Zobayed et al., 2005)**. In field conditions, MTS is more common than STS. This is due to plant homeostasis which alleviates STS by transpiration, except under severe habitat conditions **(Salvucci, 2013)**.

2.5.1.1 Effects of temperature stress on Vigna unguiculata yield

Temperature stress prevents optimal yield by affecting pollen viability, fertilization and fruit formation. Yield can also be decreased by any chronic or short-lived exposure to elevated temperature during the pollination phase, at the time of grain-filling or at fruit set **(Hatfield et al., 2008)**. During grain-fill or pod-set stage, temperature stress directly affects transportation of recently fixed C to the developing sinks **(Salvucci, 2013)**. In *V. unguiculata*, the reproductive stage is extremely sensitive to temperature stress **(Redden et al., 2013)** with elevated night temperature being more detrimental than increased day temperature **(Warrag and Hall, 1984)**. Pod set is hypersensitive to heat stress (33 °C) during the last 6 hours of the night and is exacerbated under long days than in short days **(Mutters and Hall, 1992)**.

During the night, temperature stress causes tapetal cells, essential for development of the pollen grain, to degenerate which then leads to poor pod set **(Ahmed et al., 1992)**. A limited supply of photosynthates to sinks, common under night heat-stress can also make reproductive organs fail. **Ahmed et al. (1993)** reported high night temperature of 30 °C inhibits photosynthesis and overall carbohydrates, particularly

peduncle sugars which led to floral deformation and flower abscission in heat-sensitive cowpea lines. Early heat-stress induction suppresses development and causes abortion of flower buds **(Patel and Hall, 1990)**. When inducted, during later stages of development, it causes anther indehiscence, pollen sterility and complete flower abscission **(Warrag and Hall, 1984; Mutters and Hall, 1992; Sinclair, 2004)**. However, it is important to note that yield inhibition under field conditions is a synergistic effect of intensifying temperature stress, soil water deficit and reduced rainfall. This may also include weed growth, pests and diseases which increase with the elevation in temperature **(FAO, 2016b)**.

2.5.1.2 Plant response to temperatures

Plant response to temperature varies according to three points called cardinal temperatures, namely, base or minimum temperature, optimum temperature and maximum or ceiling temperature **(Torabi et al., 2020)**. These thermal constraints allow seed germination, growth and phenology to occur within their specific ranges **(Hatfield et al., 2008)**. Any extreme in temperature outside minimum and maximum temperatures induces inhibitory effects or growth impairments. The minimum temperature is essential for breaking seed dormancy and marks the inception of seed germination and vegetative growth **(Hatfield et al., 2008)**. For example, legumes germinate poorly at low temperatures and most require temperatures above 10 °C to break seed dormancy **(DAFF, 2013)**. Plants flourish under optimum temperature because it allows activation of rapid growth through increased cell division and expansion in unstressed tissues **(Hatfield et al., 2008)**. **Balkaya (2004)** and **Motsa et al. (2015)** ascertained optimum temperature and maximum ceiling temperature in *V. unguiculata* to range between 20-25 °C and 35-36 °C, respectively.

Since plants depend on temperature and photosynthesis to sustain vegetative growth, develop and nourish reproductive organs and vegetative parameters **(Salvucci, 2013)**, leaf optimum temperature for photosynthetic performance is primarily variable, varying by a difference of 4-10 °C in just a few days to allow seasonal acclimation **(Nobel, 2009)**. With increasing biosphere temperatures, leaf optimum temperature for keeping photosynthetic performance and net CO_2 assimilation in C_3 plants is 20-35 °C and 30-45 °C for C_4 plants **(Nobel, 2009)**. Plants also adjust yield optimum

temperature to avoid early maturity which can be induced both by high temperature and photoperiod sensitivity to daylength in legumes **(Sinclair, 2004)**. Early maturity is not ideal, especially for non-perennial crops as it accounts for a shorter life cycle, smaller plants, early flowering, shorter reproductive stages and thus lower yield **(Sinclair, 2004; Hatfield et al., 2008).** Therefore, to avoid this, non-perennial crops keep yield optimum temperature less than that of vegetative growth and leaf appearance rate to delay early fruiting or maturity phase.

2.5.2 Effects of drought stress on plants

Plant sensitivity to drought or soil water deficit varies and depends on growth stage, genetic potential, plant species and duration. Although C_4 plants have higher water use efficiency than C_3 plants, the metabolism that support their growth is equally sensitive to drought stress **(Ghannoum, 2013)**. Drought impairs growth process by affecting photosynthesis (**Fig. 2.2**), respiration, hormonal metabolism, enzymatic activity and cause C-starvation **(Okunlola et al., 2017; Yan et al., 2020)** and N_2-deficiency **(Sinclair, 2004; Nguyen et al., 2017)**. During growth, drought stress first affects sensitive cell turgor, consequently disrupting cell division and enlargement **(Yan et al., 2017; Yan et al, 2020)**. Thereafter, it affects photosynthesis via C balance (i.e. C assimilation and respiration), wherein the C assimilation is often the first and largest physiological process to decline relative to respiration **(Yan et al., 2020)**. High sensitivity of photosynthesis to drought stress also leads to a decrease in total NSC reserves. This is due to photosynthesis's incapacity to meet respiratory demands, eventually leading to C starvation if consumed carbohydrates in reserves remain depleted **(Güneralp and Gertner, 2007; McDowell et al., 2008)**. In legumes, drought stress also affects seed germination, shoot and root development, harvest index and reproductive stage which is the extremely sensitive stage to water deficits, and this often results in final yield loss (**Table 2.1**) **(Nadeem et al., 2019)**.

2.5.2.1 Plant response to drought stress

Plants use drought avoidance and drought tolerance mechanisms to respond to moderate, short, extremely severe and prolonged drought stress **(Vurukonda et al., 2016)**. Generally, these mechanisms protect plants and allow adaption as well as yield to increase under hostile conditions. Drought avoidance is the ability of the plant to

regulate growth rates or selected morphological structures to improve water up-take, reduce water loss and position changeover from vegetative reproduction to reproductive growth **(Monclus et al., 2006)**. To increase water up-take capacity, plants partition more biomass to roots **(Deng et al., 2020)**. To prevent water loss, plants close stomata and avoid osmosis through osmotic adjustment. Osmotic adjustment is less severe on yield because it allows continuous root growth and demands low energy **(Daryanto et al., 2015)**. Low stomatal and mesophyll conductance to alleviate high transpiration and improve water optimization also limit photosynthesis **(Schafleitner et al., 2013)**. Closing stomata reduces leaf internal CO_2 concentration with subsequent reductions in net photosynthesis. Low internal CO_2 concentration also reduces Rubisco activity, but a greater threat is from an exacerbated elicitation of a Rubisco inhibitor called pentadiulose-1,5-bisphosphate (PDBP) resulting in oxygenation and photorespiration **(Parry et al., 1993)**.

Contrary to the above mechanism, drought tolerance refers to a secondary protection mechanism where plants elicit antioxidant systems (i.e. enzymatic and non-enzymatic antioxidants) to alleviate damage by free radicals caused by severe drought stress **(Siddhuraju and Becker, 2007; Carocho and Ferreira, 2013; Goa et al., 2020)**. Such damages are minimised through osmo-regulatory constituents that protect membrane structures and improve water absorption and uptake of water-soluble nitrate, sulphate, Ca, Mg and Si **(Reddy et al., 2004; Vurukonda et al., 2016)**. These constituents include soluble proteins, soluble sugars and proline **(Reddy et al., 2004; Vurukonda et al., 2016)**. Other plant metabolites known to suppress elicitation and the effects of the free radicals are superoxide dismutase, catalase, peroxidase and ascorbic acid glutathione (AsA-GSH) and non-enzymatic antioxidants like flavonoids and total phenols **(Carocho and Ferreira, 2013)**.

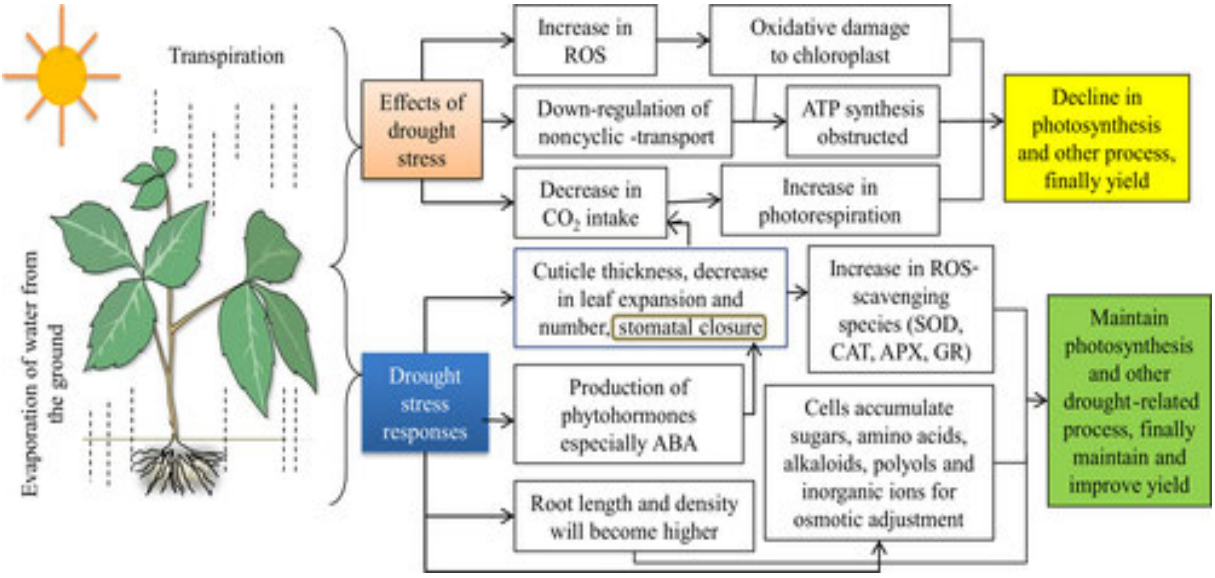

Fig. 2.2: Effects of drought stress on plants and possible responses **(Ullah et al., 2017)**.

Table 2.1: Yield losses of important legumes under drought stress.

Legume Crops	Scientific Name	Growth Stage	Yield Loss (%)	Reference
Soybean	*Glycine max*	Pod set	73-82	Wei et al., 2018
		Reproductive	46-71	Samarah et al., 2006
		Pod set	45-50	Kobraee et al., 2011
		Grain filling	42	MalekI et al., 2013
Chickpea	*Cicer arietinum*	Reproductive	45-69	Nayyar et al., 2006
		Ripening	49-54	Samarah et al., 2009
		Anthesis	27-40	Mafakheri et al., 2010
		Ripening	50	Varshney et al., 2014
Cowpea	*Vigna unguiculata*	Reproductive	60	Ogbonnaya et al., 2003
		Reproductive	34-66	Ahmed and Suliman, 2010
		Pod filling	39	Kyei-Boahen et al., 2017
Common bean	*Phaseolus vulgaris*	Reproductive	58-87	Martinez et al., 2007
		Pod filling	40	Ghanbari et al., 2013
		Flowering	49	Rosales-Serna et al., 2004
Mungbean	*Vigna radiata*	Reproductive	26	Ranawake et al., 2012
		Flowering	31-57	Ahmad et al., 2015
Faba bean	*Vicia faba*	Grain filling	68	GhassemI-Golezani and Hosseinzadeh-Mahootchy, 2009
Lentil	*Lens culinaris*	Pod development	70	Shrestha et al., 2006
		Reproductive	24	Allahmoradi et al., 2013

2.6 Plant biostimulants

According to **Du Jardin (2015)** "*A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content*". This definition is endorsed by key role players in the biostimulant industry like regulators, scientists as well as associations such as European Biostimulants Industry Council (EBIC) and the Biostimulant Coalition in USA. Based on the definition, the word biostimulant does not refer to a single substance but numerous ones. Therefore, biostimulants are best explained by categories which include humic acids and fulvic acids, protein hydrolysates and other N_2-containing compounds, seaweed extracts and botanicals, chitosan, biopolymer chitosans, inorganic compounds, and beneficial fungi and bacteria **(Kauffman et al., 2007; Du Jardin, 2015)**. These biostimulants are applied to plants as powders, microbial strains, granules, drenching solutions or liquid foliar products. For this Review Chapter, we will briefly discuss categories that are relevant to the biostimulants under study and those reported by **Poberężny et al. (2020)** as most important in the biostimulant industry (**Table 2.2**).

2.6.1 Humic substances

Humic substances are natural groups of heterogenous compounds called humins, humic acids and fulvic acids and originate from plant and animal decomposition, metabolic activity of soil microbes and microbial residues **(Du Jardin, 2015)**. They are categorized in accordance to molecular weights and solubility. They form one of the most abundant organic constituents in nature making up 60% of organic matter in the world's soil profile **(Saju et al., 2019)**. Their essential roles for plants (**Table 2.2**) include acting on soil fertility, chemical, physio-chemical, physical as well as biological properties **(Du Jardin, 2015)**. However, these variable roles depend on the sources, environmental conditions, plant species, cultivar, concentration and mode of application **(Rose et al., 2014)**. Sources of natural humic organic matter include compost, vermicompost, peat, volcanic soils and mineral deposits like leonardite **(Du Jardin, 2012)**. The science community also considers vermicompost or vermicomposting as a new sustainable way to produce a biostimulant called vermicompost leachate (VCL) rich in other active ingredients **(Joshi et al., 2015; Gutiérrez-Miceli et al., 2017)**.

2.6.1.1 Vermicompost leachate

VCL is produced from organic matter by earthworms (e.g. *Eudrilus* species, *Eisenia foetida, Lumbricus rubellus* and *L. terrestris*) and catalysed microbial activity **(Waldbillig and Brain, 2012; Ganeshnauth et al., 2018)**. During vermicomposting, worms break down organic matter, improving microbial activity and subsequently increasing availability of water-soluble trace minerals, potassium phosphate, N and Ca **(Manivannan et al., 2009)** found in VCL. As a liquid biostimulant, VCL improves plant growth by increasing the abundance of stimulants such as hormones and nutrient volumes **(Bidabadi et al., 2017)**. These include phytohormones such as cytokinins and auxins and high volumes of N, Zn, Mg, Ca, Cu, Fe, vitamins and amino acids **(Radovich and Norman-Aranco, 2011; Eskkiammal et al., 2015)**. Moreover, humic acids **(Singh et al., 2010; Joshi et al., 2015)** and fulvic acids present in VCL stimulate seed germination and regulate numerous physiological processes during plant development **(Carlos et al., 2008)** including an increase in micro- and macronutrient adsorption **(Aremu et al., 2012)**. Active principles induce other various effects on soil and plant parameters (**Tables 2.2** and **2.3**). VCL also contains active ingredients that can inhibit pathogenic infestation and diseases on plants. Foliar spray of 20% VCL solution contains enough phenolics and microbiota compounds to protect the plant from pathogens **(Joshi et al., 2015)**. *E. fetida* thrive in symbiosis with beneficial bacteria like Actinobacteria, Proteibacteria, Bacteroidetes, Firmicutes and Verrucomicrobia that have anti-fungal properties against *Fusarium moniliforme* and *Colletotrichum coccodes* **(Yasir et al., 2009)**. *Brodyrhizobium japonicum* bacteria from *L. terrestris* not only protect legumes, but also enhance nodulation in roots of *G. max* **(Joshi et al., 2015)**. However, VCL activity is highly dependent on vermicompost age, extraction method and source of material **(Radovich and Norman-Aranco, 2011)**.

2.6.2 Seaweed extracts

Archaeological records indicate that seaweeds have been used for millennia as a food source, marine fertilizer, medicine, cosmetic products, colouring dyes and textiles **(Battacharyya et al., 2015)**. Their use as food dates back 600-800 BC in China, 961 BC in Iceland and 300 AD as medicine for Asian people **(Papenfus, 2011)**. As a marine fertilizer, seaweeds were applied directly to the soil in the form of seaweed

compost or desiccated and ground seaweed meal to slowly release nutrients, condition the soil, improve soil aeration and stabilize soil aggregates **(Stirk and Van Staden, 2006)**. Alginic acid which makes up about one third of carbohydrate content in seaweeds is a principle soil conditioner in seaweed meal **(Metting et al., 1990)**. Fresh seaweed biomass normally contains the same amount of N_2, lower P and higher potassium (K), salinity and micronutrient quantities than animal manure **(Stirk and Van Staden, 2006)**. Plants cultivated in kelp-enriched soils exhibit greater tolerance to drought stress, rapid germination rate, vigorous seedling growth and establishment, and higher overall productivity **(Weiersbye et al., 2001)**.

However, seaweed composting has its own shortcomings. These include excessively high salt **(Weiersbye et al., 2001)** and sand content, a slow decomposition process, heat sensitivity and poor nutrient retention during salt removal process **(Stirk and Van Staden, 2006; Papenfus, 2011)**. This led to the development and sale of many processed seaweed products collectively called seaweed concentrates (SWCs) which are either powdery or aqueous extracts. The discovery of the simplicity of use and far-reaching positive effects of SWCs on soil, plants, stress tolerance (i.e. salinity, drought, freezing, high temperature, flooding as well as pollution), various gene expressions (e.g. *RD22, RD29, CBF3, SOS and COR15A*), disease and pathogen suppression **(Battacharyya et al., 2015)** has been recently reported in research literature. This encouraged the commercial use of SWCs and purified compounds such as alginates, laminarin and carrageenans **(Du Jardin, 2015)**. Other key active ingredients in SWCs include enzymes, proteins, amino acids (metalloproteins, glycoproteins, exogenous amino acids like glutamic acid, alanine, aspartic acid), and phytohormones (auxins, cytokinins, gibberellins, polyamines, abscisic acid, ethylene, betaine, castasterone, brassinosteroids, brasinolide), polyphenols (florotanins, floroglucinol, ecol), oligosaccharides, polysaccharides (agar, hyaluronic acid, fucans, mannitol, sorbitol and laminarin), phytalexins, vitamins (C, B_2, B_{12}, D_3, E, K, niacin, folic acid and panthotenic), macro- and microelements (Mg, Cu, Fe, Br, Zn, I, Mn), as well as unsaturated fatty acids (eicosapentenoic, arachidonic) **(Kocira et al., 2020)**.

The efficacy of seaweed extracts depends on algae species, concentration, plant growth stage, plant species, mode of application and time **(Kocira et al., 2013; Battacharyya et al., 2015)**. Liquid solutions of SWCs can be applied in low

concentrations as either a seed treatment, drenching, foliar treatment or hydroponic application. Foliar spraying has been shown to be more effective when applied in the morning while leaf stomata are open **(Battacharyya et al., 2015)**. Positive promotional effects on seed germination, plant growth and stress tolerance, among other key effects (**Table 2.2**) are largely accounted for, by hormonal effects. These are derived from phytohormones like auxins, gibberellins, cytokinins, abscisic acid and other hormone-like compounds such as sterols and polyamines present in most harvested seaweeds that belong to the family Phaeophyceae, particularly brown seaweeds of the Northern Hemisphere, especially *Ascophyllum nodosum* **(Craigie, 2011; Wally et al., 2013)**. SWCs from *A. nodosum* (which is one of the most common harvested kelps) have been widely researched in the science community. Other brown algae used today as biofertilizers from the Northern Hemisphere include *Fucus vesiculosus*, *F.* serratus, *Laminaria hyperborean* and *L. digitata*. The Southern Hemisphere marine algae most used as SWCs are from kelp species like *Durvillea potatorum* and *Ecklonia maxima* which also belong to the Phaeophyceae **(Stirk and Van Staden, 2006)**.

2.6.2.1 Commercial seaweed extract: Kelpak®

In the 1950s, the first SWC was produced using a preparation method patented by **Milton (1952)**. This preparation method entailed heating pressurised seaweed at temperatures over 100 °C and the use of alkaline chemicals to destabilize cell walls. Since then numerous companies have used various alkaline-based hydrolysis methods to produce SWCs. Kelpak® Products (Pty) Ltd. use *Ecklonia maxima* (Osbeck) Papenfuss, a brown algal species, to produce its SWC products. Unlike the method described in **Milton (1952)** which has the risk of destroying growth regulators by heat, the company uses a unique cold cell burst method that excludes the use of heat and chemicals to manufacture a commercial SWC, Kelpak® (KEL). KEL harvest starts by divers cutting *E. maxima* at the holdfast and collection of lamina and stipes from the shore in Kommetjie, Western Cape Province, South Africa. Upon arrival at the factory in Simonstown, the brown algae biomass is separated into more handleable materials, washed and minced before being placed in a chamber (with high and low pressure) wherein solid biomass is disintegrated, producing a concentrate. Natural auxins are relatively greater in concentration to cytokinins (2.20 mg/L and 0.0062 mg/L, respectively) as active components in the KEL crude stock. These

Table 2.2: Activity and anti-stress precursor of categories of plant biostimulants.

Source	Biostimulation activity	Anti-stress precursor	Reference
Humic substances	Enhance nitrate uptake, Fe and Zn solubility, cation exchange capacity, P availability, root nutrition and contribute to cell division and organ growth.	Phenolic compounds, humic and fulvic acids.	Jindo et al., 2012; Saju et al., 2019
Seaweed extracts	Improve gelling formation, water retention, cation exchange capacity, heavy metals fixation, germination, nutrition, establishment, growth, chlorophyll and delay senescence.	Antioxidants and regulators of endogenous stress-responsive genes, osmolytes (proline, sorbitol and betaines).	Calvo et al., 2014; Battacharyya et al., 2015; Du Jardin, 2015; Di Mola et al., 2019
Protein hydrolysates and N_2-containing compounds	Increase and modulate N up-take and assimilation, facilitate cross talk between C and N metabolism, increase antioxidants, increase absorption of Fe, Zn, Mn, Cu through roots and leaves via unique transporters [Amino Acid Permease 1 (AAP1) Lysine Histidine Transporter 1, LHT1 and AAP5].	Glycine betaine and proline	Du Jardin, 2015; Drobek et al., 2019; Saju et al., 2019
Beneficial fungi	Promote acquisition of macro- and micronutrients and P, contribute to water balance, protect the plant against biotic and abiotic stress, improve Pi up-take and buffer Cadmium up-take.	Strains of *Trichoderma*, drought-tolerant strain of *Glomus intraradices*	Siddiqui et al., 2008; Gianinazzi et al., 2010; Saju et al., 2019
Beneficial bacteria	Fix N_2, solubilize Pi and organic P, and induce desired root morphogenesis and increase tolerance against abiotic and biotic stresses	Siderophores	Gamalero and Glick, 2011

phytohormones stimulate cellular elongation on the below and above the ground biomass, contributing to growth improvement and root system enhancement **(Beckett and Van Staden, 1989; Kocira et al., 2013)**. KEL also improves nutrient up-take, tolerance to nutrient deficiency **(Beckett and Van Staden, 1990)** and drought **(Mooney and Van Staden, 1985)** and elicits a hormonal increase which enhances plant nutrition, health and yield **(Beckett and Van Staden, 1989)**. As a SWC biostimulant, KEL has other beneficial effects on plant morpho-physiological processes and biochemical composition as mentioned above in **Table 2.2** and is also summarized in **Table 2.3**.

2.6.3 Protein hydrolysates and N_2-containing compounds

Protein hydrolysates are another biostimulant category made up of amino acids, polypeptides and oligopeptides which are derived from protein sources through partial hydrolysis. Sources may include plant materials like crop residues and animal wastes such as epithelial tissues and collagen **(Du Jardin, 2012; Calvo et al., 2014)**. Key amino acids present in protein hydrolysates differ with respect to these sources. Animal-based protein hydrolysates contain significantly higher amino acids than plant-based hydrolysates. For example, collagen-derived protein hydrolysates comprise of high amounts of glycine, proline as well as two non-standard amino acids, viz., hydroxylysine and hydroxyproline that are found in negligible quantities in plant-based protein hydrolysates **(Saju et al., 2019)**. Aspartic acid and glutamic acid are found in high proportions in legume-based and fish-based protein hydrolysates with proline instead of aspartic acid in casein-derived protein hydrolysates **(Saju et al., 2019)**. Other N_2-containing compounds with biostimulation activity include polyamines, betaines and non-protein amino acids. Nicotianamine is a non-protein amino acid that is responsible for transporting micronutrients and has been shown to contribute to the success of other physiological processes **(Saju et al., 2019)**. Naturally, plant roots frequently secrete non-proteinogenic amino acids called phytosiderophores **(Dakora and Phillips, 2002)** in zinc or iron deficient soils which chelate Zn or Fe and improve their availability for up-take **(Ueno et al., 2007)**.

2.6.4 Plant growth-promoting microorganisms

Microorganisms form another important category covered by the word, plant biostimulant. Plant growth promoting fungi (PGPF) and plant growth promoting bacteria (PGPB) change composition of soil-borne microbe species to benefit plants.

2.6.4.1 Beneficial fungi

Fungi have co-existed with plants since the beginning of terrestrial plants by forming mutualistic symbioses with plant roots. Despite heterogeneity in PGPF, Mycorrhizal fungi are a group of taxa that establish symbioses with more than 90% of all plant species. Mycorrhizal fungi use different physical forms of interactions but the type that form arbuscules of endomycorrhiza are widely associated with horticultural plants and agricultural crops. The fungal hyphae of Glomeromycota species infiltrate root cortical cells and establish as branched structures known as arbuscules **(Behie and Bidochka, 2014)**. Arbuscular Mycorrhizal fungi also establish an exogenous network of extrametrical hyphae outside the root that comprises of 15% of the organic C and 20-30% of the total microbes found in soil **(Wilson et al., 2009)**. This mycelial network and hydrophobic secretion called glomalin improve soil structure and quality through binding actions, water retention properties and stabilizing soil aggregates **(Saju et al., 2019)**. **Jakobsen (1995)** found that an 80% reduction in phosphate fertilizer can be the result of inoculating plants with Arbuscular Mycorrhizal fungi. Other popular fungi with biostimulation activities include *Trichoderma reesei*, *Trichoderma atroviride* and *Heteroconium chaetospira* **(Drobek et al., 2019)**. To date, many fungi are currently used as biofertilizers and biocontrols with biopesticidal properties **(Du Jardin, 2015)**.

2.6.4.2 Beneficial bacteria

The word PGPR (i.e. plant-growth promoting rhizobacteria) refers to three types of soil-born bacteria, depending on their habitat lifestyle. This includes bacteria that live freely in soil by creating habitats around the root (rhizosphere), those that colonize the root surface (rhizoplane) and endophytic bacteria that thrive within roots. These bacteria can belong to any bacterial phyla (i.e. Actinobacteria, Proteobacteria and Firmicutes) including strains of genera *Bacillus, Pseudomonas, Azospirillum, Rhizobium, Agrobacterium, Azotobacter, Alcaligens, Arthobacter, Burkholderia, Comamonas, Pantoea, Variovorax* and *Serratia*. Plant-growth promoting bacteria (PGPB) have many different positive effects on plant growth including increasing yield,

growth, salt tolerance, resistance to heavy metal toxicity and have an antagonistic capacity against certain pathogens **(Saju et al., 2019)**. For example, to increase P availability, PGPB, particularly rhizosphere bacteria frequently synthesize gluconic and citric acids that chelate insoluble compounds and decrease the pH, both of which enhance P solubility from Pi or can simply release protons that lowers pH or mineralizes organic P **(Gamalero and Glick, 2011)**. Their siderophores can also increase Fe up-take and reduce chlorosis in barley (*Hordeum vulgare*) and mungbean, respectively **(Sharma et al., 2003)**.

2.6.5 Smoke-water

Fire and smoke have also been instrumental factors in seed biology and seed germination processes for many years **(Van Staden et al., 2000)**. The central role of fire in plant species is indicated immediately after burning via increased seed germination, followed by vigorous seedling establishment **(Maret and Wilson, 2000)** or apparent years post-burning by enhanced growth and improved reproductive capacity **(Pyke et al., 2010)**. For example, **De Lange and Boucher (1990)** discovered that *Audouinia capitata* seeds will only germinate after a fire. Rapid seed germination in seed banks after fire followed by rainfall can happen in numerous plant species, in particular grass species **(Gupta et al., 2019)**. Some of the reasons for elevated germination, growth and establishment post-burning include physical scarification to break seed dormancy, direct fire stimulation on the embryo and more access to light, moisture, nutrients or 'competitive release' **(Williams et al., 2003; Smith, 2006; Krock et al., 2016)**. These reasons may be supported by historical difficulties in germinating fire-adapted plant species under greenhouse settings using traditional propagation protocols **(Van Staden et al., 2000)**.

There are now more than 1 000 plant species from over 200 families showing the marked effects of smoke **(De Lange et al., 2018)**. In South Africa, smoke has shown significant improvement in seed germination in 54% of the 221 plant species tested from various families including the Asteraceae, Fabaceae, Proteaceae, Poaceae, Ericaceae, Restionaceae, Bruniaceae, Mesembryanthemaceae, Geraniacea and Rutaceae **(Brown and Van Staden, 1997; Van Staden et al., 2000)**. **De Lange and Boucher (1990)** showed that active compounds present in aerosol smoke are water soluble and their smoke-saturated solution induced prolific seed germination. It has

also been demonstrated that properties of aerosol smoke (which stimulates germination) are heat stable with a long shelf-life and remain potent for many years in storage and can be adsorbed by various structures **(Van Staden et al., 2000)**.

Subsequent research has discovered that aerosol smoke can be bubbled through and/or trapped with water to produce an aqueous biostimulant called smoke-water (SW) **(Baxter et al., 1994; Brown and Van Staden, 1997; Gupta et al., 2019)** which is equally potent as aerosol smoke. SW has arguable advantages which one can consider in addition to those of aerosol smoke. These include cost-effective, easy to prepare, simple to use, required in minute quantities, show enhanced phytoextraction of heavy metals, and applicable as germination cues and a post-germination enhancer for fire-dependent and non-fire-dependent plant species **(Light and Van Staden, 2004; Okem et al., 2015; Gupta et al., 2019)**. The compound responsible for inducing the stimulatory effect in seed germination is known as butenolide, but now referred to as karrikinolide (KAR_1). The compound was identified from burned cellulose as 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one **(Flematti et al., 2004)** and from plant-based SW **(Van Staden et al., 2004)**. An inhibitory compound, also a form of butanolide, was identified as 3,4,5-trimethylfuran-2(5*H*)-one or trimethylbutenolide (TMB). **Light et al. (2010)** reported high inhibition in seed germination and significant reduction in the KAR_1 effect when harnessed concurrently. Biostimulation activities of SW and KAR_1 in plants may range from induction of resistance to abiotic stresses (i.e. temperature, drought and salinity) to an increase in shoot and/or sink organs (**Table 2.3**) **(Kulkarni et al., 2008; 2011)**. Due to the lack of toxicity on germination and growth of crops, SW and smoke-derived compounds have potential use in agriculture, horticulture and post-mining habitat restoration **(Kulkarni et al., 2011; Okem et al., 2015)**.

Table 2.3: Effects of vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on different plant species.

