PHOTOSYNTHETIC EFFICIENCY AND BIOCHEMICAL RESPONSES OF VEGETABLE-TYPE SOYBEAN CULTIVARS UNDER DROUGHT STRESS

By

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Declaration

“I, Jeremiah Mpumelelo Hlahla, declare that the dissertation that I herewith submit for the Master of Science degree in Botany at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education”

…….. ……..

Jeremiah Mpumelelo Hlahla Date

Dedicated to:

My sisters (Dainah and Sarah) and I for working hard, believing in God, in myself, and not giving up.

“People who say it can’t be done should not interrupt those who are doing it” (George Bernard Shaw)

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(*Psalm 56:4*)

Table of Contents

List of Tables

List of Figures

List of Equations

List of Abbreviations and SI Units

1. Abscisic acid (ABA)
2. Adenosine diphosphate (ADP)
3. Adenosine triphosphate (ATP)
4. Amylase (AMY)
5. Amyloglucosidase (AMG)
6. Analysis of variance (ANOVA)
7. Asian Vegetable Research and Development Centre(AVRDC)
8. Caffeic acid O-methyltransferase (COMT)
9. Carotenoids (CRDs)
10. Centimetre (cm)
11. Chlorophyll-a (Chl-a)
12. Chlorophyll-b (Chl-b)
13. Chlorophyllide a oxygenase (CAO)
14. Crystallinity Index (CrI)
15. Farmyard manure (FYM)
16. Fourier-transform infrared spectroscopy (FTIR)
17. Glucose oxidase/peroxidase (GOPOD)
18. Glucose-6-phosphate (G-6-P)
19. Glucuroarabinoxylans (GAX)
20. Gram per mole (g/mol)
21. Grams (g)
22. Grams per hectare (g/ha)
23. High-performance liquid chromatography (HPLC)
24. Invertase (INV)
25. Kilogram (Kg)
26. Kilograms per hectare (Kg/ha)
27. Light-harvesting complex (LHC)
28. Liquid chromatography–Mass spectrometry (LC-MS)
29. Microgram per millilitre (µg/ml)
30. Milligram per millilitre (mg/ml)
31. Millimole by square meters per second (Mmol/m^2.s)
32. Molar (M)
33. Non-structural carbohydrates (NSC)
34. Per centimetre (cm^{-1})

35. Percent (%)
36. Performance index on absorption basis (PI_{abs})
37. Phosphate solubilizing bacteria (PSB)
38. Photosystem I (PSI)
39. Photosystem II (PSII)
40. Plastocyanin (PC)
41. Plastoquinone (PQ)
42. Polyethylene glycol-6000 (PEG-6000)
43. Principal component analysis (PCA)
44. Reaction centres (RCs)
45. Reactive Oxygen species (ROS)
46. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)
47. Rribulose-1,5-bisphosphate carboxylase (Rubisco)
48. Secondary cell wall (SCW)
49. South Africa (SA)
50. Sub-Saharan Africa (SSA)
51. Sucrose phosphate synthase (SPS)
52. Sucrose synthase (SuSy)
53. Thin-layer Chromatography (TLC)
54. Total performance index on absorption basis (PI_{total})
55. Trehalose-6-phosphate synthase (TPS)
56. Uridine diphosphate glucose (UDP-glucose)
57. Variable fluorescence by maximum fluorescence (Fv/Fm)
58. Water holding capacity (WHC)
59. Water use efficiency (WUE)
60. X-Ray Diffraction (XRD)

Conference Contributions and Publications

Conference contributions

1. **Jeremiah M. Hlahla, Mpho S. Mafa, Rouxlene van der Merwe and Makoena J. Moloi.** The Photosynthetic efficiency and sugar metabolites provide essential information on the drought responses of six edamame (*Glycine max*. L. Merrill) cultivars. Presented in a Plant Science and Agriculture (PSA-2021) virtual conference, hosted by STEM international organization, Belgium (25 March 2021).
2. **Jeremiah M. Hlahla, Mpho S. Mafa, Rouxlene van der Merwe and Makoena J. Moloi**. The photosynthetic efficiency and carbohydrates responses of six edamame (*Glycine max*. L. Merrill) cultivars under drought stress. Presented virtually at a Botany and Plant biotechnology postgraduate symposium hosted by the University of Johannesburg (9 – 12 November 2021).
3. **Jeremiah M. Hlahla, Mpho S. Mafa, Rouxlene van der Merwe and Makoena J. Moloi.** The photosynthetic efficiency and carbohydrates responses of edamame cultivars under drought stress. Presented in the South African Association of Botanists (SAAB), hosted by the North-West University, Potchefstroom (17- 20 January 2022).

Publications

Hlahla JM, Mafa MS, Van der Merwe R, Alexander O, Duvenhage M, Kemp G & Moloi MJ (2022) The Photosynthetic Efficiency and Carbohydrates Responses of Six Edamame (*Glycine max*. L. Merrill) Cultivars under Drought Stress. Plants. 11(3), 394, https://doi.org/10.3390/plants11030394

Abstract

Climate change accelerates drought, which negatively impacts plant growth and development by altering the normal metabolic activities, resulting in poor crop yield. Soybean, (*Glycine max* L. Merrill), also known as edamame is native to South-East Asia and is popular in many parts of the world because of its high nutritional value, health, and economic benefits. The crop was recently introduced to South Africa because of its potential to improve food security. However, there is lack of information on its physiological and biochemical responses to drought.

Previous studies on drought-stressed edamame demonstrated that some cultivars, especially the better-adapted ones, had induced accumulation of the total soluble sugars. Since the production of the sugars emanates mainly from the process of photosynthesis, this study was conducted to elucidate how edamame responds to drought stress by focusing on the photosynthetic efficiency, quantification of the non-structural (i.e., soluble sugars) and structural carbohydrates (i.e., non-soluble sugars), including lignin and total phenols. Also, it was important to determine the relationships between the photosynthetic efficiency parameters and all the carbohydrate responses. The information from this study is very crucial as it can be used to identify the physiological and biochemical markers of drought tolerance, which is critical for screening edamame cultivars for drought tolerance. Such information can ultimately be employed in the selection of cultivars to be included in the edamame drought tolerance breeding programs.

The six edamame cultivars were germinated and grown in the greenhouse under controlled conditions. Drought stress was applied at the third trifoliate (V3) leaf stage by withholding irrigation to reach 30% WHC. The photosynthetic efficiency parameters such as chlorophyll fluorescence and stomatal conductance were determined using non-invasive methods. The sampling for all the physiological and biochemical parameters was done at flowering (R2) and pod filling (R4) stages. The photosynthesis pigments were quantified using a spectrophotometer. The non-structural carbohydrates (glucose, sucrose, trehalose, and starch) were quantified according to the instructions on the Megazyme kits. The structural carbohydrates were quantified using spectroscopy, Fourier-transform infrared (FTIR), and X-ray diffraction (XRD). The statistical analysis was done using Genstat software.

Drought stress increased the quantum efficiency (Fv/Fm) of AGS429 and UVE17 by 10% and 9% respectively at the pod filling stage respectively. The Performance index on absorption basis ($PI_{abs)}$ of AGS429 and UVE17 were also increased by 72% and 71% respectively at the pod filling stage. The overall photosynthetic performance of UVE17 was reduced by drought stress because this cultivar showed a total performance index (PI_{total}) decrease of about 28% at the pod filling stage. The cultivars, AGS354 and AGS429 had a 21% and 26% increase in chlorophyll b content at pod filling. The carotenoid content was significantly reduced in UVE17 at pod filling, showing that this cultivar had the least antioxidative defence against the reactive oxygen species (ROS) produced under drought stress. Carotenoids (CRDs) positively correlated with chlorophyll-a (Chl-a), $PI_{abs,}$ and PI_{total}, which could result in the protection of photosystems. Additionally, at the pod filling stage, UVE17 had a 59% reduction in the stomatal conductance, suggesting a poor rate of CO_2 uptake and assimilation under drought stress. According to the studied photosynthesis parameters, UV17 is a poor-performing cultivar under drought stress. Drought stress-induced the accumulation of trehalose, sucrose, and starch at pod filling, but decreased glucose content of most cultivars, except UVE7. Starch had a significant positive correlation with Ch-a and the total seed mass per plant, showing its importance in improving yield under drought stress in edamame. The cell wall studies showed that drought stress did not have any significant effect on the total phenolic content of all cultivars. However, the acid-soluble lignin was significantly increased in most cultivars except UVE14 and UVE17. The FTIR and XRD results suggest that the UVE14 cell wall was the most intact during drought stress followed by UVE8 and AGS429.

In conclusion, the CRDs, stomatal conductance, performance indexes of the photosystems, starch, hemicellulose, and lignin are essential physiological-biochemical mechanisms of drought tolerance in edamame. These parameters could be used in the edamame breeding programs for drought tolerance screening. AGS429 and UVE14 are better performing cultivars under severe drought stress and should be included in the drought tolerance breeding programs.

Keywords: Carbohydrates, carotenoids, chlorophyll fluorescence, drought stress, edamame, hemicellulose, lignin, photosynthetic efficiency, starch.

Chapter 1

General introduction

The large-seeded soybean, (*Glycine max* L. Merrill), also known as edamame or vegetable-type soybean, is an annual legume that originated in South-East Asia (Badole and Bodhankar 2013). It is a popular traditional food in Asia, especially in China and Japan. The soybean became popular in the United States and other parts of the world because of its high nutritional value, health, and economic benefits (Born 2006). Edamame is rich in proteins, micronutrients, minerals, important phytochemicals, essential amino acids, vitamins, isoflavones, and dietary fibre (Chai et al. 2015; Xu et al. 2016; Basal 2017; Lara et al. 2019). Therefore, edamame is a promising source of food now and in the future to meet the increasing food demands.

Edamame is a desirable crop to promote in Africa because the continent has the highest percentage of undernourished people in the world (Fry 2017). However, farmers do not understand the crop well because it was recently introduced in South Africa (SA) (Van der Merwe 2021). Also, there has not been much research done on this crop since its introduction in the country.

Anthropogenic climate change has a substantial impact in Sub-Saharan Africa because it accelerates droughts, resulting in a huge crop loss of up to 50% and a reduction in total crop revenues (up to 90%), which negatively affect small scale farming. Because of climate change, high temperatures, and low average rainfall, SA is expected to experience a higher degree of food production instability (Keatinge et al. 2011; Azzarri and Signorelli 2020). Therefore, the introduction of nutrition-rich crops such as edamame provides hope for meeting the nutritional demands of the people, especially since the SA population is estimated to grow even further by 6.22 million people by 2030 (Stats SA 2019).

South Africa experienced one of the longest droughts seasons in the recent past. This could impact negatively on edamame production because it is a water-demanding crop, with about 40% of its yield reduced by drought stress (Shaheen et al. 2016). Therefore, there is a need to investigate the mechanisms used by edamame cultivars for drought tolerance. Previous research demonstrated that some edamame cultivars

were able to adapt to changing environmental conditions in SA (Arathoon 2015). However, a lot is not clear regarding their biochemical and physiological responses to drought conditions.

The study aimed to investigate the effects of drought stress on the photosynthetic capacity and cell wall modifications of six edamame cultivars in order to establish the physio-biochemical mechanism responsible for drought tolerance in the edamame. The specific objectives of this study were to elucidate:

1. the effect of drought stress on the photosynthetic efficiency of six edamame cultivars;
2. the effect of drought stress on the cell wall modifications of six edamame cultivars;
3. the relationships between the photosynthetic efficiency parameters and the cell wall responses of edamame.

Chapter 2

The physiological and biochemical responses to drought stress

2.1 Edamame

2.1.1 Origin and consumption of edamame

Edamame (*Glycine max*, L. Merrill), is an important crop cultivated all over the world because of its high nutritional value (e.g. it is a good source of protein (Wang et al. 2016a). The cultivated soybean was domesticated in Eastern China many years ago (approximately 5000 years ago) from *Glycine soja* Sieb. and Zucc, its wild relative (Liu et al 2020). Edamame is termed “vegetable-type soybean” because its pods and seeds can be harvested, cooked, and eaten in its premature stage when the pods are still fresh and soft (Mahoussi et al. 2020). Different people from different countries use their local names to refer to this crop. In China, they call it “Mao dou”, in Korea they call it “Poot kong” and “Edamame”, pronounced “ay-dah-MAH-may” is the name used by the Japanese to mean “bean on branch” (Mahoussi et al. 2020). In A.D. 100, the term “Sheng dadou”, a Chinese term was used to refer to edamame as “raw/fresh large bean”, where the soybean obtained its “Large-seeded soybean” (Mahoussi et al. 2020).

The vegetable soybean was first recognized in 1275 when saint Nichiren Shonin, a Japanese Buddhist wrote to thank and appreciate the gift of edamame he received from a parishioner. In America, the edamame was introduced during the world wars by Charles C. Georgeson and Willian J. Morse, who searched for affordable protein sources (Mahoussi et al. 2020). Edamame production began to be improved in Taiwan in 1950, in 1971 it gained global popularity because of the paper published by the “Asian Vegetable Research and Development Center” (AVRDC-World vegetable centre) in Taiwan entitled “immature green soybeans” (Mahoussi et al. 2020). Their further research in 1985 looked at mechanical methods to harvest edamame. The centre became a leading edamame production and research organization and through their research, edamame production increased in Taiwan and most of the harvest was exported to the United States of America (USA) and Japan (Mahoussi et al. 2020). Due to the increasing world edamame is usually transported (at low temperatures or frozen) to many parts of the world for consumption (Sugimoto et al. 2010). The USA

alone consumes about 25,000 to 30,000 tons of edamame every year. Most of this is imported from countries such as China, Taiwan, and Japan (Xu et al. 2016). Therefore, edamame is a promising source of food and protein now and in the future to meet the increasing food demands.

Although edamame is a popular food crop in Asia (Born 2006) and the USA, it is still poorly understood in Africa (Mahoussi et al. 2020). In Japan, it is simply boiled with salt water (boiled for 5-7 minutes) and served as a snack or appetizer, whereas in other countries like China, the raw, shelled edible soybeans are mixed with meat or other vegetables and cooked (Zeipina et al. 2017). Over the years, edamame cultivation eventually resulted in producing cultivars with larger inflorescences, seeds, big stems, and other better traits such as producing upright plants (Liu et al. 2020). Edamame has recently become an attractive crop globally because of its economic importance. In addition to being a major source of proteins and oils, more than a quarter of the world's proteins for food and animal feed come from this crop. Research shows that the production of soybean globally increased by13-fold from the year 1961 to 2017 (Liu et al. 2020). The increase in crop yield over the years is due to breeding efforts (Liu et al. 2020).

2.1.2 Description of edamame

The two types of soybeans (oil soybean/grain soybean and edamame) belong to the Fabaceae family (pea family) (Zeipina et al. 2017). The oil soybean is the main source of vegetable oil and some products contain soy protein whereas edamame is harvested and eaten at its immature stage or harvested prematurely and processed into different products of soy (Saldivar et al. 2011). Edamame beans are large compared to the oil soybean (Zeipina et al. 2017). The crop is an annual, diploid, and self-pollinated legume. Phylogenetically, it is a close relative of numerous tropical edible legumes such as cowpea (*Vigna unguiculate* L.), peanut (*Arachis hypogaea*), and the well-known bean (*Phaseolus vulgaris* L.) (Mahoussi et al. 2020).

The maximum growth height of edamame can be estimated to be between 20 cm and 200 cm (Badole and Bodhankar 2013). The leaves are trifoliate, each leaf contains 3-4 leaflets with dimensions of 2-7 cm in breadth and length of 6-15 cm. The leaves usually drop before seed maturation. Edamame produces purple, white, or pink big flowers. The flowers are inconspicuous and self-fertile, they are formed in the leaf axil

(Badole and Bodhankar 2013). Pods are hairy and they form in groups of 3-5. The pods are approximately 3-8 cm in length, each pod usually carries 2-4 seeds. The seeds inside the pods have a diameter of 5-11 mm. There are different cultivars with the seed coat having different colours (Badole and Bodhankar 2013), usually black, brown, green, yellow, and mottled. The beans are harvested usually at the R6-R7 growth stages when they have filled 80-90% of the pod (Born 2006). The crop requires an 80 to 100 days growth period from planting to harvesting, depending on the cultivar (Kader et al. 2017).

2.1.3 Importance of edamame

The nutritional value of edamame is what made it popular over the years. Studies showed that edamame is rich in proteins (35-40%), oils (18-22%), it also contains macronutrients and some minerals (Basal 2017). A serving of 155 g of vegetable soybeans contains a total of 189 calories, about 8 g of fat, 3 g of sugar, 16 g of carbohydrates, and 17 g of proteins (Lara et al. 2019). In addition, edamame also provides essential amino acids (Lara et al. 2019), minerals, vitamins, isoflavones, and dietary fibre (Xu et al. 2016). According to Mahoussi et al. (2020), edamame has more vitamins A, C, K, and B compared to the grain-type soybean. Edamame also contains important elements such as iron, magnesium, zinc, calcium, phosphorus, potassium, copper, manganese, and sodium, which are higher in concentration compared to grain soybeans, green peas, and snap peas (Zeipina et al. 2017).

The pods of edamame are dark in colour when maturing. Their pod texture is soft and contains large, sweet-flavoured seeds with no bean flavour (Xu et al. 2016). The sweet taste results from glutamic acid, alanine, and sucrose in the seeds (Zeipina et al. 2017).

Edamame has many health benefits. Consumption of the crop is believed to decrease cholesterol levels because it has a high content of isoflavones or phytoestrogens (polyphenols), which also prevents symptoms of menopause in women (Mahoussi et al. 2020). It is also known to help in the prevention of heart diseases, increase bone density, lower the risks of prostate and mammary types of cancers (Xu et al. 2016). Furthermore, edamame is known to reduce the risk of high blood pressure (Mahoussi et al. 2020). Since this crop contains enough iron and zinc, it could benefit children and women of reproductive age by reducing deficiency symptoms associated with

such micronutrients, especially in Sub-Saharan African countries where most diets lack iron and zinc (Mahoussi et al. 2020).

Edamame has a short life cycle, therefore if conditions are favourable, it can grow at least four times a year, which could generate a lot of income for small-scale as well as large-scale farmers (Mahoussi et al. 2020). In Africa, edamame farming has the potential advantages of creating job opportunities for the youth, exportation, and local sales in markets, and improving the income of African farmers (Mahoussi et al. 2020). Since access to fertilizers could be a problem for resource poor-farmers, edamame biomass debris can improve soil nitrogen and fertility as it decomposes in the soil. Crop rotation and intercropping can improve soil fertility (Shurtleff and Aoyagi 2009).

2.1.4 Edamame in South Africa

Food insecurity is a huge challenge for many parts of the world, where the number of undernourished increased by 5% between 2014 and 2017 (Mahoussi et al. 2020). The highest percentage of undernourished people is found in Africa, with Sub-Saharan Africa (SSA) contributing the highest percentage (92.2%). For example, undernourishment affects about 71% of the Benin population and most of this percentage (51.3%) is children who suffer from deficiencies of zinc, iron, and vitamin A (Mahoussi et al. 2020). Statistics show that about 15% of children in South Africa are born with low weight, 5% of children are ‘wasted’ because they are underweight (The world bank 2005).

Africa needs urgent solutions to fight malnutrition problems by putting efforts into providing food that is rich in important nutrients such as carbohydrates, proteins, vitamins, and minerals. One obvious way to provide such nutrients is by promoting the consumption of vegetable and legume crops rich in these nutrients (Mahoussi et al. 2020) such as edamame.

Although the nutritional values, health benefits, and economic importance of edamame are clear, there is very little research on this crop in Africa. There is not much research done on the soybean in SA, except one done by Arathoon (2015), who reported on the germination rate, the influence of seeding rate, dryland conditions effects, and the effects of fertilizers on edamame productivity. More research is still needed in the country and Africa to fully understand the crop and produce more of it.

The world population might increase by 2.3 billion people by 2050, which is a third of the current population (Keatinge et al. 2011). Most of the growth is expected to be in developing countries, and this requires an increase in global food production to reach 70% to keep up with population increase (Keatinge et al. 2011). South Africa is one of the developing countries with a population of 58.78 million and the population is expected to reach 65 million by 2030 (Stats SA 2019). With such a high population, which is still expected to rise, food security will be a problem, and this requires an increase in food production. South Africa produces about one million tons of grain type soybean every year, but the edamame is less produced (Arathoon 2015). Edamame is currently found in some selected stores in SA, but there is no recognized commercial production of the soybean type. There is, however, an initiative called the “Ethekwini Edamame development program” which encourages farmers to cultivate edamame (Arathoon 2015). Since edamame is new in SA, farmers still need to be convinced that cultivating the crop will be of benefit to them. This can only be possible if more research is done on the crop.

The effects of climate change include increases in droughts and floods, with a great impact on agriculture, resulting in crop failure and loss in production (Keatinge et al. 2011). Due to climate change, large population size, and poverty (Azzarri and Signorelli 2020), SA is expected to experience a higher degree of food production instability (Keatinge et al. 2011). The southwestern parts of SA experience a Mediterranean climate to temperate in the interior plateau (South African weather services 2019), while the northeast part of the country experiences a subtropical climate. Because of high temperatures and low average rainfall experienced in the country, most parts of SA experience drought conditions. About 70% of arable land in the world is significantly threatened by drought (Iwuala et al. 2019). This causes a significant yield loss of important crops. The adaptability of different crops to drought differs greatly, it is, therefore, important to identify edamame cultivars that are well suited, and tolerant to drought stress in SA.

2.1.5 Growth conditions of edamame

Edamame requires well-drained silt-loamy soils with good organic matter content. The optimal temperatures range from 21- 31 °C during the day (Jane 2016) and about 10 –15 ºC at night (Kader et al. 2017) with rainfall that is well distributed throughout the

year (Jane 2016). Edamame is a crop that demands a lot of water for it to grow well and produce good quality and yield. The conservation of soil-water moisture and soil temperature is necessary for cultivating edamame (Kader et al. 2017). The seeds are sown at a soil depth of between 2.5 to 5.0 or use plantlets in the greenhouse then later transplant in the soil. The seedling emergence decreases with an increase in planting depth. Seed inoculation with *Bradyrhizobium japonicum*, a *Rhizobium* strain, is required if the vegetable soybean is sown for the first time in a given field (Zeipina et al. 2017).

Moist soils promote seed germination. The rates of base fertilizers used in Japan are 50 – 80 kg/ha nitrogen, 100 – 140 kg/ha potassium, and 70 – 100 kg/ha phosphorus. It is important to note that excess nitrogen levels can have an influence on the number of pods and lead to many empty pods or pods with only one seed (Zeipina et al. 2017). The growth and development of edamame can be negatively influenced by low phosphorus concentration. Planting density is a very important factor as it influences crop yield. Darker pods are yielded for lower density planting, keeping into consideration that the most suitable pod colour is bright-green because it is a crucial parameter in terms of visual aspects. Plant development is influenced by the distance between rows rather than the space between plants in a given row (Zeipina et al. 2017). A positive correlation in pod length, seed yield, and seed weight (100 seeds) was observed by Basavaraja et al. (2005). Seed quality is a very important factor to be taken care of during edamame farming (Zeipina et al. 2017). The properties of the different varieties can be influenced by growing conditions. Research done in India to determine a well-suited nutrient management system to produce seeds of higher quality showed that good quality seeds were produced when the treatment was 30:80:37.5 kg/ha NPK; 10 t/ha Farmyard manure (FYM), 250 g/ha inoculant of *Bradyrhizobium* and 250 g/ha phosphate solubilizing bacteria (PSB). Using these combinations, the average rate of germination was determined as 85.4% (Zeipina et al. 2017).

Harvesting of edamame is usually done by hands in small-scale farming. The pods are harvested when they have a bright green colour and when the seeds are close to filling the pod (R6 and R7 growth stage) (Zeipina et al. 2017). The pods should be stored in a cool environment after harvesting to maintain freshness. It is important to take note of a few things before harvesting fresh edamame seeds, e.g., the hilum of

the edamame should be grey or light brown with white pubescence pods. The pods should have well-developed seeds, at least 2 or 3, and the pods must be at least 1.4 cm in width and 5 cm long. The pods are organized into two levels. Level A pods contain 2 to 3 seeds and are 90% filled, green, and they have a good shape and have no blots or injury (Zeipina et al. 2017). Level B pods can be a bit light green in colour, and injury can be found on a few pods. The pods are neither mature nor unripe in both stages, they must be without diseases and damaged by insects. The biochemical quality of edamame can also be strongly influenced by many factors like climate, soil type, cultivar, growing conditions, and maturity of the plant (Zeipina et al. 2017).

2.1.6 Drought stress effect on edamame

Drought stress is one abiotic stress that causes a significant decrease in the yield of important crops (Fu et al. 2011). Plants have biochemical mechanisms that they use to manage water shortages in their environment, and the study of these mechanisms is an interesting emerging area of research. Many plants use a mechanism called the "drought escape" strategy (Sharma et al. 2020). Their open stomata help control gas exchange at rates that are higher for the high efficiency of photosynthesis. They also employ a water use efficiency mechanism (WUE), which helps them utilize water well under water shortage, and this helps plants to have a high rate of metabolism and maintain cell division and expansion. Plants such as succulents store water in their fleshy organs and have a decreased metabolic rate, and their photosynthetic efficiency is slowed down by closed stomata (Sharma et al. 2020).

Plants' response to water shortage is a significant aspect of research because it is not only limited to arid/ semiarid regions, but also in tropical rainforests and temperate forest regions. The availability of water has become an important factor due to anthropogenic activities, changes in the global climate (Sharma et al. 2020). Water available for plants depends on rainfall, the capacity of the soil to hold water, and water table depth. Water taken by plant roots is contained in the plant as cellular volume. About 97% of the water leaves the plant through transpiration, 2% of the remaining water is used for plant cell expansion and 1% is for plant metabolism (Sharma et al. 2020). When there is a water shortage in the plant, many morphological and physiological changes occur. In addition, the level of water stress in plants depends

on the growing stage of the leaf, where younger leaves have better adaptability and resistance to drought stress compared to older leaves (Sharma et al. 2020).

Despite all the environmental problems, different edamame cultivars showed a good ability to adapt to changes in climate (Arathoon 2015). The responses of the edamame cultivars towards drought need to be understood in the South African context.

