Vermicompost Leachate, Seaweed Extract and Smoke-Water Alleviate Drought Stress in Cowpea By Influencing Phytochemicals, Compatible Solutes and Photosynthetic Pigments

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Research Article

Abstract

Drought is a major constraint for agricultural production worldwide and is likely to become aggravated by global warming. It can induce land degradation, exorbitant food prices and menace livelihoods. Approaches for retaining optimal yield, especially in rainfed staple crops such as cowpea (*Vigna unguiculata*) are paramount. Biostimulants; vermicompost leachate (VCL), seaweed extract [Kelpak® (KEL)] and smoke-water (SW) have exhibited effective amelioration for plants under abiotic stresses, however, research on cowpea remains scarce. Therefore, this study aimed to investigate the effects of seed priming of cowpea with VCL, KEL and SW on the growth, photosynthesis and biochemical levels in cowpea cultivated under three watering regimes. SW treatment amplified growth variables (i.e., foliage, shoot height, root length and number of flowers) of water-stressed cowpeas. KEL- and VCL-treated seeds significantly augmented shoot and nodules production by 2 and 4-fold respectively, compared to the control. Leaf carbohydrates and photosynthetic pigments in KEL- and SW-treated plants increased considerably under severe water deficits, while leaf proteins decreased by more than 3-fold. The biostimulants also lowered phenolic and flavonoid concentrations. Increasing and decreasing levels of soluble sugars, proteins, photosynthetic pigments, phenolics and flavonoids indicate stress alleviation and osmotic adjustment to water deficits. These biostimulants are a suitable alternative to improve soil fertility, growth, and yield of staple crops under water stress conditions.

Introduction

Abiotic stresses such as elevated temperature, drought stress, salinity and nutrient deficiency have adverse effects on plant growth. These environmental factors, particularly drought (Ahmad et al. 2015) and temperature stress, significantly hamper plant growth and development with deleterious effects on crop performance and production (Prasad et al. 2008; Shao et al. 2009), thus compromising plant survival and ultimately yield (Masondo 2017, **2018**). Stresses induce various physiological, biochemical and molecular changes and responses that influence different cellular processes (Prasad et al. 2008). Drought affects the transpiration rate, stomatal conductance and relative water status of the leaf (Ullah et al. 2017). 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 the major means of livelihood. From an agricultural perspective, drought is defined as a physiological and edaphic condition that occurs when accessible water to crops is inadequate to meet their transpiration demands (Tuberosa 2012) and evaporation needs (**Ilyas et al. 2020**). Also, at the farm level, over-irrigation is uneconomical and unsustainable as it wastes water, energy, is labour intensive and risks are water-logging and nutrient leaching, resulting in a low crop yield (Ahmad et al. 2015).

Drought affects photosynthesis 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) quantity, reduced Rubisco activity, damage to ATP synthesis, impaired photophosphorylation or reduced inorganic

P (Parry et al. 2002; Bota et al. 2004). 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 declines, which promotes photorespiration and reduces photosynthesis (Lea and Leegood 1999). Similar to drought, temperature stress can elicit a decrease in activation as well as the activity of Rubisco (Prasad et al. 2004). Furthermore, it triggers modifications in the functions of the cell membrane by changing membrane fluidity (Barnabás et al. 2008). Temperature and drought stress cause discernible influences on biosynthesis of soluble proteins, amino acids and carbohydrates. These stresses are prominent inducers of reactive oxygen species (ROS) accumulation (Zobayed et al. 2005) in the mitochondria, microsomes, chloroplast, cytosol and peroxisomes (Allahmoradi et al. 2013). ROS accumulation is a menace to plants and can cause lipid peroxidation, electron leakage, impaired proteins, membranes and enzymatic activities which reduce photosynthesis and stomatal closure (Sadak et al. 2020; Maksup et al. 2014).

To curb water stress, plants employ defense mechanisms such as stomatal closure, gene expression, molecular signaling pathways like changes in chlorophyll biosynthesis, osmolyte accumulation and antioxidant systems (Barnabás et al. 2008; Luo 2010; Varshney et al. 2014). These changes are regulated by specific receptors, osmosensors and transcription factors that sense alteration in cell membranes, water status, turgor and endogenous phytohormones (Barnabás et al. 2008). As a result, activated superoxide dismutase, peroxidase and catalase as well as non-enzymatic antioxidants such as reduced glutathione, ascorbic acid, carotenoids and α-tocopherols scavenge ROS (Anjum et al. 2016; Sadak et al. 2017). Increased abscisic acid (ABA), ethylene (Eth), jasmonic acid (JA), gibberellins (GA), auxins (Aux), cytokinins (CK) and salicylic acid (SA) regulate different signaling pathways including the promotion of heat shock proteins, secondary metabolites and enzymatic antioxidants (Ullah et al. 2018; **Ilyas et al. 2021**). For example, accumulating concentrations of the stress-related phytohormone−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 improves the efflux of K^+ ions in the guard cells, which causes cells to lose turgor pressure which leads 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. 2021**).

Plant biostimulants such as VCL, KEL and SW have demonstrated growth promotory effects in various plant species even under abiotic stresses (Kulkarni et al. 2011; Battacharyya et al. 2015; Islam et al. 2016; Bidabadi et al. 2017; Kocira et al. 2018). These biostimulants improve plant growth by positively influencing photosynthetic pigments, carbohydrates, proteins (Nemahunguni et al. 2019; Ngoroyemoto et al. 2019; 2020; Gupta et al. 2021), phytohormones and phytochemistry (Aremu et al. 2014; Masondo 2017, **2018;** Gupta et al. 2019a). However, research also indicates that their efficacy depends on the mode of application, plant species, growth stage and stress severity amongst other factors. The importance of cowpea in agriculture and food security cannot be overlooked if hunger and malnutrition are to be

combated under climate change conditions. Therefore, the aim of this study was to investigate the effects of VCL, KEL and SW on growth, biochemicals, photosynthetic pigments and phytochemistry of biostimulant-primed cowpea cultivated under different watering regimes using greenhouse protocols.

Materials And Methods

Source of seeds

Seeds of *V. unguiculata* (local cultivar IT18) were purchased from McDonald's Seeds, Pietermaritzburg, South Africa.

Source of vermicompost leachate (VCL)

VCL was purchased from Wizzard Worms Commercial Company Ltd., Greytown, South Africa. According to the company, 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. Phytohormones and phytochemicals of the tested VCL were quantified by Aremu et al. (2015). VCL concentration in this study was tested by diluting crude VCL with distilled water (dH_2O) in the ratio 1:20 [1 mL VCL: 20 mL dH_2O (v/v)].

