EFFECT OF A NATURAL BIO-STIMULANT ON THE PHOTOSYNTHETIC CAPACITY AND YIELD OF SPINACH UNDER DROUGHT STRESS

By

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DECLARATION

"I, Pule Clement Liatile, declare that the dissertation that I herewith submit for the Master of Science degree in Botany at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education"

..... 30/11/2021

Pule Clement Liatile Date

DEDICATION

To my mother (Mamokete Majoro), sister (Relebohile Majoro), aunt (Malehlohonolo Diatile).

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LIST OF ABBREVIATIONS

AA: Amino acid formulations

AA: Ascorbic acid

APX: Ascorbate peroxidase

ATP: Adenosine triphosphate

Br: Boron

Ca: Calcium

ChlF: Chlorophyll-*a*-fluorescence

Cu: Copper

EL: Electrolyte leakage

ENSO: El Niño Southern Oscillation

ETC: Electron transport chain

Fe: Iron

GPX: Guaiacol peroxidase

GR: Glutathione reductase

GSH: Glutathione

HFP: Hydrolysed Fish Protein

HS: Humic substances

K: Potassium

Mg: Magnesium

Mn: Manganese

Mo: Molybdenum

N: Nitrogen

NDVI: Normalized difference vegetation index

NIR: Near-infrared radiation

NPQ: Non-photochemical quenching

NPQ: Photochemical quenching

OA: Osmotic adjustment

P: Phosphorus

PAR: Photosynthetically active radiation

$PI_{ABS:}$ Performance index absorbance

PI_{Total}: Total performance index

PSI: Photosystems one

PSII: Photosystem two

Q_A: Primary acceptor quinone

Q_B: Secondary acceptor quinone

R: Visible red light

ROS: Reactive oxygen species

RWC: Relative water content

S: Sulphur

SA: South Africa

SOD: Superoxide dismutase

sSA: Sub-Saharan African

SWE: Seaweed extract

TOC: Tocopherol

TSS: Total soluble sugar

WHC: Water holding capacity

Zn: Zinc

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ABSTRACT

Spinach (*Spinacia oleracea* L.) is a green leafy vegetable that is cultivated worldwide due to its high nutritional value, containing many vitamins and minerals. Spinach is susceptible to drought stress and its cultivation and production is limited by low water availability in arid and semi-arid areas. The potential of a natural bio-stimulant (*Xcell Boost*) to improve the physiological and biochemical responses as well as vegetative growth in spinach grown under different water stress regimes was evaluated under controlled conditions in the greenhouse. The trial design was a split-plot, where the main plot was the water treatments and subplot were *Xcell Boost* treatments. Three water levels were maintained in the soil: 100% (full irrigation), 50% (mild drought stress) and 30% (severe drought stress) water holding capacity (WHC). Three levels of *Xcell Boost* treatments were used: namely the control (no bio-stimulant, BX0), single (BX1) and double (BX2) concentration of bio-stimulant. Non-invasive and invasive techniques were used to assess the photosynthetic, biochemical, and vegetative growth parameters.

Drought stress showed insignificant effect on the normalised difference vegetation index (NDVI) under optimal water treatments. Under drought stress, NDVI values were maintained above 0.68 units, indicating that plants were green and healthy. Even so, the application of BX1 further increased the NDVI values under water deficiency. Because NDVI is a measure of vegetation “greenness”, it can be associated with potentially high chlorophyll content. This was supported by an increase in Chlorophyll *a* content under drought conditions. Application of BX2 increased the levels of Chlorophyll *a, b,* and carotenoids under drought stress. Drought stress slightly reduced the stomatal conductance under severe water stress. Application of *Xcell Boost* improved the stomatal conductance across all water regimes with BX1 showing the highest increase in stomatal conductance, particularly under severe water deficiency stress, which could have resulted in a high carbon dioxide fixation and increased photosynthetic rates.

The application of BX1 enhanced the accumulation of osmoprotectants (proline and total soluble sugars (TSS) and reduced electrolyte leakage under mild stress (50% WHC) but failed to boost the levels of TSS under severe drought stress (30% WHC). Positive correlations between proline and performance index absorbance (PI_{ABS})

shows that high proline level is linked to better photosynthetic capacity of spinach. *Xcell Boost* (BX2) induced substantial increases in the activities of the reactive oxygen species (ROS) scavenging enzymes, ascorbate peroxidase (APX) and guaiacol peroxidase (GPX), under severe drought stress. BX1 was only effective at inducing these enzymes under mild drought stress.

Xcell Boost applications at both concentrations reversed the negative effects of drought stress by inducing remarkable increases in plant height, leaf area, stem dry weight, root length and root moisture. This indicates that *Xcell Boost* application enhanced spinach resilience under drought stress. Stem dry weight also had a positive correlation with APX and stomatal conductance. Also, there was a significant positive correlation between total performance index (PI_{Total}) and root moisture content, this relationship could ensure that enough water is available for transport from the roots to the leaves to participate in the photosynthesis process.

Based on the results, applying *Xcell Boost* enhances several physiological, biochemical, and vegetative parameters in spinach during drought stress. This makes *Xcell Boost* a highly beneficial bio-stimulant for improving yield in spinach, and most probably for other crops, cultivated in semi-arid and arid regions.

Keywords: Bio-stimulants, chlorophyll-*a*-fluorescence, drought stress, normalised difference vegetation index, osmoprotectants, photosynthesis, *Spinacia oleracea*, stomatal conductance, vegetative growth.

CHAPTER 1

1. INTRODUCTION

Spinach (*Spinacia oleracea* L.), a green leafy vegetable belonging to the Amaranthaceae family, has gained popularity around the world (Verma, 2018). The popularity of this crop is attributed to its high nutritional value. It contains many micronutrients, vitamins (particularly A and C) and minerals (Roughani and Miri, 2019; Salehi et al., 2019). Most of the resource poor households in Sub-Saharan Africa (sSA), particularly in the rural areas, are faced with micronutrient (iron and zinc) and vitamin A deficiencies (Oelofse and van Averbeke, 2012; Nyathi et al., 2016). Leafy vegetables can provide dietary diversity and are used as an alternative crop to alleviate malnutrition in semi-arid and arid areas (Maseko et al., 2018).

The cultivation of leafy vegetables in South Africa (SA) has gained popularity in rural and urban households, smallholder, and commercial farmers (Mahlangu et al., 2020). The popularity of leafy vegetables is a function of various factors, including short growing season, high nutritive value, ease of preparation, taste, and low agronomic requirements. Most of the leafy vegetable species are tolerant to environmental stresses such as drought (Mabhaudhi et al., 2017). However, spinach is susceptible to drought and requires a constant and uniform supply of water. Therefore, low irrigation or prolonged drought conditions will result in low yields (Reyes et al., 2018).

Climate change puts a strain on the water cycle and pose a high risk on agricultural production especially in arid and semi-arid areas (Wheeler and Von Braun, 2013). Drought conditions can be made worse by climatic elements such as high temperatures, strong winds, low soil moisture, and low relative humidity (Baudoin et al., 2017). Drought contributes to reduced water supply to the plant from the surrounding environment and leads to plant water deficit or plant water stress, which is one of the important limitations to plant productivity (Araujo et al., 2016). Exposure to abiotic stress such as water deficit or a combination of specific stresses can impose adverse effects on the growth and quality of plants, which can result in significant yield and economic losses. Mild water stress may damage the photosynthetic apparatus and cell membranes (Ali and Ashraf, 2011). Severe water stress can further impair

metabolic functions and cause irreversible damage to the plant (Das and Roychoudhury, 2014). Such damage is a consequence of excessive accumulation of the reactive oxygen species (ROS) (Mittler, 2002; Torres, 2010). Drought stress also impacts on the thylakoids, chloroplast pigments, photosystems, the electron transport chain, and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which in conjunction or separately, influence the photosynthetic capacity of plants (Huseynova, 2011).

Adverse effects of water deficit depend on the severity and length of the water stress period, while the plant's ability to recover is associated with the cultivar type, its age, and stage of development (Potopová et al., 2016; Khan et al., 2018). Plants have evolved several reactions or strategies at morphological, physiological, cellular, and molecular levels to either avoid, tolerate, escape, or recover from water stress (Avramova et al., 2015; Fang and Xiong, 2015). One of the earliest plant responses to limited water availability includes stomatal closure, which subsequently leads to a decrease in photosynthesis rates (Basu et al., 2016) while increasing the water use efficiency (Liu et al., 2003; Ache et al., 2010). Consequently, the photosynthetic responses of plants to drought stress affects the synthesis of photo assimilates (Hopkins and Hüner, 2009). Accumulation of the osmolytes during different drought stress levels may maintain osmoregulation in plants (Fang and Xiong, 2015).

Plants mediate the defence responses to drought stress conditions through modification of several cellular functions and accumulation of cellular osmolytes or osmoprotectants such as proline, glycine betaine, and non-structured sugars (Sanders and Arndt, 2012). Plants accumulate a plethora of these organic, highly soluble, low molecular weight, electrically neutral, and non-toxic compounds in their cells as a protective mechanism to protect the photosynthetic system and sustain cell structure (Fang and Xiong, 2015). The accumulation of osmoprotectants play a role in lowering water potential and maintaining cell turgor pressure and thus improving water retention in response to drought stress. This occurrence is known as osmotic adjustment, and it aids in drought resistance (Chen and Jiang, 2010; Fang and Xiong, 2015). Moreover, osmoprotectants can play a role in protecting the cell membrane and protein structures, and detoxify the ROS, thereby protecting the key enzymes (Zulfiqar et al., 2019). In an effort to further adapt to drought stress, plants may produce the

antioxidants, which may be enzymatic, such as ascorbate peroxidase, superoxide dismutase (SOD), guaiacol peroxidase (GPX), and glutathione reductase (GR) or non-enzymatic, such as ascorbic acid (AA), glutathione (GSH) and tocopherol (TOC) (Torres, 2010, Das and Roychoudhury, 2014). Other alternatives to alleviate drought stress in plants involve the application of the bio-stimulants (du Jardin et al., 2015).

The use of plant bio-stimulants is one of the new approaches to increase water use efficiency and agricultural productivity, especially under drought stress (Bulgari et al., 2015; Van Oosten et al., 2017). A bio-stimulant is a substance or compound or microorganism that can be applied externally to the foliar plant parts or at the rhizosphere zone, to enhance plant nutrition, promote plants tolerance to abiotic stress, and improve crop yield and quality (European bio-stimulants industry council, EBIC, 2012; Calvo et al., 2014; du Jardin, 2015). Four main groups of bio-stimulants exist, which include humic substances (HS), protein hydrolysate and amino acid formulations (AA), seaweed extract (SWE), and plant growth-promoting microorganisms that have shown positive effects on plant growth and yield (Calvo et al., 2014; Battacharyya et al., 2015; du Jardin, 2015).

One such a bio-stimulant is *Xcell Boost*, marked by IntroLab (Pty) Ltd., South Africa. This bio-stimulant is currently being evaluated for its bio-catalytic potential at the University of the Free State. *Xcell Boost* is a liquid plant growth stimulant containing enzymatically hydrolysed L-amino acids from fish waste and kelp (*Ecklonia maxima*), manufactured by IntroLab (Pty) Ltd., and distributed by Precision Farming Holdings Ltd. *Xcell Boost* contains plant-growth-regulators (auxins (15 mg/L), cytokinins (10 mg/L)), and free amino acids (112 g/L). The bio-stimulant can be applied to all crops including seedlings during periods of biotic and abiotic stress to promote general plant growth and improve quality and yield (IntroLab, 2020). The application of Xcell *Boost* varies with crop type and can be applied as a foliar, soil, or drench application (https://www.introlab.co.za).

The use of specific bio-stimulants and their potential effects regarding plant-growth promotion and to enhance the plant's ability to recover from biotic stresses have been extensively reviewed (Calvo et al., 2014; Colla and Rouphael, 2015; du Jardin et al 2015). However, the use of bio-stimulants for improving plant resilience to

environmental stresses such as in water-limiting environments still needs to be studied further (Van Oosten et al., 2017). Also, information on the effect of bio-stimulant applications on physiological and biochemical parameters under water-deficit conditions has not been studied in detail. Despite many investigations associated with a water deficit, there are only few studies on spinach. Ekinci et al. (2015) indicated that under cultivated conditions with increased irrigation, spinach produces fresh good quality and high-yield leaves.

In addition, the application of a bio-stimulant at low concentrations had positive effects on spinach growth and development under drought stress (Xu and Leskovar, 2015). Still, there is not enough research on the productivity of green leafy vegetables under water-limited environments (Mabhaudhi et al., 2017; Maseko et al., 2020). In this study, it was hypothesised that foliar applications of *Xcell Boost* at different concentrations on spinach seedlings will improve the plant's tolerance to drought stress.

This study aimed at investigating the effects of a new bio-stimulant, *Xcell Boost*, on the physiological and biochemical responses as well as the vegetative growth parameters of spinach growing under water-deficit conditions. The objectives were to investigate:

1.1. the effect of single and double dosages of *Xcell Boost* on the photosynthetic efficiency and biochemical parameters of spinach under water-deficit conditions.

1.2. the effect of single and double dosages of *Xcell Boost* on the vegetative growth parameters of spinach under water-deficit conditions.

1.3. the relationships between the vegetative growth parameters, physiological, and biochemical parameters when single and double dosages of *Xcell Boost* are applied under different water regimes.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Water scarcity and drought status in South Africa

South Africa (SA) is a water-scarce, semi-arid country with an average rainfall of about 495 mm, which is well below the global average of about 860 mm per year (Dennis and Dennis, 2012; Lubbe et al., 2016). The main challenge throughout SA is that rainfall is unevenly distributed geographically and seasonally (DWAF, 2004; DWAF, 2013), where the amount of rainfall is higher in the eastern than western parts of the country (Agri SA, 2019). The interior of the country receives moderate levels of rainfall (Figure 2.1). As a result, the North/Central regions vary from tropical to sub-tropical, and the South ranges between semi-arid and arid climates (Tadross and Johnston, 2012; Mabhaudhi et al., 2017).

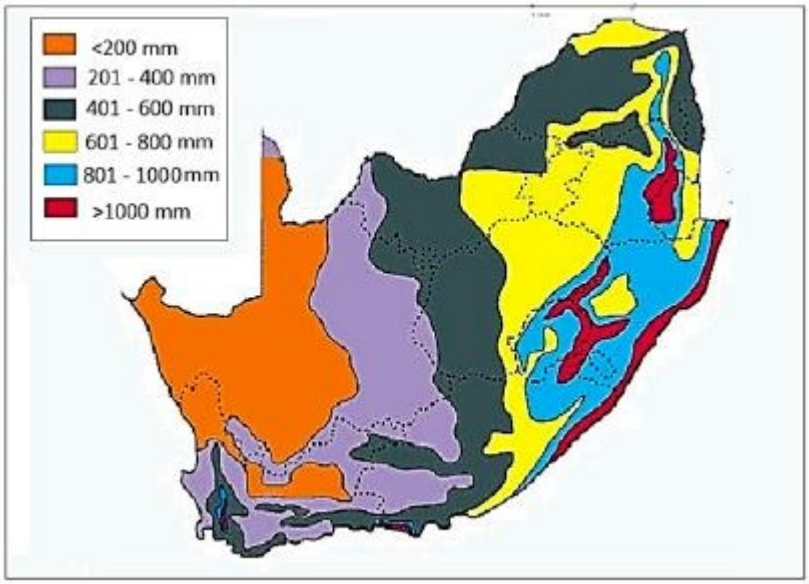

Figure 2.1: Rainfall distribution of South Africa adapted for farming and cropping systems (Mabhaudhi et al., 2017).

Water scarcity and water supply is a major issue, especially for SA. It was estimated that by 2025, water demand in SA would have exceeded the existing water availability of economically usable fresh-water resources (DWAF, 2013). Furthermore, predictions show that the global water demand for agriculture will increase by 60% in

2025. Meanwhile, the global population is expected to increase from 7.7 billion to 10.2 billion by 2050 and around 57% of the population would encounter water scarcity at least once a year by 2050 (Boretti and Rosa, 2019).

The continuously growing world population will put a strain on the water resources in the three sectors: industrial, domestic, and agriculture (Boretti and Rosa, 2019). It is estimated that over the next two decades, the global water demand will grow significantly with the agricultural sector having the largest water demand compared to industrial and domestic sectors, but the water demand for the industrial and domestic sectors will grow the fastest. Currently, the agricultural water demand accounts for 70% of global water use and most of it is used for irrigation (Boretti and Rosa, 2019). Climatic factors such as high temperature, high wind, low soil moisture, and low relative humidity may reduce the availability of surface water resources. These additional factors can significantly aggravate the severity of drought conditions (Baudoin et al., 2017).

Dry seasons, especially in semi-arid areas like SA, may result in a prolonged drought (Araujo et al., 2016). Severe drought stress can significantly reduce crop productivity due to limited water supply. Also, severe climatic changes will put a huge strain on the water cycle and pose a high risk on the agricultural production, especially in arid and semi-arid areas (Wheeler and Von Braun, 2013).

In SA, the term drought is regularly defined based on the degree of dryness and the duration of the dry period associated with natural causes or influenced by anthropogenic activities. This type of drought is known as a meteorological drought. Where less than 75% of normal rainfall is regarded as a severe meteorological drought but a shortfall of 80% of normal rainfall will cause crop and water shortages, which will ultimately affect social and economic factors. Normal rainfall for a particular place can be calculated over 30 years using previous rainfall figures (South African Weather Service, 2017).

South Africa is among the drought-prone countries in Africa. Furthermore, estimations show that Africa is most likely to experience longer and severe widespread droughts in the future (Baudoin et al., 2017; Phaduli, 2018), which could negatively impact the projections of future rainfall and temperatures in SA (Phaduli, 2018). According to Agri SA (2019), most parts of SA are threatened by drought, where the drought status

range from “severe” to “below normal”. This is due to the high temperatures and relatively low rainfall events (Agri SA, 2019).

In 2015-2016, SA experienced a severe drought, aggravated by increased temperatures (strong El Niño events) (Vogel and van Zyl, 2016). South Africa is inherently prone to droughts due to its location in the Southern African continent, caused by the El Niño Southern Oscillation (ENSO), a quasi-periodic invasion of warm sea surface waters into the central and eastern tropical Pacific Ocean. These extreme droughts take place at least once in any ten years (Baudoin et al., 2017).

The disastrous impacts of the drought in SA had an impact on the country's economy and local communities resulting in a significant decline in harvest and imposing severe to critical stress in groundwater and surface water availability, which resulted in the implementation of water restrictions (Agri SA, 2019). Drought has also affected agricultural production in neighbouring nations that rely on food imports from South Africa, such as Zimbabwe, Lesotho, and Botswana (Baudoin et al., 2017). This resulted in a reduced availability of basic foods (i.e., maize meal) in markets, thereby affecting food insecurity in the southern African region. Green leafy vegetable such as spinach is popular in SA because of its short growing cycle and high nutrition (Mabhaudhi et al., 2017; Salehi et al., 2019). However, this crop is highly sensitive to water-deficiencies (Reyes et al., 2018).

2.2 Spinach and drought

In SA, cultivation of vegetable crops is limited to low water availability, particularly for leafy vegetables since they are less tolerant to most abiotic stressors such as water limitations, saline, nutrient, and heat compared to other crops (Xu and Leskovar, 2015; Rouphael et al., 2016). The spinach crop (*Spinacia oleracea* L.) is a green leafy vegetable from the Amaranthaceae family. The cultivation and consumption of spinach has increased worldwide, and its popularity is attributed to its high nutritional value, consisting of many vitamins (A, B_1, B_2, C, E, K, niacin, and folic acid), and minerals (iron, copper, phosphorus, potassium, zinc, selenium, calcium, magnesium, and manganese). Spinach is also a rich source of fibre and phytonutrients that promote good health in humans (Salehi et al., 2019), making it the most desirable vegetable crop for consumers. The vegetative (leafy) part, including young, succulent stems of

the plant is harvested and used as a vegetable (Van Rensburg et al., 2007). Since the leaf is the consumable part, the bigger the leaves the higher the yield. Spinach can be consumed after boiling the fresh leaves, raw as a salad or in smoothies. The leaves can also be cooked as a potherb or canned or quick frozen (Eriksen et al., 2016; Roberts and Moreau, 2016; Alessa et al., 2017).

Several leafy vegetables are cultivated and utilized in SA and limited to the south-eastern areas, due to the erratic rainfall patterns. A few of these species are reported to be drought tolerant and can grow naturally in poor soils, and arid and semi-arid regions (Oelofse and van Averbeke, 2012). The popularity of these leafy vegetables is attributed to their short growing season, high nutritive value, ease of preparation, unique and acquired taste, and low agronomic requirements (Mabhaudhi et al., 2017). Therefore, they hold potential to contribute to diverse human diets and alleviate malnutrition; particularly in SA where micronutrient (iron and zinc) and vitamin A deficiencies are a major challenge, affecting poor rural communities (Oelofse and van Averbeke, 2012; Nyathi et al., 2016). Even though certain leafy vegetables are drought tolerant, water stress has been reported to reduce most physiological and biochemical processes, resulting in poor growth, and subsequently yield loss (Maseko et al., 2019). Furthermore, some leafy vegetables are susceptible to drought and require adequate amounts of water to produce marketable yields (Nyathi et al., 2016; Maseko et al., 2020).

Xu and Leskovar (2015) reported a significant reduction in spinach yields (leaf area, relative water content, and stomatal conductance) under water deficit conditions. Furthermore, greater yields were observed under increased irrigation, suggesting that spinach requires adequate water for optimum growth (Ekinci et al., 2015). Cultivation of spinach under mild water stress does not significantly affect its physiological parameters (Reyes et al., 2018). The survival of the plant depends on the growing environmental conditions, duration, and intensity of the stress, and the genotype (Fang and Xiong, 2015). Therefore, decreased irrigation or irrigation suspension decreases plant moisture and reduces growth and productivity (Reyes et al., 2018). In SA, the bulk of the water (7 836 m^3a) is used by the agricultural sector for irrigation, which is approximately 62% of the total water use (DWAF, 2004). Therefore, as the water uses for agricultural practice increases, the severity of water scarcity will deepen (Fauraes et al., 2012).

2.3 Drought effect on plant growth and productivity

Drought is a major and widespread stress factor for plants in most parts of the world, particularly in arid and semi-arid regions (Feitosa de Vasconcelos et al., 2009). During drought, water supply becomes a major limiting factor, which may impose water stress on plants. Water deficit negatively affects crop productivity and can result in significant yield and economic losses and remains a threat to food security (Oelofse and Averbeke, 2012; Araujo et al., 2016; Maseko et al., 2019). Under natural conditions, during their life cycle, plants can experience temporary water stress due to water drainage induced by gravity, soil evaporation, or absorption by plant roots irrespective of their growing region. However, plants are adapted to regulate their growth period to overcome water stress (Rahman and Hasegawa, 2012; Khan et al., 2018).

The effect of water stress on the responses, growth, and productivity of various vegetables including green leafy vegetables, have been studied. Ekince et al. (2015) found that under low irrigation levels (water deficit) plant weight, leaf number, and area of spinach decreased. Another study by Oelofse and Averbeke (2012), showed that water stress reduced the yield of most summer leafy vegetables such as Swiss chard (*Beta vulgaris*), pigweed (*Amaranthus cruentus*), dun. black nightshade (*Solanum retroflexum*), pumpkin (*Cucurbita maxima*), and walp cowpeas (*Vigna unguiculata*). The relative water content and leaf surface of these leafy vegetables was remarkable reduced by the water stress (Oelofse and Averbeke., 2012). The reduction of growth and production in plants to drought stress is influenced by the disturbances at the physiological and biochemical levels (Kabbadj et al., 2017).

