Physio-morphological and biochemical traits of dibutyldithiophosphate treated drought-stressed edamame

By

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Declaration

I, Khoza Bongiwe Minah, declare that this dissertation is my own work except where otherwise stated. It is being submitted for the degree of Master of Science in Botany at the University of Free State. I have not previously submitted it for a qualification at this or any other university.

Khoza

30 November 2022

Khoza Bongiwe Minah **Date**

Dedicated to:

My Mom,
Selinah Khoza

My Dad,
Sipho Khoza

My Sisters,
Fikile, Nelisiwe, Jabu, Nonhle Khoza

and My Brother,
Khehla Khoza

"The only rock I know that stays steady, the only institution I know that works, is the family." –Lee Iacocca

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List of Abbreviations

%	–	Percent
µg/mL	–	Microgram per millilitre
µS	–	MicroSiemens
•OH	–	Hydroxyl oxide
A	–	Absorbance
ABA	–	Abscisic acid
ADP	–	Adenosine diphosphate
ANOVA	–	Analysis of variance
APR	–	APS reductase
APS	–	Adenosine 50-phosphosulfate
APX	–	Ascorbate peroxidase
AsA-GSH	–	Ascorbate-Glutathione
ATP	–	Adenosine triphosphate
BPP	–	Branches per plant
C	–	Cultivar
CA	–	Carbonic anhydrase
Car	–	Carotenoids
CAT	–	Catalyse
Chl	–	Chlorophyll
Chl-a	–	Chlorophyll -a
Chl-b	–	Chlorophyll -b
cm	–	Centimetres
cm^{-1}	–	Per centimetre

CO_2	–	Carbon dioxide
COS	–	Carbonyl sulfide
CRI	–	Carotenoid reflective index
Cys	–	Cysteine
Cyt	–	Cytochrome
D-CDes	–	D-cysteine desulfhydrase
DHA	–	Dehydroascorbate
DHAR	–	Dehydroascorbate reductase
DW	–	Dry weight
e^-	–	Electrons
EDTA	–	Ethylenediaminetetraacetic acid
EL	–	Electrolyte leakage
FBPase	–	Fructose 1, 6-bisphosphatase
Fd Bnd	–	Iron-containing protein
Fd Sol	–	Soluble ferredoxin
Fm	–	Maximum fluorescence
FRI	–	Flavonoid reflectance index
Fv	–	Variable fluorescence
Fv/Fm	–	Ratio of variable fluorescence to maximum fluorescence
FW	–	Fresh weight
g	–	Grams
GAPDH	–	Glyceraldehyde 3-phosphate dehydrogenase
GDP	–	Gross domestic product
GPX	–	Guaiacol peroxidase

GR – Glutathione reductase

GSH – Glutathione

GSSG – Glutathione disulphide

GSSH – Oxidized glutathionine

GYY4137 – Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate

H^+ – Hydrogen ion

H_2O – Water

H_2O_2 – Hydrogen peroxidase

H_2S – Hydrogen sulfide

I – One light reaction

kg – Kilograms

KZN – KwaZulu Natal

L – Litres

LCD – L-cysteine desulphydrase

L-CDes – L-cysteine desulfhydrase

M – Molar

m/m – Mass/ mass percentage

m/v – Mass/ volume percent

MDA – Malondialdehyde

MDHA – Monodehydoascorbate

MDHAR – Monodehydroascorbate

mg/mL – Milligram per millilitre

mM – Millimole

$mmol.m^{-2}.s^{-1}$ – Millimole by square meters per second

Na^+ – Sodium

Na_2S – Sodium sulfide

Na_2SO_3 – Sodium sulfite

Na_2SO_4 – Sodium hydrogen sulfate

NAD^+ – Nicotinamide adenine dinucleotide

NADH – Nicotinamide adenine dinucleotide bonded with hydrogen

$NADP^+$ – Nicotinamide adenine dinucleotide phosphate

NADPH – Nicotinamide adenine dinucleotide phosphate hydrogen

NaHS – Sodium hydrosulfide

$NaHSO_3$ – Sodium hydrogen sulfite

NaOH – Sodium hydroxide

NBT – Nitro blue tetrazolium

NIR – Near infra-red

nm – Nanomole

NPQ – Non-photochemical quenching

O_2^- – Superoxide

O_2 – Oxygen

$O_2\bullet$ – Superoxide radicals

OA – Osmotic adjustment

OAS – O-acetylserine

OAS-TL – O-acetylserine thiol lyase

PC – Plastocyanin

PEA – Photosynthesis efficiency analyser

PH – Plant height

Pi	–	Phosphate
PI_{abs}	–	Perfomance index absorbance
PI_{total}	–	Performance index (potential)
PLP	–	5’-pyridoxal phosphate
PPP	–	Pods per plant
PQ	–	Plastoquinone
PRI	–	Photochemical reflectance index
Prol	–	Proline
PSI	–	Photosystem I
PSII	–	Photosystem II
PTM	–	Post-translational modification
PUFA	–	Polyunsaturated fatty acid
PVPP	–	Polyvinylpolypyrrolidone
R Chl	–	Relative Chlorophyll
R6	–	Reproductive stage six
R8	–	Reproductive stage eight
ROO•	–	Peroxyl
ROS	–	Reactive oxygen species
Rubisco	–	Ribulose 1,5-bisphosphate carboxylase-oxygenase
RWC	–	Relative water content
SA	–	South Africa
-SH	–	Thiol group
SiR	–	Sulfite reductase
SO_2	–	Sulphur dioxide

SO_3^{2-}	–	Sulfite
SO_4^{2-}	–	Sulfate
SOD	–	Superoxide dismutase
SSA	–	Sub-Saharan Africa
-SSH	–	Persulfide group
TBA	–	Thiobarbituric acid
TCA	–	Trichloroacetic acid
Tot Chl	–	Total chlorophyll
TSMPP	–	Total seed mass per plant
TSPP	–	Total seeds per plant
TTS	–	Total soluble sugars
TW	–	Turgid weight
USA	–	United States of America
v/v	–	Volume/ volume percent
VWC	–	Volumetric water content
W	–	Water level
w/v	–	Weight/ volume percent
WHC	–	Water holding capacity

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Equation 1: Chlorophyll-a (Chl-a) = $(12, 72 \times A_{663}) - (2, 59 \times A_{645})$

Equation 2: Chlorophyll-b (Chl-b) = $(22, 9 \times A_{645}) - (4, 68 \times A_{663})$

Equation 3: Total chlorophyll (Tot Chl) = $(20, 2 \times A_{645}) + (8, 02 \times A_{663})$

Equation 4: Carotenoids (Car) = $A_{480} + (0{,}114 \times A_{663}) – (0{,}638 \times A_{645})$

Equation 5: Chlorophyll content = chlorophyll x Volume/ Weight

Equation 6: Carotenoid content = carotenoids x Volume/ Weight

Equation 7: $\% \text{ EL} = 100 \times (C_{max} - C_0)/C_{total}$

Equation 8: $\%\text{RWC} = 100 \times (\text{TW} - \text{FW}) / \text{DW}$

Intended Research Outputs

The following research outputs will be delivered from this work in the year 2023:

1. Bongiwe Minah Khoza, Ned Bowden, Makoena Joyce Moloi. Physiological, biochemical and morphological responses of dibutyldithiophosphate treated edamame under drought stress. *48th annual conference of the South African Association of Botanists 2023, 17-20 January 2023* (Oral).

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3. Khoza, B.M., Bowden, N., Moloi, M.J. The role of hydrogen sulfide during the physiological and biochemical responses of plants under abiotic stress. *South African Journal of Botany (part of the literature review chapter is prepared for submission).*

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Abstract

Edamame (*Glycine max* L. Merrill) has recently received attention in South Africa due to its high nutritional value. Production of edamame in South Africa is limited since the country consists of arid and semi-arid regions, with insufficient water available for irrigation. Dibutyldithiophosphate, a biodegradable chemical that slowly releases hydrogen sulfide, could be a solution based on recent studies that indicate H_2S acts as a signaling molecule for tolerance induction during various environmental stresses. This study investigated the impact of dibutyldithiophosphate on the physiological, biochemical and morphological characteristics of drought-stressed edamame cultivars (UVE14 and UVE17). Furthermore, the study established the relationships between the physiological, biochemical and the morphological characteristics [plant height, branches per plant, total seeds per plant, total seed mass per plant, and pods per plant] of drought-stressed edamame under different dibutyldithiophosphate concentrations. Dibutyldithiophosphate was directly applied around the seed during sowing before drought stress (30% water holding capacity) introduction at the third trifoliate leaf stage.

Drought stress adversely affected the physiological, biochemical and morphological responses of the edamame cultivars. The efficacy of dibutyldithiophosphate differed according to the cultivars, growth stages (flowering and pod-filling), concentrations of dibutyldithiophosphate (0, 0.1, 1, and 2 mg/mL), as well as the water level (drought or optimal watering). The concentrations required to activate the physiological and biochemical responses were generally higher in drought-stressed UVE17 than UVE14 cultvar. To improve drought tolerance in edamame, dibutyldithiophosphate application upregulated the photosynthetic capacity (increased total chlorophyll content, chlorophyll-a, chlorophyll-b, and photochemical reflective index), which resulted in increased accumulation of sugars (total soluble sugars) and proline. The increase in total soluble sugars and proline indicate that dibutyldithiophosphate application increases the osmotic and antioxidative potential of drought-stressed edamame. Additionally, results of this study showed that dibutyldithiophosphate application decreased membrane damage by increasing the antioxidative capacity [superoxide dismutase, ascorbate peroxidase, flavonoid reflective index, and carotenoid reflective index] in drought-stressed edamame. Application of

dibutyldithiophosphate also improved drought tolerance in a susceptible cultivar (UVE17) by increasing pods per plant (0.1 mg/mL), branches per plant (1 mg/mL), and total seed per plant (2 mg/mL). For drought-stressed UVE14, application of dibutyldithiophosphate increased branches per plant (all concentrations), pods per plant (0.1 and 1 mg/mL) and total seed per plant (2 mg/mL). Since there were more positive correlations between the physiological, biochemical and morphological characteristics under 0.1 and 1 mg/mL, these dibutyldithiophosphate concentrations could be recommended for drought tolerance stimulation in edamame. As a result, the hypothesis that application of dibutyldithiophosphate enhances the physiological, biochemical and morphological responses of drought-stressed edamame is accepted. In addition, it is acknowledged that application of dibutyldithiophosphate on drought-stressed edamame increases the relationships between physiological, biochemical and morphological responses.

Chapter 1: General Introduction

Edamame, a vegetable-type soybean (*Glycine max*. L. Merrill) is a nutritious and high-value specialty crop that can be consumed at reproductive stage six (R6 stage) (Carneiro et al., 2021; Shurtleff and Aoyagi, 2021). It is a recently cultivated crop in South Africa (SA), which on contrary to grain soybean, can be consumed as a green vegetable (Dlamini et al., 2014; Shurtleff and Aoyagi, 2021). Compared to conventional field or grain soybeans, edamame has the benefit of having a better flavor and less allergens (Shurtleff and Aoyagi, 2021). It is rich in nutrients that are essential for human health, including proteins, minerals, and vitamins (Anda et al., 2019; Iqbal et al., 2019).

Malnutrition has become a major issue in many regions of the world, it increased from 783.7 million people in 2014 to 820.8 million in 2017 (Djanta et al., 2020). This highlights the critical need for cheaper nutrient-rich food to be provided in order to combat malnutrition issues. Consumption and promotion of edamame can help address the issue of food insecurity and malnutrition in the region of Sub-Saharan Africa (SSA), which has the highest rate of undernourished people than anywhere in the world (Djanta et al., 2020). The majority (about 87%) of agricultural land in SA is occupied by 40 000 commercial farms, with the remaining 13% by smallholder or household farmers. Currently, small-scale farmers are the sole growers of edamame in SA. As a result, their impact on edamame production is less significant (Masuda and Goldsmith, 2009).

The most prominent threat to cultivating edamame is drought, which is brought on by climate change (Moloi and van der Merwe, 2021). Climate change is accelerated by anthropological activities, and this has a significant impact on food production around the world (Moloi and van der Merwe, 2021). Severe droughts have impacted food security, production, and yield in a number of South African provinces since 2015 (Molotsoane, 2016). For the majority of crops, conditions such as water availability, soil moisture, nutrients in the soil, and carbon dioxide should be at their optimum levels to ensure the greatest amount of plant development, growth and yield (Baker and Capel, 2011; Chen et al., 2015). Smaller and fewer leaves, shorter stems, and changes to the plant's reproductive phase are all effects of the aberrant growth and development that occurs during drought stress (Anjum et al., 2011; da Silva et al., 2013). Drought stress impacts photosynthesis and other metabolic reactions in plants, which results in reduced plant production. For instance, under drought stress, the cell's basic organizational structure is disrupted, which affects the photosynthetic equipment and inhibits the absorption of carbon into the leaf (Wang et al., 2018; Kapoor et al., 2020). To enable plants to conserve water, drought stress leads to a reduction in stomatal conductance. This, however, has adverse long-term effects since it causes limited carbon dioxide (CO_2) to enter the stomata, which will reduce the rate of photosynthesis and eventually decrease plant development and productivity. In addition, severe drought stress brings damaging changes to the chlorophyll components and photosynthetic apparatus on how light is captured and the electron transport chain, which eventually lead to the inhibition of photosynthesis (Jaleel et al., 2009; Farooq et al., 2012).

One of the primary responses during drought stress at the biochemical level include the generation of reactive oxygen species (ROS) (de Calvalho, 2008; da Silva et al., 2013; Noctor et al., 2014; You and Chan, 2015; Verma et al., 2019). An excess accumulation of ROS in plants results in oxidative stress, which directly damages membrane lipids and increases lipid peroxidation (Anjum et al., 2011; da Silva et al., 2013). Oxidative stress also degrades proteins, which may lead to excessive ion leakage and plant death (Murray et al., 1989, Silva et al., 2016). Additionally, hyperactive accumulation of ROS, which damages the chloroplasts, further leads to a decrease in photosynthesis.

For plants to survive drought stress and its devastating effects, it is essential that they possess physiological and biochemical mechanisms that can counteract the damaging effects. Kapoor et al. (2020) stated that plants involve non-enzymatic and enzymatic antioxidants that scavenge for ROS and lessen the injury triggered by ROS. The reduction of ROS such as hydrogen peroxide (H_2O_2) to water (H_2O) is catalyzed by the enzyme antioxidants: ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). The capacity of a plant to scavenge for ROS and reduce their adverse effects may be correlated with drought stress tolerance (Tsugane et al., 1999). Other survival strategies in plants involve altered expression of various solutes or osmolytes (Shinozaki and Yamaguchi-Shinozaki, 2007). Most plant species respond to drought stress by lowering their osmotic potential to maintain their water levels. A buildup of secondary metabolites, such as phenols, flavonoids, proline, trehalose, mannitol, inositol, and sorbitol, is linked with drought stress tolerance (Alhaithloul, 2019). The period and severity of water stress, the species, and the genotype, all affect the composition of solutes that contribute to osmotic adjustment (Pospisilova et al., 2011).

Drought has a significant influence on agricultural plant growth and productivity, and its effects are more significant in underdeveloped countries (Jaleel et al., 2009). The recent drought in SA during 2015 lowered productivity of the agricultural industry, which affected the economy (Botai et al., 2016; Lottering et al., 2021; Meza et al., 2021). Drought affects plants productivity and yield through the modification of the biochemical and physiological characteristics, and in some cases, modification of the morphological structures (Moloi and van der Merwe, 2021; Hlahla et al., 2022). Since edamame is still a relatively new crop in SA, breeding for drought-tolerant cultivars is currently underway. Given that this is a long process, alternative approaches are required to increase plants' tolerance to drought. Hydrogen sulfide (H_2S) has recently been accepted as a significant gasotransmitter in many plants (Bindu and Snyder, 2015). During abiotic stress, it can improve plant development and crop yields. Administration of H_2S donors in numerous crops in small concentrations has been suggested to have protective benefits against heat, drought, freezing, cold, and salt stresses (Carter et al., 2019).

1.1. Problem Statement

According to Kron et al. (2008), water deficient related stress affects almost all growth stages of edamame as it has negative impacts on photosynthesis in the plant and eventually affects crop yield. The capacity of a plant to endure stressful conditions decreases as it develops from reproductive stage one (R1, beginning of flowering) to reproductive stage five (R5, seed enlargement), which causes an increase in yield loss (Rosa et al., 2020). When severe, drought stress may lead to the death of the plant (Jaleel et al., 2009). Previous research indicated that drought stress negatively affects the physiological, biochemical and yield responses in edamame (van der Merwe et al., 2018; Moloi and van der Merwe, 2021; Hlahla et al., 2022).

To enhance crop performance in drought-stressed conditions, urgent climate-smart solutions are required. The effects of such solutions must be observed at physiological, biochemical and morphological levels in plants. According to studies, H_2S plays a vital role in how plants respond, particularly under drought stress (Carter et al., 2018; Carter et al., 2019; Arif et al., 2021; Brown et al., 2021). Hydrogen sulfide functions as a signaling molecule for inducing defense reactions when delivered in miniscule amounts (Chen et al., 2011; Shan et al., 2018; Khan and Corpas, 2021).

The current concern with using compounds that slowly release H_2S is that they simultaneously release molecules that are not found in the environment naturally (Carter, 2018). A newly developed dibutyldithiophosphate, which slowly releases H_2S, is safe, non-polluting, biodegradable, and has no adverse environmental impacts (Brown et al., 2021). However, there is no information on whether such a donor can increase yield in edamame under drought stress. Nevertheless, this product is promising because it significantly increased maize production even under non-stressed environments (Carter et al., 2019). The application of H_2S-releasing dithiophosphates is anticipated to decrease the damaging impacts of drought stress by increasing harvest index yield and edamame survival through modification of the physiological and biochemical parameters. If this hypothesis is accepted, this study will be conducted on a bigger scale in the field where there are more environmental stressors to establish the effectiveness of this H_2S donor further.

1.2. Research aim

Investigate the effect of a biodegradable, slow releasing H_2S donor (dibutyldithiophosphate) that is environmentally safe on the physiological, biochemical and morphological responses of edamame cultivars under different water levels. The reason for including investigation under drought is that the product has not been tested under drought stress environment on any crop.

1.3. Objectives

The specific objectives were:

1. To investigate the effect of dibutyldithiophosphate on the physiological and biochemical responses of drought-stressed edamame.

2. To investigate the influence of dibutyldithiophosphate on the morphological responses of drought-stressed edamame, and to establish their relationships with the physiological and biochemical responses.

Chapter 2: Literature Review

The physiological, biochemical, and morphological responses of edamame and other agricultural crops to drought stress, and the improved responses due to application of hydrogen sulfide

2.1. Edamame: history, nutrition, and introduction in South Africa

Edamame (*Glycine max*. L. Merrill) is a Japanese word for vegetable-type soybean and is pronounced: "ay dah-MAH-may" (Shurtleff and Aoyagi, 2021). It is also referred to as large-seeded soybean, and belongs to the Fabaceae family (Agahiu, 2020). The crop was first cultivated in China before 2500 BC (Department of Agriculture, Forestry and Fisheries, 2010; Zeipina et al., 2017). Its production is small but increasing in South Africa (SA) (Dlamini et al., 2014; Agri4africa, 2020). It was first introduced in the KwaZulu-Natal (KZN) province in 2009 (Smit, 2019). In March of 2014, the Edamame Development Programme, a pilot project that was based in KZN, harvested its first edamame crop in Mariann hill, Monastery farm (Seid, 2014; Smit, 2019).

Edamame can be harvested four to six times a year, making it a profitable crop (Zeipina et al., 2017; Djanta et al., 2020). At reproductive stage six (R6), the beans are harvested green, shortly before they mature (R8) for use as a vegetable (Shurtleff and Aoyagi, 2021). Edamame has larger seeds that make up 80-90% of the pods with better texture and flavour than grain soybeans (Zeipiņa et al., 2017; Casas-Leal et al., 2022). Depending on the method of preparation, they can be cooked and served in or out of their pods (Shurtleff and Aoyagi, 2021).

Malnutrition affects many people in SA because of the widespread use of starchy foods and the high cost of animal protein, which is often out of reach for low-income families. Protein deficit is more common in children, pregnant women, and breast-feeding mothers. As a result, there is a need to produce and consume low-cost protein-rich meals (Khojely et al., 2018). Edamame is an ideal crop for meeting these requirements because it is high in proteins, vitamins, fibre, and minerals. Studies indicate that it is a better replacement for meat products (Djanta et al., 2020;

Engelbrecht et al., 2020). Drought stress, on the other hand, threatens production of edamame. It impacts the physiological, yield, and nutritional content of South African edamame cultivars (Moloi and van der Merwe, 2021; Hlahla et al., 2022).

2.2. Impact of drought in South Africa

The impact of climate change on crops is evident with drought and heat waves at the forefront of crop damage (Wolf et al., 2020). Significant changes in the global mean temperature, wind patterns, precipitation, and other climate variables that occur over decades can cause drought stress (Davis, 2021). Drought stress is characterized as a period of reduced rainfall and precipitation when soil moisture levels are insufficient to meet the needs of a certain crop (National Weather Service, 2021), leading to continuous evaporation or transpiration-related water loss in plants (Atta et al., 2022). Drought has a substantial effect on agricultural plant growth and productivity, and its effects are more significant in underdeveloped countries (Jaleel et al., 2009).

A substantial number of disadvantaged residents in these countries, particularly those who live in rural areas, are affected by severe economic repercussions of the agriculture industry's underperformance due to drought (Barta et al., 2019, Meza et al., 2021). Production in the agricultural industry was lowered by drought in 2015, which affected the economy of SA (Lottering et al., 2021; Meza et al., 2021). Even though the agricultural sector contributes approximately 3% of SA’s gross domestic product (GDP), it is a vital industry for economic growth (Meza et al., 2021). The sector is only growing at an annual rate of 0.5% partly because of drought conditions that affect crop yields. In 2017, the sector lost roughly 25,000 jobs because of the continuous drought that started in 2015. Sadly, most of the workers could not secure alternative jobs (Drummond, 2019; Kalaba, 2019; Pal et al., 2020), which further pressurized the economy of SA. In addition, drought conditions increased food insecurity because most rural populations in SA rely on subsistence farming for income (Makaya et al., 2020; Meza et al., 2021). Although drought has an impact on both commercial and small-scale farmers, the latter suffer the most because of limited resources (Kalaba, 2019; Meza et al., 2021). Moreover, drought has a significant impact on farmers that grow edamame, since it reduces production by

40% (Shaheen et al., 2016), affecting turnover and production of the agricultural sector.

2.3. Impact of drought on plant physiology, biochemistry, and morphology

Drought stress impacts plants at various stages of development and growth. For example, edamame plants are more sensitive to drought during the stages of ontogeny, such as seedling, flowering, and pod-filling, which result in reduced seed size and yield (Makeli et al., 2013; Kunert et al., 2016; Mwenye, 2017; Smit, 2019; Moloi and van der Merwe, 2021). Such losses emanate from the disruptions caused by drought stress at physiological and biochemical levels (Moloi and van der Merwe, 2021). The most evident impacts of drought stress are reduced germination percentage, stunted growth, severe damage to the photosynthetic machinery, a decrease in net photosynthesis, and a loss in nutrient intake (Nadeem et al., 2019). To prevent the devastating impacts of drought stress in edamame, it is critical to understand its effects on the crops' processes for long-term management. The physio-morphological and biochemical responses of plants to drought stress are discussed in the following sections.

2.3.1. Drought's impact on the physiological responses

2.3.1.1. Photosynthetic capacity

Water deficiency influences the performance of agricultural crops, which may result in stomatal closure, premature leaf senescence due to pigment degradation, inefficient photosynthesis, oxidation of chloroplast lipids, protein structural changes, and reduced leaf area (Anjum et al., 2011b).

a) Chlorophyll content

Chlorophyll is a pigment found in the chloroplasts and has a vital role during photosynthesis. It is a key component of the pigment-protein complex on the thylakoid membrane, and the most important pigment because of its role in light energy absorption for photosynthesis (Li et al., 2018; Dong et al., 2019). There are several kinds of chlorophylls, but the two most important in terrestrial plants include chlorophyll-a and -b (Kume et al., 2018). The predominant photosynthetic pigment chlorophyll-a is responsible for transmitting an electron directly to an electron transport chain during the conversion of light energy to chemical energy. Chlorophyll-b transfers absorbed light energy to chlorophyll-a and therefore is a secondary light absorption pigment that complements chlorophyll-a (Yahia et al., 2018; Qui et al., 2019).

Chlorophyll loss due to drought is recognized as a sign of oxidative stress. Photo-oxidation of chloroplast pigments obstructs biosynthesis of chlorophyll and accelerates chlorophyll degradation. Drought stress affects both chlorophyll-a and -b (Lei et al., 2006; Anjum et al., 2011b). Under severe drought, chlorophyll concentration in agricultural crops may decrease or increase (Deka et al., 2018; Karimpour, 2019; Khayatnezhad and Gholamin, 2021), depending on the crop's tolerance or susceptibility. Drought-stressed sensitive corn (*Zea mays*) (Gholamin and Khayatnezhad, 2020), wheat (*Triticum aestivum* L) (Karimpour, 2019; Khayatnezhad and Gholamin, 2021), and barley (*Hordeum vulgare*) (Hebbache et al., 2021) showed substantial decline in chlorophyll content. According to Basal et al. (2020) and Hlahla et al. (2022), drought stress reduced the chlorophyll-a content in sensitive and tolerant soybean and edamame cultivars, respectively. Total chlorophyll content was higher in non-stressed soybeans according to Makbul et al. (2011), and Chowdhury et al. (2017), however, water stress reduced chlorophyll-a/ b ratio, and total chlorophyll content in both tolerant and sensitive cultivars. Drought stress reduced chlorophyll-a more than chlorophyll b, resulting in a lower chlorophyll-a/ b ratio in drought-stressed soybeans (Chowdhury et al., 2017; Kapoor et al., 2020). Mafakheri et al. (2010) also observed the same trend in drought-stressed chickpea (*Cicer arietinum*) cultivars. The lack of a significant decrease in chlorophyll-b content showed that it was less sensitive to drought.

b) *Chlorophyll-*a *fluorescence*

Photosynthesising plants possess photosystems [photosystem I (PSI) and photosystem II (PSII)], which are responsible for absorbing light energy. Pigment molecules make up photosystems, which absorb light photons and send the captured light energy to the reaction centres (Yahia et al., 2018). Some of the absorbed light can dissipate as heat or fluorescence if not used in photochemical reactions (Helm et al., 2020). Chlorophyll-a fluorescence is primarily and effectively used to determine the potential quantum yield of PSII and photo-inhibition, especially under stress (Khalid et al., 2019). The state of PSII reflects the state of other electron transport components, which in turn, reflect the redox state of metabolic pathways within the cell. An electron transport chain starts when excitation energy reaches chlorophyll-a at the reaction centre. Each step in the electron transport chain subsequently lowers energy of each electron and utilizes it to form adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (Figure 2.1) (The biology project, 1996; Yahia et al., 2018; Khalid et al., 2019).

The measurement of chlorophyll-a fluorescence reveals information about PSII, PSI and other photosynthetic components (Ogawa and Sonoike, 2021). For instance, the Fv/Fm ratio [(Fm-F0)/Fm] is used to estimate the maximum photochemical efficiency of PSII and to detect the loss of function of PSII reaction centers (where F0 represents the minimum fluorescence, Fm represents the maximum fluorescence, and Fv represents the variable fluorescence) (Tadina et al., 2007; Gorbunov and Falkowski, 2021). A sensitive indicator of plant photosynthetic efficiency is the Fv/Fm ratio. A decrease in this index represents reduced PSII efficiency (i.e. photoinhibition) because in the thylakoid membranes, it is the most vulnerable component to damage. Photoinhibition is caused by the photo inactivation of PSII centres, which is attributable to D1 protein damage (Campos, 1998; Zlatev, 2009) and it is visible when the PSII's principal acceptor, plastoquinone (QA), is reduced to an abnormally low level (Guidi et al., 2019) and affects the level of photosynthesis in the cells. The Fv/Fm values for normal treatments can vary from 0.75 to 0.85 arbitrary units. Based on the plant species, these values can decrease under drought stress (Liatile, 2021).

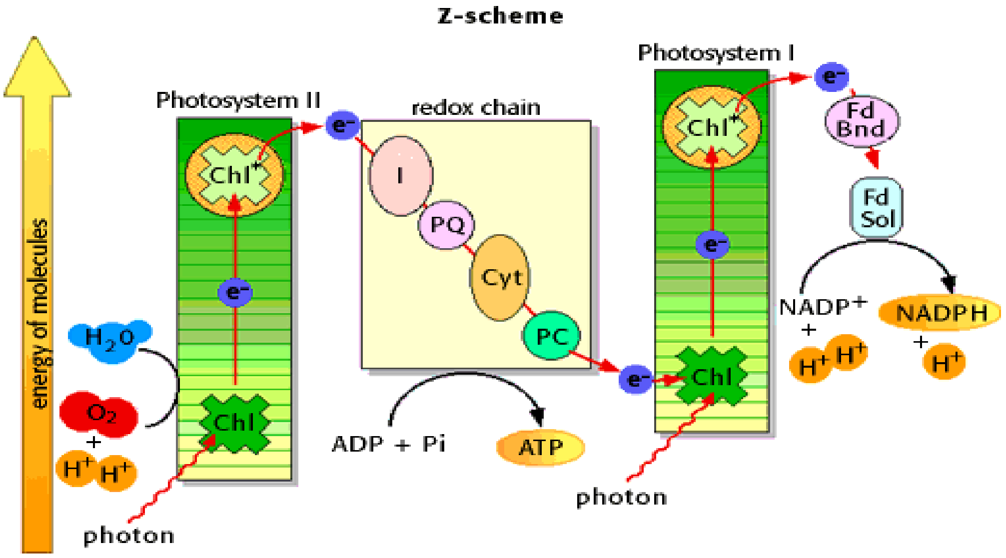


***Figure 2.1. An illustration of the electron transport chain**. When PSII absorbs light, the primary electron acceptor captures an electron excited to a higher energy level in the reaction centre. An electron transport chain connects the main electron acceptor in photosystem II to the photo excited electron acceptor in PSI. The thylakoid membrane uses the exergonic "fall" of electrons to a lower energy level to generate ATP as they go down the chain. During the Calvin cycle, the ATP created in this process will supply energy for glucose production (light-independent reactions). The excited electrons are sent to a second electron acceptor, which delivers them to an iron-containing protein (Fd Bnd), via the primary electron acceptor of photosystem I (The biology project, 1996). Chl- Chlorophyll, ADP- Adenosine diphosphate, ATP- Adenosine triphosphate, I- One light reaction, Pi- Phosphate, H^+- Hydrogen ion, $NADP^+$- Nicotinamide adenine dinucleotide phosphate, NADPH- Nicotinamide adenine dinucleotide phosphate hydrogen, O_2- Oxygen, H_2O- Water, PC- Plastocyanin, Cyt- Cytochrome, PQ- Plastoquinone, I- One light reaction, Fd Sol- Soluble ferredoxin, e^-- electrons, Fd Bnd- iron-containing protein*

In wheat (Živčák et al., 2008) and maize (Badr and Brüggemann, 2020), Fv/Fm ratio significantly decreased under severe drought stress. The Fv/Fm ratio decreased below 0.7 under drought stress in grapevine (*Vitis vinifera*) leaves (Zulini et al., 2007). In contrast, Liatile (2021) and Hlahla et al. (2022) observed that 30% drought stress had no significant impact on Fv/Fm in spinach (*Spinacia oleracea* L) and edamame during flowering, respectively. These findings indicate that drought stress affects Fv/Fm differently in different crops.