2.2 Effect of drought on the photosynthetic efficiency and non-structural carbohydrates

The process of photosynthesis is the main provider of oxygen and makes life possible on earth (Farazdaghi 2011). Not only does photosynthesis provide oxygen for humans and animals, but it is also responsible for plant growth and productivity. The products of photosynthesis are responsible for crop biomass of more than 90% (Yamori 2020). Inland plants, photosynthesis occurs in green leaves, in special organelles contained in leaf mesophyll cells called chloroplasts. Changing environments such as drought affects the process of photosynthesis which significantly influence crop growth and yield (Du et al. 2020a; Yamori 2020).

Drought stress initiates a lot of processes at the physiological level in plants. This may include increased concentrations of abscisic acid (ABA) and reductions in the stomatal aperture (Banks 2018), leading to decreased photosynthesis (due to decreased CO_2) (Ghotbi-Ravandi et al. 2014). Drought stress may also lead to overproduction of the reactive oxygen species (ROS) in plants (Farooq et al. 2018). ROS has devastating effects on the chloroplasts by destabilizing protein complexes and damage the thylakoid membranes (Farooq et al. 2018). These result in reduced leaf surface area (Banks 2018) and reduced plant growth (plants with small leaves, leaf production declines, increase in abscission, and senescence of leaves) (Kebbas et al. 2015; Farooq et al. 2018). Drought stress effects on plants depend on the species, cultivar, age, and development status of the plants being studied (Moloi et al. 2016; Liang et al. 2019).

2.2.1 Photosynthetic pigments

Chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) are photosynthetic pigments found in photosynthetic plants. Both reaction centres (RCs), photosystem I (PSI), and photosystem II (PSII) in all light-harvesting complexes (LHCs) contain Chl-a (Pareek et al. 2017). Carotenoids (CRDs) are a class of natural pigments, C_{40} terpenes, and are important as they regulate photosynthesis by increasing the wavelength range over which light can be absorbed (Wong et al. 2019). Plant pigments must be available at good ratios for photosynthesis to be possible (Kattenborn et al. 2019). However, drought stress has negative effects in that it could damage the chloroplasts leading to reduced pigment content (Banks 2018). Drought stress was reported to significantly

reduce chlorophyll pigments content in *Arabidopsis thaliana* (Jung 2004) and barley (*Hordeum vulgare*) grown under severe (10%) drought stress and in leaves of peanut (*Arachis hypogaea*) (Ghotbi-Ravandi et al. 2014; Meher et al. 2018). Chlorophyll-a was more sensitive to drought stress than Chl-b in peanut plants (Meher et al. 2018). The photosynthetic capacity is directly linked to the chlorophyll content of photosynthetic plants (Banks 2018).

Drought stress is also associated with oxidative stress, which is a condition that involves the overproduction of ROS in plants. A positive correlation between oxidative damage and declined plant growth due to drought stress was reported in honeysuckle (*Lonicera japonica*) (He et al. 2021). This oxidative stress affects the synthesis and functioning of chlorophyll pigments, which ultimately affects the whole photosynthesis system (pigments, photosystems, electron transport system, and CO_2 reduction pathways (Meher et al. 2018).

Carotenoids (lutein, violaxanthin, β-carotene, zeaxanthin, antheraxanthin) accumulation is one of the mechanisms developed by plants in mitigating ROS congestion in the chloroplast (Wang et al. 2018a; Collini 2019). Carotenoids are associated with PSll reaction centres where they deactivate the triplet chlorophyll molecular (^{3}Chl*) and prevent it from generating ROS (Wang et al. 2018a). They also dissipate excess light energy; in this way, it decreases ROS congestion. Other functions of carotenoids include antenna proteins stabilization by Lutein (Wang et al. 2018b). Increased carotenoids accumulation in drought-stressed transgenic tobacco (*Nicotiana tabacum*), alfalfa (*Medicago sativa*), and sweet potato (*Ipomoea batatas*) plants were proven to be responsible for improved membrane stability and enhanced antioxidant activity (Wang et al. 2015; Cho et al. 2016; Park et al. 2016; Wang et al. 2018a). Other studies show that drought stress leads to a decline in β-carotene levels in *Arabidopsis thaliana*, barley, strawberry, and apple trees (Ghotbi-Ravandi et al. 2014).

2.2.2 Chlorophyll fluorescence of drought stressed edamame

Excess light energy and high temperatures are associated with drought stress. Each of these factors or their combination reduces the photosynthesis rate through photoinhibition and photo-oxidation (Wang et al. 2018a). Severe drought stress was also reported to decrease D1 protein (a protein associated with PSll repairs) content

in barley which led to decreased maximum quantum yield (Ghotbi-Ravandi et al. 2014). *In vivo*, the photosynthetic efficiency is measured using a fluorimeter, which calculates the minimum and maximum quantum yield and maximum fluorescence intensity of light-adapted leaves (ΔFv/Fm). The performance index on absorption basis (PI_{abs}) is important in understanding the energy fluxes in PSII and PSI (Çiçek et al. 2015). PI_{abs} and PI_{total} are hypersensitive to environmental changes than other fluorescence parameters such as Fv/Fm (Çiçek et al. 2015). This suggests that PI may be a good parameter for monitoring PSI in drought-stressed plants (Oukarroum et al. 2009).

Drought stress significantly reduced the maximum quantum yield (Fv/Fm) of PSII by 10%, PI_{abs} by 75%, and PI_{total} in apple (*Malus*) leaves (Mihaljević et al. 2021). Drought stress had little effect on Fv/Fm in grapes (*Vitis*) cultivars grown under severe drought stress, but PI_{abs} was significantly decreased. This further supports the fact that PI_{abs} is very sensitive to abiotic stresses, making it a good parameter to use when evaluating and monitoring plants under drought stress (Wang et al. 2012). Boguszewska-Mańkowska et al. (2018) found that Fv/Fm parameter could not distinguish drought-susceptible from tolerant potato cultivars because the quantum yield was not different, but PI_{abs} had significantly decreased in drought-sensitive cultivars. In barley cultivars, Fv/Fm was reduced under severe drought stress but did not affect PI_{abs} (Oukarroum et al. 2009). This may imply that drought stress has little or no effect on PSII. The performance index on an absorption basis (PI_{abs}) depend strongly on the flow of electrons through PSI and temporal blockage of electron flow on the PSI acceptor side. PSI content and the type of plant genotype also determine PI_{abs} (Oukarroum et al. 2009).

2.2.3 Stomatal conductance

The adenosine triphosphate (ATP) and the reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced in the light-dependent reactions are used in the Calvin cycle to reduce carbon dioxide (CO_2) which enters the leaves through stomatal pores in the leaves (McKown and Bergmann 2018). The number of stomata depends on plant communities, plant species, and plant individuals. This number of stomata on a leaf is termed “stomatal frequency” or “stomatal density” (McAinsh and Taylor 2016). Other factors that contribute to stomatal frequency are environmental, such as the

availability of water, temperature, light intensity, and the concentration of CO_2 in the environment. The morphology of the leaf, cell size as well as the plant's genetic background also influence stomatal frequency. In addition, stomatal frequency increases with the decrease in the size of guard cells (McAinsh and Taylor 2016).

When a plant undergoes water shortage, one of the responses is that it closes stomata to save water, which inhibits carbon influx, Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) carboxylation activity, and activities of other enzymes of the Calvin cycle, and consequently reduce photosynthesis (Farooq et al. 2018; Alves et al. 2021; He et al. 2021). Stomatal conductance significantly decreased in *Populus x euramericana "Neva"* under drought stress (Liang et al. 2019). This decrease in stomatal conductance contributed to the plant's significant decline in internal CO_2 concentration and photosynthesis rate. This stomatal closure also led to blockage and deoxidation of the electron transport system by decreasing NADPH demand in the Calvin cycle (Liang et al. 2019). Reduced CO_2 diffusion decreases the internal fixation of CO_2, which also causes photoinhibition resulting in chloroplast damage (Banks 2018).

In barley cultivars (Yousof and Morocco) grown under mild and severe drought stress, the stomatal conductance decreased dramatically in the Morocco cultivars, but less effect was observed in the Yousof cultivar (Ghotbi-Ravandi et al. 2014). Early stomatal closure in these barley cultivars also caused a significant decrease in CO_2 assimilation rate under severe stress. Ghotbi-Ravandi et al. (2014) concluded that CO_2 assimilation is strongly influenced by stomatal closure, however, leaves may lose too much water if their stomatal conductance is high. Drought stress decreased the stomatal conductance in kidney beans (*Phaseolus vulgaris*) followed by a decreased rate of photosynthesis (Miyashita et al. 2005). Although it did not reduce the stomatal conductance in *Saussurea involucrata* transgenic plants, it was significantly reduced in the wild type (Xia et al. 2021). As a result, the transgenic plants had higher internal CO_2 concentrations and a significantly high rate of transpiration compared to the wild type. These findings showed that the higher stomatal conductance of the transgenic plants made them vulnerable to drought stress due to the increased rate of transpiration (Xia et al. 2021).

2.2.4 Non-structural carbohydrates

Non-structural carbohydrates (NSC) or soluble sugars accumulate in drought-stressed plants, in leaves experiencing osmotic stress. This happens so that the osmotic balance may be achieved again (Lu et al. 2019). Extreme temperatures, salt stress, cold, and oxidative stress induce soluble sugars accumulation in plant leaves (LU et al. 2019). The concentration of soluble sugars increased with a decrease in starch in maize (*Zea mays*) exposed to drought stress. In rice, drought-tolerant cultivars produced more soluble sugars (Lu et al. 2019). Soluble sugars accumulated in some vegetable soybean (*Glycine max*) cultivars under drought stress (Moloi and Merwe 2021). The accumulation of the sugars may be responsible for regulating the osmotic balance, oxidative stress prevention, and stabilization of plasma membranes and biomolecules to improve drought tolerance in soybeans (Moloi and Van der Merwe 2021).

2.2.4.1 Sucrose

The rate of photosynthesis is significantly reduced by drought stress in plants, leading to disruptions in the metabolism of carbohydrates in leaves. This results in little amounts of photo-assimilates available for export to sink tissues, and poor plant productivity (Liu et al. 2004). One of the important major products of the Calvin cycle of photosynthesis is sucrose. Sucrose is synthesized by sucrose phosphate synthase (SPS), from UDP-glucose and D-fructose 6-phosphate (Everard and Loescher 2016). It has many functions in the plant cells including transport, growth, and development, signal transduction, storage and it also helps to respond to different environmental stresses (Cumino et al. 2002). The sucrose content in the leaves depends on factors such as the photosynthesis rate, photosynthetic carbon partitioning between sucrose and starch, the rate at which sucrose is exported from source to sink organs, and the hydrolysis rate of sucrose at sink organs. If drought stress affects any of these factors, it will alter leaf sucrose content (Liu et al. 2004).

Sucrose, fructose, glucose, UDP-glucose, and sorbitol are known as vital osmoregulators because they maintain the leaf turgor pressure and osmotic balance during water shortage (Yang et al. 2019). Water deficit increases the activity of SPS leading to increased synthesis of sucrose. The synthesis of sucrose in *Arabidopsis thaliana* was inhibited by declining SPS under normal water conditions (Du et al.

2020a). In rapeseed (*Brassica napus*), and soybean (*Glycine max*), the sucrose content in leaves increased due to drought stress (La et al. 2019; Du et al. 2020a) but significantly decreased in grapevine (*Vitis vinifera*) (Morabito et al. 2021). Sucrose is transported from source to sink tissues where it is hydrolyzed by invertase (INV) and sucrose synthase (SuSy) to glucose and fructose. Hexoses (glucose/fructose) increase in plants exposed to drought stress because of SuSy and INV activities as they cleave sucrose (Liu et al. 2004). However, drought stress inhibited the activity of SuSy and INV in the reproductive organs of maize and rice (*Oryza sativa*) and resulted in high sucrose content (Liu et al. 2004).

2.2.4.2 Starch and glucose

Starch is synthesized from simple hexoses such as units of D-glucose. Starch has two forms, namely amylose, which is a simple linear sugar molecule or branched amylopectin that is composed of units of D-glucose joined together by α-1-4- or β-1-6 linkages (Prathap and Tyagi 2020). Starch is a polysaccharide and an important compound that stores carbon and energy for later use when needed by the plant (Du et al. 2020b). Starch is involved in drought stress response as a major storage carbohydrate. Drought tolerance in plants is indicated by an increase in starch degradation (Abdelhakim et al. 2021). Starch is synthesized by ADP-Glucose pyrophosphorylase, starch synthase, and branching and debranching enzymes (Du et al. 2020b). Starch breakdown increases in plant leaves under osmotic imbalance, this ensures soluble sugars availability to respond to drought stress (Du et al. 2020b; Ferreira et al. 2021). Starch content was significantly reduced by drought stress in rice and soybean under drought stress (Liu et al. 2004; Du et al. 2020b; Prathap and Tyagi 2020). The combined stress of heat and drought also significantly decreased starch content in wheat (Abdelhakim et al. 2021).

Glucose is an important building block of many carbohydrates in plants (Prathap and Tyagi 2020). The increase in D-glucose content due to starch degradation under drought stress supports the fact that glucose is one of the important osmoregulators of sugar (Yang et al. 2019). In soybean, a decrease in starch content due to degradation and sucrose conversion to glucose and fructose enhanced the regulation of osmotic balance under drought stress (Liu et al. 2004; Du et al. 2020b). In the grapevine, glucose content was not affected by drought stress, the concentrations

even increased as the plants recovered from drought (Morabito et al. 2021). In another study, glucose was reduced by drought stress in some wheat cultivars grown under ambient carbon dioxide conditions (Abdelhakim et al. 2021). The increase in glucose concentration may also help in regulating photosynthesis in a feedback mechanism. Other functions of glucose include the regulation of the ovary and the development of seeds (Liu et al. 2004).

2.2.4.3 Trehalose

Trehalose is a disaccharide of two glucose rings joined together by a glycosidic bond. Trehalose and its phosphorylated form, trehalose-6-phosphate (T6P) are synthesized by trehalose-6-phosphate synthase (TPS) (Morabito et al. 2021). Trehalose was first found to be protecting proteins and intracellular membranes from degradation in mosses, fungi, and ferns, and it was found to have an osmoregulation role in yeast (Morabito et al. 2021). The same observations were made in higher plants but limited in plants that overexpress nuclear TPS (Di Gioacchino et al. 2021; Morabito et al. 2021). Trehalose was also found in angiosperms at low concentrations. An increase in trehalose concentration in monocots and dicots improves tolerance to osmotic stress. Trehalose also plays important role in carbon allocation (Morabito et al. 2021). Trehalose accumulated in *desi* chickpea (*Cicer arietinum*) under drought stress and enhanced the plant's intake of carbon and maintenance of turgor pressure (Farooq et al. 2018).

Many carbohydrates accumulate in drought-stressed plants, but trehalose is the most effective disaccharide in protein and cell membrane protection against dehydration and helps plants restore their metabolic functions and survive drought stress (Di Gioacchino et al. 2021). Trehalose also acts as a free radical scavenger of ROS by binding phosphate and the protein's hydroxyl group with the polar region of intracellular membranes (Farooq et al. 2018). The exogenous application of trehalose increased the content of chlorophyll, biomass and increased the lengths of shoots under normal conditions in soybean (Asaf et al. 2017), it also improved the resistance of rice, and alfalfa roots in salt stress (Domínguez-Ferreras et al. 2009; Nounjan and Theerakulpisut 2012). Reversible water absorption is an important feature possessed by trehalose, this feature assists in protecting biomolecules from damages caused by

dehydration. The increase in accumulation of soluble carbohydrates correlated with trehalose content in pigeon pea (*Cajanus cajan*) under salt stress (Asaf et al. 2017).

2.3 Plant cell walls

The sucrose produced during photosynthesis is converted to many soluble polysaccharides (Yang et al. 2019; Du et al. 2020a). One of the precursor molecules is uridine diphosphate glucose (UDP-Glc) that undergoes a polymerization reaction catalyzed by glycosyltransferases, which make cell wall components (glucans) (Barnes and Anderson 2018). The cell wall plays a role in structural support and protection of cell components, it filters substances going in or leaving the cell. The most important function of a cell wall is that it acts as a pressure vessel to avoid cells from over-expanding when water enters them. The cell walls composition differs between species, developmental stage of the plant, and tissues of the plant. (Barnes and Anderson 2018) The cell wall of terrestrial plants is made up of polysaccharides, which include the major components cellulose (25-35%) and hemicellulose (20-35%) followed by trace amounts of pectin (O'Neill and York 2018). The non-polysaccharides content of the cell wall consists of lignin as a major component (10-30%) followed by lower amounts of proteins and ash contents (Barnes and Anderson 2018).

2.3.1 Functional properties of the cell wall during drought

The expression of carbohydrate-modulated genes in plant cells is strongly influenced by drought stress (Yang et al. 2019). One of the most important first lines of defence induced is the synthesis of cell wall sugars and phenols. The plant cell walls play very important roles to deal with water shortages in their environments (Van der Weijde et al. 2017). One of the important physiological roles played by plant cell walls during drought stress is the elasticity of the cell wall, which is a very important adaptation to drought and salinity stresses (Al-Yasi et al. 2020). The cell walls could either be tightened or loosened in response to drought stress. When there is a water shortage, elastic adjustments are happening that lead to the shrinking of plant cells, thus reducing cell volumes. The reduction of cell volume help plants save water and avoid water-potential decline (Al-Yasi et al. 2020).

Water shortage affects plant growth by preventing cell division and cell enlargement due to the stiffening of cell walls (Van der Weijde et al. 2017; Ashwin et al. 2021). Understanding plant responses to different abiotic stresses is often a complex

process. Plants responding to drought stress show increased levels of proteins related to cell walls synthesis (Ashwin et al. 2021). Such related proteins are the expansins, xyloglucan endo-β-transglycosylase/ hydrolase, and endo-1,4-β-D-glucanase (Cosgrove 2000; Cosgrove 2016). These proteins play a role in controlling cell wall extension, which helps cells enlarge and expand. Cell expansion under drought stress is made possible by expansin protein, which was first observed in hypocotyls of cucumber (*Cucumis sativus*). Expansins break the non-covalent bonds linking fibrils of cellulose and extracellular polymers and lead to an extension of the cell wall in a manner that depends on the pH called “acid growth”. Expansins also play vital roles in seed germination, development, growth of leaves and roots, the elongation of stems, regulation of opening and closing of stomata, tolerance to stress, reproduction, and ripening of fruits (Ashwin et al. 2021).

2.3.2 Role of cellulose during drought stress

Cellulose is produced by the cellulose synthase (CesA) protein complexes of the type I GT2 family in the cytoplasm plasmalemma, unlike the matrix polymers (hemicellulose and pectin) which are synthesized in the Golgi bodies (Kumar et al. 2017; Verbančič et al. 2018; Zeng et al. 2020). Cellulose is a biopolymer, made up of β-(1,4)-glucans which are highly ordered and crystalline, unbranched, and parallel, with alternating amorphous regions that are less ordered (Mafa et al. 2021). The chains are fused into microfibrils through intermolecular hydrogen bonds, and van der Waals forces, and it is the major component of plant cell walls as it accounts for about 25-35% of dry plant biomass (Mafa et al. 2020a; Mafa et al. 2021; Van Dyk and Pletschke 2012). The cellulose microfibrils give tensile strength to the wall and determine the direction of cell enlargement (Verbančič et al. 2018). In addition, the microfibrils serve as scaffolds for other polymers. The microfibrils of cellulose are connected at mechanical hotspots by hemicelluloses and packed within a matrix of pectin (Cosgrove 2016).

Drought stress affects cellulose synthesis and deposition. Ul-Allah et al. (2021) demonstrated that fibre thickness in cotton plants (*Gossypium*) was affected by cellulose synthesis and accumulation under drought stress. Drought stress may affect the metabolism of carbohydrates by lowering UDP-glucose and sucrose content, this affects secondary cell wall deposition because callose may be synthesized instead of cellulose, resulting in weak tissues (Ul-Allah et al. 2021). A group of genes were shown

to be responsible for the synthesis of cellulose synthase (CesA), if these genes were defective or mutated due to water shortage cellulose content was reduced, resulting in thinner cell walls and this causes the tracheid of the xylem to collapse (Chen et al. 2005; Wang et al. 2016b). Tracheid walls collapsed severely in pine (*Pinus*) needles under severe drought stress. Such collapsing of walls is used as a defence mechanism against drought (Chen et al. 2005). Plasmolysis (the separation of plasma membrane from cell wall) is common in drought-stressed plants and may occur under severe osmotic stress (Wang et al. 2016b). Plasmolysis may interrupt the synthesis of cellulose (Wang et al. 2016b). However, there is not much proof linking drought stress and cellulose modification (Chen et al. 2005).

2.3.3 Hemicellulose involvement in drought tolerance

Hemicelluloses are a group of heterogeneous polymers produced in the Golgi bodies and delivered into the apoplast by secretory vesicles (Barnes and Anderson, 2018). About 15-30% of plant biomass is made up of hemicellulose (Gírio et al. 2010). Hemicelluloses of the monocots are mainly composed of pentose sugars (e.g., β-D-Xylose) that form backbone-chain, while the dicots consist of hexose sugars (α-D-galactose, β-D-mannose, β-D-glucose) that form backbone-chain. The hemicellulose side chains are diverse, and they include sugars such as α-L-fucose, α-L-arabinose α-L-rhamnose, and uronic acids (α-D-4-O-methylgalacturonic acid, α-D-glucuronic acid, α-D-galacturonic acid) (Girio et al. 2010; Malgas et al. 2016; Malgas et al. 2019).

The hydroxyl groups of C2 or C3 of the pentose or hexose sugars can be substituted with one of the side chains sugars, uronic acids, or acetyl groups (Girio et al. 2010; Malgas et al. 2016). For instance, glucuroarabinoxylans (GAXs) are a type of complex hemicelluloses in monocots (Malgas 2019; Mafa et al. 2020b). GAXs backbone chain is composed of xylose moiety linked by β-1,4-glycosidic bonds and substituted with α-arabinose, α-glucuronic acid, or α-methyl-glucuronic acid at position C2 or C3, respectively (Mafa et al. 2020b). Mannans are the most common hemicelluloses for dicots (Girio et al. 2010). Malgas et al. (2015) showed that galactoglucomannan is one of the most complex hemicelluloses for dicots because it has a backbone chain made of mannan and glucose residue in a 3:1 ratio, and both mannan and glucose residue can be substituted with galactose residue. The biological function of hemicelluloses is

to form a network that joins cellulose microcrystalline fibre together, and form crosslinks with lignin to strengthen the cell wall (Atalla 2005; Mnich et al. 2020).

Several studies investigated the hemicellulose changes in plants exposed to drought stress and found that the effect of drought was variable (Schädel al. 2010; Jiang et al. 2012). White spruce (*Picea glauca*) treated with reduced water supply decreased the lignin content and increased the hemicellulose content of the cell wall (Schädel et al. 2010). In contrast, drought affected the leaf and total plant tissue in switchgrass (*Panicum virgatum*) by reducing their hemicellulose content, while lignin in the stem and the total plant tissue increased (Jian et al. 2012). In drought-stressed soybean plants, pectin, cellulose, and lignin were significantly reduced by drought stress in either shoots or roots, whereas hemicellulose significantly increased in either tissue (Al-Hakim 2006). These observations support Van der Weijde et al. (2017) hypothesis that levels of hemicellulose content in plants may increase or decrease depending on the type of crop and plant tissue.

2.3.4 Lignin and cell wall-associated phenolics

Lignin ($C_{31}H_{34}O_{11}$) is synthesized through the polymerization process of monolignols, coniferyl, *P*-coumeryl, and sinapyl alcohol (Begović et al. 2018; Mnich et al. 2020). Peroxidase and/or laccase enzymes oxidize the monolignols followed by a radical coupling of the monolignols to produce three phenylpropane units, guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) alcohols (Begović et al. 2018; Kazzaz and Fatehi 2020). The coupling of these subunits forms lignin macromolecule, and as a result, lignin is a three-dimensional irregular complex macromolecule (Kazzaz and Fatehi 2020). Some studies suggested that lignin could be the main source of phenolic compounds in plants. Due to the interlinkage of lignin to cellulose and hemicellulose, the isolation of lignin from plant cell walls proves to be highly complicated (Kazzaz and Fatehi 2020).

Lignin contents differ in plants, annual plants (herbs) contain less than 15% lignin per plant biomass, in wood, the lignin content ranges between 15 to 40%. The lignin composition also differs with the source of plant biomass, for example, there are about 80-90% G units in softwood, 50-70% S, and 25-50% G units in hardwood, grasses contain 25-50% S, 10-25% H, and 25-50% G units (Kazzaz and Fatehi 2020; Mnich et al. 2020). The synthesis of lignin is important as it is a component of the secondary

cell wall and helps to strengthen water-conducting tissues and make them more hydrophobic (Silva et al. 2019), which is important in defence under drought stress (Huisman et al. 1998; Le Gall et al. 2015).

One of the important enzymes involved in lignin biosynthesis is caffeic acid O-methyltransferase (COMT). It accumulated in maize leaves under drought stress (Vincent et al. 2005; Xie et al. 2018). However, another study done on maize leaves demonstrated decreased level of lignin biosynthesis enzymes under water shortage (Frei 2013). Lignin and phenols accumulated in the roots of drought-stressed maize plants. This increase in lignin was preceded by increased levels of lignin biosynthesis enzymes in the root zone of elongation, which ensures high inflexibility and causes growth to cease in the root (Moore et al. 2008). Drought stress affects lignin content differently in different plant genotypes and tissues. In transgenic tobacco plants (*Nicotiana tabacum*), lignin was associated with crop resistance to drought. In contrast, studies done on alfalfa (*Medicago sativa*) showed that mutated crops had lower lignin content under drought stress and as a result, they were more drought-tolerant compared to their wild type (Frei 2013).

Phenolic compounds are involved in important plant physiological and morphological processes such as growth, development, plant resistance to biotic and abiotic stresses (Mechri et al. 2020). Some of the important phenolic compounds associated with cell walls include lignans, phenolic alcohols, phenolic acids, and derivatives of hydroxycinnamic acids (Mechri et al. 2020; Mnich et al. 2020). Monomers of phenols and lignin are cross-linked to cell wall polysaccharides via oxidases, laccases, and peroxidases. This cross-linking makes the cell wall structure tight (Frei 2013, Mnich et al. 2020). Phenols act as agents for protecting the plants against the effects of UV-B radiation, which damages the photosynthesis system and some macromolecules such as proteins and DNA (Mechri et al. 2020). Mechri et al. (2020) found that drought stress significantly increased total phenolic content in the leaves of tolerant olive trees (*Olea europaea*). Depending on the type of crop and tissue, the total phenolic content might differ. Some studies demonstrated phenols accumulation in some plants under drought stress, but some studies showed that drought stress decreased phenolic content as in *Salix viminalis*, and some data showed that drought stress did not affect total phenolic content (Köhler et al. 2020).