2.4 Drought effects on photosynthesis

Plants respond to water stress via various processes at the morphological, physiological, and molecular levels (Osakabe et al., 2014). During early stages of water stress, plants respond by closing their stomata to limit water loss through transpiration (Fang and Xiong, 2015). The effect of drought on vegetable crops varies with plant species, developmental stage, and other environmental factors. Also, the intensity and duration of drought influences distinct physiological parameters such as chlorophyll content, photosynthetic parameters (chlorophyll-*a*-fluorescence), and relative water content (RWC) (Maxwell and Johnson, 2000; Strasser et al., 2010; Guo

and Tan, 2015; Gadi et al., 2019; Guidi et al., 2019). These parameters are useful in detecting adverse changes in the plant during the growth and development stages. Several photosynthetic parameters can be measured to provide information about the photosynthetic efficiency. In addition, the normalized difference vegetation index (NDVI) can also be used to measure vegetation "greenness", which can be associated with potentially high chlorophyll content (Pietragalla, 2012).

2.4.1 Photosynthetic pigments

In higher plants and green algae, photosynthesis occurs place in the chloroplast of green leaves that contains a green pigment, chlorophyll. Chlorophyll is the main pigment that absorbs photosynthetically active radiation (PAR) required for photosynthesis (Filimon and Filimon, 2016). There are four known chlorophylls molecules namely, *a*, *b*, *c*, *d.* In addition, chloroplast consists of other pigments such as the carotenoids. These pigments differ in their abundance, molecular structures, and absorptive properties (Hopkins and Hüner, 2009). Chlorophyll *a* is the primary photosynthetic pigment and occurs in all photosynthetic eukaryotes and cyanobacteria. The chemical structure of Chlorophyll *a* contains a magnesium ion held in a nitrogen-containing porphyrin ring (Scheer, 2006). Attached to the porphyrin ring is a long hydrocarbon chain (Figure 2.2). Chlorophyll *b* is found in higher plants and green algae. It is an accessory pigment that absorbs light energy and passes it to the primary pigment. The structure of Chlorophyll *b* differs from that of Chlorophyll *a,* it has a formyl group (-CHO) that replaces the methyl group ($-CH_3$) on ring. Chlorophyll *c* has similar basic structure as Chlorophyll *a,* except that it lacks the long hydrocarbon chain (Hopkins and Hüner, 2009). Chlorophyll *d* is often found in red algae or ascidians (*Lissoclinum patella*) and is also similar to chlorophyll *a,* except that the (-O-CHO) group replaces the (CH CH_2) group on the first ring (Mohr et al., 2010). Chlorophylls (*a*, *b,* and *c*) absorb light strongly in the violet, blue and red regions of the absorption spectrum. While Chlorophyll *d* absorbs light in the near-infrared region for photosynthetic light-harvesting.

Carotenoids are red, orange, or yellow lipid-soluble pigments found in chloroplasts of higher plants and in cyanobacteria (Hashimoto et al., 2016). They are accessory pigments that work in conjunction with Chlorophyll *a* by absorbing light energy at

different wavelengths in the blue-green region of the absorption spectrum where chlorophyll absorption is lower, and transfer the absorbed energy to Chlorophyll *a.* Furthermore, carotenoids play a key role in preventing photo-oxidative damage to the chlorophyll molecule and the membranes from excess light (Hashimoto et al., 2016).

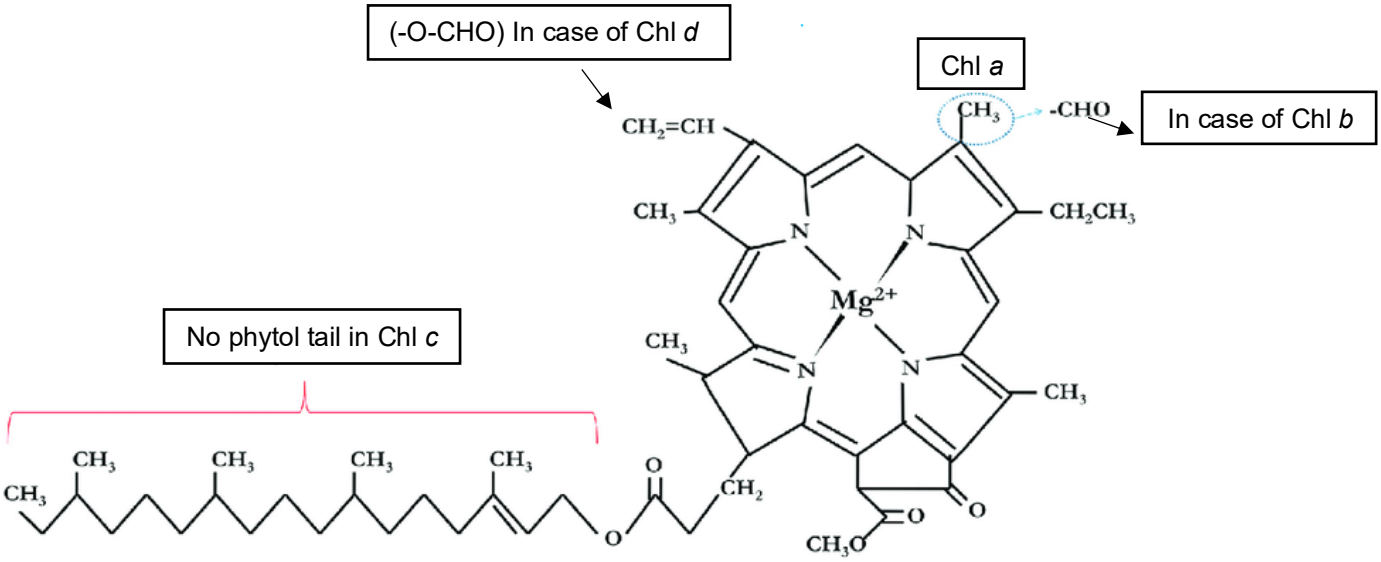

Figure 2.2: Structure of chlorophyll containing porphyrin ring to indicate the differences between Chlorophyll *a, b, c,* and *d* (Hopkins and Hüner, 2009).

Chlorophylls *a* and *b* are the main pigments in higher plants, they absorb light energy and drive photosynthetic reactions within the different chloroplast structures, thylakoid membrane, stroma, and double membrane envelope (Jensen et al., 2000). Within the chloroplast, carotenoids are also present in lipid and protein fractions, embedded in the thylakoid membranes (Hashimoto et al., 2016). The photosynthetic reactions can be divided into three steps: (1) primary reaction (absorption of light), (2) electron transport chain reactions and phosphorylation, and (3) carbon fixation (Baker, 2008; Croft et al., 2017). The light energy absorption reactions, electron transport reactions, and production of adenosine triphosphate (ATP) take place in the thylakoid membranes while the carbon fixation reactions take place in the stroma (Ahmad et al., 2019). The ratio of Chlorophyll *a* to *b* is approximately 3 to 1, and the total amount of chlorophyll content is directly related to the amount of photosynthesis per unit leaf area (Croft et al., 2017). Changes in chlorophylls ratio, and chlorophyll and carotenoids ratio can be an indication of senescence, environmental stress, and damage to the components of photosynthesis (Filimon and Filimon, 2016; Li et al., 2018).

Environmental stresses such as drought stress reduce the accumulation of chlorophyll pigments, decrease the leaf area, thereby limiting the photosynthesis of plants. Therefore, chlorophyll deficiency in plant leaves is a critical symptom of drought stress (Gebre et al., 2016). Rapid loss of chlorophyll will result in both the reduction of growth, and yield under water stress (Khayatnezhad and Gholamin, 2012, Ping et al., 2015). Leskovar and Piccinni (2005) reported that the chlorophyll content of spinach leaves was reduced significantly during water deficit stress and spinach leaves had yellowing spots. Later, Ekince et al. (2015) confirmed that chlorophyll content in spinach leaves decreased under different irrigation levels. However, Reyes et al. (2018) stated that if the plants are not subjected to intense drought stress, then chlorophyll content will not be affected.

2.4.2 Chlorophyll-*a*-fluorescence

Photosynthesis is divided into two phases namely, the light-dependent reactions and the light-independent reactions. The light-dependent reactions occur in the chloroplast's thylakoid membrane, while the light-independent reactions occur in the stroma (Larcher, 2001; Hopkins and Hüner, 2009). The light reactions consist of photosystems I (PSI) and photosystem II (PSII) that capture radiation energy and convert it into chemical energy to support photosynthesis, and this is known as photosynthetic efficiency (Maxwell and Johnson, 2000). According to these authors, when light energy from the sun is absorbed by a chlorophyll molecule at PSII, the electron configuration of that molecule is temporarily altered (unstable) and the absorbed light energy undergoes one of the three fates:

(1) the absorbed energy is used to drive photosynthesis (photochemistry), by donating an energized electron from the chlorophyll pigment to a primary electron acceptor molecule plastoquinone (Q_A) of PSII, which later passes it to a subsequent electron acceptor molecule (Q_B) in an electron transport chain. This is also known as the photochemical quenching (PQ) process:

(2) the absorbed excess energy can be dissipated as heat and does not drive photosynthesis this process is known as non-photochemical quenching (NPQ),

(3) the energy absorbed can be re-emitted as light (infra-red radiation and red/far-red radiation), which is known as chlorophyll-*a*-fluorescence (ChlF).

The three processes are linked and compete in a phenomenon known as fluorescence quenching hence, an increase in efficiency of one process will result in a decrease in the yield of the other two processes. Measuring the yield of chlorophyll-*a*-fluorescence can provide information about the changes in the efficiency of photochemistry and heat dissipation involved in the light-dependent reactions (Maxwell and Johnson, 2000; Murchie and Lawson, 2013).

Any disturbance (biotic or abiotic stress) on the photosynthetic performance of the sample, will affect the leaf's ability (reduced chlorophyll molecule and carotenoid) to absorb and channel solar radiation energy via photochemical processes, and this will eventually alter the intensity of the chlorophyll fluorescence emission (Strasser et al., 2000). Several ChlF parameters have been widely used to understand the stress responses in plants (Maxwell and Johnson, 2000; Banks, 2017). The ChlF data can provide several parameters involved in photochemistry (Figure 2.3). In this study, three specific ChlF parameters were selected to provide information on the activity of PSII and PSI under specific drought stress conditions:

(1) *Fm/Fv* ratio (quantum efficiency of photosystem II photochemistry), calculated from the following parameters, *Fo* (Initial fluorescence), *Fm* (maximum fluorescence), *Fv* (variable fluorescence = *Fm-Fo*),

(2) Performance indices (PI) involved in photochemistry; PI_{ABS} (overall functionality of the electron flow through photosystem II, PSII efficiency) and PI_{total} (overall functionality of the electron flow from photosystem II to photosystem I, thus total photosynthetic performance) (Ceusters et al., 2019).

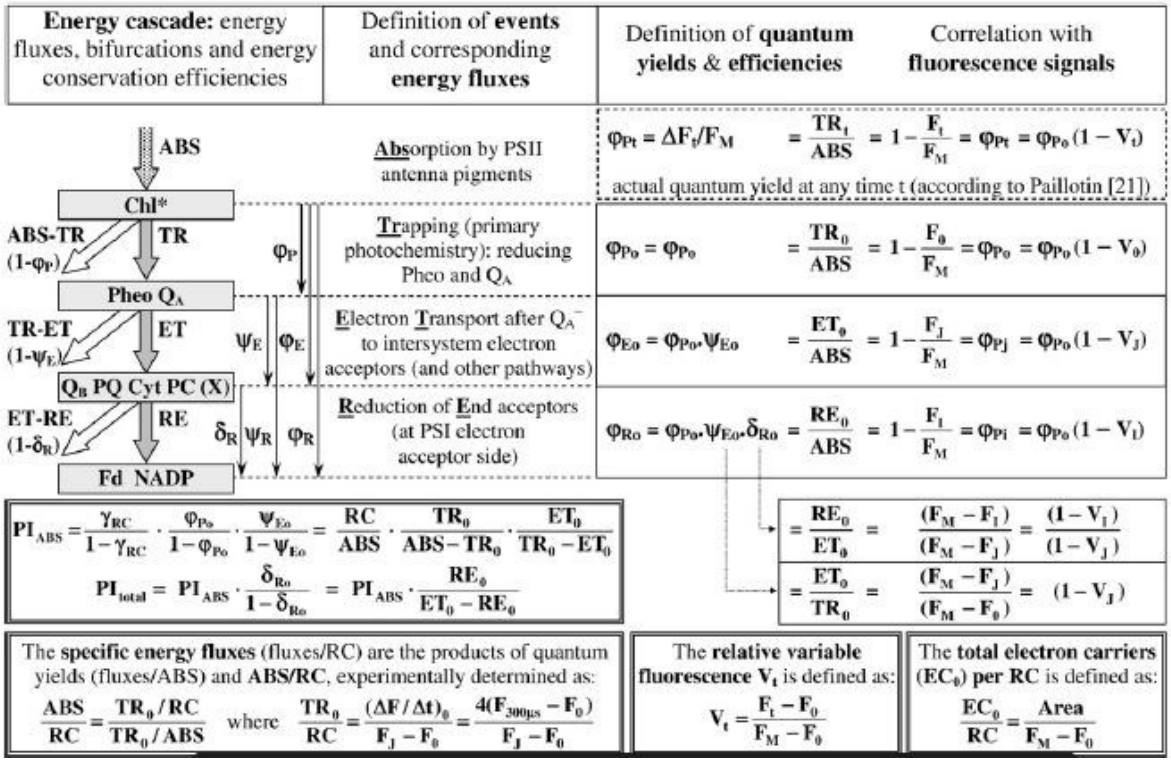

Figure 2.3: Schematic presentation of the relationship of chlorophyll-*a*-fluorescence parameters and calculations (Strasser et al., 2010).

Kautsky and colleagues were the first to notice chlorophyll fluorescence (Kautsky and Hirsch, 1931; Maxwell and Johnson, 2000). Kautsky found that transferal of a photosynthetic material from dark into light, causes the chlorophyll-*a*-fluorescence value to rise very fast from a dark-adapted ground fluorescence value (*Fo*) to a maximum fluorescence value (*Fm*) around one second, which subsequently decreases to a steady-state value for several minutes (20-30 min). This general pattern was later described as the Kautsky effect (Maxwell and Johnson, 2000). The increase in the yield of chlorophyll-*a*-fluorescence is due to the reduction of electron acceptors, namely plastoquinone (Q_A) upon light absorption, in the photosynthetic pathway downstream of PSII. After accepting an electron from PSII, Q_A should pass it to the subsequent electron acceptor Q_B (Figure 2.4). This is because Q_A cannot accept more than one electron from PSII. This results in a delay, which causes the PSII reaction centres to be in a “closed state”. Closure of the reaction centre leads to an overall reduction in the quantum efficiency of PSII hence, and subsequently increasing the yield of chlorophyll fluorescence, becomes maximum (*Fm*). This will also result in the increased heat dissipation (Maxwell and Johnson, 2000). Thereafter, the yield of

chlorophyll-*a*-fluorescence may decline over minutes, and this is controlled by a phenomenon termed fluorescence quenching, which involves a combination of two processes (photochemical quenching and non-photochemical quenching). Photochemical quenching is when there is an increase at the rate at which electrons are transferred away from PSII to the final acceptors in the carbon metabolism, which is activated by light and the opening of stomata. Non-photochemical quenching is when there is an increase in the efficiency at which energy is converted into heat (Maxwell and Johnson, 2000; Galle and Flexas, 2010; Murchie and Lawson, 2013; Bucher et al., 2018).

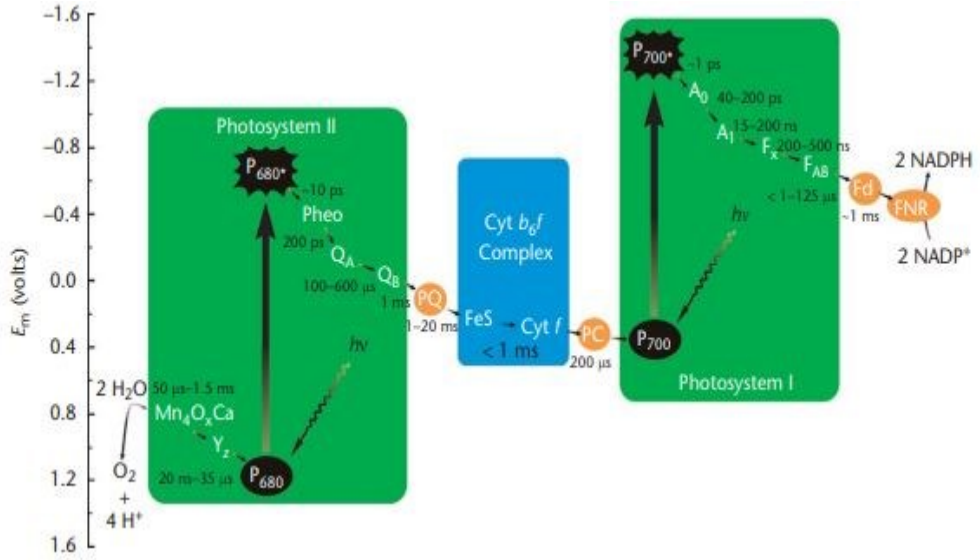

Figure 2.4: Z- scheme of photosynthetic electron transport (Govindjee et al., 2010).

During the dark adaptation both reaction centres, Q_A, and photosystem II are fully oxidised to drive photochemistry, by donating an electron from the chlorophyll pigment to an electron acceptor molecule. The reaction centres are “open”, and a minimal fluorescence yield is observed (*Fo*). After the introduction of saturated light (actinic), Q_A becomes fully reduced and can no longer accept electrons, until the accepted electrons are transferred to the next electron carrier Q_B. The reaction centres are “closed”, and the fluorescence yield increases to a maximal value (*Fm*). Any available fluorescence yield is quenched (Maxwell and Johnson, 2000). The time taken for fluorescence quenching varies significantly between plant species (Murchie and Lawson, 2013) hence, for this experiment, the spinach leaf was dark-adapted for 45 minutes as a sort of standardisation. The difference between *Fo* and *Fm* is the variable fluorescence, *Fv*, while the ratio between *Fv* and *Fm* can be expressed as *Fv/Fm* =

(*Fm-Fo/Fm*) (Maxwell and Johnson, 2000; Murchie and Lawson, 2013). Under normal conditions, the *Fv/Fm* value ranges from 0.75 to 0.85 irrespective of the plant species being studied. Under specific stress conditions, like heat stress, or water stress, *Fv/Fm* declines, indicating a reduction in the photosynthetic capacity (Galle and Flexas, 2010; Murchie and Lawson, 2013).

The OJIP curve can be used to analyse the fluorescence induction curve in the 1st second when a dark-adapted leaf is exposed to continuous light. The curve represents the initial fluorescence rises (*Fo)* and the maximum fluorescence value (*Fm*) under one second (Strasser et al., 2000; Esposito et al., 2006). The induction curve is considered a rapid process with distinct phases named O, J, I, and P. The fast chlorophyll fluorescence induction can be measured using a chlorophyll fluorimeter and the transient (OJIP) fluorescence signal is recorded, plotted on a logarithmic time scale (Figure 2.5). The transient has inflection points starting from initial fluorescence (*Fo*) to the maximal fluorescence (*Fm*). The O-point corresponds to the initial fluorescence emitted, whereas J-point and I-point corresponds to subsequent fluorescence emitted, and P-point corresponds to maximum fluorescence (Strasser et al., 2000). The curve is divided into three parts where the OJ-phase denotes the photochemical reactions in the reaction centres of PSII leading up to the reduction of Q_A. While JI-phase relates to the events of the photosynthetic pathway via electron transport chain (PSII, Cyt b_6/f complexes, and PSI). The IP-phase relates to the reduction of PSI and nicotinamide adenine dinucleotide phosphate ($NADP^+$). Where the P-phase corresponds with the maximal fluorescence, *Fm* (Strasser et al., 2010; Schansker et al., 2011; Guo and Tan, 2015).

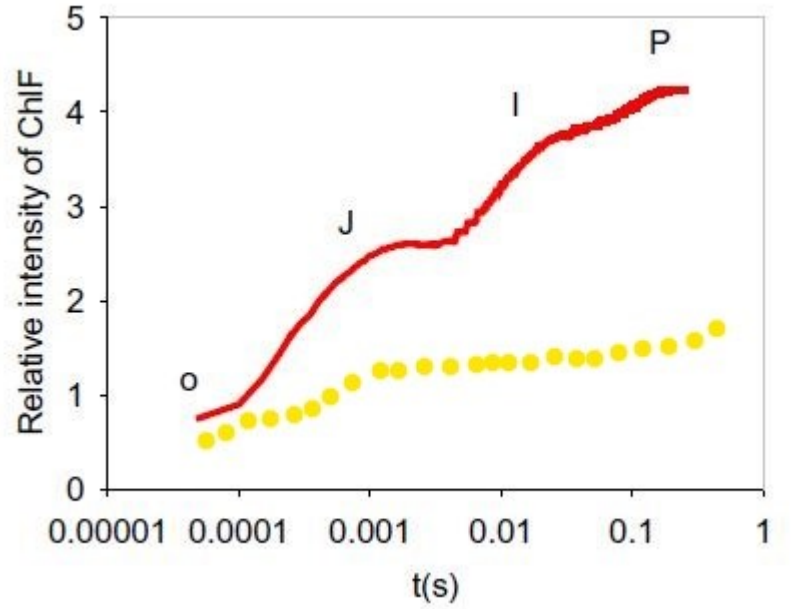

Figure 2.5: The OJIP curve indicating the Kautsky effect when a dark-adapted leaf material is excited by constant light (Maxwell and Johnson, 2000).

Although *Fv/Fm* has been extensively used as a good indicator of photosynthetic function and a good measure of water stress, there are some researchers that question the usefulness of this parameter (Strasser et al., 2000). Another chlorophyll-*a*-fluorescence parameter referred to as absorbance performance index (PI_{ABS}) was introduced, which is a product of three independent parameters (Figure 2.3) combining structural and functionality of PSII. Therefore, PI_{ABS} gives information about the density of the active reaction centres, the efficiency of electron movement to the electron transport chain (ETC), and the probability that an absorbed photon will be trapped by the reaction centres (Strasser et al., 2000). Later the total performance index (PI_{total}) parameter was introduced (Smit et al., 2009). This parameter is based on PI_{ABS} and gives information about the efficiency of electron transfer from (PSII) to the efficiency of reduction of (PSI). Thus, PI_{total} gives information about the functionality of both photosystems.

The PI_{ABS} and PI_{total} are more sensitive to external factors than *Fv/Fm*, therefore are the most preferred parameters for plant homeostasis (Živčák et al., 2014; Ceusters et al., 2019).

The application of chlorophyll-*a*-fluorescence technique on evaluating plant health is well documented, especially under abiotic and biotic stress (Maxwell and Johnson, 2000; Rohacek et al., 2008; Murchie and Lawson, 2013; Salvatori et al., 2014; Melo et al., 2017; Pérez-Bueno et al., 2019). The focus was on salt stress (Arias-Moreno et al., 2017), crop monitoring and phenotyping under water stress (Zhuang et al., 2020), nitrogen deficiency (Wu et al., 2019). Only a few chlorophyll-*a*-fluorescence applications on water stress are studied in spinach. Xu and Leskovar (2015) showed that *Fv/Fm* was not affected by water stress in spinach. While decreased *Fv/Fm* and photochemical yield was reported under water and saline stress as well as under nitrogen deficiency (Ors and Suarez, 2017; Xu and Mou, 2016). These accounts suggest that in spinach, *Fv/Fm* may not be a sensitive parameter to use or that it may be negatively affected by drought stress. Since the responses of plants are specific to the type and duration of stress (Fang and Xiong, 2015), Fv/Fm will be included in this study.