Performance index (PI) measures the energy absorption, trapping, and conversion into the electron transport process (Bano et al., 2021; Stefanov et al., 2022).

Performance index absorbance (PI_{abs}) reflects PSI functionality (performance potential for energy conservation from exciton to the reduction of intersystem electron acceptors) and provides quantitative information on the status of plants' performance under drought stress (Živčák et al., 2008). Performance index (total) (PI_{total}) is based on PI_{abs} and gives details on the efficacy of electron transfer from PSII to PSI (Liatile, 2021). As a result, PI_{total} gives information on the functionality of both photosystems (Živčák et al., 2014; Liatile, 2021). Drought stress significantly decreased PI_{abs} in wheat from commencement of water stress (Živčák et al., 2008). Studies in edamame showed that photosynthetic responses are specific to the growth stages because PI_{abs} was lower at pod-filling than flowering stage (Hlahla et al., 2022). Numerous studies revealed that chlorophyll-a fluorescence characteristics were accurate stress indicators and were substantially linked with whole-plant mortality in response to abiotic stresses (Baker and Rosenqvist, 2004; Valladares et al., 2005). Therefore, chlorophyll-a fluorescence analysis paves a way for indirect exploration of various functional levels of photosynthesis, and for evaluation of plant health status.

2.3.1.2. Stomatal conductance

Stomata are important openings on leaf surfaces that allow gas and water exchange to take place in plants. Higher carbon dioxide (CO_2) concentrations in a leaf accelerates the Calvin cycle (Kimura et al., 2020), a process that converts CO_2 and water into organic compounds through a variety of reactions involving ribulose-1,5-bisphosphate carboxylase–oxygenase (Rubisco), NADPH and ATP (Khalid et al., 2019). Closure of stomata is one of the first activities that take place during drought to prevent water loss through transpiration (Pirasteh-Anosheh et al., 2016), leading to a decrease in gas exchange. A decrease in CO_2 concentration results in reduced rates of photosynthesis (Singh and Reddy, 2014; Mwamlima et al., 2021). Limited stomatal conductance due to drought increases the likelihood of ROS production, especially H_2O_2. During oxidative stress, which is caused by overproduction of ROS, H_2O_2 inhibits Calvin cycle enzymes such as fructose 1, 6-bisphosphatase (FBPase) and can also regulate the net CO_2 assimilation rate and stomatal conductance, which negatively impacts photosynthesis (Damour et al., 2010).

Drought stress has detrimental effects to the cell's basic organizational structure, resulting in damaged photosynthetic equipment and limited carbon uptake into the leaf (Wang et al., 2018; Kapoor et al., 2020). During the drought period, endogenous abscisic acid (ABA) is rapidly produced, initiating a series of physiological reactions, such as stomatal closure, which is controlled by a signal transduction network (Osakabe et al., 2014). Water deficiency stress also increases heat dissipation in the form of non-photochemical quenching (NPQ) when the stomatal conductance decreases, leading to an increase in leaf temperature. The persistent forms of NPQ can cause photoinhibition and photorespiration, or damage to the PSII reaction centre (Najar et al., 2019; Helm et al., 2020). In soybeans, stomatal conductance and net photosynthesis were decreased under water deficit stress (Mutuva et al., 2015). Similarly, Chowdhury et al. (2016) observed that water deficit stress decreased stomatal conductance in soybeans compared to non-stressed soybeans. Additionally, Hlahla et al. (2022) observed that five drought-tolerant and drought-susceptible edamame cultivars exhibited substantial decline in stomatal conductance when exposed to drought stress.

2.3.1.3. Total soluble sugars

The photoassimilates are transported from the photosynthetic source (leaves) to the sink (roots, stems, and flowers) organs, making photosynthesis process critical to ensure plant development and productivity (Ho, 1988; Rosa et al., 2009). Drought stress leads to a significant reduction in the efficiency and rate of photosynthesis and thus, reduces the supply of soluble sugars to sink tissues (Rosa et al., 2009). During the early stages of plant development, growth depends mainly on mobilised carbohydrates in the form of soluble sugars such as glucose, sucrose, and fructose, to various organs. Up to 80% of the CO_2 absorbed during photosynthesis is allocated towards production of the soluble sugars during the Calvin cycle (Prado et al., 2000; Santos and Buckeridge, 2004).

Usually under abiotic stress, there is a level fluctuation in soluble sugars due to affected CO_2 assimilation in the leaves (Gupta and Kaur, 2005; Rosa et al., 2009). It is commonly known that the generation and accumulation of soluble sugars directly contribute to the stabilization of proteins like Rubisco, radical scavenging, osmotic adjustment, carbon storage (Ghosh et al., 2021). Sugars are the most prevalent osmolytes used by plants to regulate their osmotic balance. They also serve as a crucial source of energy for plant cells; therefore, their accumulation is vital for survival during drought stress (Khan et al., 2020).

Rahman et al. (2022) and Begum et al. (2022) showed a substantial accumulation of total soluble sugars (TSS) in water stressed soybeans compared to optimum watered soybeans. In drought-stressed edamame, a higher TSS content was observed during flowering than pod-filling (Moloi and van der Merwe, 2021). Additionally, research done on wheat (Planchet et al., 2014) and rice (*Oryza sativa* L.) (Pattanagul, 2011), showed an increase in TSS under water deficit conditions, ultimately leading to increased drought tolerance.

2.3.1.4. Osmotic adjustment and relative water content

Water is required for proper functioning of a cell's protoplasm. In general, plants respond to drought by either increasing water absorption or minimizing water loss (low stomatal conductance). This process is facilitated by a biochemical response mechanism that helps plants adapt to arid conditions known as osmotic adjustment (OA) (Sanders and Arndt, 2012). The amount of osmotically active compounds in the cell increases because of OA. The rise in solute concentration results in a greater negative osmotic potential, which can enhance cell hydration and sustain turgor in metabolically active cells. In other words, if OA happens, plants can continue to grow and maintain metabolic functions in drying soil (Basu et al., 2006; Chowdhury et al., 2017). Crops with improved OA under drought stress result in higher growth and yield. Osmotic adjustment is caused by a variety of compounds, including organic acids, carbohydrates, amino acids, and inorganic cations and anions. It is frequently linked to the deposition of solutes such as sugars, proline and glycine betaine that contain protective properties (Ashraf and Foolad, 2007). These solutes can concentrate in the cytoplasm and aid in preventing the dehydration of enzymes, cellular proteins, and membranes. It is crucial to understand that, in many species, individual solutes do not contribute substantially to OA, and that the accumulation of several diverse solutes is mostly what induces OA (Sanders and Arndt, 2012).

There is also an indication that research has undervalued leaf relative water content (RWC) when quantifying OA, which adds to the intrinsic variability in OA expression (Sanders and Arndt, 2012). For a precise evaluation of the relative capacity for OA in various plants, proper monitoring of plant water status, such as RWC, is essential. Relative water content is used to evaluate internal plant water status under water deficit stress (Gonzales and Gonzales-Vilar, 2001; Omae et al., 2007; Chowdhury et al., 2017) and it reflects the balance between leaf tissue water supply and transpiration rate (Soltys-Kalina et al., 2016). Colom and Vazzana (2003) demonstrated a correlation between the effects of water deficit stress and the photosynthetic apparatus in weeping love grass (*Eragrostis curvula*) plants. Under water deficit stress, a susceptible weeping love grass cultivar displayed a 65% reduction in RWC along with significant drops in photosynthetic rates, Fv/Fm and leaf pigment content, correlating reduced RWC with degradation of the photosynthesis apparatus (Colom and Vazzana, 2003). Drought-sensitive pea (*Pisum sativum* L.) genotypes were significantly affected by a decrease in RWC than tolerant genotypes (Upreti et al., 2000). Relative water content in soybeans grown under water stress was lower than that of plants cultivated under non-stress conditions. Susceptible soybean cultivars had the lowest RWC compared to the tolerant cultivars (Chowdhury et al., 2017). In a study by Sarker and Oba (2018), a decrease in RWC and plant growth was observed with an increase of drought stress in edible amaranth (*Amaranthus tricolor).* Therefore, RWC can be used as a parameter in studies that investigate how well plants can withstand drought stress since it is a very good indicator of the plant's water status.

2.3.2. Drought impact on the biochemical reactions

2.3.2.1. Production of reactive oxygen species

An increase in the generation of ROS during drought stress (You and Chan, 2015; Verma et al., 2019) emanate's from stomatal closure affecting photosynthesis, increased photorespiration, and the decrease of the photosynthetic electron transport chain (Hernández et al., 2012). Reactive oxygen species are produced because of a reduction in intracellular CO_2, which causes electrons to be transferred from the electron transport chain to oxygen at PSI (Rao and Chaitanya, 2016).

Excessive amount of ROS in plant cells causes oxidative stress, which results in changes to the intrinsic properties of biomolecules such as proteins, lipids, and nucleic acids, leading to cell death (da Silva et al., 2013; Nadeem et al., 2019).

Reactive oxygen species such as superoxide radicals (O_2^-), hydroxyl radicals (•OH), and H_2O_2 are highly reactive and can damage the plants' natural functions by causing lipid peroxidation, protein breakdown, DNA fragmentation, chlorophyll degradation, and cell death in the absence of effective protective mechanisms (Rao and Chaitanya, 2016; Kapoor et al., 2020). Moloi and van der Merwe (2021) reported high levels of H_2O_2 in susceptible edamame cultivars under drought stress compared to drought-tolerant ones. At lower levels, authors showed that H_2O_2 acts as an essential biochemical molecule involved in the signaling events of drought tolerance. The following subsections discuss the consequences of oxidative burst on plant cells.

a) Lipid peroxidation

The production of ROS (O_2^-, H_2O_2, and •OH) can damage membrane lipids directly, leading to an increase in lipid peroxidation. Malondialdehyde (MDA) is produced as a result, especially during droughts (Figure 2.2) (Moore and Roberts, 1998; Anjum et al., 2011b; Labudda, 2013; Laxa et al., 2019). Malondialdehyde is a by-product of oxidative lipid modification that causes cell membrane damage, including changes to the membrane's intrinsic characteristics such as permeability, ion transport, enzyme activity loss, and protein crosslinking. Cell death occurs due to these alterations (Anjum et al., 2011b; Labudda, 2013). Therefore, MDA has been utilized as a marker for lipid peroxidation in membranes. Several studies have linked high levels of lipid peroxidation (MDA content) to cell membrane damage in drought-stressed crops, with drought-sensitive cultivars having much higher levels (Gülen et al., 2008; Harish et al., 2010; Ali and Ashraf, 2011; Anjum et al., 2011a; Anjum et al., 2011b; Mohammadi et al., 2011; Singh et al., 2012; Wang et al., 2019). Under drought stress, Moloi and van der Merwe (2021) observed a significant amount of MDA in susceptible edamame compared to a more drought tolerant cultivar. Hamim et al. (2017) found a similar tendency, with non-stressed soybean plants having a lower amount of MDA than drought-affected plants. Considering that tolerant cultivars have lower MDA levels, they may have better systems for scavenging ROS, thereby minimizing oxidative stress.

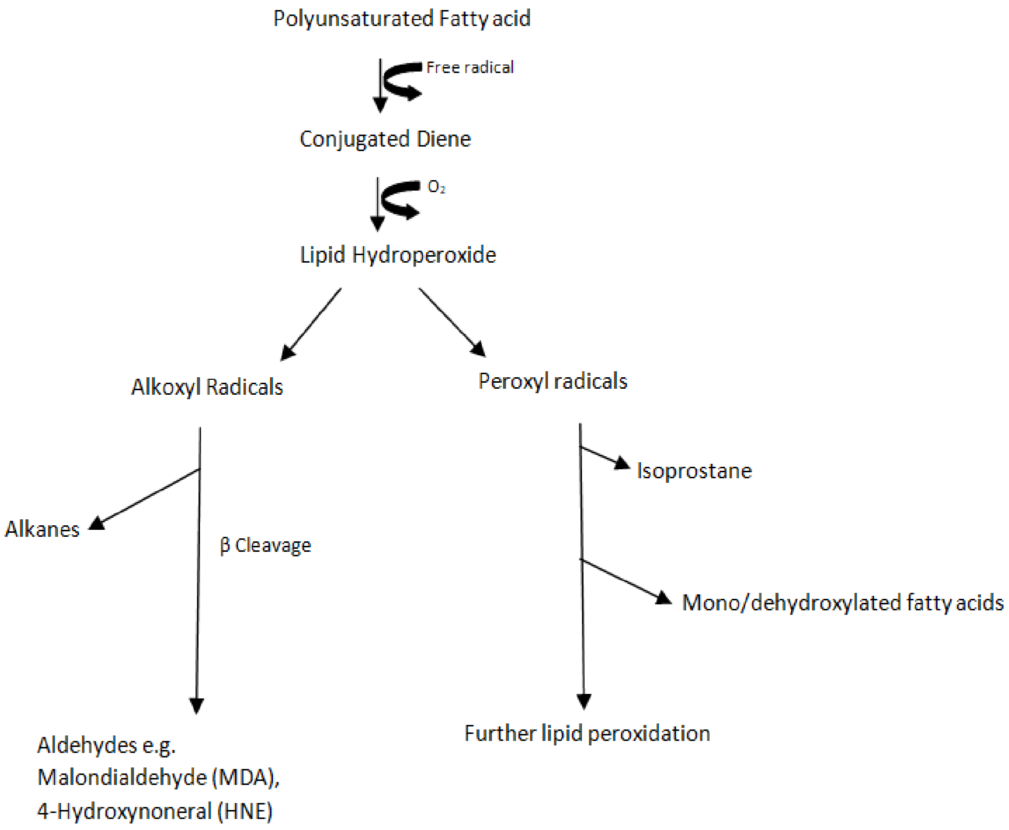


Figure 2.2. An illustration of the lipid peroxidation product's synthesis. *Membrane phospholipids' polyunsaturated fatty acid (PUFA) components are particularly vulnerable to ROS action. Hydroperoxides, lipid peroxyl radicals, and conjugated dienes are produced when hydroxyl radicals or superoxide combine with PUFA methylene groups. Aldehydes (crotonaldehyde, malondialdehyde, and acrolein), lipid alkoxyl radicals, alkanes, lipid epoxides, and alcohols are readily produced when lipid hydroperoxides decompose (Moore and Roberts, 1998; Labudda, 2013).*O_2*- Oxygen.*

b) *Electrolyte Leakage*

Cell membrane integrity is one of the best physiological indicators of drought stress tolerance (Bandurska, 2000). The integrity of the membranes is damaged under drought stress, and the tissues are damaged by ROS, which promotes severe injuries, such as lipid peroxidation and cell content leakage (Silva et al., 2016). When lipid peroxidation damages the cell membrane, cells are unable to retain the chemical composition of their contents and as a result, electrolytes are released via the damaged membranes (Murray et al., 1989). Measurement of cell electrolyte leakage (EL) can be used to determine cell membrane damage during drought (Bandurska, 2000). A low level of injuries is linked to a high level of cell membrane integrity, which ensures greater resistance to water stress (Ciulca et al., 2017). Jungklang et al. (2015) observed high levels of EL in drought-stressed siam tulip (*Curcuma alismatifolia)*. Electrolyte leakage increased in all wheat varieties, with susceptible wheat cultivars having higher EL than tolerant wheat varieties (Sayar et al., 2008). To avoid oxidative burst and cell membrane damage, H_2O_2 production must be carefully controlled (Moloi and van der Merwe, 2021).

2.3.2.2. Reactive oxygen species scavenging mechanisms

Plants have a variety of biochemical defences to endure periods of drought. Some plants have an in-built protective system that is elaborate enough to prevent ROS damage and ensure proper cellular function. To adapt to abiotic stress conditions, plants employ a variety of methods, such as accumulating protective proteins, compatible osmolytes, enzymatic systems, non-enzymatic systems, and actively producing antioxidant systems (Naz et al., 2021). An antioxidant system, which can eliminate prooxidants (ROS and lipid peroxyl radicals) and maintain a healthy cellular redox balance, is one of the most essential drought defence mechanisms. The ROS can be neutralized by antioxidants (ROS scavengers) either directly or in coordination with other antioxidants. Additionally, because antioxidants are important sensors of the cellular redox status, they set off several signaling processes aimed at maintaining a healthy cellular redox balance (Nadeem et al., 2019).

The defence mechanism against ROS in plant cells is regulated by enzymatic and non-enzymatic systems. Maintaining a high antioxidant enzyme activity is an adaptive response under drought stress. Non-enzymatic antioxidants include glutathione, ascorbate (Shan et al., 2018), tocopherols, carotenoids, phenolics, flavanols (Rao and Chaitanya, 2016), and ascorbic acid (Nadeem et al., 2019). Enzymatic antioxidants include ascorbate peroxide (APX), catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Anjum et al., 2011b; Nadeem et al., 2019).

2.3.2.2.1. Non-enzymatic mechanisms

a) Ascorbate and glutathione

Non-enzymatic antioxidative systems include metabolites such as ascorbate and glutathione, which are very important in the ascorbate-glutathione (AsA-GSH) cycle (Figure 2.3) (Shi et al., 2013; Hernández et al., 2017; Shan et al., 2018; Hasanuzzaman et al., 2019). Under drought stress, the AsA-GSH cycle plays a crucial function in protecting plants against oxidative damage and balancing the redox state of the glutathione and ascorbate (Shan et al., 2018). The AsA-GSH is a principal ROS-scavenging response in the chloroplasts, cytosol, mitochondria, apoplast, and peroxisomes. It prevents oxidative stress caused by various abiotic stresses (Caverzan et al., 2016).

b) Flavonoids

Other ROS scavengers within the non-enzymatic category include flavonols (Rao and Chaitanya, 2016). Flavonoids are produced via the phenylpropane pathway as secondary metabolites. They play a role in plant defence responses to biological and non-biological stress. The hydroxyl groups at the 3' and 4' positions of flavonoids participate in the scavenging of oxygen free radicals as non-enzymatic antioxidants, and flavonoids' significance in reducing stress-induced oxidative damage has been widely reported (Li et al., 2021). Flavonols also function as potential antioxidant molecules by donating electrons to guaiacol peroxidases for the detoxification ofsubstantial quantities of H_2O_2 formed under stress conditions. Yang et al. (2007) observed a substantial increase in flavonol content in drought-stressed *Glycyrrhiza inflata Batal* compared to the control treatments

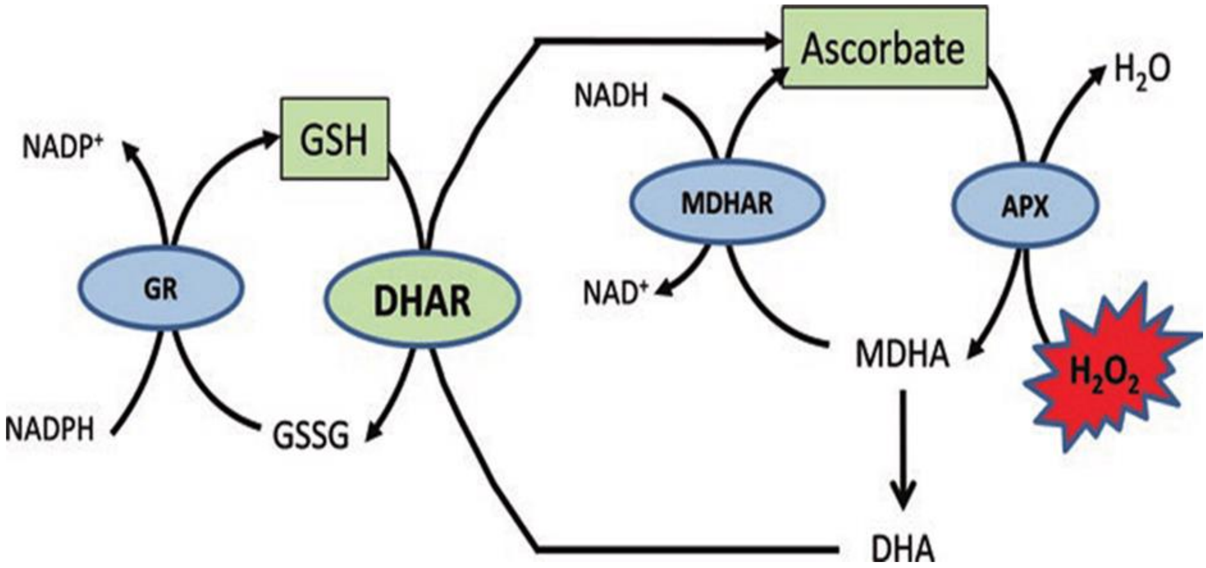


Figure 2.3. Plants' ascorbate–glutathione (AsA–GSH) cycle. *This cycle is made up of a set of reactions whose primary purpose is to scavenge* H_2O_2*. The enzyme APX uses AsA as an electron donor and transforms* H_2O_2 *into water as the first step in the AsA-GSH cycle. AsA is also transformed to MDHA. The activity of MDHAR causes MDHA to regenerate AsA once more in the second step, and some of this is naturally converted into DHA. In the third step, GSH is used to decrease DHA to AsA once more, which causes it to be oxidized to create GSSG. Finally, employing NADPH as the electron donor, this GSSG regenerates GSH by the action of GR (Shi et al., 2013; Hernández et al., 2017). GR- Glutathionine, MDHA- Monodehydoascorbate, MDHAR- Monodehydroascorbate, APX- Ascorbate peroxidase, DHAR- Dehydroascorbate reductase, DHA- Dehydroascorbate, GSH- glutathione,* NAD^+*-Nicotinamide adenine dinucleotide,* H_2O_2*- Hydrogen peroxide,* H_2O*- Water,* $NADP^+$*- Nicotinamide adenine dinucleotide phosphate, NADPH- Nicotinamide adenine dinucleotide phosphate hydrogen, GSSG- Glutathione disulfide, NADH- Nicotinamide adenine dinucleotide bonded with hydrogen ion.*

c) Carotenoids

Carotenoids are pigments that are necessary for many functions in plants and may act as antioxidants under stress. They capture light, scavenge for singlet oxygen species, dissipate excess damaging energy during stress conditions, additionally they are membrane stabilizers, and protectors of the photosynthetic apparatus (Parvaiz et al., 2010; Sarker and Oba, 2018; Uarrota et al., 2018). Photosynthetic tissues are protected through direct quenching of triplet chlorophyll by carotenoids, which defend against oxidative damage and improve plants in enduring drought conditions (Sarker and Oba, 2018). The chemical reactivity of carotenoids with free radicals like peroxyl (ROO•), hydroxyl (•OH), and superoxide radicals (O_2•) quenches singlet oxygen. At sufficient concentrations carotenoids act as photo-protectors and can prevent lipids from peroxidative degradation (Parvaiz et al., 2010; Maoka, 2020; Tanwir et al., 2021). Hlahla et al. (2022) noted that in drought tolerant edamame there was no reduction in carotenoids molecules compared to the susceptible cultivar, showing their protective role on the photosystems. Increased levels of carotenoids and antioxidant activity were observed in edible amaranth plants that were exposed to different levels of drought stress. In this study, the increase in carotenoids was associated with drought stress tolerance (Sarker and Oba, 2018).

d) Proline

Osmolytes like amino acids and their derivatives such as glutamate, glycine betaine and proline are increased in response to abiotic stresses. Among them, proline is the most essential osmolyte under water deficiency stress (Jogawat, 2019). As an antioxidant, proline aids in scavenging free radicals (Ghosh et al., 2021), buffering cellular redox potential, and maintaining sub-cellular structures under stress circumstances, in addition to osmotic adjustment (Anjum et al., 2011a; AL-Aloosy et al., 2019). Additionally, a rapid breakdown of proline might produce adequate reducing agents to promote mitochondrial oxidative phosphorylation and the production of ATP for stress recovery and damage repair (AL-Aloosy et al., 2019). Drought stress enhanced proline levels in pea cultivars, which increased their tolerance levels (Alexieva et al., 2001). Yamada et al. (2005) also observed a build-up of free proline in a drought-tolerant petunia (*Petunia hybrida*) cultivar under drought stress. Soybean increased proline levels under water deficit stress (60-80% increase compared to the controls) (Rezayian et al., 2020; Waqar et al., 2022). Likewise, under water deficit stress, there was an increase in proline content in alfalfa (*Medicago sativa)* (An et al., 2014), field elm (*Ulmus minor)* (Dias et al., 2014), mung bean (*Vigna radiata)* (Farooq et al., 2006), wheat (Sarafraz-Ardakani et al., 2014; Pal et al., 2018) and in edamame (Moloi and van der Merwe, 2021).

2.3.2.2.2. Enzymatic mechanisms

a) Ascorbate peroxidase and guaiacol peroxidase

Enzymes such as peroxidases (APX and GPX), SOD and CAT are important in scavenging the ROS. These enzymes work against oxidative stress because they convert H_2O_2 to water (AL-Aloosy et al., 2019). The APX enzyme does this within the ascorbate–glutathione cycle (Pandey et al., 2015). In addition, APX may oversee adjustments of ROS signaling since it has a higher affinity for H_2O_2 (Parvaiz et al., 2010). Increased APX during flowering and pod development stages was closely associated with drought tolerance in grain soybeans (Moloi et al., 2016).

The increased level of antioxidative enzymes differs for every cultivar and the developmental stage of a crop under environmental stress. In edamame, APX activity significantly dropped for susceptible cultivars while it increased for drought-tolerant cultivars. Similarly, there was a substantial drop in the level of GPX in drought-susceptible cultivars with an increase in a moderately tolerant cultivar. At the flowering stage of a moderately tolerant cultivar, there was a substantial increase in GPX under drought stress (Moloi and van der Merwe, 2021).

b) Superoxide dismutase

The dismutation of superoxide (O_2^-) into H_2O_2 and oxygen (O_2) is catalysed by SOD, which inhibits the synthesis of •OH, a more reactive ROS (Bartwal et al., 2013; Suman et al., 2021). High SOD is essential for providing resistance against oxidative stress (AL-Aloosy et al., 2019; Roychowdhury et al., 2019). Plants that are affected by drought stress increase their SOD activity to alleviate the detrimental effects of ROS. Improved oxidative stress tolerance in drought-stressed rice seedlings was linked to increased SOD content (Sharma and Dubey, 2005). Upregulated SOD activity also played a crucial function in protecting peony (*Paeonia lactiflora*) plants against water deficit conditions (Wang et al., 2019; Kapoor et al., 2020). Such evidence shows that an increase the activity of SOD is associated with the reduction of oxidative stress imposed by abiotic stress and is important for plant life and health.

2.3.3. Drought impact on the morphological responses

During drought stress, plant cells undergo a sequence of physiological and biochemical changes, and the resulting damage is represented in the plants' exterior appearance (Wang et al., 2019). Drought stress affects cell expansion, mitosis, and elongation resulting in reduced leaf size, reduced height, fewer leaves, stomatal closure, and alterations in the plant's reproductive phase, with ultimate effect on growth and development (Anjum et al., 2011b; da Silva et al., 2013; Modi and Mabhudhi, 2013). Drought affected height, leaf number, and leaf expansion in soybeans (Modi and Mabhudhi, 2013), mung bean (Bangar et al., 2019), maize (Su et al., 2019) and wheat (Shan et al, 2018) seedlings.

Different edamame cultivars were studied under drought stress to determine their morphological responses to water deficit conditions (Chen et al., 2021). Total pod dry weight of drought-stressed plants was considerably lower than that of the control, suggesting that the yield of edamame cultivars significantly decreases under drought stress. In addition, cultivars that showed drought sensitivity had lower plant height, fertile pod, seeds per plant, and seeds per pod. Although drought reduced yield parameters in edamame, the level of reduction differed according to drought sensitivity of the cultivars (Chen et al., 2021). Some SA cultivars such as UVE17 displayed high sensitivity to drought stress with high yield reduction, while others such as UVE14 and AGS429 showed more drought tolerance according to their physiological responses and morphological traits (van der Merwe et al., 2018; Moloi and van der Merwe, 2021; Hlahla et al., 2022).

To improve drought tolerance and crop yields under changing climates in edamame, intense plant breeding must take place to produce cultivars that can express induced biochemical and physiological responses. Since this is a long process, alternative methods are needed to improve tolerance of plants to drought and increase food security. The use of hydrogen sulfide-releasing agents is one of the recently studied strategies in plants.