Biostimulant	Application mode	Plant species	Biological effect	Reference
VCL	Foliar spraying	*Amaranthus hybridus*	Increases height, biomass weights, chlorophyll *a*, *b* and *a* + *b*, carotenoids and proteins.	Ngoroyemoto et al., 2019
	Drenching and foliar spraying	Bananas (*Musa* x *paradisiaca*) 'Williams'	Improves shoot elongation, root biomass, leaf number, leaf area and contribute to shoot:root ratio.	Aremu et al., 2012
	Not specified	French dwarf bean (*Phaseolus vulgaris*)	Increases plant height, pod length, pod number and improves lateral branching.	Ayyobi et al., 2014
	Perlite drenching	Tomato (*Solanum lycopersicon*)	Offsets negative effects of K and P deficiencies and enhances morphological appearance with increased leaf number, shoot length, biomass weight and photosynthetic pigment content.	Arthur et al., 2012
	Foliar spraying	Pomegranate (*Punica granatum*)	Increases seedling leaf area, shoot and root weight under saline and non-saline soils.	Bidabadi et al., 2017
KEL	Soil drenching and foliar spraying	*Amaranthus hybridus*	Increases height, biomass weights and enhances absolute and relative growth rates of leaf number.	Ngoroyemoto et al., 2019

	Foliar spraying	Common bean (*Phaseolus vulgaris*)	Increases pod number, seed number, seed weights, protein contents, seed polyphenols and flavonoids.	Kocira et al., 2013, 2020
	Soil drenching and stem-based application	*Pelargonium peltatum* L.	Enhances root growth, leaf number and stimulates accumulation of phenolics, proteins and photosynthetic pigments.	Krajnc et al., 2012
	Not specified	Spring barley (*Hordeum vulgare* L.)	Increases N and P uptake, root weight, grain number and yield weight.	Szczepanek et al., 2018
SW	Soil drenching	*Solanum lycopersicon*	Increases height, stem thickness, leaf emergence, fruit number, harvest index and promotes early fruiting.	Kulkarni et al., 2008
	Substrate moistening	Light sensitive lettuce (*Lactuca sativa* L. cv. Grand Rapids)	Weak dilution elicits increased germination and strong solutions inhibit germination.	Gupta et al., 2019
	Substrate moistening and vermiculite drenching	Kikuyu (*Pennisetum clandestinum*)	Improves seedling vigour index, Cd up-take and shoot length under Cd-stress soil.	Okem et al., 2015
	Substrate supplementing	*Tulbaghia ludwigiana*	Increases root number, total phenolics, flavonoids and condensed tannin contents with no toxicity effects.	Aremu et al., 2014
	Substrate moistening	*Phaseolus vulgaris* (Dwarf-Imbali), *Solanum lycopersicon*	Increases root and shoot elongation, vigour indices, biomass weights, rooting and leaf emergence.	Van Staden et al., 2006
	Prime by pre-soaking kernels and soil drenching	*Zea mays*	Improves plant height, roots, biomass weights, vigour indices and reduces plant mortality.	Sparg et al., 2006; Van Staden et al., 2006

2.7 Conclusions

It is evident that the adoption of one approach to increase food security in order to reduce hunger and malnutrition is not enough. The current chapter reveals that although *V. unguiculata* has high potential yield with remarkable nutrition it is still a less cultivated versatile crop in commercial agriculture of most countries, especially in South Africa. Furthermore, it is shown that the prevalent increase in temperature and drought episodes can significantly reduce yield of *V. unguiculata* due to the crop's susceptibility to temperature stress and water deficits. Future CO_2 enrichment in the atmosphere could also induce N and P deficiencies. These findings suggest a need to find more atmospheric and environmentally friendly way(s) to improve the crop yield and combat hunger under climate change conditions. Increasing the use of VCL, KEL and SW in crop production could be one of the possible solutions. The ability of these plant biostimulants to improve seed germination, plant growth and yield cannot only add value in food security and hunger amelioration but can also play a critical role in horticulture, habitat restoration and climate change mitigation. Their attributed influence on proline, glycine betaine, regulators of endogenous stress-responsive genes, total phenolics and other phytochemicals can protect and increase plant adaptation to habitats prone to elevated global warming. Improved photosynthesis, root growth and nutrient up-take efficiency induced by VCL and KEL means these plant biostimulants have the potential to allow C_3 plants like legumes to cope with stress in ecological niches that favour photorespiration, oxygenation reaction and inhibition of N and P acquisition. However, despite advances made in biostimulant research many studies are limited to one or two plant growth phases. Therefore, more work is still required into phase-to-phase studies in a plant's life cycle to elucidate the full potential of these biostimulants under current and future abiotic stresses, which is what this study aims to achieve with *V. unguiculata*.

Chapter 3: Effects of priming with three biostimulants on *Vigna unguiculata* seed germination, seedling establishment and potted plants under various temperature regimes

3.1 Introduction

Germination and early seedling growth are the two most sensitive stages to water deficits in many plant species. Water limitations can prevent onset, decrease the rate and uniformity of seed germination, culminating in poor plant performance and yield reduction **(Demir et al., 2006)**. However, drought stress, intense temperature regimes, oxidative stress and salinity often interact together in inducing growth impairments and in activation of cell signaling pathways and cellular responses **(Jisha et al., 2013)**. Such abiotic stresses also exacerbate the ubiquitous problems of poor seed germination, seed emergence, establishment (i.e. seed germination phase, seedling emergence phase and early seedling growth phase) and high mortality in legumes **(Smith, 2006; Maurya et al., 2020)**. Hence, various seed treatment protocols/methods have been developed in order to address problems of poor crop establishment. These methods include seed priming **(Haider et al., 2020)** and exposing seeds to either an aerosol or aqueous smoke solution **(Krock et al., 2016)**. The former method is associated with enhanced germination rates and seed vigour, thus making it possible to attain uniform and rapid seedling emergence. These are the two essential prerequisites for higher yields and crop nutrition in annual crops **(Parera and Cantliffe, 1994)**.

Seed priming is defined as a controlled hydration of seeds in either water, solid media (i.e. solid-matric priming) or in an osmotic solution that allows the seeds to imbibe water followed by drying to the original moisture level before radicle protrusion **(Murray and Wilson, 1987**; **Parera and Cantliffe, 1994)**. This helps to suspend seeds in the lag phase and can be done using various approaches such as osmopriming, chemical priming, hydropriming, biological priming, hormonal priming, solid matrix priming and redox priming **(Jisha et al., 2013)**. Before the inception of germination, which is identified by radicle emergence, seeds imbibe water in three phases. Phase I, high water imbibition by seeds; phase ll, reactivation of metabolism; and finally phase

lll, radicle protrusion **(Ali and Elozeiri, 2017)**. During phase I and lll, water imbibition is rapid as a result of swift hydration of cells, and support to the developing and protruding radicle from the seed at the end of germination. Conversely, in phase II (i.e. lag phase) water imbibition is slow. By priming seeds, this induces the most integral metabolic steps of germination to occur and when sown, the seedlings can readily emerge as a result of reduced triphasic water uptake **(Smith, 2006)**.

Nevertheless, the positive effects of seed priming are mostly exhibited in seeds exposed to stress rather than under unstressed conditions **(Parera and Cantliffe, 1994)**. These positive effects depend on numerous factors including the type of the priming agent, stress severity and crop species **(Iqbal and Ashraf, 2005)**. For example, hydropriming can increase seed germination and seedling growth by 3-4-fold under drought stress relative to non-hydropriming **(Maurya et al., 2020)**. Hence, as an indispensable defense method, seed priming refers to a physiological state wherein a plant's defence capacity is prepared for faster and better activation of response to imminent abiotic stresses **(Jisha et al., 2013)**. The increased defence response is closely associated with an abundance of inactive signal proteins which may amplify signal transduction **(Conrath et al., 2006)** while high germination percentage and seed vigour may be due to elevated DNA and RNA synthesis, mobilization of reserved food materials, activation and resynthesis of specific enzymes **(Nascimento and West, 1998)**. Priming also helps to achieve greater germination uniformity with reduced time between seed sowing and seedling emergence. It also synchronises emergence, promotes better stand establishment, vigorous allometry, early flowering, and higher grain set **(Basra et al., 2005; Farooq et al., 2008)**. This has also been reported in various legumes cultivated under adverse conditions **(Arun et al., 2020; Farooq et al., 2020; Muarya et al., 2020)**. These positive attributes hold important implications for agronomy, floriculture and horticulture **(McDonald, 2000)** and thus show the essence for finding a suitable priming agent **(Job et al., 2000)**.

The use of biostimulants to offset the effects of abiotic stresses on plants has been explored in several studies **(Calvo et al., 2014; du Jardin, 2015; Bidabadi et al., 2017)**. The induced promotory effects of vermicompost leachate (VCL) **(Arthur et al., 2012, 2016; Aremu et al., 2014)**, seaweed extract Kelpak® (KEL) **(Kocira et al., 2013, 2020)** and smoke-water (SW) **(Van Staden et al., 2006; Kulkarni et al., 2011)** in many

plant species is well-documented. However, there is still the need for more studies to explore the full potential of VCL, KEL and SW in agriculture in order to ensure food security. Therefore, the objective of the current study was to investigate priming and non-priming effects of the biostimulants; VCL, KEL and SW on *V. unguiculata* seed germination and morphology 7 days after sowing (DAS) under various temperature regimes. Also, the influence of seed-priming with VCL, KEL and SW post-germination was evaluated.

3.2 Materials and methods

3.2.1 Source of seeds

V. unguiculata (local cultivar IT18) seeds and two liquid biostimulants (i.e. VCL and KEL) were procured from different suppliers. Seeds were purchased from McDonald's Seed Company, Pietermaritzburg, South Africa.

3.2.2 Source of biostimulants

The three biostimulants were bought and/or prepared before preliminary experiments as follows;

3.2.2.1 Seaweed extract Kelpak® (KEL)

KEL was manufactured by Kelpak® Products (Pty) Ltd. Company, Simon's Town, South Africa. The KEL label indicates that it contained 2,2 mg/l natural auxins as well as 0,0062 mg/l cytokinins. Other important active ingredients present in KEL are summarized below (**Table 3.1**). The three dilutions of KEL used in this investigation were prepared as follows; as 0.6% [0.6 mL KEL: 99.4 mL distilled water (dH_2O) v/v], 0.8% (0.8 mL KEL: 99.2 mL dH_2O v/v) and 1% (1 mL KEL: 99 mL dH_2O v/v).

3.2.2.2 Vermicompost leachate (VCL)

VCL was purchased from Wizzard Worms Commercial Company Ltd., Greytown, South Africa. According to the product's label, VCL was produced from garden waste and vegetables using red wiggler earthworms (*Eisenia fetida*). The decomposed dry matter comprised of 2.26% N, 0.99% P, 0.64% K, 2.52% Ca and 631.03 Mg/kg of

sodium (Na) and the crude stock had a pH of 7.82. Further details on the preparation procedure are provided on the company website (http://www.wizzardworms.co.za). Biochemical compounds present in tested VCL including phytohormones and phytochemicals like phenolic acids which were quantified by **Aremu et al. (2015a)**. Various VCL dilutions were prepared by diluting crude VCL with dH_2O. The three dilutions were 1 mL VCL: 5 mL dH_2O v/v; 1 mL VCL: 10 mL dH_2O v/v and 1 mL VCL: 20 mL dH_2O v/v.

3.2.2.3 Smoke-water (SW)

SW was prepared according to the method of **Gupta et al. (2019)** at the Research Centre for Plant Growth and Development (RCPGD), University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The preparation protocol encompassed an apparatus in accordance to the method described by **Brown (1993a, b), Baxter et al. (1994)** and **Jäger et al. (1996)**. Twenty-five millilitre crude SW was first dispensed into 75 mL dH_2O to make a sub-dilution of (1 SW: 3 dH_2O v/v) before being further diluted to 1 mL SW: 1000 mL dH_2O v/v; 1 mL SW: 1500 mL dH_2O v/v and 1 mL SW: 2000 mL dH_2O which were the desired dilutions. Distilled water was used as the control treatment in all tested abiotic stresses. All the prepared solutions were stored in a cold room with the temperature set at 10 °C immediately after preparation.

3.2.3 Seed priming and seedling culture

The study commenced by first undertaking preliminary experiments in the RCPGD plant growth chambers (Conviron®) where the three dilutions of biostimulants were tested for efficacy on seed germination, seedling emergence and seedling growth. Dilutions which best induced biostimulation effects were further tested for stress-amelioration on potting experiments in Conviron® and a greenhouse. Only these dilutions were reported in the present study and subsequent investigations conducted under greenhouse conditions. Two groups of *V. unguiculata* seeds were sown in plant growth chambers. First set of seeds was rinsed in dH_2O for 10 s and primed for 1 h by pre-soaking in 150 mL of different dilutions of test solutions (VCL 1:20 v/v, VCL 1:5 v/v, KEL 0.6%, SW 1:1000 v/v, SW :1500 v/v and dH_2O). Each dispensed test solution had 48 seeds in a 250 mL volumetric beaker. After 1 h, seeds were air-dried under a laminar flow bench for 45 min before being allowed to reach seedling stage. The seeds

were considered to have completed germination when the hypocotyls have emerged from soil (crook stage). After 7 days (d), seedlings were harvested when have two cotyledons, primary leaves and 1st unfolded trifoliate leaf at node one, two and three, respectively. Sandy-loam soil from RCPGD botanical garden was used as substrate for all experiments except in hydroponics.

3.2.4 Seven-day seedling experiments and seed germination

Before seed sowing, the soil was first autoclaved and drenched with test solutions and subsequently left under room temperature for 36 h to reach field capacity. Thereafter, 16 air-dried seeds in triplicates per dilution were sown using a spatula and forceps. Each seed was cultivated with the hilum facing downward in a 3 cm single furrow in 128 cell trays. The same cultivation procedure was also followed when sowing non-primed seeds from the second set. To avoid cross contamination, a polyethylene collective compartment was placed underneath the cells of the tray for each dilution. After sowing, the 128 cell trays were placed in plant growth chambers with 16:8 h light and dark under constant day and night temperatures of 30/30, 35/35 and 40/40 °C. The illumination of plant growth chambers was 27± 5 µmol $m^{-2}s^{-1}$ photosynthetic photon flux (PPF). The day and night relative humidity of the three temperature regimes was 65-70%/65-70% with ambient CO_2 concentration.

The seeds were watered with 4.5 mL of dH_2O a day after sowing (DAS). The biostimulants were then applied on every second day of the week. During the days when biostimulants were not applied, 4.5 mL dH_2O per tray cell was applied to the soil to maintain moisture. All drench applications were done every morning for 7 d after recording the number of emerged seedlings as germinated seeds. Seven DAS, seedlings were harvested by first counting the number of leaves per seedling. Dicotyledon leaves and trifoliate leaflets were individually counted as leaf number only when clearly developed. This approach was adopted in all subsequent experimental trials. Seedling height/shoot length was measured from the root collar to the apical bud using a 30 cm ruler. Stem diameter readings were taken 4 cm above the root collar using a Marshal digital caliper. The seedlings were then carefully uprooted, washed and measured for root elongation from the root collar to the tip of the taproot. The fresh weight was also recorded before placing seedlings on a Li-3100 Area meter (LI-COR, Inc. Lincoln, Nebraska, USA) to measure seedling area. Dry weight was

recorded after desiccating seedlings in an oven at 55 ± 2 °C for 4 d. The entire experiment was repeated twice and the number of germinated seeds as well as morphological parameters were recorded from replicates of test solutions which had highest seed germination and seedling emergence. All the described sowing and data collection procedures were repeated in pot experiments unless stated otherwise. Furthermore, all other atmospheric conditions of the three temperature regimes in plant growth chambers were unchanged for potting and hydroponic experiments.

3.2.5 Hydroponic culture

For hydroponic experiments, 400 *V. unguiculata* seeds were hydroprimed to imbibe dH_2O and another 400 *V. unguiculata* seeds were non-hydroprimed but were also sown in separate plastic pots containing vermiculite. The vermiculite was first treated with only dH_2O prior to sowing and 2 DAS the seeds. Then, the pots were placed under constant day and night temperature of 35/35 °C in a plant growth chamber for 7 d. Seven DAS, the root system of the seedlings was cut above the root collar. Thereafter, hydroponic trials were set-up by submerging stems of the cuttings into poly vial tubes containing dH_2O, VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) and placed in plant growth chambers. Each treatment had 4 replicates with 2 cuttings. After 15 d, the cuttings were removed from the substrates and the number of new lateral roots was recorded. The entire experiment was repeated twice before recording the final data.

3.2.6 Four-week potting experiments

The potting experiments were done between August and September 2019 in plant growth chambers and only post-germination effects of three dilutions [i.e. VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v)] were further investigated for a growth period of 4 weeks. These test solutions were selected as the best dilutions and germination cues with marked effects on seed germination and morphological growth of *V. unguiculata*. Sixteen seeds were first primed by pre-soaking in the selected test solutions and sown at 3 cm depth in 72 coloured plastic pots. The pots were then placed under constant day and night temperatures of 30/30, 35/35 and 40/40 °C in plant growth chambers. Each temperature regime had 24 completely randomized 12 cm pots. There were 4 replicates per treatment each with 4 seeds. The interior base of the pot was lined with a double layer of Whatman™ No.1 (90 mmØ) filter paper.

Seven DAS, the seedlings were thinned to 2-3 plants per pot and the first 100 mL drenching of biostimulants was applied. Prior to this, each pot received 100 mL of dH_2O after 2-3 d depending on the temperature regime. Thereafter, the treatments were applied once every Monday of the second week after recording seedling height and leaf number.

Table 3.1: Amino acid and mineral composition of Kelpak®.

Amino acids	Content (mg)	Macro-/microelements	Content (%/ mg)
Alanine	150	Nitrogen	0.28
Serine	140	Phosphorus	0.72
Proline	92	Potassium	0.42
Threonine	84	Calcium	0.01
Lysine	80	Sodium	0.11
Leucine	72		
Valine	70	Boron	3.2
Glycine	70	Copper	1.8
Ornithine	63	Iron	1.2
Phenylalanine	60	Magnesium	56.4
Tyrosine	60	Manganese	0.8
Arginine	48	Zink	0.9
Isoleucine	40		
Glutamine	35		
Asparagine	31		
Asparagine	31		
Hydroxyproline	27		
Methionine	25		

(http://www.caltecag.com/products/kelpak.html)

Distilled water was used as control and drenched to all pots during the week that followed biostimulant-application week. Twenty-eight DAS, the experimental trials were terminated and parameters of the above ground biomass (i.e. seedling height, leaf number, peduncle diameter, seedling area, fresh and dry weight) were recorded.

3.2.7 Data analysis

Data was subjected to one-way and two-way analysis of variance (ANOVA), using Statistical Package for Social Sciences (SPSS®, Version 26.0, IBM, Armonk, New York, USA). The statistical significance was determined using the Duncan's Multiple Range Test (DMRT) to separate mean values at $P \leq 0.05$.

3.3 Results

3.3.1 Effect of biostimulants on seed germination (preliminary experiments)

Although the tested biostimulants did not significantly improve germination percentage, preliminary experiments, however, indicated that VCL 1:20 (v/v) improves germination percentage, seed vigour index, emergence index and elicit marked effects on *V. unguiculata* growth parameters such as root length, shoot length, leaf number and peduncle diameter under 30 and 40 °C compared to the control (**results not shown**). KEL (0.6%) and SW 1:1000 (v/v) were shown to be efficacious under all three temperature regimes. Biostimulation effects of VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) on seed germination were then evaluated on primed and non-primed *V. unguiculata* seeds against those of dH_2O (control) under 30, 35 and 40 °C for 7 d.

3.3.2 Overall average effect of priming and non-priming on seedling growth under the three temperature regimes

The overall average (i.e. an average of growth parameters derived from biostimulant-priming and hydropriming compared to an average of all non-primed growth parameters) effect of priming versus non-priming with biostimulants showed that there was significant difference between seedlings of primed and non-primed seeds in the three temperatures (**Table 3.2**). This was also noted in the test solutions wherein their effects on growth parameters such as leaf number, root number, shoot length and seedling area were significantly different ($P < 0.05$), especially at the non-optimum temperature of 40 °C.

Priming significantly improved most growth parameters of *V. unguiculata* measured in the three temperature regimes compared to non-priming. At 30 °C, priming significantly increased root length compared to non-priming, inducing the highest root elongation in the three temperatures (**Fig. 3.1B**). Highest significant increase in shoot length and number of leaves was induced by priming compared to non-priming at 35 and 40 °C, respectively (**Fig. 3.1A** and **C**). However, priming did not positively impact the number of roots of the seedlings as non-priming at 30 and 40 °C (**Fig. 3.1D**). Seed-priming was most effective for eliciting high root length, shoot length and leaf number at 30, 35 and 40 °C, respectively.

Table 3.2: Two-way analysis of variance of growth parameters of primed and non-primed *Vigna unguiculata* seedlings with main effects and their interaction.

Source of variation	Leaf No.		Shoot length (mm)		Root length (mm)		Root No.		Seedling Area (cm^2)	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
30 °C										
Soaking (Non-primed, primed)	-	-	52.476	< 0.001	7.565	< 0.006	11.399	< 0.001	1.086	> 0.299
Treatment	-	-	21.318	< 0.001	12.802	< 0.001	2.021	> 0.086	8.606	< 0.001
Soaking X treatment	-	-	12.175	< 0.000	4.323	< 0.001	2.369	< 0.048	6.568	< 0.000
35 °C										
Soaking	9.694	< 0.002	107.414	< 0.001	65.377	< 0.001	5.781	< 0.019	36.109	< 0.001
Treatment	0.284	> 0.921	6.079	< 0.001	1.814	> 0.111	3.154	< 0.012	5.175	< 0.001
Soaking X treatment	1.732	> 0.128	1.646	> 0.149	0.891	> 0.488	2.342	= 0.050	4.474	< 0.001
40 °C										
Soaking	47.345	< 0.001	334.087	< 0.001	61.330	< 0.001	33.85	< 0.001	78.036	< 0.001
Treatment	4.904	< 0.001	8.150	< 0.001	1.659	> 0.145	3.879	< 0.004	6.461	< 0.001
Soaking X treatment	1.207	> 0.307	0.416	> 0.838	2.375	< 0.040	3.416	< 0.008	3.679	< 0.003

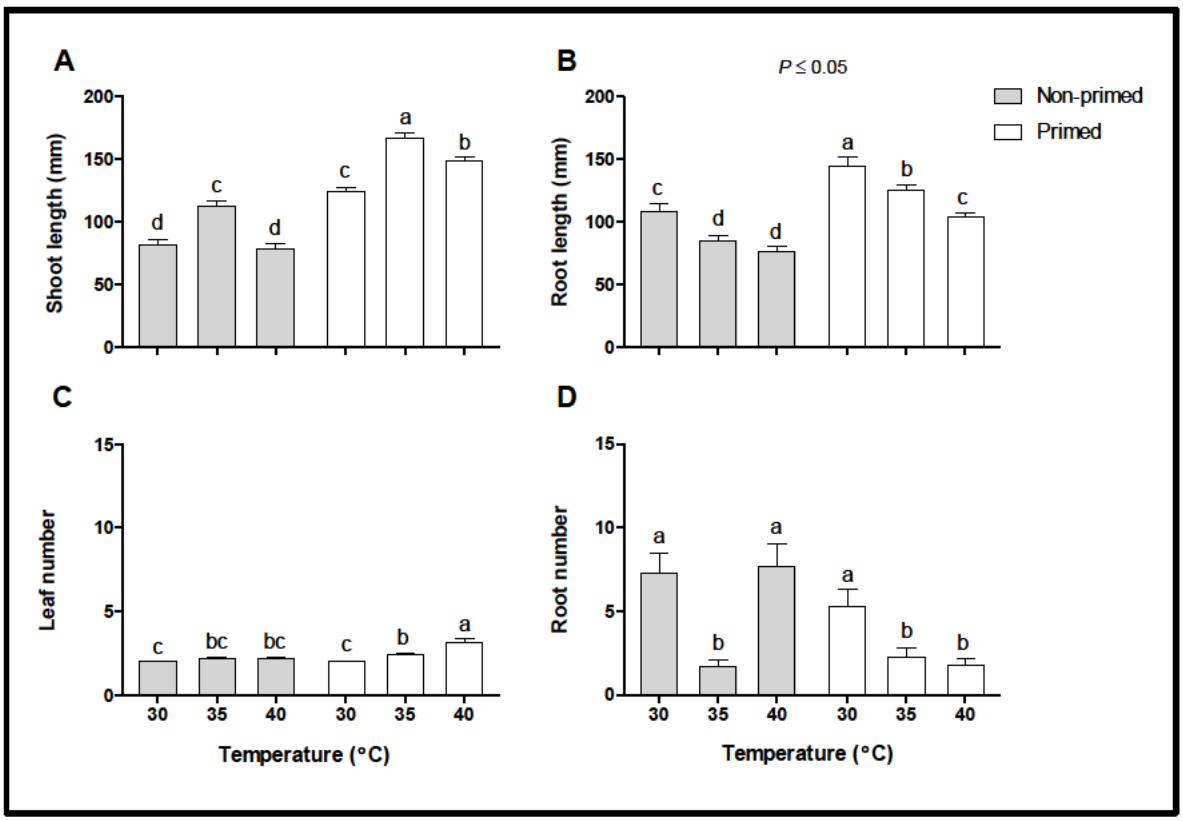

Fig. 3.1: Effects of priming and non-priming on shoot length, root length, leaf number and root number of *Vigna unguiculata* cultivated under different temperature regimes 7 days after sowing (DAS). For each temperature graph, bars (mean and ± Standard error of the mean; shoot length, *n* = 20; root elongation, *n* = 20; leaf number, *n* = 20 and root number, *n* = 7) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.3.3 Effect of biostimulant-priming on seedling growth under 30 °C

The observed growth promotory effects of priming the seeds (**Fig. 3.1**) was greatly contributed by biostimulant-priming. At 30 °C, seed priming with VCL, KEL and SW elicited the highest significant increase in root length compared to when seeds were primed with the control (i.e. hydropriming using dH_2O) (**Fig. 3.2B**). This improvement in root elongation of biostimulant-primed plants was also the greatest in all three temperature regimes (**Figs. 3.2, 3.3** and **3.4**). Priming with either VCL, KEL or SW also induced significant increase in dry weights of the seedlings cultivated under 30 °C compared to primed and non-primed controls in all three temperature regimes (**Table 3.3**). Furthermore, VCL-priming promoted a greater shoot length compared to

priming the seeds with dH_2O (control) (**Fig. 3.2A**). Despite the positive effects of biostimulant-priming, VCL, KEL and SW did not promote greater root and leaf number in primed seedlings compared to non-primed seedlings at 30 °C (**Fig. 3.2C** and **D**). Furthermore, non-priming with VCL significantly promoted higher shoot length, root length, root number and seedling area compared to other non-priming agents (**Fig. 3.2A, B** and **C; Table 3.3**).

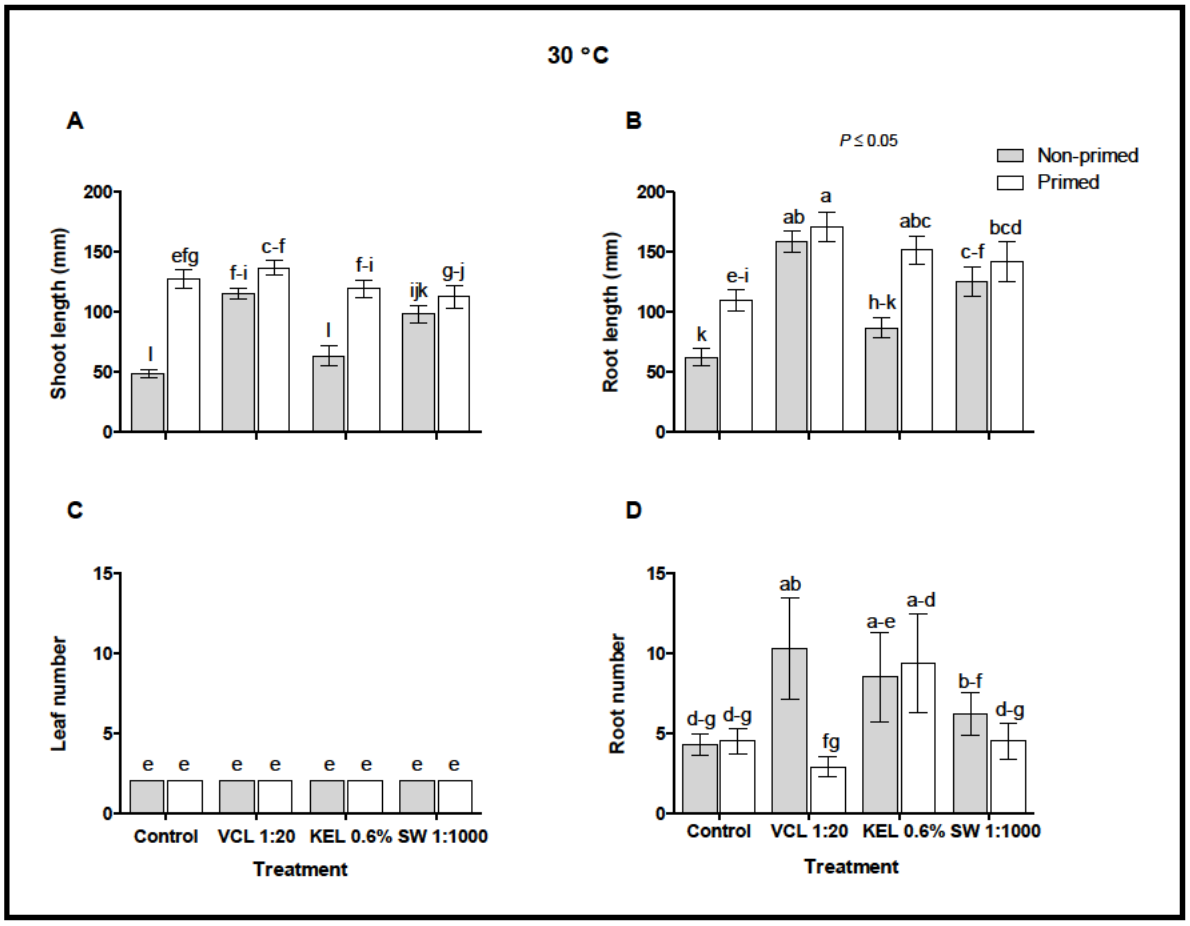

Fig. 3.2: Effects of priming and non-priming with distilled water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) on growth parameters of *Vigna unguiculata* seedlings under 30 °C 7 days after sowing (DAS). For each treatment, bars (mean and ± standard error of the mean; shoot length, *n* = 20; root elongation, *n* = 20; leaf number, *n* = 20 and root number, *n* = 7) with different letter(s) are significantly different (*P ≤ 0.05*) based on Duncan's Multiple Range Test (DMRT).

Table 3.3: Effects of priming and non-priming with distilled water (control), vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on seedling growth of *Vigna unguiculata* after incubation at 30, 35 and 40 °C for 7 days (d).

Treatment (mL)	Peduncle diameter (mm)		Fresh weight (g)		Dry weight (g)		Seedling Area (cm^2)	
	Non-primed	Primed	Non-primed	Primed	Non-primed	Primed	Non-primed	Primed
30°C								
Control	$0.32 \pm 0.01^{c-d}$	0.34 ± 0.01^{bcd}	0.77 ± 0.05^{j}	$1.50 \pm 0.10^{b-e}$	0.09 ± 0.01^{e}	$0.12 \pm 0.01^{b-e}$	14.69 ± 1.66^{f}	27.38 ± 2.59^{abc}
VCL 1:20	$0.34 \pm 0.01^{a-d}$	$0.35 \pm 0.01^{a-d}$	$1.21 \pm 0.09^{f-i}$	1.66 ± 0.06^{bc}	0.11 ± 0.01^{de}	0.17 ± 0.01^{a}	32.65 ± 2.28^{a}	28.75 ± 2.07^{abc}
KEL 0.6%	0.34 ± 0.01^{bcd}	$0.35 \pm 0.01^{a-d}$	0.98 ± 0.07^{ij}	1.70 ± 0.08^{ab}	0.11 ± 0.01^{de}	0.17 ± 0.01^{a}	14.06 ± 2.50^{f}	23.44 ± 2.22^{bcd}
SW 1:1000	$0.35 \pm 0.01^{a-d}$	0.36 ± 0.02^{abc}	1.70 ± 0.08^{ab}	$1.45 \pm 0.12^{b-f}$	0.16 ± 0.01^{ab}	0.16 ± 0.01^{a}	26.17 ± 1.64^{abc}	27.63 ± 2.43^{abc}
35°C								
Control	0.33 ± 0.01^{bcd}	0.32 ± 0.02^{d}	1.04 ± 0.05^{hi}	1.54 ± 0.11^{bcd}	0.10 ± 0.01^{e}	$0.13 \pm 0.01^{b-e}$	24.58 ± 1.84^{bcd}	28.27 ± 2.22^{abc}
VCL 1:20	$0.34 \pm 0.01^{a-d}$	0.36 ± 0.01^{ab}	1.17 ± 0.07^{ghi}	$1.47 \pm 0.08^{b-f}$	0.11 ± 0.01^{de}	$0.13 \pm 0.01^{b-e}$	22.08 ± 2.34^{cde}	26.85 ± 2.13^{abc}
KEL 0.6%	$0.34 \pm 0.01^{a-d}$	0.38 ± 0.02^{a}	$1.38 \pm 0.07^{c-g}$	1.93 ± 0.08^{a}	0.11 ± 0.01^{de}	0.16 ± 0.01^{a}	30.11 ± 1.92^{ab}	28.87 ± 1.70^{abc}
SW 1:1000	0.33 ± 0.01^{bcd}	0.36 ± 0.01^{abc}	$1.30 \pm 0.06^{d-h}$	1.66 ± 0.09^{b}	0.12 ± 0.01^{cde}	0.15 ± 0.01^{abc}	16.33 ± 1.34^{ef}	30.74 ± 1.88^{ab}
40°C								
Control	0.32 ± 0.01^{d}	0.34 ± 0.01^{bcd}	1.04 ± 0.06^{hi}	$1.43 \pm 0.09^{b-g}$	0.12 ± 0.01^{cde}	0.12 ± 0.01^{cde}	13.57 ± 1.20^{f}	27.00 ± 2.01^{abc}
VCL 1:20	0.33 ± 0.01^{bcd}	$0.35 \pm 0.01^{a-d}$	1.08 ± 0.08^{hi}	1.65 ± 0.10^{bc}	0.12 ± 0.01^{cde}	$0.13 \pm 0.01^{b-e}$	19.27 ± 2.58^{def}	26.77 ± 2.68^{abc}
KEL 0.6%	$0.35 \pm 0.01^{a-d}$	$0.35 \pm 0.02^{a-d}$	$1.25 \pm 0.08^{e-i}$	1.67 ± 1.00^{b}	0.12 ± 0.02^{cde}	$0.14 \pm 0.01^{a-d}$	22.10 ± 1.75^{cde}	29.30 ± 2.35^{abc}
SW 1:1000	$0.35 \pm 0.01^{a-d}$	$0.34 \pm 0.01^{a-d}$	$1.28 \pm 0.09^{d-h}$	1.64 ± 0.12^{bc}	$0.13 \pm 0.01^{b-e}$	$0.13 \pm 0.01^{b-e}$	23.98 ± 2.37^{bcd}	26.57 ± 2.78^{abc}

Mean values ± standard error (n = 20) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) based on DMRT.