2.4 Conclusions

Drought stress is one of the most damaging abiotic environmental stress affecting crop production globally. As a high water-demanding crop, edamame crop yield is significantly reduced by drought stress. The physiological, biochemical, and morphological features of edamame can be affected by drought stress. In this study, six edamame- cultivars are exposed to drought stress and investigated for induced physiological/ biochemical responses. The changes in plant pigments (chlorophyll-a, and accessory pigments), stomatal conductance, non-structural sugars as well as structural cell wall carbohydrates, phenols, and phenotypical changes that occur to assist the plant in dealing with drought can be used as a measure of tolerance. The successful cultivars may be used in the plant breeding program to produce more drought-resistant cultivars, which will be very useful for the present and future agriculture, plant biotechnology, and the economy of the country.

Chapter 3

The effect of drought stress on the photosynthetic efficiency components of six edamame cultivars

3.1 Abstract

Drought stress can reduce the photosynthetic efficiency, which might affect the production and partitioning of non-structural carbohydrates (NSC). Edamame is an important crop with high nutritional and health benefits. This study was conducted to investigate the effects of drought stress on the photosynthetic capacity and NSC of six edamame cultivars. The results will be used to establish the mechanisms of drought tolerance in edamame. This study will further recommend cultivars (based on their physiological/ biochemical mechanisms of drought tolerance) for the drought tolerance breeding program. Edamame cultivars were grown under controlled conditions in the greenhouse. Severe drought stress was applied by withholding irrigation to 30% soil water holding capacity (WHC) from the third trifoliate (V3) leaf stage throughout the growth cycle. The photosynthesis efficiency parameters such as chlorophyll-a fluorescence and stomatal conductance were determined using non-invasive methods. The photosynthesis pigments were quantified spectrophotometrically. The non-structural carbohydrates were quantified using Megazyme kits. The thin-layer chromatography (TLC) and starch-iodine complexation were performed for qualitative studies of NSC. Drought stress significantly increased the Fv/Fm and PI_{abs} of AGS429 and UVE17 at the pod filling stage but reduced the PI_{total} of UVE17. Chlorophyll-a was significantly reduced in AGS429 and UVE17. UVE17 had a significant decline in the chlorophyll-b content of UVE17 at flowering. At pod filling, AGS429 had increased Chl-b. The carotenoid content was significantly reduced in UVE17 at pod filling. Drought-stressed UVE17 further showed a substantial reduction in the stomatal conductance at pod filling. Drought stress significantly increased trehalose, sucrose, and starch but decreased glucose content at pod filling. Based on the results, carotenoids, PI_{total}, stomatal conductance as well as starch are the most significant physiological/ biochemical parameters for drought tolerance screening edamame. AGS429 and UVE14 have the most significant drought tolerance mechanisms induced and their inclusion in the drought tolerance breeding program is recommended.

Keywords: Carbohydrates, edamame, photosynthesis, pigments, stomatal conductance, water deficiency.

3.2 Introduction

Edamame (*Glycine max* L.) is a water-demanding crop with about 40% of its yield reduced by drought stress. Edamame is currently cultivated in many parts of the world because of its high nutritional value and health benefits (Shaheen et al. 2016; Moloi and Van der Merwe 2021). Drought stress is multidimensional environmental stress that affects the growth and development of plants by altering their normal metabolic activities and resulting in reduced crop yield (Bhardwaj and Kapoor 2021). Drought has a negative impact in Sub-Saharan Africa, resulting in huge crop losses (Keatinge et al. 2011). Plants respond to abiotic stresses like drought by modifying their morphological structures, biochemical processes, and physiological features. Moloi and Van der Merwe (2021) showed that different edamame genotypes displayed different biochemical responses to drought stress at different growth stages (flowering and pod filling stages).

Chlorophyll-a fluorescence is one of the photosynthetic parameters used to monitor linear electron transport during the light reactions of photosynthesis. A decrease in fluorescence intensity of light-adapted leaves (Fv/Fm) of drought-stressed plants suggests photo-inhibition or damage of performance of photosystem II (PSII) and that might reduce the rate of photosynthesis (Mihaljević et al. 2021). The Fv/Fm is commonly used to monitor plants under drought stress, but the performance index on an absorption basis (PI_{abs}) is more sensitive to abiotic stress. In addition, PI_{abs} can also monitor the performance of photosystem I (PSI), the final electron acceptor during the light phase of photosynthesis (Çiçek et al. 2015). Drought stress might inhibit the flow of electrons beyond PSII, meaning that the electrons might not reach and reduce PSI under drought stress. To represent the efficiency of an electron to reach PSI, the PI_{total} (total performance index on absorption basis) is used. Lower PI_{total} values mean that there is an inhibition in the flow of electrons or damage in one of the intersystem electron acceptors beyond PSII under drought stress. The PI_{total} values represent the overall performance of PSII and PSI (Dietz and Pfannschmidt 2011; Mihaljević et al. 2021).

Drought stress is associated with the production of reactive oxygen species (ROS), which cause protein destabilization, damage the thylakoid membranes in the chloroplasts, and reduce chlorophyll content (chlorophyll-a, and -b). This results in early leaf senescence, reduced surface area of the leaves, reduced rate of photosynthesis, as well as reduced plant growth and productivity (Farooq et al. 2018; Moloi and Van der Merwe 2021). Chlorophyll pigments were significantly reduced by drought stress in barley (*Hordeum vulgare*), and in peanut (*Arachis Hypogaea*), with chlorophyll-a (Chl-a) being more sensitive to drought stress than chlorophyll-b (Chl-b) (Ghotbi-Ravandi et al. 2014; Meher et al. 2018). Carotenoids (CRDs) act as antioxidants through their association with PSll reaction centres where they deactivate the triplet chlorophyll molecule from producing ROS. Therefore, carotenoids have a protective role to plasma membranes and proteins under drought stress (Wang et al. 2018b; Collini 2019). An increase in CRDs accumulation in drought-stressed transgenic tobacco *(Nicotiana tabacum*), alfalfa (*Medicago sativa*), and sweet potato (*Ipomoea batatas*) plants improved membrane stability through enhanced antioxidant activity (Cho et al. 2016; Park et al. 2016; Wang et al. 2018b).

The last step of photosynthesis involves the assimilation of carbon dioxide (CO_2) inside plant leaves through stomata (Campbell and Farrel 2012; Brotosudarmo et al. 2014). It is well documented that plants save water by closing their stomatal aperture (Miyashita et al. 2005). The closing of stomata results in low stomatal conductance under drought stress, which is associated with reduced CO_2 uptake (Miyashita et al. 2005). The reduced stomatal conductance decreases the photosynthetic efficiency in many plants such as kidney beans (*Phaseolus vulgaris*), due to low intercellular CO_2 substrate, which might lead to poor plant productivity (Miyashita et al. 2005). Therefore, the stomatal conductance can be used to predict the performance of plants under drought stress (Gorthi et al. 2019).

Non-structural carbohydrates (NSC) such as glucose, trehalose, sucrose, and starch are vital osmoregulators because they are involved in maintaining plant cell turgor pressure under drought stress (Moloi and Van der Merwe 2021). Soluble sugars such as glucose, trehalose, and sucrose also improve plant tolerance to drought through oxidative stress prevention and stabilizing biomolecules and plasma membranes (Lu et al. 2019). Starch is a storage carbohydrate that accumulates in plants under water deficit conditions so that when there is a high demand for oligosaccharides it can be

readily available and hydrolyzed to meet the demand of the sugars. Many studies have demonstrated the decrease in starch content with an increase in oligosaccharides such as glucose and maltose. Makonya et al. (2019) demonstrated that sucrose, fructose, and starch accumulated in heat-resistant chickpea (*Cicer arietinum*) genotypes.

Therefore, this study aimed to elucidate edamame's response to drought stress by focusing on the photosynthetic efficiency and quantifying the specific non-structural carbohydrates. Such information is critical for the drought tolerance screening in edamame.

3.3 Materials and methods

3.3.1. Plant materials

Six edamame cultivars (AGS354, AGS429, UVE7, UVE8, UVE14, and UVE17) were germinated in seedling trays using Hygromix seedling mix and thereafter transplanted in potting bags (10 L capacity) containing 10 kg sandy loamy soil in the greenhouse (29° 6'31.94"S; 26°11'18.95"E) at the University of the Free State, Bloemfontein Campus, South Africa. According to a study published by van der Merwe et al. (2018), the edamame cultivars have different characteristics as shown in Table 3.1. The temperature was set at 25°C during the day and 18°C at night. Plants were watered to 100% (1.6 L) soil water holding capacity (WHC) until drought stress was induced at three trifoliate leaf stages by withholding irrigation to reach 30% WHC. The amount of water required was 480 mL (i.e., the soil weighing 30% of what it weighed at 100%). The control plants were maintained at 100% WHC. Plants were weighed (Optika N 3200, Italy) daily to establish the amount of water needed to maintain the two water levels. The trial design was a randomized split-plot (main plot-water treatment, sub-plot-cultivars), three biological replications (eighteen pots/treatment).

Table 3.1. Characteristics of the six edamame cultivars under drought stress

Cultivar	**Characteristic**
AGS354	High yielding under optimal irrigation, highly unstable under drought stress
UVE8	High yielding under optimal irrigation, highly unstable under drought stress
UVE17	Susceptible under drought stress
UVE14	Stable under drought stress, but not high yielding
UVE7	Low yielding, stable under drought stress
AGS429	Stable under drought stress, low yield reduction

3.3.2. Chlorophyll fluorescence

The potential quantum efficiency of photosystem II and PSI in the six cultivars of edamame plants was determined according to Pareek et al. (2019) using a portable PEA chlorophyll fluorimeter (Hansatech Instrument, King's Lynn, UK). The top, fully expanded young leaves were dark-adapted for 30 minutes, followed by illumination with excitation light energy set at 3000 $\mu molm^{-2}s^{-1}$. The chlorophyll fluorescence was measured during two growth phases: flowering (R1) and pod filling (R2-R6) because they are important stages in plant growth and development. The photochemical efficiency of photosystem II was measured in light-saturated according to equation (1).

$$Fv/Fm = (Fm - F_0)/Fm \quad (1)$$

Where F_0 is the minimum fluorescence, Fm is the maximum fluorescence and Fv is the variable fluorescence. The performance indexes (PI_{abs}) were used to monitor the performance of PSI. PI_{total} was also used to monitor the overall photosynthetic efficiency of both photosystems. The measurements were performed between 10:00 AM to 12:00 PM during the summer season at two plant growth stages (flowering and pod filling).

3.3.3. Stomatal conductance

A leaf porometer (Li-Cor ADC BioScientific Ltd., Hoddesdon, UK), an instrument used to quantify the humidity gradient that forms between the chamber and the surroundings of the leaf surface, was used to measure the stomatal conductance. The stomatal conductance of the top fully expanded leaf was measured (abaxial) when the sun was at its peak between 10:00 to 13:00 at two growth stages (flowering and pod filling).

3.3.4. Pigments extraction and quantification

Leaf samples (top fully expanded compound leaf per pot) were collected at the beginning of flowering and pod filling stages, crushed to a fine powder in liquid nitrogen and stored at -26°C. The extraction and measurements of Chl-a, Chl-b and CRDs were performed according to Pareek et al. (2019). Briefly, 100 mg of ground tissue was homogenized in 2 mL of 80% (v/v) chilled acetone (Sigma, RSA). The homogenate was centrifuged at 5000 × *g* for 5 minutes at 4°C, and the Chl-a, Chl-b, and CRD content in the supernatant was measured spectrophotometrically according to the equations below:

$$\text{Chl-a } (mg.mL^{-1}) = [(12.7 \times A663_{nm}) - (2.69 \times A645_{nm})] \quad (2)$$

$$\text{Chl-b } (mg.mL^{-1}) = [(22.9 \times A645_{nm}) - (4.68 \times A663_{nm})] \quad (3)$$

$$\text{CRDs } (mg.mL^{-1}) = [(1000 \times A470_{nm}) - ((3.27 \times \text{Chl-a}) + (1.04 \times \text{Chl-b}))/227] \quad (4)$$

3.3.5. Extraction of non-structural carbohydrates in the leaf tissue

Sugars were extracted by weighing 100 mg of ground leaf tissue and extracted twice with 2 mL of 80% (v/v) ethanol (VWR Chemicals, France) and boiling at 80°C. The extract was centrifuged at 5000 *x g* for 5 minutes. Then 60 mg of activated charcoal (Merck, RSA) was added to the supernatant to remove other non-sugar metabolites, after mixing and waiting for 5 minutes, centrifugation was done at 3000 *x g* for 10 minutes. The clear supernatant was stored at 4°C and used for glucose, sucrose, and trehalose assays using Megazyme kits (Ireland). Starch was extracted from the remaining pellets in the tubes.

3.3.5.1. Glucose assay

The reaction was done by pipetting 1.5 mL of glucose oxidase/peroxidase (GOPOD) (Megazyme, Ireland) reagent to 0.05 mL of the clear extract sample solution and incubating at 40°C for 20 minutes in a water bath. The absorbance was read at 510 nm against the blank to get the change in absorbance of samples and glucose standards [1.0 mg/mL in 0.2% (v/v) benzoic acid] (Megazyme, Ireland) according to Table 3.2.

The glucose concentration was then calculated using equation (5), where ΔA is the change in absorbance.

$$[\text{D-Glucose } \mu g/0.1 \text{ mL}] = (\Delta A_{\text{Sample}}/\Delta A_{\text{D-Glucose standard}}) \times 100 \quad (5)$$

Table 3.2. GOPOD method determination of Glucose content

	Reagent blank	Standard	Sample
GOPOD reagent	1.5 mL	1.5 mL	1.5 mL
D-Glucose standard	-	0.05 mL	-
Sample	-	-	0.05 mL
Distilled water	0.05 mL	-	-

$$[\text{D-Glucose } \mu g/0.1 \text{ mL}] = (\Delta A_{\text{Sample}}/\Delta A_{\text{D-Glucose standard}}) \times 100 \quad (6)$$

3.3.5.2. Trehalose assay

Trehalose was quantified from the plant extract according to the manufacturer's procedure using the trehalose kit from Megazyme (Ireland). Trehalase hydrolyses trehalose to 2 glucose units which are then phosphorylated by hexokinase in the presence of Adenosine triphosphate (ATP) to produce glucose-6-phosphate (G-6-P) and Adenosine diphosphate (ADP), G-6-P gets oxidized to gluconate-6-phosphate with the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The formed NADPH is stoichiometric with the amount of D-glucose and with twice the trehalose amount as a result. Hence, the increase in NADPH absorbance at 340 nm is used for the detection of trehalose in the samples. The assay is specific to trehalose and the sample preparations were done according to Table 3.3.

Table 3.3. Determination of trehalose content

Pipette into plastic cuvettes	Blank	Sample
dH_2O (at 25°C)	1.05 mL	1.00 mL
Sample solution	-	0.05 mL
Solution 1 Buffer	0.10 mL	0.10 mL
Solution 2 ($NADP^+$/ATP)	0.05 mL	0.05 mL
Suspension 3 (HK/G-6PDH)	0.01 mL	0.01 mL
Mixed and waited 5 minutes then read the absorbance of the solutions as A_1 then started the reactions by adding trehalase		
Suspension 4 (trehalase)	0.01 mL	0.01 mL
The solutions were mixed, and absorbance was read after 5 minutes and recorded as A_2.		

To determine the change in absorbance for trehalose, A_1 was subtracted from A_2, (i.e., ΔA (trehalose) = $A_2 - A_1$), then the concentration of trehalose was calculated according to equation (6), where V = final volume [mL]; MW = Molecular weight of trehalose [g/mol]; Ɛ = extinction coefficient of NADPH at 340 nm = 6300 [L/mol x cm]; d = light path [cm] ; v = sample volume [mL]; 2 = 2 molecules of D-Glucose released from each molecule of trehalose hydrolysed.

$$[\text{Trehalose}] = (V \times MW) / (Ɛ \times d \times v \times 2) \times \Delta A_{\text{trehalose}} \quad (7)$$

3.3.5.3. Sucrose assay

Sucrose assay was performed according to Zhao et al. 2010 and modified according to Megazyme GOPOD method. Twenty microlitres of invertase (BDH biochemicals, England) in the reaction tubes containing 0.05 mL of the soluble sugar extraction and 0.150 mL GOPOD (Invertase: 30.9 U/mL), the mixture was incubated for 15 minutes at 30°C to catalyze the breakdown of sucrose to D-Glucose and D-Fructose. Glucose accumulation was quantified according to GOPOD assays as explained in section 3.3.5.1.

The glucose absorbance was subtracted from the total absorbance of sucrose breakdown to obtain the change in absorbance of sucrose. A sucrose standard (1 mg/mL) was prepared by dissolving 1 mg of sucrose (Sigma, RSA) in 1 mL dH_2O. The standard was used to calculate sucrose concentration in the samples according to equation (8):

$$[\text{Sucrose}] = ((\Delta A_{total} - \Delta A_{glucose}) / \Delta A_{Standard}) \times \text{Sucrose} \quad (8)$$

3.3.5.4. Starch assay

The starch extraction was done according to a method described by Zhao et al. (2010) with some modifications using the "Total starch HK" kit from Megazyme. A sodium acetate buffer (100 mM, pH 5.0) was prepared by adding 5.8 mL of glacial acetic acid (Merck, Germany) to 900 mL dH_2O. The pH was adjusted to 5.0 by adding a 1 M sodium hydroxide solution (NaOH) (BDH Chemicals, England). Calcium chloride (BDH Chemicals, England) (0.74 g) was dissolved in the buffer and the volume was adjusted to 1 L with dH_2O. The second buffer of sodium acetate (1.2 M, pH 3.8) was prepared by adding 68.6 mL of glacial acetic acid to 800 mL dH_2O and the pH was adjusted to 3.8 with NaOH and the final volume was made to 1 L with dH_2O. A 2M potassium hydroxide (KOH) (BDH Chemicals, England) solution was prepared by dissolving 112.2 g KOH to some dH_2O and adjusting the final volume to 1 L with dH_2O. The enzyme reagents were prepared by diluting 1.0 mL of thermostable α-amylase (AMY) to 30 mL with the 100 mM sodium acetate, pH 5.0. The diluted enzyme was stored frozen at -26°C.

The pellet in the tubes (remaining after soluble sugars extraction, as explained in section 3.3.5) was suspended in 0.2 mL of 80% (v/v) ethanol and the tubes were stirred in a vortex mixer to aid dispersion. Then, 2 mL of 2 M KOH was added to each tube. The tubes were boiled at 100°C for 1 hour in a water bath with gentle stirring within 10 minutes intervals. The tubes were removed and allowed to cool to room temperature before adding 8 mL of sodium acetate buffer (pH 3.8) to each tube and after stirring, 0.1 mL of AMY and 0.1 mL of Amyloglucosidase (AMG) were added to each tube. After mixing well, the tubes were incubated in a water bath at 50°C for 30 minutes. After the incubation, the tubes were gently mixed by hand and centrifuged at 3000 *x g* for 10 minutes. The clear undiluted supernatant was used for the starch assay according to Table 3.4.

Table 3.4. Determination of starch content

Pipette into cuvettes	Blank	Sample
dH_2O (at 25°C)	1.05 mL	1.00 mL
Sample	-	0.05 mL
Buffer, pH 7.6	0.05 mL	0.05 mL
$NADP^+$	0.05 mL	0.05 mL
After well mixing and waiting 3 minutes, the absorbance was read as A_1 at 340 nm		
HK/G-6P-PDH	0.01 mL	0.01 mL
After well mixing and waiting for 5 minutes, the absorbance was read as A_2 at 340 nm, A_1 was subtracted from A_2 to obtain the change in absorbance (ΔA) for starch.		

The concentration of starch was calculated according to equation (8), Where V = final volume (mL); MW = Molecular weight of D-Glucose (g/mol); Ɛ = extinction coefficient of NADPH at 340 nm = 6300 (L/mol x cm); d = light path (cm); v = sample volume (mL); 162/180 = Adjustment from free D-Glucose to anhydro D-Glucose (as it occurs in starch).

[Starch] = (V x MW) / (Ɛ x d x v) x 162/180 x ΔA (D-Glucose) (9)

3.3.6. Thin-layer chromatography of soluble sugars

The thin-layer chromatography mobile phase was composed of butanol: acetic acid: distilled water (2:1:1). Standard solutions of glucose (0.4 mg/mL), sucrose (0.5 mg/mL), trehalose (0.5 mg/mL) (Megazyme, Ireland) and the six samples (cultivars) were spotted on the silica gel 60G F254 HPTLC plate (Merck, Germany). The plate was allowed to develop for 2 hours. The plate was dried and stained with 0.3% (w/v) Naphthol (Merck, Germany) in 95% (v/v) ethanol using sulfuric acid (Merck, Germany) and dried until all the bands were visible with a hairdryer.

3.3.7. Microscopic qualitative determination of starch content

Ground leaf samples were dried at 60°C for 72 hours. Soluble sugars were extracted three times from the 200 mg dried ground leaf tissues with 2 mL of 80% (v/v) ethanol (Sigma, RSA) and boiled at 80°C for 5 minutes. After each extraction, the tube contents were centrifuged at 5000 × *g* for 5 minutes and the supernatant discarded. An iodine solution was prepared by dissolving 0.2 g I_2 and 2.0 g potassium iodide (KI) (B. Owen Jones, RSA) in 100 mL distilled water. The pellets were stained by adding 1.0 mL of iodine solution to each tube. The tubes were vortexed and incubated at room

temperature for 30 minutes then centrifuged at 5000 × *g* for 5 minutes and the supernatant discarded. Each sample was visualized under Olympus SZX10 microscope using 1.5X objective and resolution of 1920 x 1200. Pictures were taken for each sample of drought-stressed and control samples.

3.3.8. Statistical analysis

Statistical analysis was done on the photosynthesis parameters using Genstat Release 19 software (VSN International 2018). The data collected were tested for normality using the Shapiro-Wilkson normality test. The logarithmic (log10) transformation was done to transform the data upon detection of skewness. The combined and separate effects of water treatments and cultivars were determined using the analysis of variance (ANOVA). Means were separated using the Fischer's protected least significant difference (LSD) test at $P \leq 0.05$.

3.4. Results

3.4.1. Analysis of variance

At the flowering stage, cultivars were significant ($P \leq 0.05$) for PI_{total}, stomatal conductance, starch, and trehalose content (Table 3.4). Water treatment was significant for Chl-b, stomatal conductance, glucose ($P \leq 0.001$) and PI_{abs}, ($P \leq 0.05$). Cultivar by water treatment interaction was significant only for glucose ($P \leq 0.05$). At pod filling, cultivars were significant for Fv/Fm ($P \leq 0.05$), glucose ($P \leq 0.01$), and trehalose ($P \leq 0.001$). Water treatment was significant for all except carotenoids, PI_{total}, glucose, and starch (Table 3.4). Cultivar by water treatment interaction was significant for Chl-b, trehalose ($P \leq 0.01$), Fv/Fm, and PI_{abs} ($P \leq 0.05$).

Table 3.5. Analysis of variance representing mean square (ms) values for the biochemical parameters during the flowering and pod filling stages of the six edamame cultivars under two water treatments [(100% soil water holding capacity, WHC) and 30% soil WHC].

Variate	Flowering			Pod filling		
	Cultivar (C)	Treatment (T)	CxT	Cultivar (C)	Treatment (T)	CxT
Chl-a	0.5669	0.0036	0.1114	0.2602	2.8**	0.7264
Chl-b	8.82	211.31***	4.49	13.681	147.299***	28.959**
CRDs	0.07206	0.0729	0.0156	0.0156	0.0002	0.0702
Fv/Fm	0.0005	0.0019	0.0002	0.0015*	0.0088***	0.0002*
PI_{abs}	5.241	11.890*	1.244	1,0877	11.0412***	1.8621*
PI_{total}	20.685*	0.23	5.968	18.39	19.01	6.95
gs	21350*	712547***	10122	20468	123748*	15354
GLU	0.0976	47.676***	2.832*	8.693**	2.544	0.815
Starch	578.7*	400.9	162.5	1982	22	1233
SUC	178.8	96.7	85	60.47	2497.9***	46.81
TRE	614.9*	503.1	200.6	1231.7***	6269***	941.9**
Grand mean	2068.8984	64892.6259	962.2510	2162.209	12065.4184	1601,3720

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, Chl-a = Chlorophyll-a, Chl-b = Chlorophyll-b, CRDs = Carotenoids, Fv/Fm = Ratio of variable fluorescence to maximal fluorescence of PSII, PI_{abs} = Performance index of photosystem I (PSI) and photosystem II (PSII), PI_{total} = Total performance index of PSI and PSII, g_s = Stomatal conductance, TRE = Trehalose, GLU = Glucose, SUC = Sucrose.

3.4.2. Chlorophyll fluorescence for photosystem I and photosystem II performance

Drought stress did not have any significant effect on the chlorophyll fluorescence (Fv/Fm) for all cultivars during the flowering stage. At the pod filling stage, drought stress significantly increased the Fv/Fm of AGS429 (10%), followed by UVE17 (9%). These two cultivars were not significantly different under drought stress (same letters). UVE14 and UVE7 chlorophyll fluorescence were different from each other and to the other cultivars under drought stress. The Fv/Fm was generally higher at flowering than at the pod filling stage (Figure 3.1).