2.4. Hydrogen Sulfide (H_2S)

Hydrogen sulfide (H_2S) is a naturally occurring, colourless gas with a distinct rotten egg odour (Chen et al., 2011; Aroca et al., 2018). Although toxic at high concentrations (Chen et al., 2011), it is a naturally released gas in many plants (Wilson et al. (1978) as cited in Huo et al., 2018). In wetlands, salt marshes, and estuaries, bacteria naturally produce the gas during the anaerobic decomposition of organic sulfur compounds and it is also found in volcanoes and geothermal vents. In addition to natural sources, atmospheric H_2S is emitted by biomass, burning fossil fuels in industrial processes and by livestock (Lisjak et al., 2013; Ausma and De Kok, 2019).

In soils, sulfur occurs in organic and inorganic forms (Tabatabai, 1987). The uptake of inorganic sulfate by sulfate transporters is the initial stage of sulfur used by plants. Sulfate is carried to the central cylinder by plasmodesmata once it has entered the epidermis. Alternatively, sulfate can leak into the apoplast, which also plays an important role in the dispersion of sulfur. Once inside the cell, sulfate is either instantly metabolized to H_2S or stored in the vacuole (Calderwood and Kopriva, 2014). The following section explains how inorganic sulfate from the soil is used by the plants to synthesize H_2S.

2.4.1. Hydrogen sulfide biosynthesis

Plants can either absorb H_2S through the roots as sulfate (SO_4^{2-}) or through the leaves' stomata as H_2S, sulphur dioxide (SO_2), and carbonyl sulfide (COS) (Liu et al., 2021). Sulfate is subsequently transferred from the roots to all areas of the plant via the xylem vessels. A portion of SO_4^{2-} that enters the cells will be stored in the vacuoles, and the remaining portion will enter the chloroplast's assimilation route (Saud et al., 2022). In the chloroplast, SO_4^{2-} is converted to adenosine 50-phosphosulfate (APS) under the catalysis of ATP sulfurylase. The APS is first converted to sulfite (SO_3^{2-}) by APS reductase (APR) (Liu et al., 2021). Ferredoxin-dependent sulfite reductase (SiR) then converts SO_3^{2-} to sulfide (Huo et al., 2018). Lastly, O-acetylserine thiol lyase (OAS-TL) incorporates sulfide into the amino acid skeleton of O-acetylserine (OAS) to create cysteine, and its reverse process might release H_2S (Arif et al., 2018). L-cysteine desulfhydrase (L-CDes) and D-cysteine desulfhydrase (D-CDes) enzymes oversee the majority of endogenous cytosolic H_2S generation (Fotopoulos et al., 2015; Aroca et al., 2018; Liu et al., 2021). When stomata are the source of H_2S, SO_2 can be transformed into SO_3^{2-} and enter the assimilation pathway, and COS can be hydrolysed by carbonic anhydrase (CA) to generate H_2S (Figure 2.4) (Liu et al., 2021).

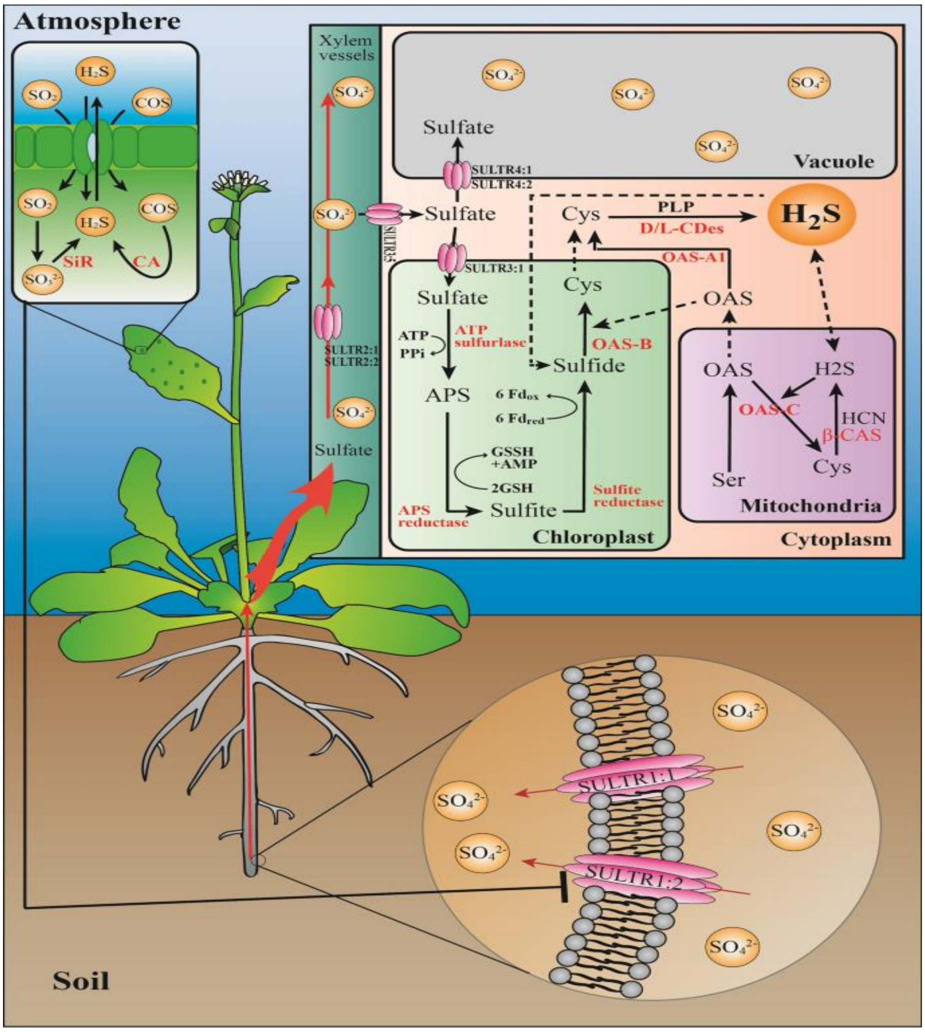


Figure 2.4. Biosynthetic pathways of hydrogen sulfide in plants. *In the presence of ferredoxin, SiR catalyses the conversion of sulfite (SO_3^{2-}) to H_2S in plant cells. The SO_2 (from the atmosphere) or sulfate (SO_4^{2-}), generated by sulfur feeding, acts as the substrate for H_2S biosynthesis (Liu et al., 2021). CA- Carbonic anhydrase, SiR- Sulfite reductase, ATP- Adenosine triphosphate, OASB-, D-CDes- D-Cysteine desulfhydrase, L-CDes – L-Cysteine desulfhydrase -, OAS- O-acetylserine, OAS-TL- O-acetylserine thiol lyase, COS- carbonyl sulfide, APS- adenosine 5'-phosphosulfate, Cys- Cysteine, Ser- Serine, Fd_{ox}- oxidized ferredoxin, Fd_{red}- reduced ferredoxin, GSSH- oxidized glutathionine, GSH- Glutathionine, PLP- 5’-pyridoxal phosphate.*

2.4.2. Hydrogen sulfide’s function in the modification of plant proteins

Hydrogen sulfide received a great deal of interest recently since it plays an important part of the plant's response to numerous biotic and abiotic stressors at low concentrations (Chen et al., 2011). Its significance as a major gasotransmitter in plants has lately been acknowledged (Shan et al., 2018; Khan and Corpas, 2021), as it demonstrated to be beneficial for plant development, survival, and harvest yield at low quantities (Zhang et al., 2008; Zhang et al., 2010b; Wang et al., 2012; Dooley et al., 2013; Carter et al., 2019; Arif et al., 2021). In addition to endogenous production, H_2S can be supplied to plants via donors such as sodium sulfite (Na_2SO_3), sodium hydrosulfide (NaHS), sodium sulfide (Na_2S), sodium sulfate (Na_2SO_4), sodium hydrogen sulfate ($NaHSO_4$), sodium hydrogen sulfite ($NaHSO_3$), sodium acetate, and morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate (GYY4137) (Zhang et al., 2009; Fotopoulos et al., 2015; Carter et al., 2019; Arif et al., 2021; Brown et al., 2021; Chen et al., 2021), and recently via dibutyldithiophosphates (Carter et al., 2019; Brown et al., 2021). Most donors release H_2S in response to hydrolysis, light, or the presence of thiols. Low dosages of H_2S are often delivered over extended periods, ranging from days, weeks, and months, using chemicals that slowly release H_2S by hydrolysis (Brown et al., 2021). During drought stress, which often releases ROS, H_2S modulates the persulfidation of cysteine (Cys) residues directly through post-translational modification (PTM), enabling H_2S to control protein activity (Wang et al., 2021). Persulfidation is a PTM of proteins in which reactive cysteine residues on target proteins are changed by converting the thiol group (-SH) into a persulfide group (-SSH); and the activities, structures, and subcellular localization of the target proteins are altered functionally because of this modification (Filipovic, 2015; Aroca et al., 2018) (Figure 2.5).

Furthermore, modification leads to a change in target protein activities. A recent study indicated that persulfidation elevated the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in thale cress (*Arabidopsis thaliana)*. In addition, protein persulfidation elevated the activity of APX in various Arabidopsis cell compartments such as cytosol, chloroplasts, mitochondria, and peroxisomes (Aroca et al., 2015). Such findings suggest that H_2S-mediated persulfidation regulates ROS toxicity by activating ROS scavenging enzymes (Khan et al., 2022). It was reported that when the endogenous level of H_2S rise in response to environmental factors, the activity of H_2S-generating desulfhydrases rises as well (Arif et al., 2021).

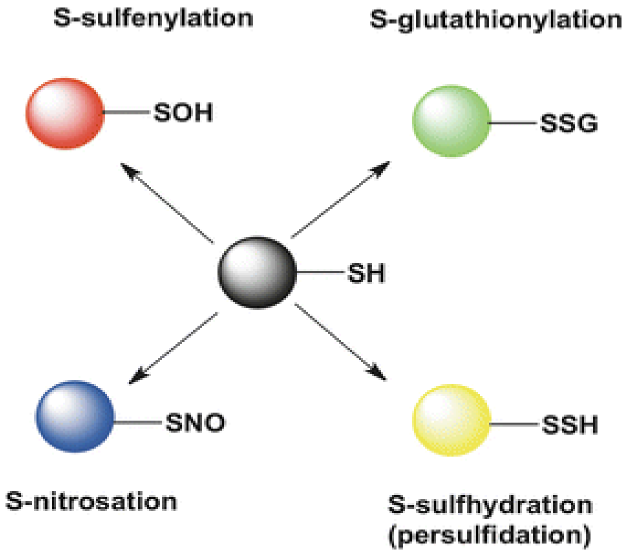


Figure 2.5. Protein cysteine residues undergo oxidative posttranslational changes that influence protein structure and function. *The grey sphere represents the protein cysteine where the thiol group (-SH) is converted into a persulfide group (-SSH) (yellow sphere) (Filipovic, 2015).*

Moreover, reports show that the use of H_2S, which comes from a variety of donors, helps crops to withstand drought stress (Carter et al., 2019; Mukherjee and Corpas, 2020; Arif et al., 2021; Brown et al., 2021; Chen et al., 2021). As a result, H_2S is now considered as one of the most important signaling molecules in agriculture (Carter, 2018; Chen et al., 2020; Chen et al., 2021).

2.4.3. The impact of hydrogen sulfide on the physiological and biochemical responses of plants

Since 2005, researchers repeatedly showed that H_2S delivered at optimal concentrations had dramatic effects on plants. It led to more than doubling the sizes of roots, protected and improved crop performance during drought, salt, heat, and cold stresses, prolonged shelf-lives of harvested fruit (Carter et al., 2018), and acted as a signaling molecule for induction of plant defence responses under stress (Carter et al., 2019; Brown et al., 2021). Exogenous application of H_2S can also suppress the burst of ROS by activating enzymatic and non-enzymatic defence components of the ascorbate-glutathione cycle, consequently averting oxidative stress damage to plants (Arif et al., 2021). In this review, important information that demonstrates a significant effect of H_2S on the physiology, biochemistry, and morphology of crop plants is discussed, with a specific focus on abiotic stress. The review shows that H_2S greatly improves plant tolerance to abiotic stress via its effects on physiology, biochemistry, and morphology.

2.4.3.1. Effect of H_2S on photosynthesis

It is widely known that increasing the activity of Rubisco can enhance the photosynthetic capacity of plants (Chen et al., 2011; Liu et al., 2021). The increase in Rubisco protein expression and activity and the involvement of proteins in thiol redox modification were the main reasons for the increase in net leaf photosynthesis produced by sodium hydrosulfide (NaHS) in a study conducted by Chen et al. (2011) on non-stressed spinach. Such H_2S donor treatment significantly boosted Fv/Fm and chlorophyll content, which further improved photosynthesis and plant biomass. Furthermore, they found that exogenous treatment of a low concentration of NaHS increased the activities of OAS-TL, and L-cysteine desulfydrase (LCD) along with the other photosynthetic properties (Chen et al., 2011). In another study, NaHS was applied in combination with rhizobia in soybeans, which led to significant increases in chlorophyll synthesis (Zou et al., 2020). Peaembryo radicle length and chlorophyll content increased after H_2S administration under non-stressed conditions (Zhang et al., 2009; Li et al., 2010). Additionally, H_2S increased stomatal conductance, leading to upregulation of photosynthesis in non-stressed rice (Duan et al., 2015). Furthermore, spinach seedlings that were treated with NaHS had increased photosynthetic rate and Rubisco activity (Chen et al., 2011). The effect of H_2S donors on the photosynthetic responses of plants under different abiotic stress conditions are recorded below.

Low temperature stress diminished chlorophyll content of blueberry (*Vaccinium cyanococcus*), which reduced its ability to absorb and utilize light energy (Tang et al., 2020). The Fv/Fm and PI_{abs} were also decreased under low temperature stress, indicating the negative effect of this stress on the PSII photochemical activity and performance of PSI, which could lead to photoinhibition. Moreover, low temperature degraded the D1 protein which is the core of the PSII reaction center and inhibited electron transport from Q_A to Q_B in the PSII acceptor side. However, exogenous application of hydrolysed NaHS increased the turnover of D1 protein and improved PSII and PSI activities, which improved chilling resistance. In addition, a smaller decrease in gaseous exchange was also observed after spraying leaves under low temperature stress with hydrolysed NaHS compared to the control, suggesting that exogenous H_2S stimulated stomatal opening, which could increase CO_2 fixation and boost the photosynthetic capability of blueberry leaves (Tcherkez and Limami, 2019; Tang et al., 2020).

Bharwana et al. (2014) studied the effects of H_2S under lead stress in cotton (*Gossypium herbaceum*) plants. Due to lead toxicity, photosynthetic pigments such as chlorophyll-a, chlorophyll-b, total chlorophyll, and carotenoids declined significantly. Interestingly, the degree of reductions in photosynthetic pigments was notably lower in NaHS-treated plants, indicating that H_2S was efficient in reducing the inhibitory effects of lead stress. Barley exposed to combined drought and heat stress had substantial decreases in chlorophyll-a, chlorophyll-b, and carotenoids. However, after NaHS treatment, these plants had upregulated contents of the photosynthetic pigments (Naz et al., 2021).

Liu et al. (2022) noted that there was a significant decrease in the biosynthesis of chlorophyll and carotenoid, which inhibits photosynthesis, thereby restricting growth of tall fescue (*Festuca arundinacea*) plants under low light stress. However, application of NaHS significantly increased chlorophyll and carotenoid contents. In addition, they found that plants under low light stress and treated with NaHS had higher net photosynthetic rates and Fv/Fm values than plants exposed to low light stress with no NaHS treatment. These findings show that H_2S improved the photosynthetic efficiency and benefited tall fescue's tolerance to low light.

Furthermore, Zhang et al. (2010a) investigated how NaHS affected soybean seedlings under drought stress and found that it reduced the damaging effects of drought on chlorophyll content. Although there are reports on the beneficial effects of H_2S on photosynthesis under different environmental stresses, and it appears that the effect of H_2S on photosynthesis under drought stress is often neglected. Evidence to show the effect of H_2S under drought stress in edamame is also lacking.

2.4.3.2. Effect of H_2S on osmolytes and total soluble sugars

Osmolytes and TSSplay a key role in plant defense under different environmental stress (Roychoudhury and Chakraborty, 2021). Proline and total soluble sugars accumulated under salt, drought, and cold stresses in bermudagrass (*Cynodon dactylon*), and exogenous application of NaHS increased these osmolytes further under stressful conditions (Shi et al., 2013). In drought-stressed barley, NaHS treatment increased proline accumulation by 316% and considerably reduced the negative effects of drought (Naz et al., 2021).

Several sugars, including glucose, sucrose, fructose, and trehalose, function as effective osmolytes during the osmotic adjustment that plants go through in response to osmotic stress. In comparison to plants grown in a non-stressed environment, stressed plants have substantially higher TSS content. It is commonly found that these sugars have an important mediating role in helping plants develop tolerance to a variety of abiotic stresses (Roychoudhury and Chakraborty, 2021). Shi et al. (2013) reported that bermudagrass plants that were subjected to salt and cold stresses had increased accumulation of TSS. Exogenous administration of NaHS induced the accumulation of these osmolytes further. Contrary to barley, exposure to drought and heat stress down regulated the accumulation of TSS. Thereafter, application of NaHS increased TSS significantly (Naz et al., 2021). By increasing the concentration of TSS, H_2S donor protected the photosynthetic apparatus and maintained the health of plants for a higher barley yield (Naz et al., 2021).

2.4.3.3. Effect of H_2S on relative water content

Drought stress significantly decreases the RWC of leaves and roots. Studies demonstrated that application of H_2S donors under temperature and water stress conditions improved RWC of plants. For example, heat and drought-stressed barley showed increased RWC after application of NaHS (Naz et al., 2021). Also, H_2S application caused an increase in the leaf RWC in strawberry (*Fragaria ananassa*) (Christou et al. 2013). In addition, Garcia-Mata and Lamattina (2010) observed that treatment of drought-stressed broad beans (*Vicia faba)* with NaHS led to a 10% improvement in RWC. Moreover, Zhang et al. (2010a) further demonstrated that soybean seedlings treated with NaHS had an increased RWC under drought conditions.

2.4.3.4. Effect of H_2S on the reactive oxygen species production and scavenging mechanisms

Hydrogen sulfide is extremely mobile across plant membranes and can be influxed into or effluxed out of the plant system as a protective mechanism against environmental stress (Liu et al., 2021). Antioxidant activities, osmoregulator accumulation, cell signaling protein, and other gene expressions all contribute to plant stress adaptation against drought stress in H_2S-treated plants. Overall, H_2S allows plants to tolerate stress factors such as drought by altering intracellular redox balance and effectively removing ROS (Hasanuzzaman et al., 2020). Abiotic stressors increase ROS generation while also accumulating endogenous H_2S, which can help to alleviate oxidative stress by encouraging antioxidative defences to scavenge ROS (Liu et al., 2021).

Pre-treatment with NaHS provided better drought tolerance in thale cress through abscisic acid (ABA) interaction in stomatal regulation and increased SOD activity (Jin et al., 2011). Zhi et al. (2018) showed that the treatment of strawberries with H_2S reduced the cell wall-modifying enzyme activities and reinforced the hydrogen bonding in polysaccharides, causing stabilisation of the cell wall structure. It also increased APX and SOD activity, which reduced ROS and MDA, leading to reduced oxidative damage under drought stress in strawberries (Arif et al., 2021). Bermuda grass that was subjected to salt and cold stresses had high accumulation of major

ROS (H_2O_2 and O_2^-), and MDA. To maintain cellular survival, application of NaHS enhanced the main ROS detoxification systems contributing to plant defence such as the enzymatic antioxidant enzymes (SOD, CAT, POD, and glutathione reductase, GR) and non-enzymatic antioxidant molecules (ascorbate and glutathione). This led to improved health as well as increased growth and yield of Bermuda grass (Shi et al., 2013).

Numerous studies also demonstrated that NaHS application on drought-stressed plants upregulated APX and SOD activities (Khan et al., 2018; Almeida et al., 2020; Antoniou et al., 2020; Batista et al., 2020; Zhou et al., 2020; Ocvirk et al., 2020; Amir et al., 2021). This evidence further suggests the importance of H_2S in the defence responses of plants against environmental stress.

2.4.3.5. Effect of H_2S on electrolyte leakage and lipid peroxidation

Naz et al. (2021) observed that electrolyte leakage, MDA, H_2O_2, and O_2^- levels were higher in barley subjected to water deficiency and heat stress. A significant rise in antioxidant activity was seen in plants that had been exposed to H_2S donor NaHS, while oxidative stress markers such as MDA and O_2^- were reduced under drought and heat stress. A study conducted by Zhang et al. (2010a) under water deficit stress, revealed that NaHS-treated soybean seedlings had much lower amounts of H_2O_2, O_2, and MDA than untreated plants, which resulted in reduced oxidative damage.

2.4.3.6. Effect of H_2S on growth and yield

Small amounts of H_2S donors dissolved in water and absorbed by seeds or roots have a major impact on key components of plant physiology and increase agricultural yields for human consumption. The application of NaHS on tomato (*Solanum lycopersicum*) seedlings greatly increased plant height, dry weight and shoot fresh weight of tomato plants. Carter et al. (2019) investigated the effects of hydrolysed GYY-4137, another H_2S donor, on maize growth. Application of this H_2S donor at the lowest concentration resulted in increased plant weight and height. In addition, Brown et al. (2021) noted that corn plants treated with another H_2S donor known as dibutyldithiophosphate, showed an increase in growth and yield, with a significant harvest weight increase. Similarly, dibutyldithiophosphate treatment resulted in a 38% increase in the weight of the corn plants (Carter et al., 2018).

Exogenous delivery of NaHS accelerated growth, biomass, and physiological processes compared to non-H_2S treated controls (Arif et al., 2021). Dooley et al. (2013) exposed the root systems and seeds of peas, wheat, and corn to varying concentrations of H_2S. They reported significant increases in biomass and yield in all plant species. Strawberries' fruit yield was assessed, and the results showed that plants treated with NaHS developed better yield under alkalinity stress than untreated plants (Bahmanbiglo and Eshghi, 2021). Kaya et al. (2020) reported an increase in plant growth, fruit yield, and essential nutrients in *Capsicum annum* L. plants treated with NaHS exposed to salinity stress. Although most H_2S donors discussed here can improve the physiology, biochemistry and growth, emphasis should be on their environmental impact.

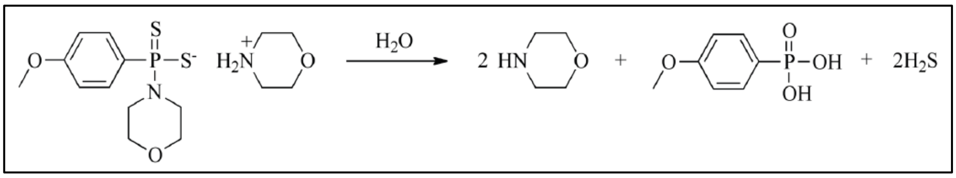
2.4.4. Dibutyldithiophosphate, a climate smart hydrogen sulfide donor

The breeding process for drought tolerance takes several years. To increase the performance of crop plants under drought stress, urgent climate-smart solutions are needed. To affect the growth, development and yield of edamame, the impacts of such solutions must be noticeable at the physic-biochemical and morphological level. Since H_2S can serve as a signaling molecule for the stimulation of defence reactions when applied or present in small concentrations (Shan et al., 2018; Khan and Corpas, 2021), it may be one of the possible solutions to improved drought tolerance in edamame.

However, chemicals used as H_2S donors need careful selection because the existing compounds that release H_2S may also release other chemicals that are not naturally occurring in the environment, constituting a pollution hazard (Carter, 2018). For example, upon hydrolysis, the commonly used H_2S donor, GYY-4137, releases morpholine and non-natural phosphate (Carter, 2018; Carter et al, 2019). Additionally, non-natural compounds are released into the environment by other H_2S donors such as Na_2S and NaHS (Corvino et al., 2021) (Figure 2.6A, B and C). These non-natural compounds impose waste to the environment and contaminate the soil (Carter, 2018).

To lessen the quantity of waste in the environment, it is essential to use biodegradable chemicals to donate H_2S (Carter, 2018). The biodegradable chemicals help to ensure that the chemicals do not stay in the soil for more than a year (Carter, 2018). Novel, slow releasing H_2S chemicals, dithiophosphates are safe, non-polluting, and biodegradable with no adverse environmental impacts have been developed. Hydrolysis of dibutyldithiophosphate results in the production of butanol and phosphoric acid, which are both natural compounds with no negative environmental impacts, especially at low levels (Carter et al., 2018; Carter et al., 2019; Brown et al., 2021). However, there is no knowledge on the responses of edamame to such dibutyldithiophosphates, especially under drought stress.

A



B

$$Na_2S + 2H_2O \longrightarrow 2NaOH + H_2S$$

C

$$NaHS + 2H_2O \longrightarrow Na^+ + H_2S$$

Figure 2.6. An illustration of H_2S donors hydrolysis *(**A**) The hydrolysis of GYY-4137 releases morpholine and non-natural phosphate (Carter, 2018). (**B**) Na_2S hydrolyses to release H_2S and sodium hydroxide. (**C**)* The hydrolysis of *NaHS releases H_2S and sodium cations (Corvino et al., 2021). Na_2S- Sodium sulfide, NaHS- Sodium hydrosulfide, H_2S- Hydrogen sulfide, Na^+- Sodium, NaOH- Sodium hydroxide.*

2.5. Conclusion

The introduction of edamame to South Africa, its history, and its nutritional value were discussed in this chapter. The chapter also highlighted the benefits of growing edamame as a nutrient-rich crop to reduce the country's food insecurity, which is a result of climate change and its consequences, such as drought. Reviewing the impact of drought stress on the physiological, biochemical and morphological responses of plants aided to better understanding of how to increase edamame harvests and production. The gathered information demonstrated that increasing some of the crucial processes such as the photosynthetic capacity, accumulation of osmolytes, enzymatic, and non-enzymatic mechanisms in the leaves can aid plants in alleviating the effects of drought stress. The review also emphasized the benefit of using biodegradable dibutyldithiophosphate in treating plants that are under drought stress. The knowledge gathered demonstrated that the negative impacts of environmental stress, such as drought stress, might be mitigated by applying small doses of H_2S donors. Experiments must be done to determine the ideal concentration of a new biodegradable H_2S donor, dibutyldithiophosphate. Therefore, it is crucial to conduct this research in drought-stressed edamame to improve growth and development. The following chapter focuses on the effects of dibutyldithiophosphate on the physiological and biochemical responses of drought-stressed edamame.

Chapter 3: The effect of dibutyldithiophosphate on the physiological and biochemical responses of drought-stressed edamame

3.1. Abstract

Hydrogen sulfide (H_2S) was recently identified as one of the signaling molecules involved in plant tolerance to various environmental stresses. This study reports on the impact of various concentrations (0, 0.1, 1, and 2 mg/mL) of dibutyldithiophosphate, a newly developed biodegradable H_2S donor on the physiological and biochemical responses on drought-stressed edamame (*Glycine max*. L. Merrill) cultivars (UVE14; drought tolerant and UVE17; drought sensitive) at flowering and pod-filling stages. The cultivars responded differently to dibutyldithiophosphate under drought stress. Upregulation of the different responses was influenced by different dibutyldithiophosphate concentrations, namely, the photosynthetic parameters increased at flowering and the antioxidative responses increased at pod-filling. In addition, the response of cultivars to dibutyldithiophosphate under drought stress depended on the concentration of this donor. Generally, UVE17 required higher concentrations of dibutyldithiophosphate than UVE14 to upregulate the physiological and biochemical responses under drought stress. The findings of this study showed that dibutyldithiophosphate application on drought-stressed edamame can increase the photosynthesis capacity of edamame, leading to increased accumulation of sugars and proline, which potentially act as antioxidants against excess ROS produced during drought stress. Depending on the cultivar and growth stage, application of dibutyldithiophosphate also upregulates other antioxidative mechanisms (superoxide dismutase, ascorbate peroxidase, flavonoid reflective index, carotenoid reflective index) in drought-stressed edamame cultivars, which lead to reduced electrolyte leakage. Therefore, such increases in the physiological and biochemical responses of drought-stressed edamame under dibutyldithiophosphate could lead to improved growth and yield, which needs to be studied further.

3.2. Introduction

Edamame (*Glycine max*. L. Merrill) is a vegetable-type soybean that was recently introduced in South Africa and is produced by small-scale farmers (Dlamini et al., 2014; Seid, 2014; Agri4Africa, 2020). It is a highly nutritious and profitable crop because it can be harvested four to six times a year (Zeipina et al., 2017; Khojely et al., 2018; Djanta et al., 2020). The South African economy has suffered significantly because of a decline in agricultural productivity caused by the recent droughts, with many people losing employment (Lottering et al., 2020; Mothapo, 2020; Meza et al., 2021). In addition, drought exacerbated by changing climate poses a severe challenge on sustainable crop production in Sub-Saharan Africa (Shaheen et al., 2016; Mothapo, 2020; Wolf et al., 2020).

In plants, tolerance responses to abiotic stress conditions such as drought involves modification of the physio-biochemical characteristics, and in some cases, modification of the morphological structures (Moloi and van der Merwe, 2021; Hlahla et al., 2022). Introduction of drought stress at the reproductive stages of flowering and pod-filling in edamame results in the formation of smaller and fewer seeds because these are the most sensitive developmental stages to environmental factors (Smit, 2019; Moloi and van der Merwe, 2021). Drought affects plants in a variety of ways including stomatal closure, early leaf senescence, ineffective photosynthesis, oxidation of chloroplast lipids, protein structural changes, and reduced leaf area (Anjum et al., 2011b). Limited stomatal conductance due to drought increases the likelihood of excessive production of the reactive oxygen species (ROS) such as superoxide anion (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) (Damour et al., 2010). Oxidative stress may lead to impairment of chlorophyll biosynthesis pathways, degradation of chlorophyll, loss of the chloroplast membrane, lipid peroxidation and electrolyte leakage (EL) (Anjum et al., 2011b; Silva et al., 2016; Nahakpam, 2017; Laxa et al., 2019). Sayar et al. (2008) reported high EL increase in drought susceptible durum wheat than tolerant wheat varieties during water stress. High cell membrane integrity was also associated with low EL and increased drought stress tolerance in wheat (Ciulca et al., 2017). In turn, such responses may reduce the photosynthetic capacity of plants through downregulation of chlorophyll content (Karimpour, 2019; Gholamin and Khayatnezhad, 2020;

Hebbache et al., 2021; Khayatnezhad and Gholamin, 2021). In edamame and grain soybeans, decreases in chlorophyll-a content were recorded during drought stress irrespective of whether they were drought tolerant or susceptible (Basal et al., 2020; Hlahla et al., 2022). Although stomatal closure prolongs the period that the plant has access to vital root zone water reserves in the soil, it also leads to reduced CO_2 assimilated in plant cells (Deka et al., 2018), which negatively affects plant growth and development.