Source of commercial seaweed extract Kelpak® (KEL)

KEL was manufactured by Kelpak® Products (Pty) Ltd., Simon's Town, South Africa and sourced from Starke Ayres (Pty) Ltd., South Africa. KEL comprised of 2,2 and 0,0062 mg/l auxins and cytokinins, respectively. Its concentration was prepared as 0.6% [0.6 mL KEL: 99.4 mL (dH_2O) v/v].

Sources of smoke-water (SW)

SW was prepared following the method of Gupta et al. (2019b). Twenty-five millilitres of crude SW were first mixed with 75 mL dH_2O to make a sub-dilution in the ratio of 1 SW: 3 dH_2O (v/v) before being further diluted to 1 mL SW: 1000 mL dH_2O (v/v).

Greenhouse experimental trials

The experiments were conducted in the Botanical Garden of the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The area has the following global coordinates: 29⬚37'S 30⬚23'E (World Atlas 2015). The greenhouse conditions were 50-60% relative humidity, day and night temperatures of 27/15°C and photosynthetic photon flux density of 450 ± 5 µmol $m^{-2}s^{-1}$. Seeds were first rinsed with dH_2O and primed by pre-soaking in VCL 1:20 (v/v), KEL (0.6%), SW 1:1000 (v/v) and dH_2O for 1 h followed by 45-min air drying on a laminar flow bench. Eight seeds were then sown in a 3 cm furrow in 20 cm plastic pots with sandy-loam soil. Thereafter, pots were placed in four rows on steel benches with 45 cm inter-row and 30 cm intra-row spaces in a completely randomized design. Sixteen days after sowing (DAS), seedlings were thinned to 4 seedlings per pot and biostimulants were applied immediately after thinning.

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

The pot substrate was moistened with 400 mL of tap water twice a week for a period of 2 weeks. Each treatment had 4 replicates per watering regime of once (1), twice (2) and thrice (3) a week. Two-hundred millilitres of treatment was applied by drenching every Monday, Wednesday and Friday morning for 13 weeks, depending on the watering regime. Bimonthly (i.e. every second week) biostimulant application was alternated with tap water application for all pots. The number of leaves was recorded every Monday morning of the second week until the termination of the experiment. To control *Pythium*, 450 mL Previcur® fungicide was applied per pot. Insects were controlled by spraying the greenhouse walls, benches and pots with Malasol® (Efekto). The fungicide was applied once every 28 DAS and fumigation was done 2 days (d) prior to the commencement of the experiments and 40 DAS using Dithane® M-45.

Measurement of morphological parameters

Morphological data were collected from 10 replicates per treatment after 13 weeks. The number of flowers was counted every Monday for 3 weeks. Plant/shoot height was measured from the root collar to the apical bud using an Air Liquide ruler. 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. Nodules were then counted before recording root length and fresh biomass weight. Leaf area of trifoliate leaves was measured using a Li-3100 Area meter (LI-COR, Inc. Lincoln, Nebraska, USA) and dry weight was recorded after desiccating plants in an oven at 55 ± 2°C for 5 d.

Biochemical Assays

Quantification of total carbohydrates

Total carbohydrate content was estimated by the method of 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 hydrolysed extract was neutralized by adding granules of sodium carbonate until the effervescence ceased. Two-hundred millilitres of dH_2O was then added to the test tubes to top up the volume to 5 mL and centrifuged at 10 000 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 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.

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 for 15 min at 15 000 rpm at 4°C. 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 proteins. Absorbance was recorded at 595 nm against a control using a UV-visible spectrophotometer (Varian Cary 50, Australia).

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 ground by homogenizing 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 3 000 rpm 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

Phytochemical assays

Sample preparation

After 5 d of oven drying, plant biomass was sorted into leaves, stems and roots. 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% MeOH 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.

Quantification of total phenolic content

The total phenolic composition of the filtrate was determined in accordance with the Folin and Ciocalteu assay following Makkar (1999) with modifications. Fifty microlitres of the plant extract were dispensed

into 950 µl dH$_2$O, 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% 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.

Estimation of flavonoids

Flavonoid composition of the leaves and roots were estimated using the colorimetric aluminium chloride procedure as described in Zhishen et al. (1999) with modifications. Two-hundred microlitres of plant extract were diluted with 800 µL of dH$_2$O. 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$_2$O were sequentially added to the reaction mixture. The colour of the reaction mixture turned pink to depict the presence of flavonoids. The absorbance of the reaction mixture was immediately measured in triplicates using a spectrophotometer 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.

Data analysis

The morphological and physiological data of *V. unguiculata* were subjected to one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS®, Version 26.0, IBM®, Armonk, New York, USA). The level of significance was determined according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Thereafter, the data were transformed into graphic figures using GraphPad Prism®.

Results

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

Priming *V. unguiculata* seeds and subsequent drenching the substrate with VCL 1:20 (v/v), KEL (0.6%) and SW 1:1000 (v/v) 14 DAS improved most of the biometric parameters in the three watering regimes. KEL and SW significantly improved shoot and root length of plants watered once-a-week compared to the control (Fig. 1A and **B**). These parameters were biologically increased to within or above the ranges of control plants watered twice-and thrice-a-week. Moreover, SW induced a significant increase in leaf area (Fig. 1C) coupled with marked improvements in stem diameter, fresh weight, dry weight and number of flowers (Table 1). Stem diameter, leaf area and number of flowers in once-a-week watered plants were similarly increased by SW to within the ranges of control plants watered twice- and thrice-a-week. In

addition to significantly greater shoot length, restricting VCL application to once-a-week increased the number of nodules by 4-fold compared to the control (Fig. 1D). VCL and SW were the only treatments that induced a higher number of nodules at the lowest watering regime. Restricting watering frequency to once-a-week resulted in all three biostimulants optimizing the number of leaves after 13 weeks. KEL delayed the leaf abortion and promoted the highest leaf number by a difference of almost five leaves relative to the control without pronounced decline (Table 1). This leaf difference also remained ubiquitous in less water stressed plants under watering regimes 2 and 3 wherein KEL induced more foliage comparatively to the corresponding controls by more than 4 and 8 leaves, respectively.

Table 1
Effects of tap water (control, Con), vermicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on morphological parameters of hydroprimed and biostimulant-primed *Vigna unguiculata* seeds at different watering regimes after 13 weeks of growth in the greenhouse.