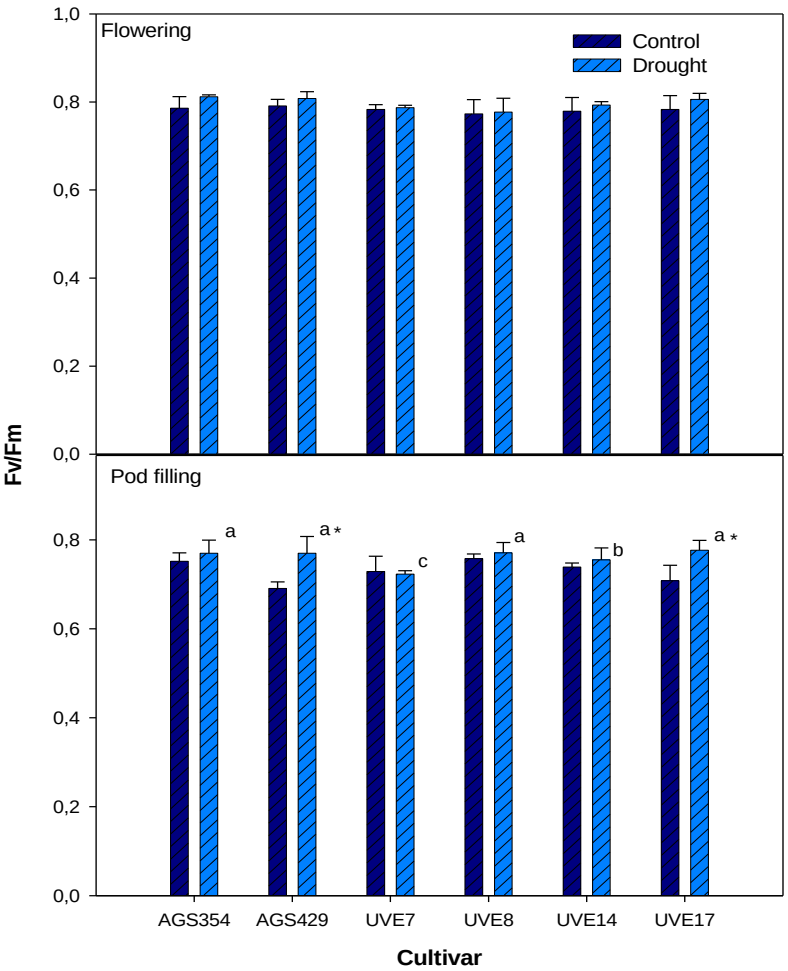

Figure 3.1. Chlorophyll fluorescence quantum efficiency of PS II (Fv/Fm) of six edamame cultivars at flowering and pod filling stages. Letters represent differences or similarities in Fv/Fm between cultivars. Asterisk represents a significant increase or decrease under drought stress at P ≤ 05. Values represent means ± SD (n= 6).

At the flowering stage, PI_{abs} increased with drought stress but the increase was not significant for all cultivars (Figure 3.2). UVE7 remained unchanged compared to the control and had the lowest PI_{abs}. At pod filling, PI_{abs} increased with drought except for UVE7, which showed a non-significant decrease with the lowest value. Drought stress significantly increased the PI_{abs} of AGS429 by 72% and UVE17 by 71%, relative to controls. UVE17 had the highest PI_{abs} and UVE7 the lowest. PI_{abs} was higher at flowering than at pod filling.

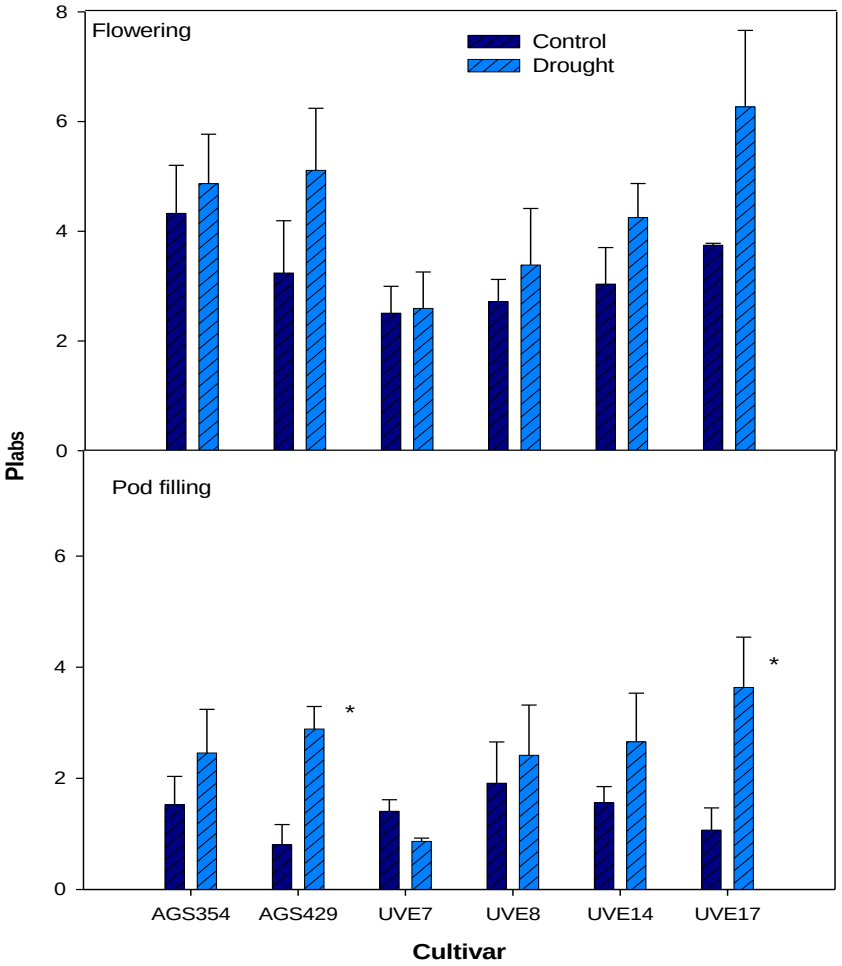

Figure 3.2. The performance Index (PI_{abs}) of six edamame cultivars at flowering and pod-filling stages. Asterisk represents a significant increase or decrease in PI_{abs} under drought stress at P ≤ 05. Values represent means ± SD (n= 6).

Drought stress increased PI_{total} for most cultivars at flowering, except for AGS354, which had a decreased PI_{total} compared to control (Figure 3.3). However, the increase of PI_{total} in other cultivars was not significant. UVE7 remained the same as the control, had the lowest PI_{total}, and was different from other cultivars under drought stress. At pod filling, drought increased the PI_{total} of most cultivars, but drought inhibited the PI_{total} of UVE17. PI_{total} was higher at flowering than at pod filling.

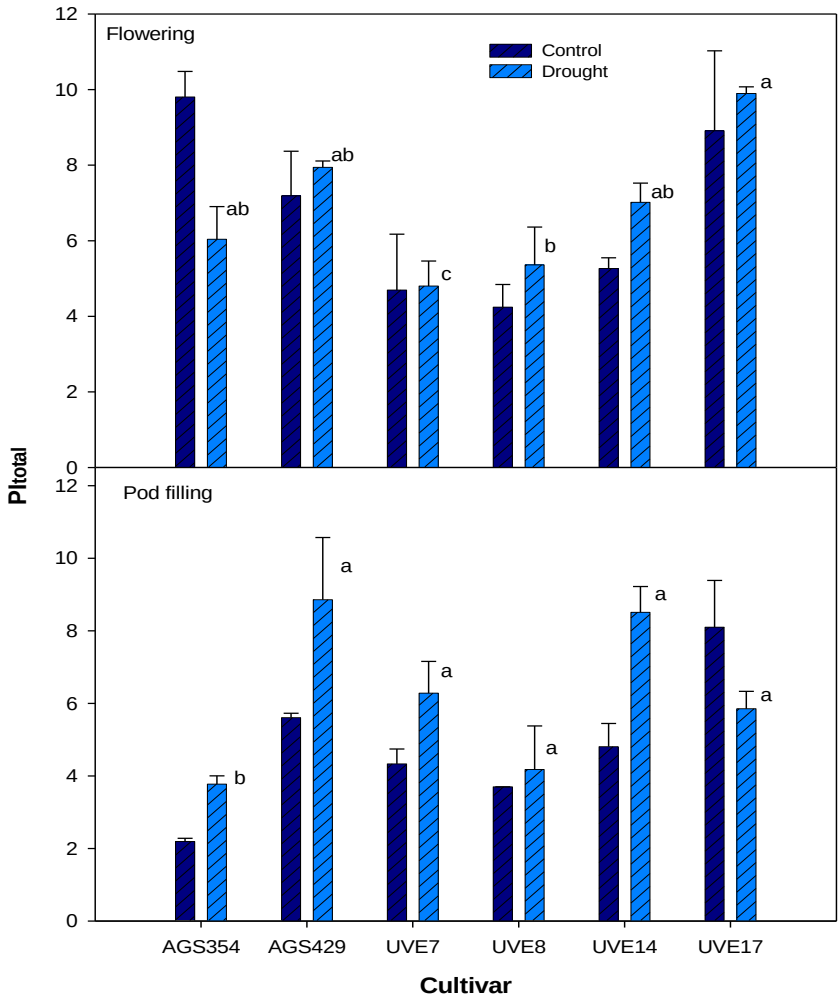

Figure 3.3. The total performance index (PI_{total}) of six edamame cultivars at flowering and pod filling stages. Letters represent differences or similarities in PI_{total} between cultivars. Asterisk represents a significant increase or decrease under drought stress at $P \leq 05$. Values represent means ± SD (n= 6).

3.4.3. Pigments

Drought stress did not have any significant effect on the Chl-a concentration of the six edamame cultivars during the flowering stage (Figure 3.4). Although not significant, Chl-a content decreased with drought treatment for UVE17. At pod filling, drought resulted in a significant reduction of chl-a concentration for AGS429 (5%) and UVE17 (6%). The Chl-a content of UVE17 was low compared to other cultivars under drought stress. Drought had no significant effect on the Chl-a content of UVE7. No notable differences in Chl-a were observed at flowering and pod filling.

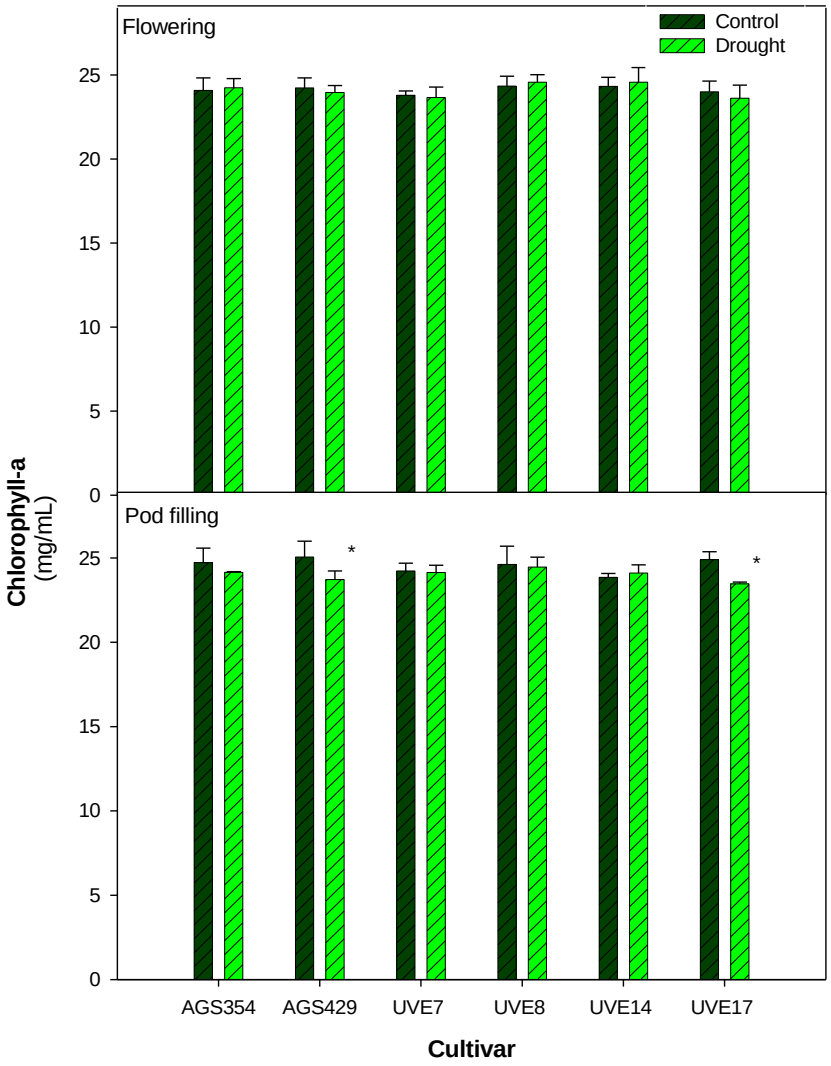

Figure 3.4. Chlorophyll-a content of six edamame cultivars at flowering and pod-filling stages. Asterisk represents a significant decrease under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

Drought stress did not have any significant effect on the Chl-b content of most drought-stressed cultivars at the flowering stage, except UVE17, which was significantly reduced by up to 20% (Figure 3.5). Drought-stressed UVE8 had the lowest Chl-b content. At pod filling, drought stress significantly increased the Chl-b contents of AGS354 and AGS429 by 21% and 26% compared to controls, respectively. In general, drought-stressed cultivars had higher Chl-b content at pod filling compared to the flowering stage.

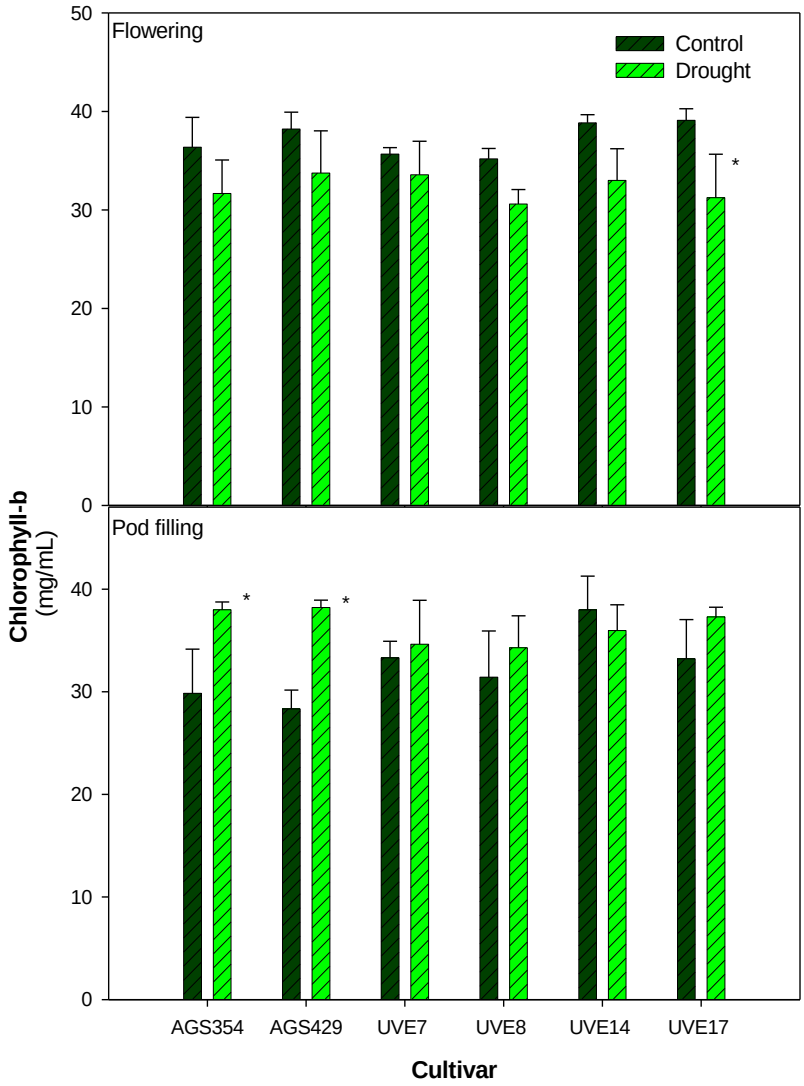

Figure 3.5. Chlorophyll-b content of six edamame cultivars at flowering and pod filling stages. Asterisks represent a significant increase or decrease under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

Drought stress did not have any significant effect on the CRDs contents of all the cultivars during the flowering stage. At the pod filling stage, drought stress significantly reduced CRDs concentration of UVE17 by 4%, the other cultivars were not significantly affected by stress (Figure 3.6). There were no notable differences in levels of CRDs observed at flowering and pod filling.

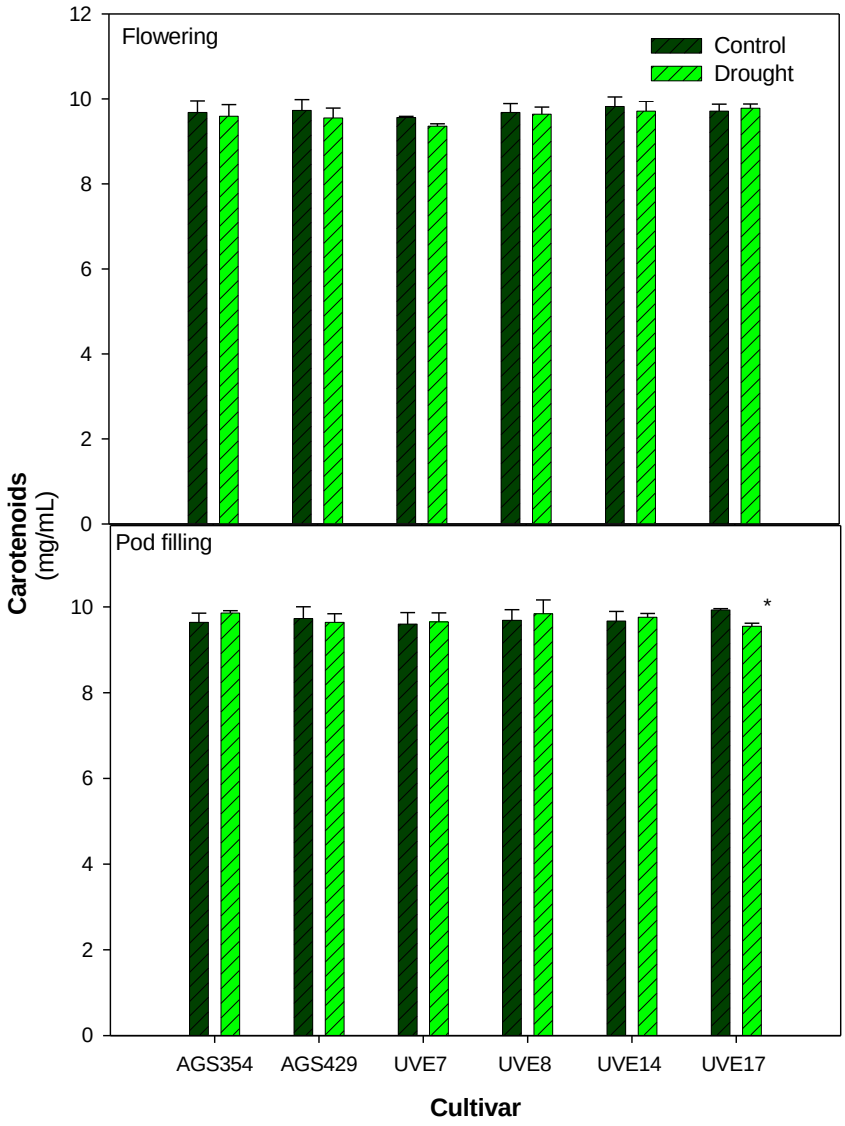

Figure 3.6. Carotenoids content of six edamame cultivars at flowering and pod filling stages. Asterisk represents a significant decrease under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

3.4.4. Stomatal conductance

Drought stress significantly reduced the stomatal conductance of all drought-stressed cultivars at the flowering stage (Figure 3.7). The stomatal conductance was reduced by 67% in AGS354, 50% in AGS429, 36% in UVE7, 59% in UVE8, 44% in UVE14, and 43% in UVE17 relative to control. UVE7, UVE14, and UVE17 were not significantly different from each other. AGS354, AGS429, and UVE8 were significantly different from each other and the other cultivars. At pod filling, drought-stressed plants had higher stomatal conductance than at flowering. Only UVE17 had a significant reduction of 59% in stomatal conductance due to drought stress.

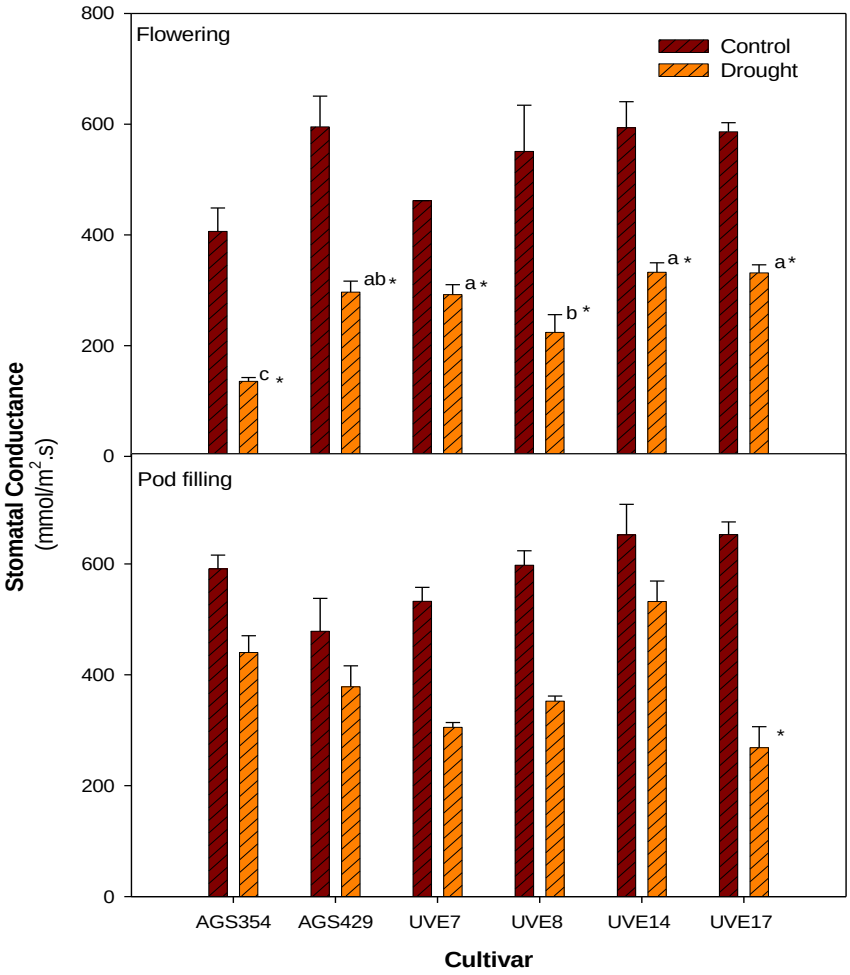

Figure 3.7. The stomatal conductance of six edamame cultivars at flowering and pod filling stages. Letters represent differences or similarities in stomatal conductance between cultivars. Asterisk represents a significant decrease under drought stress at p ≤ 05. Values represent means ± SD (n= 3).

3.4.5. Qualitative determination of non-structural carbohydrates

At the flowering stage, the controls only showed a single band of monosaccharides, which corresponded to glucose standard (Figure 3.8A). All drought-stressed cultivars, except UVE8, had a second disaccharide produced due to drought stress, even though bands of the disaccharide were not intense (Figure 3.8B). Since sucrose and trehalose have the same molecular weight (342,296 g/mol), the second band could either be trehalose or sucrose, or a combination of both.

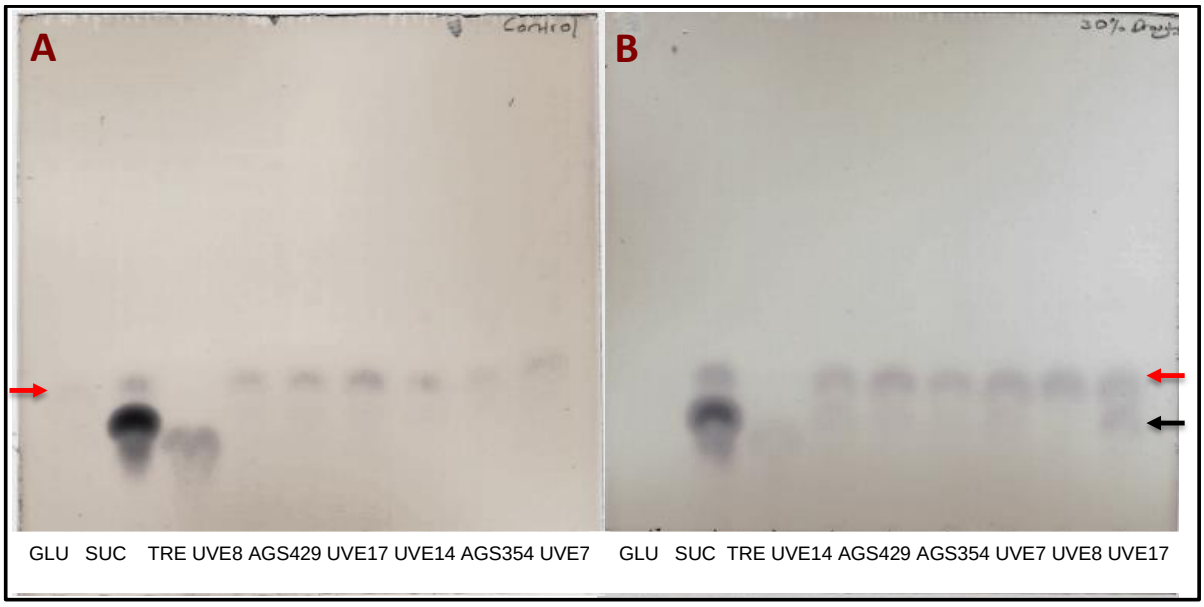

Figure 3.8. Thin-layer chromatography profiles for soluble sugars in the controls (A) and drought-stressed edamame cultivars (B) during the flowering stage. The red arrow shows the glucose (Glu), and the black arrow represents disaccharides such as sucrose (SUC) and trehalose (TRE).

At the pod filling stage, glucose intensity decreased in drought-stressed plants, and the sucrose/ trehalose spots increased in intensity (Figure 3.9B). Control cultivars also showed some concentrations of sucrose/ trehalose at the pod filling stage, but the spots intensity was the same as for glucose (Figure 3.9A). Generally, sucrose/ trehalose content is higher in drought-stressed cultivars at the pod filling stage.