3.3.4 Effect of biostimulant-priming on seedling growth under 35 °C

Seed priming with dH_2O or biostimulants benefitted more growth variables at 35 °C compared to non-priming. Although priming with either VCL, KEL or SW did not significantly improve many growth parameters, they were able to induce a considerable increase in root length compared to hydropriming (**Fig. 3.3B**). Seedlings of KEL and SW-primed seeds had more foliage (**Fig. 3.3C**) with higher root proliferation (**Fig. 3.3D**). Priming with either VCL, KEL or SW elicited the highest significant increase in peduncle diameter at 35 °C compared to hydropriming (**Table 3.3**). Biostimulant-priming with KEL also significantly improved shoot length (**Fig. 3.3A**), fresh and dry weights, resulting in taller plants with the greatest biomass (**Table 3.3**).

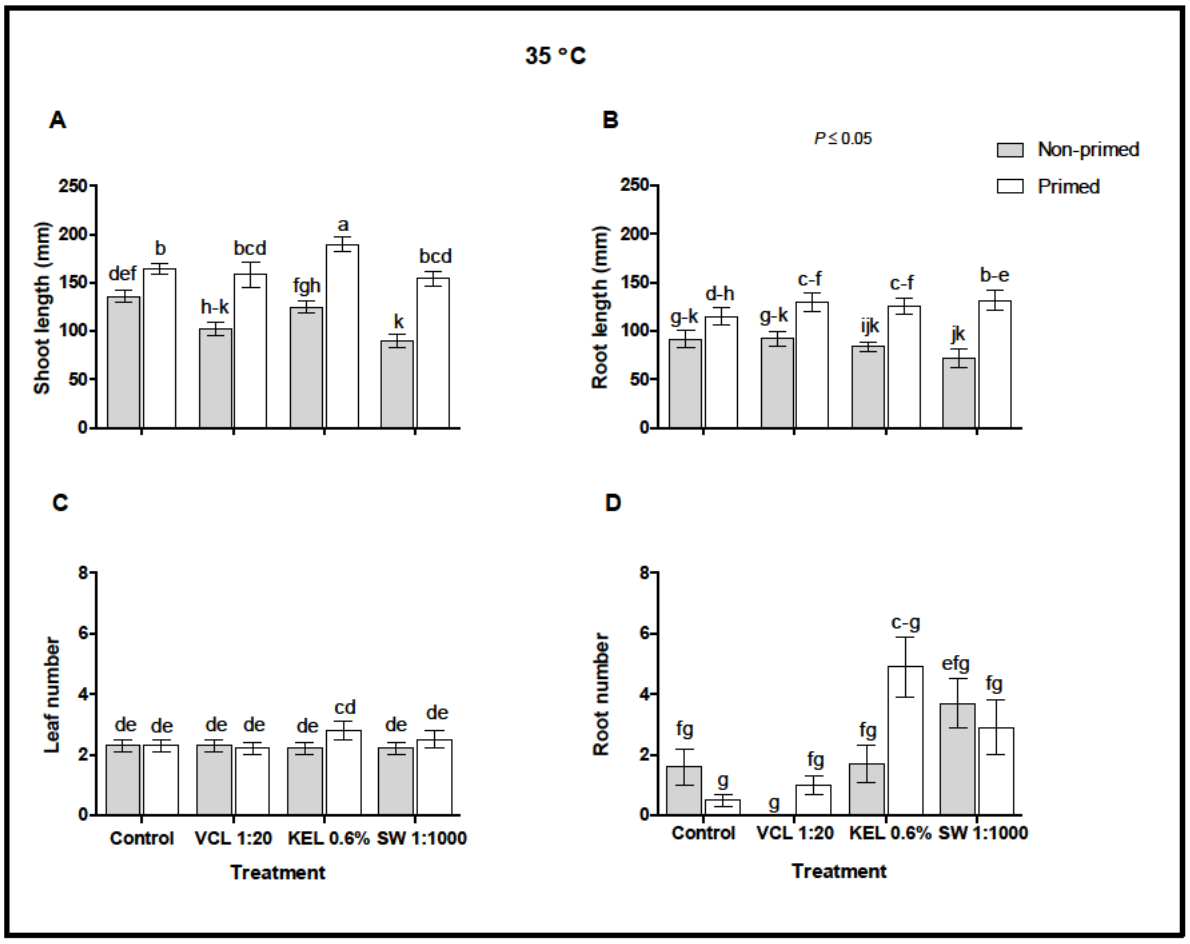

Fig. 3.3: Effects of priming and non-priming with distilled water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW

1:1000 v/v) on growth parameters of *Vigna unguiculata* seedlings under 35 °C 7 days after sowing (DAS). For each treatment, bars (mean and ± standard error of the mean; shoot length, *n* = 20; root elongation, *n* = 20; leaf number, *n* = 20 and root number, *n* = 7) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

3.3.5 Effect of biostimulant-priming on seedling growth under 40 °C

Priming *V. unguiculata* seeds using the three biostimulants or dH_2O was more beneficial in promoting shoot length, root length and the leaf number of 7 d-old seedlings compared to non-priming at 40 °C (**Fig. 3.4A, B** and **C**). Hydropriming (27.00 ± 2.01) improved seedling area of non-primed (13.57 ± 1.20) seedlings by almost 2-fold (**Table 3.3**). Biostimulant-primed seedlings had significantly increased shoot length and leaf number compared to hydroprimed and all non-primed seedlings (**Fig. 3.4A** and **C**). SW followed by KEL promoted longest shoot length and greatest number of leaves when applied as priming agents under 40 °C (**Figs. 3.4A** and **C**, **3.5**). Priming with KEL also yielded greater fresh and dry weight (**Table 3.3**). Biostimulant-priming using VCL promoted longest roots and significantly inhibited root stimulation compared to non-priming (**Fig. 3.4B** and **D**). Non-priming *V. unguiculata* seeds and subsequently drenching the seedlings' growth substrate with either SW or VCL significantly increased their shoot length and number of roots relative to non-primed controls (**Figs. 3.4D, 3.6**). The number of leaves stimulated by non-priming with SW was also significantly higher compared to non-priming with KEL and control (**Fig. 3.4C**).

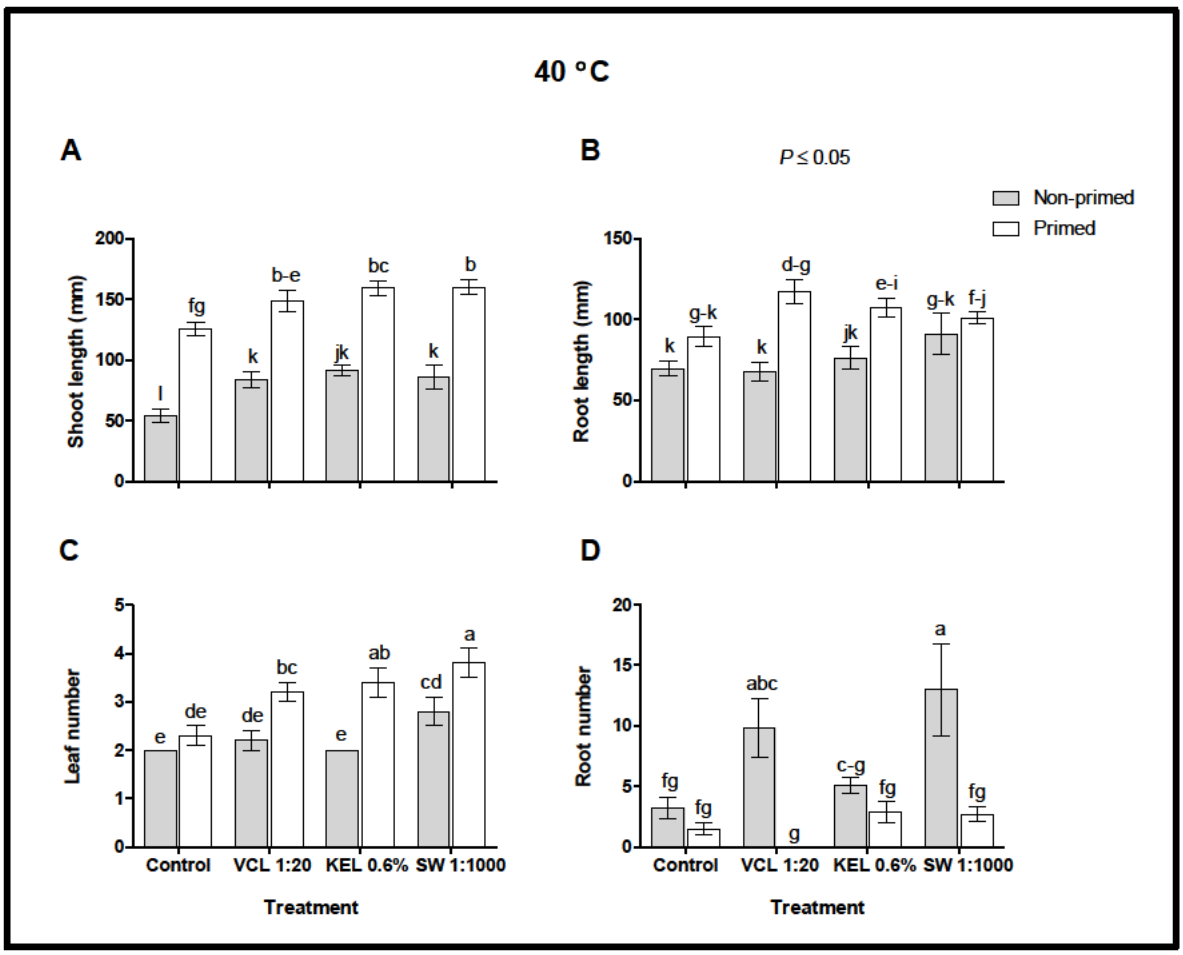

Fig. 3.4: Effects of priming and non-priming with distilled water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) on growth parameters of *Vigna unguiculata* seedlings under 40 °C 7 days after sowing (DAS). For each treatment, bars (mean and ± standard error of the mean; shoot length, *n* = 20; root elongation, *n* = 20; leaf number, *n* = 20 and root number, *n* = 7) with different letter(s) are significantly different ($P \leq 0.05$) based on based on Duncan's Multiple Range Test (DMRT).

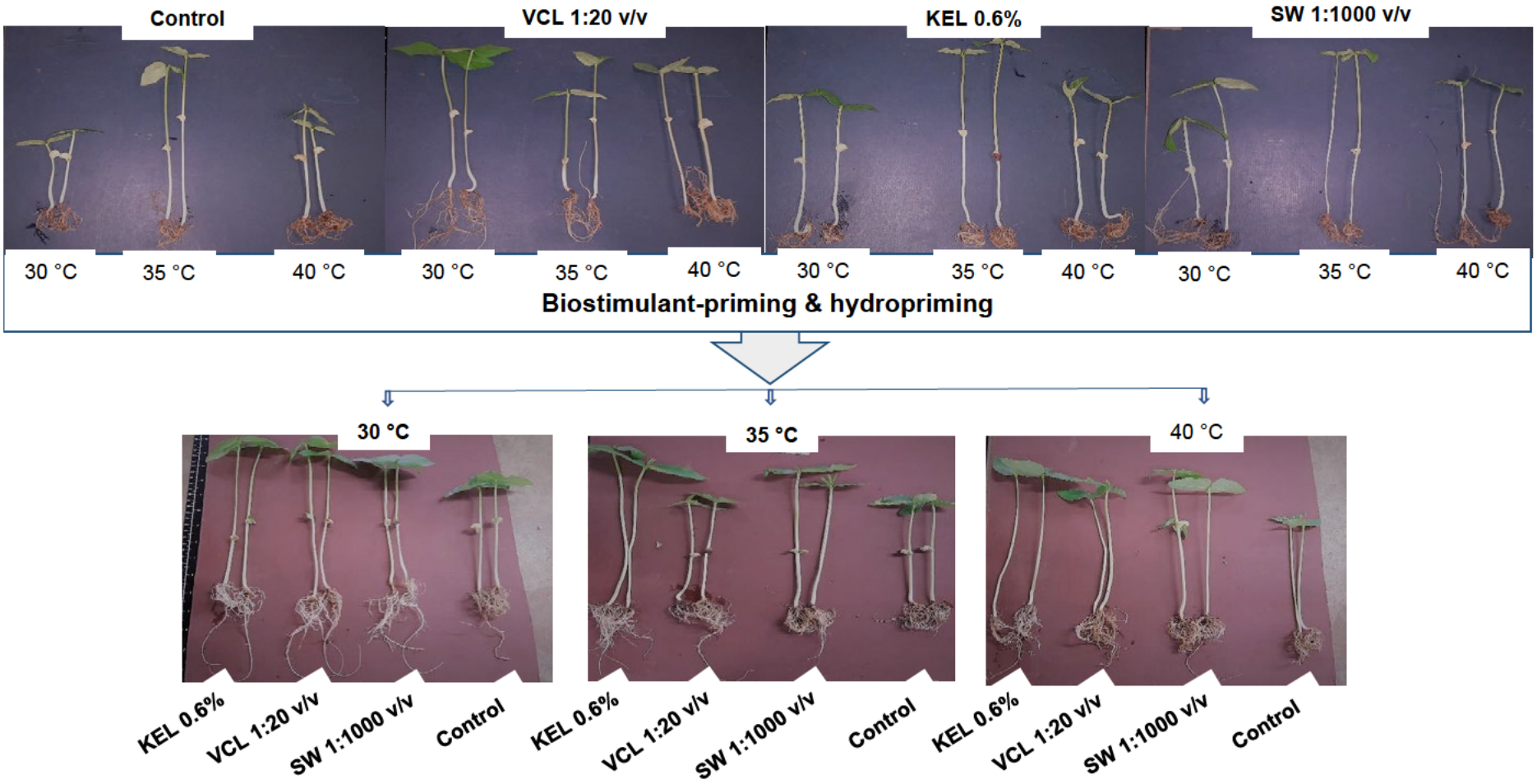

Fig. 3.5: Effects of hydropriming with distilled water and biostimulant-priming with vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) on seedling growth of *Vigna unguiculata* seeds after incubation at 30, 35 and 40 °C 7 days after sowing (DAS).

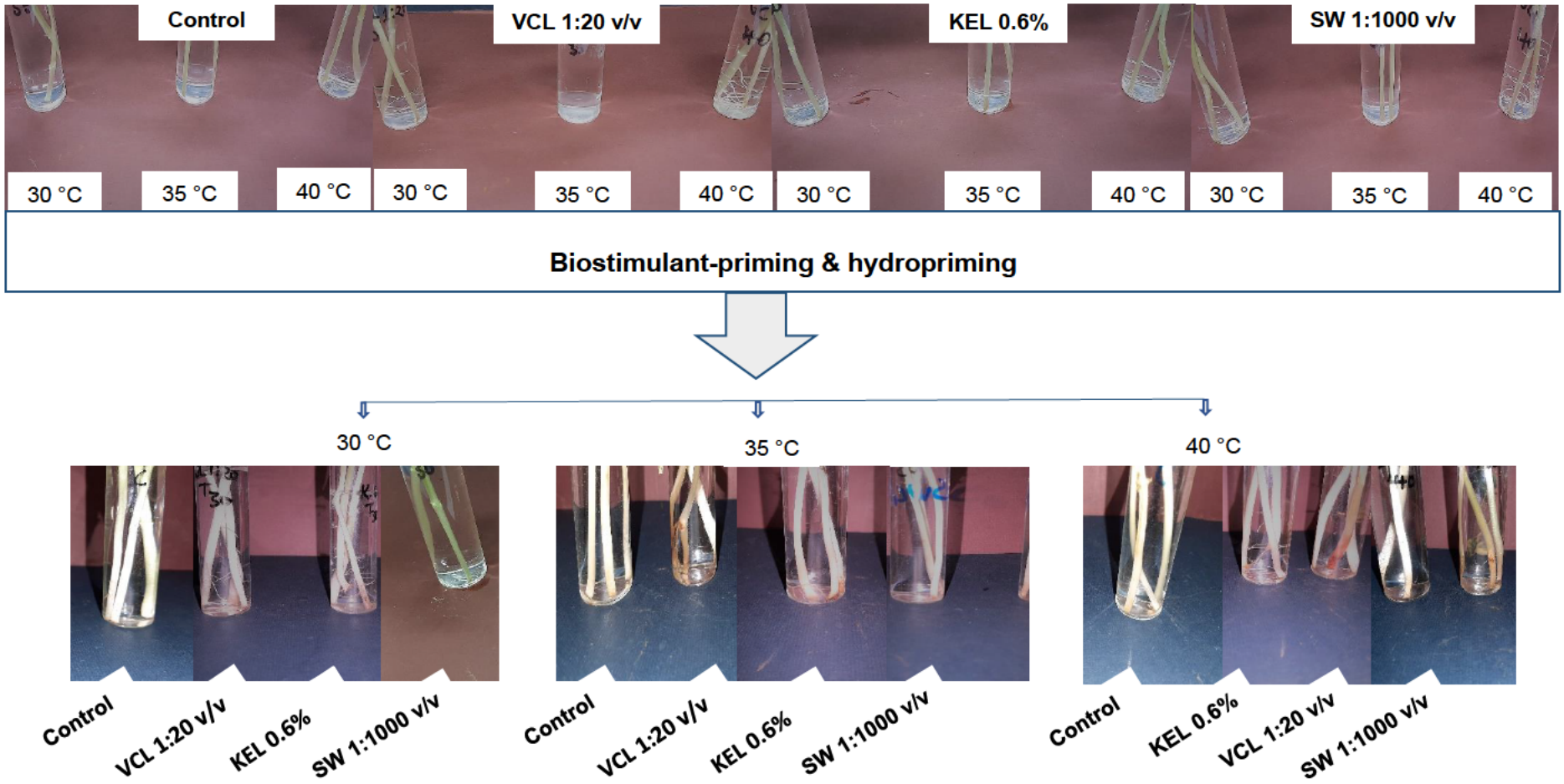

Fig. 3.6: Effects of biostimulant-priming and hydropriming on growth of foliated *Vigna unguiculata* cuttings in hydroponics at 30, 35 and 40 °C 22 days after sowing (DAS).

3.3.6 Post-germination effects of priming with vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on Vigna unguiculata plants under heat-stress of 40 °C 28 DAS.

Post-germination effects of seed priming using the three biostimulants were most pronounced at 30 °C compared to 35 and 40 °C. Growth parameters such as leaf number, shoot length, peduncle diameter, fresh and dry weight were highly influenced by the three biostimulants. For instance, seedlings derived from seeds primed with KEL and SW showed significantly increased shoot length, number of leaves (**Fig. 3.7A** and **B**), seedling area (**Table 3.4**) fresh and dry weight compared to the controls (**Fig. 3.8A**). Moreover, VCL did not only elicit significant increase in leaf number and fresh weight, but peduncle diameter was similarly influenced (**Figs. 3.7.B, 3.8A** and **B**). Cowpea seedlings did not establish post-germination response to all three biostimulants at 35 °C, except insignificant increase caused by KEL and SW during the first and final week on shoot length and leaf number, respectively (**Fig. 3.7C** and **D**).

Despite post-germination effects of priming cowpea seeds with biostimulants not showing significant increase on leaf number and shoot length at 40 °C 28 DAS, however, priming with KEL and SW improved shoot length and the number of leaves during the first and fourth week compared to the control (**Fig. 3.7E** and **F**). Peduncle diameter, fresh and dry weight were also considerably improved by priming and drenching with KEL and SW (**Fig. 3.8 A** and **B; Table 3.4**). Furthermore, priming and subsequently drenching with VCL showed marked stem elongation and early leaf stimulation on the first and second week, but this biostimulation influence was greatly reduced by heat stress of 40 °C during the subsequent weeks (**Fig. 3.7E** and **F**).

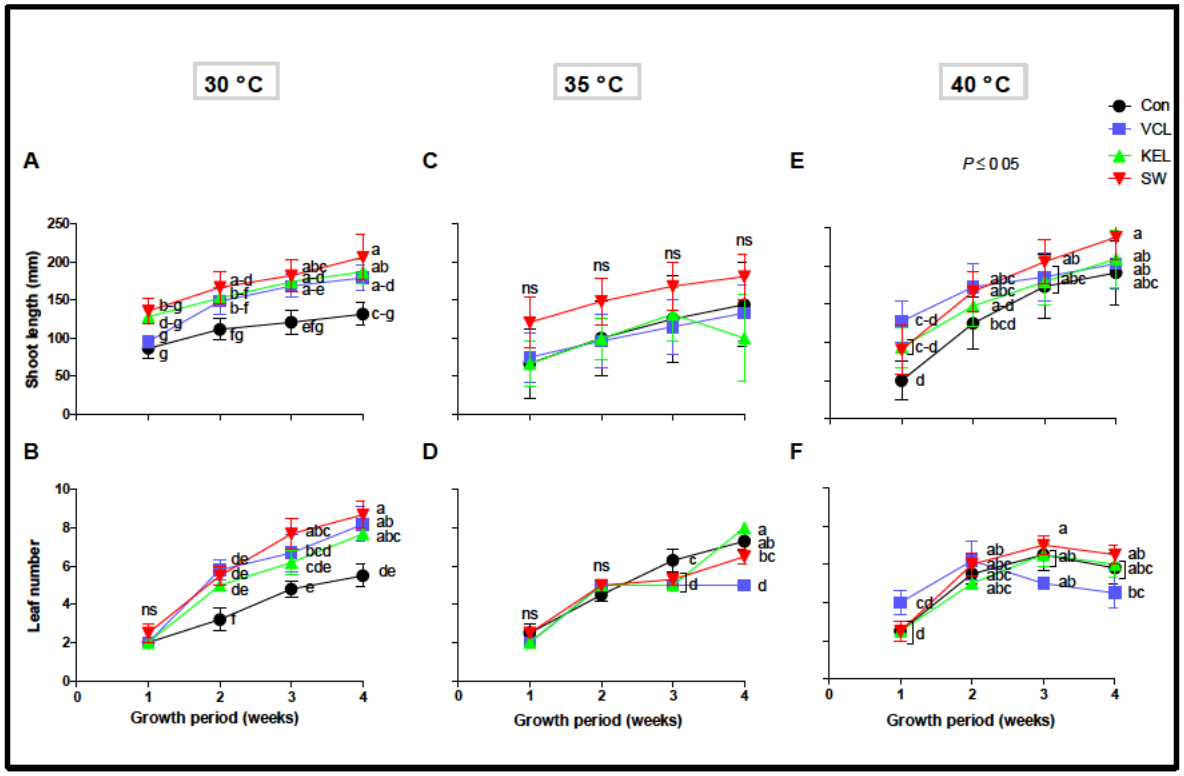

Fig. 3.7: Post-germination effects of priming with distilled water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) on growth progression of *Vigna unguiculata* shoot and leaf number under 30, 35 and 40 °C 28 days after sowing (DAS). For each treatment, error bars (mean and ± standard error of the mean; $n = 6$) with different letter(s) are significantly different ($P \leq 0.05$) and not significantly (ns) different ($P > 0.05$) based on Duncan's Multiple Range Test (DMRT).

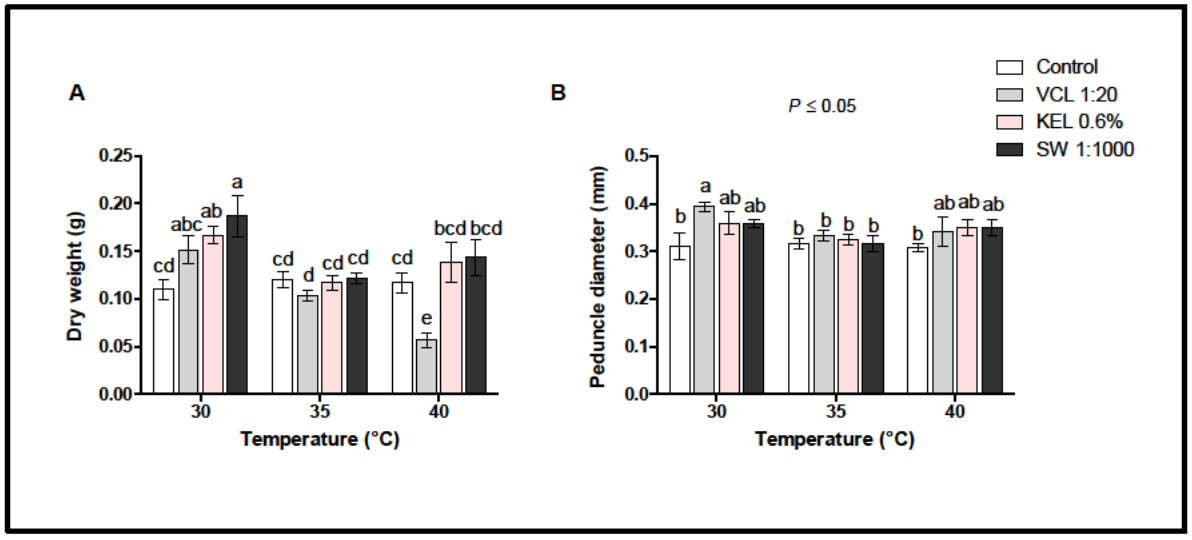

Fig. 3.8: Post-germination effects of priming by pre-soaking with distilled water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) on *Vigna unguiculata* dry weight and peduncle diameter at 30, 35 and 40 °C 28 days after sowing (DAS). For each treatment, error bars (mean and ± standard error of the mean; $n = 6$) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

Table 3.4: Post-germination effects of priming by pre-soaking with distilled water (control), vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on *Vigna unguiculata* fresh weight and seedling area at 30, 35 and 40 °C 28 days after sowing (DAS).

Priming treaments	Fresh weight (g)	Seedling area (cm^2)
30 °C		
Control	1.49 ± 0.23^{b}	29.94 ± 5.89^{bc}
VCL 1:20 v/v	2.29 ± 0.20^{a}	43.91 ± 6.54^{b}
KEL 0.6%	2.39 ± 0.13^{a}	58.77 ± 4.08^{a}
SW 1:1000 v/v	2.54 ± 0.12^{a}	56.94 ± 4.24^{a}
35 °C		
Control	1.69 ± 0.15^{b}	22.88 ± 1.41^{cd}
VCL 1:20 v/v	1.31 ± 0.13^{b}	24.71 ± 2.56^{cd}
KEL 0.6%	1.53 ± 0.06^{b}	34.90 ± 3.76^{bc}
SW 1:1000 v/v	1.72 ± 0.11^{b}	33.14 ± 2.11^{bc}
40 °C		
Control	1.56 ± 0.07^{b}	31.28 ± 3.84^{bc}
VCL 1:20 v/v	0.80 ± 0.10^{c}	15.41 ± 2.26^{d}
KEL 0.6%	1.73 ± 0.25^{b}	31.46 ± 6.92^{bc}
SW 1:1000 v/v	1.61 ± 0.20^{b}	28.71 ± 6.05^{cd}

Mean values ± standard error (n = 6) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) based on DMRT.

3.4 Discussion

As an integral component of sustainable agriculture, biostimulants elicit key morpho-physiological and molecular processes. This results in optimized crop growth, better seedling establishment, more nutrient uptake, elevated phytochemical accumulation and assimilation. Thus, improving plant yield and nutrition and promote plant tolerance to varying degrees of heat-stress, drought, salinity and nutrient deprivation **(Rouphael et al., 2018; Di Mola et al., 2019; Poberežny et al., 2020)**. Most of the induced biostimulation influence on germination, seedling growth and development exhibit a stable biological property which can remain up to maturity **(Drobek et al., 2019; Salvi et al., 2019)**. In the present study, notable beneficial effects of the biostimulants; VCL, KEL and SW were observed on the growth morphology of seedlings at optimum (30 °C), maximum (35 °C) and heat-stress (40 °C) conditions, irrespective of whether the seeds were primed or non-primed (**Fig. 3.5**). The biostimulation effects of KEL and SW remained heat stable for four weeks under 30, 35 and 40 °C. This biostimulation

may have contributed to the elevation of heat tolerance and acclimatization to heat stress, thus promoting better heat shock response in *V. unguiculata* seedlings at 40 °C (**Fig. 3.7A** and **B**).

3.4.1 Effects of biostimulant-priming and non-priming on seedling growth under 30 and 35 °C

Plants respond to temperature in accordance to cardinal temperature (i.e. minimum, optimum and maximum temperature) and any thermal extreme outside these thresholds has the potential to induce inhibitory or growth impairments **(Hatfield et al., 2008; Torabi et al., 2020)**. The established minimum temperature in *V. unguiculata* is above 10 °C while the optimum and maximum temperatures range between 20-25 °C and 35-36 °C, respectively **(Balkaya, 2004; DAFF, 2013; Motsa et al., 2015)**. In the current study the benchmark temperature which caused more than 50% germination was 10 °C (i.e. 30 °C) above optimum temperature (**results not shown**). Based on the results and sowing procedures followed, it was estimated that the optimum and maximum temperatures of the cowpea local cultivar IT18 were 30 ± 1 and 35 ± 1 °C, respectively, and heat stress range was above 35 ± 1 °C. A difference of 5 °C between cardinal temperatures was enough to change temperature threshold, induce stress and trigger a relatively different morphological response in cultivar IT18. The response was dependent on both treatment type and mode of application (i.e. biostimulant-priming, hydropriming and non-priming) (**Fig. 3.1**). Therefore, these results highlight an important insight regarding the possible morphological behaviour of *V. unguiculata* treated with VCL, KEL and SW under increasing temperature as a result of global warming.

Seed priming with a solid media, water (i.e. hydropriming) or low osmotic potential solution was reported to improve germination and growth variables in many plant species **(Murray and Wilson, 1987; Parera and Cantliffe, 1994)**. In leguminous crops, seed priming is considered the most practical technique over non-priming to improve germination, seedling growth, achieve good crop stands, increase threshold of N_2 fixation and derive extra rhizosphere benefits in habitats prone to low soil fertility, heat and drought stress **(Maurya et al., 2020)**. The findings of the current study indicate that seed priming improved shoot length, root length, stem thickness, seedling area, fresh and dry weights under optimum and maximum temperatures of 30 and 35

°C compared to non-priming. These results are in accordance with the findings of **Haider et al. (2020)** wherein mungbean (*V. radiata*) shoot height, number of branches, leaf area, stem, and leaf dry weight were significantly enhanced by hydropriming, and priming with 0.01 and 0.05 M Zn dilutions. **Arun et al. (2020)** recently reported the influence of hydropriming on seeds as well as priming with ammonium molybdate, $ZnSO_4$, $CaCl_2$, GA_3, KBr and $MgNO_3$ test solutions on *V. unguiculata* which increased trifoliate leaves, dry biomass, harvest index and number of pods.

However, the positive attributes of seed priming depend highly on numerous pertinent factors including the priming agent, stress severity, crop species and phenological stage **(Iqbal and Ashraf, 2005)**. When the morphological response of biostimulant-primed *V. unguiculata* seedlings was evaluated, the results indicate that the response is highly influenced by both temperature (**Fig. 3.1**) and priming agent (i.e. biostimulants and dH_2O, control) (**Figs. 3.2, 3.3** and **3.4**). For example, only root elongation and dry weight were significantly increased by priming using all three biostimulants at 30 °C. At 35 °C, seedling height, peduncle diameter and fresh weight were the only parameters significantly enhanced by biostimulant-priming with KEL. The number of leaves and shoot length were the only two growth parameters significantly elicited by priming with all three biostimulants under heat stress of 40 °C compared to hydropriming. These results not only reveal seed priming as an important preconditioning technique in *V. unguiculata* seeds, but also confirm the fundamental need of biostimulant-priming over hydropriming and non-priming under optimal and supraoptimal conditions. It was also evident that at each temperature cowpea responds to each biostimulant in order to achieve a specific morpho-physiological purpose. For instance, the increased root elongation by biostimulants at 30 °C showed little contribution to leaf number and shoot length but may have contributed to larger biomass. Contrarily, the induced root elongation at 40 °C may have contributed to the increased number of leaves, shoot height and fresh weight.