Treatment (mL)	Leaf (no)	Stem diameter (mm)	Fresh weight (g)	Dry weight (g)	Number of flowers
Once a week					
Con	13.90 ± 1.00 d	4.64 ± 0.15 d	14.86 ± 1.44 e	2.66 ± 0.27 efg	1.80 ± 0.80 e
VCL 1:20 (v/v)	15.10 ± 0.90 d	4.57 ± 0.18 d	12.67 ± 0.94 e	2.09 ± 0.19 fg	1.12 ± 0.23 e
KEL (0.6%)	17.60 ± 2.00 cd	4.57 ± 0.16 d	13.80 ± 1.09 e	1.89 ± 0.16 g	1.12 ± 0.23 e
SW 1:1000 (v/v)	15.00 ± 1.20 d	5.10 ± 0.14 cd	16.55 ± 0.91 de	2.78 ± 0.27 ef	2.16 ± 0.74 cde
Twice a week					
Con	20.90 ± 0.90 bc	4.90 ± 0.33 d	17.76 ± 1.42 de	3.39 ± 0.24 cde	1.08 ± 0.48 de
VCL 1:20 (v/v)	20.70 ± 1.80 bc	4.99 ± 0.77 cd	22.72 ± 2.64 bc	3.16 ± 0.26 de	4.22 ± 0.96 c
KEL (0.6%)	25.40 ± 1.90 ab	5.65 ± 0.47 ab	23.99 ± 1.88 bc	4.13 ± 0.26 bc	8.20 ± 1.02 b
SW 1:1000 (v/v)	25.80 ± 2.80 ab	4.91 ± 0.12 d	20.85 ± 2.15 cd	3.61 ± 0.32 bcd	2.40 ± 0.68 cde
Thrice a week					
Con	21.40 ± 1.60 bc	5.45 ± 0.18 bc	24.23 ± 1.94 bc	3.81 ± 0.22 bcd	3.52 ± 0.77 cd
VCL 1:20 (v/v)	23.30 ± 1.50 b	5.98 ± 0.11 a	30.30 ± 1.51 a	5.50 ± 0.36 a	13.60 ± 0.75 a
KEL (0.6%)	28.80 ± 1.20 a	5.88 ± 0.18 ab	26.50 ± 1.23 ab	4.33 ± 0.19 b	8.00 ± 1.00 b
SW 1:1000 (v/v)	24.80 ± 1.20 ab	5.72 ± 0.16 ab	24.92 ± 0.85 bc	3.95 ± 0.37 bcd	8.00 ± 0.71 b

Mean values ± standard error (leaf no, *n* = 10; stem diameter, *n* = 10; fresh weight, *n* = 10; dry weight, *n* = 10; and number of flowers, *n* = 5) in each column with different letter(s) are significantly different (*P* ≤ 0.05) according to DMRT.

Irrigation shift to 2 d a week increased more growth in biostimulant-treated plants, especially KEL- and SW-treated plants. These biostimulants markedly improved shoot length and number of leaves more than VCL and control. SW elicited a significant increase in root length and leaf area (Fig. 1B and **C**) while KEL

significantly increased stem diameter, fresh weight, dry weight and number of flowers compared to the corresponding control (Table 1). VCL not only improved root length and number of nodules but its plants also had significantly higher leaf area (Fig. 1B, C and **D**), fresh weight, dry weight and number of flowers compared to the control (Table 1).

Although raising the watering frequency to 3 d a week did not significantly increase below- ground biometrics in biostimulant plants, this transition positively impacted VCL- and KEL-treated plants (Fig. 1). VCL significantly enhanced stem diameter, fresh weight, dry weight and increased the number of flowers by approximately 4-fold as compared to the control (Table 1). Similarly, KEL and SW promoted thicker stems and more fresh and dry weights in plants watered 3 d a week. KEL- and SW-treated plants also had two times the number of flowers relative to the controls. Before taking the measurements, growth promotory effects of the three biostimulants were also evident on root biomass with ubiquitous adventitious root proliferation in plants watered once-, twice-, and thrice-a-week (Fig. 2).

Physiological effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on V. unguiculata biochemicals under different watering regimes in the greenhouse.

Carbohydrates and proteins in the leaves and roots of *V. unguiculata* were considerably influenced by different watering regimes after 13 weeks. Restricting watering frequency to once-a-week induced less leaf and more root carbohydrates in control and SW-treated plants (Fig. 3A). SW-plants significantly accrued root carbohydrates more than those of leaves and roots of any other plants in water-favourable regimes (Fig. 3A, B and **C**). This increase in root soluble sugars was 7-fold greater compared to SW-plants watered thrice-a-week and approximately 2-fold higher compared to the corresponding controls. While leaf and root soluble sugars of VCL plants remained relatively similar to the once-a-week regime, KEL markedly improved leaf soluble sugars and significantly inhibited those of the roots (Fig. 3A). Thus, root carbohydrates of VCL- and KEL-treated plants showed a minimal influence on water deficits. Raising the watering regime to twice-a-week elicited improvements in leaf carbohydrates of KEL- and SW-treated plants. Remarkably, this increase was followed by significant reductions when further raised to a watering frequency of thrice-a-week (Fig. 3C). A similar trend was established in the roots of plants treated with KEL. During this irrigation shift, the carbohydrates in roots of VCL- and SW-treated plants significantly declined at a faster rate by nearly 4-fold in VCL plants than the corresponding controls.

Restricting watering frequency to once-a-week induced less leaf and more root total protein accumulation in biostimulant-treated plants (Fig. 3D). The three biostimulants decreased leaf proteins by more than 3-fold compared to the control. Inversely, their exogenous drenching improved root proteins although they did not differ significantly from the control. Increasing watering frequency to twice-a-week resulted in still lower leaf and root proteins than those of the control marked with significant reductions in leaf proteins (Fig. 3E). The proteins in the roots of VCL- and KEL-treated plants were not only reduced but also significantly declined at this watering regime. Watering increased to thrice-a-week significantly enhanced leaf proteins of VCL plants by almost 2-fold compared to the control and declined in KEL- and SW-treated plants (Fig. 3F). No marked increase compared to the control was established in root proteins of

biostimulant-treated plants, despite the irrigation increase from twice- to thrice-a-week. However, there was a significant increase and decline in KEL and SW plants, respectively, (Fig. 3E and **F**).

Effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on V. unguiculata photosynthetic pigments under different watering regimes in the greenhouse.

Variation in watering regimes of biostimulants positively influenced chlorophyll pigments of *V. unguiculata* after 13 weeks. Chlorophyll *a* of plants watered once with KEL and SW was improved by 80.64 and 177.62 μgg^{-1}, respectively, against the control (Table 2). Raising watering frequency to twice-a-week resulted in an increase of 126.26 μgg^{-1} in chlorophyll *a* for SW-treated plants compared to the control plants. The three biostimulants significantly stimulated chlorophyll *a* production of plants watered thrice-a-week by more than 2-fold relative to the corresponding control. This increase was the result of a significant decline in chlorophyll *a* of control plants as watering frequency increased from 1-3 d (Table 2). SW was more effective in promoting chlorophyll *a* in plants watered once- and twice-a-week.