2.4.3 Stomatal conductance

Stomatal conductance refers to the rate of gas exchange (carbon dioxide intake) and transpiration (i.e., water loss through the stomata of the leaf) (Tipple and Pagani, 2007). The ratio of carbon dioxide and water vapor is determined by the opening and closing of stomata and the degree of stomatal aperture. Hence, stomatal conductivity is determined by the density, size, and degree of stomatal opening (Pietragalla, 2012; Gadi et al., 2019).

Under water-deficiency stress, stomatal conductance decreases due to plants closing their stomata in an attempt to reduce water loss through the leaf surface, thereby matching the rate at which water can be resupplied by the roots (Liu et al., 2008). The stomatal closure blocks a route for the exchange of gases (i.e., carbon dioxide, oxygen, and water vapor) and reduces the pressure gradient of carbon dioxide and water vapor (Hopkins and Hüner, 2009). This process will eventually limit the rate of photosynthesis because it is primarily controlled by stomata for carbon dioxide absorption and water vapor exchange (transpiration) (Fang and Xiong, 2015). The opposite applies under normal conditions; the more the stomata are open, the higher the transpiration rate and gas exchange rate, resulting in CO_2 gain in the parenchyma

cells (i.e., higher stomatal conductance), leading to higher photosynthetic activity (Basu et al., 2016). Stomata may directly respond to water deficit in two ways (Fang and Xiong, 2015):

(1) A direct response to air humidity in which guard cells and adjacent epidermal cells directly evaporate moisture to induce stomatal closure.

(2) Stomata respond to changes in leaf water potential by closing when the leaf water potential falls below a specific threshold.

Studies demonstrate that stomatal conductance and transpiration rate decrease with an increase in water stress (Khan et al., 2018) hence, under severe drought conditions both photosynthetic processes and gaseous exchange (alone or in combination) will be affected. Therefore, stomatal conductance is an important indication of evapotranspiration as well as plant photosynthetic ability (Gadi et al., 2019).

Previous studies suggest that stomatal conductance can also be used to estimate plant yield of certain crops under different water levels. Therefore, higher stomatal conductance can be correlated with high yield (Roche, 2015; Parkash and Singh, 2020). Measuring stomatal conductance allows the farmer or researcher to monitor the water status of a plant under drought conditions and safely deduce from the data, the productivity of a plant (Pietragalla, 2012). However, this is not entirely true for some plants (anisohydric crops) since, stomatal conductance is somewhat insensitive to certain water stress levels (Vilalta and Garcia-Forner, 2016).

2.4.4 Normalized difference vegetation index

Chlorophyll absorbs visible light at wavelengths from 400 to 700 nm, also known as photosynthetically active radiation (PAR), for photosynthesis to occur. Of this range, chlorophyll molecules strongly absorb light (radiation energy) in the violet and blue wavelengths (400 to 500 nm), as well as the red wavelength (600 to 700 nm) from the visible light spectrum. But chlorophyll molecule has a high reflectance in the green portion of the spectrum hence, leaves appear green to the human eyes (Pettorelli, 2013; Prasad and Thenkabail, 2016). While other cellular structures of the leaves strongly reflect radiation energy of larger wavelengths (700 to 1100 nm), such as near-infrared radiation, which is either reflected off the leaves on the canopy or transmitted

to the underlying leaves as it does not have sufficient energy to excite electrons in the palisade mesophyll cells and drive photosynthesis (Figure 2.6). The reflection of near-infrared radiation is facilitated in the spongy mesophyll layer cells, where the intercellular air spaces cause it to scatter at the cell wall-air interfaces inside the leaf. The mid-infrared region carries information about the absorption of this region by water, cellulose, and lignin, which can be used to detect stress in the plants that are caused by drought (Katsoulas et al., 2016). The differences in plant leaf reflectance within the visible and near-infrared wavelengths can be used to calculate Normalized Difference Vegetation Index (NDVI) index, which assumes that chlorophyll pigments have high absorption in the visible red light (R) and high reflectance in the near-infrared radiation (NIR) region (Silva et al., 2016).

The NDVI is a vegetation measure that is based on the ratio of two spectral bands, near infrared and red, where the difference between the two spectral is divided by the sum of the two spectral bands:

$$NDVI = ((NIR-R)/(NIR+R))$$

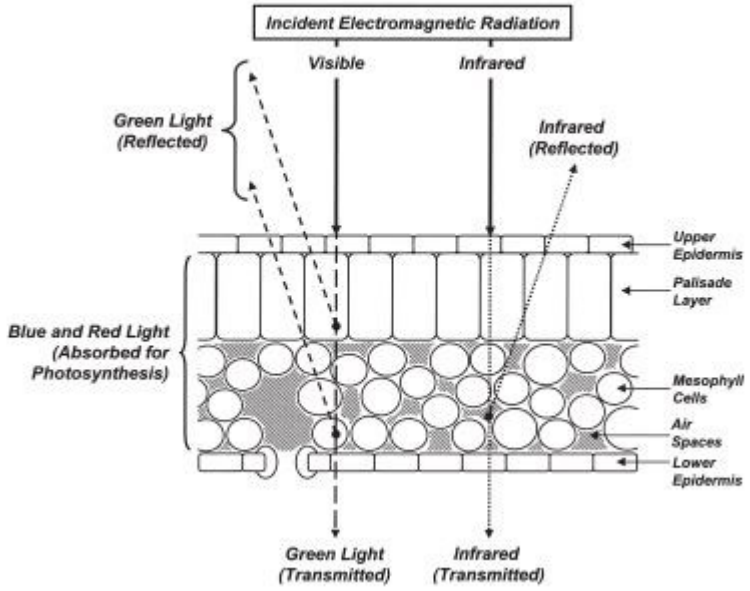

Figure 2.6: A structure of a typical plant leaf reflecting, absorbing and, transmitting an incident electromagnetic radiation in the palisade mesophyll cells (Katsoulas et al., 2016).

The NIR and R are used to calculate the NDVI value - the amount of photosynthetically active material in a spatial unit by acting as a measure of 'greenness' and represented

as a single value (Pettorelli, 2013). The NDVI values can range from -1.0 and +1.0. When determining a vegetation cover of an area, -1.0 suggests there is no vegetation and the +1.0 suggests there is a dense vegetation cover. Low values of NDVI (≤ 0.1) indicate the absence of vegetation (barren areas of rock, sand, or snow), whereas moderate NDVI values (0.2- 0.5) indicate sparse vegetation (grassland and shrubs or senescing crops). High NDVI values (0.6-0.9) indicate dense vegetation, such as temperate or tropical forests, or crops in their peak development stage (Pettorelli, 2013; Prasad and Thenkabail, 2016).

The huge difference in the amount of red light reflected by a healthy plant and a stressed plant is due to the efficiency of photosynthesis in stressed plants, or the capacity to absorb PAR (Figure 2.7). Because chlorophyll absorbs mostly blue and red light and the spongy mesophyll reflects near-infrared light, a healthy green canopy will absorb more red light and reflect near-infrared light (Pietragalla, 2012; Pettorelli, 2013).

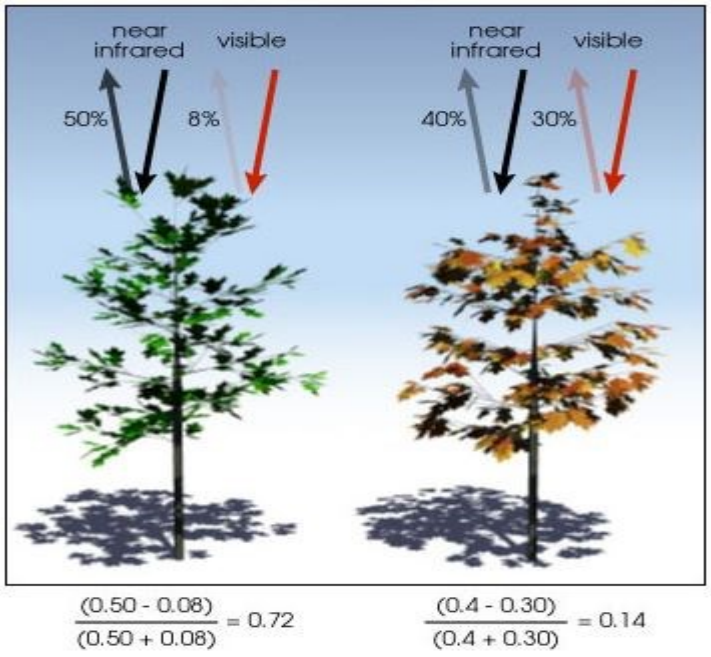

Figure 2.7: Determining plant health status using NDVI values from visible red light and near-infrared light (Earthobservatory.nasa.gov., 2000).

To date, most of the information on NDVI studies in monitoring crops and drought stress is generated from satellite remote sensing. During remote sensing, the physical

object or area under study is not in physical contact with the recording device (Pettorelli et al., 2013; Sims et al., 2014; Sruthi and Aslam, 2015). The most recent sensor technologies have been developed into portable sensors (handheld non remote sensing instruments) that can measure NDVI while in contact with the plant. Such device is the PlantPen NDVI 310 meter (Photon Systems Instruments Ltd., Czech Republic) used in this study. PlantPen NDVI 310 is a non-invasive technique that can be applied in agricultural production within field conditions and greenhouse applications.

Data collection using portable sensors is precise, fast, and less prone to interference. Also, the portable sensor is particularly advantageous in that it can produce its source of light, which allows measurements to be taken under any light condition (Pietragalla, 2012; Crusiol et al., 2016; Thapa et al., 2019). The use of such a portable sensor to obtain NDVI reading has been reported in a few studies. Crusiol et al. (2016) used a portable sensor to obtain NDVI readings in two different soybeans (*Glycine max* L. Merrill) cultivars that differed with drought sensitivities. Their results showed that NDVI of the drought-sensitive cultivar was lower than that of the less sensitive cultivar when subjected to water deficit. Thapa et al. (2019) also obtained low NDVI values in water stressed winter wheat *(Triticum aestivum* L.*)* using a handheld sensor and demonstrated that NDVI values were positively correlated with grain yield and biomass, respectively. In addition, Liu et al. (2019) established a positive correlation coefficient between NDVI and wheat grain yield using a handheld sensor. Although most reports apply for satellite imaging, the use of portable sensors to acquire NDVI data is becoming popular in agricultural production, especially in the field and greenhouse experiments.

2.5 Osmolytes and antioxidants

Generally, green plants undergo photosynthesis in the chloroplast, using sunlight to synthesize carbohydrates from carbon dioxide and water and release oxygen as a by-product (Brown and Schwartz, 2009; Jones, et al., 2013). However, the primary function of photosynthesis is the provision of enough energy and carbon that is important in the maintenance, metabolism, and growth of the photosynthetic tissues as well as the whole plant. Photoassimilates temporarily accumulate in the leaf as

either sucrose in the mesophyll vacuole or converted to starch for long-term storage in the chloroplast stroma (Mader et al., 2013). The majority of the photoassimilates are exported out of the leaf and are stored in non-photosynthetic organs and tissues (roots, tubers, and seeds). The mobilization of sucrose and starch between organs throughout the plant support respiration and other metabolic processes (Hopkins and Hüner, 2009).

Under normal conditions, vegetable crops (spinach) are high in water content (80-90% of the biomass), which act as a medium for transporting metabolites, nutrients, and facilitate various physiological processes. Water stress lowers the plant water potential and turgor, thereby restricting normal physiological functions (Rahman and Hasegawa, 2012). This can result in limiting the efficiency of plant metabolism, reduce yield and crop quality. The decrease in water potential is often associated with an increase in the soluble sugars. Soluble sugars are known to increase in different crop plants under salinity and water stress at different levels of stress (Du et al., 2020).

In drought stress, soluble sugars like sucrose and glucose play a role in cellular respiration as substrates or as osmolytes in the process of maintaining cell homeostasis (Rosa et al., 2009). Other hexoses such as fructose are not associated with osmo-protection but are related to secondary metabolites synthesis. Fructose is involved in the production of erythrose-4-P, a substrate for the formation of lignin and phenolic compounds (Fang and Xiong, 2015). Although soluble sugars are involved signalling and maintaining cell homeostasis, they have other functions in plants under abiotic stress such as, regulating protein conformation at low water potential, and facilitating stress signalling pathways (Alam et al., 2014). Soluble sugars also maintain the thylakoid membrane and influence the photosynthesis capacity (Alam et al., 2014).

The concentration, composition, and compartmentalization of osmolytes depend on the plant species, and type of abiotic stress. These osmolytes accumulate in different concentrations and different cell components such as chloroplast, cytosol and, cytoplasm (Filippou et al., 2014). For instance, to lower the osmotic potential and promote water retention in response to drought stress, natural osmolytes accumulate in various components of the cytoplasm, such as cytochylema and "cell juice". This plant adaption occurs through two events known as the osmotic adjustment (OA) and turgor regulation (Chen and Jiang, 2010; Fang and Xiong, 2015; Blum, 2016). Under

stress, protective agents such as prolines, soluble sugars, organic acids, spermines, glycine betaine, potassium, calcium, and chloride ions are produced in the plant cells to regulate turgor pressure and prevent protein denaturation and disruption of cellular structure without affecting functions of enzymes and interfering with metabolism of the plant (Zulfiqar et al., 2019).

Proline is an amphipathic molecule with hydrophobic and hydrophilic parts, which can interact with proteins and water molecules, respectively. This allows more proteins to access more water, increasing their solubility and preventing protein denaturation caused by dehydration in water-stressed conditions (Fang and Xiong, 2015). Moreover, osmoprotectants improve stress tolerance by acting as non-toxic substances that protect the biological membrane and protein structures, scavenging toxic reactive oxygen species (ROS), which need strict regulation, otherwise, uncontrolled levels lead to oxidative cellular damage (Fang and Xiong, 2015; Zulfiqar et al., 2019).

In plants, under normal conditions, ROS are by-products of cell metabolism produced at low concentrations, necessary for cellular homeostasis (Karuppanapandian et al., 2011). They are typically produced in the apoplastic region but under stress conditions and they are produced in various cellular compartments, including chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum (ER), and plasma membranes leaves (Karuppanapandian et al., 2011). Under stress conditions like salinity, drought, heat, chilling, heavy metals, nutrient deficiency, pollution, ultraviolet radiation or light stress and pathogen infection, the balance between the ROS production and elimination is disturbed in the cellular components of plants (Mittler, 2002; Miller et al., 2010). As stress severity increases, accumulation of ROS result in oxidative burst that rapidly inactivates enzymes and damage the plant cell membranes. Such impairment can be related to reduced membrane integrity and increased membrane permeability, resulting in increased solutes and ion (electrolyte) leakage (Campos et al., 2003; Masoumi et al., 2010). Furthermore, overproduction of ROS will induce the degradation of pigments, proteins, lipids, and nucleic acids, resulting in cell death (Das and Roychoudhury, 2014). Plants have developed ROS scavenging mechanisms to tolerate oxidative stress caused by the excess production of ROS such as superoxide, hydrogen peroxide and hydroxyl radical (Mittler, 2002). The mechanism involves the

upregulation of enzymatic antioxidants (superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR)) or non-enzymatic antioxidants (ascorbic acid (AA), glutathione (GSH) and tocopherol (TOC)) (Rodriguez-Serrano et al., 2009; Torres, 2010). The enzymatic antioxidants act as the major antioxidant enzymes that play a role in antioxidant defence system. While the non-enzymatic antioxidants regulate the ratio of oxidation and reduction reactions by reducing low-molecular-weight antioxidants using enzymes of the ascorbate-glutathione cycle (Foyer, 2018).

Several studies have reported that an increase in osmolytes, total soluble sugars, and enzymatic and non-enzymatic antioxidants in different vegetable crops under abiotic stress is associated with tolerance (Shafiq et al., 2015; Akram et al., 2016). For example, soluble sucrose content was increased in soybeans during R2-R6 growth stages under water stress (Du et al., 2020). In addition, Moloi and van der Merwe (2021) reported an increase in free proline content, total soluble sugars, and enzymatic antioxidants in vegetable-type soybean cultivars under water stress. However, the accumulation of osmolytes, soluble sugars and antioxidants is not only observed in water stress tolerant plants but also in water stress sensitive vegetable crops such as spinach (Jabeen et al., 2019). To improve crop yields, especially under changing climates, the cultivation and production in SA should focus on developing strategies that can improve crop water use efficiency (Ekinci et al., 2015). This can include the use of bio-stimulants.

2.6 Bio-stimulants

The application of natural bio-stimulants on vegetable crops has the ability to act directly on physiological processes, resulting in possible benefits such as improved plant growth, development, and/or abiotic stress responses (water, saline, flooding, thermal, and heavy metal toxicity) (du Jardin et al., 2015; Van Oosten et al., 2017). Plant bio-stimulants are substances, compounds, or microorganisms that can be applied externally on the foliar plant parts or at the rhizosphere. They contain beneficial substances or compounds (other than primary/ secondary nutrients), and microorganisms whose functions are to stimulate natural processes, enhance plant

nutrition, promote plant tolerance to abiotic stress, and improve crop yield and quality (EBIC, 2012; Calvo et al., 2014; du Jardin, 2015). Bio-stimulants are not considered pesticides because they have no direct effect on pests and hence do not fall within the pesticide regulatory system (EBIC, 2012). Furthermore, bio-stimulants are neither organic nor chemical fertilizers because they do not supply nutrients to soil or plants directly, instead they affect the metabolic processes in soil and plants by increasing the availability of nutrients to support both the metabolic and enzymatic processes of plants. They are biodegradable and non-polluting to the environment as they do not have negative effects on eukaryotic cells and soil ecosystems (Corte et al., 2014; Colla et al., 2015). Therefore, bio-stimulants hold the potential for organic farming as environmentally friendly strategies to alleviate drought stress and improve vegetable crop quality (Xu and Geleen, 2018; Pereira et al., 2019).

The use of bio-stimulants to enhance spinach growth, physiology and nutrition was studied under a few suboptimal conditions including, nutrient deficiency (Carillo et al., 2019), thermal stress (Craigie, 2011; Fan et al., 2013), and water stress (Xu and Leskover, 2015). Ekinci et al. (2015) reported that the application of an organic supplement at low concentrations had a positive effect on spinach yield, root diameter and length, leaf number and area. Xu and Leskovar (2015) also reported that the application of a seaweed extract as a bio-stimulant improved spinach growth (relative water content and stomatal conductance). In support, a few studies on spinach (Reyes et al., 2018; Jabeen et al., 2019), and Swiss chard (*Beta vulgaris*) (Oelofse and van Averbeke, 2012; Nyathi et al., 2016; Maseko et al., 2019) without the application of a bio-stimulant, also reported reduced crop yield under drought stress. Most of these studies however, reported on the effect of plant extract products or animal-derived products separately. No information is available on the combined (plant extract and animal-derived) effect of bio-stimulant products.

In horticulture, bio-stimulants are categorised into four main groups mainly, (1) humic substances (HS), (2) protein hydrolysate and amino acid formulations (AA), (3) seaweed extract (SWE), and (4) plant growth-promoting microorganisms (which can also be classified in its group as bio-fertilizers). These categories are based on the origin or their source and the effect of each bio-stimulant on root growth and nutrient uptake and not their chemical composition (Bulgari et al., 2019). These bio-stimulants are produced either individually or as a "cocktail" of natural or synthetic hormones or

precursors (by-products) of plant hormones or animal protein hydrolysates (Calvo et al., 2014; du Jardin, 2015; Colla and Rouphael, 2015). Important sources and precursors of bio-stimulants development include, food waste streams, compost, manure, vermicompost, aquaculture, and fish processing waste streams and sewage treated products (Xu and Geelen, 2018). These bio-stimulants are available in liquid extracts, soluble powder, and granular forms and can be applied as both foliar or soil applications (Madende and Hayes, 2020).

2.6.1 Humic substances (HS)

Humic substances comprise more than 60% of the soil's organic matter and are major components of organic fertilizers (nitrogen and carbon). They are formed due to the decomposition of microbial end-products and the chemical degradation of dead biota in soils or marine (Calvo et al., 2014; du Jardin, 2015). It is important to note that these substances do not contain microbial life but stimulate beneficial microbes and improve microbial diversity in soils, differentiating them from compost (Calvo et al., 2014). These substances are the most abundant naturally occurring organic molecules on earth (common in freshwater and soil). In soils, they control nutrient availability (i.e., ensure that existing nutrients become mobile in soils, increase the availability of phosphorus, and stabilize nitrogen and sulphur), and facilitate the exchange of carbon and oxygen between the soil and the atmosphere (Canellas and Olivares, 2014). The functional groups of these substances contain oxygen, nitrogen and sulphur that can form part of stable complexes with metal micronutrients such as iron. Humic substances have the capacity to stimulate soil bacteria and improve nutrient and water uptake in soils and enhance tolerance to environmental stress (Colombo et al., 2015).

Humic substances can be categorized according to their molecular weight, oxygen content, and ability to solubilize into humic acids, fulvic acids, and humins at specific pH values. For instance, humic acids are high-molecular-weight with a low oxygen content and fulvic acids with a higher oxygen content and low-molecular-weight (Baglieri et al., 2015). The humic acids are insoluble in pH values less than 2 but soluble in basic media and not extractable from the soil organic matter by dilute alkali and precipitate, while fulvic acids are soluble in both alkali and acid media whereas humins are insoluble at both low and high pH values (Calvo et al., 2014).

These substances can be applied in several ways, including pre-treatment of seeds or seedling plants, foliar applications, in the irrigation water, and direct applications to the soil (Halpern et al., 2015). Their application has been shown to hold the potential to improve abiotic stress tolerance (drought and saline stress) in plants (Zhang et al., 2003), increase nutrient uptake (zinc, iron, copper, phosphorus, nitrogen, and manganese) in roots (Asli and Neumann, 2010; Ertani et al., 2015; Polyakov et al., 2019). Some literature reported that application of humic substances from vermicompost increased the number of fruits and flowers in peppers, and improved fruits quality (Arancon et al., 2006). On a related note, humic extracts from vermicompost enhanced antioxidant enzymes and ROS scavenging enzymes in rice in a hydroponic study (García et al., 2012).

2.6.2 Protein hydrolysate and amino acid formulations

Protein hydrolysates are peptides mixtures, amino acids, and other nitrogen-containing compounds obtained from agricultural by-products from both plants and animals and are produced via chemical or enzymatic protein hydrolysis (du Jardin, 2012; Calvo et al., 2014; Colla et al., 2015; Halpern et al., 2015). Protein hydrolysates are categorised into two groups based on their origin, animal, or plant origin. Examples of protein hydrolysates from animal origin include leather by-products, blood meal, fish by-products, chicken feathers and casein, whereas plant origin protein hydrolysates include legumes seeds, alfalfa hay, and vegetable by-products (Colla et al., 2015). Most protein hydrolysates are produced from chemical hydrolysis of animal by-products (e.g., collagen, epithelial tissues) while enzymatically processed plant-based products (crop residues) are a recent development (du Jardin, 2012; Calvo et al., 2014; Colla et al., 2015; Halpern et al., 2015;).

Previously animal-derived protein hydrolysates were generated from chicken feathers as a bio-stimulant (Genç and Atici, 2019). Currently fish protein hydrolysates are used as bio-stimulants, the majority are derived from fish skins as well as other fish by-products including, heads, muscle, viscera, bones, tails, and fins (Madende and Hayes, 2020). The composition of fish protein hydrolysates contains many bioactive peptides and amino acids that have been demonstrated to have higher physical, chemical, and functional properties compared to its counterpart animal- and plant-derived proteins (Colla et al., 2015; Cristiano et al., 2018). Furthermore, previous literature showed that fish protein hydrolysates contain antioxidants that act against

free radicals, and antimicrobial peptides (Halim et al., 2016). These substances stimulate plant growth, increase cold and drought tolerance, and reduce the use of nitrogen fertilizers (Ugolini et al., 2015; Colla and Rouphael, 2016).