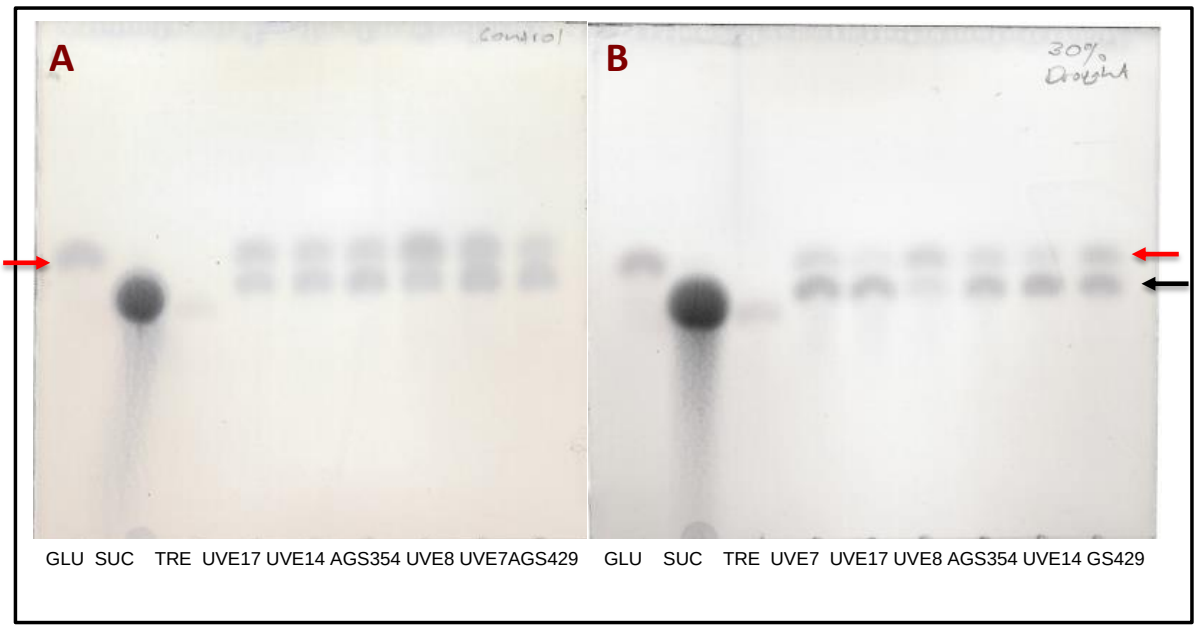

Figure 3.9. Thin-layer chromatography profiles for soluble sugars in the controls (A) and drought-stressed edamame cultivars (B) during the pod filling stage. The red arrow shows the glucose (Glu), and the black arrow represents disaccharides such as sucrose (SUC) and trehalose (TRE).

Figure 3.10 and Figure 3.11 represent the microscopy qualitative determination of starch content using the iodine complexation method. Iodine covalently binds to the amylose component of the starch to form a dark blue colour, which allows the identification of the cells that contain more starch. According to the results, the lighter the dark-blue intensity/ pale, the lesser or absence the amount of starch. As shown in Figure 3.10 and Figure 3.11, drought stress significantly reduced the starch content in most drought-stressed cultivars (seen by dark blue colour intensity, starch-iodine complex) compared to controls. Drought stress had no significant effect on the starch content in UVE14, shown by the dark starch-iodine complex intensity in Figure 3.11.

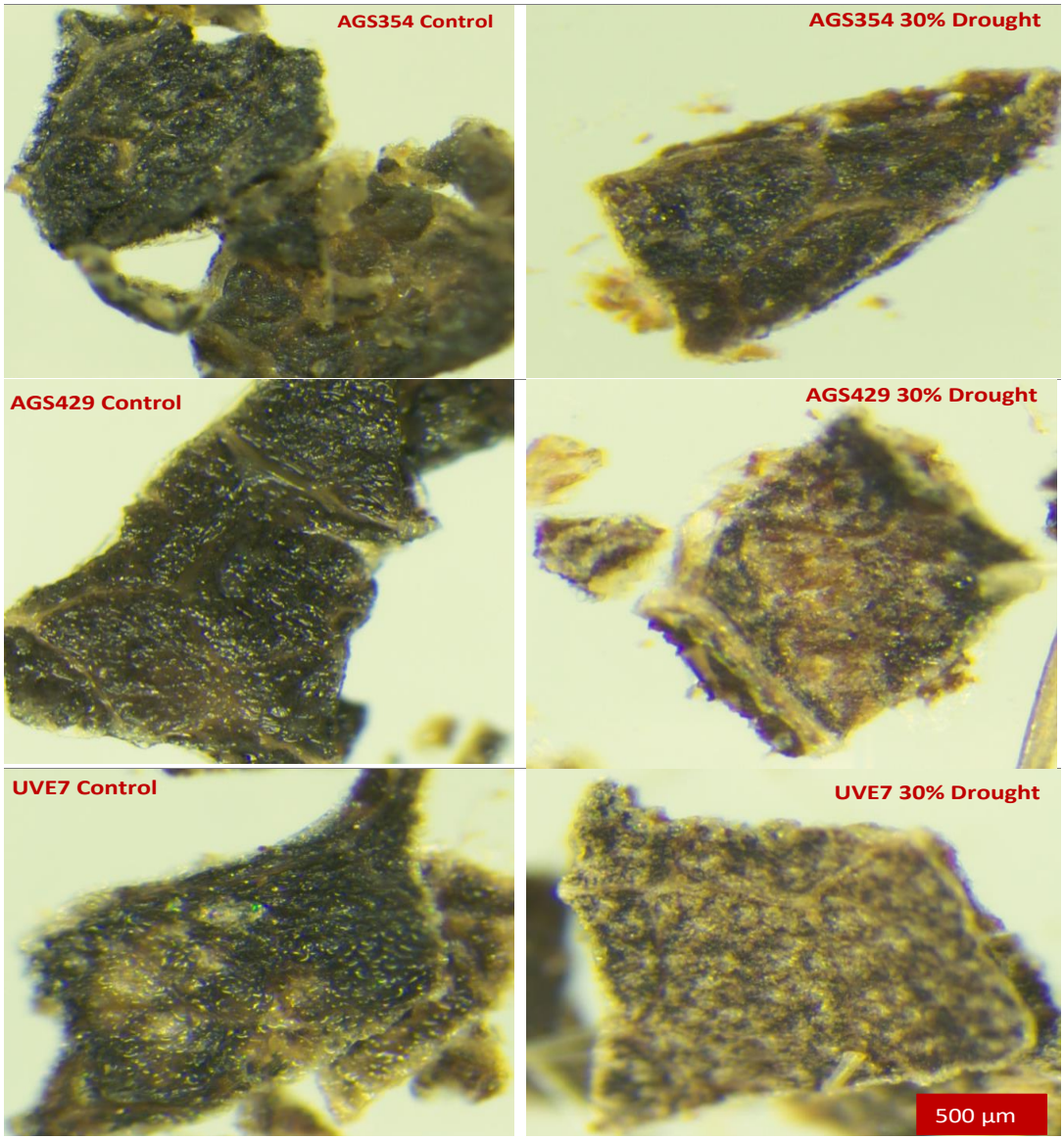

Figure 3.10. Light microscopic determination of starch content at pod filling stage in control and drought-stressed edamame cultivars. The black intensity represents starch content complex with iodine. AGS354, AGS429 and UVE7 control and drought-stressed cultivars are shown in the figure.

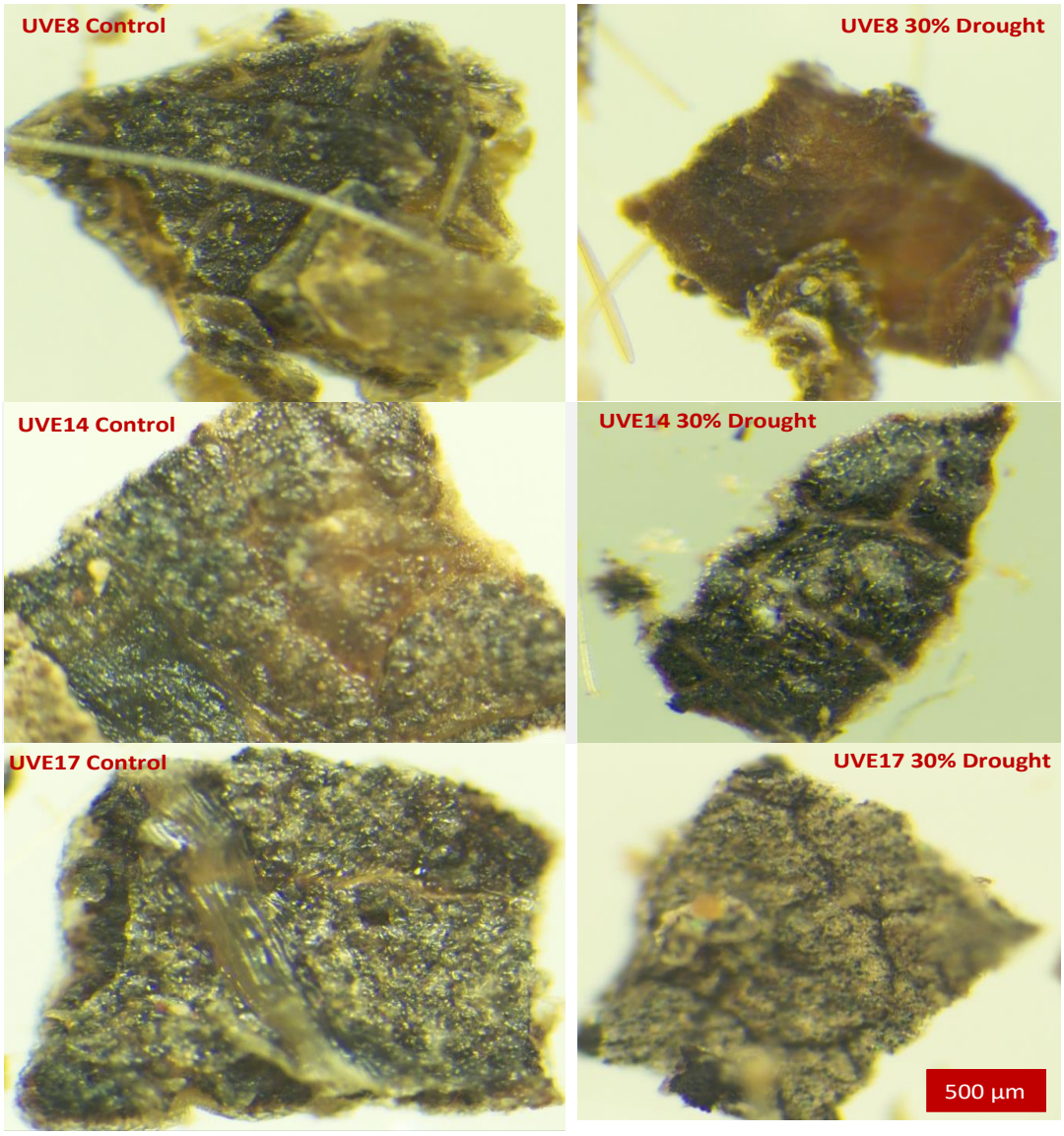

Figure 3.11. Light Microscopic determination of starch content at pod filling stage in control and drought-stressed edamame cultivars. The black intensity represents starch content complex with iodine. UVE8, UVE14 and UVE17 controls and drought-stressed cultivars are shown in the figure.

Glucose was reduced by drought stress in all cultivars (except UVE7) at the flowering stage (Figure 3.12). Drought stress reduced glucose content in UVE8 by 90% and in UVE14 by 42% relative to the control. AGS354, AGS429 and UVE17 showed glucose reductions of 29%, 20% and 25%, respectively compared to controls. Drought stress during the pod filling stage caused significant differences across cultivars. In addition, cultivars had lower glucose concentrations at pod filling compared to the flowering stage.

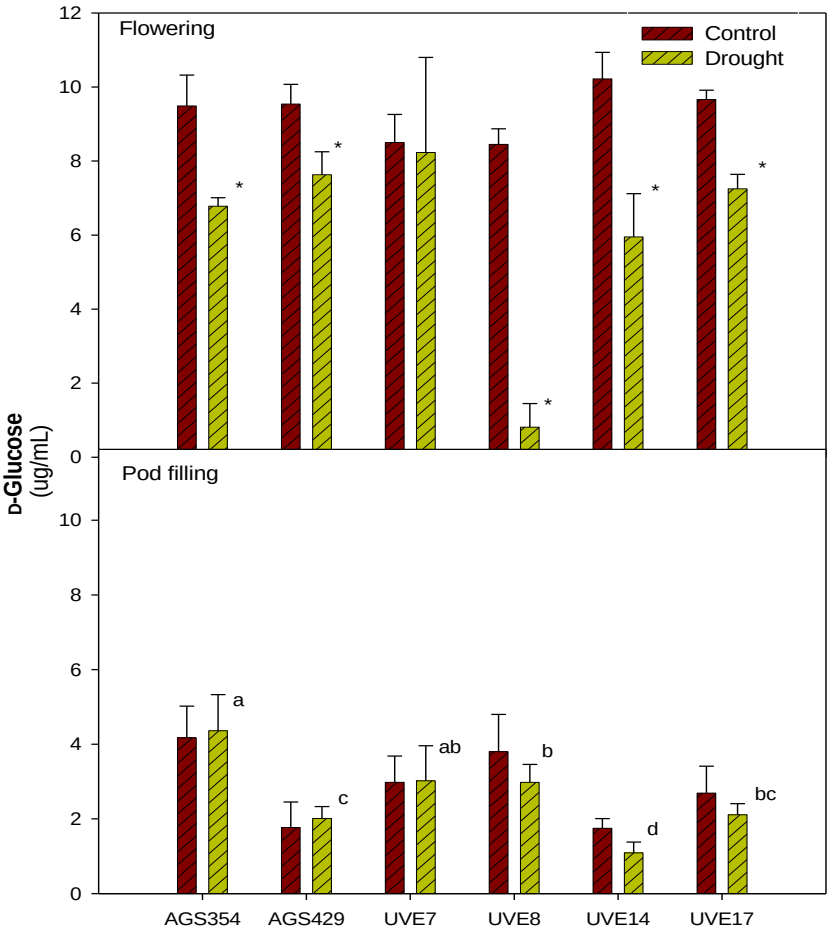

Figure 3.12. The glucose content of six edamame cultivars at flowering and pod filling stages. Letters represent differences or similarities in glucose concentrations between cultivars. Asterisk represents a significant decrease under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

Drought stress led to increases or decreases in the trehalose content at flowering, the treatments were insignificant (Figure 3.13). Cultivars were significantly different under drought stress with UVE17 showing the lowest trehalose content. UVE14 had a 3-fold higher trehalose content compared to the control at flowering stage. At the pod filling stage, drought stress significantly increased trehalose content in AGS429 (87%) and UVE17 (92%). AGS354, AGS429 and UVE17 were significantly different from each other and the other cultivars, whereas UVE7, UVE8 and UVE14 were not significantly different from each other under drought stress. The highest trehalose was recorded during the pod filling stage in drought stressed plants of AGS429 cultivar.

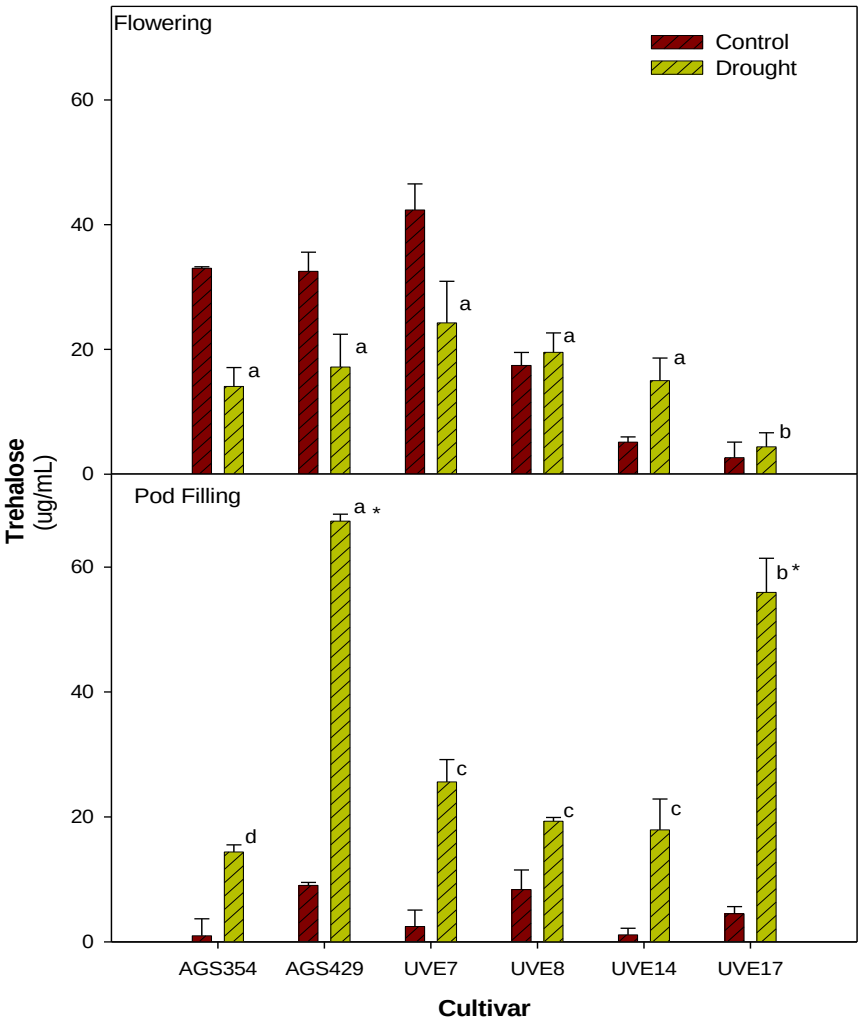

Figure 3.13. Trehalose content of six edamame cultivars at flowering and pod filling stages. Letters represent differences or similarities in trehalose content between cultivars. Asterisk represents a significant increase under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

Drought stress did not have any significant effect on the sucrose content of all the cultivars at the flowering stage (Figure 3.14). UVE17 had the lowest content of sucrose. At the pod filling stage, drought stress significantly increased the sucrose content of AGS429 (97%), UVE8 (49%), UVE14 (64%), and UVE17 (76%). The mean sucrose content was higher at pod filling than at the flowering stage.

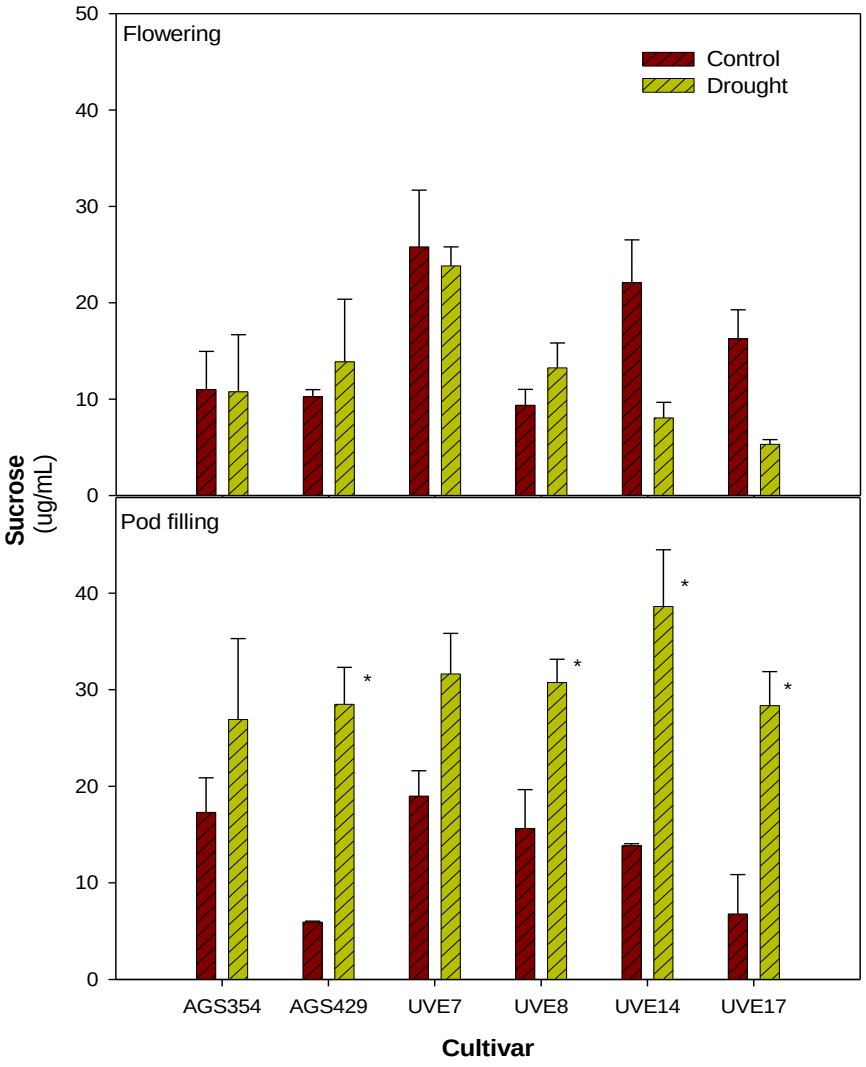

Figure 3.14. Sucrose content of six edamame cultivars at flowering and pod filling stages. Asterisk represents a significant increase under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

At the flowering stage, drought stress significantly increased the starch content by 52% in UVE14 plants relative to the control (Figure 3.15). AGS354, UVE7, UVE8, and UVE17 were not significantly different from each other. AGS429 and UVE14 were significantly different from each other and other cultivars. At the pod filling stage, drought stress reduced starch production in AGS354, UVE7, UVE8, and UVE14. However, these reductions were insignificant. In contrast, AGS429 had a significantly higher starch concentration of about 42% compared to its control. Starch content was generally higher at pod filling compared to the flowering stage.

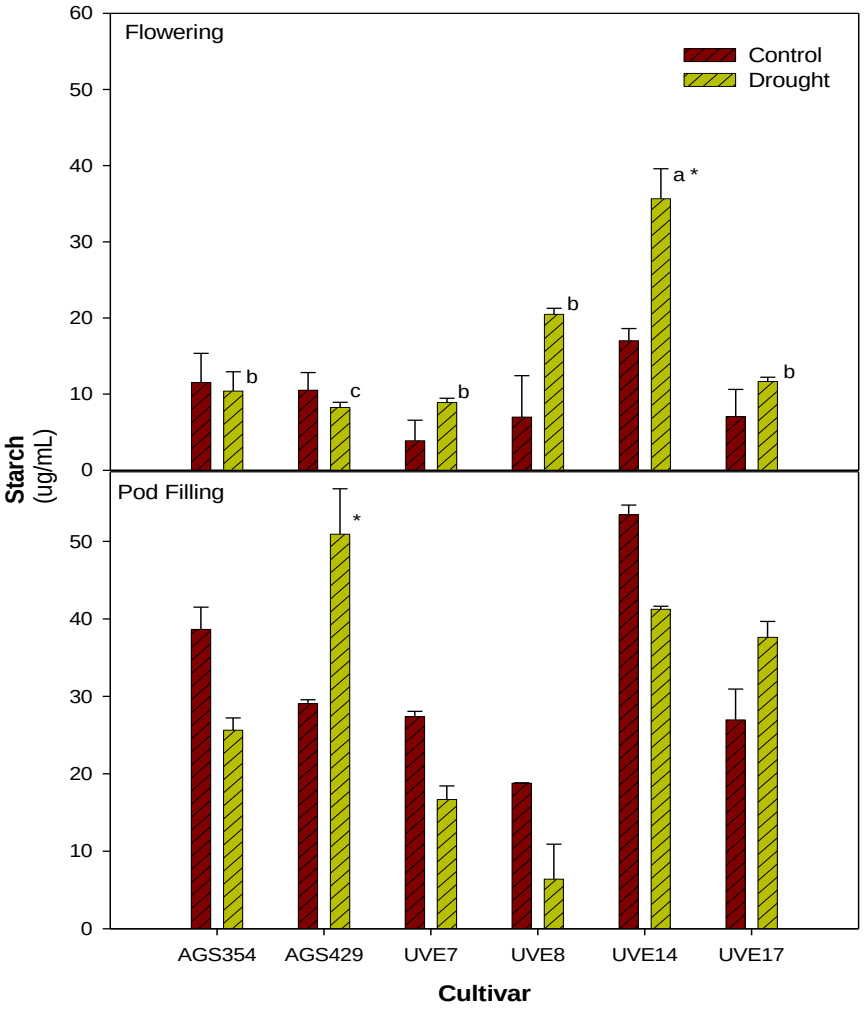

Figure 3.15. Starch content of six edamame cultivars at flowering and pod filling stages. Letters represent differences or similarities in starch content between cultivars. Asterisk represents a significant increase under drought stress at P ≤ 05. Values represent means ± SD (n= 3).

3.5. Discussion

Drought is one of the most damaging abiotic stresses causing extensive crop loss all over the world. The uptake of CO_2 during photosynthesis is coupled to linear electron transport. This linear electron transport is monitored by chlorophyll fluorescence (Yu et al. 2021). The maximum quantum efficiency is commonly used as a non-destructive procedure to monitor PSII performance under drought stress (Mihaljević et al. 2021). In this study, the Fv/Fm of the PSII photochemistry was performed to predict the leaf photosynthesis rate of drought-stressed edamame cultivars. The results of this study showed that drought stress did not affect the Fv/Fm of all cultivars at the flowering stage.

At the flowering stage, cultivars (C) were significant ($P \leq 0.05$) for PI_{total}, stomatal conductance, starch, and trehalose content. Water treatment (T) was significant for Chl-b, stomatal conductance, glucose ($P \leq 0.001$), and PI_{abs} ($P \leq 0.05$). Cultivar by water treatment interaction (CxT) was significant for glucose ($P \leq 0.05$). At pod filling, cultivars were significant for Fv/Fm ($P \leq 0.05$), glucose ($P \leq 0.01$) and trehalose ($P \leq 0.001$). Water treatment was significant for all except CRDs, PI_{total}, glucose, and starch. Cultivar by water treatment interaction was significant for Chl-b, trehalose ($P \leq 0.01$), Fv/Fm and PI_{abs} ($P \leq 0.05$). These findings are in line with Terzi et al. (2010), who found that drought stress did not have any effect on the photochemistry of PSII in common bean cultivars as it did not affect Fv/Fm in any way. In addition, cultivars were not significantly different from each other, except UVE8, which had the lowest Fv/Fm (0.77) compared to other cultivars.