Rapid emergence of deep roots serves as an important indicator for seed germination before the upper layers of the soil become dry which eventually promote better crop establishment and increase yield **(Ashraf and Foolad, 2005)**. The significantly improved root length coupled with slight stem diameter increase by VCL, KEL and SW -primed seedlings at 30 °C is an important indicator for better crop stand establishment, especially under soil prone to drought. This is essential for supporting

more foliage, inflorescence **(Chinsamy et al., 2014)** and may facilitate translocation of higher volumes of photosynthetic sugars to sinks **(Anjum et al., 2011)**. In addition, the stimulation of more lateral roots and long taproot by VCL and KEL at 30 °C as well as by SW and KEL under 35 °C (**Figs. 3.2B** and **D, 3.4B** and **D**), gives an indication that the stands of the seedlings will provide stability and be firmly anchored. Root proliferation in legumes is also a good indicator of more N_2 nodulation, increased water absorption, high nutrient hydrolysis and essential for ensuring a healthy vegetative growth. Hence, this may have influenced high fresh and dry biomass induced by VCL and KEL at 30 °C as well as SW and KEL under 35 °C (**Table 3.3**).

3.4.2 Growth effects of biostimulant-priming and non-priming on Vigna unguiculata seedling growth under heat-stress of 40 °C

Resource-poor cowpea farmers face major production constraints such as poor crop stands, source-sink relationships, high translocation-water demand and shedding of floral parts that contribute to reduced yield **(Arun et al., 2020)**. What is more alarming, is the recent prevalence in meteorological events including drought, high temperatures as well as correlated increase of pathogenic diseases and insect infestation induced as a result of global warming which has resulted in further yield reduction in cowpea. As an indispensable seed treatment technique, seed priming is known for yielding numerous benefits in plants. In legumes like alfalfa (*Medicago sativa*), white clover (*Trifolium repens*) and *V. unguiculata* it has shown to buffer and increase their tolerance to cold stress, salinity, high temperature and drought **(Tiryaki et al., 2009; Arun et al., 2020; Farooq et al., 2020)**.

Seed priming with either VCL, KEL or SW has improved germination and seedling growth by promoting seedling vigour, vegetative increase and stimulating prolific root elongation in non-leguminous crops such as tomato (*Solanum lycopersicon*), okra (*Abelmoschus esculentus*), maize (*Zea mays)* and legumes like common bean (*Phaseolus vulgaris*) under optimal conditions **(Sparg et al., 2006; Van Staden et al., 2006)**. VCL, KEL and SW have also been proposed as possible biological priming agents, germination cues, soil amendments and seedling growth elicitors on biometrical characters of *Ceratotheca triloba* under cold stress, salinity and nutrient deprivation **(Masondo et al., 2018)**. Alternatively, non-priming with VCL improved tomato and pomegranate (*Punica granatum*) morpholocal parameters under cold

stress, heat stress and salinity stress **(Chinsamy et al., 2013, 2014; Bidabadi et al., 2017)**. Despite the research advancements made on VCL, KEL and SW, to the best of my knowledge, no studies have been carried out to investigate their effects (either by seed priming or non-priming) on *V. unguiculata* under supraoptimal stress.

As hypothesised, seed priming in the present study exhibited an array of important effects to optimize growth in *V. unguiculata* under 40 °C compared to non-priming. More than 87% of the measured biometrical characters, namely leaf number, root length, shoot length, stem thickness, seedling area, fresh and dry weight were improved by seed priming. The increased number of leaves and stem thickness among other improved parameters suggested that most plants may have partitioned more growth substrates for leaf growth and peduncle elongation to support seedling growth and position the canopy higher to harvest more light and CO_2. Furthermore, the osmotic potential of some of the treatments may have leached some of the phytochemicals essential for root elongation during priming.

High root length in VCL, KEL and SW-primed seedlings at 40 °C shows that the biostimulants were highly potent in promoting root elongation which may have resulted in their significantly improved shoot length and the number of leaves. These results conform to those of **Chinsamy et al. (2013)** wherein root capacity (as an important indicator for nutrient uptake in areas prone to adverse conditions) of VCL-treated tomato seedlings under salinity, resulted in enhanced above ground biomass. In habitats prone to drought and heat stress, hydropriming has also been instrumental for increasing seed germination and seedling growth by 3-4-fold relative to non-hydropriming **(Maurya et al., 2020)**. The results of the present study indicate that hydropriming promoted leaf number, stem diameter, fresh weight, root elongation, shoot length and seedling area by approximately 2-fold compared to non-hydropriming at 40 °C (**Fig. 3.4A** and **B; Table 3.3**).

An adequate supply of nitrogen helps to improve plant tolerance to drought by eliciting an osmoregulation increase and reduced cell membrane damage **(Garcia et al., 2007)**. N_2 is also a vital component of chlorophyll and a key constituent in the biosynthesis of amino acids that make proteins. The availability of 2.26% N, 0.99% P, 0.64% K, and 2.52% Ca in VCL which facilitate N_2-fixation, nutrient solubility and phytohormonal production coupled with priming **(Azarmi et al., 2008)** may have

positively augmented the results of the present study. This suggests that VCL primed seedlings may be able to thrive in habitats prone to temperature stress and drought due to improved thermotolerance threshold, N_2-fixation, root density and nutrient uptake. A significantly high leaf number in VCL, KEL and SW-primed seedlings at 40 °C (**Fig. 3.4C**), provides an indication that cowpea cultivated under adverse global warming conditions will thrive with capacity to be harvested as a leafy vegetable and still be able to produce enough new leaves to sustain growth till maturity for a second harvest as a pulse.

The use of VCL, KEL and SW also has the potential to solve problems of photosynthesis down-regulation (i.e. acclimation of net photosynthesis to CO_2 enrichment), high photosynthate supply which may suppress N to C ratio and high P requirement under free-air carbon enrichment (FACE). **Krajnc et al. (2012)** and **Ngoroyemoto et al. (2019)** reported that KEL and VCL significantly improved photosynthetic pigments (i.e. chlorophyll *a*, *b*, *a* + *b* and carotenoids), proteins and stimulated accumulation of total phenolic in *P. peltatum* and *A. hybridus*, respectively. Negative effects of K and P deficiencies were offset, and photosynthetic pigment content was increased by VCL drenching in tomato seedlings **(Arthur et al., 2012)**. KEL enhanced N and P uptake and assimilation, grain number, root and yield weights of spring barley **(Szczepanek et al., 2018)**. Root and vegetative biomasses in bananas, *P. peltatum,* common bean, tomato and *T. ludwigiana* were improved in VCL, KEL and SW-treated plants **(Van Staden et al., 2006; Aremu et al., 2012, 2014; Krajnc et al., 2012)**. Based on these research findings and an increase in *V. unguiculata* leaf number, root length and peduncle length at 40 °C, it can be inferred that VCL, KEL and SW can buffer the seedlings and increase their productive capacity under CO_2 and future climate changes.

3.4.3 Biostimulant-rooting effects on Vigna unguiculata cuttings

Crop productivity is dependent on photosynthesis, photosynthate supply and sink activity **(Kaur et al., 2005)**. Root growth depends on a supply of photosynthate from leaves by photosynthesis. When the root system of hydroprimed seedlings was cut and cuttings examined *in vitro* hydroponics, the results showed that new root stimulation effect was significantly reduced by priming treatments, especially at 35 and

40 °C (**Fig. 3.1D**). This could have been due to further exposure of the seeds to surplus water in the growth substrate (i.e. vermiculite) after priming which may have caused premature radicle protrusion or leached some of the essential metabolites. Radicle protrusion during seed priming induces irreversible damage on embryo and seedlings, regardless of the prolific germination that may be observed **(Parera and Cantliffe, 1994)**. Furthermore, hydropriming can be problematic given that it is difficult to contain its hydration, therefore seeds are often primed with osmotic solutions to optimize hydration and avert germination **(Welbaum et al., 1998)**. These findings from the study and research literature indicate the need of properly re-drying the seeds after priming and knowing the prescribed priming duration of a specific crop species. Furthermore, they indicate that it may not be ideal to sow hydroprimed *V. unguiculata* seeds when soil has not reached field capacity, waterlogged or immediately after a long heavy rainfall.

Nevertheless, SW significantly stimulated the highest number of new lateral roots as a non-priming agent compared to other priming and non-priming treatments at 40 °C. These new lateral roots were also longer and thicker compared to fewer and thinner new roots at 30 °C (**Fig 3.6**). This was an evident health indicator normally observed under ideal field conditions. In a study by **Van Staden et al. (2006)** not only rooting stimulatory effects were marked by SW and 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, a cellulose and SW-based compound called KAR_1 **(Flematti et al., 2004; Van Staden et al., 2004)** on maize seedlings, but KAR_1-treated kernels had greater adventitious root development. Cytokinins in rice inhibit lateral root initiation but promote root elongation **(Debi et al., 2005)**. In peas, both lateral root initiation and development is regulated at 10^{-4} M optimum concentration by natural and synthetic auxins **(Wightman and Thimann, 1980)**. Root formation in mungbean (*V. radiata*) Wilczek was enhanced by smoke extract, showing that active smoke constituents could have significantly contributed to root promotory formation **(Taylor and Van Staden, 1996)**. Additionally, the induced root biostimuli by SW as a non-priming agent may have been instrumental in eliciting the highest improvement in peduncle length, number of leaves, seedling area, fresh and dry weight biomass at 40 °C (**Fig. 3.4C; Table 3.3**). New roots of non-primed cuttings were also significantly elicited by VCL at 30 °C, 40 °C and greatly reduced at 35 °C. However, it was evident that VCL was more potent and suitable for non-priming at 30 °C due to the significant improvement of root elongation, shoot

length and seedling area. Stem thickness is an important biometrical character that provides a good indication of sturdy plants and an improved carrying capacity at fruiting stage **(Chinsamy et al., 2014)**. An increase in peduncle diameter induced by priming and non-priming with the three biostimulants, particular VCL and KEL, provides an indication that these biostimulants can offer *V. unguiculata* better stability and carrying capacity to cater for its increased above-ground biomass and possible yield under heat stress.

3.4.4 Post-germination effects of biostimulant-priming on Vigna unguiculata plants under heat-stress of 40 °C 28 DAS

When post-germination effects of priming the cowpea seeds with VCL, KEL and SW were evaluated on above-ground biomass, the number of leaves was greatly reduced at 40 °C relative to 30°C 28 DAS (**Fig. 3.7B** and **F**). A difference of 10 °C increase between the two temperature regimes was enough to induce more dicotyledon abscission at 40 °C which might have largely contributed to the leaf reduction. However, the plant height was considerably increased by biostimulant-priming although this did not differ significantly. This may be due to reported benefits of hydropriming, especially under heat stress which were also more pronounced in the present study on leaf number, fresh and dry weights, especially at 35 and 40 °C (**Figs. 3.7D** and **F, 3.8A; Table 3.4**). Hence, all biostimulants significantly increased number of leaves and plant height but only at 30 °C (**Fig. 3.7A** and **B**). Remarkably, seedlings of VCL-primed seeds were longer with the highest leaf number at 40 °C for only 2 weeks. After this period, shoot length and the number of leaves drastically dropped below those of the other two biostimulants while SW elicited more foliage and peduncle elongation. This suggests that the biological capability of VCL to increase *V. unguiculata* temperature tolerance under heat stress was temperature dependent, periodic and limited to the seedling stage. Evidence for this suggestion was a decline in dry biomass and peduncle diameter and an approximate 3-fold decrease in fresh biomass and seedling area relative to 30 °C.

Despite the above-mentioned findings, SW elicited the highest leaf number and shoot length at 30 and 40 °C. This provided a suggestion that SW was able to delay leaf abscission post-germination and successfully inhibited heat “stroke” at 40 °C on *V. unguiculata* plants 28 DAS. A similar biostimulation effect was established on primed

and non-primed seedlings 7 DAS wherein SW enhanced the highest root length, root number, shoot length, leaf number (**Fig. 3.4A, B, C** and **D**), seedling area, fresh and dry biomass under 40 °C (**Table 3.3**). **Sparg et al. (2005)** demonstrated that smoke effects are not limited to only the germination stage but can induce marked improved seedling vigour. **Blank and Young (1998)** concurred with these findings, wherein seedling emergence and growth of different grass species were increased by aerosol smoke. Maize plants from KAR_1 and SW pre-soaked kernels showed higher root biomass weights, shoot fresh and dry weights and had more leaf number compared to the control, an indication of promotory effects of SW and KAR_1 on plant growth post-germination **(Van Staden et al., 2006)**. The long lasting, water soluble, heat stable properties of KAR_1 **(Van Staden et al., 2004)** coupled with high retention activity and long storage life characteristics of SW **(Van Staden et al., 2000; Gupta et al., 2019)** may have been transferred or induced to morpho-physiological mechanisms to establish better acclimatization and lasting heat tolerance on four-week-old *V. unguiculata* plants under heat stress. This could have played an integral role in inducing the observed highest dry weight and peduncle diameter (**Fig. 3.8A** and **B**). Significantly increased fresh (**Table 3.4**) and dry weights of KEL-treated plants showed the second highest biomass accumulation with wider stems after plants treated with SW at 30 °C. But interestingly, these parameters were least reduced at 40 °C. This could have been due to exogenous application of compactible solutes like proline, ammonium compounds, sucrose, polyols and trehalose that are known to protect the plant by reactive oxygen species detoxification, contributing to cellular osmotic adjustment, membrane integrity protection and stabilizing enzymes/protein **(Hayat et al., 2012)**. These solutes are in significant quantities in seaweed concentrates (SWC's) including KEL (**Table 3.1**) and when applied to plants they promote plant growth, development, yield and induce stress tolerance **(Kocira et al., 2020; Craigie et al., 2011; Drobek et al., 2019)**.

3.5 Conclusions

The findings of the present study reveal that an elevation of 5-10 °C in temperature induced growth-reduction and completely changed the morphological response, contributing to small and shorter cowpea plants. However, this inhibitory effect is highly

dependent on temperature regime, type of biostimulant, application mode (i.e. priming or non-priming) which affect different parameters in different ways. The present study also demonstrated that VCL, KEL and SW improve *V. unguiculata* seedling growth under constant day/night of 30/30 °C, 35/35 °C and at a heat stress of 40/40 °C, regardless of whether seeds were primed or not primed. However, in order to optimize seedling growth and maximize yield productivity to its highest levels possible in heat-stressed cowpea, VCL, KEL and SW seed priming will be an indispensable first line defence mechanism over hydropriming. The biostimulation influence of priming with VCL on seedlings under heat stress was ascertained to be time dependent and can last only 14 DAS. The biostimulation effect of SW and KEL on *V. unguiculata* seedling growth increases with an increase in heat stress, thus promoting plant tolerance, acclimation and improved growth for more than 4 weeks. These findings demonstrate the need to adopt biostimulant-priming with VCL, KEL and SW in small-scale and commercial agriculture as an additional agronomic tool/practice to enhance crop tolerance to heat stress in an era of climatic changes. Additionally, this will not only encourage farmers to derive maximum crop yield from organic farming at a lower cost but will also challenge agricultural and food science stakeholders to subscribe to an environmentally sustainable technology that promotes combating climate change and dismissing land degradation activities. However, more research is still needed on post-germination effects to establish the full potential of biostimulant-priming with VCL, KEL and SW on crop yield under heat stress and drought.

Chapter 4: Effects of different watering regimes of three biostimulants on *Vigna unguiculata* growth and flowering

4.1 Introduction

Despite the predicted upsurge in global temperature of 2-4 °C towards the end of the 21st century **(Vadez et al., 2012; Kiprotich et al., 2015)**, yield inhibitory impacts of climate change on crops are already being noticed. The occurrence of irregular meteorological events such as an increase in the number of hotter-than-normal days during the growing season, extreme temperature regimes, unusual rainfall, floods, drought episodes and land degradation are unprecedented in agriculture **(Singh, 2008)**. These environmental factors, particularly drought (which serves as a constraining factor for major crops) **(Ahmad et al., 2015)**, significantly hampers plant growth and development and induce deleterious effects on crop performance and production **(Shao et al., 2009)**, thus compromising plant survival and ultimately yield **(Masondo, 2017)**. Drought affects transpiration rate, stomatal conductance, relative water status of the leaf and water potential **(Ullah et al., 2017)**. Hence, this challenges available agronomic endeavours of addressing hunger, food insecurity and malnutrition, especially in countries where subsistence farming **(Varshney et al., 2014)** and commercial agriculture are major means of livelihoods. From an agricultural perspective, drought is defined as a physiological edaphic condition which occurs when the amount of accessible water (derived from rainfall or irrigation) to crops is inadequate to meet their transpiration demands **(Tuberosa, 2012)** and soil evaporation needs **(Ilyas et al., 2020)**. Also, at farm level, over-irrigation is uneconomical and unsustainable as it wastes water, energy, is labour intensive and bears the risk of water-logging and nutrient leaching, resulting in a lower crop yield **(Ahmad et al., 2015)**.

To ensure crops cope with drought stress, with current and future climatic changes, their available adaptation and stress protective mechanisms which regulate water deficits and elicit increase drought resistance are imperative. These mechanisms include stomatal closure, gene expression, root-shoot signaling as well as biochemical and molecular signaling pathways like changes in chlorophyll biosynthesis, osmolyte

accumulations, enzymatic and non-enzymatic antioxidant systems. Generally, plants evolved to use these sophisticated defense mechanisms to mitigate water stress and optimize limited available water via three ways, namely drought escape, drought avoidance and drought tolerance **(Varshney et al., 2014)**. To protect their susceptible growth stages, the first mechanism pertains to the plant capacity to reduce life cycles to avoid dehydration **(Ilyas et al., 2020)**. The second mechanism serves the key role of ensuring a continuous regulation in key plant structures to support physiological processes such as stomatal regulation, osmotic adjustment, deposition of epicuticular waxes, root elongation and root system development **(Barnabás et al., 2008)**. The third mechanism is responsible for the plant's ability to resist dehydration by using osmotic adjustment derived from osmoprotectants **(Luo, 2010)**. Some plants also exhibit a fourth defense response mechanism after exposure to severe drought episodes known as drought recovery. Hence, plant acclimatisation to water deficits is a consequence of different events that culminate in adaptation via induced changes in physio-biochemical and plant growth processes **(Duan et al., 2007)**. These events are regulated by water stress specific receptors, osmosensors and transcription factors that sense alteration in cell membranes, water status, turgor, bound water and phytohormones **(Barnabás et al., 2008)**.

Drought-induced morpho-physiological responses include reduced transpiration by accumulating waxy and thick foliar cuticle layers **(Ullah et al., 2017)** and the development of xeromorphic capabilities coupled with changes in plant structures such as increased trichome number, smaller stomata and reduced stomatal density, well-established vascular tissues **(Iqbal et al., 2013)**, long roots and increased density of the root system **(Comas et al., 2013)**. Plants also improve water uptake in drought-induced substrates by means of accumulating solutes like sucrose, soluble carbohydrates, proline and glycine betaine in the cytoplasm **(Anjum et al., 2011)**. They also activate enzymes like superoxide dismutase, peroxidase, catalase, and ascorbate as well as non-enzymatic (e.g. reduced glutathione) substances **(Anjum et al., 2016)**. Antioxidants serve as reactive oxygen species (ROS)-scavenging agents to protect the plant against oxidative stress caused by ROS accumulation as the result of drought and temperature stress. Furthermore, drought stress elicits an increase in the levels of endogenous phytohormones **(Ilyas et al., 2020)** such as Abscisic acid (ABA), Ethylene (Eth), Jasmonic acid (JA), Gibberillins (GA), Auxins (Aux), Cytokinins

(CK) and Salicylic acid (SA) **(Ullah et al., 2018)**. These phytohormones serve a key role in the regulation of plant growth development and stress signaling protection under water deficits. They induce different signaling pathways and as a result, elicit enhanced heat shock proteins, secondary metabolites and enzymatic antioxidant production. For example, accumulating concentrations of ABA in roots is the dominant stress regulator that controls growth, transpiration and promotes root hydraulic conductivity via root-shoot signaling pathways under drought stress **(Barnabás et al., 2008; Anjum et al., 2011)**. ABA transported by the xylem improves the efflux of K^+ ions in the guard cells, which cause cells to lose turgor pressure which subsequently lead to stomatal closure **(Anjum et al., 2011)**. ABA also regulates other physiological and developmental processes such as gene expression, embryo morphogenesis, seed dormancy, cell growth and biosynthesis of storage lipids and proteins **(Kalladan et al., 2017; Ilyas et al., 2020)**.

Growth promotory effects of VCL, KEL and SW have been reported in various plant species including leguminous crops **(Van Staden et al., 2006; Kocira et al., 2013; Islam et al., 2016)**. These include growth improvements and yield-reduction amelioration against effects of biotic and abiotic stresses **(Kulkarni et al., 2011; Battacharyya et al., 2015; Bidabadi et al., 2017; Kocira et al., 2018)**. However, research also indicates that the efficacy of these biostimulants depends on source of active constituents, mode of application, plant growth stage and plant cultivar. The importance of *V. unguiculata* in subsistence farming, livelihoods, food security and its limited tolerance threshold to drought cannot be overlooked in agriculture if hunger, poverty and malnutrition are to be alleviated under the prevailing climate change conditions. Therefore, the objective of this study was to investigate effects of VCL, KEL and SW on growth and flowering of *V. unguiculata* after 13 weeks under different watering regimes using greenhouse protocols.

4.2 Materials and methods

4.2.1 Experimental set-up

The experiments commenced in February 2020 and were conducted in the RCPGD greenhouses. The area has the following global coordinates: 29º37’S 30º23’E **(World Atlas, 2015)**. The greenhouse conditions included 50-60% relative humidity, day and

night temperatures of 27/15 °C and an average PPF of 450 ± 5 µmol $m^{-2}s^{-1}$. Based on Chapter 3 data, seeds were primed by pre-soaking in VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) dilutions according to the procedure described in Chapter 3, tap water was used as the control. Three-hundred and thirty-six primed seeds were sown in a 3 cm furrow in 20 cm plastic pots containing non-autoclaved sandy-loam soil. The pots were placed in four rows on greenhouse steel benches with 45 cm inter-row and 30 cm intra-row spaces in completely randomized design. The base of the pots was placed on polyethylene trays.

4.2.2 Vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) application, leaf number collection and applied agrochemicals

Primed seeds were sown in pots and the soil contained in the pots was moistened with 400 mL of tap water twice a week for a period of 2 weeks. Sixteen days after sowing (DAS), *V. unguiculata* seedlings were thinned to 4-5 seedlings per pot and biostimulants were first applied immediately after thinning (**Fig. 4.1**). Each treatment had 4 pot replicates per watering regime once (1) a week, twice (2) a week and thrice (3) a week. Two-hundred millilitres of treatment was applied by drenching every Monday morning, Wednesday morning and Friday morning for 3-4 months, depending on the watering regime. Bimonthly (i.e. every second week) biostimulant application was alternated with tap water application in all pots. The number of leaves was recorded every Monday morning of the second week until the termination of the experiment. Four-hundred and fifty millilitres of Previcur® fungicide was applied per pot to contain fungi. Insects were contained by spraying the greenhouse walls, benches and pots with Malasol® (Efekto). The fungicide was applied once 28 DAS and fumigation was done 2 days (d) prior to the commencement of the experiments and 40 DAS using Dithane® M-45.

4.2.3 Measurement of morphological parameters

Eleven weeks after seed sowing (i.e. reproductive stages; R7-R8), morphological data were collected from 10 plants per treatment. The number of leaves and flowers were counted, and plant height was measured with a 65 cm Air liquide ruler. The number of flowers were counted every Monday for 3 weeks (from the 11^{th}-13^{th} week). Plant height

was measured from the root collar to the apical bud. Stem diameter readings were taken 4 cm above root collar using Marshal digital calipers. The plants were then carefully removed from the pots, washed and air dried. After aerial drying, nodules were counted before recording root length and fresh biomass weights. Also, the leaf area of 3 trifoliate leaves was measured using a Li-3100 Area meter (LI-COR, Inc. Lincoln, Nebraska, USA).

Fig. 4.1: Uniformity in biostimulant-primed and hydroprimed *Vigna unguiculata* seedlings cultivated using greenhouse protocols before the first application of vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) 14 days after sowing (DAS).

4.2.4 Data analysis

Data were subjected to one-way and two-way ANOVA using Statistical Package for Social Sciences (SPSS®, Version 26.0, IBM, Armonk, New York, USA). Statistical significance was determined using the Duncan's Multiple Range Test (DMRT) to separate mean values at $P \leq 0.05$.

4.3 Results

4.3.1 Effects of different watering regimes on key morphological parameters of Vigna unguiculata

The growth of *V. unguiculata* was considerably influenced by different watering regimes after 3-4 months. For instance, limiting watering frequency by a factor of 200% (i.e. a 400 mL difference between watering regime 3 and 1) per week significantly reduced the number of leaves and root length by 22.96% and 8.26%, respectively (**Fig. 4.2A** and **B**). Soil water deficits also significantly decreased the number of flowers after 3-4 months by 6-fold (**Fig. 4.2C**). More interestingly, the number of nodules was not significantly decreased by reducing biostimulant or water application (**Fig. 4.2D**). In fact, increasing water availability by a factor of 100 and 200% reduced the overall number of nodules by 17.40 and 14.07%, respectively. Overall, ANOVA results pertaining to growth parameters indicated that there was a significant difference between plants cultivated under all three watering regimes. This was also noted among the treatments and their interaction wherein their effects on leaf number, leaf area, plant height, root length, number of nodules and flowers were significantly different from each other (**Table 4.1**). Overall, there was a significant decline in cowpea growth with limitation in watering frequency after 13 weeks. However, this was depending on growth variable.

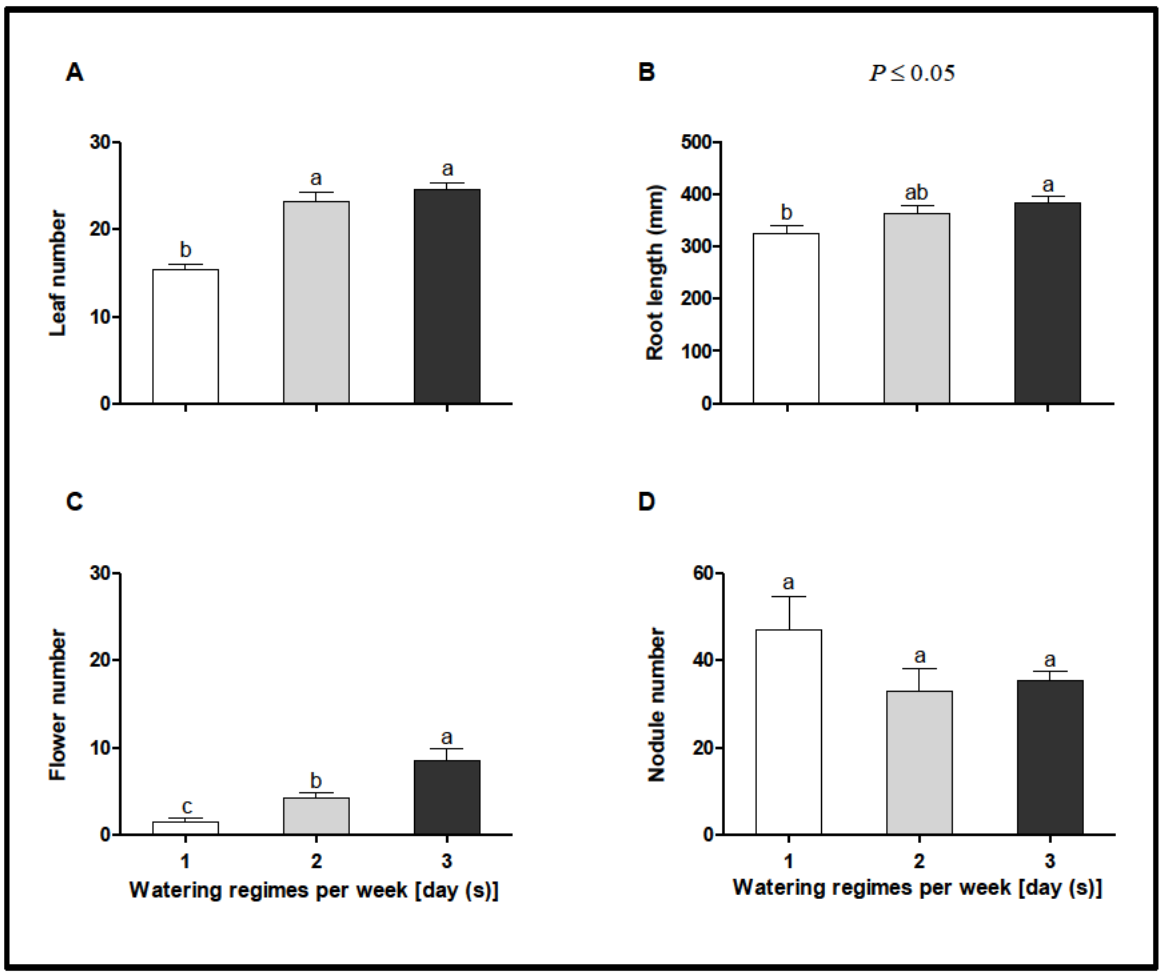

Fig. 4.2: Effects of different watering regimes per week on leaf number, root length, flower number and nodule number of *Vigna unguiculata* after 13 weeks of growth in the greenhouse. In each *Vigna unguiculata* graph parameter, bars with different letter(s) represent mean values ± standard error (n = 10) and are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

Table 4.1: Overall (two-way) analysis of variance of growth parameters of *Vigna unguiculata* plants from primed seeds with main effects and their interactions. Watering regimes in days (d) per week.

Source of variation	Leaf No.		Leaf area (cm^2)		Shoot length (mm)		Root length (mm)		Nodule No.		Flower No.	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
Watering regime (WR, 1, 2 & 3 d)	34.4	< 0.000	39.80	< 0.000	15.11	< 0.000	4.47	< 0.014	2.20	< 0.116	27.92	< 0.000
Treatment (T)	5.7	< 0.001	7.28	< 0.001	11.75	< 0.000	3.59	< 0.016	7.34	< 0.000	6.27	< 0.001
WR X T	0.68	> 0.667	2.31	> 0.067	5.04	< 0.000	2.121	> 0.057	2.80	< 0.014	4.69	< 0.001

Mean values ± standard error in each column per variation source with different letter(s) are significantly different ($P \leq 0.05$) based on DMRT. Days (d).

4.3.2 Effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on Vigna unguiculata grown under different watering regimes after 13 weeks.

Priming *V. unguiculata* seeds using VCL, KEL and SW, improved plant height, root length, the number of nodules, leaf area and the number of leaves in all three watering regimes, especially when watered once and twice a week. All three biostimulants significantly improved shoot length compared to the control under lowest watering regime (**Fig. 4.3**). Plants watered three times a week using KEL had significantly higher shoot length compared to those treated with other treatments in all watering regimes. When KEL and SW were applied 2 d a week, this promoted greater shoot length than VCL and control. All three biostimulants did not significantly promote root elongation when applied 3 d a week (**Fig. 4.4**). However, when applied once and twice a week, SW enhanced root elongation with KEL eliciting a high increase in root length under the lowest watering regime compared to other treatments. When VCL application was restricted to once a week it increased the number of nodules by almost 4-fold compared to the control (**Fig. 4.5**). VCL was the only treatment which induced an increase in number of nodules in all three watering regimes.

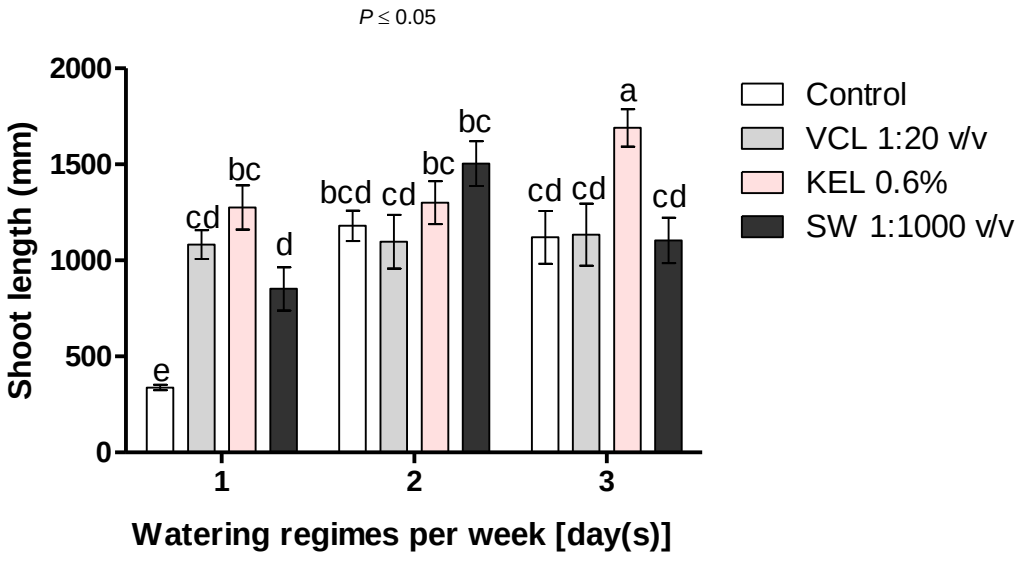

Fig. 4.3: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on shoot length of *Vigna unguiculata* after 3 months of growth in the greenhouse. In each *Vigna unguiculata* graph parameter, bars with different letter(s) represent

mean values ± standard error (*n* = 10) and are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT).