Safety assessments by Corte et al. (2014) indicated that there was no genotoxicity or phytotoxicity based on bioassays observed using yeasts and plants as test organisms, concluding that protein hydrolysates do not have negative effects on eukaryotic cells and soil ecosystems. This showed that, protein hydrolysates can be utilized in both conventional and organic agriculture without causing harm to people or the environment (Corte et al., 2014; Colla et al., 2017). However, the European Regulation No. 354/2014, still restrict the use of animal protein hydrolysates (including fish protein hydrolysates and fish meal) on edible parts of organic crops, disputing that chemical hydrolysis can produce compounds toxic to cells and the environment (Colla et al., 2017). Thus, suggesting that animal-derived protein hydrolysates can be applied as bio-stimulant mainly on non-edible parts (seeds, roots, and leaves) of organic crops in horticulture.

The application of protein hydrolysates as bio-stimulants has been studied in several crops including lettuce (Xu and Mou, 2017), tomato (Colla et al., 2014), and peppers (Ertani et al., 2014), where it was found to increase leaf number, chlorophyll content, root and shoot dry mass, relative water content, gas exchange, and stomatal conductance under climatic stresses or plant diseases. However, protein hydrolysates application did not increase the leaf area and chlorophyll fluorescence of lettuce (Xu and Mou, 2017).

2.6.3 Seaweed extract (SWE)

Seaweeds are macroscopic, multicellular marine algae, also known as marine macroalgae, large algae that grows in coastal regions of ocean water. Seaweeds make up 10% of the marine productivity and comprise of about 10 000 species, which is subdivided into three primary groupings based on their coloration (pigmentation) (Battacharyya et al., 2015). The three main groups include, Phaeophyta (brown), Rhodophyta (red), and Chlorophyta (green), with brown seaweeds (*Ascophyllum nodosum*, *Fucus Laminaria*, and *Durvillaea potatorum*) being the most common group, followed by red algae (*Lithothamnium calcareum*), and green *(Linnaeus spp.)*. Seaweed extracts have been utilized as sources of organic matter and as fertilizer

ingredients for many years in agriculture (Khan et al., 2009; Craigie, 2011). Currently seaweed extracts have been widely used in crops as bio-stimulants to promote plant growth, tolerance to salinity, drought, heat, and nutrients deficiencies (du Jardin et al., 2015; EL Boukhari et al., 2020). Seaweed extracts contain several plant-growth-stimulating compounds (plant hormones such as auxins, cytokinin, abscisic acid, and amino acids), growth-promoting constituents such as micro-and macronutrients, N-containing compounds like betaines, and sterols, and other natural biochemicals such as antioxidants and acids (du Jardin et al., 2015; Rasyid, 2017).

Xu and Leskovar (2015) reported that the application of a *Ascophyllum nodosum* seaweed extract to spinach via leaf and soil increased leaf relative water content and leaf area, respectively under water stress. Rouphael et al. (2016) observed that foliar application of *Ecklonia maxima* seaweed extract on zucchini squash resulted in higher marketable yield, dry shoot biomass and crop quality under saline stress. According to Sharma (2019), the application of *Gracilaria dura* seaweed extract in wheat plants under water stress improved plant biomass and yield significantly. Furthermore, the author reported that the seaweed extract participated in the abscisic acid mechanisms, which conserves water during drought stress.

There are no studies on the effects of combined protein hydrolysate and seaweed extracts on the physiological, biochemical and growth responses of spinach under water deficiency stress. The application of such a natural bio-stimulant could enhance drought stress tolerance in spinach, which could be a possible eco-friendly solution under the current climatic changes.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Study site and plant material

The experiments were conducted over a two-year period, from August to December 2020 (Trial 1) and from February to June 2021 (Trial 2), at the greenhouse facility of the University of the Free State, Bloemfontein (29° 6'31.94"S, 26°11'18.95"E) at 25°C (day) and 18°C (night) temperatures, under natural light. Spinach seeds (*Spinacia oleracea* L.) cv., Fordhook Giant, purchased from a local nursery (Bloempark Kwekery - Nursery, Free State, South Africa) were used in this study. This cultivar was used because of its broad leaves, and it is also a popular commercial cultivar in SA.

3.2 Experimental setup

3.2.1 Germination, transplantation, and fertilisation

Seven litre (7 L) pots (25 cm diameter and 20 cm height) were prepared beforehand, using the following procedure: A nylon mesh was cut into a circle (4 g) to line the bottom of the pot. Coarse gravel (590 g) was placed on the nylon mesh at the base of the pots, followed by loamy sandy red soil (7000 g) to fill the pots to 3 cm to the brim.

Spinach seeds were germinated in seedling trays filled with a seedling mix, Hygromix (Hygrotech (Pty) Ltd., Pretoria, South Africa) and watered daily. Ten to fourteen days after germination, seedlings were transplanted into the pots (one per pot). The seedlings were allowed to establish at soil field capacity (section 3.2.2.1 explains the volume of water needed to maintain soil at field capacity). To avoid nutrient deficiencies, the seedlings were fertilised with half strength nutrient solution, followed with full-strength nutrient solution every two weeks thereafter for the duration of the experimental period. The nutrient solution consisted of macro- and micro-nutrients as listed in Table 3.1.

The fertilization protocol used was from Hydrotech, SA. This product consists of two nutrient stock mixtures, namely (A) Hygroponics and (B) Solu-Cal (calcium nitrate). By mixing these stock mixtures according to the manufacturer’s guidelines, a balanced nutrient solution was obtained for pot trials. Half strength nutrient solution consisted of

0.5 g/L Hygroponics and 0.4 g/L Solu-Cal and full strength 1 g/L Hygroponics and 0.8 g/L Solu-Cal.

Table 3.1. Macro- and micronutrient content of full strength Hygroponic and Solu-Cal stock mixtures.

Essential element	**Nutrient regime composition (g/kg)**
Macronutrients	
Nitrogen (N)	210
Phosphorus (P)	42
Potassium (K)	208
Calcium (Ca)	160
Magnesium (Mg)	30
Sulphur (S)	64
Micronutrients	
Boron (Br)	0.373
Copper (Cu)	0.02
Iron (Fe)	1.49
Manganese (Mn)	0.3
Molybdenum (Mo)	0.037
Zinc (Zn)	0.05

3.2.2 Water treatments

For Trial 2 (2021), plants were grown under 30% (severe drought), 50% (mild drought) and 100% (control) water levels (as explained in section 3.2.2.2). Each water treatment consisted of 12 plants. The moisture in the pot plants was maintained with daily hand-irrigation using tap water to field capacity (see equations 1 - 3 for the amount of water required).

3.2.2.1 Soil holding capacity

Soil field capacity or maximum (100%) soil water holding capacity (WHC) is referred to as the amount of water that is held by soil particles against the force of gravity, after the soil has been saturated and allowed to drain under gravity for approximately 24 hours (Ojo et al., 2016). The amount of water held in the soil sample is weighed against the dry weight of the sample to determine the water holding capacity (Nayeema et al. 2016).

To determine how much water was needed to bring the soil to field capacity (i.e., 100% water holding capacity, WHC), at least three pots containing air-dried loamy sandy soil, gravel, and sieve net (total weight of 7.8 kg; Table 3.2) were irrigated with water to the point of saturation and allowed to drain under gravity. The pots were covered on top with plastic to minimize surface evaporation and were weighed until a constant weight (9.3 kg) was obtained, which represents soil weight at field capacity. An analytical balance (Optika N3200, Italy) was used for weighing.

The amount of water held in the soil at field capacity was then calculated (equation 1).

Equation 1: Determining soil holding capacity

Water required for 100% WHC

9.3 Kg (total weight of wet pot) – 7.8 Kg (total weight of dry pot) = 1.5 Kg (1.5 L of water is needed to reach soil field capacity, i.e., 100% WHC).

Table 3.2 Determination of field capacity

Apparatus	**Weight (g)**
Pot	194
Dry soil	7000
Gravel	590
Sieve net	4
Average weight of a dry pot	**7788**
Average weight of a pot at field capacity	**9300**

3.2.2.2 Drought stress regimes

Using information from the field capacity determination (100% WHC) (section 3.2.2.1); the amount of water required to create and maintain the 30% and 50% WHC in the pots was calculated from equation 2. To induce water deficiency (drought) stress, irrigation was withdrawn until the pots reached the desired water level: for 50% WHC, the weight was maintained at 8.538 Kg (equation 2a); for 30% WHC weight was maintained at 8.238 Kg (equation 2b). Water deficiency stress was initiated four weeks after transplantation.

Equation 2: Water stress regimes

a) Water required for a 50% drought regime (i.e., 50% WHC)

1.5 → 100% WHC

∴ 50% WHC = 0.75 L of water

Pots must be weighed and maintained at 7.788 Kg + 0.75 Kg = **8.538 Kg**

b) Water required for a 70% drought regime (i.e., 30% WHC)

1.5 → 100% WHC

∴30% WHC = 0.45 L of water

Pots must be weighed and maintained at 7.788 Kg + 0.45 Kg = **8.238 Kg**

In Trial 1 (2020), mild drought stress was represented by 70% WHC while severe drought stress was represented by 50% WHC. However, the water treatments were adjusted for the 2nd trial (2021) because for most of the measured parameters, plants at 70% WHC treatment did not show any symptoms of drought stress, showing that the 70% WHC was not representing drought stress in spinach (see Appendix 1 for the trial 1 (2020 results)). For the results (i.e., the 2021 data) presented in this study, drought stress was represented by 50% (mild drought stress) and 30% (severe drought stress) soil WHC.

3.2.3 Bio-stimulant preparation and treatments

Xcell Boost was supplied by IntroLab SA (Pty) as 100% Hydrolysed Fish Protein (HFP) and 100% Kelp stock solutions. According to the manufacturer guidelines, the recommended dilution dosage is 80:20 ratio between HFP and Kelp. An HFP/Kelp working solution at recommended dosage (single concentration *Xcell Boost*, (BX1)) was prepared by adding 2 mL HFP and 0.5 mL Kelp stock solutions to 247.5 mL distilled water (i.e., 2 mL HFP and 0.5 mL Kelp extract with 247.5 mL distilled water to prepare 250 mL solution). A double concentration *Xcell Boost* dosage (BX2) was prepared by adding 4 mL HFP and 1 mL Kelp stock solutions to 245 mL distilled water (i.e., 4 mL HFP and 1 mL Kelp extract with 245 mL distilled water to prepare 250 mL solution).

The plants were sprayed directly on the leaves (five weeks after transplantation) to the point of "drip-off" (forming droplets) with the single concentration (BX1) and double concentration (BX2) solutions, and thereafter every 3rd week (i.e., 21 days) during the trial period. The control plants were sprayed with water only. The controls were separated during the bio-stimulant spraying, to avoid contamination.

3.3 Data collection

3.3.1 Determination of the physiological parameters using non-destructive techniques

3.3.1.1 Determination of the photosynthetic parameters

Non-invasive techniques were used to assess the photosynthetic efficiency and vegetation "greenness" of plants. Portable (hand-held) devices were used for measuring chlorophyll-*a*-fluorescence, chlorophyll content, normalized difference vegetation index (NDVI), and stomatal conductance values. Measurements were conducted every week between 9:00 AM and 12:00 AM when there was increased light intensity. A total of 10 weeks data points was collected and used to calculate the average values for each physiological parameter. Measurements were carried out on a fully expanded mature leaf from the middle region of the plant on the upper surface of the leaf. In spinach, central stem grows, and mature, and new leaves grow in the axils of the stem leaves eventually forming a rosette (crown) leaf arrangement with inner, middle, and outer leaves.

Leaves in the inner region were young and folded, while the middle leaves were mature and fully expanded. Lastly the outer leaves were old and eventually shed from the crown.

3.3.1.2 Chlorophyll-a-fluorescence

Lightweight leaf clips were used to dark-adapt the leaves for 45 minutes. The clip was opened, placed on one representative leaf per plant and the shutter plate on the clip was closed to exclude light, creating a dark adaptation. Chlorophyll-*a*-fluorescence was measured using a Pocket PEA chlorophyll fluorimeter (Hansatech Instrument Ltd., North American), by opening the clip and attaching the fluorimeter onto the clip. The fluorimeter automatically calculates the photochemical efficiency of photosystem (PS) II parameters (maximum PSII quantum yield (*Fv/Fm*), performance index absorbance (PI_{ABS}) of PSII, and total performance index (PI_{Total}), which give information about the efficiency of both PSI and PSII centres). The data recorded on the fluorimeter was downloaded to the (PEA Plus, version 1.10; Hansatech) software via a laptop/computer Bluetooth wireless communication to export different physiological parameters.

3.3.1.3 Normalized difference vegetation index (NDVI)

The NDVI measurements were taken using a Plant Pen NDVI 310 meter (Photon Systems Instruments Ltd., Czech Republic) on the sample leaf. The NDVI devices use the difference in reflected light at two different wavelengths, 660 nm (red light, RED), and 760 nm (near infra-red light, NIR) to calculate the NDVI index as follows:

NDVI = (NIR – RED) / (NIR + RED)

3.3.1.4 Determination of stomatal conductance

Stomatal conductance was measured weekly during the vegetative growth period between 10:00 AM and 11:00 AM using a SC-1 Leaf Porometer (Meter Group Inc.). This time interval was based on Wang et al. (2018) and Gadi et al. (2019) suggestions, stating that variations in relative humidity, temperature, and radiant energy are relatively low between 10:00 AM and 12:00 PM. Measurements were made on the fully expanded mature leaves from the middle region of the plant receiving sunlight on the upper (abaxial) surface of the leaf. Leaf area expansion can cause changes in stomatal number. Young spinach leaves can be difficult to work with due to their

uneven leaf surface. A leaf is clamped to an open chamber on the porometer, trapping relative humidity between the surroundings of the chamber and the leaf surface. A leaf porometer calculates the stomatal conductance from the relative humidity gradient (Gadi et al., 2019).

3.3.2 Determination of physiological and biochemical parameters using destructive techniques

To determine the biochemical parameters, destructive methods were used, which involved the sampling of a representative leaf every three weeks during the experiment for a period of 10 weeks. The average of three data points (3-, 6-, 9-weeks) in four replications, was used to represent a single value on a bar graph.

The chemicals used for the different biochemical analyses were supplied by the following:

a) Sigma-Aldrich, St Louis: acetone, anthrone, ninhydrin, polyvinyl pyrolidone (PVP), L-glutathione oxidised (GSSG).

b) Sigma-Life Sciences, St Louis: ascorbic acid, guaiacol.

c) Merck, Darmstadt Germany: dihydronicotinamide adenine dinucleotide phosphate ($NADPH_2$) tetrasodium salt, hydrogen peroxide (H_2O_2), glacial acetic acid, sulphuric acid, proline.

d) BDH VWR chemicals, France: ethanol.

e) BDH chemicals, Poole England: triton-X 100.

f) UnivAR, SAAChem, Krugersdorp South Africa: glucose, ethylenediamine tetraacetic acid (EDTA).

3.3.2.1 Determination of electrolyte leakage

Electrolyte leakage (EL), a measure of membrane stability, was measured according to the method described by Rolny et al. (2011). Ten (10) fresh leaf discs (0.8 cm) were allowed to float in 15 mL deionized water in a test tube, and conductivity was measured afterwards using a conductivity meter (Hanna Instruments, Inc.). This represented an

initial (C_0) electrolyte leakage reading. The leaf discs were then incubated for 3 h at room temperature and the conductance was re-recorded (C_{max}) afterwards. The discs were then boiled in a water bath for 10 minutes and allowed to cool to room temperature and the final conductance was recorded (C_{total}).
Electrolyte leakage was calculated as a percentage: % EL = 100 x ($C_{max} - C_0$) / C_{total}.

3.3.2.2 Determination of chlorophyll and carotenoid content

The chlorophyll content [Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*) and carotenoids] from frozen spinach leaves were determined according to the method of Lichtenhaler (1997). Leaf tissue (100 mg) was crushed in liquid nitrogen and extracted with 5 mL 80% (v/v) aqueous acetone. The homogenate was centrifuged at 3000 *x g* for 5 minutes at 4°C. The supernatant was transferred to quartz cuvettes and the absorbance read at 663 nm (Chl *a*), 645 nm (Chl *b*), and 470 nm (carotenoids) on Cary 100 Bio (Varian, Australia) and the contents calculated from absorbance values.

Chlorophyll and carotenoid content was calculated as follows:
Total chlorophyll (mg/g) = $(20.2*Abs_{645)} + (8.02*Abs_{663})$
Chlorophyll *a* (mg/g) = $(12.7*Abs_{663)} – (2.69*Abs_{645})$
Chlorophyll *b* (mg/g) = $(22.9*Abs_{645)} – (4.68*Abs_{663})$
Carotenoids (mg/g) = $[(1000*Abs_{470}) – ((3.27*Abs_{663}) + (1.04*Abs_{645}))] /227$

3.3.2.3 Determination of total soluble sugars

Total soluble sugar (TSS) content was measured using a modified method described by Irigoyen et al. (1992). Spinach leaves were oven-dried for 72 hours at 76°C to obtain dry tissue. Samples (0.2 g) dry leaves were extracted in 5 mL ethanol (96%, v/v). The extract was incubated in a water bath for 10 minutes at 80°C, where after the test tubes were cooled down and centrifuged for 10 minutes at 4000 *x g* at 4°C. The supernatant (ethanoic extract) was collected and used to determine the total soluble sugars by reacting 0.1 mL of the ethanoic extracts with 2.9 mL Anthrone reagent (150 mg Anthrone dissolved in 100 mL of 72% (v/v) sulphuric acid). The reaction mixtures were vortexed and incubated at in a water bath at 80°C for 15 minutes. A blue green colour developed, and the tubes were cooled down. The reaction mixtures (3 mL) were vortexed again and the change in absorbance was measured at 625 nm (Cary 100

Bio, Varian, Australia) using plastic cuvettes. The TSS content was expressed as mg glucose g^{-1} dry leaf tissue using a standard curve prepared from pure glucose.

3.3.2.4 Proline determination

Proline content was determined using a method described by Carillo and Gibon (2011). A leaf sample (0.3 g) was crushed in liquid nitrogen on ice and mixed with 4 mL 70% (v/v) ethanol. The homogenate was centrifuged at 3000 *x g* for 10 minutes at 4°C and the supernatant was collected and transferred into a clean test tube. Supernatant (500 µL) was transferred into a 2 mL Eppendorf tube and mixed with 500 µL 20% (v/v) ethanol and 500 µL of 1% (w/v) ninhydrin reagent prepared in 100 mL 60% (v/v) glacial acetic acid. The mixture was vortexed and incubated at 95°C in a water bath for 20 minutes and allowed to cool down. After cooling, the reaction mixture was centrifuged at 10 000 *x g* for 10 minutes. The change in absorbance was measured at 520 nm (Cary 100 Bio, Varian, Australia) against a blank of 70% (v/v) ethanol in plastic cuvettes. The free proline was determined using an L-Proline standard curve and expressed as mg proline g^{-1} fresh weight.

3.3.3 Enzyme assays

Enzyme extractions were done using a modified method of Pukacka and Ratajczak (2005). Frozen leaf material (0.5 g) was ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. The ground powder was mixed with 5 mL of extraction buffer (50 mM potassium phosphate buffer, pH 7.0, containing 0.1% (v/v) Triton X-100, 2% (w/v) polyvinylpyrrolidone (PVP), 1 mM ascorbate, and 1 mM EDTA). The homogenate was centrifuged (15 000 *x g*) at 4°C for 20 minutes, and the supernatant obtained served as the enzyme extract. All steps were carried out on ice.

3.3.3.1 Determination of protein concentration

The protein content from enzyme extracts were determined according to the method of Bradford (1976) using gamma-globulin as a standard (1.5 mg mL^{-1}). The absorbance was read at 595 nm on a microplate detector (Anthos Labtech Inc. GmbH, Salzburg, Austria) using a micro plate reader (Greiner Bio-One, Kremsmunster, Austria) and results were expressed per mg^{-1} protein.

3.3.3.2 Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was determined using a modified method of Mishra et al. (1993). The enzyme assay mixture (1 mL) contained 550 µL 50 mM phosphate buffer (pH 7.0), 200 µL 100 mM H_2O_2, 150 µL 0.5 mM ascorbate, 50 µL 0.1 mM EDTA, and 50 µL enzyme extract. The decrease in absorbance was measured at 290 nm (Cary 100 Bio, Varian, Australia) for 5 minutes at 20°C using quartz cuvettes. The ascorbate activity was calculated using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ and expressed as (mmol ascorbate mg^{-1} prot. min^{-1}).

3.3.3.3 Guaiacol peroxidase assay

Guaiacol peroxidase (GPX) activity was determined using the method of Zieslin and Ben-Zaken (1991). The assay solution (1 mL) contained 500 µL 80 mM phosphate buffer (pH 5.5), 50 µL 200 mM H_2O_2, 100 µl 50 mM guaiacol, 340 µL distilled H_2O, and 10 µL enzyme extract. Using plastic cuvettes, the increase in absorbance was measured at 470 nm (Cary 100 Bio, Varian, Australia) for 3 minutes at 30°C. The guaiacol peroxidase activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1} and expressed as (mmol tetraguaiacol mg^{-1} prot. min^{-1}).

3.3.3.4 Glutathione reductase assay

Glutathione reductase (GR) activity was determined by monitoring the oxidized glutathione (GSSG)-dependent oxidation of NADPH at 25°C for 3 minutes at 340 nm (Cary 100 Bio, Varian, Australia) as described by Foyer and Halliwell (1976). The reaction mixture (1 mL) contained 470 µL 100 mM potassium phosphate buffer (pH 7.8), 30 µL 2.0 mM EDTA, 230 µL 0.5 mM oxidized glutathione (GSSG), 230 µL 0.2 mM NADPH, and 40 µL enzyme extract. The glutathione reductase activity was calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ and expressed as (mmol oxidised glutathione mg^{-1} prot. min^{-1}).

3.3.4 Determination of vegetative parameters (height, mass, and morphological measurements)

The growth parameters (root length, dry weight, fresh weight, moisture content, and leaf area) were assessed at termination of the experiment, except for the plant height, which was assessed during the experiment until the end of the experiment. All plants were harvested, and the leaves, shoots, and roots were separated using a penknife. In addition, the leaf numbers per pot plant were recorded by visually counting the green leaves per pot plant.

3.3.4.1 Plant height

The plant height was measured from the base of the pot plant to the tip of the highest stem using a measuring tape to the nearest centimetre (cm). Plant height data was recorded every two weeks, from the beginning of the water stress treatment until termination of the experiment. A total of 5 data sets were collected over a 10-week period and averaged to a single data point for each treatment.

3.3.4.2 Root length

The roots were gently rinsed in tap water to remove the soil and dried using a paper towel. The roots length of each plant was measured using a measuring tape to the nearest cm. The measurements were taken from the collar (the point where the root and stem meet) to the tip of the root.

3.3.4.3 Moisture content (fresh and dry mass)

The moisture content was measured using a modified method described by Reyser et al. (2008). On the day of termination, all leaves, shoots, and roots were weighed separately to determine plant fresh mass (FM) using a Shimadzu (AUW 320) analytical balance. All vegetative structures were then oven-dried at 75°C (Labotec) for five days to a constant weight and the dry mass (DM) of each structure was recorded. The moisture content was calculated using equation 3:

Equation 3: Moisture Content (%)

Moisture content (%) = 100 - (*Dry mass/ Fresh mass) X 100*

3.3.4.4 Determination of relative water content (RWC)

The relative water content was determined according to the method described by González and González-Vilar (2001). Collection of leaves were done between 10:00 am and 12:00 pm. Fully expanded mature leaves from the middle region of the plant were used (because they were not too old or too young) for RWC measurement. Leaf samples were cut, and immediately sealed in airtight plastic bags to prevent moisture loss. The plastic bags were transferred to the laboratory on ice, leaves were quickly removed, and 10 fresh leaf discs were punched out using an 8 mm diameter cork-borer. The fresh weight (FW) of discs was recorded. The leaf discs were then hydrated in test tubes filled with distilled water (10 mL) for 24 hours in a dark cold room at (4°C). After hydration, the discs were quickly removed and blot-dried with tissue paper, and the turgid weight (TW) was recorded. To obtain the dry weight (DW), leaf discs were then oven-dried for 72 hours at 76°C.