At the pod filling stage, AGS429 and UVE17 had an increase in Fv/Fm ($P \leq 0.05$) due to drought stress compared to their controls. This study shows that the quantum efficiency of the PSII photochemistry under drought stress is influenced by the developmental stage and the genotype. Also, the responses may differ with the plant type because, in dry beans, drought reduced the Fv/Fm substantially (Mathobo et al. 2017). The increase in Fv/Fm in AGS429 and UVE17 at the pod filling stage suggests the up-regulation of photosynthesis under drought stress, which is in agreement with Mathobo et al. (2017). Our findings further show that the recorded Fv/Fm for drought-stressed cultivars range between 0.78 – 0.81 at flowering and 0.72 – 0.78 at the pod filling stage, indicating a decline in chlorophyll fluorescence from flowering to pod

filling. However, the decline was not substantial, indicating that there was minimal photoinhibition and/ or photodamage of some photosystems (Mihaljević et al. 2021). It is important to note that the duration of drought stress also plays a significant role in edamame PSII photochemistry. The Fv/Fm was reduced by a long period of drought stress in *Lilium* plants (Yu et al. 2021). Although quantum efficiency of PSII was significant for selected cultivars in drought-stressed edamame, some studies have reported that drought stress has little effect on Fv/Fm and that it is less sensitive for analysis of PSII performance of drought-stressed plants (Mihaljević et al. 2021).

The OJIP transient test of performance index on absorption basis and total performance index (PI_{abs} and PI_{total}) is hypersensitive to abiotic stresses and a good technique to monitor the performance of both PSI and PSII (Oukarroum et al., 2009; Çiçek et al. 2015). During the flowering stage, drought stress increased the PI_{abs} in all cultivars. The PI_{abs} was increased by 40% in UVE17, followed by AGS429 (37%). UVE7 had the lowest PI_{abs} (2.59) and remained unchanged compared to the control. AGS429 and UVE17 had increased (72% and 71%) PI_{abs} due to drought stress at the pod filling stage. The low PI_{abs} of UVE7 at flowering and pod filling suggests the inactivation of PSII reaction centres (RCs) under drought, while the increased PI_{abs} in AGS429 and UVE17 under drought stress shows that these cultivars may have maintained their PSII RCs and avoided photo-inhibition (Boguszewska-Mańkowska et al. 2018; Jia et al. 2020). In general, PI_{abs} was lower at the pod filling stage compared to flowering, suggesting the inactivation of PSII RCs in most cultivars with the growth stage. The inactivation of RCs causes the absorbed light energy to dissipate, leading to a reduced rate of photosynthesis (Mihaljević et al. 2021). Boguszewska-Mańkowska et al. (2018) noted a significant decrease in PI_{abs} of potato cultivars, which were sensitive to drought stress. Under long-term drought stress, ROS accumulate to high levels, resulting in membrane injury, lipid peroxidation, and photoinhibition of both the photosystems and/ or damage PSII (Jia et al. 2020). Even though RCs may have been inhibited in most cultivars at the pod filling stage, AGS429 and UVE17 seem to have maintained most of their PSll RCs.

Photosystem l is the final electron acceptor during the light phase of photosynthesis. It is reduced by plastocyanin (PC) through a series of electron transport (Dietz and Pfannschmidt 2011). The PI_{total} is used to represent the values of the efficiency of energy flow beyond PSll in the electron transport chain of photosynthesis (Mihaljević

et al. 2021). The PI_{total} is the product of PI_{abs} and RE0/ET0, which is the possibility that an electron will move sequentially from plastoquinone (PQ) to cytochrome b6f and plastocyanin (PC), to finally reduce PSl [i.e., PI_{total} = PI_{abs}*(RE0/ET0)] (Dietz and Pfannschmidt 2011; Mihaljević et al. 2021). In this study, drought stress increased the PI_{total} for most cultivars at flowering and pod filling stages except AGS354, which showed a 38% decline at the flowering stage. An increase in the PI_{total} values for most cultivars indicates the normal and efficient flow of electrons beyond PSll to PSl under drought stress. UVE7 remained unchanged compared to its control and had the lowest PI_{total}. UVE17 had a 28% decline in PI_{total} at the pod filling stage. Decreases in PI_{total} of AGS354, UVE7, and UVE17 in the respective growth stages suggest that the flow of electrons was disturbed by drought stress in these cultivars, reducing the rate of photosynthesis. The PI_{total} of drought-stressed cultivars was generally lower at the pod filling stage compared to flowering, meaning that the photosynthesis rate in edamame is specific to the growth stage. These observations were in agreement with Jia et al. (2020), who also found that PSl and PSll activities declined with drought stress duration in maize (*Zea mays*). Generally, cultivars had high PI_{total} at the two growth stages, but AGS354 and UVE17 had their PI_{total}l decreased at the flowering and pod filling stage, respectively. These results indicate that for the light-dependent reactions of photosynthesis, AGS429 had better performance under drought stress.

The light-dependent reactions depend on the light energy harvested by the chloroplast pigments (Fiedor et al. 2019). There is a direct relationship between chlorophyll content and photosynthetic efficiency in higher plants. The rate of photosynthesis is reduced by the osmotic stress, which results from drought because it affects the content of chlorophyll pigments (Meher et al. 2018). Drought stress leads to the accumulation of ROS, of which excess can damage chloroplasts, leading to early leaf senescence and reduced chlorophyll content (Terzi et al. 2010; Kalaji et al. 2018).

In this study, drought stress did not have any effect on the Chl-a content of drought-stressed cultivars at the flowering stage. Although the Chl-a content of drought-stressed cultivars remained unchanged compared to their controls, UVE17 had the lowest Chl-a content (followed by UVE7) and was different from other cultivars. UVE8 and UVE14 had the highest Chl-a content. At the pod filling stage, UVE8 and UVE14 maintained the highest chl-a content under drought stress. There was a reduction in the Chl-a content of AGS429 and UVE17. The reduced Chl-a content in these two

cultivars could be attributed to the inhibition of synthesis or degradation of the chl-a pigment because of the longer duration of drought stress. Also, the chloroplasts are big ROS sources under drought stress, which can cause lipid peroxidation, further resulting in the degradation of Chl-a (Dias et al. 2018; Meher et al. 2018). The degradation of this main photosynthetic pigment has serious consequences on plant growth and productivity (Ustin et al. 2009; Fiedor et al. 2019).

All cultivars had reduced Chl-b content at flowering, but this reduction was only significant in UVE17 (showed 20% reduction). In all drought-stressed cultivars, AGS429 had the highest Chl-b content at flowering. High Chl-b is important in that it can be converted to form Chl-a, which will enable this cultivar to perform photosynthesis despite unfavourable conditions (Shimoda et al. 2012). At pod filling, all drought-stressed cultivars (except UVE14) had increased chl-b relative to their controls, with AGS354 and AGS429 having increased Chl-b content. Chl-a concentrations were generally lower than chl-b at both growth stages. In addition, the Chl-a contents were further slightly (not notable) reduced by drought stress at pod filling with an increase in Chl-b. This suggests that drought stress duration induced the degradation of Chl-a with the synthesis of chl-b in edamame by utilizing the produced ROS to convert chl-a to Chl-b through the activity of chlorophyllide *a* oxygenase (CAO) (Teng et al. 2021). This could also be a strategy to neutralize ROS in the drought-stressed edamame. Drought stress has more devastating effects on chl-a than chl-b in *Tilia cordata* (Kalaji et al. 2018). Our findings coincide with Hassanzadeh et al., (2009) who reported increased chl-b with a decrease in chl-a under drought stress in sesame (*Sesamum indicum*). Chlorophyll-b did not increase in UVE14, suggesting that there was less conversion of chl-a to chl-b in this cultivar, whereas the decreased Chl-a with Chl-b increase in AGS429, UVE17, and AGS354 at pod filling suggests the interconversion of the pigments.

The CRDs are very important metabolites formed in plants as accessory pigments during photosynthesis. Furthermore, CRDs accumulation in drought-stressed plants can scavenge different ROS forms. The antioxidant activity of CRDs helps the thylakoid and plasma membranes to become more strong and rigid under drought stress (Ahluwalia et al. 2021). Different plant species produce and regulate CRDs differently during water-deficit conditions. In rice plants, the CRDs content was reduced by up to 33% due to drought stress (Nasrin et al. 2020), whereas in chickpea

(*Cicer arietinum*), the accumulation of CRDs improved the plant's tolerance to drought stress (Vir et al. 2018). Therefore, CRDs content monitoring is often used as a biological marker of plants affected by drought (Cicevan et al. 2016). In this study, drought stress did not have any effect on the CRDs content of most cultivars at the flowering stage. UVE7 was different from other cultivars at flowering stage, and it had the lowest CRDs content (9.36 mg/mL). At pod filling, UVE17 had a reduction in CRDs content. All other cultivars had slightly increased CRDs content under drought stress at the pod filling stage. The reduction in CRDs content in UVE17 at the pod filling stage suggests the lack of protection against ROS in this cultivar, making it susceptible to drought stress. However, the increased production or maintenance of CRDs in other cultivars suggests that they have better ROS scavenging capacity compared to UVE17.

CRDs scavenge ROS by quenching the singlet oxygen ($^1O_2$) produced by the triplet chlorophyll. CRDs may directly quench the triplet chlorophyll molecule or through a de-epoxidation reaction which converts violaxanthin to zeaxanthin, a reaction occurring in the light-harvesting complex 1 (LHC1) protein (Wehner et al. 2004; Ahluwalia et al. 2021). This reaction ensures that the chlorophyll triplet is not formed and the $^1O_2$ is not produced. This reaction leads to chloroplasts' protection against photo-oxidative damage under drought stress and helps sustain photosynthesis (Wehner et al. 2004). The CRDs reduction in UVE17 matches its reduction in chl-a, Chl-b, and PI_{total}. The susceptibility of this cultivar to drought stress might lead to reduced photosynthesis and poor productivity. The reduction in CRDs contents in drought-stressed ornamental (*Magnoliophyta*) cultivars was associated with reduced plant growth (Cicevan et al. 2016). In edamame cultivars, low CRDs content reduced the rate of photosynthesis and resulted in a poor photo-protective capacity of the photosystems (do Rosário et al. 2020).

Plants usually save water by closing their stomatal aperture to increase their water use efficiency (WUE) under drought stress. However, the closing of stomata reduces their stomatal conductance under drought stress. This might also reduce the photosynthetic efficiency due to low intercellular CO_2 substrate, resulting in poor crop yield (Gorthi et al. 2019). In this study, it was found that all cultivars had a reduction in stomatal conductance at the flowering stage, with AGS354 having the lowest stomatal conductance (135.54 mmol/m².s). This cultivar was also different from others in

stomatal conductance. The decrease in stomatal conductance of all cultivars at the flowering stage due to water shortage suggests the decreased rate of photosynthesis. The flowering stage is very crucial in plant growth because it is where pollination, fertilization, and seed setting occur. The reduction of photosynthesis rate at this stage due to drought-induced stomatal closure could disturb these important growth processes and result in poor crop yield (Liu et al. 2020). AGS354 was previously reported to have its yield significantly reduced by drought stress (Van der Merwe et al. 2018; Moloi and Van der Merwe 2021). The lowest stomatal conductance of AGS354 at the flowering stage in this study suggest that this cultivar had the most reduction in internal CO_2 substrate under drought stress, explaining its high yield reduction. Early stomatal closure was associated with a decrease in CO_2 assimilation under drought stress in barley (*Hordeum vulgare*), and that led to poor productivity (Ghotbi-Ravandi et al. 2014).

Noteworthy is that at the pod filling stage, only UVE17 had a huge reduction in stomatal conductance by up to 59%. All other cultivars had increased stomatal conductance at the pod filling stage. This cultivar showed the lowest stomatal conductance (268.58 mmol/m².s). The reduction in stomatal conductance in UVE17 at pod filling shows that there was less CO_2 fixation in this cultivar. This could be an additional reason for its susceptibility because reduced stomatal conductance means that transpiration will be less, which could increase the leaf temperature and further inhibit the process of photosynthesis, but it could also be it's coping mechanism to reduce water loss. Photo-damage of the photosystems in plant leaves is directly linked to stomatal closure under drought stress (Chaturvedi et al. 2019). The highest stomatal conductance at the pod filling stage was recorded in UVE14 (532.25 mmol/m².s), followed by AGS354 (440.18 mmol/m².s) and AGS429 (378.37 mmol/m².s), showing that they had the highest CO_2 fixation capacity under drought stress.

The NSCs (i.e., glucose, sucrose, trehalose, and starch) serve as sources of energy for the cell and the allocation of carbon in the plant cells. Also, they serve as osmolytes (Makonya et al. 2019). The NSCs were previously reported to maintain the cell membrane integrity of plants under drought stress (Fu et al. 2011). Similarly in edamame, they were reported to play a role in drought stress tolerance (Moloi and Van der Merwe 2021). The findings of the current study showed that drought stress reduced the glucose content of all cultivars (except UVE7) at the flowering stage. The

reduction was substantial in UVE8 (90%). The findings match the reduction in stomatal conductance of all cultivars at the flowering stage, which is related to a reduction in photosynthetic efficiency in drought-stressed cultivars, leading to poor crop yield.

Thin-layer chromatography (TLC) results also support these findings. At the pod filling stage, glucose was lower compared to flowering; all drought-stressed cultivars had reduced glucose content as shown by very faint bands in the TLC plate. The reduction of glucose content at both growth stages suggests that glucose was in high demand during the entire development of the edamame plants under stress because it was used as a vital osmoregulator under osmotic stress (Yang et al. 2019). Glucose is required by plants for important growth processes such as seed germination, cotyledon expansion, development of leaves, flowering onset, and senescence. As a result, glucose tends to decrease in drought-stressed plants (Zahid et al. 2018). Very low glucose levels, as observed in UVE8 at the flowering stage may negatively affect the plant growth, development, and yield. The other contributing factor for low glucose levels, especially at the pod filling stage could be the lack of supply from starch and sucrose hydrolysis (Liu et al. 2004), as was observed in pigeon pea (*Cajanus cajan*) (Du et al. 2020b).

Drought stress did not affect the trehalose content of all cultivars at the flowering stage in this study. However, AGS354, AGS429, and UVE7 controls showed increased trehalose content. UVE17 had the lowest trehalose content at flowering (4.38 µg/mL) followed by AGS354 (14.06 µg/mL). At the pod filling stage, drought-stressed cultivars had elevated trehalose content, with AGS429 and UVE17 showing 87% and 92% increases. The increase in trehalose content at pod filling in drought-stressed cultivars is because this disaccharide is very effective in improving plant tolerance to drought by protecting membrane lipids, proteins, and biological structures against dehydration during water shortage (Alam et al. 2014; Di Gioacchino et al. 2021). However, for UVE17, this increase may not be sufficient to improve the drought tolerance of edamame. Low trehalose content in drought-tolerant cultivar, UVE14 (17.92 µg/mL), further shows that this metabolite may not necessarily be used as a biochemical marker of drought tolerance in edamame. In contrast, Farooq et al. (2018) found that trehalose accumulation in chickpeas improved tolerance to drought stress.

Trehalose is a disaccharide of glucose units while sucrose is a disaccharide of glucose and fructose. Both trehalose and sucrose have a molecular weight of 342. 296 g/mol, thus, they migrated to the same position on the TLC. Plants depend on sucrose as an energy source for metabolic processes and the biosynthesis of amino acids for the development and growth of seeds (Du et al. 2020a). In this study, drought stress did not have any effect on the sucrose content at the flowering stage. UVE17 had the lowest sucrose content (5.31 µg/mL), and this cultivar was different from others, suggesting that sucrose might have a vital role in drought tolerance in edamame. At the pod filling stage, there was an overproduction of sucrose in stressed plants leaves. The sucrose content was high in AGS429, UVE8, UVE14, and UVE17, with UVE14 showing the highest sucrose content (38.62 µg/mL). The TLC plate verified the results, showing the intense second band (indicated by the black arrow). In agreement with our findings, sucrose was induced under drought stress in grain soybean (*Glycine max*) at the pod filling stage (Du et al. 2020a).

High sucrose content is necessary for edamame leaves at pod filling because this stage influences the composition, size, and weight of the seeds, which ultimately determines the yield quality and quantity (Du et al. 2020a)., Therefore, the mobilization of carbohydrates is very crucial during the pod filling stage. The increase in sucrose content at pod filling in this study might be directly linked to the requirement of the sugar for transport to form seeds at the pods, and less to do with drought tolerance. The overproduction of sucrose was also reported to be less correlated with drought stress compared to glucose accumulation (Zahid et al. 2018). Similarly, Du et al. (2020) found that sucrose accumulated in soybean leaves under drought stress. AGS429 and UVE14, which had high sucrose content at pod filling produced better yield (in terms of seed numbers and weight) compared to other cultivars (Appendix, Figure S6, Figure S7). These two cultivars (including AGS354) also had the highest stomatal conductance at the pod filling stage, which relates to their high rate of photosynthesis and CO_2 fixation under drought stress at the pod filling stage.

The starch content was only high for UVE14 at the flowering stage. Other cultivars had low starch content at the flowering stage. At pod filling, drought stress increased the starch content of AGS429. The starch content of other cultivars decreased relative to their controls. The iodine-starch complexation microscopy results support these findings. Some studies have shown that due to glucose being in high demand during

drought stress, starch gets hydrolyzed, and sucrose is actively transported from the source and broken down to glucose and fructose at sink tissues to meet the glucose demands (Liu et al. 2004). This reduction in starch due to breakdown under drought stress was reported to be a mechanism used by plants to cope with drought stress (Abdelhakim et al. 2021). Most cultivars (except UVE14) had very low starch content at flowering, which might be due to its hydrolysis to meet glucose demands in the plants at this growth stage, and this accounts for the high glucose content at the flowering stage in most cultivars (except UVE8). It is also clear that generally, starch decreased with glucose increase at flowering stage and vice versa at pod filling.

A study by Du et al. (2020) also showed a decrease in starch content for drought-stressed edamame cultivars, and the decrease was due to starch hydrolysis to form glucose. It is also important to note that starch serves as an energy source, and its accumulation in leaves indicates the capacity of the leaf source to form starch as a storage sugar under drought stress (Liu 2004; Du et al. 2020b). Therefore, starch as an energy source is hydrolyzed depending on soluble sugars requirements. At the pod filling stage of this study, the increase in starch means that it was not hydrolyzed, which might be due to less glucose demand at pod filling than flowering. The highest starch content in UVE14 at flowering and AGS429 at pod filling indicates that these two cultivars' leaves were better sources of starch under drought stress, and when there is high demand for soluble sugars their starch reserves could be readily available. The ability of AGS429 and UVE14 to produce high starch content at the pod filling stage might be linked to their drought tolerance.

3.6. Conclusions

The photosynthetic efficiency studies showed that AGS429 had better photosynthetic capacity due to its high Fv/Fm, $PI_{abs,}$ and PI_{total}. The accumulation of CRDs in all cultivars (except UVE17) also suggests that this pigment played a vital role in scavenging ROS under drought stress, which has damaging effects in the photosystems. On the other hand, the decline in CRDs in UVE17 suggests that this cultivar lacked PSII and PSI protection against ROS. This matched its decline in PI_{total} and very low stomatal conductance at the pod filling stage, suggesting its susceptibility to drought stress. The highest stomatal conductance in UVE14 and ability to produce high starch, and sucrose content (together with AGS429) under drought stress, implies

that these cultivars were less sensitive to drought stress compared to other cultivars. Therefore, CRDs, PI_{total}, stomatal conductance as well as starch are the most significant physiological/ biochemical parameters for drought tolerance and are recommended for drought tolerance screening in edamame. Cultivars AGS429 and UVE14 have better physiological and biochemical drought tolerance responses.

Chapter 4

The effect of drought stress on the cell wall modifications of six edamame cultivars

4.1 Abstract

Drought stress can affect the synthesis of plant cell wall carbohydrates, lignin, and phenols. These molecules can improve the integrity of cell walls, which might improve edamame tolerance to drought stress. This study aimed to investigate the effects of drought stress on the cell wall modifications of six edamame cultivars. The structural carbohydrates were determined using Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). Acid soluble lignin and the total phenolic compounds hydrolysis were determined spectrophotometrically. Drought stress did not have any significant effect on the total phenolic content of all cultivars, but the acid-soluble lignin was significantly increased in most cultivars except UVE14 and UVE17. The FTIR and XRD results suggest that the UVE14 cell wall was the most intact during drought stress followed by UVE8 and AGS429. Lignin and hemicellulose can be used as additional drought tolerance mechanisms in edamame.

Keywords: Cell walls, hemicellulose, lignin, microcrystalline cellulose.

4.2 Introduction

Edamame (vegetable type soybean) is an important crop to promote in South Africa due to its high nutritional value and health benefits. South Africa produces about one million tons of commodity type soybean every year, but edamame is less produced (Arathoon 2015). In addition, drought stress is accelerated by climate change, which leads to crop loss and total crop revenue drop in Sub-Saharan Africa (Keatinge et al. 2011). Edamame research is more intense in East Asia and North America, however, the effect of drought stress on the biochemical and physiological responses of edamame cultivars needs to be investigated and understood in the South African context, because there is not much information on how these influence crop yield.

Sucrose produced during the Calvin cycle of photosynthesis is transported throughout the plant to sink tissues (Yang et al. 2019). In the sink tissues, sucrose can be converted to many carbohydrates, one of them is the uridine diphosphate glucose (UDP-glucose) which undergoes polymerization to make cell wall components

(glucans) such as cellulose, hemicelluloses (Barnes and Anderson 2018), and phenolic compounds including lignin, which are synthesized in the phenylpropanoid pathway (Poovaiah et al. 2014; Silva et al. 2019). Lignin as phenol is an important component of the secondary cell wall as it strengthens the water-conducting tissues and makes them more hydrophobic (Silva et al. 2019). Plants maintain cell wall integrity while regulating plant growth, communication between cells, plant development, adhesion, and defence against abiotic stress (Barnes and Anderson 2018).

Drought stress can affect the synthesis of plant cell wall polysaccharides. These polysaccharides are important because they can improve the cell wall integrity of drought-stressed plants (Le Gall et al. 2015), thereby improving drought tolerance. The cross-linking of lignin and phenols in drought-stressed plants adds strength to the cell walls (Frei 2013). Phenols also act as reducing agents and are effective in neutralizing the reactive oxygen species (ROS) under drought stress (Sánchez-Rodríguez et al. 2011). It is, therefore, important to study the cell wall sugars (cellulose and hemicellulose), total phenolic compounds, and lignin because they are used as the first line of defence (Le Gall et al. 2015). This will enrich our understanding of the biochemical defence mechanisms in edamame under drought stress. It will also aid in selecting cultivars with the best cell wall modifications for breeding purposes. Therefore, this chapter investigated the effects of drought stress on the cell wall modifications of six edamame cultivars.

4.1. Materials and methods

4.1.1. Plant materials

The six edamame cultivars used for this study are as described in Chapter 3, section 3.3.1. The cell wall studies were done using pod filling samples only, according to Stolle-Smits et al. (1999), because the cell wall precursors such as sucrose increased more at pod filling of edamame development. In agreement, Moloi and Van der Merwe (2021) also found larger increases in the biochemical parameters at pod filling as compared to flowering in edamame. Therefore, the pod filling stage was selected to represent the cell wall integrity of all cultivars.

4.1.2. Lignin and total phenolic content

The total phenolic content and lignin were determined according to Sluiter et al. (2008), by oven drying 60 mg of ground leaf tissues followed by extraction in 2 mL of 97% (v/v) sulphuric acid (H_2SO_4) (Sigma Aldrich, USA). After incubation at 30°C for 1 hour, the tube contents were neutralized to 4% H_2SO_4 with calcium carbonate ($CaCO_3$) (BDH Chemicals, England), and autoclaved at 121°C for 20 minutes.

Total phenols were determined by first preparing a standard curve using 1 mg/mL gallic acid (Sigma, China) as standard and using Folin–Ciocalteu (F–C) reagent (Sigma, Switzerland), which was diluted 3X with dH_2O, and 35% (w/v) sodium bicarbonate (Sarchem, RSA) were used for detection. The absorbance of the samples and standards were measured at 700 nm and the concentrations of samples (5X with dH_2O) were determined using the standard curve (Appendix, Figure S3).

Acid soluble lignin percentage was determined by measuring the absorbance at 320 nm and the percentage was calculated using equation (9) (Sluiter et al. 2008).

$$\% \text{ Lignin} = [(A320 \times V \times D)/ (\text{Ɛ} \times l \times ODW) \times 100] \quad (10)$$

Where A320 = absorbance at 320 nm, V = extract volume, D = dilution factor, Ɛ = extinction coefficient at 320 nm, and ODW = oven-dried weight.

4.1.3. Fourier-transform infrared spectroscopy analysis of cell wall components

The Fourier-transform infrared (FTIR) spectroscopy was done using a method described by Trilokesh and Uppuluri (2019). Leaf samples were dried for 72 hours at 60°C in an oven. About 1 mg of the dried tissue was mixed with 200 mg of oven-dried potassium bromide (KBr) (Sigma, France) to make a transparent disk using the finely ground mixture of a leaf sample with the aid of a hydraulic press. KBr was used as the blank during the FTIR procedure. The FTIR micro-spectroscopic imaging system used was “Nicolet Continuum Infrared Microscope” (Thermo Scientific, USA), using omnic series software. Each sample was scanned 16x and collected in absorbance mode in the 4000 cm^{-1} - 800 cm^{-1} region at 4 cm^{-1} spectral resolution.

4.1.4. X-Ray diffraction analysis of biomass

The x-ray diffraction (XRD) was done using a method described by Trilokesh and Uppuluri (2019). The Bruker diffractometer (USA) was used to do XRD for all ground oven-dried samples (dried for 72 hours at 60°C) using CuK*a* radiation generated at 40 kV and 130 mA at Coupled 2θ/Theta scanning angle and a speed of 0.5°/ minute. A single-channel analyzer was used to filter out the CuKα radiation from the data so that it does not contribute to the final data. The crystallinity index of cellulose was represented as percentages.

4.1.5. Statistical analysis

Statistical analysis was done for acid-soluble lignin and total phenols using Genstat Release 19 software (VSN International 2018), as described in Chapter 3, section 3.3.8.

4.2. Results

4.2.1. Analysis of variance

Water treatment and cultivar by water interaction were significant for acid-soluble lignin ($P \leq 0.01$) and ($P \leq 0.05$), there was no significance for total phenol content (Table 4.1).