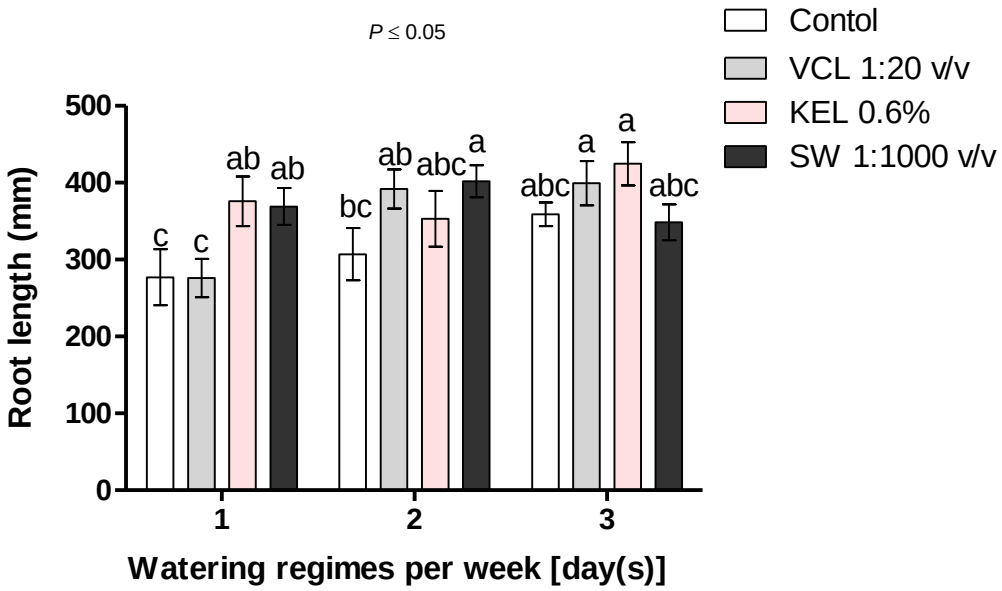

Fig. 4.4: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on root elongation of *Vigna unguiculata* after 3 months of growth in the greenhouse. In each *Vigna unguiculata* graph parameter, bars with different letter(s) represent mean values ± standard error (*n* = 10) and are significantly different (*P* ≤ 0.05) based on Duncan's Multiple Range Test (DMRT).

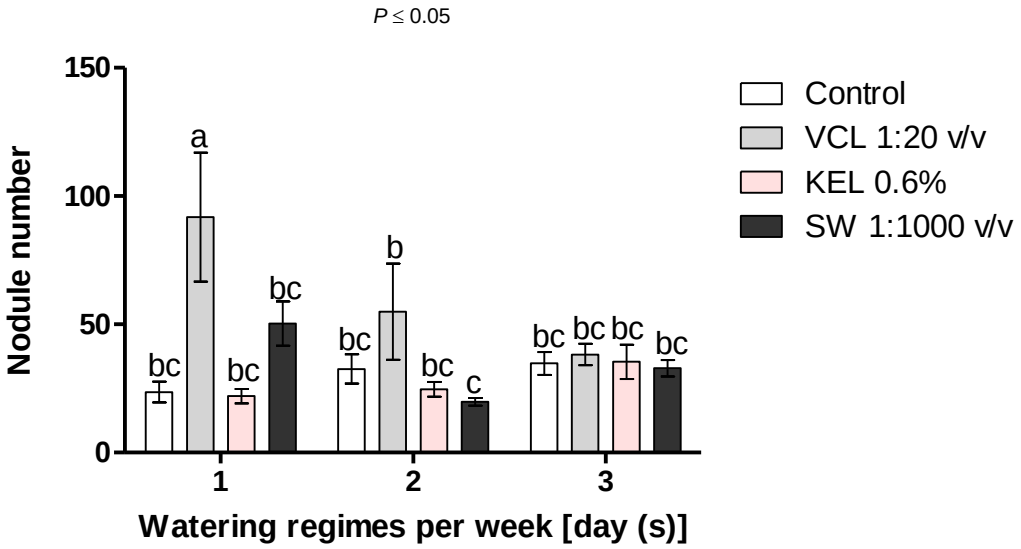

Fig. 4.5: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on number of nodules in *Vigna unguiculata* after 3 months of growth in the greenhouse. In each *Vigna unguiculata* graph parameter, bars with different letter(s) represent mean values ± standard error (n = 10) and are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

The three biostimulants induced greater leaf stimulation and growth after 11 weeks, thus indicating the biostimulants' capacity to optimize growth. Restricting watering frequency to 1 d a week resulted in all three biostimulants improving the number of leaves and leaf area (**Fig 4.6; Table 4.2**). KEL perpetually delayed leaf abortion and instantaneously promoted the highest leaf number by a difference of almost five leaves relative to the control without pronounced decline for almost 3 months. This leaf discrepancy was also induced under ideal edaphic conditions of watering regime 2 and 3 wherein KEL-treated plants had more foliage comparatively to the corresponding controls by more than 4 and 8 leaves, respectively.

When the biostimulants were applied 2 d a week, both KEL and SW enhanced leaf number with KEL promoting a significant greater number of leaves when watered thrice per week (**Fig 4.6**). Growth promotory effects of the two biostimulants were also indicated on below ground biomass. After 11 weeks, KEL and SW promoted long and many adventitious roots under all three watering regimes followed by VCL when applied once and twice a week (**Fig. 4.7**). Despite slightly poor lateral root growth in

plants watered three times a week with VCL, these plants had significantly wide stems, greater fresh and dry weights than the control (**Table 4.2**). KEL and SW also promoted thicker stems and accumulation of more fresh and dry weights in plants watered 2 and 3 d a week (**Table 4.2**).

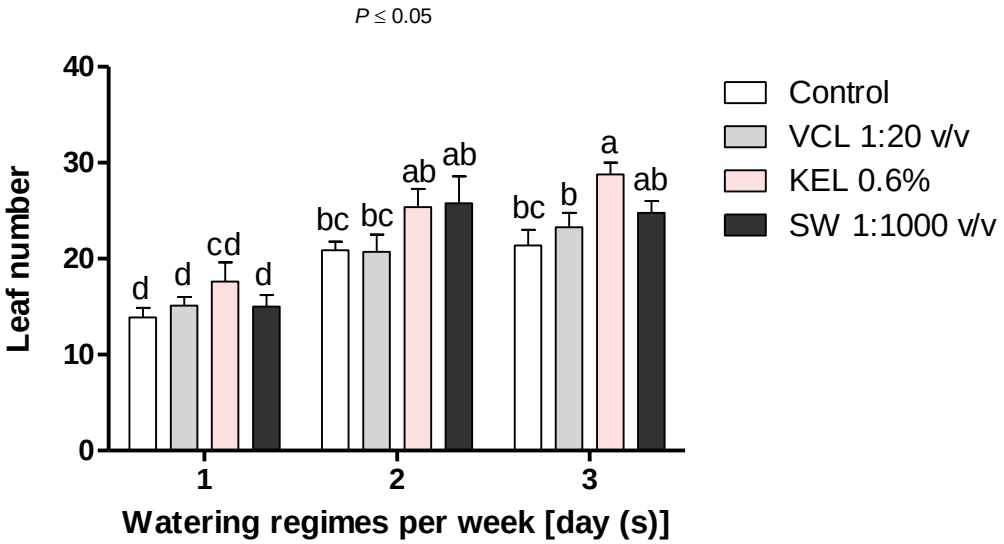

Fig. 4.6: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on leaf number of *Vigna unguiculata* after 3 months of growth in the greenhouse. In each *Vigna unguiculata* graph parameter, bars with different letter(s) represent mean values ± standard error (n = 10) and are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT).

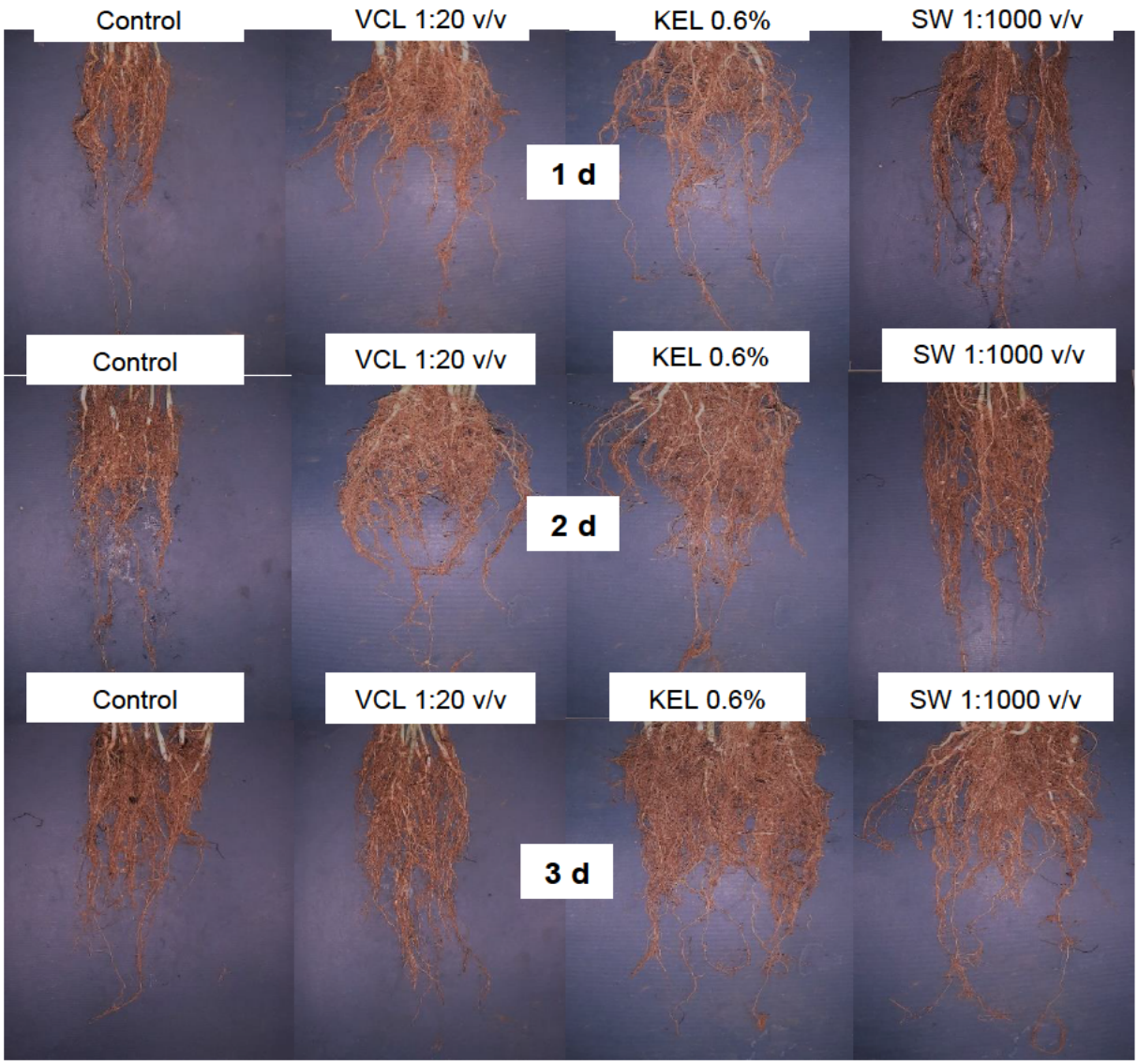

Fig. 4.7: *Vigna unguiculata* roots grown under different watering regimes per week after 11 weeks under greenhouse conditions. Watering regimes per week: once (1 d), twice (2 d) and thrice (3 d). Small letter d represents day(s).

Table 4.2: Effects of tap water (control), vermicompost leachate (VCL), Kelpak® (KEL), and smoke-water (SW) on growth parameters of primed *Vigna unguiculata* seeds at different watering regimes after 3 months in the greenhouse.

Treatment (mL)	Stem thickness (mm)	Fresh weight (g)	Dry weight (g)	Leaf Area (cm^2)
Watering regime 1				
Control	4.64 ± 0.15^{d}	14.86 ± 1.44^{e}	2.66 ± 0.27^{efg}	137.03 ± 21.24^{e}
VCL 1:20 v/v	4.57 ± 0.18^{d}	12.67 ± 0.94^{e}	2.09 ± 0.19^{fg}	148.63 ± 9.71^{de}
KEL 0.6%	4.57 ± 0.16^{d}	13.80 ± 1.09^{e}	1.89 ± 0.16^{g}	152.11 ± 15.61^{de}
SW 1:1000 v/v	5.10 ± 0.14^{cd}	16.55 ± 0.91^{de}	2.78 ± 0.27^{ef}	184.31 ± 6.36^{cd}
Watering regime 2				
Control	4.90 ± 0.33^{d}	17.76 ± 1.42^{de}	3.39 ± 0.24^{cde}	154.02 ± 12.01^{de}
VCL 1:20 v/v	4.99 ± 0.77^{cd}	22.72 ± 2.64^{bc}	3.16 ± 0.26^{de}	195.37 ± 4.08^{bc}
KEL 0.6%	5.65 ± 0.47^{ab}	23.99 ± 1.88^{bc}	4.13 ± 0.26^{bc}	222.84 ± 4.52^{abc}
SW 1:1000 v/v	4.91 ± 0.12^{d}	20.85 ± 2.15^{cd}	3.61 ± 0.32^{bcd}	235.42 ± 33.52^{ab}
Watering regime 3				
Control	5.45 ± 0.18^{bc}	24.23 ± 1.94^{bc}	3.81 ± 0.22^{bcd}	225.24 ± 2.79^{abc}
VCL 1:20 v/v	5.98 ± 0.11^{a}	30.30 ± 1.51^{a}	5.50 ± 0.36^{a}	261.78 ± 7.42^{a}
KEL 0.6%	5.88 ± 0.18^{ab}	26.50 ± 1.23^{ab}	4.33 ± 0.19^{b}	220.87 ± 9.85^{abc}
SW 1:1000 v/v	5.72 ± 0.16^{ab}	24.92 ± 0.85^{bc}	3.95 ± 0.37^{bcd}	246.87 ± 11.38^{a}

Mean values ± standard error (n = 10) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) according to DMRT.

4.3.3 Effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on Vigna unguiculata flowering under different watering regimes after 3 weeks.

V. unguiculata flowering was highly influenced by different watering regimes and biostimulants. Restricting watering frequency to once a week inhibited flowering post-11 weeks of growth (**Fig. 4.8A**). When the plants were watered twice a week, only VCL-treated plants did not flower while marked flowering emergence was established in plants watered three times a week using the three biostimulants. In the 12th week, plants treated three times per week using VCL, KEL and SW had a significantly greater number of flowers compared to the control (**Fig. 4.8B**). The number of flowers in VCL, KEL and SW-treated plants was increased by 4, 4 and 3-fold, respectively. KEL also stimulated flowering by 5-fold compared to the control when applied twice a week. However, its biostimulant capacity was inhibited to zero flowering when watered once a week. Although the biostimulants did not significantly increase the number of flowers when applied once a week during the 13th weeks, however, a considerable increase in flower number was induced by SW (**Fig. 4.8C**). Raising watering frequency of VCL from one to two and three times per week significantly triggered an increase of 4-fold in number of flowers, respectively, compared to the corresponding controls. This shift in watering regimes resulted in a 8 and 3-fold increase in flower number in KEL-treated plants. Plants watered 3 d a week with SW also increased their number of flowers by 3-fold. Increasing watering frequency from 1-3 d per week improved flower number in controls by 2-fold while in VCL, KEL and SW the increase was 12, 7 and 4-fold, respectively.

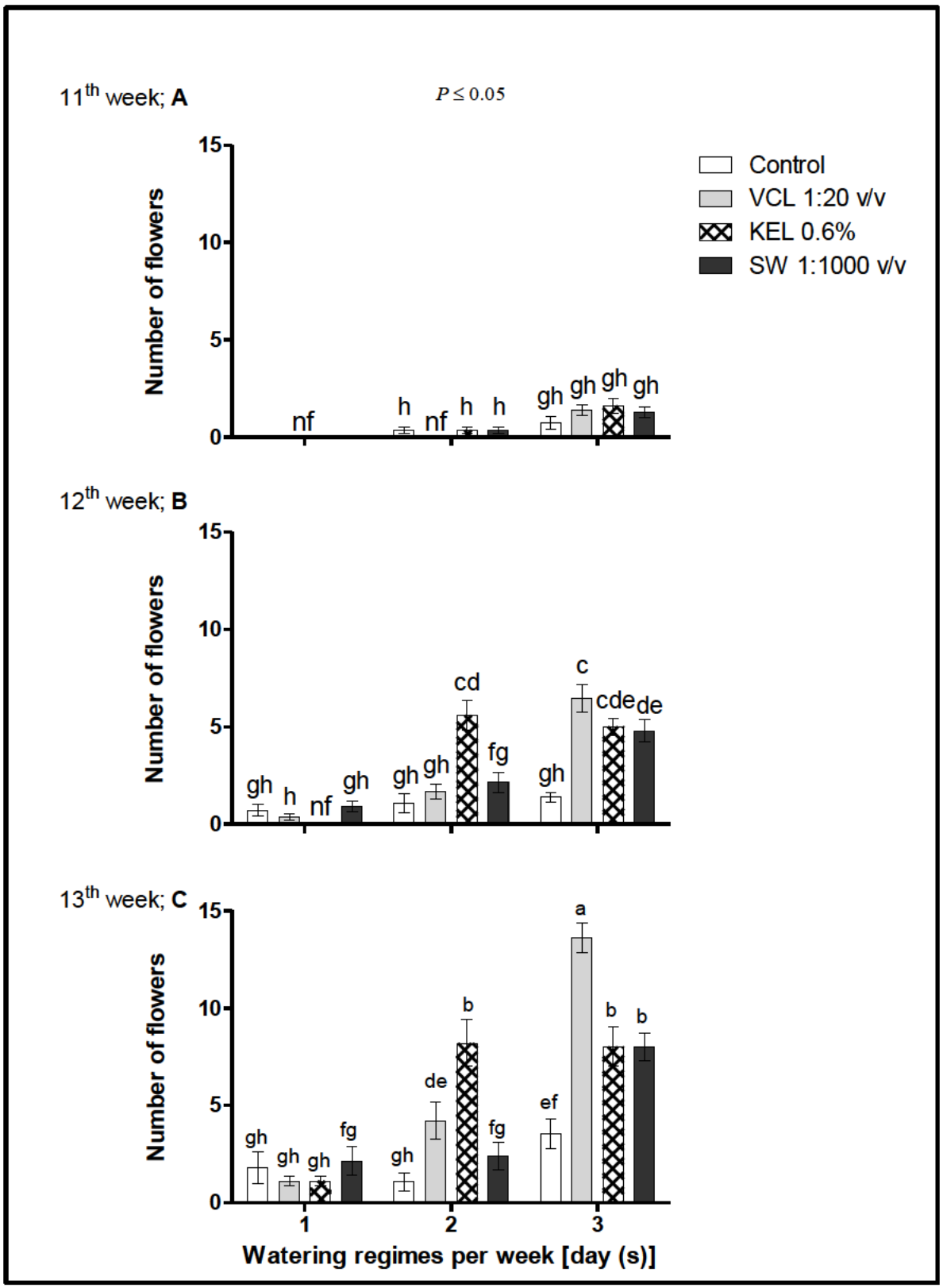

Fig. 4.8: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) on the number of *Vigna unguiculata* flowers during 11th, 12th and 13th weeks of growth in

the greenhouse. For each treatment, error bars (mean and ± standard error of the mean; flower number, n = 5) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). No flowering (nf).

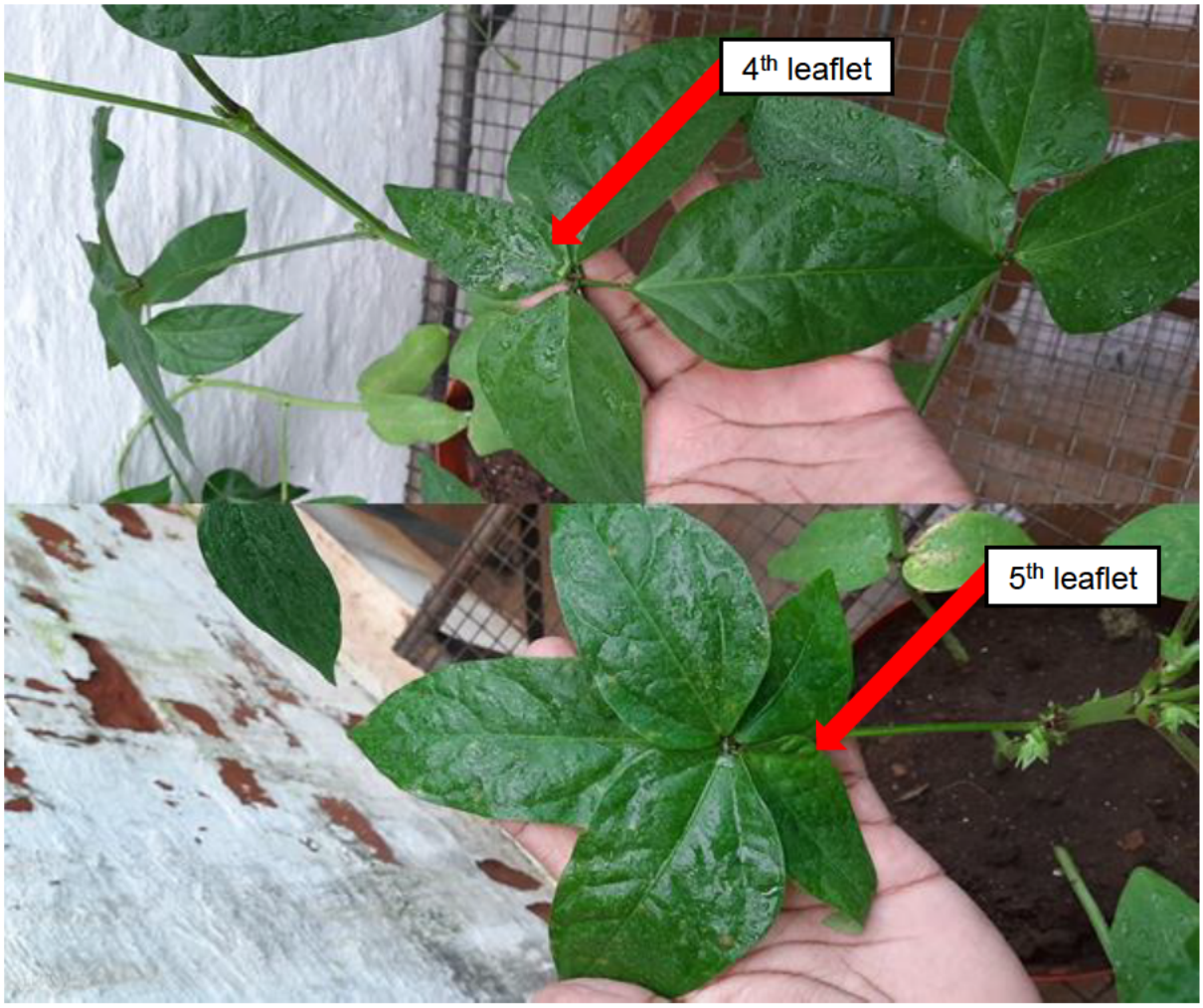

Fig. 4.9: *Vigna unguiculata* trifoliated leaf with an extra 4th and 5th leaflets treated with Kelpak® (KEL 0.6%) grown under greenhouse conditions.

4.4 Discussion

4.4.1 Growth effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on Vigna unguiculata cultivated under different watering regimes after 13 weeks.

Water limitations reduced 90% of the measured morphological parameters in *V. unguiculata*. The induced reduction was more marked on control plants relative to

biostimulant plants watered once and twice a week. Restricted soil water potential results in reduced mitosis, cellular elongation and poor expansion which reduces growth by affecting variables essential for optimising yield **(Hussain et al., 2008)**. Among the reduced growth biometrics is a reduction in leaf longevity, leaf number and leaf size **(Anjum et al., 2011)**. This may explain restrained growth observed on the number of leaves and root length (**Fig. 4.2A** and **B**). These are common symptoms exhibited when roots struggles to extract enough water or when there is an upsurge in transpiration rate **(Anjum et al., 2011)**. Hence, a widespread root system is an important requirement to ensure plant growth at an early growth stage via improved water extraction from shallow soil layers which would be lost to evaporation **(Ashraf and Foolad, 2005; Chinsamy et al., 2013)**. The significantly high root elongation and widespread adventitious roots elicited by KEL and SW at lowest watering regime (**Figs. 4.4** and **4.7**) means these biostimulants have a biological capacity to induce better root establishment and to allow *V. unguiculata* to thrive under drought stress.

Roots are important triggers of a signal cascade to the foliage through xylem-based physiological changes to establish the degree of adaption to stress. Root-to-shoot signaling is a prominent event that causes stomatal closure under drought stress via ABA increase which prevent further water loss to the transpiration stream **(Barnabás et al., 2008)**. However, stomatal closure prevents CO_2 fixation, decreasing leaf internal CO_2 concentration and promotes pentadiulose-1,5-bisphosphate (Rubisco inhibitor) production and photorespiration, eventually leading to a reduction in photosynthesis rate **(Parry et al., 1993; Schafleitner et al., 2013; Daryanto et al., 2015)**. Hence, the increased root growth in KEL and SW-treated plants at the highest water deficiency (watering regime 1) level could mean that their biostimulation capabilities facilitated the minimization of root-to-shoot stress signal by regulating production of phytohormones like ABA, JA and SA to reduce potential effects induced by stomatal closure, thus allowing the harvesting of more light, CO_2, alleviating high C_3 photorespiration and elevating net photosynthesis rate. This could have largely contributed to the increased shoot length and leaf area of KEL and SW-treated plants and considerably high stem thickness, fresh and dry weight biomatrics induced by SW (**Fig. 4.3; Table 4.2**). KEL and VCL down-regulated the production of most phytohormones like ABA and subsequently increase *Ceratotheca triloba* photosynthesis activity, growth and survival rate exposed to salinity stress **(Masondo,**

2017). The findings of **García et al. (2014)** established that the capacity of biostimulants to alleviate stress during ABA biosynthesis is via ABA independent metabolic pathway. Therefore, the capability to curb ABA biosynthesis can be linked with exogenous drenching with VCL and KEL which contain numerous active plant growth regulators **(Stirk et al., 2014; Aremu et al., 2015a, b; Kocira et al., 2020)**.

A long and extensive root system is also advantageous in legumes for more nodule establishment. Furthermore, the high number of healthy nodules is a good measure of an increased N_2-fixation threshold from the rhizosphere and balanced source-sink symbiotic interplay wherein leaves provide nodule rhizobia bacteria with sorbitol and sucrose **(Wang et al., 2019)** in exchange for ureides allantoin, allantoic acids, amides glutamine and asparagine, depending on the leguminous crop **(Redden et al., 2013)**. Root development in the present study was not the only biometric promoted by biostimunlants at the lowest watering regime. By limiting watering regime to once a week together with VCL significantly improved the number of nodules compared to all other treatments in all watering regimes (**Fig. 4.5**). The nodules elicited in the VCL-treated plants were greater in number and smaller in size relative to fewer and larger in watering regimes 1 and 3. The high-water flux demand of the xylem for transporting less soluble ureides in cowpea nodules **(Sprent, 1980)** could have been met by additional water uptake induced by elongated lateral roots in VCL-treated plants (**Fig. 4.7**) and by long tap roots in KEL and SW-treated plants (**Fig. 4.4**). These results unravel the potential of VCL in ensuring that *V. unguiculata* may flourish under climate change-induced water deficiencies via eliciting greater nodulation and enhanced leaf N. Significantly longer tap roots of water-stressed plants treated with KEL and SW imply better adaptation and endurance to water deficits due to increased nutrient extraction and water uptake provisions in the root system.

4.4.2 Growth effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on Vigna unguiculata flowering under different watering regimes after 3 weeks.

Drought stress reduces yield in legumes by inducing reductions in number of flowers produced and impair anthesis **(Nadeem et al., 2019)**. At flowering and anthesis, the yield of chickpea, common bean and mungbean is reduced by 27-40, 49 and 31-57%, respectively **(Rosales-Serna et al., 2004; Mafakheri et al., 2010; Ahmad et al.,**

2015). The findings of the current study showed that as water deficits intensify as a result of lowering watering regimes, the number of flowers significantly drop (**Fig 4.2**). Restricting watering frequency to once per week reduced *V. unguiculata* flowering by 100 and 69% during the 11th and 13th week, respectively (**Fig. 4.8A** and **C**). This decrease is physiologically instigated by drought sensitivity of the flowering stage as there is a decline in assimilate partitioning and enzyme activities involved in sucrose and starch synthesis **(Anjum et al., 2011)**. However, the decrease in number of flowers of *V. unguiculata* was biostimulant and watering regime dependent. For instance, restricting watering frequency to once a week in the 13th week considerably improved flowering in plants treated with SW compared to other treatments. Conversely, when watering frequency was raised to twice per week, VCL and KEL significantly promoted more flowering while when applied thrice a week VCL, KEL and SW significantly increased the number of flowers by 4, 2 and 2-fold, respectively, compared to the control. Varying morphological responses to biostimulants under various watering regimes were also established by foliage wherein KEL stimulated highest leaf number under both unrestricted and restricted watering regimes after 11 weeks (**Fig. 4.6**). This high leaf number was also contributed by extra 4th and 5th leaflets stimulated on trifoliate leaves of KEL treated plants (**Fig. 4.9**).

Allowing drought stress to coincide with flowering and inflorescence development results in anthesis delay or its inhibition **(Barnabás et al., 2008)**. The flowers of biostimulant-treated plants in the current study had an early anthesis compared to those of the control, depending on watering regime. During the 11th week, all three biostimulants promoted both early flowering and anthesis when applied 3 d a week (**Fig. 4.8A**). Their elicited number of flowers was slightly greater to that of the control by 2-fold. However, this biostimulation influence on flowering was inhibited when plants were watered 1 and 2 d a week. Early flowering and anthesis were rapid and significantly increased in all biostimulant-treated plants when applied 3 d a week in the 12th week and KEL also stimulated significant increase in 2 d watered plants. Restricting watering frequency to 1 d a week resulted in significantly lowered number of flowers during the 11th-13th week with only SW eliciting considerably high flowers compared to the control. However, an irrigation shift from 1 to 2 and 3 d a week, resulted in a manifold significant increase of flowers in VCL, KEL and SW-treated plants. These findings establish *V. unguiculata* flowering stage as highly sensitive to

water deficits and this high sensitivity cannot be offset by hydropriming as it has been reported in other crops **(Maurya et al., 2020)**. Thus, efficient irrigation and biostimulant-application will be critical in ensuring more foliage, early flowering and anthesis in cowpea production under areas predisposed to climate change-induced aridity. Additionally, the long peduncle as the unique feature in *V. unguiculata* can bear 2-3 pods, or even 4 or more pods under favourable conditions **(Davis et al., 1991; Masenya, 2016)**. Hence, based on ascertained data in the present study, it can be deduced that VCL and KEL are prolific pod and foliage elicitors under optimum field conditions and when soil water is depleted, this feature may be harnessed for foliar accumulation, peduncle elongation, root system establishment and leaf expansion.

4.5 Conclusions

The findings in the present study reveal that water deficiencies will induce mild inhibitory effects on *V. unguiculata* growth due to its considerable drought threshold and existing defensive morpho-physiological mechanisms. However, these indispensable defence mechanisms will not be enough to buffer *V. unguiculata* and ameliorate drought effects at the flowering stage due to high water sensitivity of this reproduction stage. Thus, this will potentially result in a more than 69% catastrophic reduction in yield. Furthermore, despite significantly high leaf number elicited by biostimulant-priming and exogenous application of VCL and KEL, this showed little contribution or delayed promotory effects on the flowering stage.