In response to excessive ROS generated by drought stress, plants may activate non-enzymatic (e.g., flavonoids, ascorbic acid, glutathione, carotenoids) or enzymatic antioxidant systems [e.g., superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and catalase]. Such systems can maintain ROS balance and prevent destruction of the plant cells (AL-Aloosy et al., 2019; González-Orenga et al., 2019). The loss of soil moisture during drought stress also causes a drop in relative water content (RWC) of plant cells. A biochemical mechanism known as osmotic adjustment (OA) allows plants to adapt to dry conditions by increasing their RWC (Sanders and Arndt, 2012). Several molecules, such as organic acids, carbohydrates, amino acids such as proline, and inorganic cations and anions contribute to OA (Ashraf and Foolad, 2007).

In the wake of the ongoing climate change crisis, researchers attempt to find novel sustainable methods to enhance crop productivity. Hydrogen sulfide (H_2S) application at low dosage is one of the most recent methods that needs to be explored because of its potential in improving the physiological, biochemical and morphological responses of plants to environmental stress (Zhang et al., 2008; Zhang et al., 2010; Chen et al., 2011; Wang et al., 2012; Dooley et al., 2013; Carter et al., 2019; Arif et al., 2021). Garcia-Mata and Lamattina (2010) found that treating drought-stressed broad beans with a H_2S donor (sodium hydrosulfide, NaHS) resulted in increased RWC. Similarly, Zhang et al. (2010) showed that treatment of drought-stressed soybean seedlings with NaHS induced RWC. Zhi et al. (2018) further showed that treatment of strawberries with this H_2S donor increased APX and SOD activity, which led to reduced ROS and malondialdehyde (MDA), and ultimately, reduced oxidative damage under drought stress. Numerous studies also demonstrated that NaHS application on drought-stressed plants upregulated APX and SOD activities. Application of NaHS enhanced main ROS detoxification systems contributing to plant defence (Khan et al., 2018; Almeida et al., 2020; Antoniou et al., 2020; Batista et al., 2020; Ocvirk et al., 2020; Zhou et al., 2020; Amir et al., 2021). Treatment of barley with NaHS led to increased antioxidative capacity and reduced oxidative stress markers such as MDA and O_2^- during drought and heat stress (Naz et al., 2021). Similarly, a study by Zhang et al. (2010) revealed that NaHS-treated drought-stressed soybean seedlings had much lower amounts of H_2O_2, O_2^-, and MDA than untreated plants, which resulted in reduced oxidative damage. Furthermore, Zhang et al. (2010) showed that such treatment reduced the damaging effects of drought by increasing chlorophyll content. Although there are reports on the beneficial effects of H_2S on photosynthesis under different abiotic stresses (Bharwana et al., 2014; Tcherkez and Limami, 2019; Tang et al., 2020; Naz et al., 2021; Liu et al., 2022), it appears that an extensive effect of H_2S on photosynthesis under drought stress is often neglected.

Dibutyldithiophosphate is a biodegradable H_2S donor with no detrimental environmental consequences. Hydrolysis of dibutyldithiophosphate results in the production of butanol and phosphoric acid, which are both natural compounds (Carter et al., 2018; Carter et al., 2019; Brown et al., 2021). However, there is no knowledge on the responses of edamame to such H_2S donor, especially under drought stress. Additionally, it also is difficult to find data demonstrating how H_2S affects edamame growth under drought stress. Therefore, this study was aimed at establishing the effect of different concentrations of dibutyldithiophosphate on the physiological and biochemical responses of drought-stressed edamame. The studied physiological responses included non-destructive (chlorophyll-a fluorescence parameters, chlorophyll content index, photochemical reflective index, stomatal conductance, flavonoid reflective index, and carotenoid reflective index) and destructive physiological and biochemical measurements (quantification of photosynthesis pigments, MDA, EL, total soluble sugars, proline, antioxidative enzymes, ROS, and RWC). It is hypothesised that different concentrations of dibutyldithiophosphate would upregulate the physiological and biochemical responses of drought-stressed edamame.

3.3. Materials and Methods

3.3.1. Plant material, application of dibutyldithiophosphate, germination and experimental setup

Two edamame genotype seeds (UVE14; drought tolerant and UVE17; drought sensitive) (Moloi and van der Merwe, 2021) were germinated in seedling trays containing Hygromix seedling mixture [Hygrotech (Pty) Ltd., Pretoria, South Africa] under controlled conditions (25°C day and 18°C night under natural light) in the glasshouse of the University of the Free State, Bloemfontein (29°6′31.94″ S; 26°11′18.95″ E). The selection of genotypes was made based on previous findings, where UVE14 exhibited tolerance to drought at physiological and biochemical level with minimal yield reduction, while UVE17 exhibited drought sensitivity with significant yield reduction with decreased physiological and biochemical responses under drought conditions (van der Merwe et al., 2018; Moloi and van der Merwe, 2021; Hlahla et al., 2022).

At the onset of plantation (Day 0), 10 mL of dibutyldithiophosphate (a H_2S donor synthesised at the Chemistry department, Iowa state university, Iowa City, Iowa, United States of America) was administered around the seed in the seedling mixture at doses of 0, 1, 10, and 20 mg/10 mL using a syringe. The three concentrations were selected after a preliminary trial was conducted to establish dosage range that will not have negative effects on seed germination. Results of this preliminary experiment showed that application of dibutyldithiophosphate at the concentrations ranging from 0 to 2 mg/mL (i.e., 0 mg/mL, 0.1 mg/mL, 1 mg/mL, 2 mg/mL) had no impact on seed germination, but concentrations beyond 2 mg/mL reduced germination. In addition, the preliminary experiment showed that 1 to 2 mg/mL concentration range increased shoot length, root length, total fresh and dry weight compared to the control (Table 3.1). During germination, seeds were tap watered daily using a 10 mL syringe to prevent leaching out of dibutyldithiophosphate. After germination at unifoliate leaf stage, seedlings were transplanted, one seedling per pot to 9 L potting bags containing 10 kg of loamy sandy soil watered to 100% soil water holding capacity (WHC, i.e., optimal watering). At 100% WHC, the soil weighed 11.6 kg, meaning that it contained 1.6 L of water. Hydrosense II (Campbell Scientific, Stellenbosch, South Africa) fitted with a CS659 (12 cm) portable soil-water probe was utilised to determine how much water was required every day to maintain soil at 100% WHC. The volumetric water content (VWC) of the soil at 100% WHC was 20.9%.

To avoid nutrient deficiencies, at the first trifoliate leaf stage, seedlings were fertilized using 200 mL full strength of nutrient solution consisting of essential macro- and micronutrients every second week for the duration of the experimental period. To obtain a balanced nutrient solution, Hygroponics and Solu-Cal (calcium nitrate) were mixed according to the manufacturer's guidelines (Hygrotech, SA); Hyperfeed (20 g) and Ca_2NO_3 (16 g) were dissolved in 20 L of tap water. Hyperfeed is a water-soluble fertilizer mix that can be used on growing plants in addition to other soluble fertilizer mixtures including KNO_3, $MgNO_3$, and $CaNO_3$.

Drought stress was applied after plants reached the third trifoliate stage by withdrawing water to 30% soil WHC. The VWC of the soil at this point was recorded to be 6.3%. Each day, soil VWC was measured in order to determine the amount of water needed to maintain soil water level at either 100% (control) or 30% (drought stress) WHC. The experiment was done in three replications with 2 pots per treatment in a split-plot randomized complete block design. Data sampling was done at flowering and pod-filling stages. The trial ran from 16 November 2021 to 28 January 2021, and 14 March to 11 July 2022.

Table 3.1: Shoot length, root length, total fresh weight, total dry weight, and germination percentage from the preliminary trial.

Dibutyldithiophosphate (H_2S) concentrations (mg/mL)	**Shoot length (cm)**	**Root length (cm)**	**Total fresh weight (g)**	**Total dry weight (g)**	**Seed germination (%)**
0	11,25	6,45	3,12	0,38	100%
0.1	13,33	6,43	3,65	0,41	100%
1	13,68	7,95	3,45	0,39	100%
1.5	13	7,75	3,23	0,4	100%
2	13,6	7,75	4,10	0,43	100%
5	8,5	7,67	1,98	0,32	75%
8	2	0	1,30	0,27	25%

Values represent means of three replications

3.3.2. Determination of the physiological and biochemical parameters using non-destructive techniques

Chlorophyll-a fluorescence, chlorophyll content, photochemical reflective index, stomatal conductance, flavonoid reflective index, and carotenoid reflective index were all measured using portable (hand-held) instruments. Data was collected between 9:00 AM and 12:00 AM, when the light intensity was at its highest on a young but fully expanded trifoliate leaf.

3*.3.2.1. Chlorophyll-a fluorescence*

Light weight leaf clips were placed on the representative leaves with a closed shutter to allow dark adaptation for 30 minutes. Afterwards, pocket photosynthesis efficiency analyser (PEA) chlorophyll fluorometer (Hansatech Instrument Ltd., North American) was attached to the leaf clip, followed by shutter opening and radiation (3500 µmol m^{-2} s^{-1}). Thereafter, the following parameters were downloaded using PEA Plus version 1.10, Hansatech; maximal photosystem (PS) II quantum yield [ratio of variable fluorescence to maximum fluorescence (Fv/Fm)], PSI performance index absorbance (PI_{abs}), and PSII total performance index (PI_{total}), which provides data on the efficiency of both PSII and PSI.

3.3.2.2. Photochemical reflective index, flavonoids reflective index, carotenoids reflective index

Photochemical reflectance index (PRI), flavonoids reflective index (FRI) and carotenoids reflective index (CRI), were evaluated using CI-710 leaf spectrometer (CID Bio-Science, WA, USA).The reading were taken between 10:00 AM and 12:00 PM. In a broad range of wavelengths, including visible and near infra-red (NIR) light (360-1100 nm), the leaf spectrometer simultaneously analyses the transmission, absorption, and reflection of light by biological substances.

3.3.2.3. Chlorophyll content index

A CL-01 Chlorophyll Meter (Hansatech Instruments Ltd) was used to measure the chlorophyll content index (CCI). Chlorophyll meter measures the dual wavelength (620 and 940 nm wavelength) optical absorbance from leaf samples. The meter measures relative chlorophyll concentration between 0 and 2000 units.

3*.3.2.4. Stomatal conductance*

A SC-1 leaf porometer (Li-Cor ADC BioScientific Ltd., Hoddesdon, UK) was used to measure stomatal conductance (g_s) between 10:00 AM and 12:00 PM. According to Wang et al. (2018) and Gadi et al. (2019), changes in relative humidity, temperature, and radiant energy are minimal between 10:00 AM and 12:00 PM. 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).

3.3.3. Physiological and biochemical parameters determination using destructive techniques

To evaluate the physiological and biochemical parameters, invasive techniques were used. Young fully expanded trifoliate leaves were collected (same leaf position for all treatments), and ground to a fine leaf powder in liquid nitrogen for different assays and stored at -20°C. Fresh leaves were used for the physiological investigations such as EL, RWC, and light microscopy).

3.3.3.1. Chlorophyll-a, -b, and carotenoid content

A modified Lichtenhaler (1987) method was used to determine total chlorophyll content (Tot Chl), chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), and carotenoids. Frozen leaf powder (0.04 g) was homogenized in 2 mL of 80% (v/v) acetone on ice. The homogenate was centrifuged for 10 minutes at 14 881 x *g*. The supernatant was used to measure the carotenoids (Car) (480 nm), Chl-b (645 nm), and Chl-a (663 nm) contents against a blank containing 80% acetone (Cary 100 Bio, Varian, Australia). The subsequent formulas were used to determine specific pigments:

Chlorophyll-a (Chl-a) = (12, 72 x A_{663}) - (2, 59 x A_{645})

Chlorophyll-b (Chl-b) = (22, 9 x A_{645}) - (4, 68 x A_{663})

Total chlorophyll (Tot Chl) = (20, 2 x A_{645}) + (8, 02 x A_{663})

Carotenoids (Car) = A_{480} + (0,114 x A_{663}) – (0,638 x A_{645})

Chlorophyll content = chlorophyll x Volume/ Weight

Carotenoid content = carotenoids x Volume/ Weight

The chlorophylls and carotenoids contents were expressed as mg g^{-1} fresh weight.

3.3.3.2. Electrolyte leakage

Electrolyte leakage was measured according to Rolny et al. (2011). Ten fresh leaf discs of 1.6 cm diameter were placed in a test tube and left to float in 15 mL of double distilled water. The initial water conductivity (C_0, µS) was recorded immediately after putting the leaf discs using a conductivity meter (Hanna Instruments, Inc.). After 3 hours of shaking at room temperature, conductivity was measured again (C_{max}). Thereafter, tubes were incubated in a water bath at 85°C for 10 minutes. After cooling to room temperature, the final conductance was measured (C_{total}). The percentage of electrolyte leakage was calculated:

% EL = 100 x (C_{max}- C_0)/C_{total}

3.3.3.3. Relative water content (RWC)

The method described by Gonzales and Gonzales-Vilar (2001) was used to determine RWC. Leaf collections took place between 10:00 AM and 12:00 PM. To prevent moisture loss, leaf samples were placed in sealed containers on ice. Leaf discs (10) were punched out using a 1.6 cm diameter cork borer. The discs' fresh weight (FW) was recorded. Thereafter, the leaf discs were transferred to a test tube containing 10 mL of distilled water and hydrated for 24 hours in a dark cold room (4°C). Thereafter, the discs were dried on a blotting paper, and the turgid weight (TW) was recorded. The leaf discs were dried in an oven for 72 hours at 76°C to get the dry weight (DW). The percentage of RWC was calculated:

%RWC = 100 x (TW- FW) / DW

3.3.3.4. Lipid peroxidation

A modified method of Heath and Packer (1968) was used for malondialdehyde (MDA) assay, a by-product of lipid peroxidation. Frozen leaf (0.2 g) was homogenised in 2 mL of 20% (m/v) trichloroacetic acid (TCA) before centrifugation at 3 500 *x g* for 20 minutes at 4°C. The aliquot (0.5 mL) was added to 0.5 mL of 20% TCA (m/v*)* containing 0.5% (m/m) thiobarbituric acid (TBA), vortexed, and incubated at 95°C for 30 minutes. The mixture was immediately cooled on ice to stop the reaction. Spectrophotometric measurements of the MDA-TBA product's absorbance were made at 532 and 600 nm (Cary 100 Bio, Varian, Australia). The MDA content was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}. The MDA content was expressed as mmol MDA g^{-1} fresh weight.

3.3.3.5. Hydrogen peroxide content

For determining H_2O_2 content, a modified methodology as published by Velikova et al. (2000) was used. The frozen leaf powder (0.2 g) was ground to a fine paste in 1.3 mL of ice-cold 0.1% TCA (m/v), and centrifuged at 12 000 *x g* for 15 minutes at 4°C. The supernatant (0.5 mL) was combined with 1 mL of 1 M potassium iodide and 0.5 mL 10 mM potassium phosphate buffer (pH 7.0). For the blank, 0.1% (m/v) TCA was used. The mixtures were incubated for an hour in the dark, and at 390 nm (Cary 100 Bio, Varian, Australia) absorbance was measured. The H_2O_2 standard, which was used for the estimation of the H_2O_2 of the leaf samples, was also subjected to similar conditions. The H_2O_2 content was expressed as mmol H_2O_2 g^{-1} fresh weight.

3.3.3.6. Total soluble sugars

A modified method of Irigoyen et al. (1992) was used to determine the TSS. Leaf powder (0.05 g) was homogenized in 1.25 mL of 96% (v/v) ethanol. Following a 10 minute incubation period at 80°C, the homogenate was centrifuged at 4 000 *x g* for 10 minutes at 4°C. Anthrone reagent (1.5 mg/mL) was prepared in 72% (v/v) sulphuric acid. The mixture of 50 µL supernatant and 1450 µL anthrone reagent was vigorously vortexed and incubated at 80°C for 15 minutes. The mixture was cooled down at room temperature before the spectrophotometric measurement of the absorbance at 625 nm (Cary 100 Bio, Varian, Australia). A glucose standard was used to estimate the TSS and expressed as mg glucose g^{-1} fresh weight.

3.3.3.7. Proline

The proline (Prol) assay was conducted using a modified Carillo and Gibon (2011) method. Leaf powder (0.15 g) was homogenized to a fine paste on ice using 2 mL of 70% ethanol (v/v). The homogenate was centrifuged at 3 000 *x g* for 10 minutes. The reaction mixture containing the supernatant (500 µL), 20% ethanol (500 µL), and 1% (m/v) acidic ninhydrin (prepared in 60%, v/v, glacial acetic acid) was vigorously vortexed before incubation at 95°C for 20 minutes. Samples were centrifuged at 10 000 *x g* for 10 minutes after cooling. A spectrophotometer (Cary 100 Bio, Varian, Australia) was used to measure the absorbance at 520 nm. Proline was calculated from $_{\text{L}}$-proline standard curve and represented as mg proline g^{-1} fresh weight.

3.3.3.8. Antioxidative enzymes

A modified method of Pukacka and Ratajczak (2005) was used to prepare enzyme extracts. Using mortar and pestle on ice, 0.1 g of leaf powder was homogenized to a fine paste in 1 mL of potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 2% (m/m) polyvinylpolypyrrolidone (PVPP), 0.1% (m/v) Triton X-100, and 1 mM ascorbate (to oxidise intracellular H_2O_2). At 4°C, the homogenate was centrifuged for 20 minutes at 15 000 *x g*. The resulting supernatant was used as the enzyme extract.

Mishra et al. (1993) modified method was used to prepare the APX assay. A volume of 1 mL of the reaction mixture contained 500 µL 50 mM phosphate buffer (pH 7.0), 200 µL 0.1 mM H_2O_2, 150 µL 0.5 mM sodium ascorbate, 50 µL 0.1 mM EDTA, and 100 µL enzyme. Using a blank, which had phosphate buffer instead of the enzyme, the ascorbate oxidation-related decrease in absorbance was measured at 290 nm (Cary 100 Bio, Varian, Australia) for 3 minutes at 20°C. The enzyme activity was determined using an extinction coefficient of 2.8 mM^{-1} cm^{-1}, and expressed as mmol acorbate mg^{-1} prot. min^{-1}.

Zieslin and Ben-Zaken (1991) modified method was used to prepare the GPX assay. The reaction mixture (1 mL) contained 50 µL 0.2 M H_2O_2, 100 µL 50 mM guaiacol, 340 µL distilled H_2O, 500 µL 80 mM phosphate buffer (pH 5.5), and 10 µL enzyme. The formation of tetraguaiacol was observed as an increase in absorbance at 470 nm (Cary 100 Bio, Varian, Australia) for 3 minutes, at 30°C. The enzyme activity was determined using an extinction coefficient of 26.6 mM^{-1} cm^{-1} and expressed as mmol tetraguaiacol mg^{-1} prot. min^{-1}.

Total SOD activity was assayed using nitro blue tetrazolium (NBT) method as described by Abedi and Pakniyat (2010). The reaction mixture consisted of 1280 µL 100mM phosphate buffer (pH 7.8), 40 µL 55 mM methionine, 50 µL 0.75 mM NBT, 15 µL enzyme and 30 µL 0.1 mM riboflavin. For the blank and control, the enzyme was replaced with 15 µL 1 mM EDTA (prepared in 100mM phosphate buffer; pH 7.8). The cuvettes containing the reaction mixtures were irradiated for 30 minutes with two light bulbs at 100 kilowatts (28 cm high) placed opposite each other. The blank was placed in the dark for 30 minutes to prevent light oxidation. The spectrophotometer (Cary 100 Bio, Varian, Australia) was used to determine the absorbance of the radiated and non-radiated reactions at 560nm. The amount of the enzyme that inhibited the reduction of NBT by 50% at 560 nm was equal to one unit of SOD. Enzyme activity was expressed as SOD units mg^{-1} prot. min^{-1}.

Using gamma-globulin as a standard (1.5 mg/mL), Bradford’s (1976) method for determining protein concentration was used. A microplate reader (Greiner Bio-One, Kremsmunster, Austria) was used to measure the absorbance at 595 nm on a microplate detector (Anthos Labtech Inc. GmbH, Salzburg, Austria), and the results were expressed in mg^{-1} protein.

3.3.3.9. Light microscopy for superoxide anion staining

A cork borer was used to cut 0.5 cm leaf discs from the representative leaves from the pod-filling stage. The leaves were vacuum infiltrated using a 10 mL syringe with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.05% (m/m) NBT. After that, leaves were incubated for an hour in the NBT solution. The NBT reaction was stopped by incubating the leaves in ethanol (Doke, 1983). The leaves were further incubated in ethanol at room temperature overnight to remove chlorophyll. The leaf discs were observed under a light microscope (Zeiss stereo Discovery. V8, Aalen, Germany) fitted with the Zeiss Axiocam 105 color camera. Images were taken with the Zen Core V2.8 software.

3.3.4. Data analysis

In order to determine the individual and combined effects of the concentrations and water treatments, data obtained on all physiological and biochemical parameters analyzed in this study were subjected to analysis of variance (ANOVA) using TIBCO Statistica version 13.5.0.17. In instances where the ANOVA indicated significant effects, the Turkey HSD test at $p = 0.05$ was used to separate the means.

3.4. Results

Table 3.2 shows the effect of dibutyldithiophosphate (H_2S donor), cultivar, water level and their interaction (analysis of variance, ANOVA) on the physiological and biochemical traits of edamame at flowering and pod-filling stages. Since the physio-biochemical effects of drought on cultivar and the water level was already studied (Moloi and van der Merwe, 2021; Hlahla et al., 2022), the focus from this table was only on (i) dibutyldithiophosphate [H_2S (H)] effect as well as (ii) the interaction between H, cultivar (c), and water level (w).

The effect of dibutyldithiophosphate alone was significant on PI_{total} ($p \leq 0.01$), Chl-a ($p \leq 0.05$), Chl-b ($p \leq 0.001$), R Chl ($p \leq 0.001$), PRI ($p \leq 0.001$), TSS ($p \leq 0.05$), FRI ($p \leq 0.05$), and SOD ($p \leq 0.05$) at flowering. At pod-filling it was significant for PI_{abs} (p

≤ 0.01), PI_{total} ($p \leq 0.01$), Chl-a ($p \leq 0.01$), Chl-b ($p \leq 0.05$), Tot Chl ($p \leq 0.001$), CRI ($p \leq 0.05$), PRI ($p \leq 0.01$), RWC ($p \leq 0.01$), Prol ($p \leq 0.001$), TSS ($p \leq 0.05$), EL ($p \leq 0.001$), APX ($p \leq 0.01$), GPX ($p \leq 0.05$), and SOD ($p \leq 0.01$).

At flowering, cultivars responded in a significantly different manner under different water levels and H_2S (i.e., their interaction) for PI_{abs} ($p \leq 0.05$), PI_{total} ($p \leq 0.05$), Chl-a ($p \leq 0.05$) and -b ($p \leq 0.001$), carotenoid ($p \leq 0.05$), CRI ($p \leq 0.01$), PRI ($p \leq 0.01$), TSS ($p \leq 0.0\ 1$), H_2O_2 ($p \leq 0.05$) and APX ($p \leq 0.01$). Similarly, at pod-filling, the interaction was significant for PI_{abs} ($p \leq 0.001$), PI_{total} ($p \leq 0.001$), Chl-b ($p \leq 0.01$), PRI ($p \leq 0.05$) and TSS ($p \leq 0.01$). Furthermore, it was significant for g_s ($p \leq 0.01$), Tot Chl ($p \leq 0.01$), Prol ($p \leq 0.05$), FRI ($p \leq 0.01$), EL ($p \leq 0.001$) and SOD ($p \leq 0.01$).

Table 3.3 shows the effect of different dibutyldithiophosphate concentrations on the physiological and biochemical responses of edamame. At flowering, PI_{total} was significantly reduced under 1 mg/mL. Chl-a and -b were significantly induced by this treatment, with the highest increase observed under 0.1 mg/mL. Similarly, CCI that was measured non-destructively had a corresponding increase under 1 mg/mL. However, treatment with the highest concentration (2 mg/mL) led to a decrease in CCI. In contrast, CRI, PRI and TSS were significantly increased under this high concentration. The RWC was significantly increased under 1 mg/mL treatment. The highest increase in FRI was observed under 0.1 and 1 mg/mL treatments. The SOD activity increased significantly under 1 and 2 mg/mL treatments.

At pod-filling, PI_{total} was substantially inhibited under 1 mg/mL dibutyldithiophosphate treatment, which was similar to the response at flowering stage. Chlorophyll-a and -b were significantly increased under 1 and 2 mg/mL treatments with the latter inducing the highest chlorophylls. Overall reduction in Tot Chl was however observed under 2 mg/mL treatment. During pod-filling, CRI was induced by all treatments while PRI was only significantly induced under 1 mg/mL. The RWC was also significantly induced under all treatments. Although, not significantly different to 0.1 and 1 mg/mL, 2 mg/mL concentration induced the highest increase in RWC. Treatment with 1 mg/mL induced the most significant increase in TSS accumulation. There was a substantial reduction in EL under 1 mg/mL while 2 mg/mL led to an increase. There was an overall reduction in the activities of the antioxidative enzymes under different concentrations of dibutyldithiophosphate.

For all interactions, focus will only be on the significant traits observed in Table 3.2. The terms “significant” and “substantial” refer to the different means according to the Tukey HSD homogeneity test (i.e., significantly different means are followed by different letters). At flowering, drought stress increased PI_{abs} in UVE17 (15.5%). Treatment of these plants (drought-stressed UVE17) with different concentrations of dibutyldithiophosphate led to substantial reduction of PI_{abs}. In contrast, dibutyldithiophosphate treatment (2 mg/mL) on optimally watered UVE17 increased PI_{abs} by 22.7% (flowering) and 130.3% (pod-filling). Although drought stress did not affect PI_{abs} in UVE14 cultivars, their treatment with 0.1 mg/mL induced a 24.7% increase in this parameter at flowering. At pod-filling, drought stress also increased PI_{abs} substantially (92.9%) in UVE17. Treatment of these (drought-stressed UVE17) with different concentrations of H_2S donor led to reduction of this parameter. Similarly, treatment of drought-stressed UVE14 with different doses of this H_2S donor did not effectively increase PI_{abs}. A similar decrease was observed in optimally watered UVE14 under different dibytyldithiophosphate treatments (Figure 3.1).

Table 3.2. The effect of dibutyldithiophosphate, cultivar and water (analysis of variance, ANOVA) on the physiological and biochemical traits of edamame at flowering and pod-filling stages.

	Flowering				**Pod-filling**			
	H_2S concentration (H)	**Cultivar (C)**	**Water level (W)**	**Interaction (H×C×W)**	**H_2S concentration (H)**	**Cultivar (C)**	**Water level (W)**	**Interaction (H×C×W)**
Fv/Fm	$0,0109^{ns}$	0,0416*	0,0145*	$0,1800^{ns}$	$0,0524^{ns}$	$0,7073^{ns}$	0,0075**	$0,1593^{ns}$
PI_{abs}	$0,6376^{ns}$	0,0003***	0,0033**	0,0342*	0,0019**	$0,8674^{ns}$	0,0000***	0,0000***
PI_{total}	0,0099**	0,0000***	0,0000***	0,0109*	0,0013**	$0,4105^{ns}$	0,0000***	0,0000***
Chl-a	0,0312*	$0,2565^{ns}$	0,0414*	0,0349*	0,0078**	0,0107*	0,0104*	$0,7637^{ns}$
Chl-b	0,0001***	0,0002***	0,0136*	0,0006***	0,0173*	0,0006***	$0,4202^{ns}$	0,0016**
Tot Chl	$0,1114^{ns}$	$0,0684^{ns}$	$0,3154^{ns}$	$0,0547^{ns}$	0,0000***	$0,3020^{ns}$	$0,5949^{ns}$	0,0011**
CCI	0,0001***	0,0000***	$0,1693^{ns}$	$0,4532^{ns}$	$0,1601^{ns}$	0,0028**	$0,7326^{ns}$	$0,9016^{ns}$
Car	$0,1635^{ns}$	$0,3386^{ns}$	$0,2268^{ns}$	0,0434*	$0,6697^{ns}$	0,0144*	$0,5392^{ns}$	$0,5966^{ns}$
CRI	$0,0064^{ns}$	$0,9508^{ns}$	$0,1873^{ns}$	0,0040**	0,0284*	$0,2940^{ns}$	0,0024**	$0,1572^{ns}$
PRI	0,0006***	0,0004***	0,0064**	0,0034**	0,0057**	$0,6410^{ns}$	0,0226*	0,0139*
g_s	$0,0012^{ns}$	$0,0859^{ns}$	0,0000***	$0,1994^{ns}$	$0,5578^{ns}$	$0,3005^{ns}$	0,0000***	0,0086**
RWC	$0,0448^{ns}$	$0,0076^{ns}$	$0,5415^{ns}$	$0,1593^{ns}$	0,0024**	0,0164*	$0,6158^{ns}$	$0,2250^{ns}$
Prol	$0,5244^{ns}$	$0,6790^{ns}$	0,0066**	$0,2241^{ns}$	0,0004***	$0,0888^{ns}$	0,0000***	0,0290*
TSS	0,0285*	0,0000***	0,0000***	0,0004***	0,0294*	0,0000***	0,0320***	0,0078**
FRI	0,0120*	0,0002***	$0,2027^{ns}$	$0,0606^{ns}$	$0,3396^{ns}$	0,0204*	0,0044**	0,0067**
MDA	$0,0548^{ns}$	0,0000***	$0,5304^{ns}$	$0,7303^{ns}$	$0,1987^{ns}$	0,0139*	0,0009***	$0,3079^{ns}$
EL	$0,0716^{ns}$	$0,7849^{ns}$	$0,9929^{ns}$	$0,1909^{ns}$	0,0001***	0,0010**	$0,5425^{ns}$	0,0893***
H_2O_2	$0,2158^{ns}$	0,0281*	$0,1031^{ns}$	0,0451*	$0,7608^{ns}$	$0,4603^{ns}$	$0,6305^{ns}$	$0,0849^{ns}$
APX	$0,2421^{ns}$	0,0005***	$0,5781^{ns}$	0,0021**	0,0077**	0,0115*	0,0100**	$0,0852^{ns}$
GPX	$0,4140^{ns}$	$0,1631^{ns}$	0,0415*	$0,4307^{ns}$	0,0499*	$0,2802^{ns}$	$0,7771^{ns}$	$0,5924^{ns}$
SOD	0,0227*	$0,8806^{ns}$	$0,3170^{ns}$	$0,4316^{ns}$	0,0086**	0,0016*	0,0265*	0,0014**

*The numbers represent the p values from the ANOVA. Asterix represents * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Fv/Fm = ratio of variable fluorescence to maximum fluorescence, PI_{abs} = performance index on absorbance basis, PI_{total} = total performance index, PRI = photochemical reflective index, CCI = chlorophyll content index, CRI = carotenoids reflective index, Chl-a = chlorophyll-a, Chl-b = chlorophyll-b, Tot Chl = Total chlorophyll, g_s = stomatal conductance, Car = carotenoids, MDA = malondialdehyde, H_2O_2 = hydrogen peroxide, EL = electrolyte leakage, Prol = proline, TSS = total soluble sugars, APX = ascorbate peroxidase, GPX = guaiacol peroxidase, SOD = superoxide dismutase, RWC = relative water content of the soil.*

Table 3.3. The effect of different dibutyldithiophosphate concentrations on the physiological and biochemical traits of edamame at flowering and pod-filling stages.