Equation 4: Determination of relative water content (RWC)

RWC (%) = (FW – DW) / (TW – DW) * 100

Where FW is the initial fresh weight, TW is the turgid fresh weight, DW is the dry weight, and RWC is the relative water content.

3.3.4.5 Leaf area

The leaf area was determined using the modified method specified by Garnier et al. (2001b). An 8 mm diameter cork-borer was used to obtain 10 leaf discs randomly from each potted-plant leaves. The fresh mass of the discs was weighed using a Smart Super Hybrid Sensor (SHS) analytic balance. Leaf area was determined on the day of termination of the experiment.

The fresh mass of the discs combined with the total leaf mass (3.3.4.3) was used to calculate the leaf area of each plant using the following equation:

Equation 5: Leaf Area (LA)

Leaf area = Fresh of all leaves x (surface area x *n* discs)/ Fresh mass of *n* discs

Example:

Area of discs = 3.14 x $(0.4)^2$ x 10 = 5.024 cm^2

Total leaf mass = 42.35 g

Disc mass = 0.162 g

42.35 x 5.024 cm/ 0.162 g = 1313.37 cm^2

3.3.5 Statistical analysis

The experimental layout was a split-plot design with complete randomisation and four replications. The main plot was the water treatment, and the subplot was *Xcell Boost* treatment. Data was analysed using a two-way analysis of variance (ANOVA) on GenStat statistical software (Version 18, VSN International, Hertfordshire, UK). Mean differences between water treatments and bio-stimulant treatments were tested using Tukey's test at the significance level $P \leq 0.05$. Figures were plotted using SigmaPlot 7.0 software (Systat Software Inc., San Jose, Carlifornia, USA), with significant differences indicated by different letters on top of bars in each figure. Correlation analysis was performed to determine a relationship between the vegetative, physiological, and biochemical parameters under different water levels at different bio-stimulant concentrations using Statistical Analysis System (SAS, version 13.2; SAS Institute Inc, Cary, NC).

CHAPTER 4

4. RESULTS

The data presented in this chapter represents the findings of the 2021 trial. The 50% water holding capacity (WHC) represents mild water deficiency (drought) stress (50%), while the 30% WHC represents a severe water deficiency (drought) stress (70%). The 100% WHC represents well-watered soil (i.e., soil at field capacity) and served as the control. Results for the 2020 trial are presented separately in Appendix 1.

The data presented are the averages of four replicates for each treatment over the whole experimental period.

4.1 The photosynthetic and biochemical responses of *Xcell Boost* treated spinach under water deficiency stress.

4.1.1 Determination of photosynthetic efficiency

The *Fv/Fm* for the 50% and 30% WHC treatment remained unchanged (> 0.80) compared to the optimal water (field capacity) treatment (100% WHC). Within each water stress treatment, *Xcell Boost* applications had insignificant effect on the *Fv/Fm* irrespective of the concentration. All *Fv/Fm* means were above 0.8 (Figure 4.1).

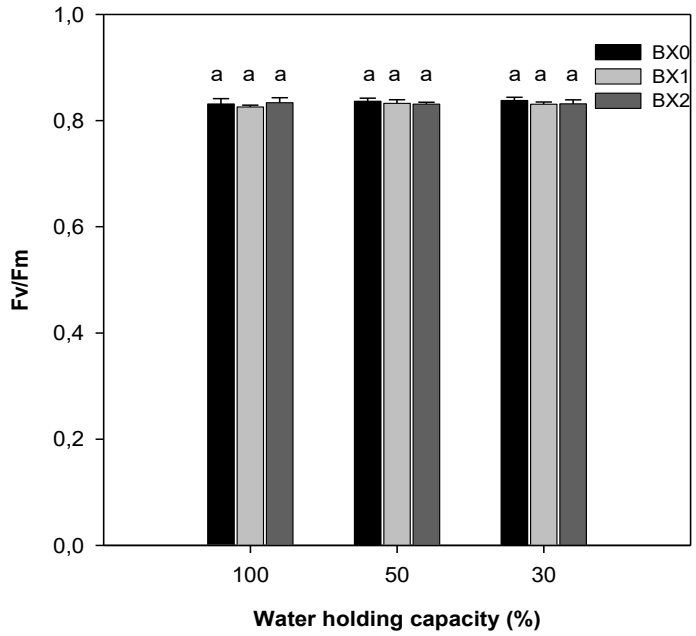

Figure 4.1: Maximum PSII quantum yield (*Fv/Fm*) of spinach grown under different water and *Xcell Boos*t levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

Compared to the 100% WHC treatment, the 50% and 30% soil WHC treatment stimulated an increase in the PI_{ABS} (23% and 14% increase respectively). Under well-watered conditions, application of single concentration Xcell *Boost* (BX1) significantly reduced the PI_{ABS}. Although slight reductions in this parameter were observed under 50% and 30% WHC, these were not substantial (Figure 4.2).

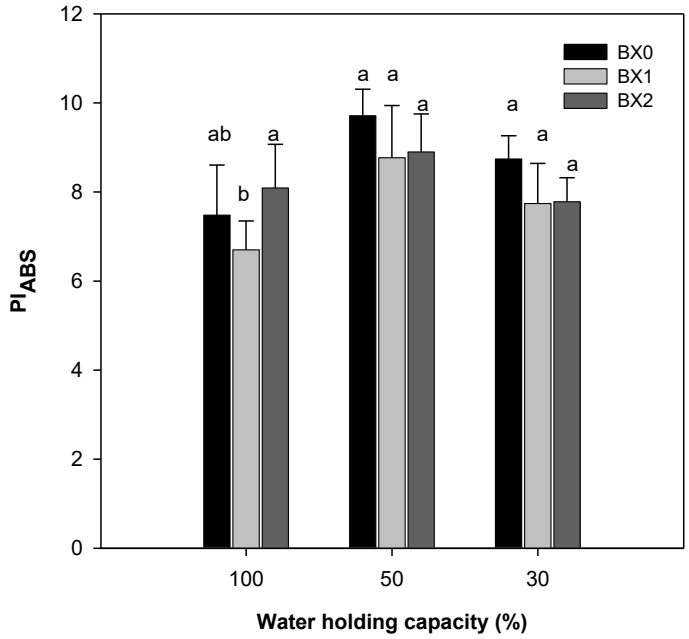

Figure 4.2: Performance index absorbance (PI_{ABS}), of spinach grown under different water and *Xcell Boos*t levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

Like PI_{ABS}, PI_{Total} also increased with stress application, especially at 50% WHC. There were insignificant differences between the different concentrations of bio-stimulant at 100% WHC and 30% WHC. The application of *Xcell Boost* (both concentrations) led to significant decreases in the PI_{Total} value of plants under 50% WHC (Figure 4.3).

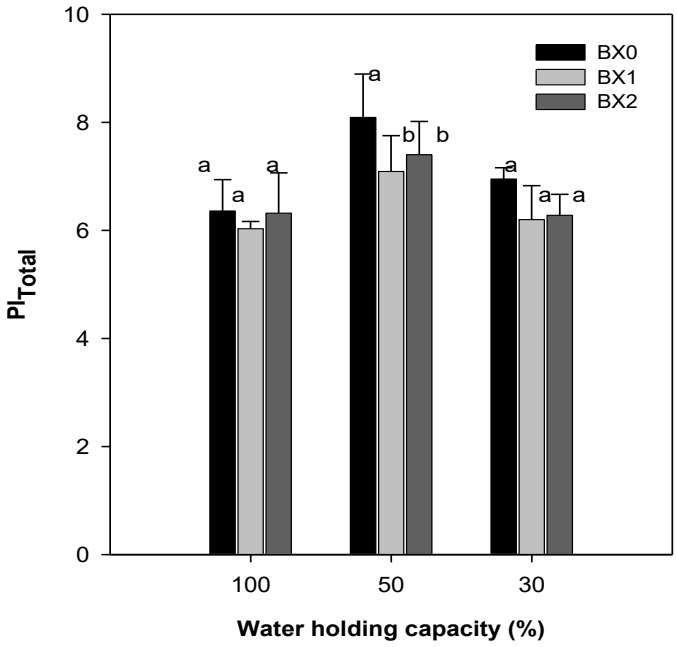

Figure 4.3: Total performance index (PI_{Total}) of spinach grown under different water and *Xcell Boos*t levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

The NDVI did not change with changing soil water content, as it was above 0.6 for all treatments. Under optimal watering, *Xcell Boost* did not have any effect on the NDVI values. Application of BX1 on the plant under 50% WHC treatment led to significantly increased NDVI. The NDVI values were significantly higher in BX1 than BX2 treatments under 30% WHC (Figure 4.4).

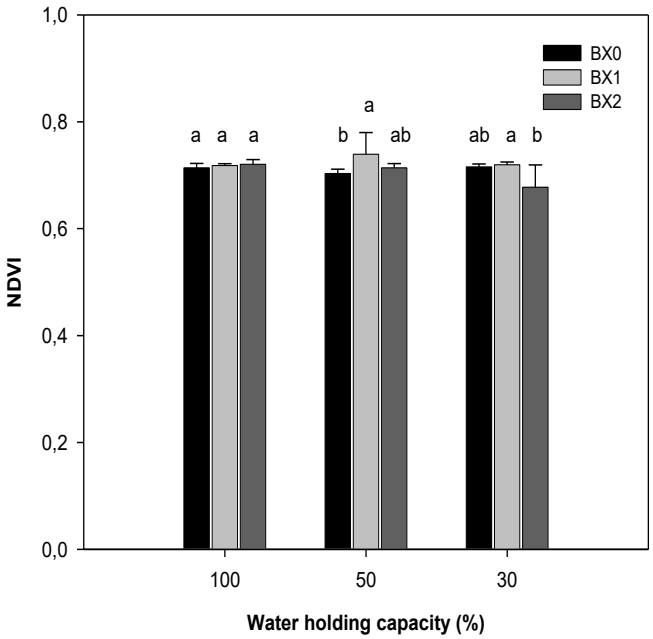

Figure 4.4: Normalised difference vegetation index (NDVI) of spinach grown under different water and *Xcell Boos*t levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

Reduction in the stomatal conductance was evident under 30% WHC treatment (13% reduction). Application of BX1 increased the stomatal conductance significantly for the well- watered plants. The concentration of BX1 showed the highest significant increase in the stomatal conductance under 30% WHC. The BX2 application also induced significant increase in this parameter, but the increase was lower than that of the BX1 treatment (Figure 4.5).

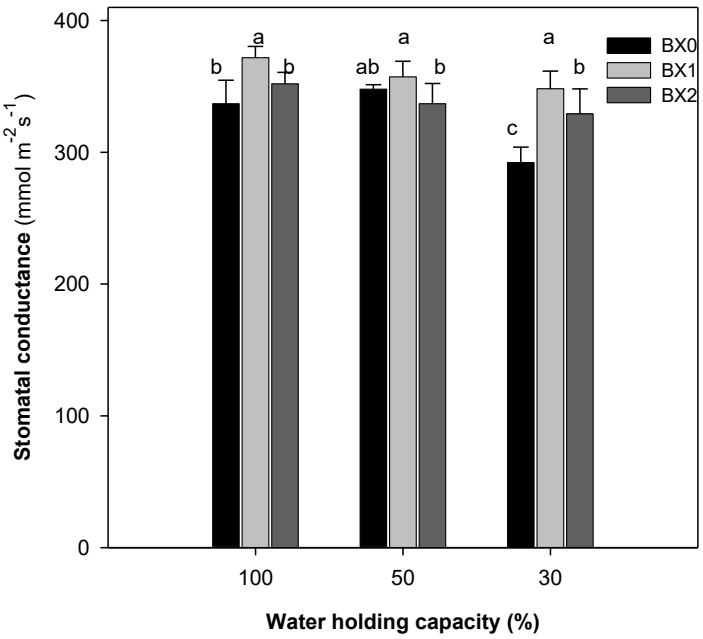

Figure 4.5: Stomatal conductance of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

The total chlorophyll content increased with the increase in the water-deficiency stress. The BX1 treatment was only significant under 100% WHC. The BX2 treatment induced significant increases in the total chlorophyll content for the 50% WHC (14% increase) and 30% WHC (14% increase) treatments (Figure 4.6). The Chlorophyll *a* content also increased with an increase in the water deficiency levels.

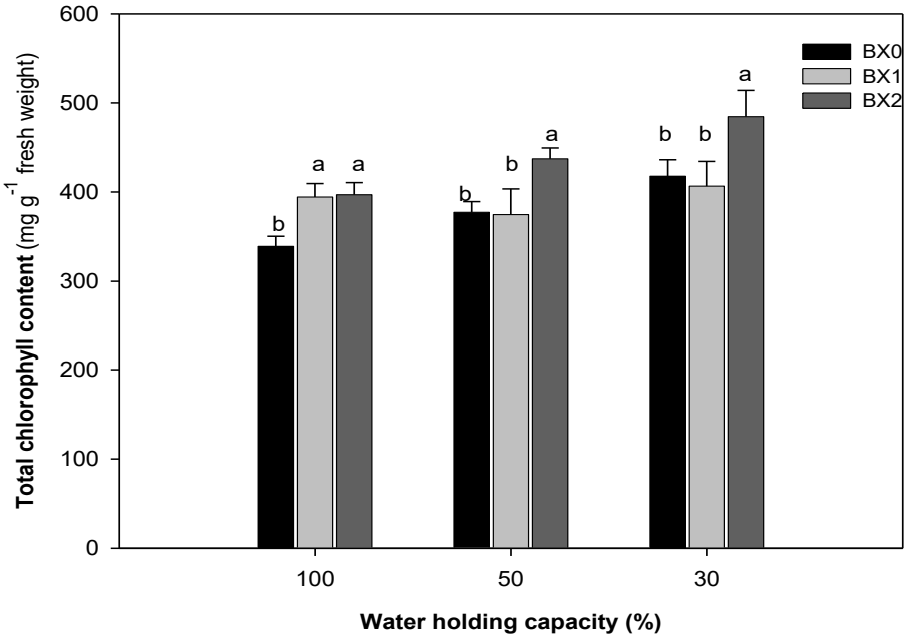

Figure 4.6: Total chlorophyll of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

The BX1 treatment was only significant under 100% WHC for Chlorophyll *a* content. The BX2 significantly increased the Chlorophyll *a* content for all water levels with the highest increase at 30% WHC (16% increase) (Figure 4.7).

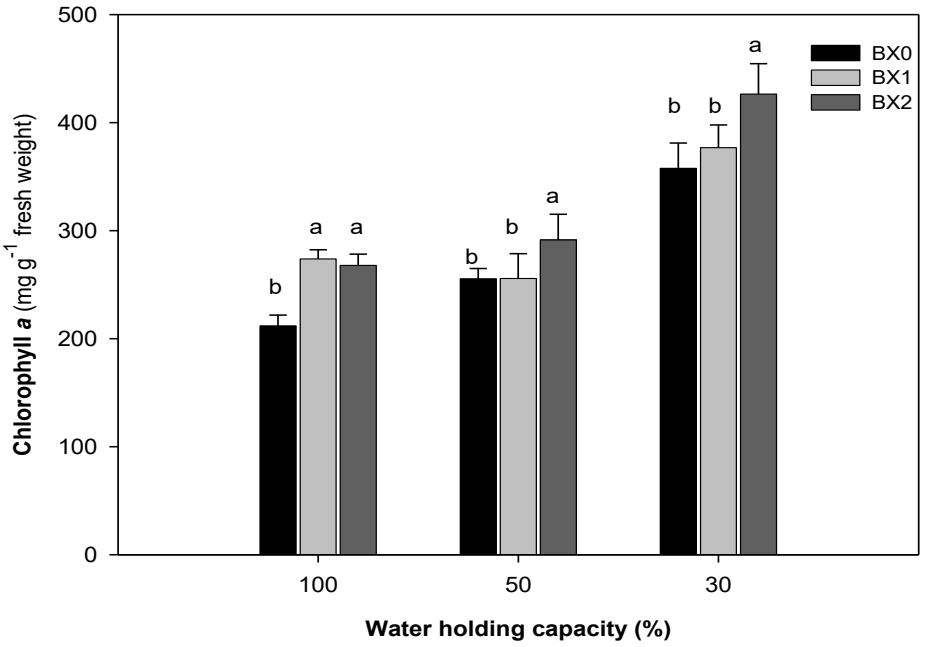

Figure 4.7: Chlorophyll *a* content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant).

The Chlorophyll *b* content declined with water deficiency treatments. Although the BX1 treatment reduced the Chlorophyll *b* content under 100% WHC, it led to significant increases under 30% WHC. The BX2 treatment induced the highest Chlorophyll *b* content under both water deficiency treatments (Figure 4.8).

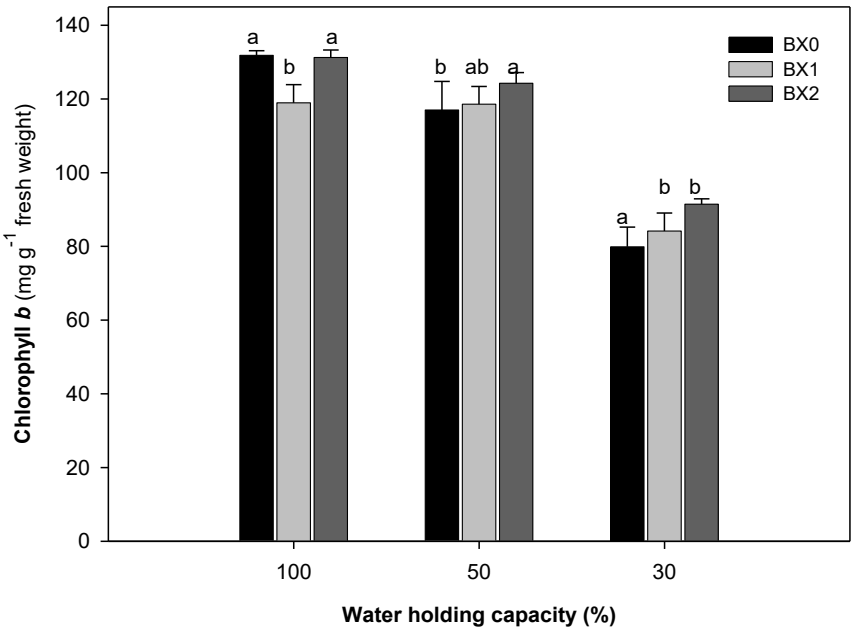

Figure 4.8: Chlorophyll *b* of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

The carotenoid content was slightly reduced under 30% WHC. Treatment of plants with BX1 was only effective under optimal water supply conditions (100%). The double dosage concentration of BX2 led to significant increases in carotenoid content at 50% and 30% WHC (13% and 19%) (Figure 4.9).

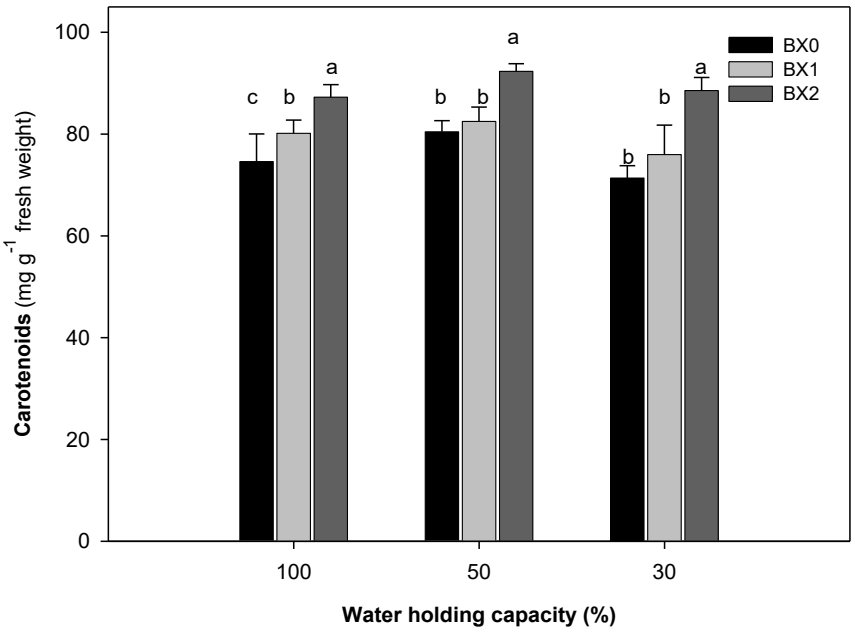

Figure 4.9: Carotenoid content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

4.1.2 Determination of osmolytes and electrolytes leakage

Figure 4.10 shows that the total soluble sugars (TSS) increased with the severity of drought. The BX1 induced significant increase in the TSS content under 50% WHC only. The BX2 treatment induced increases in the TSS accumulation under all water levels, with the 50 and 30% WHC showing the highest increases (37% and 22%).

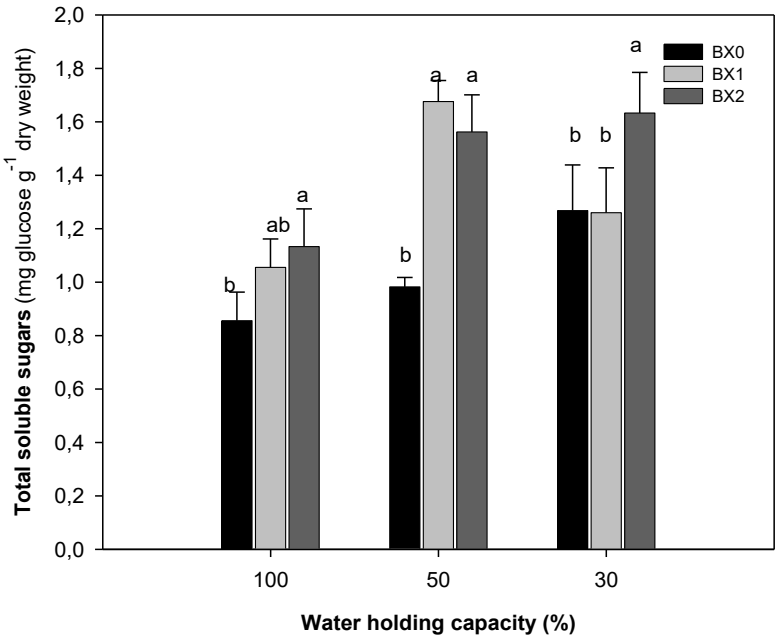

Figure 4.10: Total soluble sugar content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

Water deficiency stress only reduced proline content slightly. The BX1 treatment was only significant under 50% WHC. The BX2 treatment stimulated the highest levels of proline at 100% and 30% WHC (Figure 4.11).