Nevertheless, seed biostimulant-priming of *V. unguiculata* with SW followed by its exogenous application, will not only significantly promote larger leaves, longer roots and taller plants at the flowering stage when exposed to drought stress, but will also elicit a high number of flowers. This demonstrates that SW has potential to allow cowpea to flourish under a prolonged drought episode by improving water sensitivity and stimulating greater flower number. This study also failed to totally discount the biostimulation influence of VCL and KEL under water deficits as these biostimulants promoted a high number of leaves, plant height and widespread root systems. This provided an indication that cowpea may have harnessed the effects of VCL and KEL to partition more photosynthates for foliar accumulation and denser canopy to ensure

growth and survival which may have cost or delayed its early flowering. This is evident by early flowering and a significantly higher elicited number of flowers at watering regime 2 wherein VCL and KEL increased flowers by 4 and 7-fold, respectively, relative to watering regime 1. They also improved flower number by more than 12 and 7-fold at the highest irrigation frequency. Therefore, efficient irrigation or coinciding flowering stage with the rainfall season will be key in growing VCL and KEL-treated cowpea in areas with low rainfall. Also, farmers subsisting in areas less affected by climate change will harvest more than 7 pods with VCL and KEL-treated plants relative to the ones in drought-prone regions, assuming each flower equates to one pod. Proliferated root system in VCL and SW-treated plants and high number of nodules induced by VCL under dry conditions suggested that the plants were able to capacitate high water extraction, nutrient uptake, nodulation and fixed more N_2 from the rhizosphere, important measures for high number and nutritious seeds. These important high-yield indicators present a motivation for cowpea farmers to adopt these biostimulants. However, despite the existing potential of VCL, KEL and SW on growth and flowering ascertained in the present study, there is still a need for more studies that will focus on the reproductive stage under field conditions to ascertain their full potential on yield promotion.

Chapter 5: Effects of three biostimulants on *Vigna unguiculata* phytochemicals, photosynthetic pigments and biochemicals under different watering regimes

5.1 Introduction

Aerial and edaphic stresses such as elevated temperature, drought stress, salinity and nutrient deficiency induce adverse effects on plant growth, development and survival. These environmental factors often coincide and are encountered by plants during different stages of their life cycles at different growing seasons. Many of these abiotic stresses are directly or indirectly related to water status **(Verslues et al., 2006)**. Drought and temperature stresses are the two significant abiotic stresses that restrain crop growth and yield **(Prasad et al., 2008)**. They induce various physiological, biochemical and molecular changes and responses which influence different cellular and the entire plant's processes **(Prasad et al., 2008)**. Nevertheless, their induced effects on photosynthesis, growth, developmental and physiological processes can vary greatly.

Photosynthesis is affected by drought stress in two ways, either by causing pathway regulation via stomatal closure which lowers CO_2 assimilation and leaf CO_2 density or by having a direct negative influence on metabolic activities **(Farquhar et al., 1989)**. Direct major metabolic changes include a decrease in the regeneration of ribulose bisphosphate (RuBP) and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein quantity, reduced Rubisco activity, damages on ATP synthesis, impaired photophosphorylation or reduced inorganic P **(Parry et al., 2002; Bota et al., 2004)**. Water deficits also promote leaf senescence by decreasing N and chlorophyll *a* + *b* content **(Ghanbari et al., 2013)**. Drought-sensitive processes of photosynthesis exhibit more heat tolerance and stability at the temperature range of 30-35 °C, depending on plant species **(Prasad et al., 2008)**. However, when heat stress elevates above 40 °C, oxygen solubility relative to CO_2 will drop, which promotes photorespiration and reduce photosynthesis **(Lea and Leegood, 1999)**. Temperature stress also elicits a decrease in activation as well as the activity of Rubisco **(Prasad**

et al., 2004). Moreover, it triggers modifications in the functions of the cell membrane by changing membrane fluidity **(Barnabás et al., 2008)**.

Both temperature and drought stresses have marked influences on molecular compounds such as soluble proteins, amino acids, carbohydrates and their biosynthesis. They induce oxidative stress in plants through enhancing the accumulation of reactive oxygen species (ROS) **(Zobayed et al., 2005)** in mitochondria, microsomal, chloroplast, cytosol and peroxisomes **(Allahmoradi et al., 2013)**. ROS accumulation not only changes the activities of enzymes but also decrease photosynthesis and stomatal closure **(Sadak et al., 2020)**. The high concentration of ROS is a menace to cells since this induces lipid peroxidation, electron leakage, followed by damage to membranes, proteins and nucleic acids **(Maksup et al., 2014)**. To offset such damage, plants use enzymatic antioxidants like superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase or non-enzymatic antioxidants such as ascorbic acid, carotenoids, glutathione and α-tocopherols **(Sadak et al., 2017)**. Other stress-induced phytochemicals in plant tissues which increase plant tolerance to heat and drought include phenolic compounds **(Rice-Evans et al., 1997)**.

Legumes contain significant amounts of phenolic compounds including phenolic acids and flavonoids that are beneficial for optimizing plant growth and human health **(Silva et al., 2017)**. In plants, these secondary metabolites serve important roles such as disease resistance chemicals and provision of protection against pests and pathogens **(Kumar et al., 2017)**. In humans, phenolic compounds scavenge ROS and reactive nitrogen species, thus preventing or mitigating the frequency of degenerative diseases by protecting lipids (i.e. inhibit lipid peroxidation), proteins and deoxyribonucleic acid (DNA) **(Amarowicz and Shahidi, 2018; Limmongkon et al., 2017)**. Phenolic compounds are classified into three types, namely free phenolics which exist primarily as flavonoids and phenolic acids; soluble bound phenolics commonly known as esterified phenolics; and those that are found in the cell wall in combination with lignin, cellulose and arabinoglycan, known as insoluble bound phenolics **(Xu et al., 2018)**. The phenolic classes range from phenolic acids (i.e. hydroxybenzoic and hydroxycinnamic acids and their derivatives), flavonoids, stilbenes, lignans to tannins **(Amarowicz and Shahidi, 2017)**.

Flavonoids and hydroxycinnamic acids are also ubiquitous plant bioactive compounds that belong to the group polyphenols. Flavonoids are secondary plant phenolics that have a flavan nucleus and low molecular weight **(Heim et al., 2002)**. To date, more than 4 000 flavonoids have been reported to be found in various plant parts such as leaves, flowers, seeds and bark. These compounds exist in both fruits and vegetables mainly as 3-*O*-glycosides and polymers **(Heim et al., 2002)**. The main molecular constituents of flavonoids are glycosides called kaempferol and quercetin as well as chlorogenic acid polymers **(Neugart et al., 2017)**. The protective effect of plant phenolics are associated with the flavonoid chemical structure. According to **Rice-Evans et al. (1996)**, flavonoids have strong phytochemical activity compared to non-flavonoids, and when existing in combined forms such as glycosides, they present lower activity than in free forms. Compatible solutes such as proline, amino acids, sucrose, polyols, among others, also accumulate in plant tissues to alleviate oxidation stress by regulating osmotic adjustment **(Ghanbari et al., 2013)**.

Treatments of an exogenous nature for plant growth active ingredients have demonstrated the potential to improve plant tolerance to temperature and drought stressess. Biological treatments such as VCL, KEL and SW are plant biostimulants reported to increase abiotic stress threshold in many plant species **(Kulkarni et al., 2011; Battacharyya et al., 2015; Bidabadi et al., 2017; Kocira et al., 2018)**. These biostimulants are reported to improve photosynthesis, carbohydrates, proteins **(Ngoroyemoto et al., 2019)**, phytohormones and phytochemistry **(Aremu et al., 2014; Masondo, 2017)**. Therefore, the objective of the current study was to investigate the effects of VCL, KEL and SW on photosynthetic pigments, phenolics, flavonoids, total carbohydrates and proteins of biostimulant-primed *V. unguiculata* cultivated under various watering regimes.

5.2 Materials and methods

5.2.1 Experimental set-up and seed sowing

5.2.1.1 Greenhouse experimental trials

V. unguiculata seeds and the three biostimulants (e.i. VCL, KEL and SW) were sourced and prepared as described in Chapter 3 (**Section 3.2.1** and **3.2.2**). Similarly,

seeds were sown using greenhouse protocols as detailed in Chapter 4 (**Section 4.2.1** and **4.2.2**). The plants had a growing period of 13 weeks before termination and final data collection.

5.2.2 Biochemical assays

5.2.2.1 Quantification of total carbohydrates

Total carbohydrate content was determined using the method described by **Sadasivam and Manickam (2008)** with slight modifications. Two-hundred milligrams of a fresh leaf or root material was hydrolysed in 5 mL of 2.5 N hydrochloric acid (HCL) and kept in a water bath with boiling water for 3 h before being cooled to room temperature. The hydrolyzed extract was neutralized by adding granules of sodium carbonate until the effervescence ceased. Two-hundred millilitres of distilled water (dH_2O) was then added to the test tubes to top up the volume to 5 mL and centrifuged at 10000 $rmin^{-1}$ (rpm) for 15 min. One-hundred microlitres of the supernatant was then pipetted out, and the volume was topped up to 1 mL by adding dH_2O before 4 mL of Anthrone reagent (prepared at mixing rate of 200 mg/100mL of 98% H_2SO_4) was added. Thereafter, solutions were reheated in boiling water for 8 min, followed by cooling in running tap water. The absorbance of the reaction mixture was read at 630 nm as the colour changed from green to dark green using a UV-visible spectrophotometer (Varian Cary 50, Australia). A standard curve was prepared using 0-100 µg glucose.

5.2.2.2 Quantification of total proteins

Total proteins were quantified using bovine serum albumin (BSA) as a standard **(Bradford, 1976)**. Two-hundred milligrams of a fresh leaf or root sample was weighed and homogenized in an ice-chilled mortar and pestle containing 6 mL ice-cold phosphate-buffer saline (PBS) [8 g NaCl (137 mM), 0.2 g KCL (2.7 mM), 1.44 g NA_2HPO_4 (10 mM), 0.24 g KH_2PO_4 (1.8 mM) in 1 L of dH_2O (pH 7.2)]. Thereafter, the homogenate was centrifuged (Avanti® J-E Centrifuge, Beckman Coulter, Ireland) for 15 min at 15000 $rmin^{-1}$ at 4 °C. A JA 20.1 rotor size was utilized. One-hundred microlitres of the supernatant was pipetted out into test tubes before topping up the

volume to 1 mL with PBS. One millilitre of Bradford dye was then added. The solutions were vortexed and left in a state of immobility for 5 min. The mixture turned blue as the red dye binds to the proteins. Absorbance was recorded at 595 nm against a control using a UV-visible spectrophotometer (Varian Cary 50, Australia).

5.2.2.3 Quantification of chlorophyll content

The photosynthetic pigments [chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*) and carotenoids] were quantified following **Lichtenthaler (1987)** as detailed in **Amoo et al. (2014)**. One-hundred milligrams of a fresh leaf or root material was homogenized into 5 mL ice-cold acetone. The extract solution was filtered using Whatman No. 1 filter paper. The filtrate was then centrifuged (Hettich Universal, Tuttlingen, Germany) at 3000 $rmin^{-1}$ for 10 min under room temperature. The absorbance of the three supernatants per sample was read at 470, 645 and 662 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). Thereafter, chlorophyll *a*, *b*, total chlorophyll (*a* + *b*) and carotenoid contents were calculated using the following formulas **(Lichtenthaler, 1987)**;

Chlorophyll *a* = 11.23A662 - 2.04A645

Chlorophyll *b* = 20.13A645 - 4.19A662

Chlorophyll *a* + *b* = 7.05A662 + 18.09A645

Total carotenoids = (1000A470 - 1.90Chla-63.14Chlb)/214

5.2.3 Phytochemical assays

5.2.3.1 Sample preparation

After 5 days (d) of oven drying*, V. unguiculata* plant biomass was sorted into leaves, stems and roots. Dry leaves were pulverized using a mortar and pestle. The roots were also pulverized similarly. The powdery samples (1 g) were then extracted with 20 mL of 50% (v/v) methanol (MeOH) and 50% dH_2O in a sonication bath (Branson Model 5210, Branson Ultrasonics B.V., Soest, Netherlands) for 20 min with the water

temperature being maintained cold by adding ice. Thereafter, methanolic extracts were filtered through Whatman™ No.1 (70 mmØ) filter paper, and the filtrates were immediately used for quantification of total phenolics and flavonoids.

5.2.3.2 Quantification of total phenolic content

The total phenolic composition of the filtrate was determined in accordance with Folin and Ciocalteu assay following **Makkar (1999)** with minor modifications. Fifty microlitres of the plant extract (from leaves or roots) were dispensed into 950 µl dH_2O, and 500 µl of 1 N Folin and Ciocalteu's phenol reagent and 2.5 mL of 2% sodium carbonate were added to the reaction mixture. The reaction mixtures were then covered by metal foil and incubated in a darkroom at room temperature for 40 min. Thereafter, absorbance was measured in triplicates using a spectrometric assay on a Cary 50 UV–visible spectrophotometer (Varian, Australia) read at 725 nm wavelength. Test tubes were first placed on a BV 1000 vortex mixer (Benchmark Scientific Inc., USA) for 2-5 s before measuring the absorbance. A reaction mixture that contained 50% aqueous MeOH instead of samples was used as a blank. Subsequently, a standard curve of gallic acid equivalents (GAE) was used to convert the measured absorbance readings to phenolic compound concentrations per g of extract.

5.2.3.3 Estimation of flavonoids

Flavonoid composition of the leaves and roots were estimated using the colourimetric aluminium chloride procedure as described in **Zhishen et al. (1999)** with minor modifications. Two-hundred microlitres of plant extract were diluted with 800 µL of dH_2O. Seventy-five microlitres of 5% sodium nitrate, 75 µL of 10% aluminium chloride, 500 µL of sodium hydroxide as well as 600 µL of dH_2O were sequentially added to the reaction mixture. The colour of the reaction mixture turned pink-like to depicts the presence of flavonoids. The absorbance of the reaction mixture was immediately measured in triplicates using spectrometric assay on a Cary 50 UV-visible spectrophotometer (Varian, Australia) read at 510 nm wavelength. Methanolic solution (50%) instead of the reaction mixture was used as a blank. Flavonoids composition of the plant samples was expressed as mg/g catechin equivalent (CE) against a standard curve.

5.2.4 Data analysis

The physiological data of *V. unguiculata* plants cultivated under different watering regimes in the greenhouse were subjected to one-way ANOVA using Statistical Package for Social Sciences (SPSS®, Version 26.0, IBM, Armonk, New York, USA). The level of significance was determined according to DMRT at $P \leq 0.05$.

5.3 Results

5.3.1 Effects of watering regimes on total carbohydrate, protein, phenolic and flavonoid concentrations of Vigna unguiculata after 3 months of growth in the greenhouse.

Carbohydrate, protein, phenolic and flavonoid contents in the leaves and roots of *V. unguiculata* were considerably influenced by different watering regimes after 3 months of growth (**Table 5.1**). Increasing watering frequency from 1-2 day(s) (d) per week improved overall leaf carbohydrates, but when further raised to 3 d per week they declined to almost equal to those of plants watered once a week. Interestingly, increasing watering regime from 1-3 d per week significantly lowered overall root carbohydrates by 3-fold in the 3-d-watered plants compared to 1-d-watered plants. The roots of plants had significantly higher protein concentrations compared to the leaves and remained relatively the same as watering frequency shifted from 1-2 and 2-3 d a week. As this transition was occurring, the concentration of leaf proteins were significantly lowered but their phenolics were significantly greater compared to roots. Leaf phenolics decreased with an increase in watering frequency while the root phenolics increased with an increase in watering frequency. A similar trend was established in flavonoids.

Table 5.1: Average effects of different watering regimes per week on leaf and root carbohydrates, proteins, total phenolics and flavonoids of *Vigna unguiculata* after 3 months of growth in the greenhouse.

Watering regimes per week [day(s)]	Carbohydrates (μgg^{-1} FW)		Proteins (μgg^{-1} FW)		Phenolics (mg GAEg^{-1} DW)		Flavonoids (mg CEg^{-1} DW)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Water regime 1 (1 d)	83.91 ± 06.4^{a}	121.67 ± 21.7^{a}	59.97 ± 13.2^{b}	91.85 ± 2.4^{a}	45.77 ± 3.1^{a}	29.42 ± 1.4^{b}	39.05 ± 1.5^{ab}	33.02 ± 1.6^{bc}
Water regime 2 (2 d)	105.30 ± 11.3^{a}	108.78 ± 8.6^{a}	55.50 ± 10.2^{bc}	87.43 ± 6.9^{a}	43.25 ± 1.5^{a}	31.99 ± 1.1^{b}	32.62 ± 1.2^{bc}	39.02 ± 3.9^{ab}
Water regime 3 (3 d)	87.05 ± 09.1^{a}	43.91 ± 10.6^{b}	31.50 ± 07.8^{c}	88.30 ± 8.2^{a}	40.64 ± 1.2^{a}	34.17 ± 1.8^{b}	29.96 ± 0.8^{c}	45.11 ± 2.4^{a}

Mean values ± standard error (n = 12) in a column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Letter d represents the number of day(s) per week, FW and DW mean fresh weight and dry weight, respectively.

5.3.2 Effects of plant biostimulants on total carbohydrates of Vigna unguiculata under different watering regimes after 3 months of growth in the greenhouse.

Increasing SW application from once to twice a week resulted in a 2-fold increase in leaf carbohydrates compared to the control, followed by a significantly decline in plants watered thrice a week (**Fig. 5.1A, B** and **C**). KEL and VCL significantly elicited higher leaf carbohydrates when applied twice and thrice a week, respectively. Reducing water application to once a week increased root carbohydrates by 3 and 2-fold in SW-treated plants relative to those of leaves and roots of control plants, respectively (**Fig. 5.1A**). This root carbohydrate increase in SW plants was 7-fold greater compared to those watered thrice a week and 2-fold higher when compared to the corresponding the controls (**Fig. 5.1A** and **C**). Although KEL induced significant increase in root carbohydrates of the plants watered 2 d a week (**Fig. 5.1B**), but the increase was significantly lowered either by restricting or increasing watering frequency. Root carbohydrates of VCL-treated plants showed a minimal influence of water deficits under once and twice a week regime and declined by 5-fold in 3-d-watered plants.

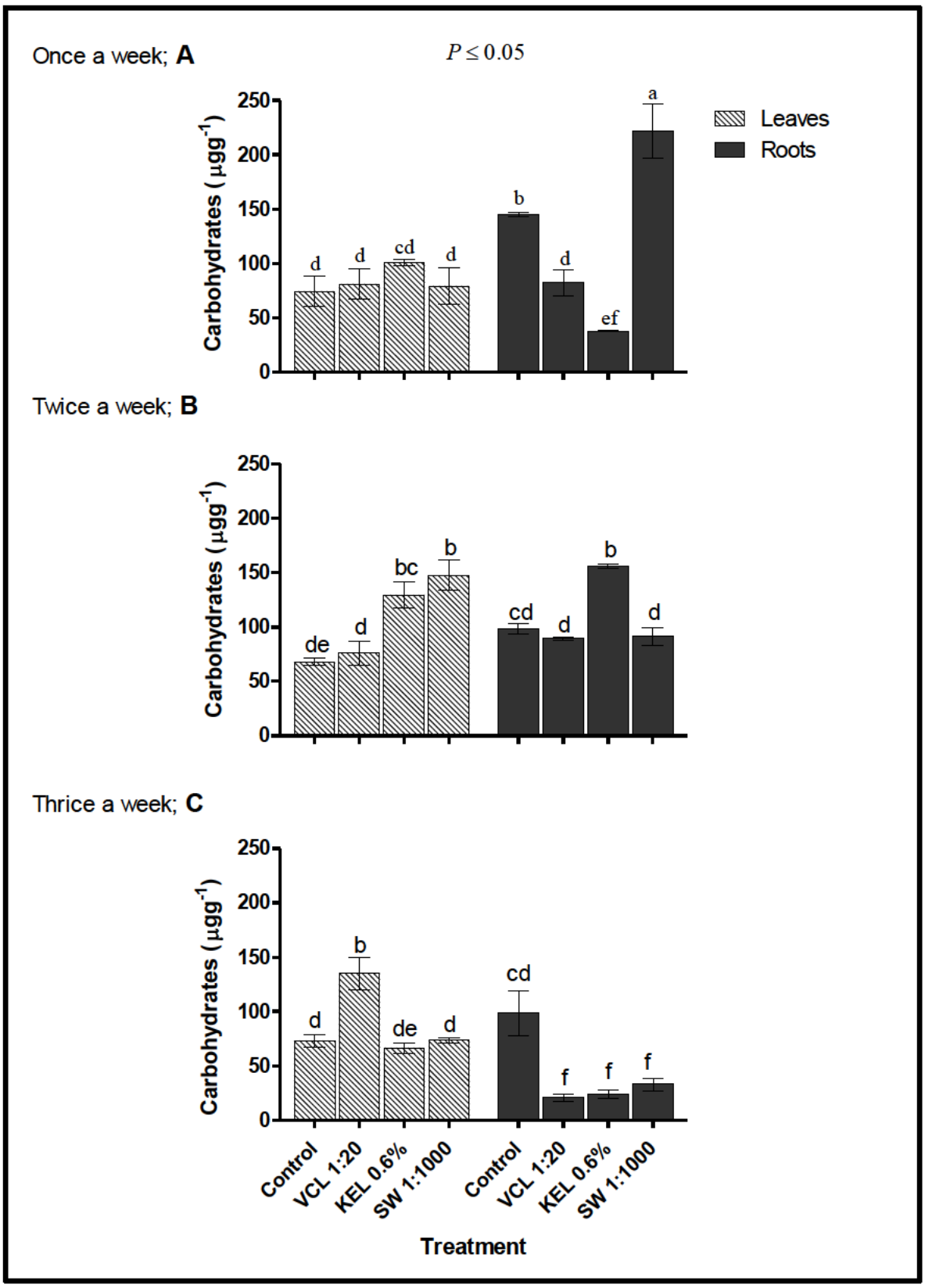

Fig. 5.1: Effects of watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on leaf and root total carbohydrates of *Vigna unguiculata* after 3 months of growth in the

greenhouse. In each *Vigna unguiculata* graph, bars with different letter(s) represent mean values ± standard error and are significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test (DMRT). Once, twice and thrice a week represent watering regime 1, 2 and 3 in day(s), respectively.

5.3.3 Effects of three biostimulants on total proteins of Vigna unguiculata under different watering regimes after 3 months of growth in the greenhouse.

Restricting watering frequency to once a week lowered the proteins of the leaves in plants watered with biostimulants compared to the control (**Fig. 5.2A**). Leaf proteins of biostimulant-treated plants were not altered despite increasing watering application to twice a week (**Fig. 5.2B**). Root proteins of VCL and KEL-treated plants were significantly reduced at this watering regime. When the watering frequency was raised to thrice a week, VCL significantly increased leaf proteins by 2-fold compared to the control while those in KEL and SW-treated plants were lowered (**Fig. 5.2C**). No marked increase compared to the control was established in root proteins of all biostimulant-treated plants, despites the irrigation increase in watering regime 2 to 3.

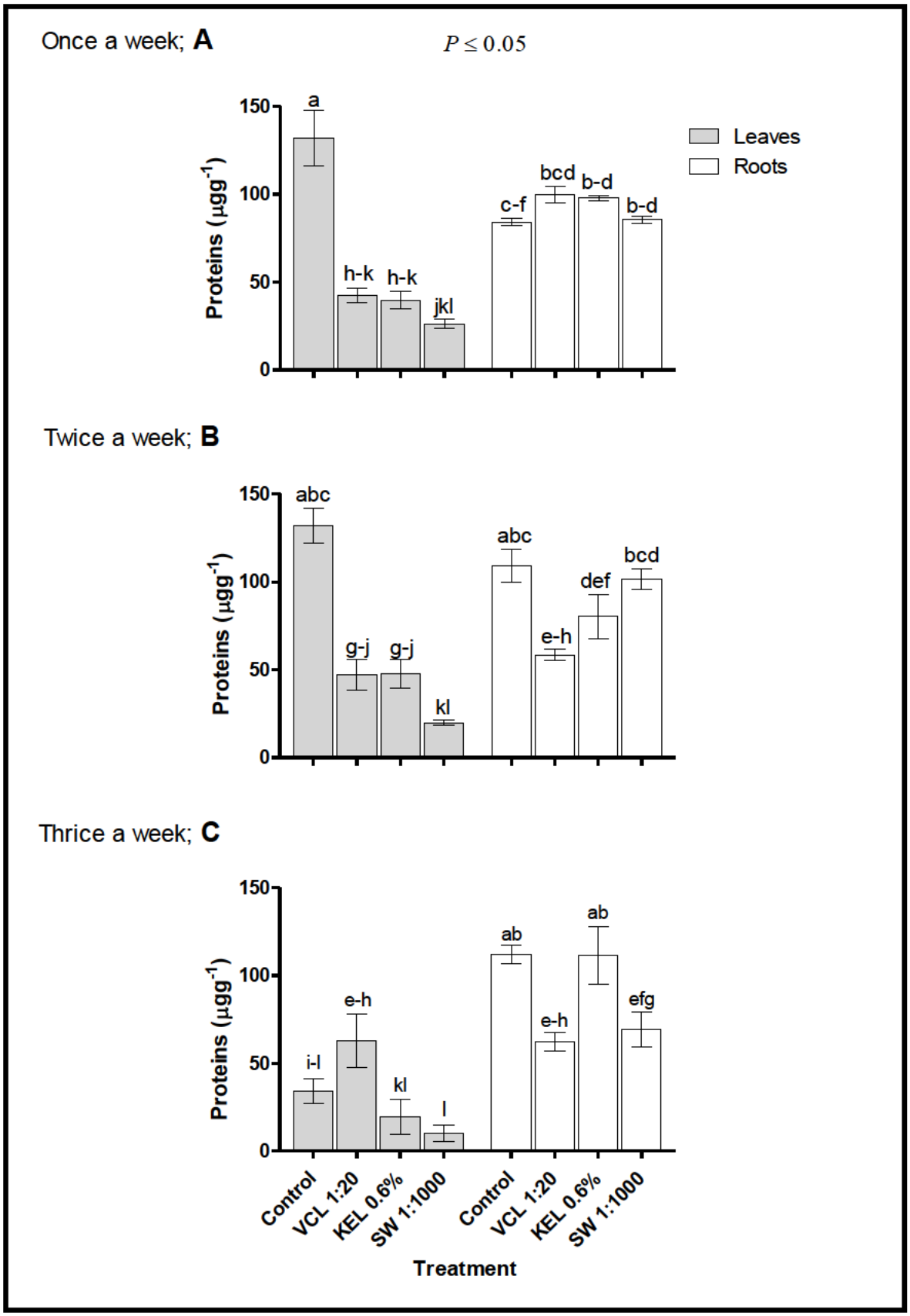

Fig. 5.2: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per

week on leaf and root total proteins of *Vigna unguiculata* after 3 months of growth in the greenhouse. In each *Vigna unguiculata* graph, bars with different letter(s) represent mean values ± standard error (n = 3) and are significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test (DMRT). Once, twice and thrice a week represent watering regime 1, 2 and 3 in day(s), respectively.

5.3.4 Effects of biostimulants on chlorophyll pigments of Vigna unguiculata under different watering regimes after 3 months of growth in the greenhouse.

Variation in watering regimes of biostimulants positively influenced chlorophyll pigments of *V. unguiculata* after 3 months of growth in the greenhouse. Chlorophyll *a* of KEL and SW-treated plants was improved by 80.64 and 177.62 $\mu g g^{-1}$, respectively, against the control in watering regime 1 (**Table 5.2**). The three biostimulants significantly stimulated chlorophyll *a* production by more than 2-fold relative to the corresponding control when applied thrice a week. This increase was the result of a significant decline in chlorophyll *a* of control plants as watering frequency increased from 1-3 d (**Table 5.2**). SW was more effective in promoting chlorophyll *a* under once and twice a week with VCL in 3-d-watered plants. Restricting KEL and SW application to once a week, promoted chlorophyll *b* by a margin of 170.09 and 114.40 $\mu g g^{-1}$, respectively, compared to the control (**Table 5.2**). Increasing irrigation frequency to twice and thrice a week did not significantly improved chlorophyll *b* production, however, VCL and SW-treated plants had higher chlorophyll *b* content under watering 2.

Chlorophyll *a* + *b* was considerably improved by all biostimulant-application at watering regime 1 and 3 compared to the controls (**Table 5.2**). At watering regime 2, only VCL and SW promoted high chlorophyll *a* + *b* contents. SW induced highest chlorophyll *a* + *b* content when watered once and twice a week while KEL was most efficacious in watering regime 3 followed by VCL. Limiting watering frequency to once a week also resulted in KEL plants having greater carotenoid contents compared to other treatments. No marked biostimulant influence was established under watering regime 2. Lastly, the three biostimulants elicited higher carotenoids in plants watered three times a week compared to the controls.

Table 5.2: Effects of tap water (control), vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on chlorophyll pigments of *Vigna unguiculata* at different watering regimes after 3 months of growth in the greenhouse.

Treatments by watering regimes per week [day (s)]	Chlorophyll pigments			
	Chlorophyll *a* µgg^{-1} FW	Chlorophyll *b* µgg^{-1} FW	Chlorophyll *a* + *b* µgg^{-1} FW	Carotenoids µgg^{-1} FW
Watering regime 1				
Control	814.47 ± 118.71^{a}	395.95 ± 48.70^{c}	1210.42 ± 167.09^{ab}	331.32 ± 63.50^{ab}
VCL 1:20 v/v	832.45 ± 97.43^{a}	389.25 ± 42.06^{c}	1221.70 ± 139.49^{ab}	289.45 ± 33.86^{b}
KEL 0.6%	895.11 ± 138.10^{a}	566.04 ± 68.66^{b}	1461.15 ± 95.63^{ab}	381.66 ± 78.89^{ab}
SW 1:1000 v/v	989.09 ± 43.76^{a}	510.35 ± 34.17^{bc}	1499.43 ± 76.81^{ab}	356.14 ± 11.70^{ab}
Watering regime 2				
Control	922.07 ± 111.19^{a}	463.66 ± 35.99^{bc}	1385.73 ± 141.58^{ab}	381.34 ± 22.64^{a}
VCL 1:20 v/v	961.10 ± 5.86^{a}	510.77 ± 11.36^{bc}	1471.87 ± 9.60^{ab}	388.51 ± 11.27^{a}
KEL 0.6%	857.59 ± 123.50^{a}	466.72 ± 45.93^{bc}	1324.30 ± 168.94^{ab}	368.55 ± 12.01^{ab}
SW 1:1000 v/v	1046.33 ± 96.68^{a}	527.10 ± 29.92^{bc}	1573.43 ± 126.58^{a}	363.77 ± 15.20^{ab}
Watering regime 3				
Control	398.67 ± 37.06^{b}	690.73 ± 64.21^{a}	1089.40 ± 101.27^{b}	335.73 ± 17.98^{ab}
VCL 1:20 v/v	938.18 ± 14.36^{a}	471.86 ± 12.83^{bc}	1410.04 ± 27.05^{ab}	386.33 ± 4.83^{a}
KEL 0.6%	901.69 ± 106.23^{a}	520.65 ± 21.42^{bc}	1422.34 ± 118.29^{ab}	392.82 ± 3.39^{a}
SW 1:1000 v/v	846.24 ± 131.08^{a}	516.46 ± 52.00^{bc}	1362.70 ± 183.07^{ab}	349.22 ± 9.05^{ab}

Mean values ± standard error (n = 3) in a column with different letter(s) are significantly different ($P \leq 0.05$) based on DMRT. Fresh weight (FW). Watering regime 1, 2 and 3 represent 1 d, 2 d and 3 d a week, respectively.

5.3.5 Effects of plant biostimulants on total phenolics and flavonoids of Vigna unguiculata under different watering regimes after 3 months of growth in the greenhouse.

As depicted in **Table 5.1**, there were significantly greater phenolics detected in leaves of *V. unguiculata* than roots in the three watering regimes. Restricting watering regime to once a week inhibited an increase of leaf phenolics in biostimulant-treated plants and lowered those of the roots in plants of VCL and SW compared to the control (**Fig. 5.3A**). Increasing watering frequency to twice a week promoted leaf phenolics in all biostimulant-treated plants (**Fig. 5.3B**). VCL also stimulated an increase in root total phenolics. An irrigation shift from once to thrice a week only improved leaf and root phenolics of plants watered with KEL (**Fig. 5.3C**).