Table 2
Effects of tap water (control, Con); 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 13 weeks 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)
Once a week				
Con	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
Twice a week				
Con	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
Thrice a week				
Con	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 (Chlorophyll *a*, *n* = 3; chlorophyll *b*, *n* = 3; chlorophyll *a* + *b*, *n* = 3; and carotenoids, *n* = 3) in each column with different letter(s) are significantly different ($P \leq 0.05$) based on DMRT.

Restricting KEL and SW exogenous application to once-a-week promoted chlorophyll *b* by a margin of 170.09 and 114.40 µgg^{-1}, respectively, compared to the control (Table 2). Applying VCL and SW twice-a-week also markedly improved chlorophyll *b* although the increased margins did not differ significantly from the control. However, despite the improved chlorophyll *b* content by biostimulants in 1- and 2-d-watered plants, their capability to stimulate this pigment was significantly inhibited in plants watered thrice-a-week. Chlorophyll *a* + *b* was considerably improved by KEL and SW in plants watered once- and thrice-a-week. VCL and SW positively impacted chlorophyll *a* + *b* of the plants watered twice-a-week. The potency of the former biostimulant remained statistically similar post watering shift to thrice-a-week. Limiting watering frequency to once-a-week resulted in KEL plants having slightly greater carotenoid content against other treatments. The three biostimulants did not positively affect the carotenoids of the plants watered twice-a-week. A similar influence was established in plants watered thrice-a-week, except VCL- and KEL-treated plants.

Effects of vemicompost leachate (VCL), Kelpak® (KEL) and smoke-water (SW) on V. unguiculata total phenolics and flavonoids under different watering regimes in the greenhouse.

As depicted in Fig. 4A, B and **C**, there were generally greater total phenolics detected in the leaves of *V. unguiculata* than roots in the three watering regimes. Leaf total phenolics and flavonoids of control plants increased as irrigation was reduced from thrice- to once-a-week (Fig. 4). Inversely, such transition significantly inhibited leaf phenolics of KEL (40.78 ± 4.52 b-e) plants watered once-a-week against the control (52.38 ± 3.12 a). The transition also lowered leaf and root phenolics in SW-treated cowpeas. Nevertheless, increasing watering frequency to twice-a-week slightly promoted leaf phenolics in all biostimulant-treated plants instead of being lowered (Fig. 4B). VCL also stimulated an increase in root total phenolics. Further raising the watering regime to thrice-a-week induced a decrease in leaf phenolics of control (40.30 ± 4.1 b-f), VCL (39.68 ± 1.6 c-g) and SW (39.41 ± 2.6 c-g) plants compared to once-a-week control (52.38 ± 3.12 a). Moreover, increasing water application from once- to thrice-a-week triggered a general increase in root phenolics of all plants (Fig. 4A, B and **C**).

Leaf flavonoids were relatively higher than roots of the plants watered once-a-week until the irrigation shift to twice- and thrice-a-week which both markedly decreased leaf flavonoids. Conversely, root flavonoids increased as this transition was occurring. Limiting watering frequency to only once-a-week-lowered leaf flavonoids of biostimulant plants with a significant decrease in plants drenched with VCL compared to the control (Fig. 4D). SW significantly improved leaf flavonoid concentrations by a margin of 7.52 mg CEg^{-1} comparative to the control when watered 2 d a week (Fig. 4E). There were no marked biostimulant effects on leaf flavonoids of the plants watered thrice. Inversely, root flavonoids were significantly increased by VCL, KEL and SW application relative to the control under once-a-week regime (Fig. 4D). This biostimulant capability was even more remarkable in plants watered 2 and 3 d a week. In these plants, VCL and SW enhanced root flavonoids by almost 2-fold (Fig. 4E and **F**).

Discussion

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

Water limitations reduced 90% of the measured morphological parameters with reductions being more pronounced in control plants than biostimulant-treated plants. Reductions in the plant's biometric characters occur under water deficits due to water/nutrient deprivation or high transpiration rate (Anjum et al. 2011). Thus, a widespread root system is a requisite to improve water extraction from shallow soil horizons before being lost to evaporation (Ashraf and Foolad 2005; Chinsamy et al. 2013). The significantly longer root length and widespread adventitious roots elicited by KEL and SW (Figs. 1B and 2) resulted in improved nutrient uptake and water availability under drought (Georgiev 2004; **Ilyas et al. 2021**). This may have contributed to their augmented leaf growth and 3-fold increase in shoot length. Water and nutrient availability regulate shoot length increase. Among the required nutrients is N_2 that increases nodes and water increases the internodal distances (Maleki et al. 2013), nutrient hydrolysis and averts dehydration. Furthermore, an enzyme known as acid invertase is linked with plant growth (**Sturm 1999**). The increased invertase activities in biometric characters of primed chickpea (*Cicer arietinum* cv. GPF-2) elevated hexose supply which may have increased energy and growth of the above-ground biomass (Kaur et al. 2005).

Root-to-shoot signaling is a prominent event that causes stomatal closure under drought stress via ABA increase which prevents further water loss to the transpiration stream (Barnabás et al. 2008). However, stomatal closure prevents CO_2 fixation, decreasing leaf internal CO_2 and promotes pentadiulose-1,5-bisphosphate (Rubisco inhibitor) and photorespiration, eventually culminating to a reduction in photosynthesis rate (Parry et al. 1993; Schafleitner et al. 2013; Daryanto et al. 2015). Hence, the increased root system caused by biostimulants in Fig. 2 could mean that biostimulants capacitated the less watered plants with growth promotors to curtail root-to-shoot stress signal. This may have played a major role in the increasing of shoot length, number of leaves and leaf area (Fig. 1A and **C**; Table 1) to prioritize vegetative growth, light interception and CO_2 absorbability. KEL and VCL down-regulated the production of ABA and increased photosynthesis activity, growth and survival rate of *Ceratotheca triloba* (Masondo 2017). García et al. (2014) showed that the capacity of biostimulants to alleviate stress during ABA biosynthesis is via ABA independent metabolic pathways. Therefore, the capability of VCL and KEL to curb ABA biosynthesis could be similar to exogenous applications which induces active plant growth (Stirk et al. 2014; Aremu et al. 2015a, b; Kocira et al. 2020). Moreover, the present study indicates that limiting watering frequency to once-a-week improved the number of nodules in SW- and VCL-treated plants by almost 2- and 4-fold, respectively (Fig. 1D), translating into an enhanced N modulation.