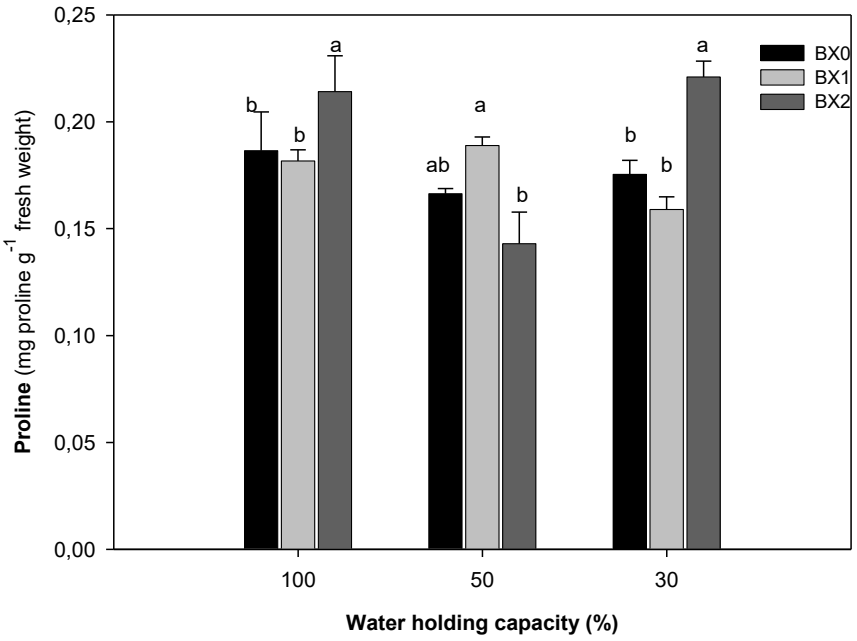

Figure 4.11: Proline content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

Water deficiency stress increased the electrolyte leakage according to Figure 4.12. The application of *Xcell Boost* significantly prevents the electrolyte leakage levels across all treatments, with the BX2 treatment showing the lowest electrolyte leakage (Figure 4.12).

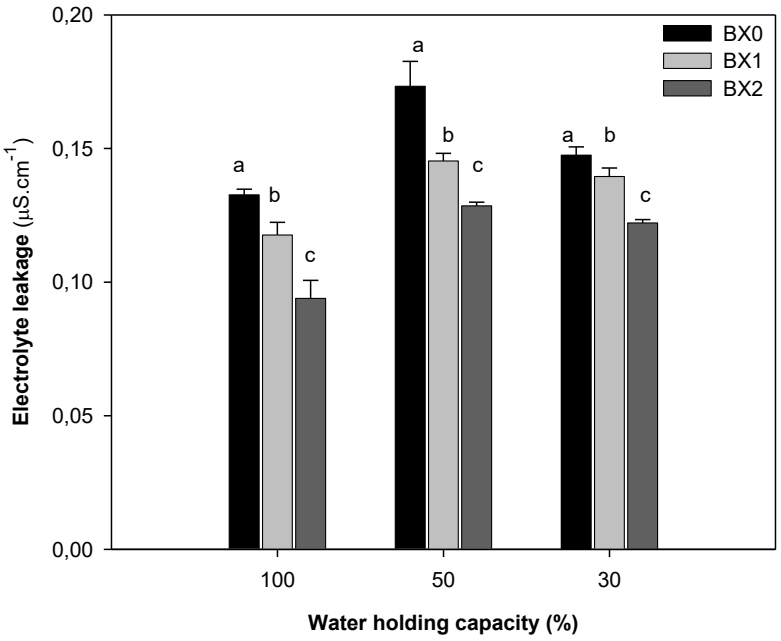

Figure 4.12: Electrolyte leakage content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

4.1.3 Determination of antioxidant enzyme activities

According to Figure 4.13, severe water deficiency (30% WHC) significantly reduced the ascorbate peroxidase (APX) activity. Treatment of plants with *Xcell Boost* induced the activity of APX under water deficient conditions only. The BX1 application increased APX activity under both water deficiency levels. The BX2 treatment induced the highest significant increase (21%) under 30% WHC.

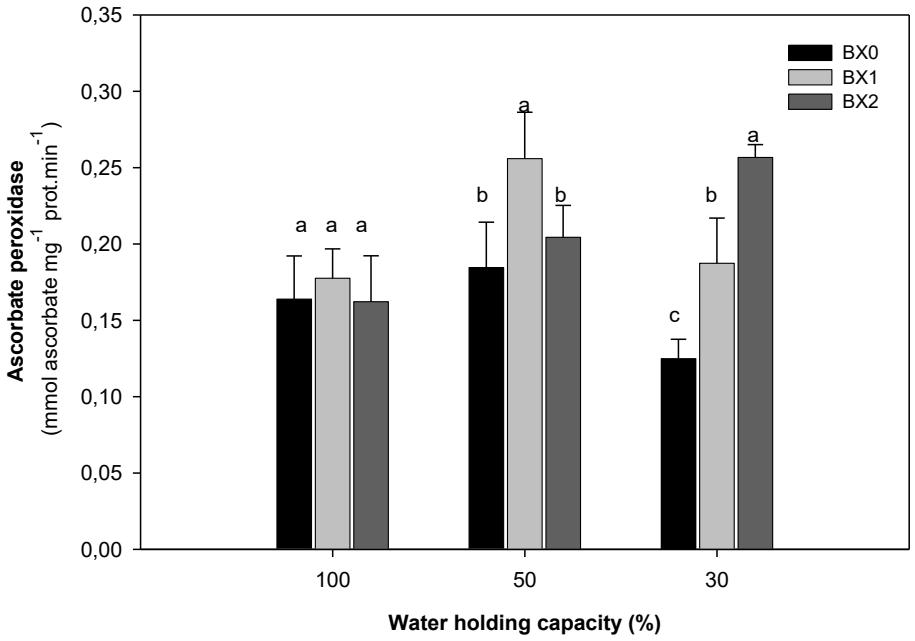

Figure 4.13: Ascorbate peroxidase activity of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

The guaiacol peroxidase (GPX) activity was substantially reduced under 30% WHC (53%). The two *Xcell Boost* concentrations had no effect on the activity of this enzyme under optimal water treatment but induced it under 50% and 30% WHC. BX2 had the highest significant increase at 30% WHC (53.5%) (Figure 4.14).

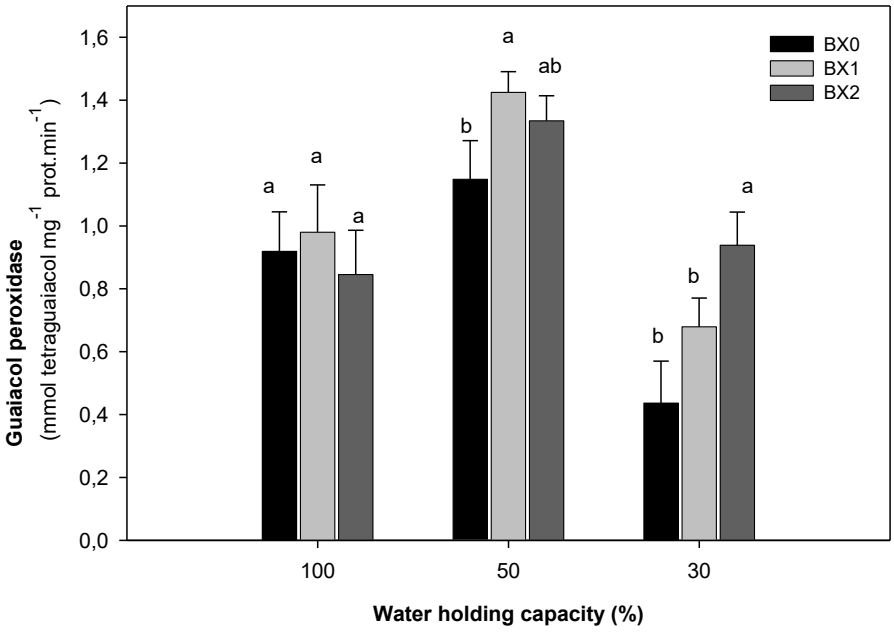

Figure 4.14: Guaiacol peroxidase activity of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

The activity of glutathione reductase (GR) increased with an increase in water deficiency. A substantial increase (3-fold increase) was observed under severe drought stress (i.e., 30% WHC). The application of *Xcell Boost* significantly decreased glutathione reductase activity at 100% and 50% WHC. Concentration of *Xcell Boost* increased glutathione reductase activity under 30% WHC, but this increase was not significant (Figure 4.15).

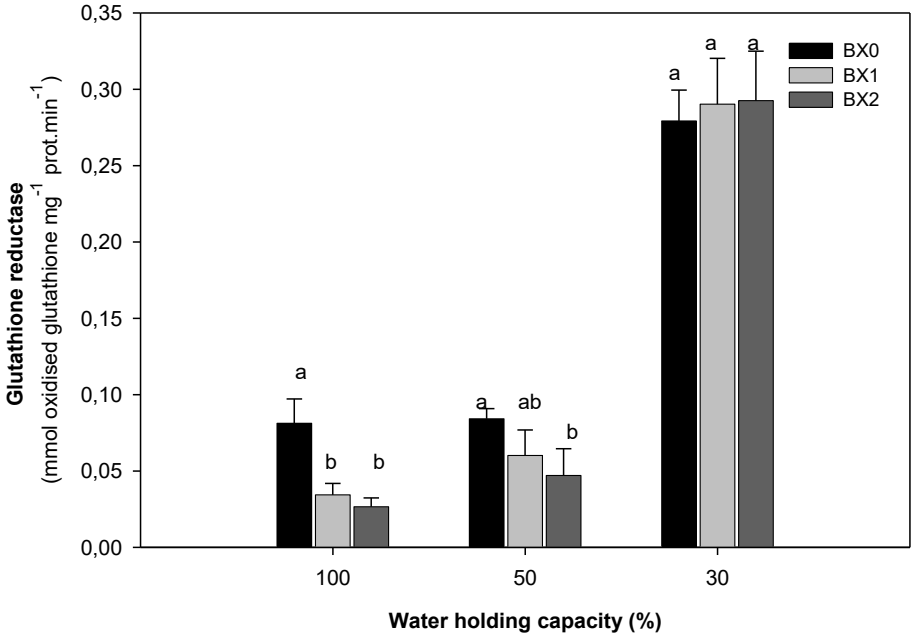

Figure 4.15: Glutathione reductase activity of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

4.2 The vegetative growth responses of *Xcell Boost* treated spinach under water deficiency stress.

Severe drought stress (30% WHC) reduced the plant height (22%). The application of BX1 significantly increased plant height under 30% WHC. Root length was reduced under 30% WHC treatment. There were insignificant differences in the root length under different concentrations of *Xcell Boost* at 100% and 50% WHC compared to the controls. The application of *Xcell Boost* significantly increased the root length under 30% WHC, with the concentration of BX2 showing the highest significant increase (25%) in root length (Table 4.1). The leaf surface area was substantially reduced under 50% and 30% WHC treatment. There was a significant difference in leaf surface area for the different concentrations of bio-stimulant at 50% and 30% WHC, but not at 100% WHC. The BX1 and BX2 treatments stimulated significant increases in the leaf surface area under 50% WHC. Under 30% WHC, only BX2 treatment showed a significant

increase in the leaf surface area. Water deficiency did not have a significant effect on the leaf numbers per plant. There were insignificant differences in the number of leaves for *Xcell Boost* concentrations under all water treatments. The 30% WHC treatment reduced the leaf dry weight substantially (24%). Addition of the *Xcell Boost* at both concentrations induced the leaf dry weights, but not significantly for both 50% and 30% WHC. A significant effect was observed for the BX2 under optimal watering. The stem dry weight was reduced under both water deficient conditions. The different concentrations of *Xcell Boost* had no effect on this parameter under 100% and 30% WHC. The BX2 treatment significantly increased the stem dry weight of spinach. Severe drought did not affect the root dry weight. The application of *Xcell Boost* had insignificant effect on root dry weight under both 100% WHC and 30% WHC. There was a decrease in the relative water content (RWC) under water deficient conditions. The BX1 treatment induced the RWC under drought stress conditions only. The concentration of BX2 had the highest significant increase in relative water content across all water levels. This treatment boosted the RWC to the level close to that of optimal water supply. Stem moisture content remained unchanged under water deficiency stress compared to the optimal water treatment. All treatments had a moisture content above 85%. The application of *Xcell Boost* had insignificant effects on the stem moisture content across all the treatments. Root moisture content decreased with an increase in severity of water stress. The application of BX1 concentration slightly increased root moisture under 100% WHC but significantly reduced the root moisture content under 50% WHC (7% decrease) and 30% WHC (19% decrease). The concentration of BX2 significantly reduced root moisture content under 100% WHC but increased root moisture content under water deficiency stress, showing the highest increase under 30% WHC (9% increase).

Table 4.1: Vegetative growth parameters of *Xcell Boost* treated spinach under water deficiency stress.

WHC (%)	[*Xcell Boost*]	Plant height	Root length	Leaf surface area	Leaf number	Leaf dry weight	Stem dry weight	Root dry weight	Relative water content	Stem moisture	Root moisture
100	BX0	23,5 ± 2,1a	26,5 ± 3,7a	0,8165 ± 87,18a	7,5 ± 0,65a	4,59 ± 0,6b	5,21 ± 0,69a	5,3 ± 1,21a	76,59± 0,82b	90,02 ± 1,04a	69,4 ± 1,28ab
	BX1	24,65 ± 2,2a	31,7 ± 2,25a	1365 ± 62,31a	6,33 ± 0,47a	5,48 ± 0,58ab	5,43 ± 0,50a	5,35 ± 1,31a	78,31 ± 1,07b	90,96 ± 0,41a	71,5 ± 2,79a
	BX2	23,7 ± 0,53a	29 ± 4,65a	1465 ± 79,1a	8,25 ± 0,75a	5,86 ± 0,45a	5,97 ± 0,48a	6,92 1,22a	80,29 ± 1,97a	90,25 ± 1,40a	65,8 ± 1,78b
50	BX0	23,4 ± 0,54a	28, ± 2,16a	899 ± 65,35b	7,33 ± 0,8a	4,39 ± 0,41a	3,84 ± 0,42b	7 ± 1,1a	72,73± 0,89c	88,94 ± 1,90a	60,6 ± 2,26a
	BX1	24,8 ± 2,03a	32 ± 2,62a	1290 ± 56,31a	6,75 ± 0,5a	4,62 ± 0,47a	4,54 ± 0,53ab	7,5 ± 1,2a	75,4 2 1,54b	89,38 ± 1,32a	56,2 ± 4,1b
	BX2	22,65 ± 1,65a	32,25 ± 4,24a	1321 ± 54,2a	7,75 ± 0,85a	5,38 ± 0,4a	5,23 ± 0,63a	7,43 ± 1,15a	78,93± 1,04a	88,83 ± 0,52a	62,5 ± 1,02a
30	BX0	18,4 ± 0,43b	24 ± 3,6b	723 ± 17,76b	7 ± 0,58a	3,5 ± 0,34a	3,15 ± 0,76a	4,92 ± 1,23a	68,35± 1,98c	89,75 ± 1,22a	56,2 ± 1,8b
	BX1	21,57 ± 1,2a	27 ± 1,41ab	799 ± 127,5ab	6,5 ± 0,625a	3,78 ± 0,15a	3,24 ± 0,66a	5,65 ± 1,2a	72,6± 0,63b	89,69 ± 1,26a	45,6 ± 4,24c
	BX2	19,3 ± 0,65ab	32 ± 0,8165a	843 ± 41,2a	7,87 ± 0,85a	4,32 ± 0,39a	3,41 ± 0,73a	6,17 ± 1,23a	76,49 ± 0,69a	88,11 ± 0,78	61,82 ± 1,93a

The values are means of four replicates ± standard error. Letters in the column indicate significant differences within each water treatment at $P \leq 0.05$. WHC: Water holding capacity. BX0 represents no bio-stimulant (*Xcell Boost*), while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

4.3 The correlations between the photosynthesis, vegetative and biochemical responses of *Xcell Boost* treated spinach under water deficiency stress.

The correlations between the photosynthetic capacity, biochemical responses, and vegetative growth parameters of *Xcell Boost* treated spinach were evaluated under severe water deficiency (30% soil WHC) treatment. The correlations for the 100% and 50% soil WHC are not presented because there were more significant changes under severe water deficiency stress.

Application of a single concentration bio-stimulant (BX1) under severe water deficiency indicated significant negative correlations between leaf dry weight (LDW) and guaiacol peroxidase (GPX), stem dry weight (SDW) and leaf moisture content (MC leaf), PI_{Total} with carotenes and Chlorophyll *a* (Chl-*a*). Significant positive correlations for this treatment included leaf dry weight (LDW) and leaf number, stomatal conductance (SC) with ascorbate peroxidase (APX) and glutathione reductase (GR), PI_{ABS} and proline, root moisture content (MC root) and PI_{Total} (Table 4.2).

In contrast, application of a double concentration bio-stimulant (BX2) under severe water deficiency indicated a high number of significant positive correlations and a few negative correlations ($P < 0.05$). The relative water content (RWC) negatively correlated to stomatal conductance (SC) and stem dry weight (SDW) while guaiacol peroxidase (GPX) negatively correlated to plant height. PI_{ABS} negatively correlated with Chlorophyll *b* (Chl-*b*). Significant positive correlations ($P < 0.05$) between root length (RL) with leaf moisture content (MC leaf), total chlorophyll (chl-total) and leaf dry weight (LDW) were observed. Other significant positive correlations under BX2 were between stomatal conductance (SC) with stem dry weight (SDW) and ascorbate peroxidase (APX), stem dry weight (SDW) and ascorbate peroxidase (APX) as well as glutathione reductase (GR) and normalised difference vegetation index (NDVI) (Table 4.2).

Table 4.2 The Pearson correlation analysis for the photosynthetic capacity, biochemical responses, and vegetative growth parameters of *Xcell Boost* treated spinach under severe water deficiency (30% soil WHC) stress.

	RL	LDW	SDW	RDW	APX	GPX	GR	Fv/Fm	Plabs	PItotal	SC	NDVI	Carotene	Chl-b	Chl-a	Chl-total	Proline	TSS	EL	RWC	Height	Leaf no	Leaf are	MC Stem	MC Root
RL	1	**0,9872**	-0,5	-0,3162	-0,3961	-0,0709	0,8627	0,5	-0,1906	-0,7693	-0,4059	0,8	0,07881	0,16134	-0,7528	**0,95934**	-0,7071	-0,7273	.	0,354	0,06325	0,4781	-0,5669	0,43778	**0,9781**
LDW	-0,0519	1	-0,6297	-0,2464	-0,5326	0,058	0,9023	0,4269	-0,2444	-0,7751	-0,5447	0,857	0,2321	0,1996	-0,6676	0,90723	-0,649	-0,7481	.	0,49347	-0,0351	0,495	-0,6878	0,29199	**0,97482**
SDW	0,7962	0,4208	1	-0,3162	**0,9902***	-0,5669	-0,647	0	0,2668	0,5585	**0,9943***	-0,7	-0,9004	-0,1613	0,03587	-0,28449	0	0,431	.	**-0,9833**	0,37947	-0,2391	0,9449	0,55909	-0,51
RDW	-0,1095	0,8044	0,513	1	-0,4384	-0,1505	-0,477	0,3162	0,6966	-0,3366	-0,3606	-0,4427	0,57636	-0,761	0,04446	-0,29519	0,89443	0,7156	.	0,46643	0,4	-0,8315	3E-05	-0,65883	-0,45618
APX	0,641	-0,626	0,046	-0,8322	1	-0,5503	-0,534	0	0,1789	0,53735	**0,9954***	-0,5941	-0,94648	-0,0697	-0,0188	-0,18755	-0,14	0,3041	.	**-0,9990***	0,33816	-0,1184	0,8982	0,65124	-0,3928
GPX	0,2294	**-0,977**	-0,3088	-0,8651	0,7762	1	0,4268	-0,82	-0,8117	0,34857	-0,6175	0,5345	0,63354	0,75583	0,7043	-0,34624	-0,0787	-0,4901	.	0,55935	**-0,9623**	0,5941	-0,7117	-0,62967	0,09531
GR	0,3974	-0,427	-0,1925	-0,8543	0,8962	0,5774	1	0	-0,637	-0,4466	-0,5843	**0,9921***	0,28332	0,5949	-0,3191	0,69665	-0,7625	-0,947	.	0,49836	-0,4501	0,7991	-0,8151	0,17006	0,9408
Fv/Fm	.	.	.	.	.	.	.	1	0,747	-0,8219	0,07302	-0,1	-0,18779	-0,7484	-0,921	0,68347	0	0,2155	.	-0,0197	0,88544	-0,4781	0,189	0,44833	0,31328
Plabs	-0,1849	-0,022	-0,5449	-0,585	0,3781	0,0996	0,7476	.	1	-0,3969	0,27263	-0,6921	-0,14147	**-0,9939***	-0,442	0,02521	0,60902	0,8096	.	-0,1669	0,92603	-0,9356	0,556	0,05928	-0,38992
PItotal	0,3791	0,143	0,022	-0,4488	0,5892	0,0325	0,8337	.	0,8195	1	0,48665	-0,3878	-0,30466	0,45315	0,82038	-0,7958	0,11177	0,1615	.	-0,5128	-0,4859	0,165	0,4022	-0,1342	-0,63187
SC	0,5265	-0,476	-0,0701	-0,8428	**0,9551**	0,6364	**0,9857**	.	0,6262	0,78415	1	-0,6486	-0,93939	-0,1653	-0,0616	-0,1809	-0,0775	0,3764	.	**-0,9932***	0,42119	-0,2071	0,9326	0,64267	-0,42218
NDVI	0,562	0,0075	0,7866	0,4555	-0,0634	0	-0,471	.	-0,9168	-0,5338	-0,3173	1	0,37156	0,64266	-0,2074	0,60615	-0,7071	-0,9429	.	0,56246	-0,5439	0,8128	-0,8693	0,05696	0,89213
Carotene	-0,1258	-0,193	0,186	0,427	-0,4342	0,0548	-0,768	.	-0,9247	**-0,9654**	-0,6779	0,7249	1	0,03336	0,29292	-0,13602	0,39244	-0,0847	.	**0,96**	-0,4027	-0,0267	-0,7814	-0,8613	0,08599
Chl-b	-0,311	-0,738	-0,3723	-0,2298	-0,0312	0,5937	-0,291	.	-0,4848	-0,7663	-0,241	0,2741	0,75076	1	0,43388	-0,03316	-0,6433	-0,7945	.	0,05707	-0,8965	0,9401	-0,4659	0,02292	0,36316
Chl-a	-0,4654	0,0365	-0,0197	0,5832	-0,736	-0,2188	-0,918	.	-0,7837	**-0,9806**	-0,8896	0,4703	0,916	0,64582	1	-0,90049	0,388	0,157	.	0,05298	-0,6977	0,1038	-0,0365	-0,66344	-0,61764
Chl-total	-0,3018	0,776	0,3357	0,9806	-0,9246	-0,8702	-0,902	.	-0,5321	-0,5131	-0,915	0,3324	0,443	-0,1504	0,65767	1	-0,6723	-0,5644	.	0,14477	0,31683	0,3094	-0,3193	0,61622	0,89775
Proline	-0,0978	0,0977	-0,4213	-0,4999	0,3641	0	0,7386	.	**0,9884**	0,88064	0,62232	-0,8704	**-0,96903**	-0,6111	-0,8303	-0,46841	1	0,8762	.	0,18078	0,26833	-0,8452	0,2673	-0,69744	-0,8011
TSS	0,3363	0,8349	0,8179	0,8808	-0,4849	-0,7929	-0,541	.	-0,4806	-0,0963	-0,4936	0,5493	0,17373	-0,5196	0,20651	0,77677	-0,3484	1	.	-0,2697	0,5895	-0,9466	0,6822	-0,27352	-0,85425
EL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RWC	0,2779	0,5194	0,7798	0,8682	-0,5274	-0,5433	-0,764	.	-0,8524	-0,5347	-0,67	0,8353	0,6335	-0,03	0,58368	0,78108	-0,7685	0,8675	.	1	-0,3421	0,0917	-0,8845	-0,68467	0,35051
Height	-0,5656	-0,748	-0,704	-0,3994	-0,0332	0,5917	-0,133	.	-0,1387	-0,5898	-0,1455	-0,1208	0,49223	0,92121	0,49519	-0,26514	-0,2873	-0,7456	.	-0,3553	1	-0,7408	0,5977	0,42199	-0,13109
Leaf no	0,0726	**0,9677**	0,5936	0,8999	-0,6381	-0,9487	-0,548	.	-0,2606	-0,0144	-0,5573	0,2582	0,00978	-0,6306	0,17292	0,84497	-0,1348	0,9422	.	0,71574	-0,738	1	-0,5421	0,24145	0,64918
Leaf area	0,8321	0,5023	0,8774	0,2885	0,2562	-0,3261	0,1883	.	-0,0761	0,49265	0,26288	0,4075	-0,30821	-0,7272	-0,4663	0,10904	0,06529	0,7073	.	0,44579	-0,9221	0,5703	1	0,42663	-0,63678
MC Stem	0,2885	-0,913	-0,0588	-0,5578	0,5599	0,8989	0,2109	.	-0,3465	-0,3357	0,31915	0,4	0,46226	0,77887	0,14464	-0,58403	-0,4364	-0,5403	.	-0,1392	0,62636	-0,7822	-0,283	1	0,41403
MC Root	0,3496	-0,002	-0,0797	-0,5786	0,6703	0,171	0,9032	.	0,855	**0,9884**	0,85337	-0,5771	**-0,95732**	-0,6608	**-0,9916***	-0,63107	0,8955	-0,2421	.	-0,6422	-0,4657	-0,1654	0,3902	-0,22023	1

Bold represents significance at $P < 0.05$ while bold plus asterisk represents significance at $P < 0.01$. RL= Root length, LDW= Leaf dry weight, SDW= Stem dry weight, RDW= Root dry weight, APX= Ascorbate peroxidase, GPX= Guaiacol peroxidase, GR= Glutathione reductase, SC= Stomatal conductance, Chl-*a* Chlorophyll a, Chl-*b*= Chlorophyll *b*, Chl-total= Chlolophyll total, TSS= Total soluble sugars, EL= Electrolyte leakage, RWC= Relative water content, MC stem= Stem moisture content, MC root= Root moisture content. A dot "." represents non-variable data.