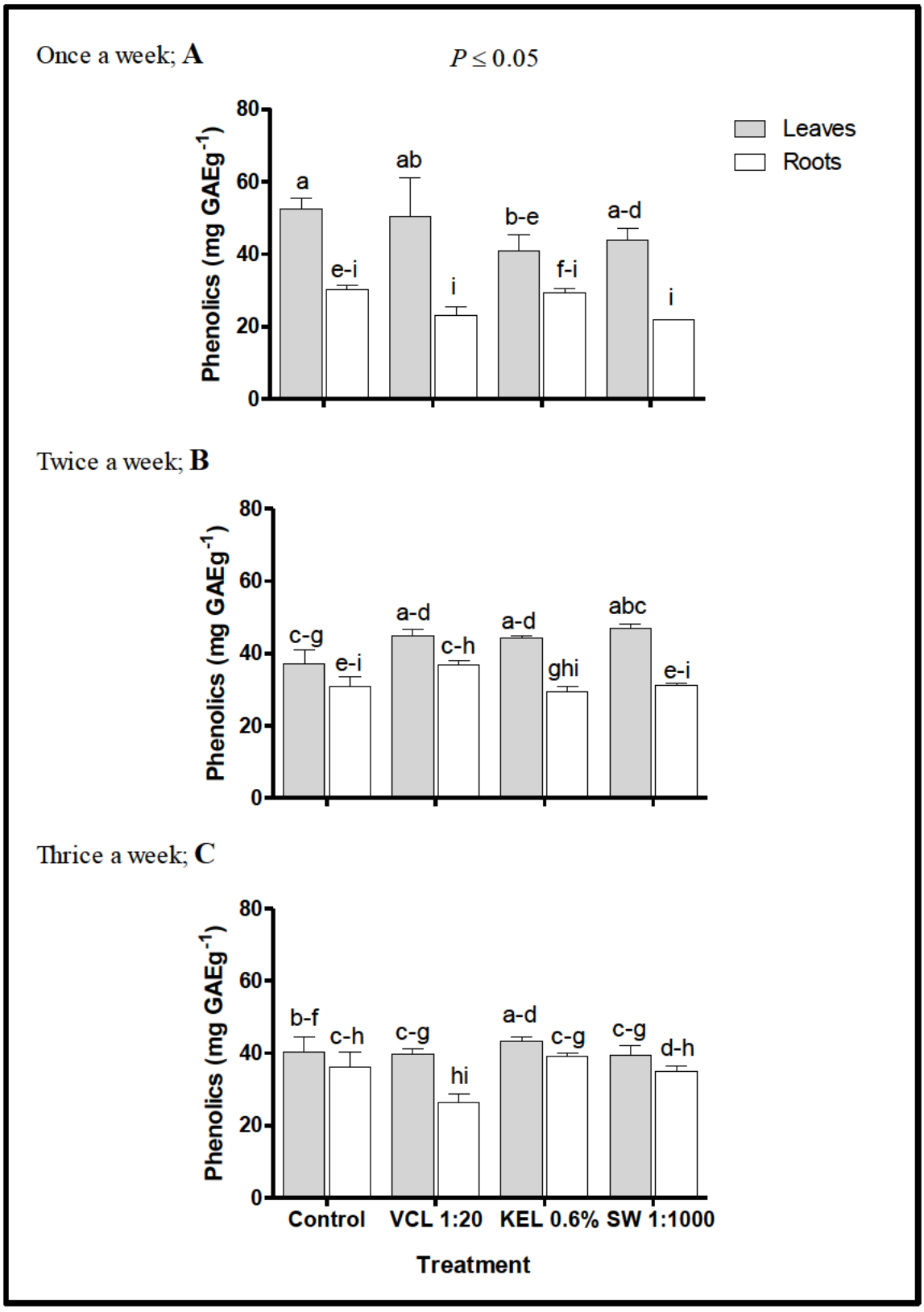

Fig. 5.3: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on leaf and root total phenolics of *Vigna unguiculata* after 3 months of growth in

the greenhouse. In each *Vigna unguiculata* graph, bars with different letter(s) represent mean values ± standard error (n = 3) and are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Once, twice and thrice a week represent watering regime 1, 2 and 3 in day(s), respectively. GAE represents gallic acid equivalents.

Leaves from plants watered once a week with biostimulants had lower flavonoid quantities compared to the control (**Fig. 5.4A**). SW significantly improved leaf flavonoids by a margin of 7.52 mg CEg^{-1} when watered 2 d a week (**Fig. 5.4B**). There were no marked biostimulant effects on leaf flavonoids in watering regime 3. Inversely, root flavonoids were significantly increased by application of these biostimulants once a week (**Fig. 5.4A**). This biostimulant capability was more remarkable in plants watered once and thrice a week wherein the VCL and SW significantly enhanced root flavonoids (**Fig. 5.4B** and **C**). Three-fold increase in flavonoids was detected in the roots of the plants treated twice a week with SW. Next to this increased margin relative to the control was that of VCL, SW and KEL in plants watered thrice a week (**Fig. 5.4C**).

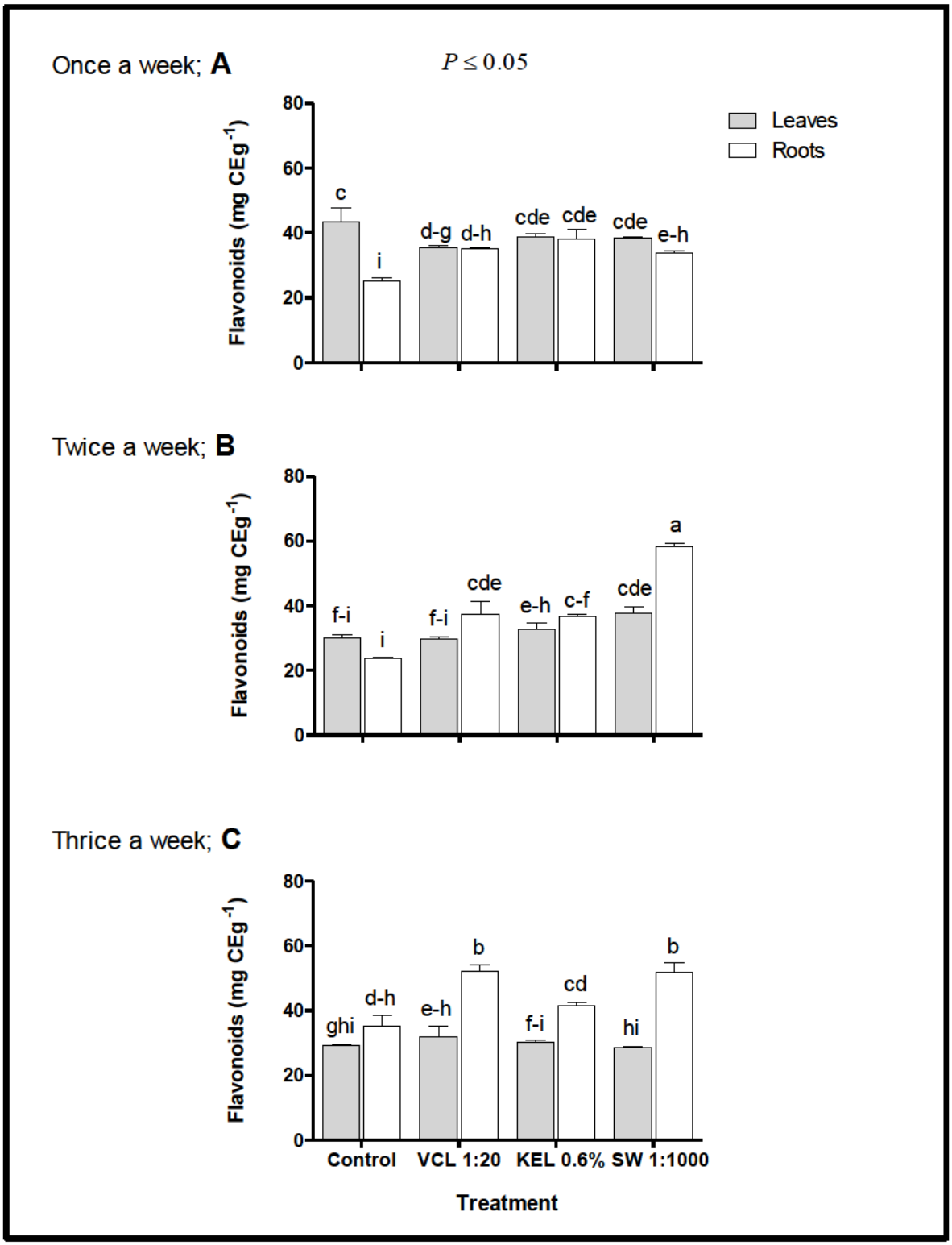

Fig. 5.4: Effects of different watering regimes of; tap water (control), vermicompost leachate (VCL 1:20 v/v), Kelpak® (KEL 0.6%) and smoke-water (SW 1:1000 v/v) per week on leaf and root flavonoids of *Vigna unguiculata* after 3 months of growth in the greenhouse. In each *Vigna unguiculata* graph, bars with different letter(s) represent

mean values ± standard error (n = 3) and are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Once, twice and thrice a week represent watering regime 1, 2 and 3 in day(s), respectively. CE represents catechin equivalent.

5.4 Discussion

5.4.1 Effects of plant biostimulants on concentration of total carbohydrates of Vigna unguiculata cultivated under different watering regimes in the greenhouse.

Plants synthesize and export carbohydrates to sinks as sucrose, sucrose-based molecules or polyols **(Lemoine, 2000)**. In sinks, sucrose is stored as starch or metabolised sucrose, an energy reserve molecule for growth and survival **(Lemoine, 2000)**. At earlier growth stages, roots and juvenile leaves are major sinks with higher demand for soluble sugars but this photosynthate demand shift to fruits, tubers and seeds at reproductive stages **(Wardlaw, 1990)**. However, due to drought disruptive influence which alter sugar metabolism and phloem loading mechanisms, leaf initiation and leaf expansion can diminish while roots continue to grow due to lesser water sensitivity and high sink demand of the root system **(Lemoine et al., 2013)**. Sucrose and hexose levels increase, whereas starch contents decline **(Pelleschi et al., 1997)**. These biochemical changes are now used as an indicator of induced sucrose synthesis and starch hydrolysis **(Lemoine et al., 2013)**. Restricting irrigation to once a week slightly improved concentration of leaf carbohydrates in biostimulant-treated plants and significantly raised those of roots of SW and control plants (**Fig. 5.1A**). In cotton (*Gossypium hirsutum*), accruing leaf sucrose and hexose is regarded as an energy supply insurance to ensure cell survival or adjustment for osmosis to maintain leaf metabolic activity under adverse conditions **(Burke, 2007)**. Raising watering frequency to twice a week, increased leaf carbohydrates of KEL and SW plants against control by 2-fold and decline by similar amount in plants watered thrice a week (**Fig. 5.1B and C**). Such transition also induced 2-fold increase in foliage carbohydrates of VCL plants, demonstrating promotory effects under water stress and favourable conditions. These results are in disagreement with the findings reported by **Ngoroyemoto et al. (2019; 2020)** and **Bidabadi et al., (2016)** wherein carbohydrate concentrations of *A. hybridus* and *Stevia rebaudiana*, respectively, under ideal conditions did not differ significantly following application of KEL, SW and VCL. This

could be due to the efficacy of biostimulants to optimize growth and alleviate stress depending on the type of plant species or cultivar **(Du Jardin, 2015)**. In seaweed extracts and VCL, this may further depend on the growth stage of the plants, application mode, method of extraction and plant/earthworm species used **(Radovich and Norman-Aranco, 2011; Battacharyya et al., 2015)**.

However, carbohydrates of the roots in the control and SW-treated plants were significantly greater under once a week, indicating high sensitivity to rhizosphere water deficits. Leaves and fruits of tomato seedlings were shown to have drought-predisposed symptoms via increased accumulation of compatible solutes such as proline, glucose, fructose and sucrose **(Nahar and Gretzmacher, 2002)**. Amongst other key functions of glucose and sucrose is osmotic adjustment under adverse conditions **(Parida et al., 2002)**. More interestingly, root carbohydrates of the plants watered with VCL and KEL under the same water deficits were within the ranges of the plants watered twice and thrice per week, respectively (**Fig. 5.1A, B** and **C**). Given the ability of sucrose to regulate the expression (using sugar-sensing pathways) of numerous genes involved in plant growth and to act as a stress signal molecule **(Koch, 2004; Muller et al., 2011)**, this provided a strong indication that VCL and KEL may have inhibited drought signaling activities by improving drought sensitivity of cowpea. Additional contributions from below-ground biometrics such as elongated lateral roots, longer taproots, increased root number and diameter may have also been integral in achieving this. These results conform with the findings of **Chinsamy et al. (2014)** wherein carbohydrates and proline content of VCL-treated tomato seedlings were increased with an increase in watering regimes but dropped significantly with water limitations.

The significantly higher root carbohydrates in SW-treated cowpeas indicates that SW may have been successful in increasing a priority system between the sinks of already water-stressed plants in favour of the roots system (**Fig. 5.1A**). Similar findings have been reported by **Chinsamy et al. (2013)** in tomato seedlings under salt stress (which is common in drought-proned areas) but using VCL. The authors point to the conversion of starch to sugars or reduced breakdown of stored starch by plant tissues as a possible explanation. While the findings of this study do not disagree with these suggestions, they add that the increased root total sugars in cowpea could have also been due to the increased root sink demand, not water stress, since the plants were

found to have significantly longer and many lateral roots to facilitate increased water extraction (results shown in **Chapter 4**, **section 4.4.2**).

5.4.2 Effects of plant biostimulants on concentration of proteins of Vigna unguiculata grown under different watering regimes in the greenhouse.

A plant's reaction to drought stress occurs when roots have trouble accessing available water or when the rate of transpiration exceeds root water supply **(Anjum et al., 2011)**. Roots may be the first sink organ to sense water deficits and respond by sending stress signals to the shoot to establish an immediate response **(Ilyas et al., 2020)**. As an addition to stomatal closure, increased phytohormone production (Abscisic acid, Auxins, Jasmonic acid, Ethylene, Salicylic acid etc.), cuticle thickening, root elongation, plant cells accumulate compatible solutes such as inorganic ions, sugars, polyols, soluble proteins, amino acids and alkaloids to establish osmotic adjustment **(Reddy et al., 2004; Barnabás et al., 2008; Vurukonda et al., 2016; Ilyas et al., 2020)**. In the present study, restricting water application to once a week induced a significant increase of 4-fold in leaf proteins of control plants compared to when applied thrice a week (**Fig. 5.2A** and **C**). Additionally, when the watering frequency was raised to twice a week, the protein content began to drop, suggesting the release of water stress under relative soil ambient conditions (**Fig. 5.2B**). High amino acid concentrations in drought-stressed chickpea could have been due to protein hydrolysis **(Ashraf and Iram, 2005)**. According to **Aranjuelo et al. (2011)**, drought-stressed plants are likely to partition significant amounts of C and N resources to promote leaf biosynthesis of osmoprotectants such as proline for osmotic adjustment and turgor maintenance. **Ghanbari et al. (2013)** reported high leaf N, seed proteins and proline content in drought-tolerant genotypes of Red beans and Chitti beans when exposed to water shortage. According to the whole-plant N budget, leaves are allocated more N for photosynthesis and protein synthesis and drought stress reduces leaf N levels which can diminish photosynthesis **(Nakayama et al., 2007)**. However, given that legumes are N self-sufficient plants and high proline accumulation in addition to the increased concentrations of amino acids keep leaf N levels in check under water deficiency **(Cardenas-Avila et al., 2006; Ghanbari et al., 2013)**. Thus, this can make it possible to increase photosynthesis and protein synthesis by increasing leaf N availability, depending on water stress level. Proline accumulation and N availability

are positively correlated **(Sánchez et al., 2007)**. Increased proline can be linked with the proliferation of other compatible solutes like proteins or amino acids providing a possibility that the significantly greater concentration of proteins in leaves of water-stressed control could have been coupled with increased proline contents and amino acids.

More interestingly, restricting watering frequency to once a week, induced more than 3-fold decline in leaf proteins of VCL and KEL-treated plants (**Fig. 5.2A**). The decline was 5-fold greater in plants drenched with SW, indicating high leaf protein hydrolysis and their translocation to sinks. This provided a strong suggestion that the three biostimulants may have been successfully protecting the plants by inhibiting water stress effects to a level whereby drought sensors and root:shoot signals were not triggered. Evidently, these biostimulant-induced protein declines in leaves fall within the range of those found in plants watered thrice a week (**Fig. 5.2C**). Similarly, the concentration of root proteins in biostimulant plants watered once were within the range of control plants watered twice and thrice a week. Protein contents of the leaves and root in SW-treated plants were least significantly affected by reducing water from 3 d (9.89 ± 4.50^{l}) to 2 d (19.67 ± 1.52^{kl}) and 1 d (26.22 ± 2.58^{jkl}). A close connection between protein synthesis and C metabolism is integral to avoid C starvation in developing tissues wherein protein synthesis contributes in the biosynthesis of new biomass **(Smith and Stitt 2007; Piques et al., 2009)**. **Bidabadi et al. (2016)** reported that VCL significantly strengthens this link between the two processes in *S. rebaudiana*. Therefore, the significantly low leaf proteins in biostimulant-treated cowpea under low and high watering regimes suggests that protein synthesis may have been harnessed for growth and biomass accumulation rather than synthesis of protein-based compatible solutes.

5.4.3 Effects of three plant biostimulants on chlorophyll pigments of Vigna unguiculata after 13 weeks of growth under different watering regimes in the greenhouse.

Low water potential (as a result of drought and salinity) compromises plant water and nutrient uptake. This can induce oxidation and osmotic stress and promote Na^{+} ion production which perturbs the cell ionic equilibrium; distressing enzyme functions, inhibit mitosis and growth and ultimately reducing the rate of photosynthesis **(Mahajan and Tuteja, 2005)**. Drought can also degrade photosynthetic capacity by

compromising the integrity of biometrics responsible for CO_2 absorbability either by reducing stomatal density, leaf expansion, promoting premature leaf senescence or modifying chlorophyll pigments, proteins or even oxidation of lipids in chloroplasts **(Menconi et al., 1995)**. Decreased chlorophyll molecules in drought-predisposed plants can now be used as an indicator of oxidative stress induced by chlorophyll degradation and pigment photooxidation **(Anjum et al., 2011)**. Chlorophyll *a* and *b* are highly susceptible to water deficits **(Farooq et al., 2009)**. Confronted with this evidence, **Cramer et al. (2011)** reported high chlorophyll pigments in more drought-tolerant transgenic maize. A metabolic flux study of *Arabidopsis* demonstrated that photosynthesis is less inhibited by water limitations than leaf expansion and an increased root:shoot vegetative partitioning is possible given a larger pool of available C for exportation to the root system **(Hummel et al., 2010)**. The results of the present study showed that the control plants grown under water deficits of once a week had significantly greater chlorophyll *a* content by a 2-fold relative to control plants watered thrice a week (**Table 5.2**). These results suggest a high dehydration-threshold in the tested *V. unguiculata* (local cultivar IT18) **(Clark, 2007; Sheahan, 2012; Cruz et al., 2014; Masenya, 2016)**.

The marked improvement in chlorophyll *a* under the highest water deficits (watering regime 1) owed to VCL, KEL and SW application suggests that not only can these biostimulants promote morphological parameters during water depletion but also photosynthesis. Also, the increased chlorophyll *a* as the result of exogenous application with the biostimulants was statistically the same regardless of changes in watering regimes and significantly above the range of control plants under the most favourable soil conditions (i.e. watering regime 3) (**Table 5.2**). Foliar application with KEL and VCL significantly enhanced chlorophyll *a*, *b* and *a* + *b* of *A. hybridus* under favourable edaphic conditions **(Ngoroyemoto et al., 2019)**. KEL replenished depleted chlorophyll pigments, total proteins and carbohydrates in *A. hybridus* inoculated with *Bacillus licheniformis* and *Pseudomonas fluorescens,* establishing evidence of an induced increase in photosynthetic activity, protein synthesis **(Ngoroyemoto et al., 2020)** and carboxylation of the leaves. Confronted with these findings and changes in chlorophyll *b*, and *a* + *b* in plants treated with SW and KEL under the lowest watering frequency, it can be deduced that these biostimulants have potential to allow cowpea to thrive under drought episodes by preventing oxidative stress caused by chlorophyll

degradation and pigment photooxidation, thus promoting the harvest of more light and CO_2. Seaweed extracts not only can improve chloroplast biogenesis, prevent chlorophyll degradation so to increase chlorophyll contents in response to abiotic stress but also delay senescence **(Battacharyya et al., 2015)**. **Jannin et al. (2013)** observed down-regulation of cysteine proteases linked with senescence and up-regulation of expressed genes associated with stress response, increased photosynthesis, N and cellular metabolism due to the application of *Ascophyllum nodosum*. Cytokinin properties detected in KEL and VCL **(Arthur et al., 2001; Stirk and van Staden, 2006)** protect chloroplasts, membranes and increase chloroplast division **(Battacharyya et al., 2015)**. In addition to the promotion of nodule development and cell division, cytokinins increase drought tolerance by contributing five mechanisms namely, protection provision to photosynthetic apparatus, establishing an increase in phytochemicals, and regulation of stress-related hormones, water equilibrium and plant growth **(Ilyas et al., 2020)**. VCL and SW also promoted a marked increase in chlorophyll *a* + *b* content in more favourable conditions of weekly application of 2 and 3 d. Such improvement was also established in carotenoids, especially in plants watered with SW, VCL and KEL in watering regimes 1, 2 and 3, respectively. These findings suggest that the three biostimulants can induce more promotory effects under more favourable conditions, but as water deficits intensify, some may lose their efficacy depending on stress severity and chlorophyll pigment.

5.4.4 Effects of three plant biostimulants on total phenolics and flavonoids of Vigna unguiculata under different watering regimes in the greenhouse.

Water availability is one of the most critical environmental factors that affects plant growth, development and food security. In this investigation, quantities of total phenolics and flavonoids in *V. unguiculata* were highly influenced by different watering regimes of VCL, KEL and SW. Phenolics are antioxidant molecules accrued in plant tissue as mostly tannins, flavonoids and lignin precursors to scavenge toxic ROS induced by drought stress **(Rice-Evans et al., 1997)**. In the present study, leaves of all treatments had markedly greater phenolics relative to roots (**Fig. 5.3**). Restricting watering to once a week significantly increased leaf phenolics in control plants compared to KEL-treated plants (**Fig. 5.3A**). The increased phenolics under water

deficiencies are ascribed to stress-induced disturbances in various metabolic processes of the cells **(Keutgen and Pawelzik, 2009)**. These cellular disturbances in numerous metabolic processes may have been down-regulated to negligible concentrations in KEL-treated plants using independent antioxidant pathways. Abscisic acid (ABA) regulates various morpho-physiological and biochemical processes to acclimatize and adapt to drought, temperature stress, cold stress, salinity, and osmotic stress **(Ilyas et al., 2020)**. However, the capacity of biostimulants to alleviate stress during ABA biosynthesis is via ABA independent metabolic pathway **(García et al., 2014)**. Therefore, the capability of KEL to curb ABA biosynthesis can be associated with its exogenous drenching, which induces numerous active plant growth regulators in plants **(Stirk et al., 2014; Kocira et al., 2020)**. Another markedly decline in phenolic concentrations under once a week application was induced by SW both in leaves and roots. The phenolics measured did not change much despite immense changes made in watering regimes (**Fig. 5.3B** and **C**). This could have been due to inhibitory properties of a SW compound called 3,4,5-trimethylfuran-2(5*H*)-one or trimethylbutenolide (TMB) or other plant growth regulators in SW such as karrikinolide (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one) that have been reported by **Kulkarni et al. (2008, 2011)** as stress alleviators. The leaf phenolics of VCL-treated plants increased with a decrease in watering regimes.

Flavonoids as secondary metabolites that accrue in plant tissue are essential for ensuring plant development and response to biotic and abiotic stresses **(Battacharyya et al., 2015)**. In the present study, leaf flavonoids of plants drenched with biostimulants were considerably decreased under the lowest watering regime (1 d) compared to those detected in the control foliage (**Fig. 5.4A**). Furthermore, leaf flavonoids of VCL-treated plants were significantly lower relative to the control. This provided a suggestion that the leaves of biostimulant-treated plants were less prone to high water deficits. However, their roots had significantly greater flavonoids, indicating that the growth of the above-ground biomass may have been protected by accruing more flavonoids in the root system. Flavonoids alleviate stress by acting on enzymes and metabolic pathways **(Araújo et al., 2008)**. Enzymatic activity of chalcone isomerase increased following seaweed extract application **(Battacharyya et al., 2015)**. Chalcone isomerase, as a key enzyme, catalyses the biosynthesis of flavanone precursors and phenylpropanoid protective compounds **(Sun et al., 2019)**. Similarly,

VCL and SW induced significantly greater root flavonoids compared to leaves and roots of the corresponding controls in the two more water favourable regimes (**Fig. 5.4B** and **C**). This may have been established to ensure protection, root growth and nodule survival for efficient N_2-fixation. As antioxidant molecules, phenolic compounds, in particular, flavonoids act as cell protectant and can induce apoptosis to ensure a plants survival **(Ndhlala et al., 2010)**.

5.5 Conclusions

The findings of the present study show a general increase in average root carbohydrates and proteins as water application was restricted and vice versa. Similarly, proteins of the leaves followed the similar trend while their carbohydrates decreased with water limitations. This demonstrated a high sink demand of photosynthates by *V. unguiculata* root system in order to ensure growth under water deficiencies. Exogenous application of VCL, KEL and SW alleviate water stress in cowpea by either promoting accumulation of compactible solutes and phytochemicals or inhibit their production to within or below the range of the plants found in more water favourable conditions. These biostimulants can protect above-ground biomass in legumes by promoting carbohydrate synthesis and inhibition of leaf phenolics and flavonoids. Water stress in below-ground biomass may be regulated by partitioning of more proteins and root flavonoids. SW can improve weakened translocation of carbohydrates to sinks (e.g. roots) by almost 2-fold in leguminous crops under water stress conditions. Legumes treated with VCL, KEL and SW can flourish even under drought episodes due to increased photosynthesis which positively influence the synthesis of carbohydrates and proteins including translocation to yield-related sinks. Despite this existing potential of VCL, KEL and SW to promote growth in *V. unguiculata* and other legumes under both water stress and normal conditions, there is still a need for more studies that will focus on the type and properties of growth regulators responsible for such promotory effects.

Chapter 6: General conclusions

Although *V. unguiculata* can thrive in harsh ecological niches, its growth remains susceptible to temperature stress and drought stress **(Gómez, 2004**; **Masenya, 2016)**. This compromises optimum growth, productivity and reduces yield. Yield reductions in cowpea as a staple crop for under-developed communities around the world escalate the challenge of meeting global food productivity needs. This also limits access to a versatile and nutritious crop with 23-32% crude protein **(Cruz et al., 2014)**, 50-65% carbohydrates and 1-2% fat **(FAO, 2012; Kirse and Karklina, 2015)** which can promote malnutrition. In an attempt to establish better understanding of how biostimulants can optimize *V. unguiculata* growth and yield under climate change, this study was undertaken to investigate effects of VCL, KEL and SW on seed germination, seedling/plant growth, flowering and morpho-physiological responses of *V. unguiculata* under heat and water stress. Given a predicted rise of 2-4 °C in global temperature **(Kiprotich et al., 2015; Vadez et al., 2012)**, temperature stress is one of the common abiotic stresses with unprecedented threats to plant growth, food security and livelihoods. As an indispensable technique, seed-priming with VCL, KEL and SW exhibited an array of growth benefits on *V. unguiculata* cultivated under optimum (30 °C), maximum temperature (35 °C) and heat stress of 40 °C. Biostimulant-priming optimizes germination and growth under temperature stress via inducing early emergence, longer roots, taller seedlings and greater leaf stimulation (**Chapter 3**). This contributes to larger stems, greater fresh biomass, heavier dry weights, less mortality and faster growth. Non-priming with the three biostimulants is also an integral component of exogenous application of biostimulants to plants under temperature stress as it can significantly elicites shoot height, leaf number and root proliferation (**Chapter 3**). Furthermore, these biostimulants can improve the aforementionned growth variables under normal temperatures. These findings mean that VCL, KEL and SW can optimize germination and growth of plants as either priming or non-priming agents even under heat stress conditions.

Nevertheless, the promotory effects of the three biostimulants at germination phase are not enough to make more precise inferences regarding yield. Thus, four-and thirteen-week studies were needed to ascertain biostimulant post-germination effects on cowpea plants exposed to heat (**Chapter 3**) and water stress (**Chapters 4** and **5**).

The above-ground biomass showed that the three biostimulants were most potent in promoting shoot length, number of leaves and peduncle diameter for 4 weeks at 30 °C (**Chapter 3**). KEL and SW also promoted these biometrics at 40 °C while VCL was most effective for only 2 weeks before declining markedly. This contributed to a significant decrease in seedling area, fresh and dry weights of VCL-plants. After 13 weeks of growth under high water deficits, the three biostimulants improved shoot and root length to within /or above the range of control plants found in more water favourable conditions (**Chapter 4**). Vermicompost leachate increased shoot growth and the number of nodules by more than 3-fold. Furthermore, the biostimulants improved relative leaf growth by more than 2 leaves and delayed leaf abortion and senescence. SW promoted early flowering, anthesis and elicited flower number that falls within the expected range in water-stressed cowpeas. VCL and KEL induced more flower stimulation in plants watered twice and thrice a week. These findings aggree with the consensus that VCL, KEL and SW not only improve plant growth post-germination and yield under temperature stress, but also in water-stressed plants.

Although the findings of this investigation reveal the ability of VCL, KEL and SW to act on growth and yield variables, the vast majority of their effects on biochemicals, photosynthesis and phytochemicals remain elusive, especially under abiotic stresses. The biostimulants increased photosynthesis in water-stressed plants by improving chlorophyll *a, b*, *a* + *b* and carotenoids to within the range of the plants cultivated in more water favourable substrates (**Chapter 5**). The increased photosynthesis was associated with an improved leaf carbohydrate in biostimulant plants and high translocation capacity in SW-treated plants. Minimal root carbohydrates of water-stressed plants drenched with VCL and KEL implied poor carbohydrate (polyols) translocation to major sinks (e.g. growing flowers, nodules or roots), increased root system starch hydrolysis, high sink demand or lack/disruption of transporting agents which may have reduced yield by inhibiting flower stimulation. This can be corrected by efficient irrigation or coinciding flowering stage with rainfall season. As water deficits intensify due to climate change, the three biostimulants can enhance leaf protein hydrolysis by 3-fold and improve proteins' transportation to plant tissues where they ensure biomass growth. With water deficiencies in the rhizosphere, the biostimulants buffered the plants and inhibited the accumulation of foliage phenolics and flavonoids while promoting an upsurge in flavonoids of the root system (**Chapter 5**). Both

increasing and decreasing trends of soluble sugars, proteins, photosynthetic pigments, phenolics and flavonoids owed to biostimulants' exogenous application indicate stress alleviation and osmotic adjustment to water deficits.

Despite the undertaken investigation on the effects of VCL, KEL and SW on *V. unguiculata*'s seed germination, seedling growth, post-germination growth, flowering, phytochemicals and biochemicals exposed to heat stress and water stress many questions still remain unanswered. If adoption of these biostimulants is to be accelerated in commercial agriculture during the era of climate change and global warming, it is imperative to answer questions such as: Which are the specific growth regulators responsible for biostimulation?; What is their complex interplay on photosynthesis, protein synthesis, phytochemistry, stress signalings and gene expression?; How do they alleviate various stresses, and what are their properties on nodulation, specific enzymes, plant growth promoting fungi/bacteria? Precise answers to these questions will provide a better understanding regarding the associated mechanisms and pathways, improving our understanding of plant metabolism. Therefore, undertaking studies that address such questions can give more physiological insights into how biostimulant-treated *V. unguiculata* may be able to optimize growth and flourish under heat and water stress.

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Appendix A (Chapter 3)

Table 3.5: Effects of priming and non-priming with distilled water (control) or biostimulants on leaf number, shoot length, root length and root number of *Vigna unguiculata* cultivated under different temperature regimes 7 days after sowing (DAS).

Temperature (°C)	Leaf Number (No.)		Shoot length (mm)		Root length (mm)		Root (No.)	
	Non-primed	Primed	Non-primed	Primed	Non-primed	Primed	Non-primed	Primed
30	2.00 ± 0.00^{c}	2.00 ± 0.00^{c}	81.45 ± 4.23^{d}	124.05 ± 3.93^{c}	108.15 ± 6.70^{c}	144.75 ± 6.69^{a}	7.31 ± 1.15^{a}	5.31 ± 0.95^{a}
35	2.23 ± 0.79^{bc}	2.41 ± 1.04^{b}	113.14 ± 3.79^{c}	166.60 ± 4.84^{a}	84.90 ± 4.09^{d}	125.36 ± 4.63^{b}	1.74 ± 0.36^{b}	2.34 ± 0.54^{b}
40	2.23 ± 0.09^{bc}	3.16 ± 0.16^{a}	78.63 ± 3.76^{d}	148.28 ± 3.69^{b}	76.29 ± 4.11^{d}	103.74 ± 3.19^{c}	7.74 ± 1.32^{a}	1.77 ± 0.36^{b}

Mean values ± standard error (leaf number, n = 20; shoot length, n = 20; root length, n = 20; and root number, n = 7) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Table 3.6: Effects of priming and non-priming with distilled water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) on seedling growth of *Vigna unguiculata* after incubation at 30, 35 and 40 °C for 7 days (d).