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

Drought impairs yield before and post-anthesis (Nadeem et al. 2019). At flowering and anthesis, drought reduces yield by 27-57% in chickpea (*C. arietinum*), common bean (*Phaseolus vulgaris*) and mungbean (*Vigna radiata*) (Rosales-Serna et al. 2004; Mafakheri et al. 2010; Ahmad et al. 2015). The present study

showed that water deprivation can reduce flowering in cowpea by 69%, depending on biostimulant and irrigation regime. For instance, only flowers of SW-plants were improved considerably under once-a-week regime. Conversely, raising watering frequency to twice- and thrice-a-week increased flowers in all biostimulant plants by more than 2-fold. Biostimulants also induced more immediate anthesis in all watering regimes, demonstrating efficacy against inhibitory effects of water stress reported by Barnabás et al. (2008).

Physiological effects of biostimulants on V. unguiculata biochemicals under different watering regimes.

Disruptive influences of drought alter sugar metabolism, phloem loading mechanisms and reduces leaf growth while roots continue to grow due to less water sensitivity and high sink demand (Lemoine et al. 2013). Sucrose and hexose levels shift by increasing, whereas starch contents decline (Pelleschi et al. 1997). Such biochemical changes are used as an indicator of induced sucrose synthesis and starch hydrolysis (Lemoine et al. 2013). Restricting irrigation to once a week slightly improved leaf carbohydrates of biostimulant-treated plants and significantly raised carbohydrates of roots of SW and control plants (Fig. 3A). In cotton (*Gossypium hirsutum*), accrued sucrose and hexose act as an energy supply to safeguard cells or for adjustment for osmosis under adverse conditions (Burke 2007). Tripling water availability induced 2-fold increase in foliage carbohydrates of VCL plants, demonstrating its promotory effects under water stress and favourable conditions (Fig. 3C). These results disagree with the findings of Ngoroyemoto et al. (2019; 2020) and Bidabadi et al. (2016) where carbohydrates of *Amaranthus hybridus* and *Stevia rebaudiana* did not differ significantly following VCL application. This could be due to the efficacy of biostimulants depending on the type of biostimulant, plant species or cultivar (Du Jardin 2015). In seaweed extracts and VCL, this may further depend on the growth stage, application mode, extraction method and algae/earthworm species (Radovich and Norman-Aranco 2011; Battacharyya et al. 2015). Tomato (*Solanum lycopersicon*) exhibited susceptibility to drought via increased accumulation of proline, glucose, and sucrose (Nahar and Gretzmacher 2002). 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. This may be due to elevated root sink demand (Hummel et al. 2010), conversion of starch to sugars or reduced breakdown of stored starch by plant tissues (Chinsamy et al. 2013). 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. 3A, B and **C**). These results conform with the findings of Chinsamy et al. (2014) wherein carbohydrate content of VCL-treated tomato seedlings was increased with an increase in watering regimes but dropped significantly with water limitations.

As an addition to stomatal closure, increased cuticle thickening, plant cells accumulate 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. 2021). 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. 3D and **F**). High concentrations of amino acids in water-stressed chickpea could have been due to protein hydrolysis (Ashraf and Iram 2005). According to Aranjuelo et al. (2011), water-stressed

plants can 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, proteins and proline content in drought-tolerant genotypes of Red beans and Chitti beans under water deficits. Interestingly, restricting watering frequency to once-a-week induced more than a 3-fold decline in leaf proteins in VCL- and KEL-treated plants (Fig. 3D). The decline was 5-fold in plants drenched with SW, indicating even higher leaf protein hydrolysis and /or translocation to sinks. The induced protein declines fall within the range of those found in plants watered thrice-a-week (Fig. 3F). A link between protein synthesis and C metabolism is integral to avert C starvation in developing tissues where protein synthesis contributes to the biosynthesis of new biomass (Smith and Stitt 2007; Piques et al. 2009). VCL promoted this relationship between the two processes in *S. rebaudiana* (Bidabadi et al. 2016). Therefore, the significantly reduced leaf proteins in the least and highly watered biostimulant-plants could mean that their protein synthesis may have been harnessed for growth and biomass accumulation rather than synthesis of protein-based compatible solutes.

Physiological effects of biostimulants on V. unguiculata photosynthetic pigments under different watering regimes in the greenhouse.

Drought induces low water potential, disrupting plant and nutrient uptake which results in osmotic stress and promotion of Na^+ ions that perturbs the cell ionic equilibrium; enzymatic functions, mitosis and reduces the rate of photosynthesis (Mahajan and Tuteja 2005). It also modifies chlorophyll pigments, proteins or even oxidation of lipids in chloroplasts (Menconi et al. 1995). Chlorophyll *a* and *b* are highly susceptible to water deficits (Farooq et al. 2009; Ghanbari et al. 2013). Decreased chlorophyll biomolecules in water-stressed plants is an indicator of oxidative stress caused by chlorophyll degradation and pigment photooxidation (Anjum et al. 2011). Confronted with this evidence, Cramer et al. (2011) reported high chlorophyll pigments in drought-tolerant transgenic maize (*Zea mays*). In the present study control plants watered once-a-week had 2-fold greater chlorophyll *a* with chlorophyl *a + b* and carotenoids remaining similar to that of the control plants watered thrice-a-week. These findings suggest a remarkable dehydration-threshold in cowpea as reported by Clark (2007), Cruz et al. (2014) and Masenya (2016). Moreover, chlorophyll *a, a + b* and carotenoids increased immensely under once- and thrice-a-week owed to biostimulant applications which translates into greater improvement of photosynthesis (Table 2). KEL and VCL significantly enhanced chlorophyll *a*, *b* and *a + b* of *A. hybridus* (Ngoroyemoto et al. 2019). KEL replenished depleted photosynthetic pigments, proteins and carbohydrates in *A. hybridus* inoculated with *Bacillus licheniformis* and *Pseudomonas fluorescens*, thus improving photosynthetic activity, protein synthesis (Ngoroyemoto et al. 2020) and carboxylation. In response to abiotic stress, seaweed extracts promote chloroplast biogenesis and delay senescence (Battacharyya et al. 2015). Jannin et al. (2013) observed down-regulation of cysteine proteases linked with senescence and up-regulation of genes associated with stress response, increased photosynthesis, N and metabolism following *Ascophyllum nodosum* application. Cytokinins in KEL and VCL protect chloroplasts, membranes, increase chloroplast division (Arthur et al. 2001; Stirk and van Staden 2006;

Battacharyya et al. 2015) and promote regulation of stress-related phytohormones and water equilibrium (Ilyas et al. 2021).

Physiological effects of biostimulants on V. unguiculata total phenolics and flavonoids under different watering regimes in the greenhouse.