Table 4.1. Analysis of variance representing mean square (ms) values for the cell wall parameters during the pod-filling stage of the six edamame cultivars under two water treatments [100% water holding capacity, (WHC) and 30% WHC].

Variate	Cell Wall		
	Cultivar (C)	**Treatment (T)**	**CxT**
ASL	0.2414	2.4620**	0.7018*
TP	0.415	0.265	1.224
CV%	13.22	80.77	27.11
Grand Mean	0.6564	1.36	0.9629

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ASL = Acid soluble lignin, TP = Total phenols, CV = Coefficient of variation

4.2.2. Acid soluble lignin and total phenolic content

Drought stress significantly increased soluble lignin content in the cell walls of AGS354 (21%), AGS429 (27%), UVE7 (20%), and UVE8 (17%) (Figure 4.1).

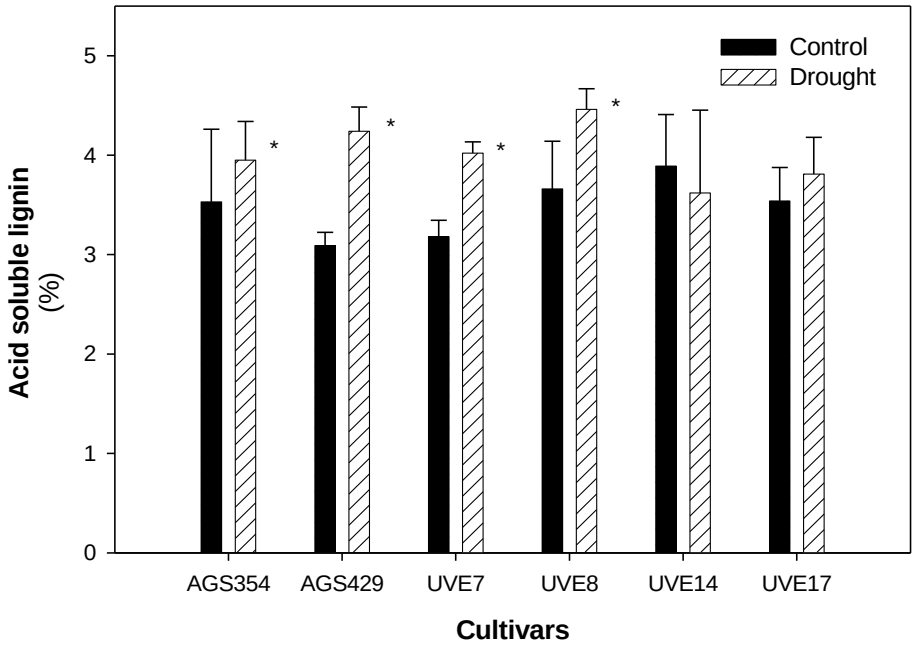

Figure 4.1. The acid-soluble lignin content of six edamame cultivars at pod filling stage. Asterisk represents a significant increase under drought stress at $P \leq 0.05$. Values represent means ± SD (n= 3).

Drought stress did not have any significant effect on the total phenol content of all cultivars (Figure 4.2).

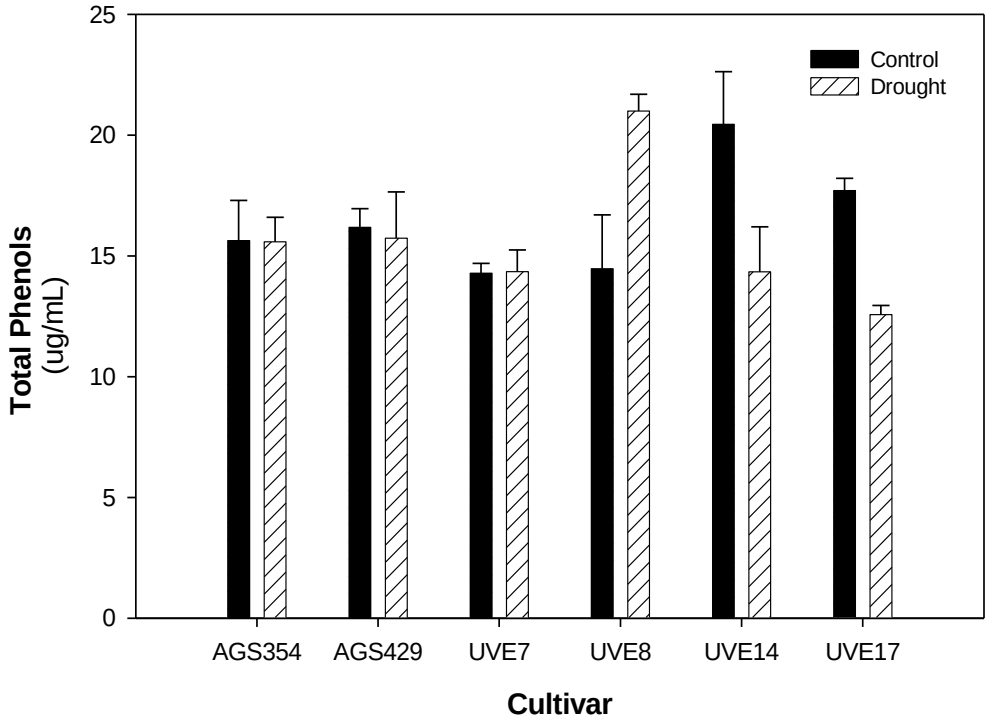

Figure 4.2. Total phenolic content of six edamame cultivars at pod filling stage. Values represent means ± SD (n= 3).

4.2.3. Fourier-transform infrared spectroscopy

Drought stress significantly reduced the crystalline cellulose at 3800 – 3000 cm^{-1}, lignin (1458 cm^{-1}), lignin/holocellulose (1450-1300; 1300-1000 cm^{-1}), unconjugated hemicellulose (1733 cm^{-1}), cellulose (2850-2918 cm^{-1}) of drought-stressed AGS354 (Figure 4.3). A similar pattern was observed from the spectra of UVE17. The amide (amino acid functional group) stretching at 2300 cm^{-1} was significantly reduced by drought in AGS354, but it was not reduced in UVE17. Drought stress slightly reduced the crystalline cellulose at 3800 – 3000 cm^{-1}, amide (2300 cm^{-1}), lignin (1458 cm^{-1}), and lignin/holocellulose (1450-1300 cm^{-1}; 1300-1000 cm^{-1}), but unconjugated hemicellulose (1733 cm^{-1}), cellulose (2850-2918 cm^{-1}) regions were not reduced in AGS429. In UVE7, drought stress only significantly reduced the crystalline cellulose (3800-3000 cm^{-1}) and the lignin/holocellulose (1450-1300 cm^{-1}; 1300-1000 cm^{-1}). The amide stretching at 2300 cm^{-1} was also lower in drought-stressed UVE7. Drought stress only reduced the crystalline cellulose (3800 – 3000 cm^{-1}) significantly, other regions such as the amides, lignin/holocellulose, unconjugated hemicellulose, and cellulose were not reduced in UVE8. In UVE14, drought stress slightly increased all

cell wall sugars (lignin/holocellulose, unconjugated hemicellulose, and cellulose) but reduced amides (2300 cm^{-1}) (Figure 4.3).

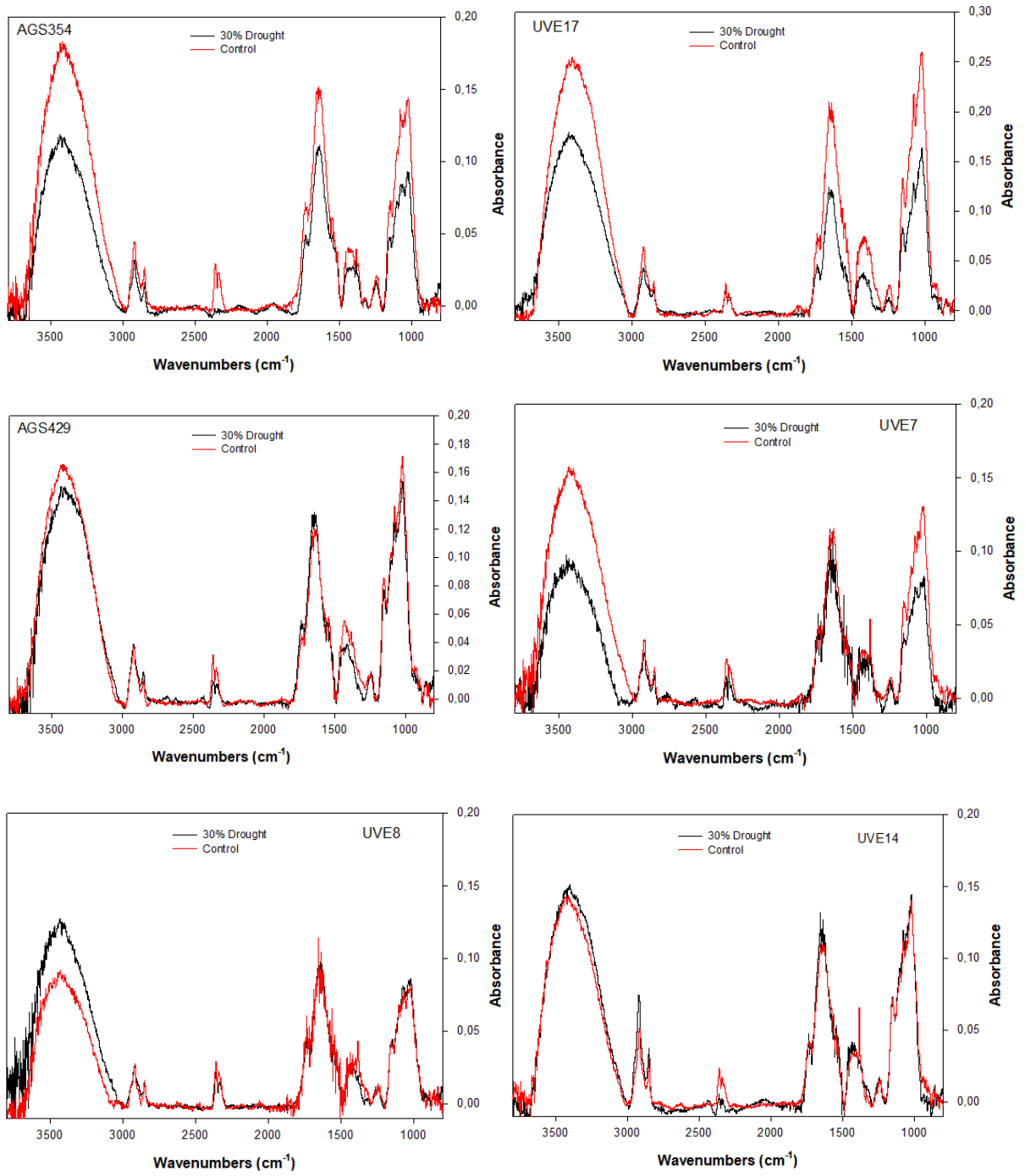

Figure 4.3. FTIR spectra of six edamame cultivars exposed to drought stress and the controls. Spectrum for cell-wall composition was performed in the region between 4000 cm^{-1} - 800 cm^{-1} at 4 cm^{-1} spectral resolution. Each sample was scanned 16x and collected in absorbance mode.

4.2.4. X-ray diffraction analysis of cellulose

Drought stress increased the crystallinity index of AGS354 by 3.3%, UVE14 (9%), and UVE17 by 12%. On the other hand, drought-stress decreased the crystallinity index of AGS429, UVE7, and UVE8 by 5.6%, 4.1%, and 1.3%, respectively (Table 4.2).

Table 4.2. X-ray diffraction (XRD) analysis of cellulose crystallinity index during the pod filling stage of the six edamame cultivars under two water treatments [100% water holding capacity, (WHC) and 30% WHC].

Cultivar	**Crystallinity index (%)**	
	Control (100% WHC)	**30% WHC**
AGS354	38.7	**42.0**
AGS429	42.0	36.4
UVE7	38.7	34.2
UVE8	32.7	31.4
UVE14	32.1	**42.0**
UVE17	26.3	**38.3**

4.3. Discussion

Plant cell walls are essential in protecting plant cells against a wide variety of biotic and abiotic stresses. The main plant cell wall component involved in aiding resistance is lignin, produced in the phenylpropanoid pathway (Zhao et al. 2021). The cross-linking of lignin and phenols add strength to cell walls (Frei 2013). Previous reports suggested the involvement of lignin and the total phenols in the drought tolerance responses (Puente-Garza et al. 2017). In this study, water treatment and cultivar by water treatment were significant ($P \leq 0.01$) and ($P \leq 0.05$) for acid-soluble lignin. Drought stress increased the ASL of AGS354, AGS429, UVE7, and UVE8. The acid-soluble lignin for UVE14 was slightly reduced by drought.

The increase in acid-soluble lignin in AGS429 suggests the involvement of lignin in edamame drought resistance. In addition, the reduced lignin content in UVE14 can be linked to higher stomatal conductance because lignin prevents water loss (Silva et al.

2019). Therefore, the reduced lignin content in UVE14 is in line with its high stomatal conductance values, suggesting that transpiration occurred during drought in this cultivar. The increase in acid-soluble lignin in most cultivars suggests that drought triggered the synthesis of high lignin content to strengthen the cell walls of the plants and prevent water loss. This is an important mechanism displayed by the edamame cultivars to prevent water loss and maintain cell wall rigidity under drought stress in agreement with Mnich et al. (2020), and Naderi et al. (2020). The increase in lignin content under drought stress was also reported in tea (*Camellia sinensis*) plants by Gu et al. (2020).

In addition to cross-linking with lignin, phenols have antioxidative properties. They can act as reducing agents by donating hydrogen to detoxify ROS under drought stress (Sánchez-Rodríguez et al. 2011). It was well documented that total phenols increase in drought-stressed plants (Gharibi et al. 2016). However, in this study, drought stress did not have any effect on the total phenolic content of all cultivars under drought stress, and a non-significant decrease was observed in UVE14 (30%) and UVE17 (29%). UVE8 had the highest total phenolic content (21.0 µg/mL), followed by AGS429 (15.74 µg/mL). These two cultivars also had the highest lignin content as shown. The non-significant changes (decrease/increase) in total phenolic content suggest that the cultivars had strong cell walls cross-linking bonds, to aid in drought resistance and these findings match acid-soluble lignin results. Furthermore, the findings of total phenols also suggest that at first the plants used a redox mechanism to detoxify ROS and then increased lignin levels to add strength to their cell walls in agreement with Sánchez-Rodríguez et al. (2011), and Naderi et al. (2020). The total phenolic content findings in this study coincide with Puente-Garza et al. (2017), who found insignificant effects in total phenolic levels in *Agave salmiana* under drought, and contrary to Naderi et al. (2020) who found a significant increase in total phenolic content in wheat. This means that the total phenolic content might differ with plant genotype under drought stress.

The stiffening of cell walls is a primary plant response to drought stress, which results in limited cell extension and improves structural resistance (Van der Weijde et al. 2017). Cellulose, a fundamental cell wall component, is a high molecular weight straight-chain polymer. It is made up of D-glucose units which are linked together with β-(1→4) glycosidic bonds (Hindi and Hindi 2016; Mnich et al. 2020). The

microcrystalline cellulose is very stable, stiff, and hydrophobic due to the inter-and intra-chain hydrogen bodies that form a 3-dimensional structure (Mnich et al. 2020). The hydrophobic, self-interacting, and inherent strength nature of microcrystalline cellulose in plants can reduce water loss under drought stress. Other regions of cellulose microfibrils, known as the amorphous regions are weak due to their weak internal hydrogen bonding (Ruel et al. 2012). The holocellulose, which is all the hemicelluloses and cellulose were previously reported to negatively correlate with lignin in tree rings of oak trees (*Quercus petraea*) (Potts et al. 2004).

The analysis of cell wall biomass using FTIR and XRD provides essential information in understanding the edamame's responses to drought stress. Drought stress reduced most cell wall structural carbohydrates (cellulose and hemicellulose) in AGS354, UVE7, and UVE17 as shown in the FTIR spectrum. The crystalline cellulose, amorphous cellulose, hemicellulose, lignin, lignin/holocellulose regions of these cultivars showed reduction due to drought stress. These findings corroborate the liquid chromatography-mass spectrometry (LC-MS) results (Appendix, Figure S1, Figure S2), showing decreases in glucose and xylose in AGS354 and UVE17, which is linked to low cellulose and hemicellulose content in these cultivars. The reduced amorphous celluloses, microcrystalline cellulose, and hemicelluloses are accountable for the reduced holocellulose (1450-1300; 1300-1000 cm^{-1}) in AGS354, AGS429 (slightly), UVE7, and UVE17.

The CrI findings from XRD showed that the crystallinity of microcrystalline cellulose was increased by 3.3% in AGS354 and 12% in UVE17, which is not in line with the FTIR spectra at 3800 – 300 cm^{-1}. The microcrystalline cellulose is embedded in a network of amorphous polymer structures of pectin, hemicelluloses, and lignin to strengthen the cell wall (Mnich et al. 2020). Any changes that occur to this network affect the cell wall crystallinity and analysis of microcrystalline cellulose (Mafa et al. 2020a; De Caroli et al. 2021; Song et al. 2021). AGS354 and UVE17 were previously reported to be very unstable under drought stress (Van der Merwe et al. 2018). The unstable cell wall structure of these cultivars analyzed in this study might be another reason for their susceptibility to drought stress.

The hemicellulose is mostly composed of xylose, which can be converted to ethanol easily, leading to weak cell walls (Emery et al. 2020). The xylose conversion under

drought stress might be accountable for the decrease in unconjugated hemicellulose in AGS354 and UVE17, as shown in the FTIR spectrum and supplementary results (Appendix, Figure S2). The CrI of microcrystalline cellulose was increased by 9% in UVE14, which is in line with the UVE14 FTIR spectra and LC-MS findings (Appendix, Figure S1, Figure S2) that showed an increase in glucose and xylose, meaning that there was a high holo-cellulolytic content under drought stress in this cultivar. Analysis of the whole FTIR spectrum of UVE14 and UVE8 suggest that these two cultivars had the most intact cell walls under drought stress due to elevated cell wall components. Drought stress decreased cellulose, and lignin but increased hemicellulose content in soybean plants (Al-Hakim 2006). van der Weijde et al. (2017) also found a decrease in cellulose content with an increase in hemicelluloses in M*iscanthus* genotypes under drought stress.

4.4. Conclusions

The FTIR, and XRD, suggest that the UVE14 cell wall was the most intact during drought stress due to high lignocellulosic biomass, followed by UVE8, and AGS429 which only had a slight insignificant decrease in crystalline cellulose and holocellulose. The other peaks of AGS429 were the same as its control, suggesting a strong cell wall defence mechanism. Other cultivars, AGS354, UVE7, and UVE17 had reduced lignocellulosic biomass, suggesting the weak nature of their cell walls under drought stress. The accumulation of lignin and hemicelluloses in UVE14, UVE8, and AGS429 suggest that these can be used as additional drought tolerance mechanisms in edamame.

Chapter 5

The relationships between the photosynthetic efficiency parameters and the cell wall responses

5.1 Abstract

Edamame is a water-demanding crop with about 40% of its yield reduced by drought stress. This study aimed to establish the relationships between the photosynthetic efficiency parameters (Fv/Fm, PI_{abs}, PIt_{total}, Chl-a, Chl-b, CRDs, glucose, trehalose, sucrose, and starch), and the cell wall responses (lignin, and total phenolic content). Such information is valuable to establish the physiological and biochemical mechanisms of drought tolerance in edamame. The correlations between photosynthesis and cell wall parameters were established under drought stress. The principal component analysis was also performed under drought stress to visualize the relationships between the parameters and cultivars. The CRDs, performance indexes (PI_{abs} and PI_{total}), starch, and total seed mass per plant had significant positive correlations and they were closely associated with AGS429, UVE14, and UVE8. Lignin and hemicellulose also accumulated in cultivars that were less sensitive to drought stress (AGS429, UVE14, and UVE8). Therefore, the CRDs, PI_{abs}, PI_{total}l, starch, lignin, and hemicelluloses could be considered as additional physiological mechanisms of drought tolerance in edamame.

Keywords: Carotenoids, chlorophyll-a, chlorophyll-b, glucose, hemicellulose, lignin, starch.

5.2 Introduction

Anthropogenic climate change has a negative impact in Sub-Saharan Africa, because it accelerates droughts, resulting in huge crop losses of up to 50% and total crop revenues drop by 90%. These negatively affect small scale farmers (Keatinge et al. 2011). The introduction of crops such as edamame, with a high nutritional value, is important (Keatinge et al. 2011). However, edamame demands a lot of water crop with about 40% of its yield reduced by drought stress (Shaheen et al. 2016). There has not been much research done on edamame in South Africa since its introduction in the country. More research needs to be done to determine some biochemical, physiological, and morphological responses to abiotic stress because they have a significant influence on the yield in plants.

Plants respond to abiotic stresses such as drought by modifying their morphological structures, biochemical processes, and physiological features (Moloi and Van der Merwe 2021). Previous studies in edamame showed that some cultivars had induced biochemical and physiological features against abiotic stress (Arathoon 2015). Photosynthesis parameters, such as pigments, the quantum efficiency of PSII (Fv/Fm), absorption in performance index of PSII and PSI (PI_{abs}, and PI_{total}) and stomatal conductance have been widely used to monitor plants' photosynthetic capacity under drought stress (Ustin et al. 2009; Gorthi et al. 2019; Mihaljević et al. 2021). Moloi and van der Merwe, (2021) demonstrated that some of the drought tolerance responses include the accumulation of the total soluble sugars, which are the products of photosynthesis. The accumulation of lignin and phenols were also linked to drought tolerance, but the responses of cellulose and hemicelluloses might differ depending on the plant genotype (Sánchez-Rodríguez et al. 2011; Le Gall et al. 2015).

It is complicated to identify suitable biochemical and physiological mechanisms of edamame tolerance responses because different cultivars respond differently to drought stress. The use of statistical tools such as correlations and principal component analysis (PCA) are excellent in helping to identify suitable cultivars and biochemical parameters for drought tolerance. Chapter 3 and Chapter 4 of this work focused on establishing the photosynthesis capacity and the cell wall responses of edamame when exposed to severe drought stress. The work identified specific physiological and biochemical responses that are associated with drought tolerance in edamame as well as cultivars with more drought tolerance responses. However, the relationships between the physiological and biochemical responses are not known. Therefore, this study aimed to establish the relationships between the photosynthetic efficiency parameters [Fv/Fm, PI_{abs}, PI_{total}, chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), carotenoids (CRDs), stomatal conductance, glucose, trehalose, sucrose, and starch], the cell wall responses (lignin, and total phenolic content), and how the different cultivars relate to these parameters using the PCA.

5.3 Materials and methods

5.3.1 Plant materials and methods

The six edamame cultivars, growth conditions as well as water treatments are used for this study are described in chapter 3, section 3.3.1. The photosynthesis capacity and cell wall methods are described in Chapter 3 and Chapter 4. Data used for this chapter is extracted from Chapters 3 and Chapter 4.

5.3.2 Statistical analysis

The analysis of variance (ANOVA) for photosynthesis, cell walls, and yield parameters was done using Genstat Release 19 software (VSN International 2018), as described in Chapter 3, section 3.3.8. The relationships between the parameters were determined using Pearson's correlations for determining the significant positive and negative relationships of photosynthesis and cell wall parameters, to identify positively correlated parameters because they might contribute to drought tolerance in edamame. The PCA was performed to represent principal component 1 (PC1) and principal component 2 (PC2) used to determine to show the contribution of photosynthetic, cell walls and yield parameters to the variability of the six edamame cultivars at 30% drought stress at the flowering and pod filling stages, respectively.

5.4 Results

5.4.1 Correlations and principal component analysis

Table 5.1 and Table 5.2 represent the correlations between the photosynthesis, cell wall, and morphological parameters analyzes at the flowering and pod filling stage, under severe drought stress conditions. At the flowering stage (Table 5.1), CRDs had significant positive correlations ($P \leq 0.05$) with Chl-a, $PI_{abs,}$ and PI_{total}. PI_{abs} had a significant and strong positive correlation ($P \leq 0.01$) with Fv/Fm and PI_{total}. Chl-a had a significant positive correlation with starch ($P \leq 0.05$), while on the other hand, it had a significant negative correlation with Chl-b ($P \leq 0.05$). The correlation between PI_{abs} and trehalose was strong and significantly negative ($P \leq 0.01$). At the pod filling stage (Table 5.2), Chl-a showed a strong and significant positive correlation with carotenoids ($P \leq 0.01$) and a significant positive correlation with the total phenols ($P \leq 0.05$). PI_{abs} showed a strong and significant positive correlation with Fv/Fm ($P \leq 0.01$). There was

a significant and positive correlation between glucose and acid-soluble lignin ($P \leq 0.05$).

Table 5.1. Correlations during the flowering stage of drought-stressed edamame at 30% water holding capacity, WHC

	100 SM	Chl-a	Chl-b	CRDs	Fv/Fm	GLU	PI_{abs}	PI_{total}	ASL	Starch	g_s	SUC	TPP	TSMP	TSP	TP	TRE
100 SM	-																
Chl-a	0.119	-															
Chl-b	-0.094	**-0.492***	-														
CRDs	0.146	**0.503***	0.025	-													
Fv/Fm	0.201	-0.366	0.254	-0.001	-												
GLU	0.014	-0.029	-0.002	-0.045	0.027	-											
PI_{abs}	0.143	-0.170	0.202	**0.494***	**0.684****	0.002	-										
PI_{total}	0.167	-0.014	0.0582	**0.480***	0.282	0.000	**0.810****	-									
ASL	-0.458	0.075	-0.083	-0.131	-0.090	0.422	-0.170	-0.253	-								
Starch	0.270	**0.484***	-0.359	0.185	-0.248	-0.347	-0.087	0.064	-0.323	-							
g_s	0.266	-0.176	-0.041	-0.070	-0.467	0.161	-0.234	0.137	-0.297	0.183	-						
Suc	-0.265	-0.189	0.208	-0.253	-0.262	-0.097	-0.394	-0.105	0.091	-0.085	0.225	-					
TPP	-0.137	-0.131	-0.174	-0.354	-0.042	-0.193	-0.165	-0.145	0.318	-0.313	-0.317	0.027	-				
TSMP	-0.114	**-0.489***	0.199	-0.426	0.163	-0.0600	-0.109	-0.240	-0.008	**0.62****	0.026	0.201	0.266	-			
TSP	0.129	-0.095	0.183	-0.308	0.050	-0.227	-0.187	-0.232	-0.059	-0.014	0.121	0.186	0.036	**0.553***	-		
TP	-0.447	0.114	0.152	0.162	-0.022	0.010	0.147	0.136	-0.137	-0.103	-0.289	-0.118	-0.075	0.031	-0.22	-	
TRE	-0.389	0.208	-0.280	-0.434	-0.332	0.186	**-0.628****	-0.391	0.121	0.054	0.079	0.503	-0.112	0.060	0.017	0.159	-

* P ≤ 0.05, ** P ≤ 0.01, 100 SM = 100 seed mass, Chl-a = Chlorophyll-a, Chl-b = Chlorophyll-b, CRDs = Carotenoids, Fv/Fm = ratio of variable fluorescence to maximal fluorescence of PSII, PI_{abs} = performance index of PSI and PSII, PI_{total} = total performance index of PSI and PSII, PS = photosystem, ASL = acid soluble lignin, gs = stomatal conductance, TPP = total pods per plant, TSMP = total seed mass per plant, TSP = total seeds per plant, TP = total phenols, TRE = trehalose, GLU = glucose, SUC = sucrose.