	Flowering				**Pod-filling**			
	0 mg/mL	**0.1 mg/mL**	**1 mg/mL**	**2 mg/mL**	**0 mg/mL**	**0.1 mg/mL**	**1 mg/mL**	**2 mg/mL**
Fv/Fm	0,82^{a}	0,82^{a}	0,81^{a}	0,82^{a}	0,83^{a}	0,83^{a}	0,82^{a}	0,83^{a}
PI$_{abs}$	4,79^{a}	4,74^{a}	4,47^{a}	4,74^{a}	6,52^{c}	5,84^{ab}	5,47^{a}	6,25^{bc}
PI$_{total}$l	4,04^{a}	3,93^{a}	3,40^{b}	3,95^{a}	5,29^{bc}	5,03^{ab}	4,50^{a}	5,78^{c}
Chl-a	969,19^{a}	1084,38^{b}	1066,37^{ab}	1037,35^{ab}	850,11^{a}	858,07^{a}	937,38^{ab}	1010,11^{b}
Chl-b	14,84^{a}	15,66^{ab}	18,02^{c}	16,31^{b}	15,13^{a}	15,22^{a}	15,33^{ab}	16,93^{b}
Tot Chl	81,34^{a}	79,75^{a}	78,75^{a}	78,60^{a}	81,22^{a}	82,02^{a}	80,86^{a}	78,63^{a}
CCI	12,38^{a}	13,62^{b}	12,97^{ab}	10,91^{c}	13,70^{a}	15,77^{a}	13,86^{a}	15,09^{a}
Car	3,32^{a}	3,33^{a}	3,35^{a}	3,37^{a}	3,32^{a}	3,32^{a}	3,34^{a}	3,31^{a}
CRI	0,03^{a}	0,03^{a}	0,03^{a}	0,032^{b}	0,028^{ab}	0,03^{a}	0,03^{a}	0,03^{a}
PRI	0,03^{a}	0,03^{a}	0,03^{a}	0,04^{b}	0,02^{a}	0,02^{a}	0,03^{b}	0,02^{a}
g_s	252,69^{b}	321,00^{ab}	377,40^{b}	338,04^{b}	297,00^{a}	299,11^{a}	289,34^{a}	326,98^{a}
RWC	49,46^{a}	66,19^{ab}	68,37^{b}	59,37^{ab}	24,99^{b}	35,12^{a}	30,50^{ab}	37,59^{a}
Prol	0,18^{a}	0,18^{a}	0,20^{a}	0,19^{a}	0,13^{a}	0,06^{b}	0,12^{a}	0,15^{a}
TSS	61,05^{ab}	65,58^{ab}	60,72^{a}	69,29^{b}	65,69^{ab}	61,42^{a}	72,52^{b}	62,32^{ab}
FRI	0,63^{a}	0,96^{b}	0,92^{b}	0,84^{ab}	0,82^{a}	0,81^{a}	0,77^{a}	0,92^{a}
MDA	25,28^{a}	26,85^{a}	27,53^{a}	23,40^{a}	22,72^{a}	22,59^{a}	21,11^{a}	20,32^{a}
EL	2,66^{a}	4,20^{a}	2,99^{a}	3,14^{a}	3,77^{bc}	2,94^{ab}	2,44^{a}	4,38^{c}
H_2O_2	3,86^{a}	4,08^{a}	4,13^{a}	4,38^{a}	6,60^{a}	7,03^{a}	7,13^{a}	6,97^{a}
APX	0,13^{a}	0,11^{a}	0,10^{a}	0,10^{a}	0,09^{b}	0,08^{ab}	0,06^{a}	0,06^{a}
GPX	0,41^{a}	0,40^{a}	0,42^{a}	0,36^{a}	0,49^{b}	0,39^{a}	0,44^{ab}	0,45^{ab}
SOD	24,15^{ab}	23,05^{a}	25,58^{ab}	28,71^{b}	22,79^{a}	18,59^{b}	22,06^{a}	20,14^{ab}

Values represent means (n = 18). For each growth stage (flowering and pod-filling), different row letters represent significant differences between the means at $p \leq 0.05$. Fv/Fm = ratio of variable fluorescence to maximum fluorescence, PI_{abs} = performance index on absorbance basis, PI_{total} = based on the PI, PRI = photochemical reflective index, CCI = chlorophyll content index, CRI = carotenoids reflective index, Chl-a = chlorophyll-a, Chl-b = chlorophyll-b, Tot Chl = Total chlorophyll, g_s = stomatal conductance, Car = carotenoids, MDA = malondialdehyde, H_2O_2 = hydrogen peroxide, EL = electrolyte leakage, Prol = proline, TSS = total soluble sugars, APX = ascorbate peroxidase, GPX = guaiacol peroxidase, SOD = superoxide dismutase, RWC = relative water content of the soil.

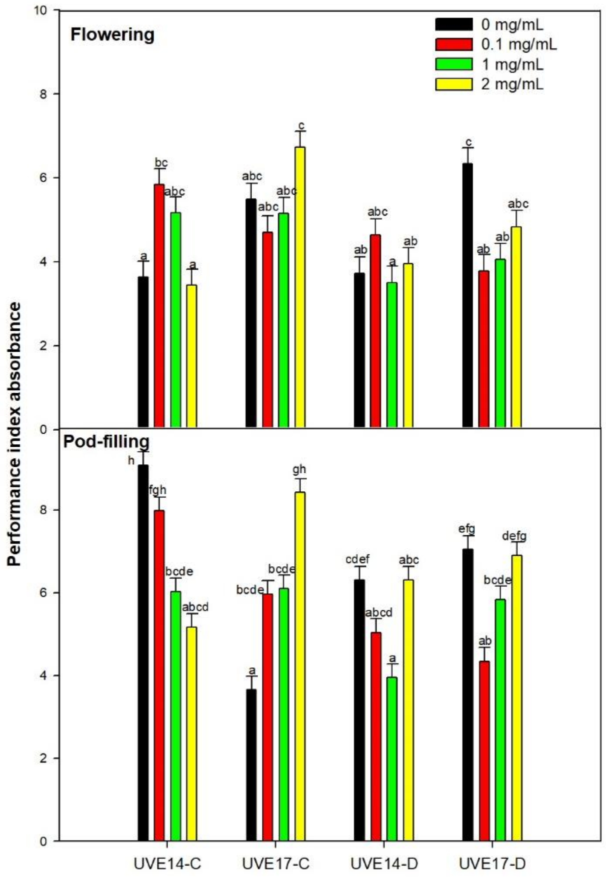


Figure 3.1. *The PI_{abs} of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

Drought stress reduced the PI_{total} of UVE17 (16.1%) at flowering stage. Treatment with increasing concentrations of dibutyldithiophosphate led to further decreases under drought stress for both growth stages of UVE17, with the exception of the 2 mg/mL concentration at pod-filling where an increase was observed (15.5%). Although there was a decrease in PI_{total} at both reproductive stages in UVE14 under drought stress, treatment with 2 mg/mL dibutyldithiophosphate induced a 15.5% at flowering (Figure 3.2).

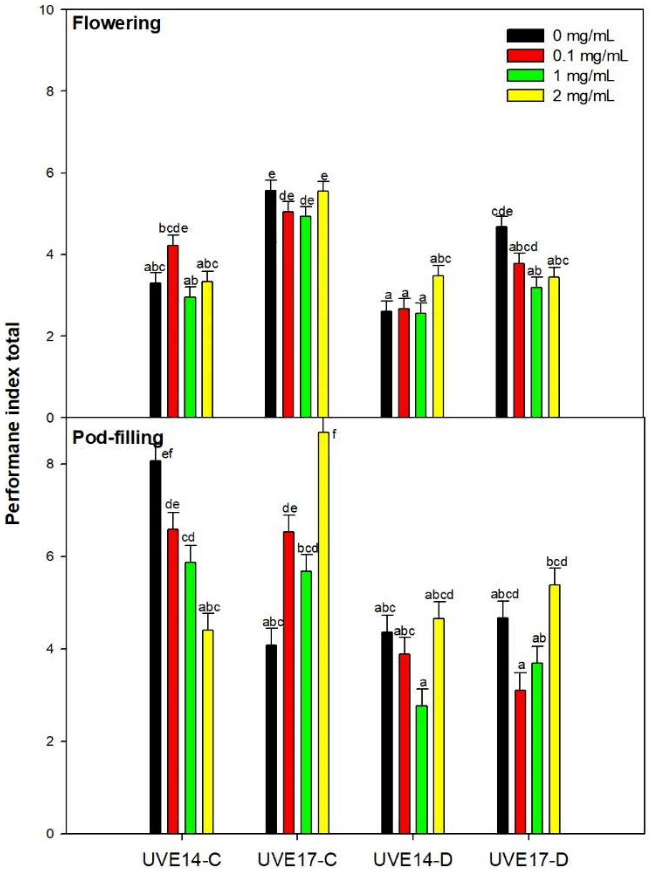


Figure 3.2. *The PI_{total} of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

At flowering, there was a decline in the PRI (25.3% decrease) of drought-stressed UVE17. Application of 0.1, 1, and 2 mg/mL dibutyldithiophosphate increased the PRI by 25.7%, 28.4% and 41.2% increase, respectively in drought-stressed UVE17. Under optimal conditions however, such treatments decreased the PRI in UVE17 during flowering. During pod-filling, drought stress induced the PRI of UVE17. However, treatment with different concentrations of dibutyldithiophosphate did not have a substantial effect on the PRI. For UVE14, treatment of drought-stressed UVE14 with 0.1 mg/mL dibutyldithiophosphate increased PRI substantially (34.3% increase) during flowering. Although dibutyldithiophosphate application was more effective under drought stress for both cultivars, UVE17 had the highest increase in PRI at flowering stage (2 mg/mL). Treatment of drought-stressed UVE14 with 1 mg/mL H_2S donor induced high PRI (88.3% increase) compared to the drought-stressed control during pod-filling. Although increases in PRI were observed in optimally watered plants treated with different H_2S donor concentrations, there were more significant increases under drought stress for both cultivars (Figure 3.3).

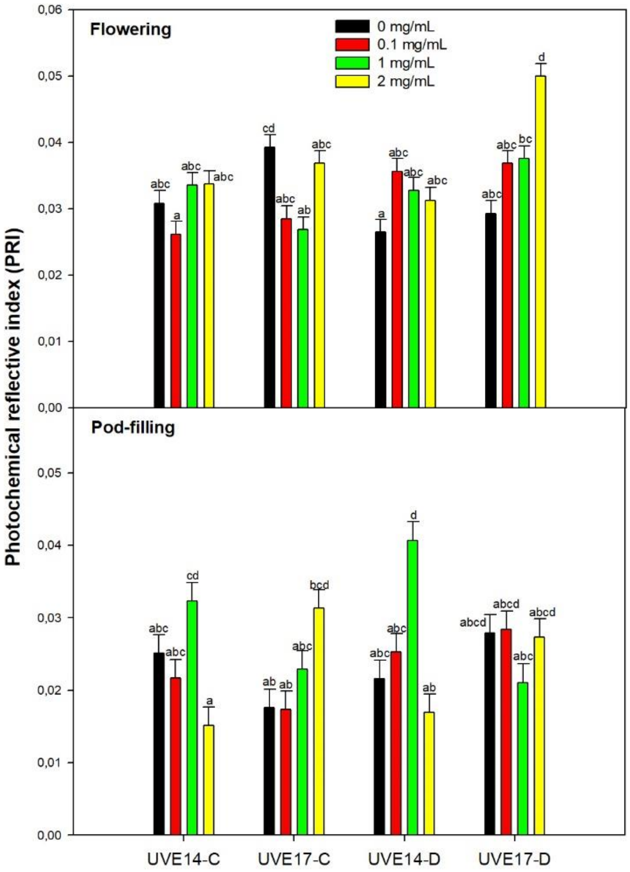


Figure 3.3. *The PRI of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

Drought stress significantly reduced g_s for UVE17 and UVE14 at both developmental stages. During flowering, 1 mg/mL increased g_s by 98.5% in UVE17 under drought stress. At pod-filling, treatment of drought-stressed UVE17 with different concentrations of dibutyldithiophosphate decreased the stomatal conductance. In contrast, treatment of optimally watered UVE17 with 1 and 2 mg/mL dibutyldithiophosphate during flowering increased this parameter by 65.1% and

42.2%, repectively compared to 0 mg/mL treatment. Treatment of UVE14 with the different concentrations of dibutyldithiophosphate had no substantial effect on this parameter under drought stress. In contrast, treatment of optimally watered UVE14 with dibutyldithiophosphate (0.1 and 2 mg/mL) induced significant increases in g_s (Figure 3.4).

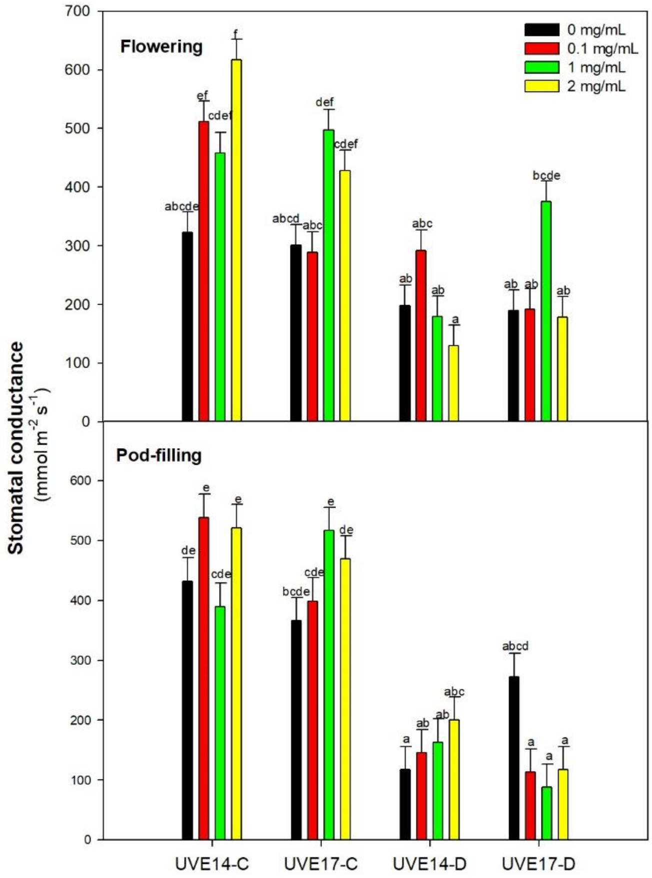


Figure 3.4. *The stomatal conductance of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

Drought stress leads to an increase in the Chl-a content of UVE17 at flowering (10.6%) stage. Treatment with dibutyldithiophosphate (0.1 and 2 mg/mL) induced 20.4% and 8.8% increases in the Chl-a content of drought-stressed UVE17. Under optimal watering, the highest increase was observed under 1 mg/mL treatment. The Chl-a content for UVE14 was not affected by drought stress. Treatment with dibutyldithiophosphate had no significant effects irrespective of its water status at flowering stage (Figure 3.5).

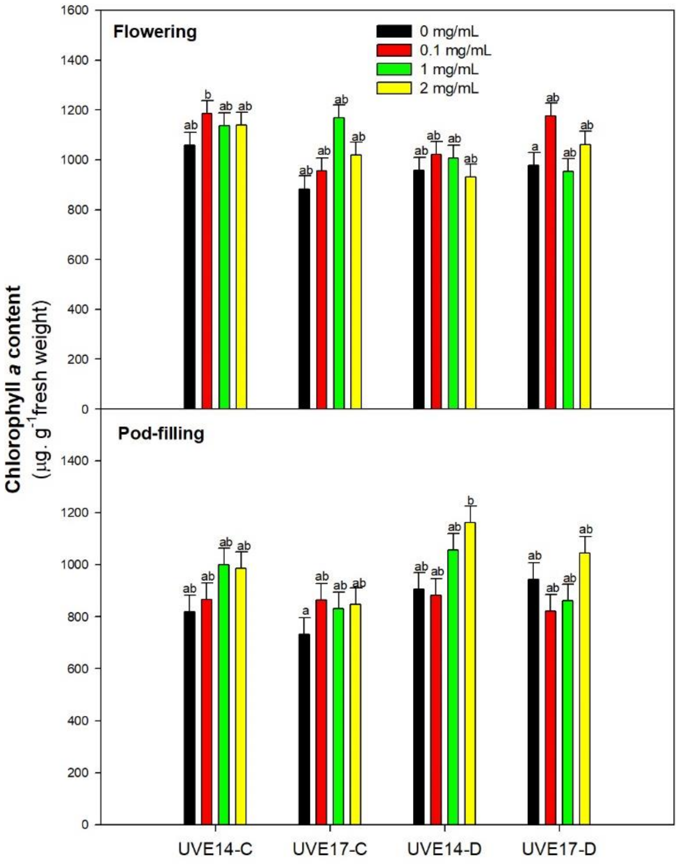


Figure 3.5. *The chlorophyll-a content of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

Drought stress had no substantial impact on the Chl-b content of UVE17 during flowering and pod-filling stages. Treatment of UVE17 with different concentrations of dibutyldithiophosphate was not efficient in increasing Chl-b content irrespective of the water level at both growth stages. In contrast, drought stress reduced Chl-b of UVE14 at flowering while application 1 and 2 mg/mL dibutyldithiophosphate increased its accumulation (33.2% and 38.2%). In addition, optimally watered UVE14 treated with 1 mg/mL H_2S donor had high Chl-b content compared to

0 mg/mL treatment. Furthermore, 2 mg/mL induced the high Chl-b under optimal watering in UVE14 (Figure 3.6).

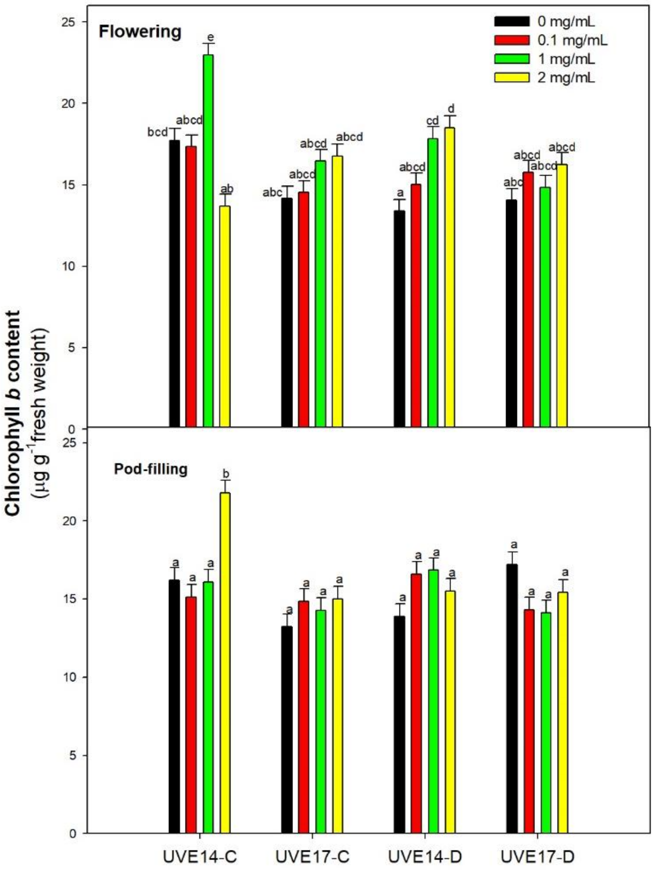


***Figure 3.6**. The Chl-b content of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

The treatment of drought-stressed UVE14 with dibutyldithiophosphate had no significant impact on the total chlorophyll content during flowering and pod-filling (Figure 3.7).

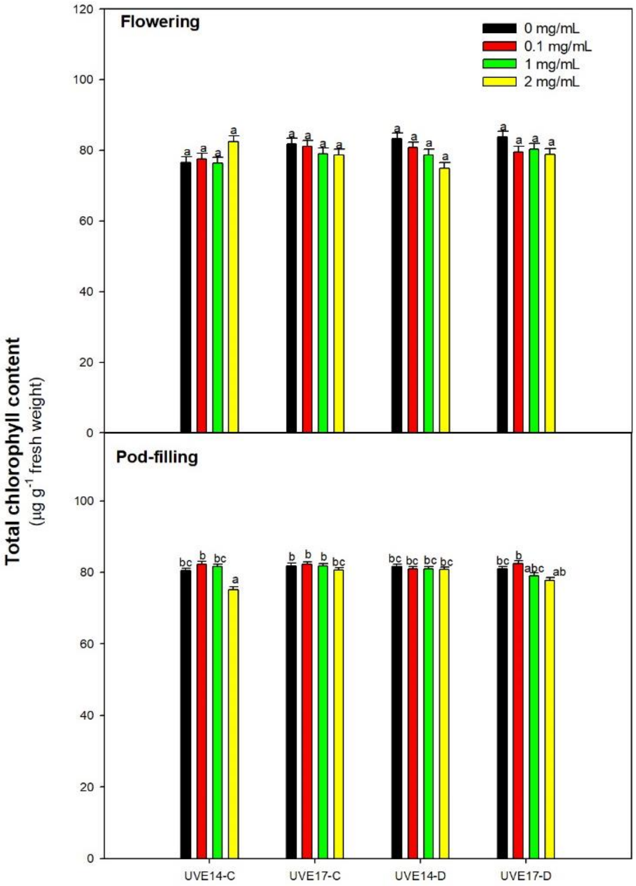


***Figure 3.7**. Total chlorophyll content of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

The H_2O_2 content for UVE17 was higher under normal than drought-stressed conditions for both growth stages, while that of UVE14 was increased under drought stress at flowering. At flowering, application of dibutyldithiophosphate reduced H_2O_2 content of optimally watered UVE17 (11.3% reduction at 0.1 mg/mL and 18.5% at 2 mg/mL). For drought-stressed UVE17, application of H_2S donor at 2 mg/mL increased H_2O_2 content (36.2%). Application of 1 and 2 mg/mL H_2S donor on optimally watered UVE14 increased H_2O_2 accumulation (19.4% and 24.8%, respectively). At pod-filling, application of 0.1 mg/mL H_2S donor reduced this parameter for drought-stressed UVE14. Under optimal watering however, dibutyldithiophosphate (0.1 and 2 mg/mL) increased H_2O_2 content for UVE14 during flowering. For drought-stressed UVE17, dibutyldithiophosphate increased H_2O_2 at 2 mg/mL concentration during flowering. There was no effect of dibutyldithiophosphate for optimally watered UVE17 (Figure 3.8).

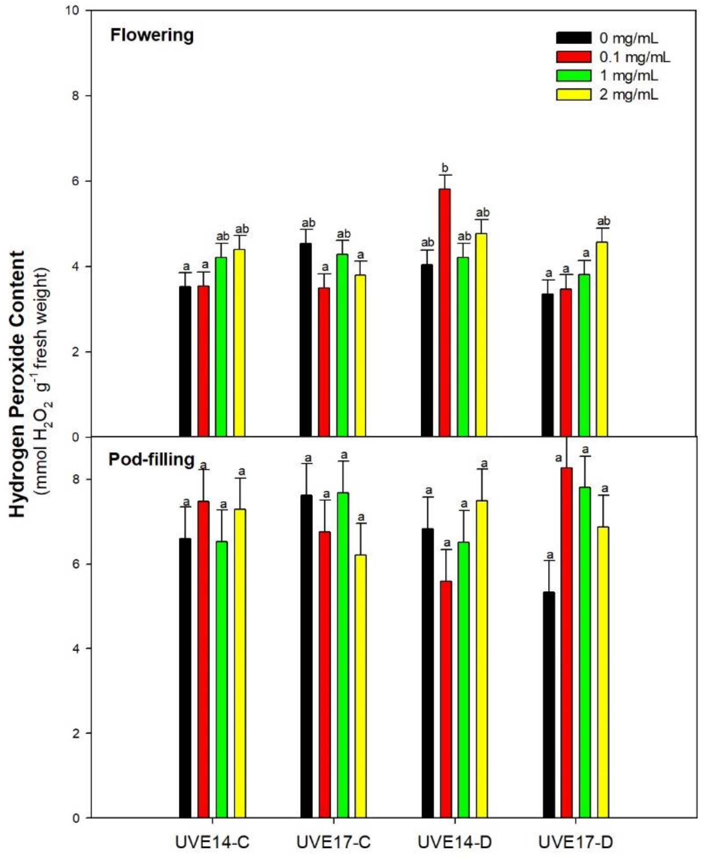


Figure 3.8. *Hydrogen peroxide (H_2O_2) content of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

At flowering, drought stress did not have a significant impact on the EL of both cultivars (Figure 3.9). Application of dibutyldithiophosphate had no significant influence on the EL of drought-stressed UVE17 but increased EL under 2 mg/mL. Application of H_2S donor at 1 mg/mL on drought-stressed and optimally watered UVE14 decreased EL. At pod-filling, drought stress increased EL for UVE14 (7.5%) and decreased EL for UVE17 (35.2%). The application of H_2S donor at 0.1 (28.4%) and 1 mg/mL (24.8%) significantly decreased EL for UVE17 under drought stress. For optimally watered UVE17, both concentrations also reduced EL but increased under

2 mg/mL. The treatment of cultivar UVE14 with H_2S donor at 1 mg/mL substantially reduced EL under drought stress (30.3%) and at optimal watering (Figure 3.9).

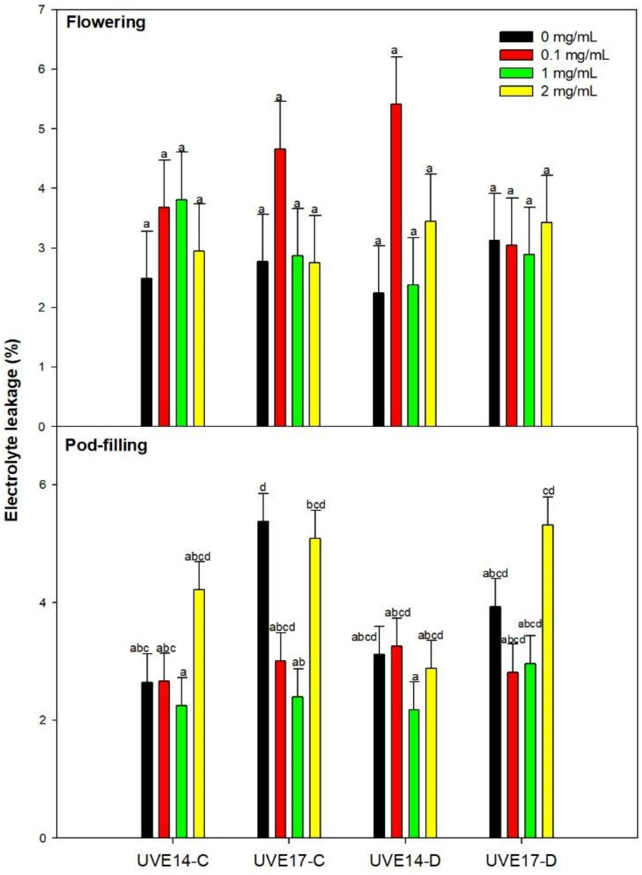


Figure 3.9. *Electrolyte leakage (EL) of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

The APX activity for drought-stressed UVE17 was significantly lower (35.8%) than that of the control (100% WHC). Although not significant, application of 0.1 and 2 mg/mL H_2S donor on drought-stressed UVE17 increased APX activity (34.1 and 24.3%, respectively) during flowering. Application of H_2S donor (2 mg/mL) on optimally watered UVE14 insignificantly increased (48%) APX activity during flowering. At pod-filling, the effect of drought stress was insignificant for UVE17 but significantly decreased for UVE14. For drought-stressed UVE14, application of H_2S significantly increased APX activity with the highest increase at 0.1 mg/mL

(34.3%). Application of dibutyldithiophosphate at all concentrations on optimally watered UVE14 however, reduced APX activity. Similarly, for drought-stressed and optimally watered UVE17, treatment with different H_2S donor concentrations reduced APX activity (Figure 3.10).