Phenolics accrue in plant tissue as tannins, flavonoids and lignin precursors, to scavenge toxic ROS induced by drought stress (Rice-Evans et al. 1997; Battacharyya et al. 2015). In the present study, leaves of all plants accumulated greater phenolic concentrations relative to roots. Restricting watering regime to once-a-week significantly increased leaf phenolics in control plants compared to KEL-treated plants (Fig. 4A). The increased phenolic compounds are ascribed to stress-induced disruptions in metabolic processes of the cells (Keutgen and Pawelzik 2009) which may have been down-regulated in KEL-treated plants using an independent antioxidant pathways. Although ABA regulates various morpho-physiological processes to acclimatize plants to abiotic stresses (Ilyas et al. 2021), the capacity of biostimulants to alleviate stress during ABA biosynthesis is via ABA independent metabolic pathways (García et al. 2014). Therefore, the capability of KEL to curb ABA biosynthesis can be associated with its exogenous application which introduces active plant growth regulators to the plant (Stirk et al. 2014; Kocira et al. 2020). Additionally, SW induced marked declines in phenolics under similar water deficits as KEL (Fig. 4A). This may be linked to inhibitory properties of trimethylbutenolide (3,4,5-trimethylfuran-2(5H)-one) or stress-related growth regulators of SW reported by Kulkarni et al. (2008, 2011).

Flavonoids activate better plant response to abiotic stresses including drought (Battacharyya et al. 2015). In the present study, leaf flavonoids in water-stressed plants drenched with biostimulants decreased considerably, reaching significant concentrations in VCL-treated plants (Fig. 4D). There was also a decreasing 'mirror-image' trend in root flavonoids of biostimulant-treated plants as drenching frequency was reduced from thrice- to once-a-week. These findings suggested an inhibition in cowpea's susceptibility to drought. Flavonoids alleviate stress by acting on enzymes, metabolic pathways (Araújo et al. 2008) and as cell protectants against apoptosis (Ndhlala et al. 2010). Enzymatic activity of chalcone isomerase increased following seaweed extract application (Battacharyya et al. 2015). Chalcone isomerase catalyses the biosynthesis of flavanone precursors and phenylpropanoid protective compounds (Sun et al. 2019).

Conclusions

The findings of the present study demonstrate that with seed-priming and exogenous drenching with VCL, KEL and SW, growth and yield reducing effects of drought can be alleviated via an induced increase in foliage, biomass accumulation, shoot length, root elongation and flower initiation. The three biostimulants can improve plant tolerance and osmotic adjustment to drought by promoting or inhibiting compatible solutes and phytochemicals and can have pronounced promotory effects on photosynthesis. While there is still more research required, this study indicates the capability of VCL, KEL and SW can be harnessed to offset growth reducing effects on plants under drought and temperature stress. These

biostimulants are not only convenient and simple to use, but also economical and toxic-free which is suitable for farmers who subsist in areas prone to climate change, drought conditions and land degradation.

Declarations

Acknowledgements

Contributions

MPV, MGK, SG and NG conceived the research idea. MPV performed the experiments and wrote the manuscript. JFF, JVS, MGK, SG and NG contributed in editing the manuscript. JVS supervised the research and is the corresponding author. All authors have read and approved the final manuscript.

Conflict of interest

It is declared that the authors have no conflict of interest in the publication of this article. Neither the manuscript nor its main contents have been published or submitted elsewhere.

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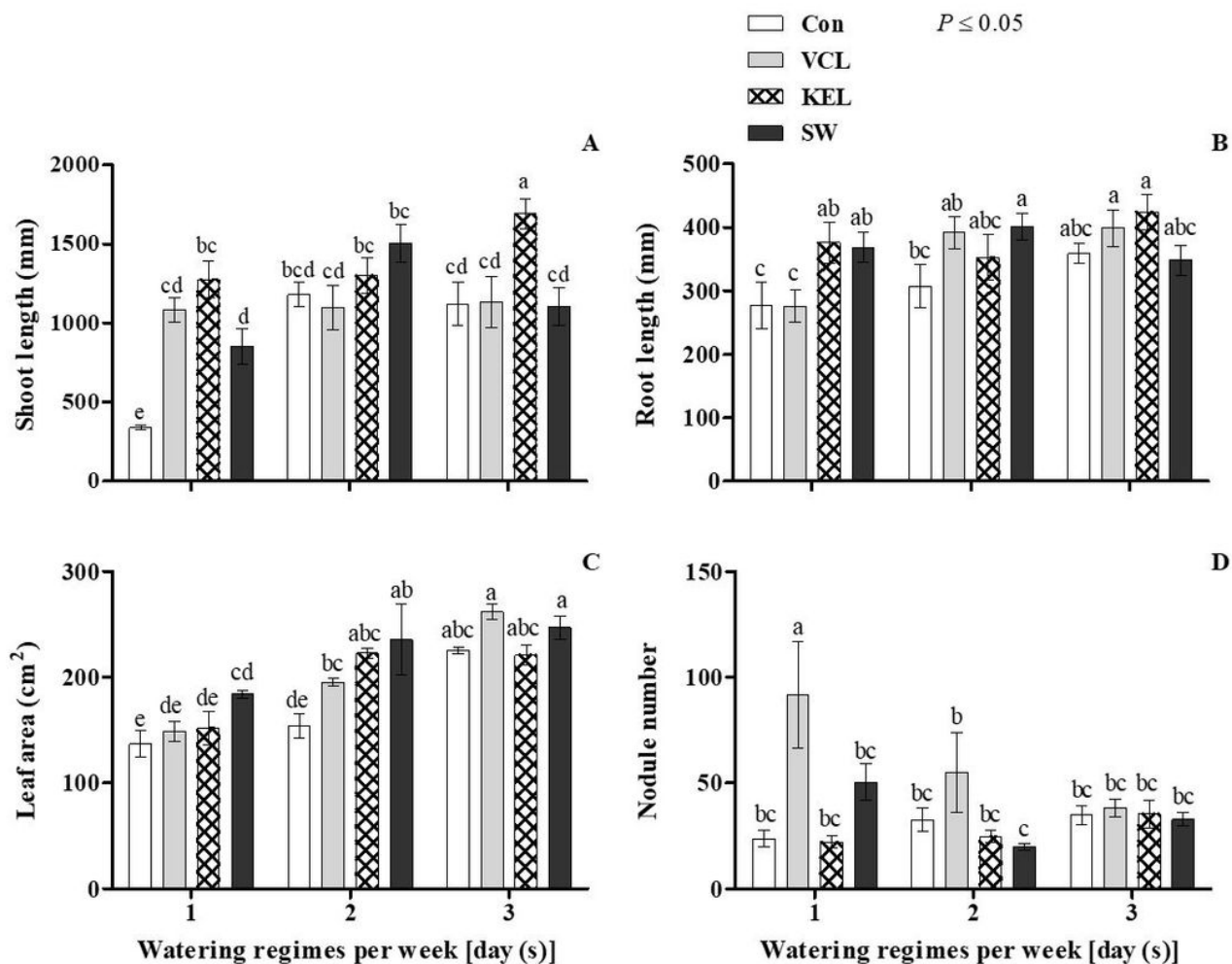
Figures