Treatment	Leaf (No.)	Root (No.)	Shoot length (mm)	Peduncle diameter (mm)	Root length (mm)	Fresh weight (g)	Dry weight (g)	Seedling area (cm^2)
Non-primed								
30 °C								
Control	2.0 ± 0.00^{e}	4.3 ± 0.70^{d-g}	48.6 ± 3.70^{l}	0.32 ± 0.01^{cd}	62.2 ± 7.10^{k}	0.77 ± 0.05^{j}	0.09 ± 0.01^{e}	14. 69 ± 1.66^{f}
VCL 1:20	2.0 ± 0.00^{e}	10.3 ± 3.20^{ab}	115.4 ± 4.40^{f-i}	0.34 ± 0.01^{a-d}	158.6 ± 9.00^{ab}	1.21 ± 0.09^{f-i}	0.11 ± 0.01^{de}	32.65 ± 2.28^{a}
KEL 0.6%	2.0 ± 0.00^{e}	8.5 ± 2.80^{a-e}	63.6 ± 8.00^{l}	0.34 ± 0.01^{bcd}	86.7 ± 8.20^{h-k}	0.98 ± 0.07^{ij}	0.11 ± 0.01^{de}	14.06 ± 2.50^{f}
SW 1:1000	2.0 ± 0.00^{e}	6.2 ± 1.30^{b-f}	98.3 ± 7.20^{ijk}	0.35 ± 0.01^{a-d}	125.7 ± 12.40^{c-f}	1.70 ± 0.08^{ab}	0.16 ± 0.01^{ab}	26.17 ± 1.64^{abc}
35 °C								
Control	2.3 ± 0.20^{de}	1.6 ± 0.60^{fg}	136.2 ± 6.00^{def}	0.33 ± 0.01^{bcd}	92.0 ± 9.20^{g-k}	1.04 ± 0.05^{hi}	0.10 ± 0.01^{e}	24.58 ± 1.84^{bcd}
VCL 1:20	2.3 ± 0.20^{de}	0.0 ± 0.00^{g}	102.3 ± 7.00^{h-k}	0.34 ± 0.01^{a-d}	92.1 ± 8.20^{g-k}	1.17 ± 0.07^{ghi}	0.11 ± 0.01^{de}	22.08 ± 2.34^{cde}
KEL 0.6%	2.2 ± 0.20^{de}	1.7 ± 0.60^{fg}	124.7 ± 6.20^{fgh}	0.34 ± 0.01^{a-d}	83.8 ± 5.20^{ijk}	1.38 ± 0.07^{c-g}	0.11 ± 0.01^{de}	30.11 ± 1.92^{ab}
SW 1:1000	2.2 ± 0.20^{de}	3.7 ± 0.80^{efg}	89.5 ± 6.80^{k}	0.33 ± 0.01^{bcd}	71.7 ± 9.30^{jk}	1.30 ± 0.06^{d-h}	0.12 ± 0.01^{cde}	16.33 ± 1.34^{ef}
40 °C								
Control	2.0 ± 0.00^{e}	3.2 ± 0.90^{fg}	53.8 ± 5.60^{l}	0.32 ± 0.01^{d}	69.7 ± 4.50^{k}	1.04 ± 0.06^{hi}	0.12 ± 0.10^{cde}	13.57 ± 1.20^{f}
VCL 1:20	2.2 ± 0.20^{de}	9.8 ± 2.40^{abc}	84.1 ± 6.70^{k}	0.33 ± 0.01^{bcd}	68.1 ± 5.80^{k}	1.08 ± 0.08^{hi}	0.12 ± 0.01^{cde}	19.27 ± 2.58^{def}
KEL 0.6%	2.0 ± 0.00^{e}	5.1 ± 0.70^{c-g}	91.1 ± 4.50^{jk}	0.35 ± 0.01^{a-d}	76.2 ± 7.00^{jk}	1.25 ± 0.08^{e-i}	0.12 ± 0.02^{cde}	22.10 ± 1.75^{cde}
SW 1:1000	2.8 ± 0.30^{cd}	13.0 ± 3.80^{a}	85.6 ± 9.70^{k}	0.35 ± 0.01^{a-d}	91.2 ± 12.70^{g-k}	1.28 ± 0.09^{d-h}	0.13 ± 0.01^{b-e}	23.98 ± 2.37^{bcd}
Primed								
30 °C								
Control	2.0 ± 0.00^{e}	4.5 ± 0.80^{d-g}	127.5 ± 7.60^{efg}	0.34 ± 0.01^{bcd}	110.1 ± 8.80^{e-i}	1.50 ± 0.10^{b-e}	0.12 ± 0.01^{b-e}	27.38 ± 2.59^{abc}
VCL 1:20	2.0 ± 0.00^{e}	2.9 ± 0.60^{fg}	136.9 ± 6.30^{c-f}	0.35 ± 0.01^{a-d}	170.7 ± 12.10^{a}	1.66 ± 0.06^{bc}	0.17 ± 0.01^{a}	28.75 ± 2.07^{abc}
KEL 0.6%	2.0 ± 0.00^{e}	9.4 ± 3.10^{a-d}	119.4 ± 7.40^{f-i}	0.35 ± 0.01^{a-d}	151.7 ± 11.70^{abc}	1.70 ± 0.08^{ab}	0.17 ± 0.01^{a}	23.44 ± 2.22^{bcd}
SW 1:1000	2.0 ± 0.00^{e}	4.5 ± 1.10^{d-g}	112.5 ± 9.30^{g-j}	0.36 ± 0.02^{abc}	142.1 ± 16.70^{bcd}	1.45 ± 0.12^{b-f}	0.16 ± 0.01^{a}	27.63 ± 2.43^{abc}
35 °C								

Control	2.3 ± 0.21^{de}	0.5 ± 0.20^{g}	164.6 ± 8.10^{b}	0.32 ± 0.02^{d}	115.2 ± 8.70^{d-h}	1.54 ± 0.11^{bcd}	0.13 ± 0.01^{b-e}	28.27 ± 2.22^{abc}
VCL 1:20	2.2 ± 0.20^{de}	1.0 ± 0.30^{fg}	158.4 ± 13.20^{bcd}	0.36 ± 0.01^{ab}	129.6 ± 9.50^{c-f}	1.47 ± 0.08^{b-f}	0.13 ± 0.01^{b-e}	26.85 ± 2.13^{abc}
KEL 0.6%	2.8 ± 0.30^{cd}	4.9 ± 1.50^{c-g}	189.3 ± 7.40^{a}	0.38 ± 0.02^{a}	125.4 ± 8.40^{c-f}	1.93 ± 0.08^{a}	0.16 ± 0.01^{ab}	28.87 ± 1.70^{abc}
SW 1:1000	2.5 ± 0.30^{de}	2.9 ± 0.90^{fg}	154.2 ± 7.40^{bcd}	0.36 ± 0.01^{abc}	131.4 ± 10.50^{b-e}	1.66 ± 0.09^{b}	0.15 ± 0.01^{abc}	30.74 ± 1.88^{ab}
40 °C								
Control	2.3 ± 0.21^{de}	1.5 ± 0.50^{fg}	125.4 ± 5.80^{fg}	0.34 ± 0.01^{bcd}	89.4 ± 6.20^{g-k}	1.43 ± 0.09^{b-g}	0.12 ± 0.01^{cde}	27.00 ± 2.01^{abc}
VCL 1:20	3.2 ± 0.30^{bc}	0.0 ± 0.00^{g}	148.5 ± 8.70^{b-e}	0.35 ± 0.01^{a-d}	117.2 ± 7.80^{d-g}	1.65 ± 0.10^{bc}	0.13 ± 0.01^{b-e}	26.77 ± 2.68^{abc}
KEL 0.6%	3.4 ± 0.30^{ab}	2.9 ± 0.90^{fg}	159.3 ± 6.30^{bc}	0.35 ± 0.02^{a-d}	107.7 ± 5.80^{e-i}	1.67 ± 1.00^{b}	0.14 ± 0.01^{a-d}	29.30 ± 2.35^{abc}
SW 1:1000	3.8 ± 0.30^{a}	2.7 ± 0.60^{fg}	160.0 ± 6.10^{b}	0.34 ± 0.01^{a-d}	100.8 ± 3.60^{f-j}	1.64 ± 0.12^{bc}	0.13 ± 0.01^{b-e}	26.57 ± 2.78^{abc}

Mean values ± standard error (n = 20) in each column per temperature regime with different letter(s) are significantly different ($P \leq$ 0.05) based on Duncan's Multiple Range Test.

Table 3.7: Post-germination effects of priming with distilled water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) concentrations on growth progression of *Vigna unguiculata* shoot length/plant height under 30, 35 and 40 °C 28 DAS.

Treatment	Temperature (°C)	1st Week	2nd Week	3rd Week	4th Week
Control	30	86.7 ± 13.20^{g}	111.5 ± 14.20^{fg}	121.0 ± 15.30^{efg}	131.5 ±14.90^{c-g}
	35	146.7 ± 18.20^{a}	160.0 ± 19.30^{a}	170.0 ± 22.80^{a}	177.5 ± 22.00^{a}
	40	140.0 ± 10.00^{d}	170.0 ± 13.50^{bcd}	189.3 ± 16.90^{abc}	196.5 ± 16.70^{abc}
VCL 1:20	30	95.8 ± 8.30^{g}	148.3 ± 17.40^{b-f}	168.3 ± 14.90^{a-e}	178.8 ± 16.90^{a-d}
	35	150.0 ± 12.90^{a}	158.5 ± 13.90^{a}	166.0 ±14.30^{a}	173.2 ± 15.00^{a}
	40	171.0 ± 10.90^{cd}	189.0 ± 12.40^{abc}	194.3 ± 12.50^{abc}	201.2 ± 13.40^{ab}
KEL 0.6%	30	128.3 ± 6.0^{d-g}	152.5 ± 7.3^{b-f}	173.8 ± 7.3^{a-d}	186.7 ± 15.80^{ab}
	35	146.7 ± 11.9^{a}	159.8 ± 10.8^{a}	172.5 ± 13.7^{a}	165.0 ± 22.80^{a}
	40	157.5 ± 10.5^{cd}	179.0 ± 10.6^{a-d}	191.7 ± 12.3^{abc}	203.8 ± 14.90^{ab}
SW 1:1000	30	135.8 ± 16.6^{b-g}	166.5 ± 20.3^{a-e}	182.0 ± 21.3^{abc}	206.2± 29.90^{a}
	35	168.3± 13.0^{a}	179.3 ± 12.2^{a}	187.2 ± 12.4^{a}	192.3 ± 12.00^{a}
	40	156.2 ± 13.0^{cd}	186.7 ± 10.7^{abc}	202.3 ± 11.8^{ab}	215.2± 11.5^{a}

Mean values ± standard error (n = 6) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Table 3.8: Post-germination effects of priming with distilled water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) concentrations on growth progression of *Vigna unguiculata* leaf number under 30, 35 and 40 °C 28 DAS.

Treatment	Temperature (°C)	1st Week	2nd Week	3rd Week	4th Week
Control	30	2.0 ± 0.00^{f}	3.2 ± 0.6^{f}	4.8 ± 0.40^{e}	5.5 ± 0.60^{de}
	35	2.5 ± 0.50^{e}	4.5 ± 0.3^{d}	6.3 ± 0.60^{c}	7.3 ± 0.50^{ab}
	40	2.5 ± 0.50^{d}	5.5 ± 0.5^{abc}	6.5 ± 0.80^{ab}	5.8 ± 0.80^{abc}
VCL 1:20	30	2.0 ± 0.00^{f}	5.8 ± 0.5^{de}	6.7 ± 1.00^{bcd}	8.2 ± 0.90^{ab}
	35	2.0 ± 0.00^{e}	5.0 ± 0.0^{d}	5.0 ± 0.00^{d}	5.0 ± 0.00^{d}
	40	4.0 ± 0.60^{cd}	6.2 ± 1.0^{bc}	5.0 ± 0.00^{abc}	4.5 ± 0.80^{bc}
KEL 0.6%	30	2.0 ± 0.00^{f}	5.0 ± 0.0^{de}	6.2 ± 0.60^{cde}	7.7 ± 0.30^{abc}
	35	2.0 ± 0.00^{e}	5.0 ± 0.0^{d}	5.0 ± 0.00^{d}	8.0 ± 0.00^{a}
	40	2.5 ± 0.50^{d}	5.0 ± 0.0^{abc}	6.5 ± 0.60^{ab}	6.0 ± 0.70^{abc}
SW 1:1000	30	2.5 ± 0.50^{f}	5.5 ± 0.5^{de}	7.7 ± 0.80^{abc}	8.7 ± 0.70^{a}
	35	2.5 ± 0.20^{e}	5.0 ± 0.0^{d}	5.3 ± 0.30^{d}	6.5 ± 0.40^{bc}
	40	2.5 ± 0.50^{d}	6.0 ± 0.6^{abc}	7.0 ± 0.50^{a}	6.5 ± 0.50^{ab}

Mean values ± standard error (n = 6) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Table 3.9: Post-germination effects of priming by pre-soaking with distilled water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) concentrations on *Vigna unguiculata* stem thichness/peduncle diameter, fresh weight, dry weight and seedling area at 30, 35 and 40 °C 28 DAS.

Treatment	Stem thickness (mm)	Fresh weight (g)	Dry weight (g)	Seedling Area (cm^2)
30 °C				
Control	0.31 ± 0.03^{b}	1.49 ± 0.23^{b}	0.11 ± 0.01^{cd}	29.94 ± 5.89^{bc}
VCL 1:20	0.39 ± 0.01^{a}	2.29 ± 0.20^{a}	0.15 ± 0.01^{abc}	43.91 ± 6.54^{b}
KEL 0.6%	0.36 ± 0.03^{ab}	2.39 ± 0.13^{a}	0.17 ± 0.01^{ab}	58.77 ± 4.08^{a}
SW 1:1000	0.36 ± 0.01^{ab}	2.54 ± 0.12^{a}	0.19 ± 0.02^{a}	56.94 ± 4.24^{a}
35 °C				
Control	0.32 ± 0.01^{b}	1.69 ± 0.15^{b}	0.12 ± 0.01^{cd}	22.88 ± 1.41^{cd}
VCL 1:20	0.33 ± 0.01^{b}	1.31 ± 0.13^{b}	0.10 ± 0.01^{d}	24.71 ± 2.56^{cd}
KEL 0.6%	0.33 ± 0.01^{b}	1.53 ± 0.06^{b}	0.12 ± 0.01^{cd}	34.90 ± 3.76^{bc}
SW 1:1000	0.32 ± 0.02^{b}	1.72 ± 0.11^{b}	0.12 ± 0.01^{cd}	33.14 ± 2.11^{bc}
40 °C				
Control	0.31 ± 0.01^{b}	1.56 ± 0.07^{b}	0.12 ± 0.01^{cd}	31.28 ± 3.84^{bc}
VCL 1:20	0.34 ± 0.03^{ab}	0.80 ± 0.10^{c}	0.06 ± 0.01^{e}	15.41 ± 2.26^{d}
KEL 0.6%	0.35 ± 0.02^{ab}	1.73 ± 0.25^{b}	0.14 ± 0.02^{bcd}	31.46 ± 6.92^{bc}
SW 1:1000	0.35 ± 0.02^{ab}	1.61 ± 0.20^{b}	0.14 ± 0.02^{bcd}	28.71 ± 6.05^{cd}

Mean values ± standard error (n = 6) in each column per temperature regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Appendix B (Chapter 4)

Table 4.3: Effects of different watering frequencies per week on leaf number, root length, flower number and nodule number of *Vigna unguiculata* after 13 weeks of growth in the greenhouse. In each *Vigna unguiculata* graph parameter, bars with different letter(s) represent mean values ± standard error (n = 10) and are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Watering regimes	Leaf (No.)	Nodule (No.)	Shoot Length (mm)	Root length (mm)	Leaf Area (cm^2)	Flower (No.)
Once a week (1 d)	15.40 ± 0.68^{b}	46.93 ± 7.90^{a}	887.25 ± 70.59^{b}	324.50 ± 16.28^{b}	155.52 ± 7.12^{c}	1.55 ± 0.28^{c}
Twice a week (2 d)	23.20 ± 1.13^{a}	33.00 ± 5.23^{a}	1270.50 ± 59.93^{a}	363.50 ± 15.50^{ab}	201.91 ± 12.16^{b}	4.18 ± 0.72^{b}
Thrice a week (3 d)	24.58 ± 0.79^{a}	35.33 ± 2.33^{a}	1262.00 ± 74.38^{a}	382.90 ± 12.74^{a}	238.69 ± 6.17^{a}	8.54 ± 1.28^{a}

Mean values ± standard error (n = 40) in each column per watering regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Table 4.4: Effects of tap water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) concentrations on growth parameters of primed *Vigna unguiculata* seeds at different watering regimes after 13 weeks in the greenhouse.

Watering regimes								
Treatment	Leaf (No.)	Nodule (No.)	Shoot length (mm)	Root length (mm)	Stem diameter (mm)	Fresh weight (g)	Dry weight (g)	Leaf area (cm^2)
Once a week								
Control	13.9 ± 1.0^{d}	23.6 ± 3.0^{bc}	338.0 ± 13.7^{e}	277.0 ± 35.5^{c}	4.64 ± 0.15^{d}	14.86 ± 1.44^{e}	2.66 ± 0.27^{efg}	137.03 ± 21.24^{e}
VCL 1:20	15.1 ± 0.9^{d}	91.8 ± 25.1^{a}	1 083.0 ± 74.8^{cd}	265.0 ± 25.1^{c}	4.57 ± 0.18^{d}	12.67 ± 0.94^{e}	2.09 ± 0.19^{fg}	148.63 ± 9.71^{de}
KEL 0.6%	17.6 ± 2.0^{cd}	22.0 ± 2.8^{bc}	1 276.0 ± 115.5^{bc}	376.0 ± 32.3^{ab}	4.57 ± 0.16^{d}	13.80 ± 1.09^{e}	1.89 ± 0.16^{g}	152.11 ± 15.61^{de}
SW 1:1000	15.0 ± 1.2^{d}	50.3 ± 8.6^{bc}	852.0 ± 112.0^{d}	369.0 ± 23.9^{ab}	5.10 ± 0.14^{cd}	16.55 ± 0.91^{de}	2.78 ± 0.27^{ef}	184.31 ± 6.36^{cd}
Twice a week								
Control	20.9 ± 0.8^{bc}	32.6 ± 5.7^{bc}	1 180.0 ± 79.4^{bcd}	307.0 ± 33.9^{bc}	4.90 ± 0.33^{d}	17.76 ± 1.42^{de}	3.39 ± 0.24^{cde}	154.02 ± 12.01^{de}
VCL 1:20	20.7 ± 1.8^{bc}	54.9 ± 18.8^{b}	1 094.0 ± 139.9^{cd}	392.0 ± 25.2^{ab}	4.99 ± 0.77^{cd}	22.72 ± 2.64^{bc}	3.16 ± 0.26^{de}	195.37 ± 4.08^{bc}
KEL 0.6%	25.4 ± 1.9^{ab}	24.7 ± 2.8^{bc}	1 301.0 ± 111.7^{bc}	353.0 ± 36.2^{abc}	5.65 ± 0.47^{ab}	23.99 ± 1.88^{bc}	4.13 ± 0.26^{bc}	222.84 ± 4.52^{abc}
SW 1:1000	25.8 ± 2.8^{ab}	19.8 ± 1.5^{c}	1 504.0 ± 116.2^{bc}	402.0 ± 20.9^{a}	4.91 ± 0.12^{d}	20.85 ± 2.15^{cd}	3.61 ± 0.32^{bcd}	235.42 ± 33.52^{ab}
Thrice a week								
Control	21.4 ± 1.6^{bc}	34.8 ± 4.5^{bc}	1 120.0 ± 137.5^{cd}	359.0 ± 15.3^{abc}	5.45 ± 0.18^{bc}	24.23 ± 1.94^{bc}	3.81 ± 0.22^{bcd}	225.24 ± 2.79^{abc}
VCL 1:20	23.3 ± 1.5^{b}	38.2 ± 4.2^{bc}	1 134.0 ± 161.7^{cd}	399.5 ± 28.7^{a}	5.98 ± 0.11^{a}	30.30 ± 1.51^{a}	5.50 ± 0.36^{a}	261.78 ± 7.42^{a}
KEL 0.6%	28.8 ± 1.2^{a}	35.4 ± 6.7^{bc}	1 690.0 ± 98.0^{a}	424.6 ± 28.2^{a}	5.88 ± 0.18^{ab}	26.50 ± 1.23^{ab}	4.33 ± 0.19^{b}	220.87 ± 9.85^{abc}
SW 1:1000	24.8 ± 1.2^{ab}	32.9 ± 3.2^{bc}	1 104.0 ± 118.5^{cd}	348.5 ± 23.2^{abc}	5.72 ± 0.16^{ab}	24.92 ± 0.85^{bc}	3.95 ± 0.37^{bcd}	246.87 ± 11.38^{a}

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

Table 4.5: Effects of different watering regimes of, tap water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) per week on leaf number of *Vigna unguiculata* after 3 months of growth in the greenhouse.

Treatment	Watering regimes per week [day(s)]	3rd Week	5th Week	7th Week	9th Week
Control	Once a week	5.0 ± 0.00^{f}	7.8 ± 0.20^{e}	9.4 ± 0.40^{d}	10.8 ± 0.60^{c}
	Twice a week	5.0 ± 0.00^{g}	8.0 ± 0.00^{f}	12.8 ± 0.50^{de}	14.9 ± 0.60^{bc}
	Thrice a week	5.0 ± 0.00^{g}	8.5 ± 0.30^{f}	11.5 ± 0.50^{de}	14.9 ± 0.7^{c}
VCL 1:20	Once a week	5.0 ± 0.00^{f}	8.3 ± 0.10^{de}	11.9 ± 0.60^{bc}	14.6 ± 0.60^{a}
	Twice a week	5.0 ± 0.00^{g}	8.0 ± 0.00^{f}	12.3 ± 0.50^{de}	15.5 ± 0.70^{b}
	Thrice a week	5.0 ± 0.00^{g}	8.7 ± 0.30^{f}	12.6 ± 0.40^{d}	16.4 ± 0.70^{b}
KEL 0.6%	Once a week	5.0 ± 0.00^{f}	8.1 ± 0.10^{de}	12.7 ± 0.60^{b}	15.7 ± 0.70^{a}
	Twice a week	5.0 ± 0.00^{g}	11.5 ± 0.20^{e}	14.2 ± 0.50^{bc}	19.1 ± 0.80^{a}
	Thrice a week	5.0 ± 0.00^{g}	10.7 ± 0.40^{e}	14.7 ± 0.30^{c}	18.9 ± 0.60^{a}
SW 1:1000	Once a week	5.0 ± 0.00^{f}	8.9 ± 0.30^{de}	12.3 ± 0.40^{b}	11.6 ± 0.70^{bc}
	Twice a week	5.0 ± 0.00^{g}	8.6 ± 0.30^{f}	13.3 ± 0.60^{cde}	19.0 ± 1.90^{a}
	Thrice a week	5.0 ± 0.00^{g}	9.1 ± 0.40^{f}	14.0 ± 0.00^{c}	17.5 ± 0.90^{b}

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

Table 4.6: Effects of different watering regimes of tape water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) on *Vigna unguiculata* number flowers during 11th, 12th and 13th week of growth in the greenhouse.

Treatment	Watering regimes per week [day(s)]	11th Week	12th Week	13th Week
Control	Once a week	0.00 ± 0.00^{h}	0.72 ± 0.32^{gh}	1.80 ± 0.80^{gh}
	Twice a week	0.36 ± 0.16^{h}	1.08 ± 0.48^{gh}	1.08 ± 0.48^{gh}
	Thrice a week	0.72 ± 0.32^{gh}	1.40 ± 0.25^{gh}	3.52 ± 0.77^{ef}
VCL 1:20	Once a week	0.00 ± 0.00^{h}	0.36 ± 0.16^{h}	1.12 ± 0.23^{gh}
	Twice a week	0.00 ± 0.00^{h}	1.68 ± 0.37^{gh}	4.22 ± 0.96^{de}
	Thrice a week	1.40 ± 0.25^{gh}	6.48 ± 0.71^{c}	13.60 ± 0.75^{a}
KEL 0.6%	Once a week	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	1.12 ± 0.23^{gh}
	Twice a week	0.36 ± 0.16^{h}	5.60 ± 0.75^{cd}	8.20 ± 1.02^{b}
	Thrice a week	1.60 ± 0.40^{gh}	5.00 ± 0.45^{cde}	8.00 ± 1.00^{b}
SW 1:1000	Once a week	0.00 ± 0.00^{h}	0.93 ± 0.27^{gh}	2.16 ± 0.74^{fg}
	Twice a week	0.36 ± 0.16^{h}	2.16 ± 0.50^{fg}	2.40 ± 0.68^{fg}
	Thrice a week	1.28 ± 0.29^{gh}	4.80 ± 0.58^{de}	$8.00 \pm .71^{b}$

Mean values ± standard error (n = 5) in each column per watering regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test.

Appendix C (Chapter 5)

Table 5.3: Effects of watering regimes of tap water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) per week on leaf and root total carbohydrates, total proteins, total phenolics and flavonoids of *Vigna unguiculata* after 3 months of growth in the greenhouse.

Treatment by watering regime	Total carbohydrates (µgg^{-1} FW)		Total proteins (µgg^{-1} FW)		Total phenolics (mg GAEg^{-1})		Flavonoids (mg GAEg^{-1})	
	leaves	roots	leaves	roots	Leaves	Root	Leaves	Roots
Watering regime 1								
Control	74.50 ± 14.24^{d}	145.04 ± 2.09^{b}	131.84 ± 15.67^{a}	84.21 ± 2.08^{c-f}	52.38 ± 3.12^{a}	30.27 ± 1.13^{e-i}	43.33 ± 5.41^{c}	25.15 ± 1.09^{i}
VCL 1:20 v/v	81.07 ± 14.16^{d}	82.24 ± 11.90^{d}	42.16 ± 4.09^{h-k}	99.77 ± 4.62^{bcd}	50.53 ± 10.65^{ab}	23.07 ± 2.27^{i}	35.60 ± 0.55^{d-g}	35.18 ± 0.36^{d-h}
KEL 0.6%	100.86 ± 2.92^{cd}	37.89 ± 0.78^{ef}	39.66 ± 5.00^{h-k}	97.85 ± 1.64^{b-e}	40.78 ± 4.52^{b-e}	29.45 ± 1.04^{f-i}	38.74 ± 1.11^{cde}	38.02 ± 2.97^{cde}
SW 1:1000 v/v	79.18 ± 16.96^{d}	221.51 ± 24.86^{a}	26.22 ± 2.58^{jkl}	85.56 ± 1.89^{b-e}	43.80 ± 3.53^{a-d}	21.69 ± 0.07^{i}	38.53 ± 0.21^{cde}	33.72 ± 0.75^{e-h}
Watering regime 2								
Control	68.12 ± 3.12^{de}	98.43 ± 4.80^{cd}	107.64 ± 9.98^{abc}	109.37 ± 9.16^{abc}	37.21 ± 3.81^{c-g}	30.76 ± 2.71^{e-i}	30.17 ± 0.97^{f-i}	23.67 ± 0.55^{i}
VCL 1:20 v/v	76.03 ± 10.84^{d}	89.17 ± 1.86^{d}	47.15 ± 8.51^{g-j}	58.48 ± 3.07^{e-h}	44.83 ± 1.92^{a-d}	36.73 ± 1.27^{c-h}	29.75 ± 0.55^{f-i}	37.37 ± 4.13^{cde}
KEL 0.6%	129.50 ± 12.28^{bc}	156.01 ± 2.09^{b}	47.53 ± 8.11^{g-j}	80.37 ± 12.67^{def}	44.14 ± 0.60^{a-d}	29.31 ± 1.58^{ghi}	32.88 ± 1.79^{e-h}	36.65 ± 0.75^{c-f}
SW 1:1000 v/v	147.56 ± 13.98^{b}	91.51 ± 8.42^{d}	19.67 ± 1.52 ^{kl}	101.50 ± 6.00^{bcd}	46.82 ± 1.33^{abc}	31.17 ± 0.68^{e-i}	37.69 ± 2.17^{cde}	58.38 ± 0.96^{a}
Watering regime 3								
Control	73.15 ± 5.89^{d}	98.43 ± 20.13^{cd}	34.09 ± 6.97^{i-l}	112.06 ± 5.16^{ab}	40.30 ± 4.11^{b-f}	36.25 ± 3.89^{c-h}	29.12 ± 0.55^{ghi}	35.18 ± 3.45^{d-h}
VCL 1:20 v/v	134.87 ± 14.96^{b}	20.70 ± 3.25^{f}	62.70 ± 15.23^{e-h}	61.94 ± 5.30^{e-h}	39.68 ± 1.61^{c-g}	26.43 ± 2.34^{hi}	32.05 ± 3.09^{e-h}	52.11 ± 2.02^{b}
KEL 0.6%	66.50 ± 4.88^{de}	23.58 ± 3.98^{f}	19.30 ± 9.87^{kl}	111.29 ± 16.31^{ab}	43.18 ± 1.38^{a-d}	39.13 ± 0.86^{c-g}	30.17 ± 0.72^{f-i}	41.45 ± 1.09^{cd}
SW 1:1000 v/v	73.69 ± 2.12^{d}	32.94 ± 5.79^{f}	9.89 ± 4.50^{l}	69.23 ± 9.95^{efg}	39.41 ± 2.61^{c-g}	34.87 ± 1.69^{d-h}	28.50 ± 0.55^{hi}	51.69 ± 3.08^{b}

Mean values ± standard error (n = 3) in each column per watering regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test. FW represent fresh weight.

Table 5.4: Effects of tap water (control); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%); and smoke-water, SW 1:1000 (v/v) concentrations on chlorophyll pigments of *Vigna unguiculata* at different watering regimes after 3 months of growth in the greenhouse.

Treatment by watering regimes per week [day (s)]	Chlorophyll pigments			
	Chlorophyll *a* µgg^{-1} FW	Chlorophyll *b* µgg^{-1} FW	Chlorophyll *a* + *b* µgg^{-1} FW	Carotenoids µgg^{-1} FW
Watering regime 1				
Control	814.47 ± 118.71^{a}	395.95 ± 48.70^{c}	1210.42 ± 167.09^{ab}	331.32 ± 63.50^{ab}
VCL 1:20 v/v	832.45 ± 97.43^{a}	389.25 ± 42.06^{c}	1221.70 ± 139.49^{ab}	289.45 ± 33.86^{b}
KEL 0.6%	895.11 ± 138.10^{a}	566.04 ± 68.66^{b}	1461.15 ± 95.63^{ab}	381.66 ± 78.89^{ab}
SW 1:1000 v/v	989.09 ± 43.76^{a}	510.35 ± 34.17^{bc}	1499.43 ± 76.81^{ab}	356.14 ± 11.70^{ab}
Watering regime 2				
Control	922.07 ± 111.19^{a}	463.66 ± 35.99^{bc}	1385.73 ± 141.58^{ab}	381.34 ± 22.64^{a}
VCL 1:20 v/v	961.10 ± 5.86^{a}	510.77 ± 11.36^{bc}	1471.87 ± 9.60^{ab}	388.51 ± 11.27^{a}
KEL 0.6%	857.59 ± 123.50^{a}	466.72 ± 45.93^{bc}	1324.30 ± 168.94^{ab}	368.55 ± 12.01^{ab}
SW 1:1000 v/v	1046.33 ± 96.68^{a}	527.10 ± 29.92^{bc}	1573.43 ± 126.58^{a}	363.77 ± 15.20^{ab}
Watering regime 3				
Control	398.67 ± 37.06^{b}	690.73 ± 64.21^{a}	1089.40 ± 101.27^{b}	335.73 ± 17.98^{ab}
VCL 1:20 v/v	938.18 ± 14.36^{a}	471.86 ± 12.83^{bc}	1410.04 ± 27.05^{ab}	386.33 ± 4.83^{a}
KEL 0.6%	901.69 ± 106.23^{a}	520.65 ± 21.42^{bc}	1422.34 ± 118.29^{ab}	392.82 ± 3.39^{a}
SW 1:1000 v/v	846.24 ± 131.08^{a}	516.46 ± 52.00^{bc}	1362.70 ± 183.07^{ab}	349.22 ± 9.05^{ab}

Mean values ± standard error (n = 3) in each column per watering regime with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test. FW represent fresh weight.