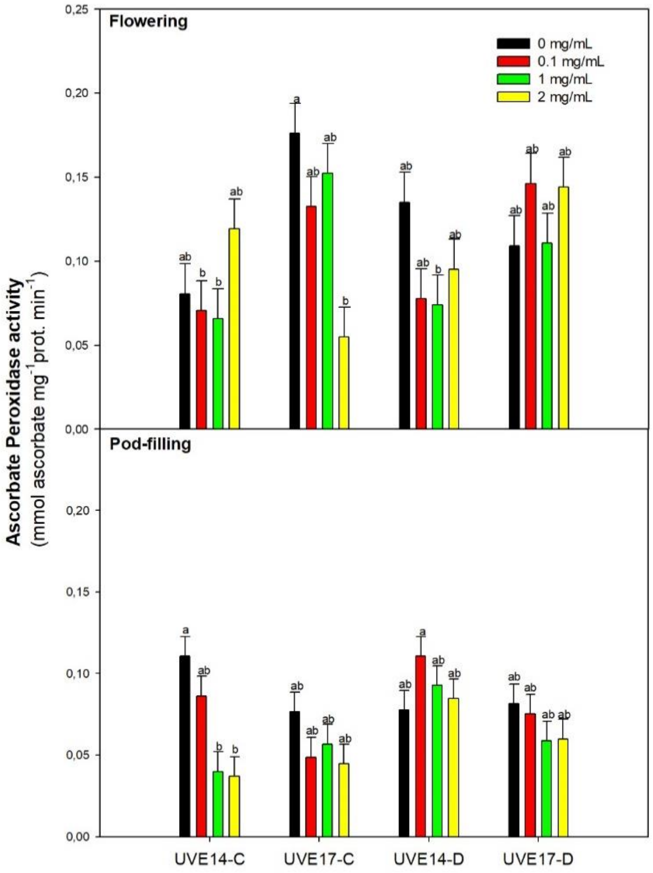


Figure 3.10. *Ascorbate peroxidase (APX) activity of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

The blue colour was more intense in the optimally watered UVE17 at 0 and 2 mg/mL dibutyldithiophosphate treatments, showing more presence of the superoxide radicals than under 0.1 and 1 mg/mL during pod-filling (Figure 3.11).

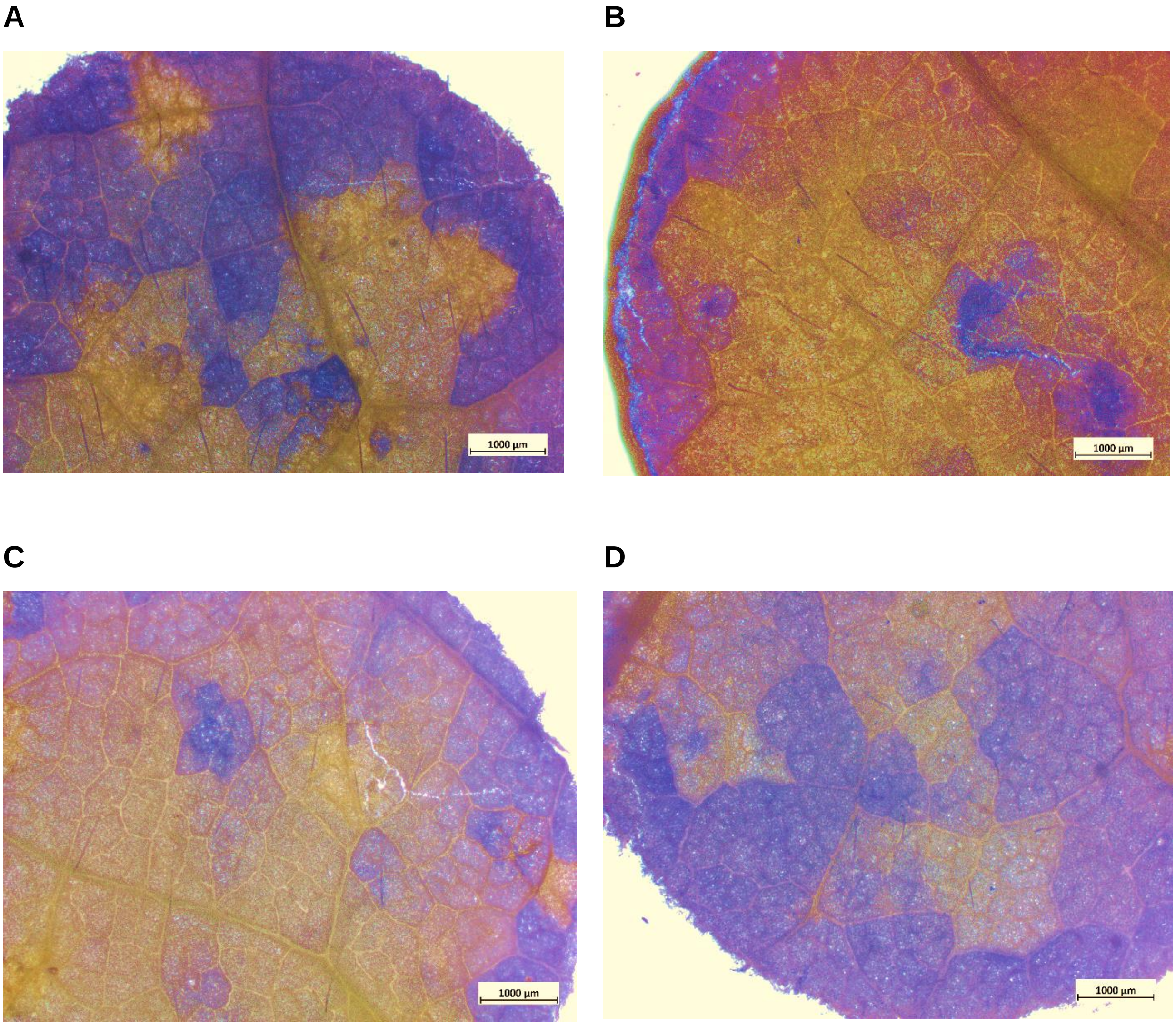


Figure 3.11. *Nitro blue tetrazolium (NBT) reduction in drought-stressed UVE17 (30% water holding capacity, WHC) at 0 mg/mL (A), 0.1 mg/mL (B), 1 mg/mL (C) and 2 mg/mL (D). Scale bar: 1000 µm. The blue colour represents the presence of* O_2^- *when NBT interacts with superoxide radicals. Samples were taken at pod-filling.*

The blue was more intense in the drought-stressed UVE14 (0 mg/mL) and 1 mg/mL concentration. However, treatment with 0.1 and 2 mg/mL dibutyldithiophosphate reduced the blue colour intensity, signifying less O_2^- formation (Figure 3.12).

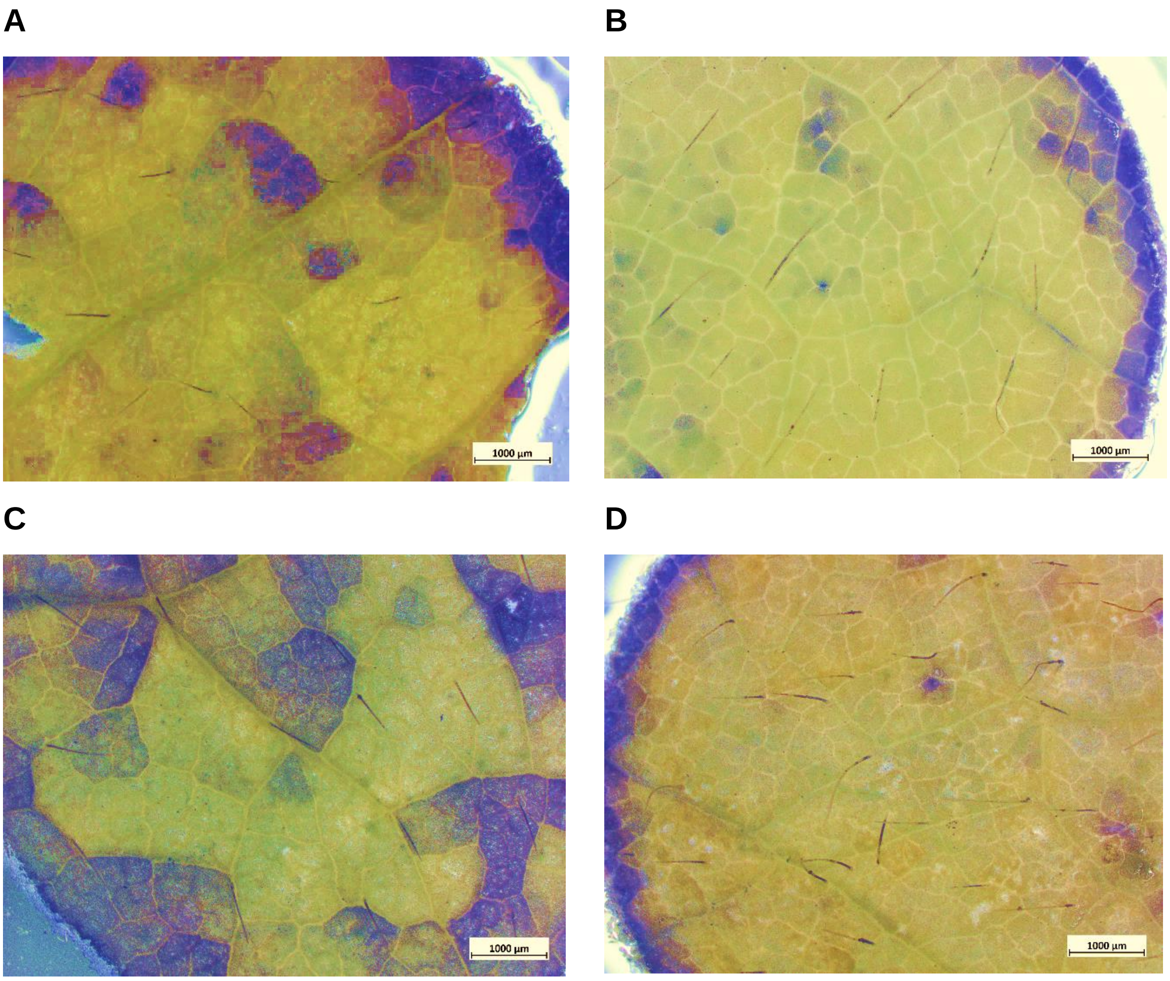


Figure 3.12. *Nitro blue tetrazolium (NBT) reduction in drought-stressed UVE14 (30% water holding capacity, WHC) at 0 mg/mL (A), 0.1 mg/mL (B), 1 mg/mL (C) and 2 mg/mL (D). Scale bar: 1000 µm. The blue colour represents the presence of* O_2^- *when NBT interacts with superoxide radicals. Samples were taken at pod-filling.*

Compared to the control, blue colour intensity (O_2^-) remained unchanged after application of different concentrations of dibutyldithiophosphate in UVE17 (Figure 3.13).

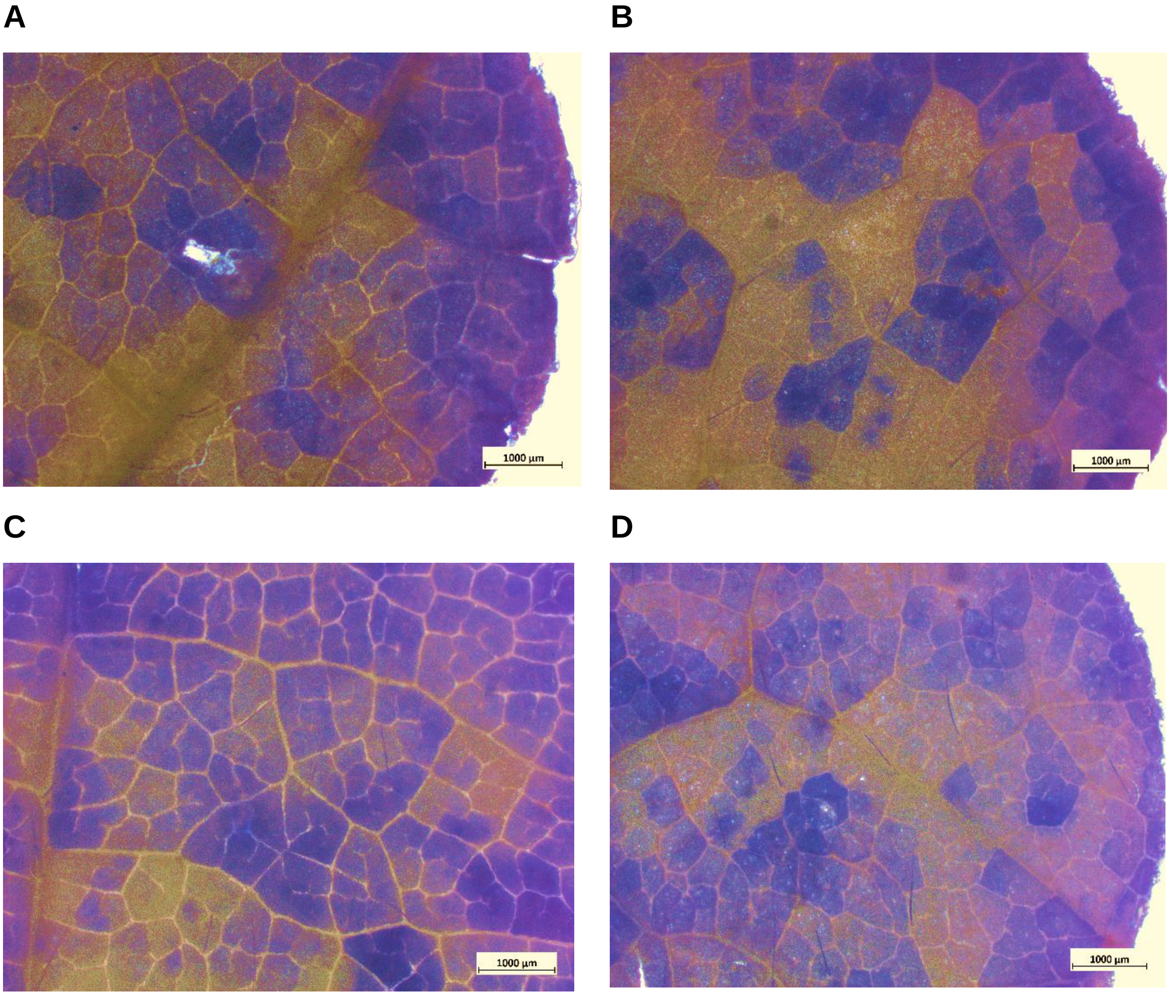


Figure 3.13. *Nitro blue tetrazolium (NBT) reduction in optimally watered UVE17 (100% water holding capacity, WHC) at 0 mg/mL (A), 0.1 mg/mL (B), 1 mg/mL (C) and 2 mg/mL (D). Scale bar: 1000 µm. The blue colour represents the presence of* O_2^- *when NBT interacts with superoxide radicals. Samples were taken at pod-filling.*

There was less O_2^- in optimally watered UVE14 under no dibutyldithiophosphate treatment. The differences in the colour intensity were not very clear under different H_2S donor treatments (Figure 3.14).

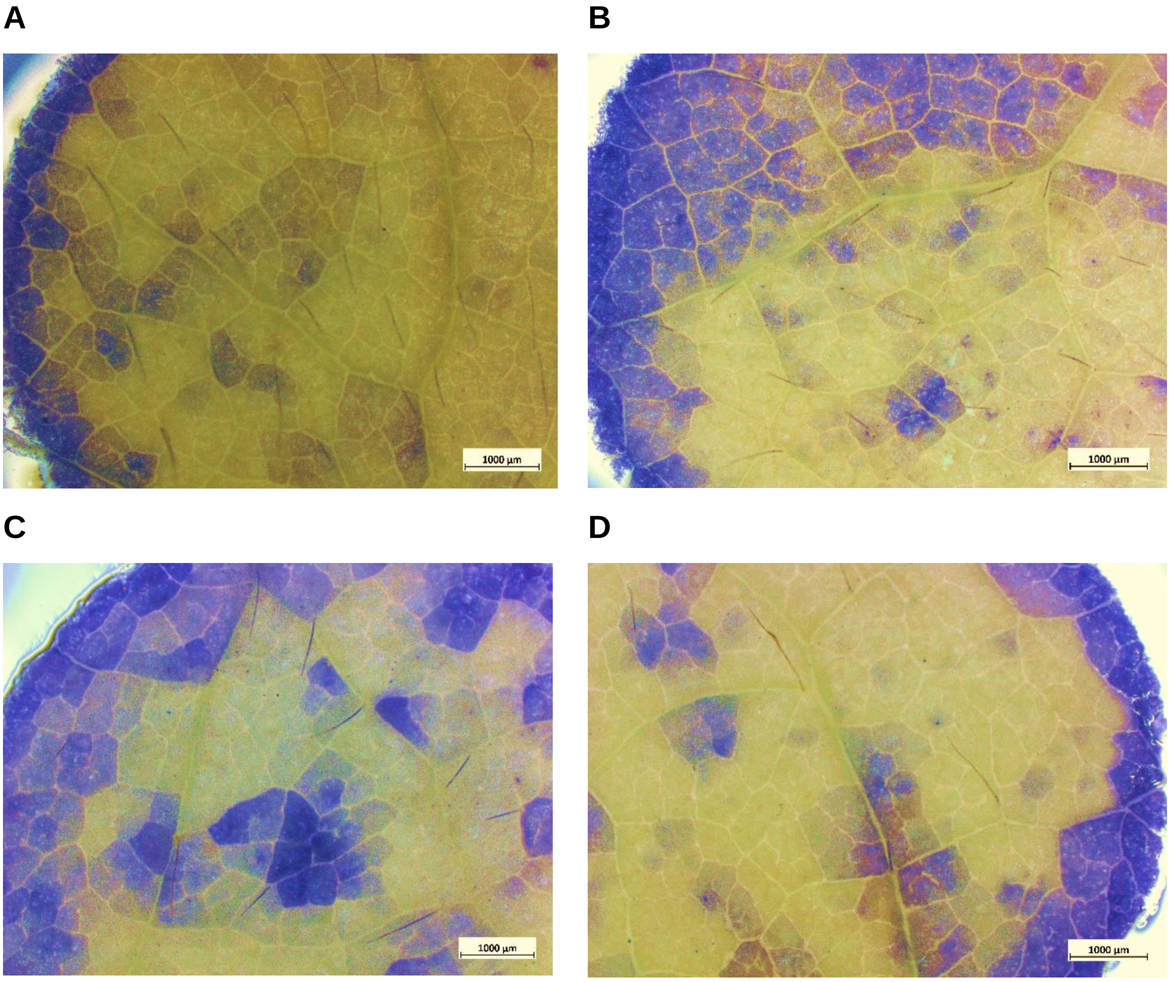


Figure 3.14. *Nitro blue tetrazolium (NBT) reduction in optimally watered UVE14 (0% water holding capacity, WHC) at 0 mg/mL (A), 0.1 mg/mL (B), 1 mg/mL (C) and 2 mg/mL (D). Scale bar: 1000 µm. The blue colour represents the presence of* O_2^- *when NBT interacts with superoxide radicals. Samples were taken at pod-filling.*

At flowering, application of dibutyldithiophosphate on drought-stressed and optimally watered cultivars had no significant effect on the SOD activity. Drought stress significantly reduced the SOD activity (30.3%) of UVE14 at pod-filling. Treatment of drought-stressed UVE14 with 0.1 mg/mL dibutyldithiophosphate led to 21.5% increase in SOD activity. Under optimal watering, treatments with H_2S donor reduced the SOD activity in UVE14 at 0.1 and 2 mg/mL. Treatment of drought-stressed UVE17 with 1 mg/mL increased SOD (23.7%) activity. No substantial increase in SOD activity was observed when H_2S was applied on optimally watered UVE17, however, significant decrease was observed at 0.1 mg/mL (Figure 3.15).

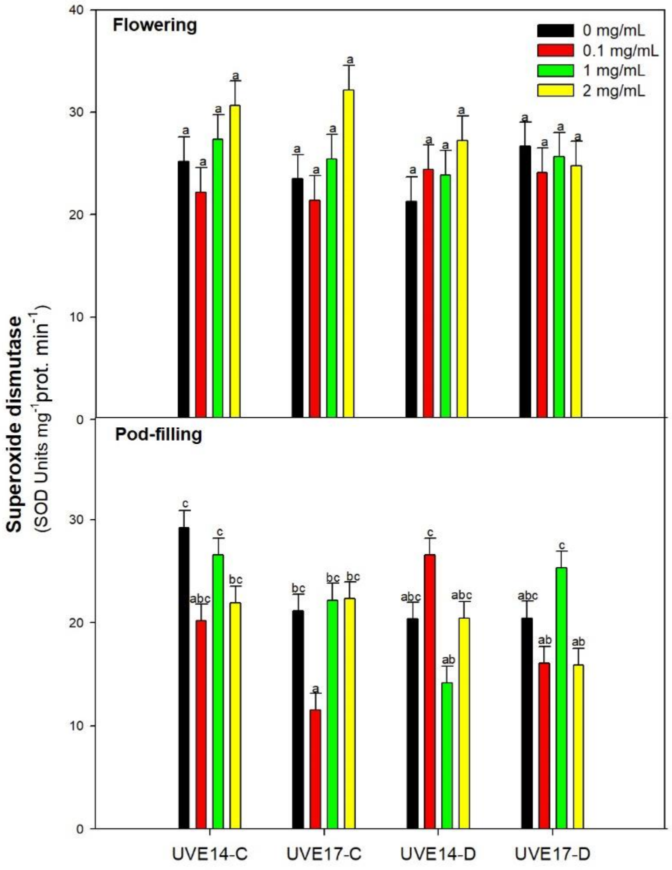


Figure 3.15. *Superoxide dismutase (SOD) activity of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

Drought stress induced increased FRI in both cultivars at flowering and pod-filling except for UVE14 during flowering. At flowering, application of dibutyldithiophosphate at all concentrations increased the FRI for drought-stressed UVE17, with 0.1 mg/mL inducing the highest increase (126.1%). Application of H_2S donor at all concentrations on optimally watered UVE17 led to significant increases in the FRI with the highest observed under 1 mg/mL (128.1%) during flowering. At pod-filling, treatment of optimally watered UVE17 with 0.1 and 2 mg/mL dibutyldithiophosphate increased FRI substantially (46.7% and 76.7%, respectively). Application of dibutyldithiophosphate on drought-stressed UVE14 resulted in substantial increases in FRI at flowering [0.1 (110.6%) and 2 mg/mL (138.7%)] and pod-filling (2 mg/mL). Application of this H_2S donor at (1 and 2 mg/mL) on optimally watered UVE14 induced the highest increases in FRI at pod-filling (Figure 3.16).

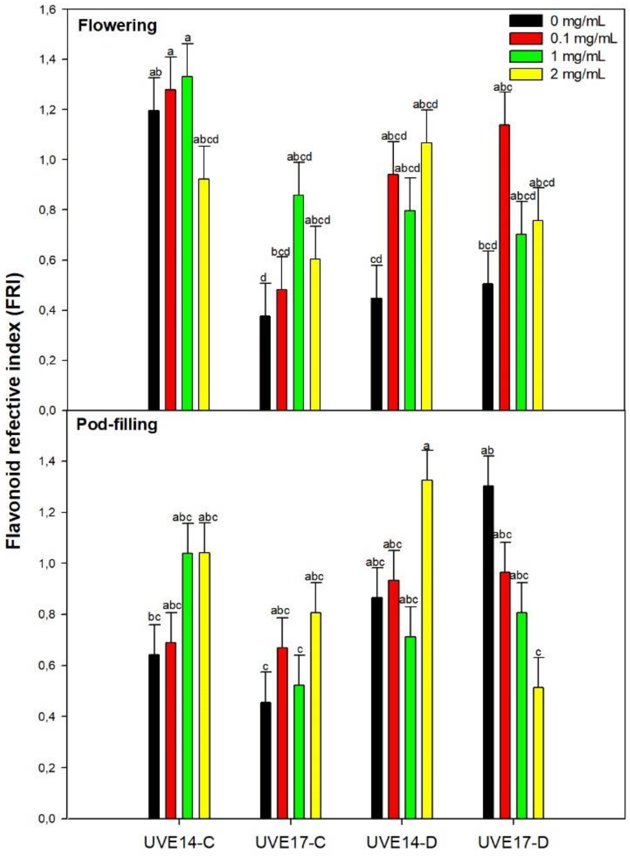


Figure 3.16. *Flavonoid reflective index (FRI) of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

Although there were no significant differences in the CRI within the different H_2S donor treatments at both growth stages, there were notable differences compared to when no H_2S donor was applied. At flowering, treatment of drought-stressed UVE17 with 0.1 and 2 mg/mL induced high CRI (24.9% and 29%). A similar pattern was observed at pod-filling for this treatment. For optimally watered UVE17, increased CRI was observed under 1 and 2 mg/mL treatments at flowering. Drought-stressed UVE14 treated with 1 mg/mL H_2S donor showed a 14.7% increase in the CRI at the flowering stage. Optimally watered UVE14 treated with 2 mg/mL showed a 17.3% increase in CRI (Figure 3.17).

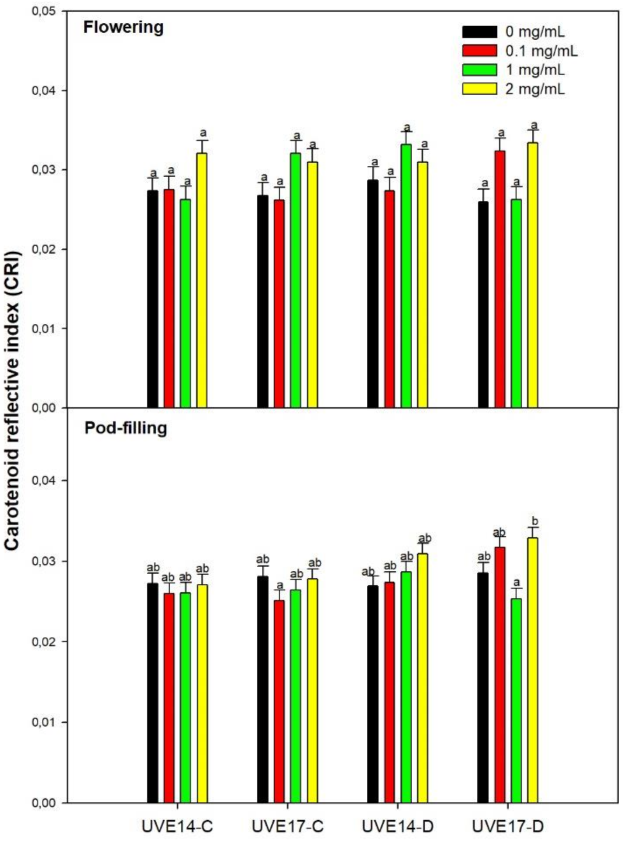


Figure 3.17. *Carotenoid reflective index of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments (p ≤ 0.05).*

Drought stress substantially increased the TSS content of UVE14 at flowering (67.2%) and pod-filling stages (63.4%). Dibutyldithiophosphate application on drought-stressed UVE14 did not promote further increases in accumulation of TSS for both growth stages. However, at flowering, under optimal watering, 0.1 and 2 mg/mL application increased TSS in this cultivar. At pod-filling, it increased under 1 mg/mL (66.6%) and 2 mg/mL. At flowering, 0.1 mg/mL increased the TSS (32.7%) content in drought-stressed UVE17. Under optimal watering, 2 mg/mL increased TSS for this cultivar. All concentrations had no significant effects on the TSS of UVE17 at pod-filling (Figure 3.18).

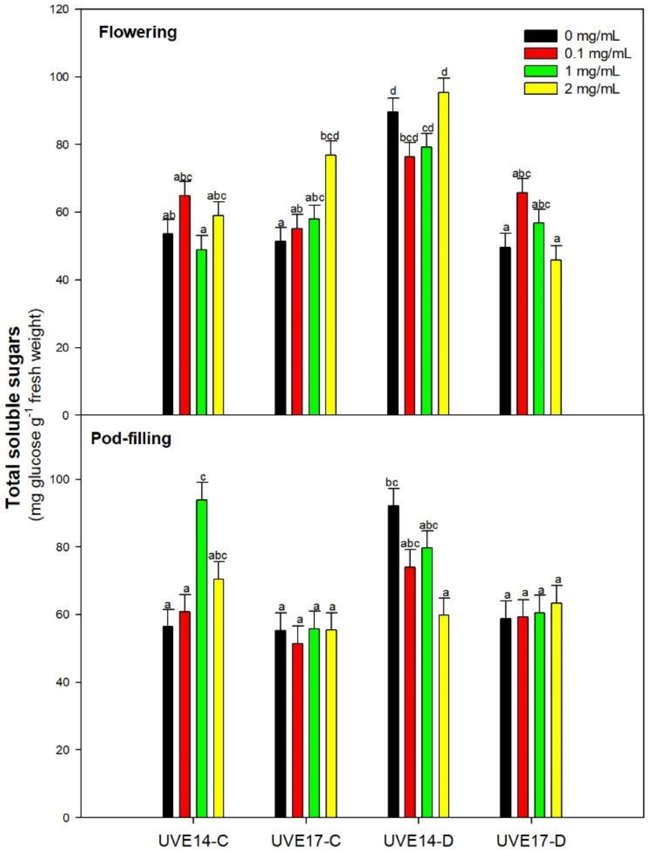


Figure 3.18. *Total soluble sugar (TSS) content of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/ similarities are represented by letters within various treatments ($p \leq 0.05$).*

Drought stress reduced proline content (27.6%) of UVE17 during flowering, and a 30.5% decline during pod-filling. Application of different H_2S donor treatments on drought-stressed UVE17 induced proline content at flowering and pod-filling stages. There was a significant increase in the proline of drought-stressed UVE17 at 0.1 mg/mL treatment (58.9% increase). Such treatments did not lead to increases in proline under optimal watering for this cultivar. During pod-filling, 1 and 2 mg/mL dibutyldithiophosphate treatments induced significant increases in proline content (140.8% and 115%) of UVE17 under drought stress. Similarly, treatment of UVE14 with 1 and 2 mg/mL dibutyldithiophosphate under drought stress at flowering, substantially upregulated proline (54% and 73% increases). At pod-filling, a more substantial increase in proline for optimally watered UVE14 under 2 mg/mL. There were no substantial increases in proline for drought-stressed UVE14 under dibutyldithiophosphate treatments (Figure 3.19).