Figure 1

Effects of different watering regimes of tap water (control, Con); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%) and smoke-water, SW 1:1000 (v/v) per week on shoot length, root length, leaf area and nodule number of Vigna unguiculata after 13 weeks of growth in the greenhouse. For each treatment, bars (mean and ± standard error of the mean; shoot length, n = 10; root elongation, n = 10; leaf area, n = 3 and nodule number, n = 10) with different letter(s) are significantly different (P ≤ 0.05) based on DMRT.
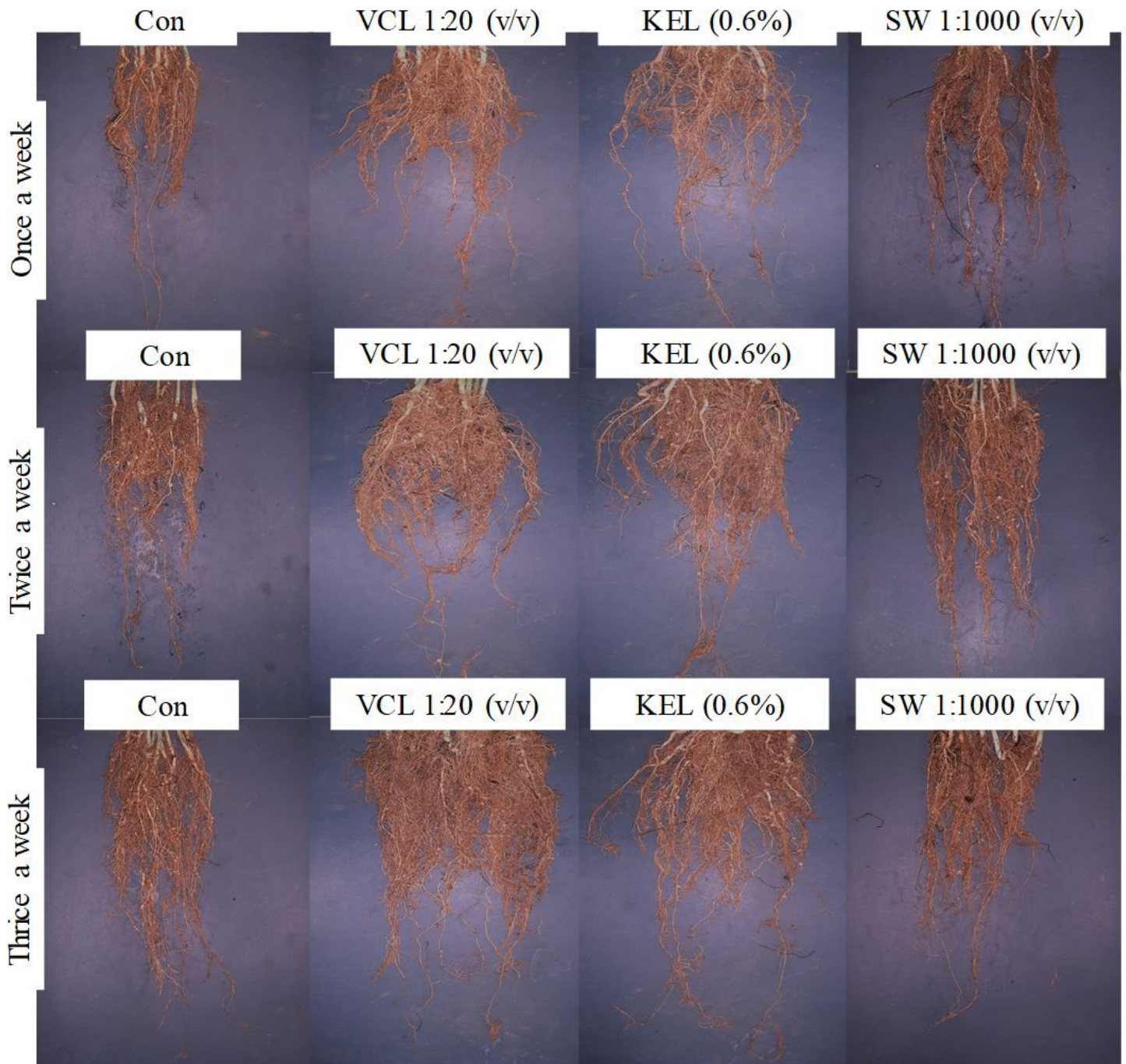
Figure 2