The top right shaded triangle represents the correlations under double concentration (BX2) *Xcell Boost* treatment. The non-shaded bottom triangle represents the correlations under single concentration (BX1) *Xcell Boost* treatment.

CHAPTER 5

5. DISCUSSION

The *Fv/Fm* ratio is a reliable chlorophyll-*a* fluorescence parameter for evaluating plant health and detecting disturbances within the photosynthetic system under abiotic stresses such as drought (Krause and Weis, 1991; Banks, 2017). Previous studies illustrated a decrease in chlorophyll-*a*-fluorescence under drought stress (Li et al., 2018; Zhuang et al., 2020). Decreased chlorophyll-*a*-fluorescence indicates a potential damage to PSII and inhibition of primary reactions of photosynthesis (Lichtenthaler and Miehé, 1997). However, in this study, the *Fv/Fm* ratio slightly increased under water deficiency stress, contrary to the above findings. The application of *Xcell Boost* had insignificant effect on *Fv/Fm* (Figure 4.1). Different plant species usually have *Fv/Fm* that fluctuates between 0.75 and 0.86 units (Melo et al., 2017). The *Fv/Fm* of above 0.80 units in all treatments (Figure 4.1) indicates good health according to the international maximum standard value (Melo et al., 2017). Therefore, the non-significant effect of *Fv/Fm* to *Xcell Boost* treatment under all water treatments shows that this bio-stimulant does not affect the quantum efficiency of PSII irrespective of the water treatment.

Ceusters et al. (2019) reported the negative effects of drought stress on photosynthetic performance when studying chlorophyll fluorescence parameters in Crassulacean acid metabolism (CAM) orchid *Phalaenopsis*. Their results indicated that *Fv/Fm* remained unaffected while the PI_{ABS} parameter was lower in drought stressed plants compared to the control plants. In this study, substantial reduction of PI_{ABS} under 50% and 30% soil WHC (23% and 14% respectively) shows that drought stress has a negative effect on the PSII efficiency of spinach (Figure 4.2). Like *Fv/Fm,* the application of *Xcell Boost* had insignificant effect on the function of PSII under water deficiency stress, suggesting that it does not prevent the reduction of the photochemical reactions associated with PSII under drought stress. Although no substantial effect of *Xcell Boost* was observed under drought stress, a strong positive correlation ($r = 0.9884$, $P < 0.05$, Table 4.2) between PI_{ABS} and proline under BX1 treatment and severe water deficiency stress (30% soil WHC) shows that it influences proline accumulation, a very important molecule that improves tolerance of plants under drought stress

(Suprasanna et al., 2016). Therefore, although not directly involved in the photochemical reactions, *Xcell Boost* application plays an important role in improving photosynthetic capacity of spinach under drought stress.

In Figure 4.3, a similar pattern was observed for the 50% and 30% soil WHC treatments, where PI_{Total} increased with drought stress. Both concentrations of *Xcell Boost* showed insignificant effect under 100% WHC and 30% WHC. The application of both concentrations showed a significant decrease in the PI_{Total} under 50% WHC, which further shows no direct benefit of *Xcell Boost* on the photochemical reactions of the photosystems in agreement with $PI_{ABS.}$ Application of protein hydrolysates did not alter chlorophyll fluorescence in lettuce under full irrigation (Xu and Mou, 2017). Furthermore, Xu and Leskovar (2014) reported that leaf fluorescence is not affected under mild drought stress, and that the application of *Ascophyllum nodosum* seaweed extract had no effect on fluorescence parameters and function of PSII. According to the literature, there are limited studies on the application of seaweed extracts on the function of PSII. Similarly, there are no records of the application of both seaweed extracts and protein hydrosylates on the overall photosynthetic capacity. Although *Xcell Boost* does not have a direct effect on the total performance index of the photosystems under 30% WHC, it significantly correlated ($r = 0.9884$, $P < 0.05$, Table 4.2) with the root moisture content under single concentration (BX1) application. This coincides with the PI_{ABS} findings, where it was positively correlated to proline (Table 4.2).

The chlorophyll-*a* fluorescence parameters can be paired with other measurements to create a complete concept of the photosynthetic system (Murchie and Lawson, 2013; Banks, 2017). The NDVI parameter is used to estimate plant health, plant biomass, net primary productivity, and leaf area index under abiotic stresses such as water stress and nutrient deficiency (Pettorelli, 2013). Plants with NDVI values between 0.6 - 0.9 are considered healthy (Gandhi et al., 2015; Sentera, 2017). According to Cruisol et al. (2016), NDVI values of two soybean cultivars with contrasting responses to drought (drought-sensitive cultivar and less drought sensitive) were similar with slight differences observed under water deficit during the vegetative growing period. However, it was found that when both cultivars were subjected to water deficit at the reproductive stage, NDVI values of less drought sensitive were higher than that of drought-sensitive cultivar. In this study, the NDVI values did not change with the

application of drought stress (Figure 4.4). Under mild water stress, BX1 had NDVI value of (0.74) units compared to the control plants (0.71) units, showing an improvement of the spinach health. The application of *Xcell Boost* (BX1) significantly increased the NDVI values under water deficiency stress but had no effect on the NDVI values under full irrigation.

A decrease in the stomatal conductance is one of the earliest and common responses to water stress in plants (Liu et al., 2008; Wang et al., 2012). Leskovar and Xu (2014) reported that stomatal conductance was lower under mild water stress in cabbage. Also, according to Pazzaglia et al. (2016), stomatal conductance was significantly lowered under irrigation deficit in tomato plants. The combined effect of water deficiency and salinity stress significantly decreased stomatal conductance in spinach (Ors and Suarez, 2017). In agreement, the stomatal conductance was significantly reduced (13%) under severe drought stress in this study. The application of *Xcell Boost* prevents this by increasing the stomatal conductance under severe drought stress with BX1 showing the highest increase in significance. However, the BX2 concentration was also effective at inducing the stomatal conductance under drought stress (Figure 4.5). *Xcell Boost* clearly shows bio-stimulantory properties by reducing the negative impact of water stress deficiency, allowing plants to increase their stomatal conductance (increased CO_2 uptake), which could potentially lead to a higher photosynthetic potential. This higher photosynthetic capacity was clearly evident in increased PI_{ABS} (Figure 4.2) and PI_{Total} (Figure 4.3) levels under water stress conditions. Enhanced photosynthetic capacity will result in a higher yield potential and yield (Basu et al., 2016). Similarly, Ekinci et al. (2015) reported that application of a bio-stimulant induced the stomatal conductance in spinach under different irrigation levels. Therefore, it can be assumed that application of *Xcell Boost* at both concentrations is beneficial for spinach under drought stress.

Chlorophyll content is the main physiological index that can be directly related to the photosynthetic capacity in plants and provide insight on the integrity of the photosynthetic apparatus (croft and Gholamin, 2012; Croft et al., 2017). The total chlorophyll content and Chlorophyll *a* content increased with the severity of water deficiency (Figure 4.6 and Figure 4.7). A few studies also reported an increase in the chlorophyll content under mild water deficiency stress in purslane (Rahdari et al.,

2012), and mild saline water stress in spinach (Xu and Mou, 2016; Ors and Suarez, 2017). The application of different concentrations of *Xcell Boost* significantly increased total chlorophyll and Chlorophyll *a* content, with BX2 inducing the highest increase under drought stressed conditions. This suggests that *Xcell Boost* application (BX2) could increase the light energy harvested by the plants under drought stress, further increasing the photosynthetic capacity of spinach (Wu et al., 2019). However, Chlorophyll *a* negatively correlated with PI_{Total}.

The amount of Chlorophyll *b* decreased with the severity of drought stress (Figure 4.8). Contrary to the above findings, drought stress can also decrease the chlorophyll content (Khayatnezhad and Gholamin, 2012; Ping et al., 2015; Gebre et al., 2016). Plants adjust the amount of Chlorophyll (Chl *a*, Chl *b*, Chl *a*+*b*, chl *a*/*b*), carotenoids and anthocyanin pigments in order to adapt to different abiotic stresses and optimize their photosynthetic capacity (Li et al., 2018). According to Al Kharusi et al. (2019), salt-tolerant plants can protect their photosynthetic systems from abiotic stress by altering the amounts of pigments produced in their leaf tissues. Although application of BX1 and BX2 efficiently induced the Chlorophyll *b* content under 50% and 30% WHC, the BX2 was more effective in inducing the synthesis of Chlorophyll *b*. Since Chlorophyll *b* is an accessory pigment that transfers the light energy to Chlorophyll *a*, its accumulation maximises the photosynthesis capacity of spinach (Filimon and Filimon, 2016). Therefore, BX2 application improves tolerance of spinach under drought stress. The application of protein hydrolysates also increased chlorophyll content in lettuce (Naroozlo et al., 2019). In another experiment, Al-Ghamdi and Elansary, (2018) reported a significant increase in the total chlorophyll content of asparagus treated with *Ascophyllum nodosum* seaweed extract under saline irrigation.

In addition to their role as accessory pigments (Hashimoto et al., 2016), carotenoids preserve the chlorophyll molecules from photo-oxidative damage (Ramel et al., 2013). In the present study, the carotenoid content was slightly low under severe water deficiency stress (Figure 4.9). When screening for drought tolerant sorghum cultivars, Devnarain et al. (2016) reported that carotenoid content was not significantly reduced under both water stressed and non-stressed plants. The results of this study are almost similar, since the carotenoid content was slightly lower under severe water deficiency stress compared to the optimal water treatment. The carotenoid content increased to the level of non-stressed control plants when treated with double

concentration *Xcell Boost* solution, which further shows the importance of *Xcell Boost* in drought tolerance of spinach.

Since plants are sensitive to the environmental stresses, they have developed strategies to protect their metabolic functions. Plants adapt to water stress by upregulating the synthesis of soluble sugars and other osmolytes to increase their osmotic potential and improve water retention (Rosa et al., 2009; Fang and Xiong, 2015). The accumulation of the total soluble sugars (TSS) significantly increased with the severity of water deficiency (Figure 4.10). Proline content slightly decreased with the severity of drought (Figure 4.11). Proline plays a key role in plant defense as an osmoprotectant and can act as an antioxidant due to its reactive oxygen species (ROS) scavenging abilities (Akram et al., 2016). Proline also plays a crucial role in plant metabolism (Liang et al., 2013), hence its low accumulation is not necessarily due to a stress response only. Our findings are not entirely in accordance with previous results that show that drought stress increases soluble sugars and free proline content in plants (Liang et al., 2013; Yan, 2015; Suprasanna et al., 2016). Application of BX1 showed a general trend by significantly increasing TSS (Figure 4.10) and proline content (Figure 4.11) at 50% WHC. The application of BX2 resulted in a significant increase in the TSS content under water deficiency treatments, as well as in proline content under 100% and 30% WHC. Yuan et al. (2019) and Song et al. (2019) used wheat seedlings (*Triticum aestivum* L.) and rice seeds (*Oryza sativa* L.) respectively, to study the effect of polysaccharides from seaweed extracts, *Lessonia nigrescens* and *Grateloupia filicina*, respectively, under salt stress. Their findings also indicated that the use of seaweed extracts enhance sugars and proline accumulation. Accumulation of proline and TSS play a crucial role as osmoprotectants in response to drought stress and help protect cells from ROS (Zulfiqar et al., 2019).

Drought stress results in excessive production of ROS causing oxidative stress. Oxidative stress damages the plasma membrane, resulting in high solute and electrolyte (ion) leakage (Masoumi et al., 2010). The level of electrolytes leakage (EL) significantly increased with an increase in water deficiency stress (Figure 4.12), suggesting more membrane damage. Ekinci et al. (2015) reported that EL significantly increased with a decrease in irrigation amounts. However, the application of *Xcell Boost* at double concentration significantly prevents EL across all treatments, indicating its bio-stimulant properties by making spinach more tolerant under drought

stress. Feitosa Vaconcelos et al. (2009) stated that the application of humic acids on common bean (*Phaseolus vulgaris* L.) plants subjected to saline stress, showed reduced membrane damage. Similarly, Patel et al. (2018) reported that a seaweed (*Kappaphycus alvarezii*) extract application reduced EL and lipid peroxidation (malondialdehyde) under saline and drought stress in three *Triticum durum* varieties. In order to reduce the EL, plants need to possess active ROS scavenging systems. Since *Xcell Boost* application effectively reduced the EL, it is worth investigating if this bio-stimulant may upregulate the antioxidative capacity of spinach under drought stress. Therefore, the involvement of antioxidative enzymes in *Xcell Boost* treated spinach is discussed.

The antioxidant defense systems play important roles in the drought tolerance of different plant species such as in radish (Shafiq et al., 2015), cabbage (Yan, 2015), and edamame (Moloi and van der Merwe, 2021). In this study, the activities of ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) were substantially inhibited under severe water deficiency (30% WHC) stress (Figure 4.13 and Figure 4.14). Similarly, Sahin et al. (2018) found that the antioxidative enzyme activities of cabbage decreased with an increase of drought. However, the responses differ because glutathione reductase (GR) activity substantially increased (3-fold-increase) under severe drought stress. The application of *Xcell Boost* had insignificant effect on the activity of APX and GPX under optimal water treatment. However, it induced the activity of the enzymatic antioxidant enzymes under 50% and 30% WHC. The concentration of BX2 showed the highest significant increase at 30% WHC for APX (21%) and GPX (53.5%). This study agreed with the findings of Trivedi et al. (2018) in maize using *Kappaphycus alvarezi* seaweed extract under drought stress. Al-Ghamdi and Elansary (2018) also reported an increase in asparagus using *Ascophyllum nodosum* seaweed extract under saline conditions. This is an indication that *Xcell Boost* has a potential of improving the antioxidant capacity of spinach against oxidative stress under water deficiency stress. The strong, positive correlation between the APX and SC (Table 42), further indicates that *Xcell Boost* plays an important role in the improvement of plant performance under severe drought stress. In contrast to the other antioxidative enzymes, drought stress increased the activity of glutathione reductase (GR) (Figure 4.15), suggesting increased ROS scavenging ability of spinach. *Xcell Boost* treatment (irrespective of the concentration) had no effect on the

GR activity (activity stayed the same as that of the control), suggesting that the observed reduction in EL under treatment with this bio-stimulant was not influenced by this enzyme. Interestingly under severe drought stress GR positively correlated with the NDVI (under BX2) and stomatal conductance (under BX1) (Table 4.2). Therefore, although GR is not directly involved in making spinach tolerant to water stress under *Xcell Boost* treatment, it is indirectly involved in improving spinach greenness (health), leading to improved CO_2 fixation and a high photosynthetic rate.

The relative water content (RWC) is a useful drought stress indicator that is closely associated with the leaf water status and cell turgor (Ihuoma and Madramootoo, 2017). Several studies have reported that RWC decreased with increased levels of water stress in various vegetable crops such as squash (Abd El-Mageed and Semida, 2015), tomato (Bahadur et al., 2015), and cabbage (Sahin et al., 2018). In this study, RWC decreased under water deficient conditions (Table 4.1). The application of *Xcell Boost* significantly increased RWC under drought stress conditions especially at high concentrations (BX2). This increase could possibly be a result of increased TSS and proline under BX2 and 30% WHC (reported above), which are involved in osmo regulation (Zulfiqar et al., 2019), thereby improving the water content of the leaves. Xu and Leskovar (2015) reported that RWC was significantly reduced under mild water stress in spinach and the application of an *Ascophyllum nodosum* seaweed extract improved RWC. The increased RWC in drought stressed spinach treated with *Xcell Boost* could emanate from increased carotenoid content (Table 4.2).

Plant dry weights (leaf and stem) were negatively affected by water deficient conditions, except for root dry weight (Table 4.1). Severe drought stress reduced the vegetative parameters such as plant height and root length (Table 4.1). Leaf surface area was reduced under water deficient stress, but the number of leaves was not significantly affected (Table 4.1). Previous studies conducted on spinach agree that leaf growth is inhibited under abiotic stresses such as salinity and nutrient deficiency (Xu and Mou, 2016), salinity and water deficiency stress (Ors and Suarez, 2017; Jabeen et al. 2019). The application of *Xcell Boost* had insignificant effect on leaf dry weight (50% and 30% WHC), stem dry weight (100% and 30% WHC), and root dry weight (100% and 50% WHC). However, the BX2 concentration significantly increased leaf dry weight under optimal irrigation, stem dry weight under mild stress, and induced

root dry weight under mild stress but not significant. The different concentrations of *Xcell Boost* significantly increased plant height (BX1) and root length (BX2) under 30% WHC, respectively. Both concentrations of *Xcell Boost* significantly increased the leaf surface area under mild water stress but showed the least significant increase under severe water stress, except for the BX2 concentration. The application of *Xcell Boost* had insignificant effect on the number of leaves under all water treatments. Xu and Leskovar (2015) reported that the application of an *Ascophyllum nodosum* seaweed extract improved spinach leaf growth under drought stress. Furthermore, this study shows that *Xcell Boost* treatment increases the leaf dry weight along with increased root length (under BX2) and increased leaf numbers (under BX1) (Table 4.2). This implies that *Xcell Boost* application further benefits spinach growth and yield, especially under severe drought stress.

The determination of moisture content is also an indicator of plant water status, which is essential in understanding plant growth (productivity) and development. When evaluating stem moisture content (Table 4.1), it was found that all plants' stems (vegetative structures) had a moisture content above 85% for all treatments. This is an indication that enough water was stored in the stem tissue to support transpiration (optimal), which can be linked to photosynthesis and dry matter production. Stem and root development are important under water stress for increased water and nutrient uptake and transport to other parts of the plant (sink to source) (Hopkins and Hüner, 2009). In contrast to stem moisture content, root moisture decreased with the severity of water stress (Table 4.1). The application of *Xcell Boost* had no effect on stem moisture content. The single concentration of *Xcell Boost* only had a positive effect on root moisture under the optimal water treatment. While the double concentration had a positive effect on root moisture content only under water deficiency stress. This shows that plants' responses to *Xcell Boost* are influenced by the external environment.

CHAPTER 6

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The single concentration *Xcell Boost* (BX1) application increases plant health (NDVI) more under water deficiency stress. Furthermore, BX1 improves the photosynthetic efficiency of spinach through increased Chlorophyll *a* (only at optimal irrigation) and stomatal conductance under all water treatments. Application of BX1 improves the accumulation of important osmoregulators with antioxidative properties, proline, and total soluble sugars (TSS), under mild stress. It is also noteworthy that BX1 further increased the antioxidative (ascorbate peroxidase, APX and guaiacol peroxidase, GPX) enzyme activities of mild drought stressed spinach. For glutathione reductase (GR), the bio-stimulant effectively induces the activity of GR only under optimal and mild drought stress. Stomatal conductance is positively correlated with APX under drought stress. The BX1 also stimulates vegetative growth and biomass under water deficiency stress in spinach (increases plant height under severe drought stress, increases stem moisture content under both 50% and 30% water holding capacity (WHC), and increases root dry mass under mild stress), showing that BX1 treatment benefits spinach under drought stress.

The application of *Xcell Boost* (BX2) improves the photosynthetic efficiency (PI_{Total}, total chlorophyll, Chl *a*, Chl *b*, carotenoids) under water deficiency stress. Carotenoids protect the cells, therefore increased concentrations under drought stress mean more protection. This application also increases the relative water content (RWC) and root moisture under water deficiency stress. Significant positive correlations with the RWC shows that stimulation of carotenoids by *Xcell Boost* is crucial in the management of water usage under drought stress (especially under severe stress) in spinach. The application of BX2 further contributes to osmoregulation and water usage through enhanced levels of TSS and proline, especially under severe water deficiency stress. The BX2 treatment induces the activities of antioxidative enzymes, especially APX and GPX, under drought stress, leading to substantially reduced electrolyte leakage (EL) under severe drought stress. Not only does BX2 application improve spinach production at physiological level, but it also improves its growth under drought stress. On a more practical note, application of BX2 results in marketable increases in spinach

yield and biomass (leaf area, leaf dry weight, stem dry weight and root length) under severe water stress.

The result of this study proves beyond doubt that application of *Xcell Boost* is beneficial for spinach because it improves performance at physiological and biochemical levels, which substantially contributes to vegetative growth. The results show that the benefits of this bio-stimulant on spinach varies with the concentration, soil water level and the parameters studied (BX2) responded positively for most (not all) parameters under drought stress.

6.1.2 Recommendations

Results show that BX1 improved fewer parameters under severe drought stress but significantly improved several parameters under mild water stress and under optimal water treatment. Therefore, BX1 may be applied with optimal irrigation or applied during short periods of water stress.

Although *Xcell Boost* (BX1) is effective under optimal and drought stress, more benefits are reaped when spinach is sprayed with an increased BX2 concentration. Therefore, under drought stress, a BX2 treatment is more likely to be promoted.

This study could be cemented by investigating more physiological/ biochemical responses, such as glycine betaine, lipid peroxidation, starch, and soluble carbohydrates. Investigating the best application method (foliar/ soil) is also worth exploring.

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8. APPENDIX 1

The results of the 2020 trial showing the effects of *Xcell Boost* on the different physiological, biochemical, and vegetative growth responses of spinach under different water levels. Optimal/maximum soil water holding capacity was represented by 100% WHC, mild drought stress was represented by 70% WHC and severe drought stress represented 50% WHC.