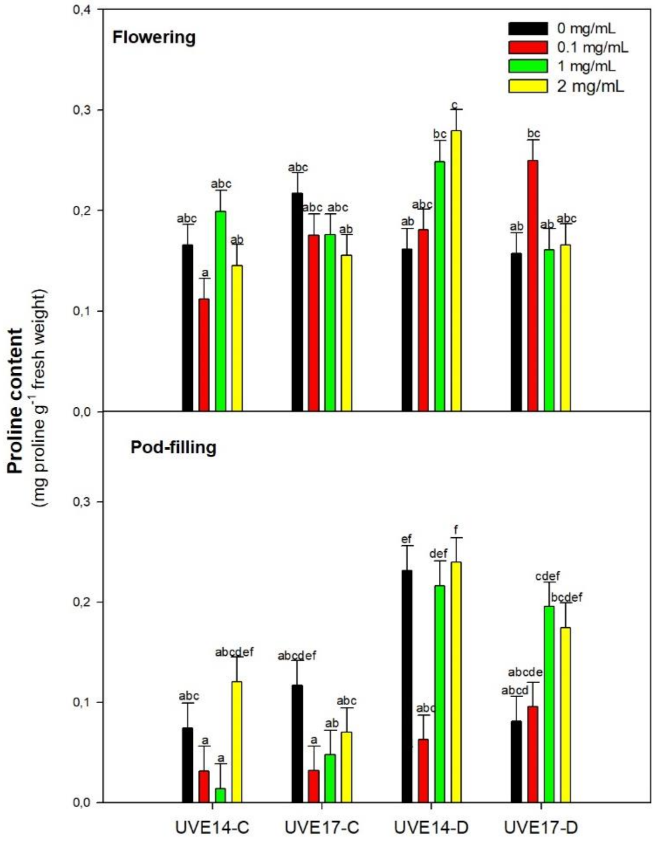


Figure 3.19*. Proline content of edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels (100% and 30% soil water holding capacity, WHC) at different reproductive stages (flowering and pod-filling). Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

3.5. Discussion

Drought stress affects the physiological and biochemical reactions in edamame (Moloi and van der Merwe, 2021; Hlahla et al., 2022). In this study, it was hypothesised that the application of dibutyldithiophosphate would improve the physiological and biochemical responses of edamame under drought stress. Results showed that the physio-biochemical responses differed according to the cultivars (UVE14 and UVE17), growth stages (flowering and pod-filling), concentrations of dibutyldithiophosphate (0.1, 1, and 2 mg/mL) as well as the water level (drought or optimal watering). During drought stress, photosynthesis is one of the most affected physiological mechanisms with catastrophic consequences on crops (Wang et al., 2018). Information from a variety of physiological responses such as gas exchange (stomatal conductance), chloroplast pigment contents, and chlorophyll-a fluorescence, is used to evaluate the photosynthetic capacity of plants (Chen et al., 2021; Khruschev et al., 2022). Drought stress significantly decreased the Fv/Fm ratio in wheat (Živčák et al., 2008), maize (Badr and Brüggemann, 2020), and grapevine leaves (Zulini et al., 2007). In contrast to this study, Fv/Fm was insignificantly affected by drought stress in both cultivars (UVE14 and UVE17), and application of different concentrations of dibutyldithiophosphate were ineffective for both edamame cultivars under drought stress because it did not increase their quantum efficiency at any growth stage (Table 3.2 and 3.3).

The performance indices, (PI_{abs}) and total performance index (PI_{total}), are hypersensitive to environmental stress (Çiçek et al., 2015). Treatment of drought-stressed edamame with dibutyldithiophosphate selectively increased the performance indices at flowering, showing that under H_2S treatment, growth stage matters for these parameters. The results also showed that under drought stress, these performance indices differ according to the H_2S donor concentration and cultivar. For example, during flowering drought-stressed UVE14 treated with 0.1 mg/mL dibutyldithiophosphate was the only cultivar with significantly increased PI_{abs}, while 2 mg/mL was the only concentration that could induce significant increases in the PI_{total} for both cultivars (Figure 3.2). When these concentrations were changed, the performance of the photosystems was reduced. In addition, during pod-filling the effect to dibutyldithiophosphate under optimal watering increased the performance indeces of UVE17; however, UVE14 had decreased performance indices at this stage (Figures 3.1 and 3.2). This indicates that under drought stress, 0.1 mg/mL application of dibutyldithiophosphate could increase the PSI performance in UVE14, while 2 mg/mL treatment increased performance for both photosystems in drought-stressed UVE14 and UVE17. Therefore, application of dibutyldithiophosphate at specific concentrations on drought-stressed edamame could prevent photoinhibition of both photosystems (Smit et al., 2009; Živčák et al., 2014; Liatile, 2021; Hlahla et al., 2022).

Photochemical reflectance index, which can also be correlated with non-photochemical quenching (Inamullah and Isoda, 2005; Hikosaka and Tsujimoto, 2021), is one of the reliable indicators of thermal dissipation of excess excitation energy used in this study to determine plant's health (Thenot et al., 2002; Inamullah and Isoda, 2005; Helm et al., 2020). Drought stress reduced the PRI of UVE17 in agreement with Inamullah and Isoda (2005), where four water treatments (100%, 50%, 25% and 10% WHC) caused a decrease in PRI with increased severity of the stress in soybean. In this study, it was observed that the application of different concentrations of dibutyldithiophosphate on drought-stressed UVE17 and UVE14 increased PRI during flowering stage, while at pod-filling, increase was only observed in UVE14, showing that growth stage matters. Since UVE17 is more susceptible to drought than UVE14, it seems that it needed more dibutyldithiophosphate (2 mg/mL) to increase the highest photochemical efficiency than UVE14, which only required 1 mg/mL. The xanthophyll cycle, which is associated with how efficiently plants utilize light (Gamon et al., 1992; Filella et al., 1996; Gamon et al., 1997), was activated in water stressed soybeans (Inamullah and Isoda, 2005), and as a result, an increase in PRI in our study may also indicate that dibutyldithiophosphate enhanced the xanthophyll cycle. Although increases in PRI were also observed in optimally watered edamame plants, application of dibutyldithiophosphate was insignificant (Figure 3.3). Therefore, for this parameter, application of dibutyldithiophosphate is more effective under drought stress both cultivars. Whether this could translate into increased growth and yield remains to be tested.

Carbon dioxide assimilation into the leaves is proportional to the stomatal conductance. Different concentrations of dibutyldithiophosphate were able to increase the stomatal conductance of drought-stressed UVE17 (1 mg/mL) and UVE14 (0.1 mg/mL) at flowering. Similar to PRI, drought susceptible edamame (UVE17) required more dibutyldithiophosphate to increase the stomatal conductance. At pod-filling, dibutyldithiophosphate significantly increased PRI, however at increased concentration (1 mg/mL). The ineffective response of dibutyldithiophosphate treatment at pod-filling in drought-stressed UVE17, further showed that the efficacy of this H_2S donor on the stomatal conductance depends on the developmental stage. Application of dibutyldithiophosphate on optimally watered plants was effective in increasing the stomatal conductance irrespective of the growth stage (Figure 3.4). These results show that application of dibutyldithiophosphate increases the stomatal opening in edamame, which is beneficial for increasing photosynthetic capacity and decreases the likelihood of ROS formation (Damour et al., 2010).

Degradation of chlorophyll pigments under drought stress is usually caused by the overproduction and accumulation of ROS and early leaf senescence (Terzi et al., 2010; Kalaji et al., 2018). In this study, drought stress increased Chl-a content in cultivar UVE17 (Figures 3.5).

Although not in the same plant species, these findings are in agreement with Liatile's (2021) observation that spinach's Chl-a content increased as the intensity of the drought stress increased. This contradicted Hlahla et al. (2022) who observed no increase in Chl-a but an increase in Chl-b for drought-stressed UVE17. Dibutyldithiophosphate application (0.1 and 2 mg/mL) to drought-stressed UVE17 led to significant increases in Chl-a during flowering. The same concentration (2 mg/mL) increased Chl-a content for drought-stressed UVE14 at pod-filling (Figure 3.5). Chl-b for UVE17 did not respond positively to dibutyldithiophosphate at any growth stage irrespective of the water level. This suggests that for H_2S donor to increase Chl-b, the concentration of dibutyldithiophosphate might have to be increased beyond 20 mg/mL. For UVE14, application of dibutyldithiophosphate at the concentrations of 1 and 2 mg/mL effectively increased Chl-b content independent from the growth stage (Figure 3.6). Non-significant impact of dibutyldithiophosphate on Tot Chl under well-watered and drought stress conditions was observed (Figure 3.7). Therefore, based on Chl-a and Chl-b results it appears that dibutyldithiophosphate increases the photosynthesis pigments more under drought stress in edamame.

Overproduction of ROS caused by drought stress results in oxidative stress, which can be detected by evaluating the levels of MDA, EL and H_2O_2 (Naz et al., 2021). There were significant differences in the responses of edamame cultivars to drought stress with high H_2O_2 accumulation in UVE14 and low H_2O_2 in UVE17 at flowering (Figure 3.8). In partial agreement, barley plants under drought stress had greater levels of H_2O_2, MDA, and EL. Interestingly, application of H_2S alleviated oxidative stress by reducing the levels of H_2O_2, MDA, and EL (Naz et al., 2021). Also, H_2S (NaHS) application maintained relatively low H_2O_2 concentrations compared to untreated strawberries (Christou et al., 2013). In the current study, treatment of optimally watered UVE14 with higher concentration of dibutyldithiophosphate increased H_2O_2 under optimal watering while lower concentration reduced it. This shows that high concentrations of dibutyldithiophosphate could possibly damage the membranes for optimally watered edamame. Under drought stress, 2 mg/mL dibutyldithiophosphate induced high EL in UVE17, which corresponded to increased MDA at this concentration. Lower concentrations of dibutyldithiophosphate (0.1 and 1 mg/mL) during pod-filling in UVE17 reduced EL under drought stress, which could contribute to increased membrane integrity and therefore increased tolerance to drought stress. This was in line with the results of Khan et al. (2018), who demonstrated that the application of NaHS to drought-stressed *Eruca sativa* significantly reduced EL. Considerable reduction in EL, which is necessary for plant tolerance to drought stress (Balsam and Bush, 2022), ensured membrane integrity in edamame. For UVE14 under drought stress, lower concentration of dibutyldithiophosphate (0.1 mg/mL) increased EL, further showing that tolerant edamame is more sensitised to dibutyldithiophosphate (Figure 3.9). The accumulation of ROS can cause damage to membrane lipids directly, leading to an increase in MDA (Moore and Roberts, 1998; Cunhua et al., 2010; Anjum et al., 2011b; Labudda, 2013; Laxa et al., 2019). Christou et al. (2013) showed that the pre-treatment of strawberry plants with NaHS resulted in low MDA concentrations. In this study, the MDA content was not significant for the interactions during flowering and pod-filling (Table 3.2). The application of the different dibutyldithiophosphate concentrations had no significant effect on the edamame cultivars (Table 3.3), which contradicts the results on H_2O_2 and EL above. It might be necessary to conduct more research on the impact of H_2S delivered through the dibutyldithiophosphate on MDA on edamame cultivars. In contrast, Kolupaev et al. (2019) found that applying NaHS to drought-stressed wheat resulted in a significant reduction in MDA content.

Superoxide dismutase, APX, and GPX are part of plants' antioxidative systems that function to keep ROS levels low (Garbero et al., 2011). In the current study, not all antioxidative enzymes studied were influenced significantly by different concentrations of dibutyldithiophosphate. The GPX activity remained unchanged under different H_2S donor treatments irrespective of the developmental stage (Table 3.3). Drought stress dramatically decreased APX activity in UVE17 during flowering and significantly decreased APX activity in UVE14 during pod-filling. However, application of dibutyldithiophosphate did not increase APX significantly if UVE17, irrespective of whether there was drought stress or not. The only effective response to dibutyldithiophosphate treatment was observed for drought-stressed UVE14 at 0.1 mg/mL during pod-filling (Figure 3.10). This shows that for APX under drought stress, efficacy of dibutyldithiophosphate is cultivar and concentration dependent. It also appears that this H_2S donor can only increase this parameter under stress because both cultivars had substantially reduced APX when dibutyldithiophosphate was applied on optimally watered edamame. Similarly, NaHS application on drought-stressed rice upregulated APX activity (Zhou et al., 2020). These results imply that H_2S enhanced the expression of APX to further promote tolerance of UVE14 to oxidative stress caused by drought stress through conversion of H_2O_2 to water (AL-Aloosy et al., 2019).

The negative effects of drought stress on SOD activity were evident at pod-filling for both edamame cultivars (Figure 3.15). According to the results, water level affects the efficiency of SOD activity which was substantially reduced under drought stress. Like APX, efficacy of dibutyldithiophosphate was only observed at pod-filling, where it increased SOD activity of drought-stressed UVE17 (1 mg/mL) and UVE14 (0.1 mg/mL). This supports earlier suggestions that drought susceptible edamame needs more dibutyldithiophosphate than drought tolerant to upregulate the physiological responses. Increased SOD activity due to dibutyldithiophosphate treatment may also promote plant health through maintenance of cell during drought-stressed plants (Shi et al., 2013). Since SOD enzyme positively correlates with H_2O_2 during pod-filling, findings of this study explains that during drought stress, treatment of UVE14 with 0.1 mg/mL dibutyldithiophosphate increased SOD activity, thereby converting O_2^- to H_2O_2 (because the same concentration increased H_2O_2 in drought-stressed UVE14). These results corresponded with the histochemical studies where 1 mg/mL H_2S treatment on drought-stressed UVE17 had lesser O_2^-, supporting that this treatment increased SOD activity to convert O_2^- to H_2O_2, hence less staining. In addition, there was more O_2^- for drought-stressed UVE17 under 2 mg/mL dibutyldithiophosphate treatment (Figure 3.11), further showing that reduced SOD activity (Figure 3.15) under this treatment, inhibited conversion of these radicals to form H_2O_2. Similarly for drought-stressed UVE14, there was less detection at 0.1 mg/ml dibutyldithiophosphate, showing that this H_2S donor increased the antioxidative capacity of both cultivars at pod-filling in agreement with Jin et al. (2011), who found that pre-treatment of *thale cress* with NaHS provided better drought tolerance by increasing SOD activity.

Non-enzymatic antioxidants include molecules such as the flavonoids, carotenoids and compatible solutes such as proline. Similar to the enzymatic antioxidative system, these molecules are responsible for increasing the plant defence responses by scavenging free radicals (Parvaiz et al., 2009; Sarker and Oba, 2018; Uarrota et al., 2018; Ghosh et al., 2021; Li et al., 2021). Flavonoids are physiologically active compounds that play an important role against plant stresses. In this study, drought stress induced substantial increases in the flavonoid content in both cultivars at both stages except for UVE14 during flowering. The application of the dibutyldithiophosphate substantially increased the flavonoid content in both cultivars, at any water level, and all concentrations especially at 0.1 and 1 mg/mL (Figure 3.16). In the context of our experiment, the treatment of the H_2S donor resulted in the activation of secondary metabolism, which is seen by the increase in flavonoid content. However, little is understood about how H_2S affects the amount of flavonoid compounds in plants (Kolupaev et al., 2018).

In addition to their role in photosynthesis, carotenoids enhance plant defences under drought conditions and protect photosynthetic tissues (Sarker and Oba, 2018). Drought stress had no significant effect on the CRI on the edamame cultivars. According to Devnarain et al. (2016), carotenoid content in both water-stressed and optimally hydrated sorghum cultivars did not decrease significantly. Application of dibutyldithiophosphate (2 mg/mL) was only influential on drought-stressed UVE17, where it increased CRI, suggesting that this H_2S donor increased the antioxidative capacity selectively (Figure 3.17). It is possible that H_2S functions as a signaling molecule, raising accumulation of the carotenoids, further increasing the ROS scavenging capacity and improving the drought tolerance of drought-stressed UVE17, which could agree with Liu et al. (2022).

During drought stress, the accumulation of TSS directly supports the radical scavenging, osmotic adjustment, and protein structure stabilization (Ghosh et al., 2021). A drought-tolerant edamame cultivar demonstrated an increase in TSS when under drought stress (Moloi and van der Merwe, 2021). In water-stressed plants, the soluble sugars assist in enhancing osmotic potential (Liatile, 2021) and providing drought tolerance (Moloi and van der Merwe, 2021). This study had no significant effect on the TSS accumulation in UVE17 (flowering) while it increased in UVE14 (flowering and pod-filling). These findings back up Moloi and van der Merwe's (2021) assertion that TSS contributes to edamame tolerance. Application of dibutyldithiophosphate increased TSS under optimal watering for both growth stages except UVE17 at pod-filling. Under drought stress, application of dibutyldithiophosphate at all concentrations reduced TSS accumulation in UVE14 while it increased TSS in UVE17 at flowering stage (0.1 and 1 mg/mL), showing that cultivars respond differently to H_2S donor (Figure 3.18). These results show the importance of considering cultivars when it comes to dibutyldithiophosphate application for improving the TSS accumulation. These results agree with Naz et al. (2021), who found that treatment of drought and heat stressed barley with NaHS (H_2S) upregulated the TSS. Increased TSS for drought-stressed UVE17 treated with dibutyldithiophosphate implies that H_2S increased the photosynthetic capacity of this cultivar, which corresponded to increased PI_{total}. Also, such increase implies that this cultivar had increased potential of taking up water from the soil under drought stress, thereby increasing its potential for survival under drought stress. Furthermore, since TSS increases the antioxidative capacity of plants (Ghosh et al., 2021), application of dibutyldithiophosphate increased the antioxidative ability of a drought susceptible edamame cultivar, which could impact positively on yield.

In addition to the soluble sugars, molecules such as proline contribute to osmotic adjustment under drought stress (Ashraf and Foolad, 2007; Roychoudhury and Chakraborty, 2021). To adapt to the adverse effects of drought, plants can modulate their stress tolerance response and osmotic adjustment through H_2S signaling and plant metabolism (Roychoudhury and Chakraborty, 2021). According to Zhang et al. (2010), drought-stressed soybean seedlings treated with NaHS had elevated RWC. In contrast, in this current study RWC was not significant for the interactions during flowering and pod-filling (Table 3.2). During both developmental stages, treatment with H_2S led to significant increases in RWC (1 mg/mL at flowering and 2 mg/mL at pod-filling) (Table 3.3). According to Table 3.3, the high TSS accumulation during flowering and pod-filling could be the cause of the elevated RWC at these concentrations (Roychoudhury and Chakraborty, 2021). In addition to its role in osmotic adjustment, proline maintains sub-cellular structure and acts as an antioxidant under drought stress (Anjum et al., 2011a; AL-Aloosy et al., 2019). Previous research showed that under drought stress, plants accumulate proline in their cells (Rezayian et al., 2020; Moloi and van der Merwe, 2021; Waqar et al., 2022). In this experiment, however, drought stress decreased proline except for UVE14 at pod-filling (Figure 3.19). When applied on drought-stressed UVE17, dibutyldithiophosphate increased proline accumulation significantly during flowering (0.1 and 2 mg/mL) and pod-filling. Additionally, the proline content during flowering at 1 and 2 mg/mL substantially increased when the H_2S donor was applied to drought-stressed UVE14. This shows that for both cultivars, dibutyldithiophosphate can increase drought tolerance in edamame. Similarly, NaHS treatment significantly decreased the detrimental effects of drought and increased proline accumulation in drought-stressed barley (Naz et al., 2021). The effectiveness of dibutyldithiophosphate depended on the water level because under optimal watering, it reduced proline accumulation for both cultivars.

3.6. Conclusion

This study highlighted the response of edamame cultivars to different water levels and the efficacy of using dibutyldithiophosphate to provide drought stress tolerance. The results showed that drought stress significantly reduced the photosynthetic capacity of the cultivars (especially in UVE17), stomatal conductance, APX, SOD and proline. Under optimal watering, dibutyldithiophosphate effectively upregulated fewer physio-biochemical responses while more responses were increased under drought stress for both cultivars. In addition to the water level, efficacy of dibutyldithiophosphate on the physiological and biochemical responses varied according to the concentration, cultivar used as well as the developmental stage. Although the responses varied with the cultivars, different concentrations of dibutyldithiophosphate increased the photosynthetic capacity, reduced ROS accumulation, and increased the antioxidative capacity of drought-stressed edamame. The concentrations required to trigger the defence responses were mostly higher for UVE17 than for UVE14. The effect of dibutyldithiophosphate on the photosynthetic capacity is evident at flowering stage while that of the antioxidative capacity is observed at pod-filling. Therefore, this study provides ground-breaking discovery showing that dibutyldithiophosphate application on drought-stressed edamame can increase the photosynthesis capacity of edamame, leading to increased accumulation of sugars and proline, which act as antioxidants against excess ROS produced during drought stress. It also increases the antioxidative capacity (SOD, APX, FRI, CRI) of drought-stressed edamame, contributing to reduced membrane damage. These increases could upregulate the physiological and biochemical responses and increase growth and yield. This will be covered in the next chapter.

Chapter 4: The influence of dibutyldithiophosphate on the morphological responses of drought-stressed edamame and their relationships with the physiological and biochemical responses

4.1. Abstract

Edamame (*Glycine max* L. Merrill) is a drought sensitive crop and its production in South Africa is influenced by unpredictably dry weather and thus impact negatively on growth and yield. This study sought to investigate the effect of a biodegradable hydrogen sulfide (H_2S donor), dibutyldithiophosphate, at various concentrations (0, 0.1, 1 and 2 mg/mL) on the morphological traits of drought-stressed edamame cultivars (UVE14 and UVE17). It also aimed to establish the relationships between the physio-biochemical and the morphological characteristics [plant height (PH), branches per plant (BPP), total seeds per plant (TSPP), total seed mass per plant (TSM), and pods per plant (PPP)] of drought-stressed edamame under different dibutyldithiophosphate concentrations. The morphological characteristics were reduced under the drought stress for both edamame cultivars. Treatment of edamame with dibutyldithiophosphate significantly reduced the negative impacts of drought stress. Performance of the yield parameters differed according to the cultivars and concentration of H_2S donor. In a drought-stressed UVE17, application of dibutyldithiophosphate increased PPP (0.1 mg/mL), BPP (1 mg/mL), and TSPP (2 mg/mL). For drought-stressed UVE14 application of dibutyldithiophosphate increased BPP (all concentrations), PPP (0.1 and 1 mg/mL) and TSPP (2 mg/mL). Results further showed that dibutyldithiophosphate increased the yield responses (BPP, PPP and TSPP) of drought-stressed edamame through upregulation of the photosynthetic, osmotic, and antioxidative capacity of drought-stressed edamame. This study provides novel information on the improvement of edamame drought tolerance and needs to be studied further under uncontrolled field conditions.

4.2. Introduction

High consumption of starchy foods alone is usually associated with malnutrition in vulnerable populations in sub-Saharan Africa (SSA), including South Africa (SA). Not only are protein and energy insufficiency common among South Africans, but so are the deficiencies in micronutrients such as vitamin A, iron, and zinc, which are present in meat and eggs (Taylor, 2017). Therefore, promotion of edamame (*Glycine max* L. Merrill) consumption and production in SA can combat malnutrition because it is considered a better and cheaper replacement for meat and eggs as a source of protein (Palupi et al., 2022). Although not fully accessible, edamame is slowly gaining popularity as a healthy food snack in SA (Smit, 2019).

However, SA is a semi-arid country, implying that the edamame growing areas frequently have unpredictable dry weather, making it a challenging task to grow edamame (van der Merwe et al., 2018). Edamame yield is substantially reduced by drought stress throughout the growing cycle, with the highest effect experienced during the reproductive stages (Shaheen et al., 2016, Moloi and van der Merwe, 2021), leading to substantial yield losses for the resource poor smallholder farmers, who are the main producers of edamame in SA (Meza et al., 2021). Chen et al. (2021) demonstrated a decrease in total seeds per plant, pod dry weight, plant height, fertile pods, seeds per plant and seeds per pod in drought-stressed edamame cultivars. South African cultivars such as UVE17 displayed high sensitivity to drought stress with high yield reduction, while others such as UVE14 displayed more drought tolerance (van der Merwe et al., 2018; Moloi and van der Merwe, 2021; Hlahla et al., 2022).

Drought tolerant edamame cultivars alter a variety of physiological and biochemical responses such as total soluble sugars (TSS), proline, ascorbate peroxidase (APX), guaiacol peroxidase (GPX), carotenoids, and cell wall carbohydrates that lead to modification of the morphological structures (Moloi and van der Merwe, 2021; Hlahla et al., 2022). The morphological characteristics are frequently decreased because of these responses (Shahzad et al., 2016). Although breeding for drought tolerance is successful, the process is lengthy. Therefore, urgent climate wise solutions are required to make edamame cultivars drought tolerant, which can contribute to food security in SSA. Several studies show that application of hydrogen sulfide (H_2S) donors in small amounts can positively affect growth and yields in plants under different environmental stresses (Carter et al., 2019; Kaya et al., 2020; Arif et al., 2021; Bahmanbiglo and Eshghi, 2021; Brown et al., 2021).

A novel, biodegradable H_2S donor named dibutyldithiophosphate, is a recently synthesised chemical that has shown to slowly release H_2S over weeks to months in water. The responses of plants to this donor were established under optimal growing conditions and led to improved yields (Carter et al., 2019). However, no study explored the effects of H_2S donor under drought conditions, let alone in edamame. The goal of this study was to ascertain the effects of various dibutyldithiophosphate concentrations on the morphological characteristics of edamame under drought stress, and to establish the relationships between the morphological and the physio-biochemical responses under these conditions. The studied morphological characteristics included plant height (PH), branches per plant (BPP), total seed mass (TSM), total seed mass per plant (TSPP), and pods per plant (PPP). It was hypothesised that dibutyldithiophosphate would increase growth and yield in drought-stressed edamame. It was also hypothesised that there is a close relationship between the increased physio-biochemical responses (chapter 3) and the mentioned morphological responses under different dibutyldithiophosphate concentrations for drought-stressed edamame.

4.3. Materials and Methods

4.3.1 Plant material, application of dibutyldithiophosphate, germination and experimental setup

The plant material, study design, water and dibutyldithiophosphate treatments were described previously (chapter 3, section 3.3.1).

4.3.2. Determination of the morphological parameters

Growth and yield parameters (shoot height, number of pods, number of branches per plant, total number of seeds per plant and total seed mass per plant) were measured at termination of the experiment (reproductive stage, R8) (28 January 2021 and 11 July 2022). Although edamame is consumed at R6 stage, yield and growth parameters were only measured at maturity for seed production, because the study did not focus on the nutritional quality. Plant height was measured using a measuring tape. The numbers of pods, number of branches per plant, total seeds per plant were manually counted. Seed mass was weighed on a scale (AND Company limited, FX-3000i) in grams (g).

4.3.3. Data analysis

To determine the individual and combined effects of the concentrations and water treatments, data obtained on all morphology parameters analysed in this study were subjected to analysis of variance (ANOVA) using TIBCO Statistica version 13.5.0.17. In instances where the ANOVA indicated significant effects, the Tukey HSD test at $p = 0.05$ was used to separate the means. A correlation analysis between the physio-biochemical (chapter 3) and morphological traits was also conducted using TIBCO Statistica version 13.5.0.17, with significance measured at $p \leq 0.05$.

4.4. Results

Table 4.1 shows the effect of dibutyldithiophosphate on cultivar, water level and their interaction (analysis of variance, ANOVA) on the morphological traits of edamame at R8 stage. Since the effect of drought on the morphological traits of UVE14 and UVE17 were already studied (Moloi and van der Merwe, 2021; Hlahla et al., 2022), the focus will only be on (i) the effect of varying concentrations of dibutyldithiophosphate, (ii) the interaction between dibutyldithiophosphate, cultivar and water for the different morphological responses.

The effect of dibutyldithiophosphate was significant for plant height (PH, $p \leq 0.05$), branches per plant (BPP, $p \leq 0.001$), total seeds per plant (TSPP, $p \leq 0.05$) and pods per plant (PPP, $p \leq 0.001$) (Table 4.1). Cultivars responded in a significantly different manner under different water levels and H_2S (i.e., their interaction) for BPP ($p \leq 0.001$), TSPP (total seed per plant, $p \leq 0.001$), and PPP ($p \leq 0.001$) (Table 4.1).

Table 4.1. The effect of dibutyldithiophosphate (H_2S), cultivar and water (analysis of variance, ANOVA) on the morphological traits of edamame at R8 stage.

Variable	H_2S concentration (H)	Cultivar (C)	Water level (W)	Interaction (H×C×W)
PH	0,0499*	0,0005***	0,0186*	$0{,}2106^{ns}$
BPP	0,0046***	$0{,}4262^{ns}$	0,0000***	0,0009***
TSM	$0{,}0855^{ns}$	0,0041**	0,0000***	$0{,}4691^{ns}$
TSPP	0,0182*	0,0000***	0,0000***	0,0365*
PPP	0,0002***	0,0000***	0,0000***	0,0000***

*The numbers represent the p values from the ANOVA. Asterix represents * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. PH = Plant height, BPP = Branches per plant, TSM = Total seed mass, TSPP = Total seed mass per plant, PPP = Pods per plant.*

Table 4.2 shows the effect of different dibutyldithiophosphate concentrations on the morphological traits of edamame. The shoot height was positively affected by the application of dibutyldithiophosphate at all the concentrations, with 2 mg/mL inducing the highest increase in shoot height. Branches per plant were increased upon the application of H_2S even though the differences were not significant between the concentrations. The application of H_2S had no significant difference in the TSM. There was a significant increase in PPP and TSPP under 0.1 and 1 mg/mL dibutyldithiophosphate concentrations.