Roots of Vigna unguiculata plants primed and drenched with tap water (control, Con); vermicompost leachate, VCL 1:20 (v/v); Kelpak®, KEL (0.6%) and smoke-water, SW 1:1000 (v/v) at different watering regimes per week after 13 weeks under greenhouse conditions. Watering regimes per week: once a week (1 d), twice a week (2 d) and thrice a week (3 d).
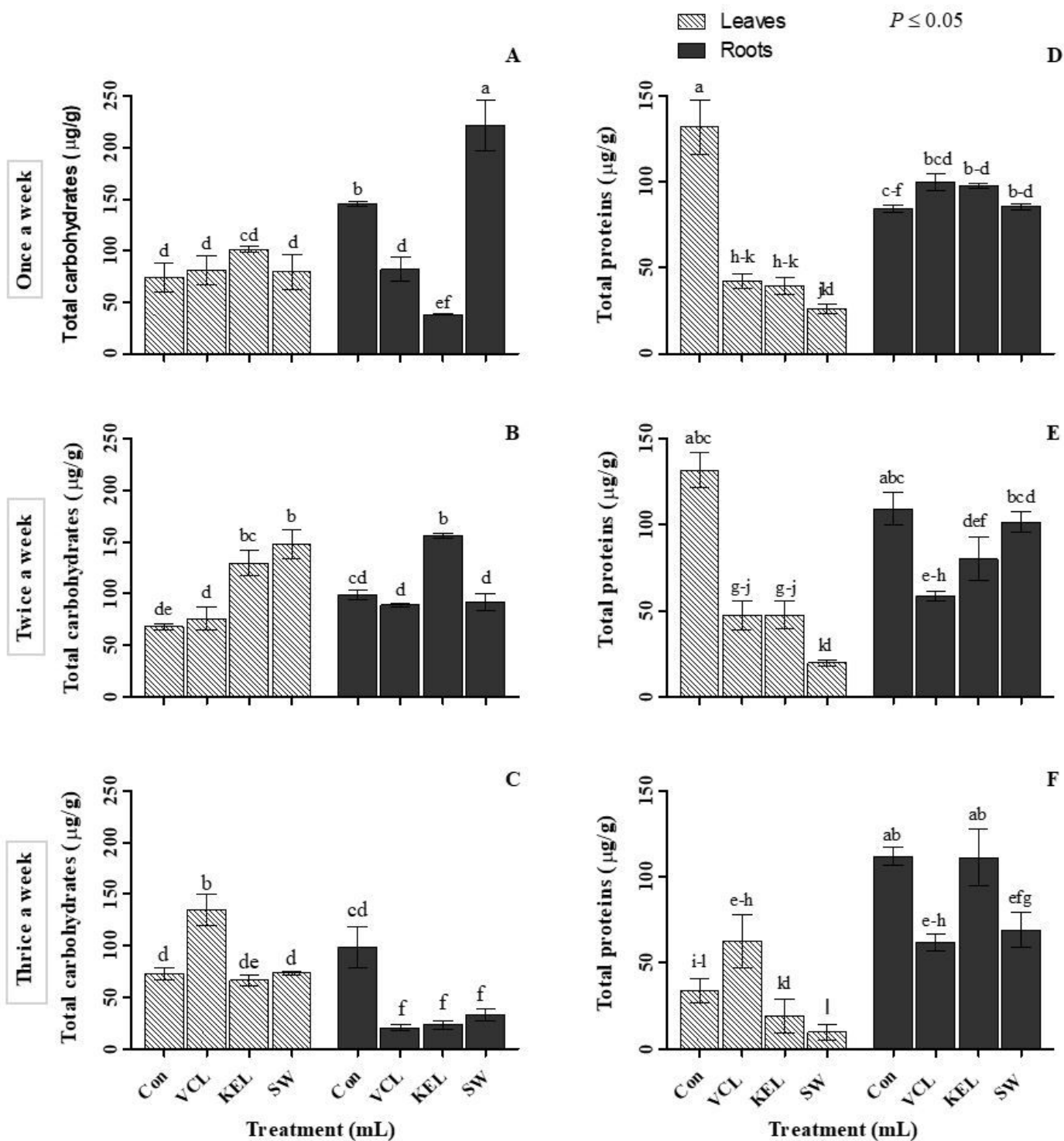
Figure 3

Effects of watering regimes of tap water (control, Con); vermicompost leachates, 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 and total proteins of V. unguiculata after 13 weeks 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
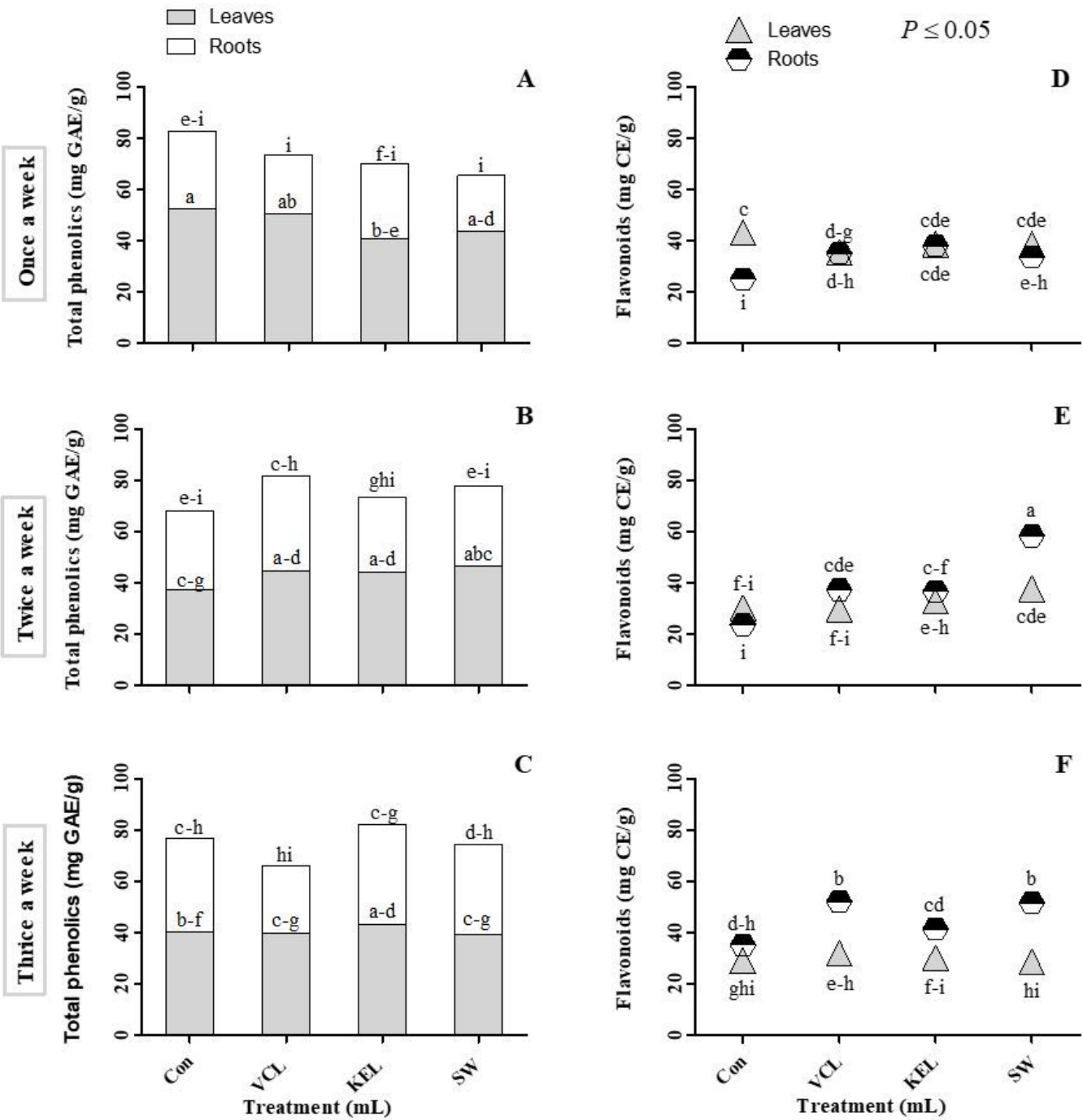
Figure 4

Effects of watering regimes of tap water (control, Con); vermicompost leachates, 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 and flavonoids of V. unguiculata after 13 weeks 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 ≤ 0.05)

according to DMRT. Once, twice and thrice a week represent watering regime 1, 2 and 3 in day(s), respectively.