8.1 The photosynthetic and biochemical responses of *Xcell Boost* treated spinach under water deficiency stress.

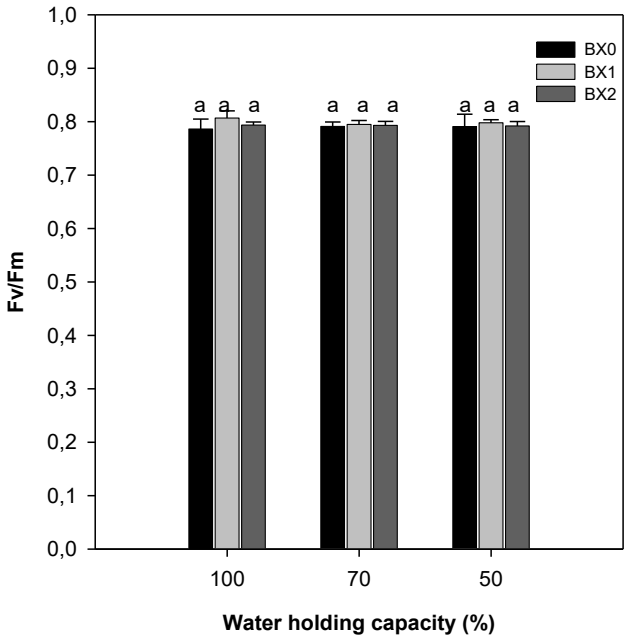

Figure A1. Maximum PSII quantum yield (*Fv/Fm*) of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

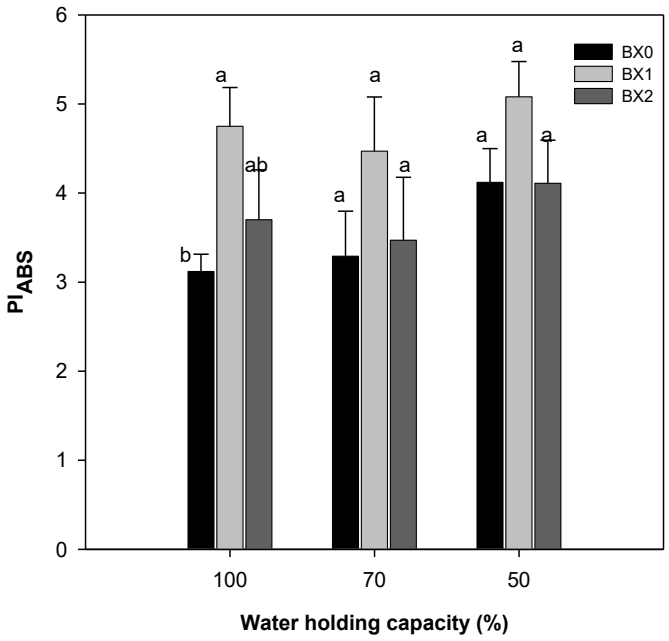

Figure A2. Performance index absorbance (PI_{ABS}) of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

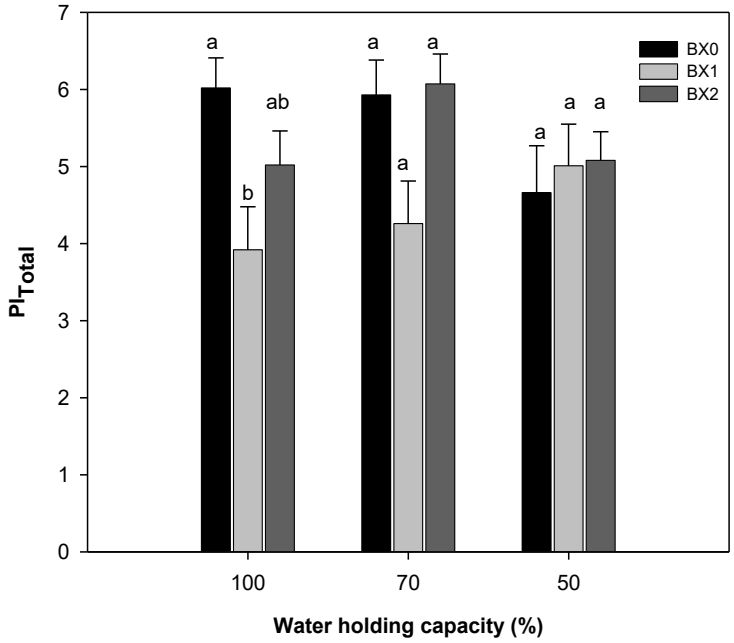

Figure A3. Total performance index (PI_{Total}) of spinach under grown different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

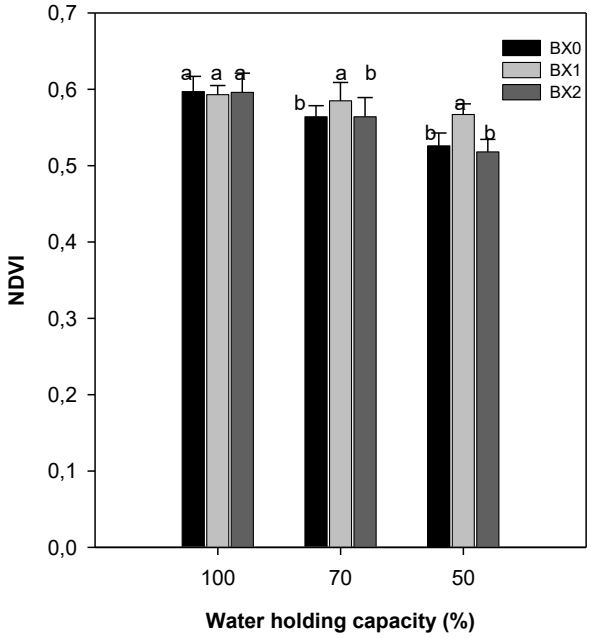

Figure A4. The NDVI of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

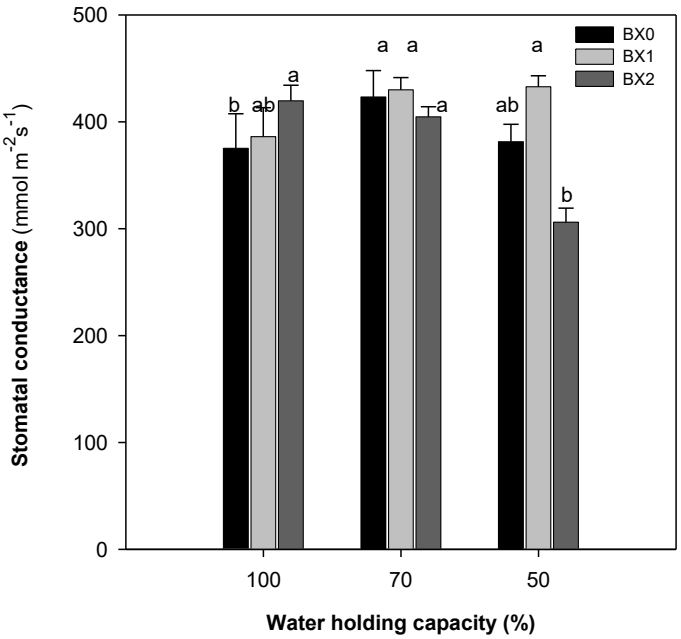

Figure A5: Stomatal conductance of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

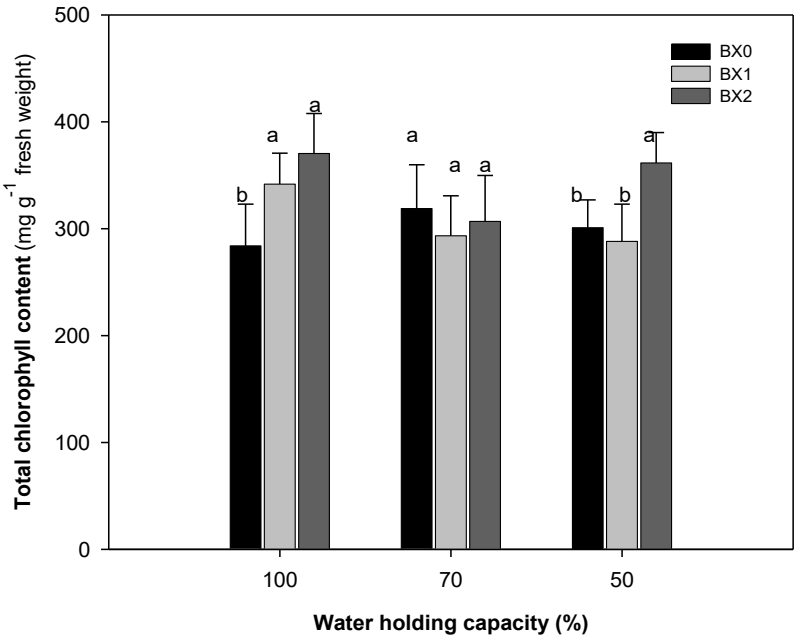

Figure A6: Total chlorophyll of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

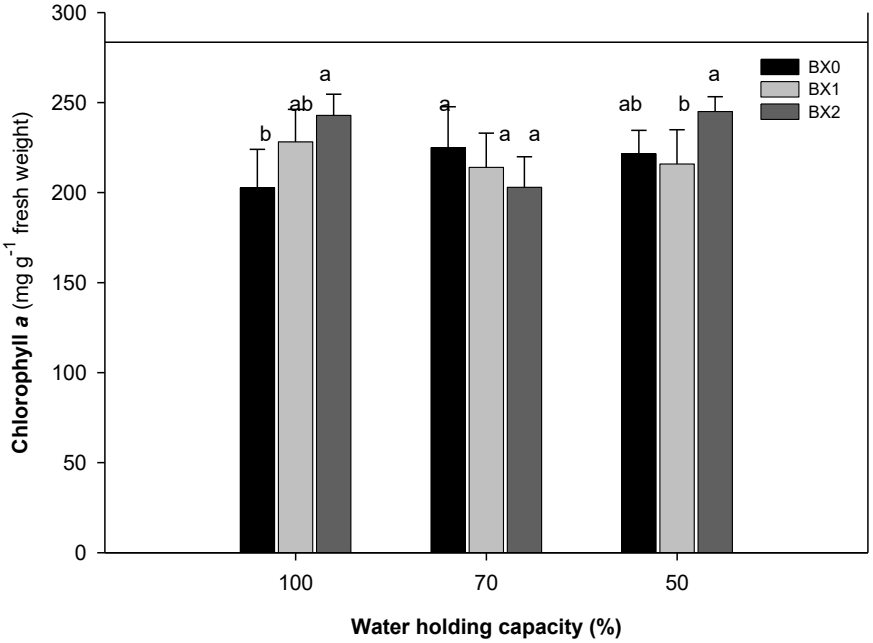

Figure A7: Chlorophyll *a* of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

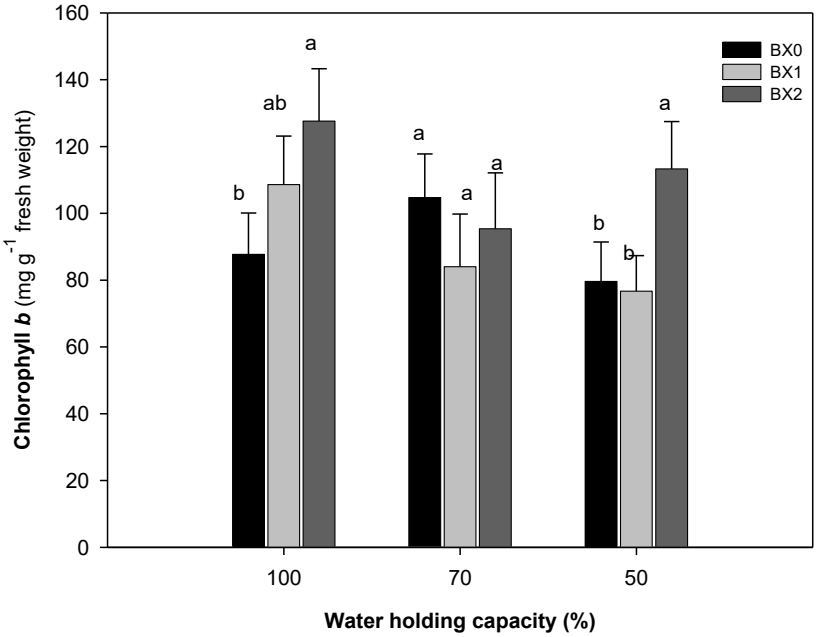

Figure A8: Chlorophyll *b* of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

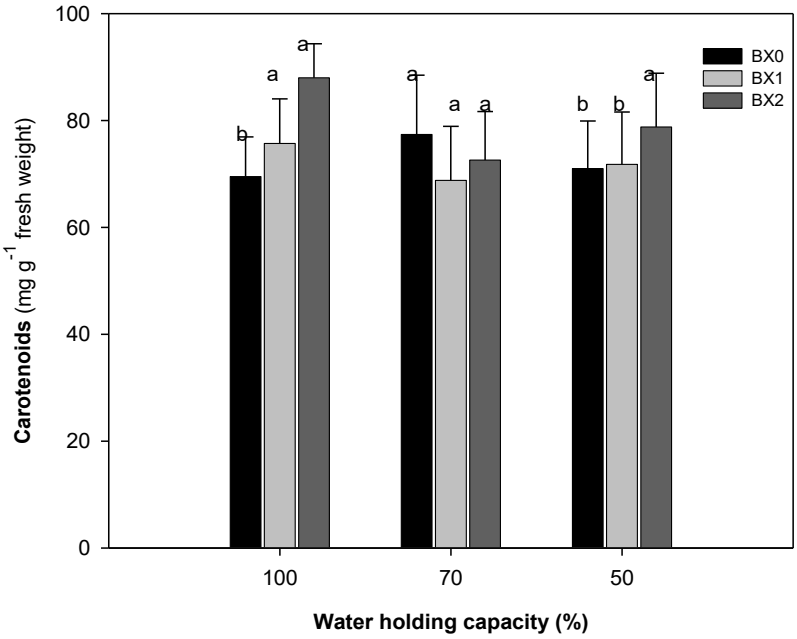

Figure A9: Carotenoid content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

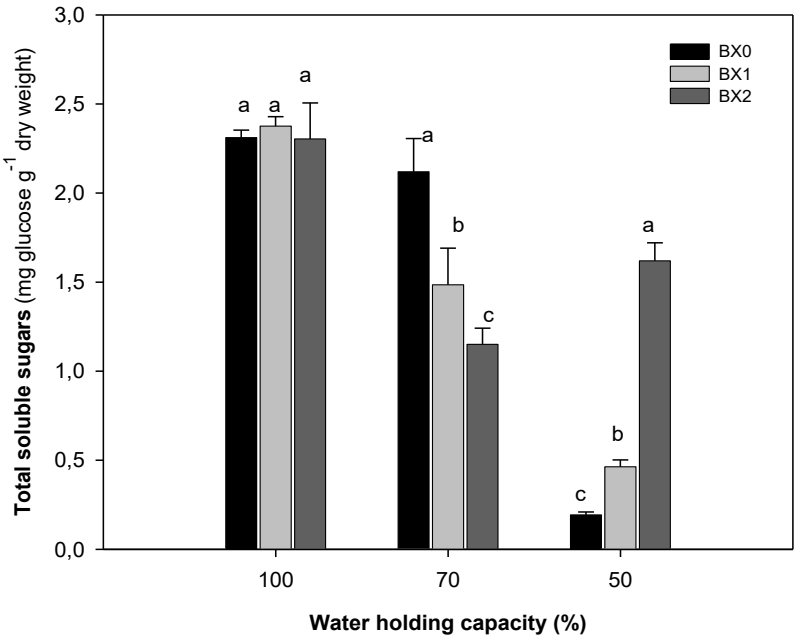

Figure A10: Total soluble sugar content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

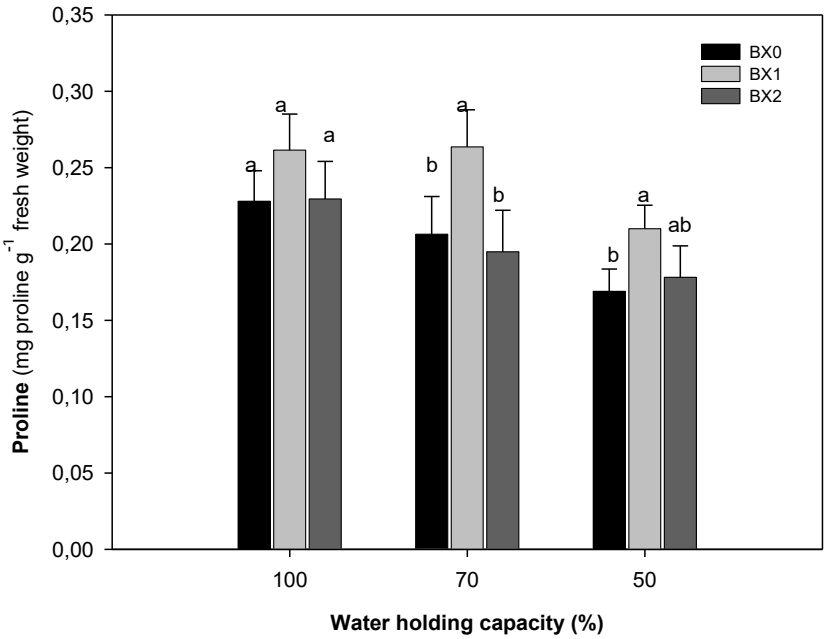

Figure A11: Proline content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

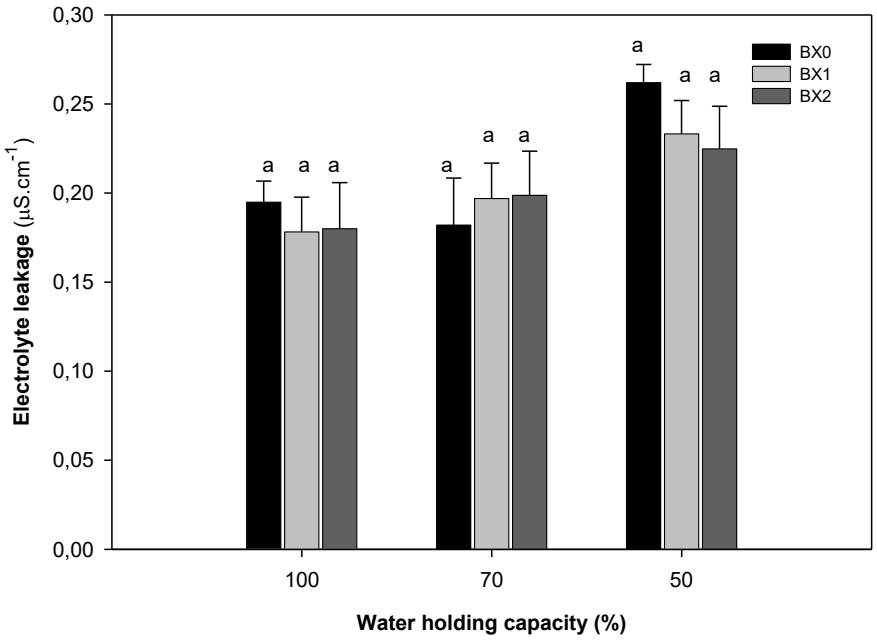

Figure A12: Electrolyte leakage content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

8.2 The vegetative growth responses of *Xcell Boost* treated spinach under water deficiency stress.

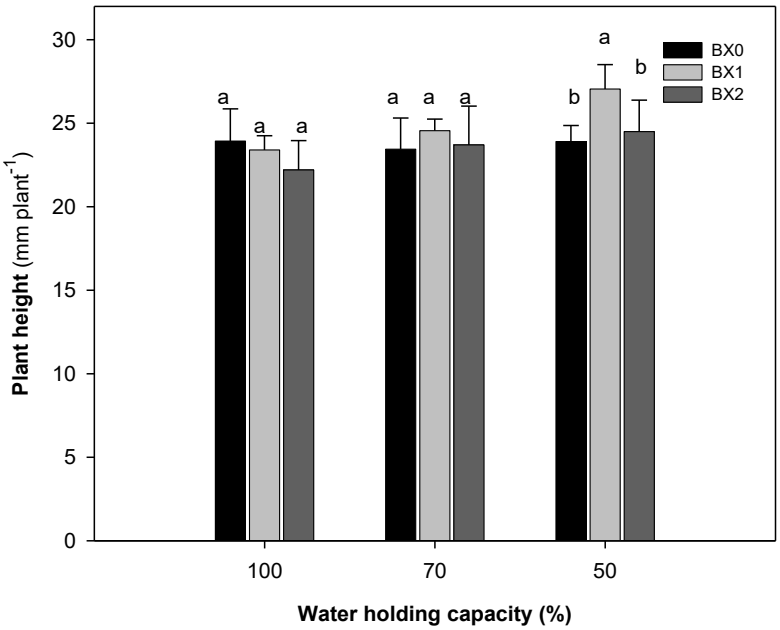

Figure A13: Plant height of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

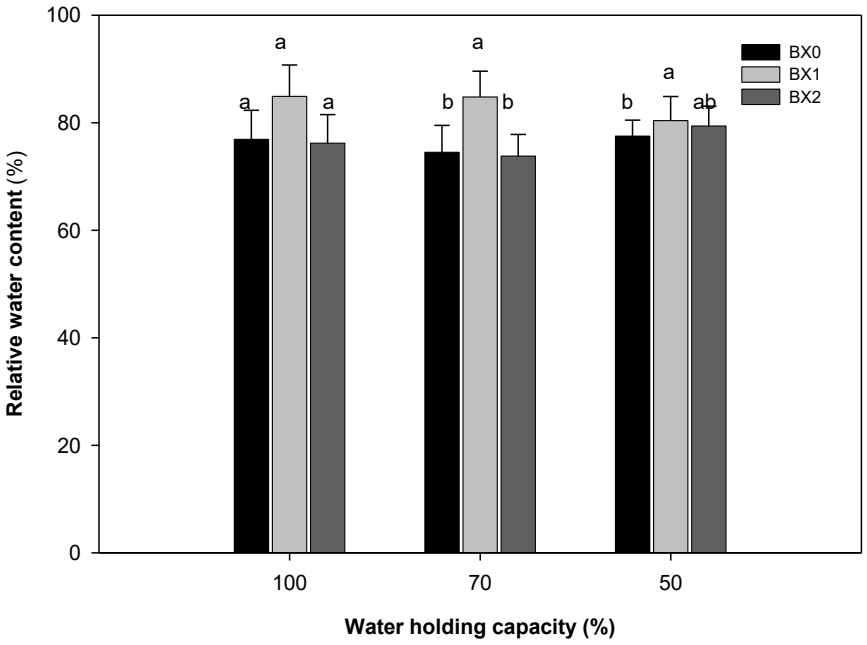

Figure A14: Relative water content of spinach grown under different water and *Xcell Boost* levels. The values are means of four replicates ± standard error. Letters on top of bars indicate significant differences within each water treatment at $P \leq 0.05$. BX0 represents no bio-stimulant, while BX1 represents single concentration and BX2 double concentration of the bio-stimulant.

Table A1: A summary of the parameter responses under different water levels and different *Xcell Boost* treatments. Red indicates a significant increase, green denotes a significant decrease, black represents a non-significant effect.

Parameters	100% WHC		50% WHC		30% WHC	
	BX1	BX2	BX1	BX2	BX1	BX2
Fv/Fm	X	X	X	X	X	X
PI_{ABS}		X		X		X
PI_{Total}		X		X		X
NDVI	X		X		X	
Stomatal conductance	X		X		X	
Total chl		X		X		X
Chl a	X			X		X
Chl b		X		X		X
Carotenoids		X		X		X
TSS		X	X			X
Proline		X	X			X
EL		X		X		X
APX	X		X			X
GPX	X		X			X
GR		X		X		X
Plant height	X		X		X	
Root length	X			X		X
Leaf area		X		X		X
Leaf no		X		X		X
Leaf dry		X		X		X
Stem dry		X		X		X
Root dry		X	X			X
RWC		X		X		X
MC stem	X		X		X	
MC root	X			X		X