Table 4.2. The effect of different dibutyldithiophosphate concentrations on the morphological traits of edamame at R8 stage under 30% WHC.

Variable	0 mg/mL	0.1 mg/mL	1 mg/mL	2 mg/mL
PH	$63{,}70^{a}$	$69{,}30^{ab}$	$65{,}80^{ab}$	$72{,}40^{b}$
BPP	$6{,}50^{b}$	$6{,}88^{a}$	$6{,}94^{a}$	$6{,}81^{a}$
TSM	$2{,}15^{a}$	$1{,}91^{a}$	$1{,}82^{a}$	$2{,}07^{a}$
TSPP	$6{,}00^{a}$	$6{,}19^{ab}$	$6{,}43^{ab}$	$7{,}06^{b}$
PPP	$5{,}56^{a}$	$6{,}81^{b}$	$6{,}38^{b}$	$5{,}63^{a}$

Values represent means (n = 32). For each growth stage, different row superscript letters represent significant differences between the means at $p \leq 0.05$. PH = Plant height, BPP = Branches per plant, TSM = Total seed mass, TSPP = Total seed mass per plant, PPP = Pods per plant

Drought stress significantly decreased BPP in edamame by 16.7% and 4.0% in cultivar UVE17 and UVE14, respectively. When applied on drought-stressed UVE17, dibutyldithiophosphate only increased BPP at 1 mg/mL. For drought-stressed UVE14 however, all concentrations of of dibutyldithiophosphate significantly increased this parameter, with 0.1 and 2 mg/mL inducing the highest increases. Similarly under optimal watering, all concentrations increased BPP significantly for UVE14 [0.1 mg/mL (16%), 1 mg/mL (19.2%), and 2 mg/mL (12%)] but had no noticeable effects on UVE17 (Figure 4.1).

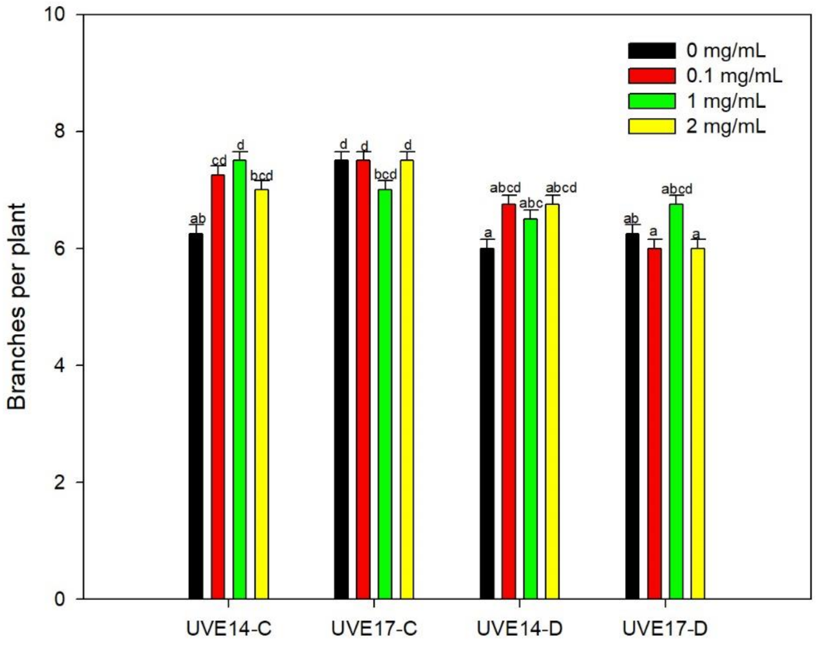


Figure 4.1. *Number of branches per plant for edamame cultivars (UVE14 and UVE17) treated with different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at the reproductive stage 8, R stage. Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments (p ≤ 0.05).*

Pod production per plant decreased significantly under the effects of drought stress [22.2% (UVE17) and 21.7% (UVE14)]. When the H_2S donor was applied on drought-stressed edamame, PPP for UVE17 significantly increased (4.8%) only at 0.1 mg/mL and decreased at higher concentrations. However, for drought-stressed UVE14, PPP was substantially increased under 0.1 (16.7%) and 1 mg/mL (11.1%) dibutyldithiophosphate treatment. Treatment of optimally watered UVE17 with dibutyldithiophosphate (0.1 and 1 mg/mL) increased high PPP (51.9%). For optimally watered UVE14, PPP was significantly increased at 0.1 (8.7%) and 2 mg/mL (26.1%) (Figure 4.2).

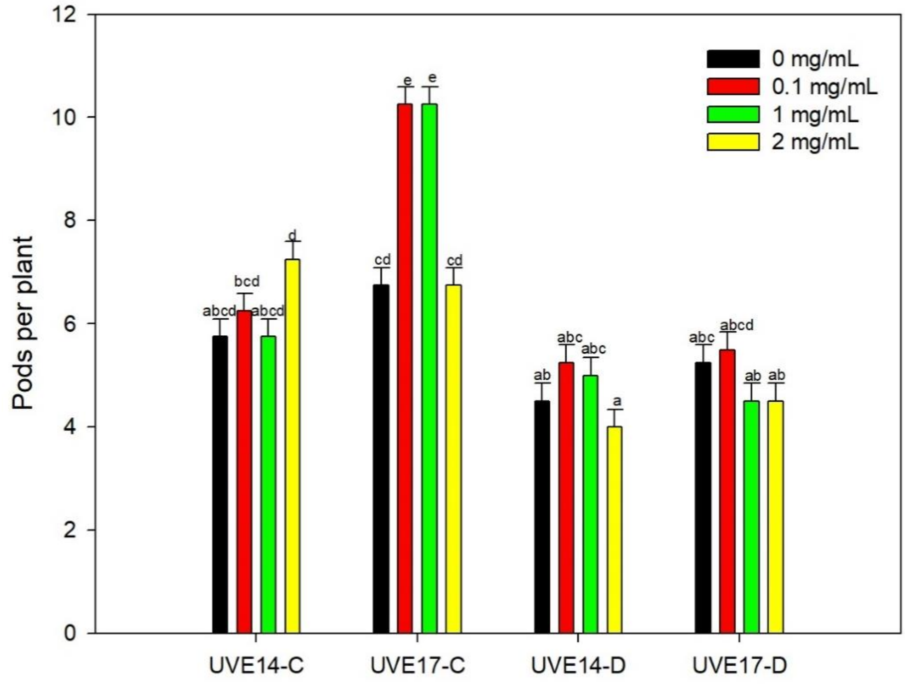


Figure 4.2. *Pods per plant of edamame cultivars (UVE14 and UVE17) treated different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at R8 stage. Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

The application of dibutyldithiophosphate at all concentrations increased TSPP in optimally watered UVE17 [20.8% (0.1 mg/mL), 4.2% (1 mg/mL), and 37.5% (2 mg/mL)], and drought-stressed UVE 17 at 2 mg/mL (11.1 %) concentration. For the UVE14 cultivar, application of dibutyldithiophosphate on optimally watered plants significantly increased TSPP (14.3% at 0.1 mg/mL). Under drought stress, dibutyldithiophosphate (1 and 2 mg/mL) increased TSPP (6.7 and 19.2%, respectively) in this cultivar (Figure 4.3).

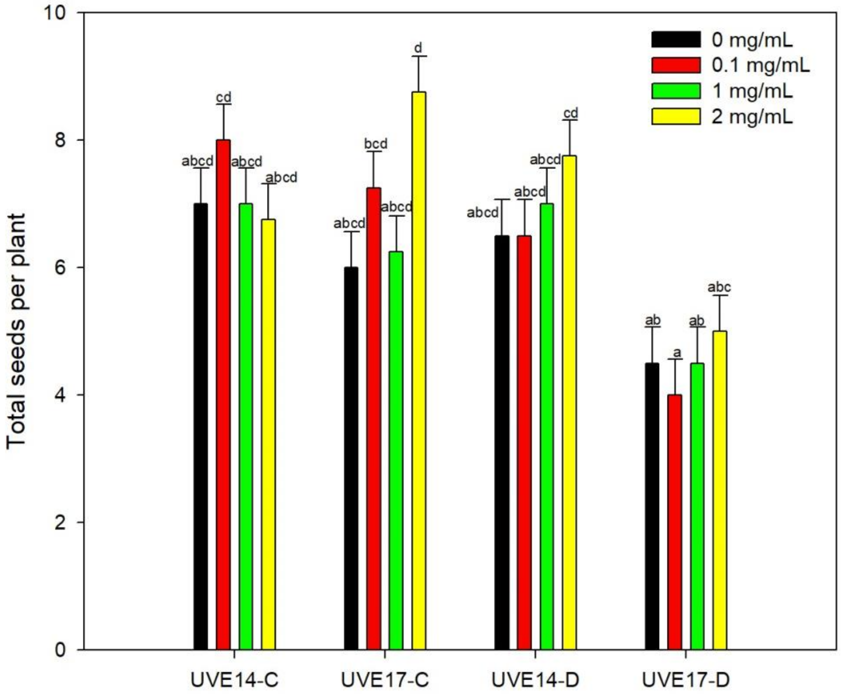


Figure 4.3. *Total seeds per plant of edamame cultivars (UVE14 and UVE17) treated different dibutyldithiophosphate concentrations under different water levels [100% (control, C) and 30% (drought, D) soil water holding capacity, WHC] at R8 stage. Values represent means ± SD, n = 3. Significant differences/similarities are represented by letters within various treatments ($p \leq 0.05$).*

The correlations between the physio-biochemical and morphological responses under different concentrations of dibutyldithiophosphate were only assessed for drought stress edamame to establish if dibutyldithiophosphate application increases the relationships between these responses. The physio-biochemical responses used for this assessment were extracted from chapter 3. Table 4.3 shows the correlations between the physio-biochemical [performance index on absorbance basis (PI_{abs}), total performance index (PI_{total}), photochemical reflective index (PRI), carotenoids reflective index (CRI), chlorophyll-b (Chl-b), total chlorophyll (Tot Chl), stomatal conductance (g_s), carotenoids (Car), hydrogen peroxide (H_2O_2), electrolyte leakage (EL), proline (Prol), total soluble sugars (TSS), guaiacol peroxidase (GPX)] and morphological responses of drought-stressed edamame under no application of dibutyldithiophosphate. The PPP correlated positively with PI_{total} (flowering) and MDA (pod-filling). Total seeds per plant positively correlated with GPX during flowering. The TSM correlated positively with PI_{abs} (pod-filling). The BPP positively correlated with EL and PRI (pod-filling). The PH correlated positively with TSS (flowering) and FRI (pod-filling).

Table 4.3. The relationships between the physio-biochemical and morphological parameters of drought-stressed edamame under no (0 mg/mL) dibutyldithiophosphate application.

	PPP	*TSPP*	*TSM*	*BPP*	*PH*
Fv/Fm Flow	0,7644	0,0464	0,3117	0,7516	**-0,9649***
PI_{abs} Flow	0,8962	0,2772	0,5227	0,5856	**-0,9538***
PI_{abs} Pod	0,9300	**-0,9750***	**0,9583***	-0,1728	-0,5193
g_s Pod	0,8154	0,1273	0,3750	0,6985	**-0,9970***
PI_{total}l Flow	**0,9578***	0,4348	0,6516	0,4418	-0,9205
EL Flow	0,8318	0,1463	0,3996	0,6907	**-0,9911***
EL Pod	-0,0191	-0,7398	-0,5340	**0,9616***	-0,5761
CRI Flow	-0,0857	0,6658	0,4633	**-0,9748***	0,7019
FRI Pod	-0,7095	0,0281	-0,2215	-0,7922	**0,9945***
PRI Pod	0,1459	-0,6248	-0,4106	**0,9845***	-0,7439
GPX Flow	-0,9208	**0,9630***	**-0,9593***	0,1578	0,4800
MDA Pod	**0,971***	0,5879	0,782	0,2526	-0,7739
H_2O_2 Pod	**-0,987***	-0,5466	-0,7444	-0,3247	0,8619
Chl-b Pod	0,6817	-0,0823	0,1859	0,8345	**-0,9706***
Car Flow	**-0,9663***	-0,4625	-0,6813	-0,4141	0,8927
Prol Pod	-0,8713	-0,2208	-0,4705	-0,6339	**0,974***
TSS Flow	-0,7927	-0,0906	-0,3398	-0,7231	**0,9991***

Asterix represents significant correlation coefficients at $p \leq 0.05$. Fv/Fm = ratio of variable fluorescence to maximum fluorescence, PI_{abs} = performance index on absorbance basis, PI_{total} = total performance index, PRI = photochemical reflective index, CRI = carotenoids reflective index, Chl-b = chlorophyll-b, Tot Chl = Total chlorophyll, g_s = stomatal conductance, Car = carotenoids, MDA = malondialdehyde, H_2O_2 = hydrogen peroxide, EL = electrolyte leakage, Prol = proline, TSS = total soluble sugars, GPX = guaiacol peroxidase, PH = plant height, BPP = Branches per plant, TSM = Total seed mass, TSPP = Total seed per plant, PPP = Pods per plant, Flow = flowering, Pod = pod-filling.

Table 4.4 represents the correlations between the physio-biochemical responses and morphological responses of drought-stressed edamame under 0.1 mg/mL dibutyldithiophosphate treatment. The PPP only correlated positively with PRI (pod-filling). The TSPP correlated positively with Chl-b, TSS, SOD (pod-filling), H_2O_2, MDA, and APX (flowering). There were significant correlations between TSM and Fv/Fm, PI_{abs}, Chl-b (pod-filling), APX (flowering). For BPP, there were positive correlations with MDA (flowering) and SOD (pod-filling). Significant positive correlations were also observed between PH with g_s and Tot Chl (flowering).

Table 4.4. The relationships between the physio-biochemical and morphological parameters of drought-stressed edamame under 0.1 mg/mL dibutyldithiophosphate application

	PPP	***TSPP***	***TSM***	***BPP***	***PH***
Fv/Fm Pod	-0,4606	0,9117	**0,9881***	0,7903	0,9082
PI_{abs} Pod	-0,3817	0,8912	**0,9809***	0,7368	0,8685
g_s Flow	-0,7707	0,7941	0,8520	0,8813	**0,9616***
PRI Pod	**0,9513***	-0,5427	-0,3739	-0,8241	-0,6718
APX Flow	-0,5216	**0,9967***	**0,9529***	0,8735	0,8853
APX Pod	0,4275	**-0,9829***	**-0,9782***	-0,8160	-0,8663
SOD Pod	-0,3712	**0,9715***	0,9430	0,7797	0,8097
MDA Flow	-0,7477	**0,9646***	0,8761	**0,9722***	0,9319
MDA Pod	0,7647	**-0,9653***	-0,9220	**-0,9815***	**-0,9819***
H_2O_2 Flow	-0,6043	**0,9880***	0,9086	0,9113	0,8869
Chl-b Pod	-0,5278	**0,9973***	**0,9533***	0,8770	0,8886
Tot Chl Flow	-0,6962	0,8787	0,9326	0,8934	**0,9791***
Prol Flow	0,5972	**-0,9849***	-0,9010	-0,9058	-0,8773
TSS Pod	-0,7292	**0,9655***	0,8709	**0,9630***	0,9181

Asterix represents significant correlation coefficients at p ≤ 0.05. Fv/Fm = ratio of variable fluorescence to maximum fluorescence, PI_{abs} =Performance index on absorbance basis, PRI = Photochemical reflective index, Chl-b = Chlorophyll-b, Tot Chl = Total chlorophyll, g_s = Stomatal conductance, MDA = Malondialdehyde, H_2O_2 = hydrogen peroxide, TSS = Total soluble sugars, APX = Ascorbate peroxidase, , SOD = Superoxide dismutase, PH = Plant height, BPP = Branches per plant, TSM = Total seed mass, TSPP = Total seed per plant, PPP = Pods per plant, Flow = flowering, Pod = pod-filling.

Table 4.5 represents the correlations between the physio-biochemical and morphological responses of drought-stressed edamame under 1 mg/mL dibutyldithiophosphate. The PPP significantly correlated with Chl-a, Chl-b, MDA (flowering), Chl-b, TSS and PRI (pod-filling). There were positive correlations for TSPP with CRI, Chl-a, Chl-b, MDA, APX (flowering), PRI, Chl-b and TSS (pod-filling). Positive correlations existed between TSM and CRI, MDA, Chl-b (flowering), Chl-b and TSS (pod-filling). There were no significant correlations between BPP and all the physio-biochemical responses. The PH correlated positively with Chl-b (flowering), Chl-b, PRI and TSS (pod-filling).

Table 4.5. The relationships between the physio-biochemical and morphological parameters of drought-stressed edamame under 1 mg/mL dibutyldithiophosphate application

	PPP	*TSPP*	*TSM*	*BPP*	*PH*
Fv/Fm Flow	**-0,9644***	-0,8830	-0,8238	0,6917	-0,8873
Fv/Fm Pod	**-0,9767***	-0,8850	-0,8256	0,7279	-0,9322
g_s Flow	**-0,9672***	**-1,0000***	**-0,9908***	0,3512	-0,9307
CRI Flow	0,8528	**0,9600***	**0,9858***	-0,0740	0,8407
PRI Flow	**-0,9902***	-0,9170	-0,8684	0,6781	**-0,9748***
PRI Pod	**0,9964***	**0,9750***	0,9468	-0,5254	**0,9790***
APX Flow	0,9444	**0,9590***	0,9358	-0,3945	0,8448
SOD Flow	-0,9499	**-0,9990***	**-0,9956***	0,2946	-0,9067
SOD Pod	**-0,9733***	**-0,9750***	**-0,9596***	0,4459	**-0,9847***
MDA Flow	**0,9518***	**0,9970***	**0,9953****	-0,3069	0,9351
Chl-a Flow	**0,9792***	**0,9750***	0,9474	-0,4658	0,9066
Chl-b Flow	**0,9924***	**0,9870***	**0,9641***	-0,4760	**0,9673***
Chl-b Pod	**0,9528***	**0,9610***	**0,9503***	-0,4179	**0,9832***
Tot Chl Flow	**-0,9927***	**-0,9870***	**-0,9638***	0,4772	**-0,9664***
Car Pod	-0,8660	**-0,9690***	**-0,9909***	0,0923	-0,8359
TSS Pod	**0,9743***	**0,9800***	**0,9656***	-0,4345	**0,9808***

Asterix represents significant correlation coefficients at $p \leq 0.05$. Fv/Fm = ratio of variable fluorescence to maximum fluorescence, PRI = Photochemical reflective index, CRI = Carotenoids reflective index, Chl-a = Chlorophyll-a, Chl-b = Chlorophyll-b, Tot Chl = Total chlorophyll, g_s = Stomatal conductance, Car = Carotenoids, MDA = Malondialdehyde, TSS = Total soluble sugars, APX = Ascorbate peroxidase, SOD = Superoxide dismutase, PH = Plant height, BPP = Branches per plant, TSM = Total seed mass, TSPP = Total seed per plant, PPP = Pods per Plant, Flow = flowering, Pod = pod-filling.

Table 4.6 represents the correlations between the physio-biochemical and morphological responses of drought-stressed edamame under 2 mg/mL dibutyldithiophosphate. The positive correlations for PPP were observed with Fv/Fm and carotenoids (flowering). For TSPP, there were more negative but no positive correlations with the physio-biochemical responses. The TSM correlated positively with APX (flowering), SOD and Tot Chl (pod-filling). There were positive correlations between BPP with Chl-b and TSS (flowering). The PH correlated positively with MDA, Chl-b and TSS (flowering).

Table 4.6. The relationships between the physio-biochemical and morphological parameters of drought-stressed edamame under 2 mg/mL dibutyldithiophosphate application.

	PPP	***TSPP***	***TSM***	***BPP***	***PH***
Fv/Fm Flow	**0,9982***	-0,7420	-0,8881	-0,5213	-0,6208
PI_{abs} Flow	0,6087	**-0,9670***	-0,8782	-0,8943	**-0,9961***
PI_{abs} Pod	0,7948	**-0,9970***	-0,9462	-0,9131	**-0,9684***
FRI Flow	0,8535	**-0,9720***	**-0,9915***	-0,8048	-0,9411
PRI Flow	0,6916	**-0,9890***	-0,9201	-0,9033	**-0,9973***
APX Flow	**-0,9708***	0,8780	**0,9816***	0,6574	0,7993
APX Pod	0,7596	-0,9940	-0,9140	-0,9442	**-0,9653***
SOD Pod	**-0,9760***	0,8680	**0,9770***	0,6437	0,7854
MDA Flow	-0,4858	0,9230	0,8015	0,8802	**0,9771***
Chl-b Flow	-0,4961	0,9360	0,7542	**0,9876***	**0,9597***
Tot Chl Flow	0,7573	**-1,0000***	-0,9329	-0,9269	**-0,9815***
Tot Chl Pod	-0,8793	**0,9700***	**0,9928***	0,8081	0,9284
Car Flow	**0,9910***	-0,8370	**-0,9503***	-0,6250	-0,7381
Prol Pod	**-0,9954***	0,8080	0,9273	0,6007	0,6993
TSS Flow	-0,4575	0,9230	0,7326	**0,9806**	**0,9556***

Asterix represents significant correlation coefficient at $p \leq 0.05$. Fv/Fm = ratio of variable fluorescence to maximum fluorescence, PI_{abs} = Performance index on absorbance basis, PRI = Photochemical reflective index, Chl-b = Chlorophyll-b, Tot Chl = Total chlorophyll, Car = Carotenoids, MDA = Malondialdehyde, Prol = Proline, TSS = Total soluble sugars, APX = Ascorbate peroxidase, SOD = Superoxide dismutase, RWC = Relative water content, PH = Plant height, BPP = Branches per plant, TSM = Total seed mass, TSPP = Total seed per plant, PPP = Pods per plant, Flow = flowering, Pod = pod-filling.

4.5 Discussion

The effects of drought stress on plants have been reported to include significant reductions in plant height, leaf area, stem diameter, and biomass in a variety of field crops (Khan et al., 2015; Hussain et al., 2018) including edamame (Shaheen et al., 2016; Chen et al., 2021; Moloi and van der Merwe, 2021). Studies have highlighted the potential of H_2S application in improving the drought tolerance in major crops by improving their morphological traits. The potential of H_2S application in improving the morphological traits of crops under different environmental stresses were reported (Kaya et al., 2020; Arif et al., 2021; Bahmanbiglo and Eshghi, 2021). In this study, the yield responses (BPP, TSPP and PPP) of edamame cultivars (UVE14 and UVE17) to dibutyldithiophosphate treatment differed according to the soil water level (Table 4.1). The yield responses of the edamame cultivars increased when the H_2S donor was applied.

Drought stress significantly decreased the number of BPP in cultivar UVE17. Application of dibutyldithiophosphate significantly alleviated drought stress effects on both cultivars. Treatment of H_2S donor at all concentrations significantly improved BPP in the optimally watered and drought-stressed UVE14. Treatment of optimally watered UVE17 with dibutyldithiophosphate had no significant effect on BPP, but under drought stress, a concentration of 1 mg/mL was significantly beneficial leading to increased BPP in this cultivar (Figure 4.1). This shows that for UVE17, water level and concentration of dibutyldithiophosphate matters the most. Based on this study, the physio-biochemical responses under drought stress, increased BPP is more likely to be associated with increased PRI (Figure 3.3), stomatal conductance (Figure 3.4), SOD (Figure 3.15), FRI (Figure 3.16), TSS (Figure 3.18) and proline (Figure 3.19) because these physio-biochemical parameters were increased at this concentration in drought-stressed UVE17. Positive correlations between BPP and TSS at 0.1 and 2 mg/mL dibutyldithiophosphate strongly suggests that this donor increases the antioxidative capacity and osmotic potential of a drought-stressed edamame (Christou et al., 2013; Shi et al., 2013; Naz et al., 2021), ultimately leading to increased BPP under drought stress. In addition, increased BPP under drought stress was linked to increased Chl-b accumulation under dibutyldithiophosphate treatment (2 mg/mL), showing that this treatment increased the light harvesting capacity of drought-stressed edamame. To further show that dibutyldithiophosphate was associated with increased BPP through increased physio-biochemical responses, when drought-stressed edamame was not treated with dibutyldithiophosphate, there was high electrolyte leakage (signifying membrane damage) and low CRI (signifying low antioxidative capacity) (Tables 4.3, 4.4 and 4.6). In support, elevation of physiological and biochemical parameters was associated with improved drought tolerance in many plants (Inamullah and Isoda, 2005; Singh and Reddy, 2014; Sarker and Oba, 2018; AL-Aloosy et al., 2019; Moloi and van der Merwe, 2021; Mwamlima et al., 2021).

Drought stress significantly decreased the PPP compared to the optimally watered plants. Under both water levels, application of 0.1 mg/mL dibutyldithiophosphate significantly increased PPP in both edamame cultivars irrespective of the water level (Figure 4.2). This could emanate from increased Chl-a (Figure 3.5), Chl-b (Figure 3.6), stomatal conductance (Figure 3.4), and FRI (Figure 3.16) because these parameters were also upregulated under this H_2S donor treatment (0.1 mg/mL). The 1 mg/mL concentration increased PPP in drought-stressed UVE14 and optimally watered UVE17 (Figure 4.2). Elevated parameters under these concentrations included PRI (Figure 3.3), stomatal conductance (Figure 3.4), Chl-a (Figure 3.5), Chl-b (Figure 3.6), SOD (Figure 3.15), FRI (Figure 3.16), CRI (Figure 3.17), TSS (Figure 3.18) and a decrease in EL (Figure 3.9), which may have caused the edamame to produce more pods per plant due to the effect of H_2S, indicating drought tolerance (Damour et al., 2010; Ciulca et al., 2017; Basal et al., 2020; Ghosh et al., 2021; Li et al., 2021; Naz et al., 2021). In support, increased strong positive correlations observed between Chl-a, Chl -b, PRI, TSS and carotenoid show that dibutyldithiophosphate increased the photosynthesis and antioxidative capacity, thereby increasing the PPP of drought-stressed edamame.

The application of dibutyldithiophosphate on UVE17 and UVE14 (optimally watered and drought-stressed) showed positive increases in TSPP. For this parameter, efficacy of dibutyldithiophosphate varied according to water levels. Application of dibutyldithiophosphate (1 and 2 mg/mL) effectively increased TSPP in drought-stressed UVE17 and UVE14 (Figure 4.3), which corresponded to increased APX (Figure 3.10), SOD (Figure 3.11) and TSS (Figure 3.18). No correlations between TSPP and the physio-biochemical parameters under no (0 mg/mL) H_2S donor application provides evidence that dibutyldithiophosphate improves drought tolerance through increased physio-biochemical responses in edamame. To prove this further, increasing the concentration of dibutyldithiophosphate (0.1 mg/mL) increased more correlations between TSPP and APX, SOD, TSS, as well as Chl-b, which corresponds to the above findings. Interestingly, most significantly positive correlations between TSPP and the physio-biochemical responses were induced under 1 mg/mL (Chl-a, Chl-b, PRI, TSS, CRI, APX, SOD) than 2 mg/mL (Tot Chl at pod-filling, and the rest negatively correlated to TSPP) (Tables 4.3 - 4.6). This suggests that while dibutyldithiophosphate is essential for upregulation of the physio-biochemical responses under drought stress, leading to increased TSPP, its concentration is important. These results further confirm that this H_2S donor increases the antioxidative and photosynthetic capacity of drought-stressed edamame.

4.6 Conclusion

This study highlighted the response of morphological traits of edamame cultivars to different water levels and the efficacy of using dibutyldithiophosphate to provide drought stress tolerance. The findings revealed that drought stress indeed had a significantly negative impact on the morphological parameters of the edamame cultivars. Treatment of drought-stressed edamame with dibutyldithiophosphate improved the yield responses (BPP, PPP and TSPP) through upregulation of the photosynthetic (Chl-a, Chl-b, Tot Chl, PRI) and antioxidative (APX, SOD, CRI, TSS) capacity. Application of dibutyldithiophosphate at 0.1 and 1 mg/mL on drought-stressed edamame increased more significantly positive relationships between the physio-biochemical responses and the morphological characteristics compared to 2 mg/mL, further showing the importance of H_2S donor concentration. The hypothesis that application of dibutyldithiophosphate improves the morphological responses of drought-stressed edamame, and that such H_2S donor application on drought-stressed edamame increases the relationships between the physio-biochemical and morphological responses is accepted.

Chapter 5: General Conclusion

5.1. General conclusion and future perspectives

Taken together, results from this study indicated a significant reduction in the physio-biochemical and morphological responses of the two edamame (UVE14 and UVE17) cultivars under drought stress. Efficacy of dibutyldithiophosphate on the physiological and biochemical responses differed based on the concentration of this H_2S donor, cultivar utilized, developmental stage, and water level. Dibutyldithiophosphate treatment was less efficient when applied on optimally watered edamame because fewer physio-biochemical responses were upregulated for both edamame cultivars. High EL under no dibutyldithiophosphate (0 mg/mL) treatment during pod-filling indicates that drought significantly increased oxidative damage, while reduced EL under dibutyldithiophosphate shows that this H_2S donor stimulates the antioxidative mechanisms in drought-stressed edamame.

Different dibutyldithiophosphate concentrations enhanced drought tolerance in edamame through induced osmotic adjustment (TSS and proline) leading to increased RWC, upregulation of the photosynthetic capacity (Chl-a, Chl-b, PRI and g_s), and activation of oxidative defence responses (FRI, CRI, TSS, proline, GPX, SOD and APX) at different reproductive stages. Treatment of drought-stressed edamame cultivars with 0.1-1 mg/mL dibutyldithiophosphate concentrations led to remarkable increases in the yield parameters (PPP, BPP, and TSPP). Furthermore, the study showed that increased physio-biochemical responses of drought-stressed edamame due to dibutyldithiophosphate treatment led to the improvement of the morphological characteristics. The results of this study are novel in that they indicate that application of dibutyldithiophosphate triggers the physio-biochemical defence responses leading to increased drought tolerance in edamame. To establish the effect of dibutyldithiophosphate as a drought stress reliever, it may be worth exploring different application methods (foliar and seed dressing), and to test efficacy of dibutyldithiophosphate on drought stress relief under field conditions.

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