Table 5.2. Correlations during pod filling stage of drought-stressed edamame at 30% water holding capacity, WHC

	100 SM	Chl-a	Chl-b	CRDs	Fv/Fm	GLU	PIabs	PItotal	ASL	Starch	gs	SUC	TPP	TSMP	TSP	TP	TRE
100 SM	-																
Chl-a	**-0.513***	-															
Chl-b	0.259	-0.389	-														
CRDs	-0.424	**0.87****	0.010	-													
Fv/Fm	-0.040	-0.236	0.125	-0.201	-												
Glu	-0.308	0.225	-0.079	0.204	-0.162	-											
PIabs	0.234	-0.465	0.108	-0.386	**0.874****	-0.401	-										
PItotal	0.244	-0.012	-0.156	-0.163	0.098	-0.383	0.210	-									
ASL	-0.458	-0.021	0.105	0.057	-0.252	**0.487***	-0.366	-0.232	-								
Starch	0.360	-0.415	0.442	-0.311	0.159	-0.139	0.231	0.206	-0.176	-							
gs	0.370	0.118	-0.099	0.068	0.237	0.278	0.124	0.038	-0.298	0.174	-						
SUC	0.064	-0.027	-0.057	-0.012	0.035	-0.410	0.171	0.407	-0.281	0.082	-0.325	-					
TPP	-0.137	-0.315	0.035	-0.431	0.196	0.413	0.079	-0.186	0.318	0.060	0.045	-0 .098	-				
SMP	-0.114	-0.0800	-0.234	-0.219	0.056	0.155	0.049	-0.047	-0.008	0.219	0.085	-0.407	0.266	-			
TSP	0.129	-0.220	-0.295	-0.304	0.146	-0.029	0.212	0.253	-0.059	0.322	0.270	0.110	0.036	**0.553***	-		
TP	-0.447	**0.588***	-0.149	0.459	0.199	-0.003	0.026	-0.126	-0.136	-0.133	-0.091	-0.035	-0.075	0.031	-0.217	-	
TRE	0.057	-0.449	0.173	-0.391	0.146	-0.252	0.243	0.080	0.167	0.402	-0.116	-0.367	-0.166	0.450	0.286	-0.327	-

* P ≤ 0.05, ** P ≤ 0.01, 100 SM = 100 seed mass, Chl-a = Chlorophyll-a, Chl-b = Chlorophyll-b, CRDs = Carotenoids, Fv/Fm = ratio of variable fluorescence to maximal fluorescence of PSII, PI_{abs} = performance index of PSI and PSII, PI_{total} = total performance index of PSI and PSII, PS = photosystem, ASL = acid soluble lignin, gs = stomatal conductance, TPP = total pods per plant, TSMP = total seed mass per plant, TSP = total seeds per plant, TP = total phenols, TRE = trehalose, GLU = glucose, SUC = sucrose.

Results from the PCA biplot are presented in Figure 5.1 and Figure 5.2 for the flowering and pod fillings stages, respectively. At flowering, PC1 and PC2 together explained 61.84% of the variation at 30% soil water holding capacity (WHC) (Figure 5.1). Chl-b, glucose, acid-soluble lignin, sucrose, total phenols, and trehalose showed positive loadings with PC1. Fv/Fm, PI_{abs}, PI_{total} and stomatal conductance showed positive loadings with PC2. The positive associations observed in the biplot are in accordance with the significant correlations observed in Table 5.1. For example, the significant correlations between Chl-a and CRDs; Chl-a and starch; PI_{abs} and CRDs; PI_{total} and CRDs; Fv/Fm and PI_{abs}; PI_{abs} and PI_{total}. In addition, the negative associations between some parameters such as Chl-a and Chl-b; PI_{abs} and trehalose are in accordance with the significant negative correlations in Table 5.1. At flowering, AGS354 is at average with almost all the parameters tested. UVE14 showed a positive association with starch content.

At pod filling (Figure 5.2), PC1 and PC2 together explained 62.01% of the variation at 30% drought stress. Chl-b, Fv/Fm, PI_{abs}, PI_{total}, stomatal conductance, sucrose, starch, and trehalose showed positive loadings with PC1. Glucose, total phenols, chl-a and CRDs showed positive loadings with PC2. The positive associations observed between Chl-a and CRDs, Fv/Fm and PI_{abs}, acid-soluble lignin and glucose are in accordance with results obtained in Table 5.2. At pod filling, AGS354 is close to average with all the parameters tested. This cultivar previously showed high contents of Chl-b and acid-soluble lignin content (Table 5.2). Similarly, AGS429 had significantly high Fv/Fm, PI_{abs}, Chl-b, sucrose, trehalose, starch, and acid-soluble lignin.

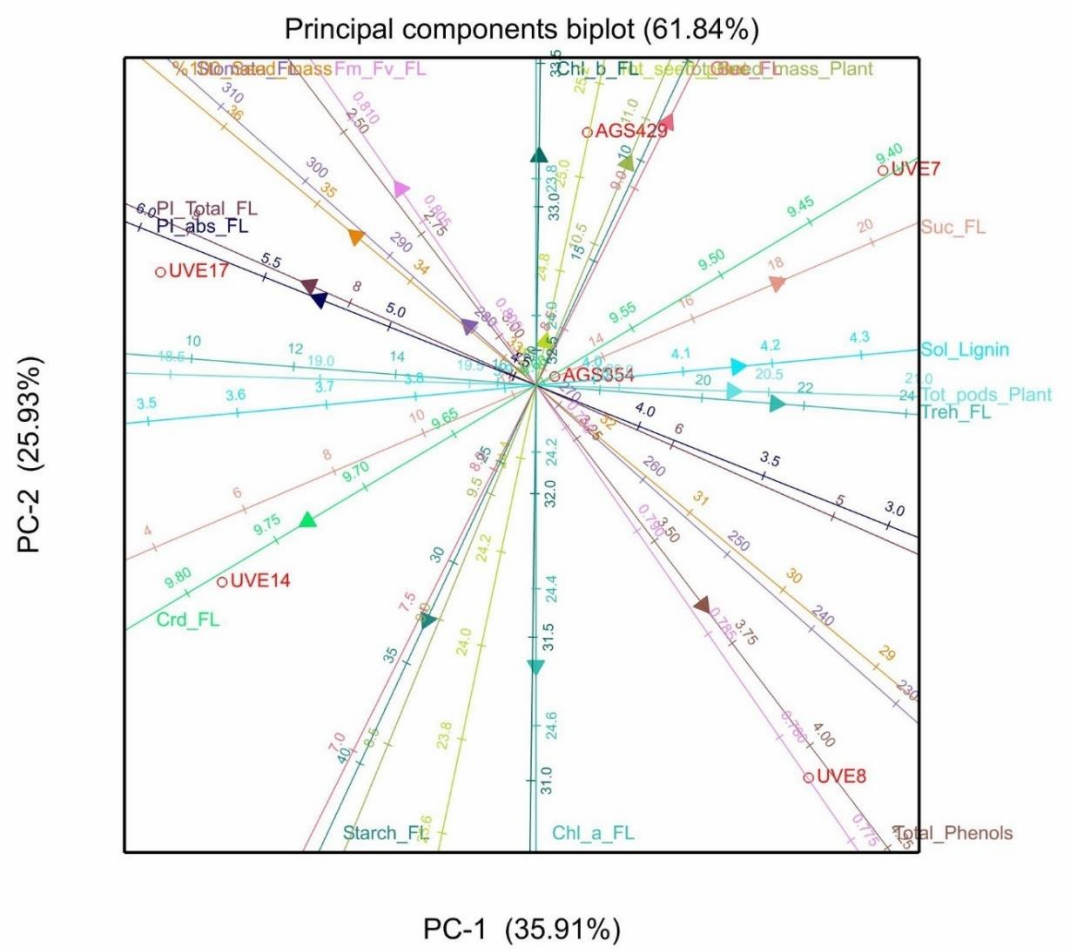

Figure 5.1. Principal components analysis biplot representation of PC1 and PC2 showing the contribution of photosynthetic, cell walls and yield parameters to the variability of six edamame cultivars under drought stress [30% water holding capacity, (WHC)] at the flowering stage.

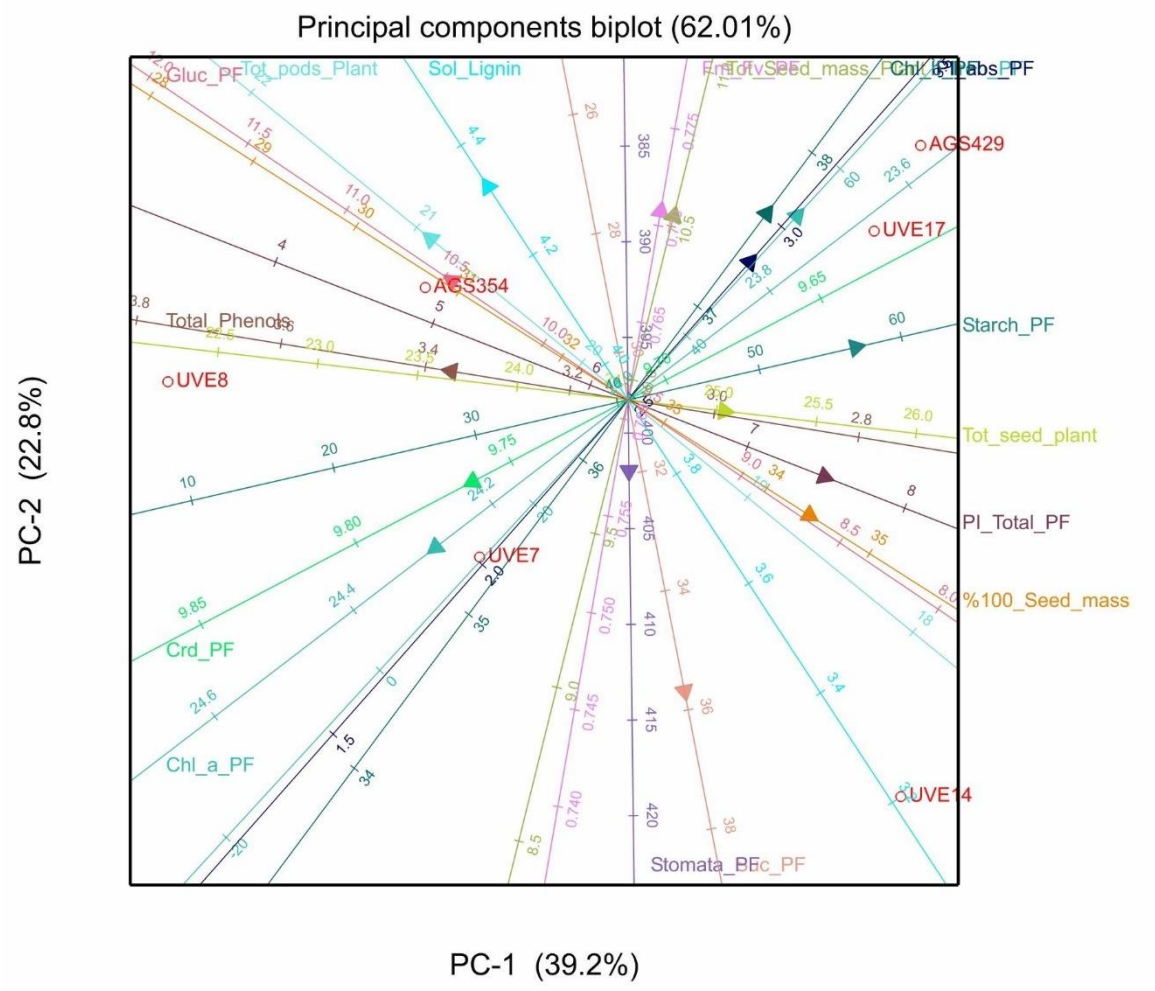

Figure 5.2 Principal components analysis biplot representation of PC1 and PC2 showing the contribution of photosynthetic, cell walls and yield parameters to the variability of six edamame cultivars under drought stress [30% water holding capacity, (WHC)] at the pod filling stage.

5.5 Discussion

There was a significant positive relationship ($P \leq 0.05$) between Chl-a, and starch at flowering with UVE14 and UVE8 showing high Chl-a, and starch contents. The relationship between these two cultivars for Chl-a and starch was further confirmed by the PCA. These findings coincide with Zhang et al. (2021) who found that chlorophyll content directly correlated with plant productivity in a grassland. In addition, the strong positive correlation ($P \leq 0.01$) between starch and total seed mass per plant also supports the fact that starch accumulation at flowering had a significant role in plant productivity in edamame under drought stress. Chl-a was also positively and significantly correlated to the carotenoids both at flowering and pod filling ($P \leq 0.01$). The significant negative relationship between Chl-a and Chl-b was due to the decrease of Chl-a with Chl-b increase (as observed in Chapter 3), suggesting that under drought stress Chl-a was possibly converted to Chl-b (Teng et al. 2021).

The PCA at flowering showed that UVE14 was closely associated with high CRDs and Chl-a content, whereas AGS429 was closely associated with high Chl-b and high yield. AGS354 lies at the centre of the PCA, suggesting that almost all the parameters investigated were halved under drought stress in this cultivar. Hence, it is not stable under drought conditions as stated by Van der Merwe et al. (2018). The increase in CRDs content under drought stress at flowering was significantly associated with the PI_{abs} and PI_{total}, which suggests the protective role of CRDs on the photosystems. The high CRDs content, therefore, assisted cultivars to have better photosystems performance under drought stress at the flowering stage. At pod filling, the activity of the photosystems declined, and this was represented by the negative correlations between CRDs and fluorescence parameters (i.e., Fv/Fm, PI_{abs} and PI_{total}). This was also shown by the weak negative association in the PCA. The significant positive relationship between Fv/Fm and PI_{abs} under drought stress, confirms that the photosynthesis increased with an increase in quantum efficiency of PSII at both growth stages.

AGS429 was still associated with high Chl-b content and yield parameters at pod filling, whereas UVE14 was closely associated with high stomatal conductance and sucrose content. This shows that there is no universal drought tolerance mechanism, and it differs with genotype.

Although this study did not focus on the agronomic parameters, these were included in the relationships because, ultimately, it is important to see how they relate to the physiological and biochemical responses under drought stress. AGS429 and UVE14 produced the highest number of seeds per plant compared to other cultivars, and this was positively correlated with the total seed mass per plant. AGS429 and AGS354 showed the highest seed mass. Results showed that AGS429 was closely associated with Fv/Fm, Chl-b, trehalose, sucrose, starch, and lignin at pod filling, suggesting that this cultivar had the most improved photosynthetic efficiency at pod filling compared to flowering, which led to the accumulation of non-structural carbohydrates. In addition to its association with high stomatal conductance and sucrose at pod filling, UVE14 had a stable cell wall compared to other cultivars. Furthermore, AGS429 and UVE14 had better seed yield quantity (number of seeds per plant) and quality (total seed mass per plant) (Appendix: Figures S6 and S7). Van der Merwe et al. (2018) reported AGS429 to have better stability and to be a good yielding cultivar under drought stress. Moloi and Van der Merwe (2021), reported UVE14 to be a stable cultivar under drought stress. This study also confirmed that UVE14 is stable by elucidating the cell wall polysaccharides of this cultivar. UVE17 was the most unstable cultivar under drought stress as it was closely associated with significantly reduced stomatal conductance (at both growth stages), reduced PI_{total}, Chl-a and -b at flowering, and CRDs reduction at pod filling. The reduction of these important photosynthesis parameters suggests reduced photosynthetic capacity, and this instability was associated with a huge yield reduction. Our findings are in accordance with Van der Merwe et al. (2018) and Moloi and Van der Merwe (2021), who reported this cultivar to be susceptible to drought stress.

The cell wall carbohydrates of UVE17 were also reduced by drought stress. AGS354 and UVE7 performed moderately under drought stress. In addition, AGS354 has its seed yield significantly reduced by drought, this cultivar was also reported to be susceptible to drought stress (Moloi and Van der Merwe 2021). In addition, AGS354, UVE7 and UVE17 had many empty pods that resulted in cultivars having low seed yield (Appendix: Figures S4, S5, and S6).

5.6 Conclusions

The CRDs, PSII and PSI performance indexes (PI_{abs} and PI_{total}), stomatal conductance, starch, hemicellulose, and lignin could be used as additional physiological selection criteria of drought tolerance in edamame because their accumulation under drought stress and significant positive relationships suggest that they were directly responsible for drought tolerance and yield in edamame. The CRDs are directly involved in the protection of the photosystems under drought stress in edamame. UVE14 and AGS429 display high performance based on these parameters and are important for drought tolerance breeding.

Chapter 6

General conclusions and recommendations

Results from this study demonstrated that AGS429 had better photosynthetic efficiency under drought stress because it had high Fv/Fm, PI_{abs} and PI_{total}. The CRDs accumulated in all cultivars, but UVE17 had a significant decline in CRDs content; suggesting this cultivar lacked protection against ROS under drought stress, which might have damaged the photosystems in this cultivar. The low CRDs content in UVE17 matched its reduced PI_{total}, suggesting an interruption in electron transport beyond PSII to PSI. UVE17 also had very low stomatal conductance at pod filling. The findings on this cultivar mean that it had the poorest photosynthetic capacity under drought stress, which might be linked to its susceptibility to drought. The fluorescence (Fv/Fm, PI_{abs}, PI_{total}) parameters had significant positive relationships with CRDs, which suggest that CRDs were involved in protecting the photosystems under drought stress.

The highest stomatal conductance in UVE14 and AGS429 and their ability to produce and store high starch, and sucrose content under drought stress means less sensitivity to drought stress in these cultivars compared to others. More photosynthesis changes occurred at pod filling, as compared to flowering, which means that the photosynthesis rate in edamame might be growth stage-specific, or the duration of drought stress might have led to induction of more biochemical changes.

The cell wall studies showed that the cell wall of UVE14 was the firmest under drought stress, followed by UVE8 and AGS429 because these cultivars had high lignocellulosic biomass, especially hemicelluloses and lignin. This suggested a strong cell wall defence mechanism under drought stress. Cultivars AGS354, UVE7, and UVE17 had reduced lignocellulosic biomass, which suggested a weak nature of their cell walls under drought stress. The slight decreased microcrystalline cellulose and amorphous cellulose in AGS429 means that cellulose might not be involved in drought tolerance in edamame.

AGS429 and UVE14 had the best photosynthesis performance under drought stress, and they are recommended to be used in the breeding programme. UVE8 had moderate photosynthesis capacity and its cell walls were intact. AGS354 and UVE7

also performed moderate, but they had poor cell walls modifications, which suggest that they were susceptible to drought stress. UVE17 had the poorest photosynthetic performance and had reduced cell wall carbohydrates. It is further recommended that CRDs, PSII and PSI performance indexes (PI_{abs} and PI_{total}), starch, hemicellulose, and lignin can be used as additional physiological selection criteria of drought tolerance in edamame due to their accumulation under drought stress and significant positive correlations. This further suggests that they were directly responsible for drought tolerance and yield in edamame under drought stress.

Future studies in edamame cultivars can explore additional abiotic stress, such as heat, which intensifies drought stress. Plasmolysis (the separation of plasma membrane from the cell wall) is common in drought-stressed plants and lead to reduced cellulose synthesis. It can be a good approach to study the membrane stability of edamame under stress, which could help in understanding the reduced cellulose content, especially in susceptible cultivars such as UVE17, AGS354, and UVE7.

Appendix

Supplementary results

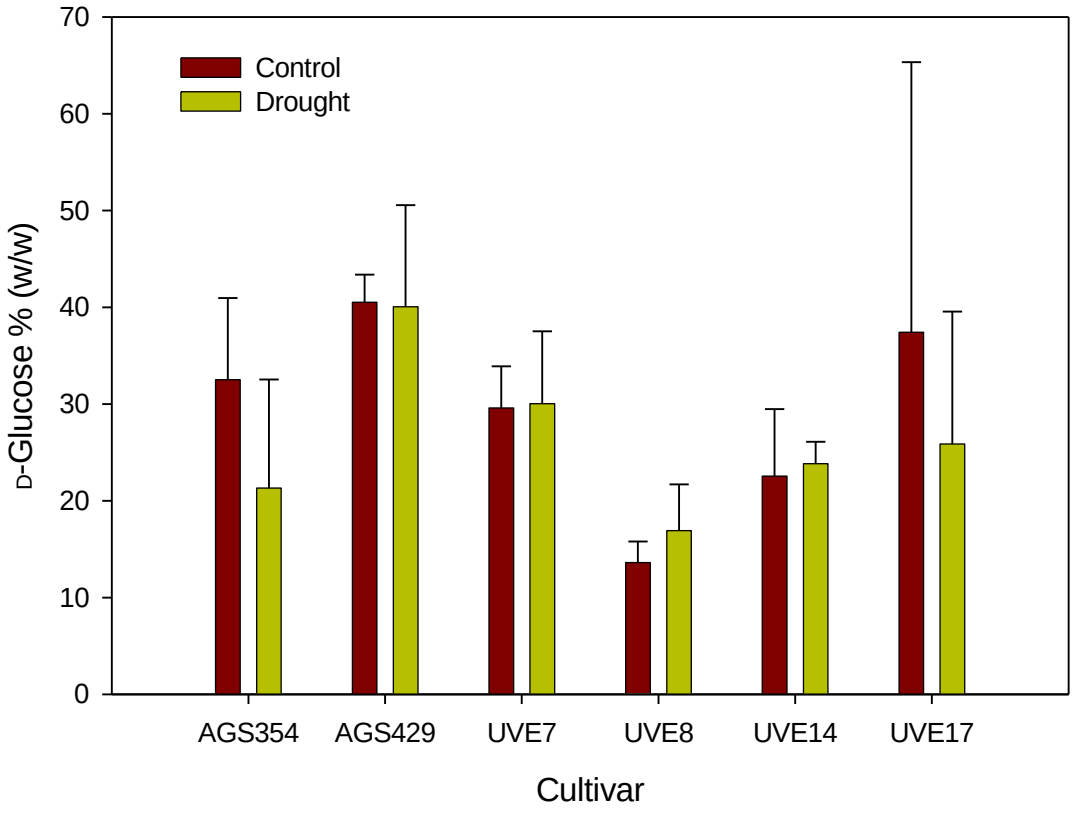

Figure S1. The glucose (making up cellulose through β(1→4) glycosidic linkage) content of six edamame cultivars under drought stress. Values represent means ± SD (n= two replications).

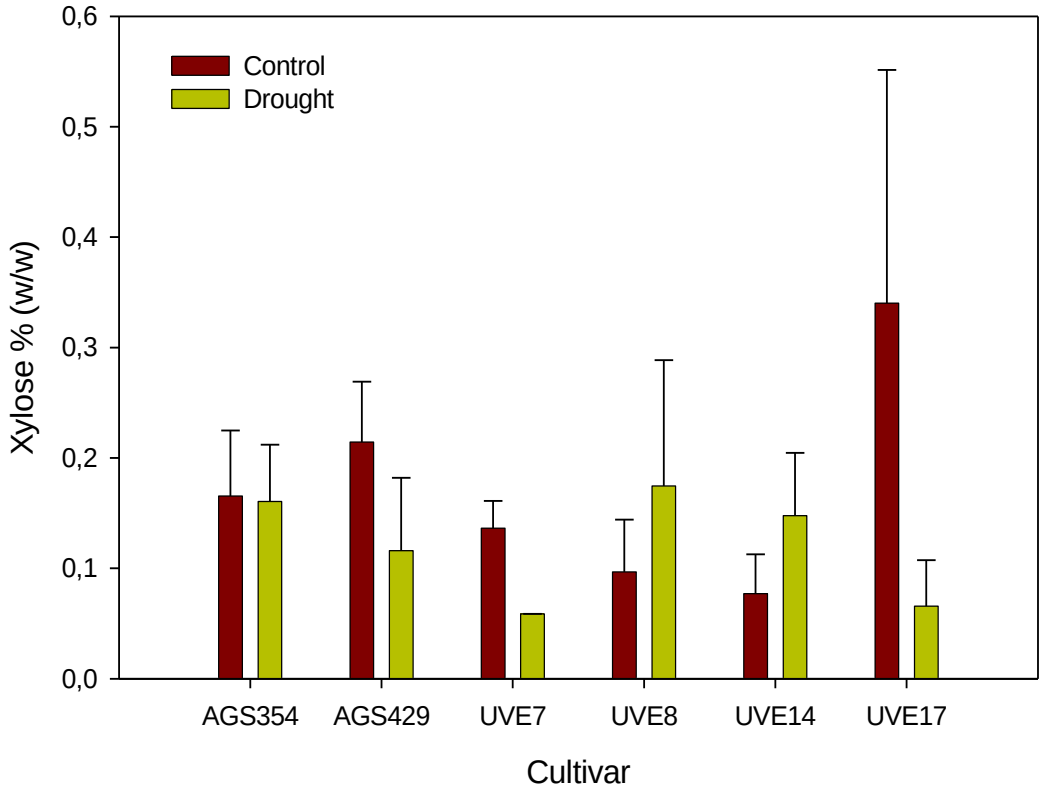

Figure S2. The xylose (making up hemicellulose) content of six edamame cultivars under drought stress. Values represent means ± SD (n= two replications).

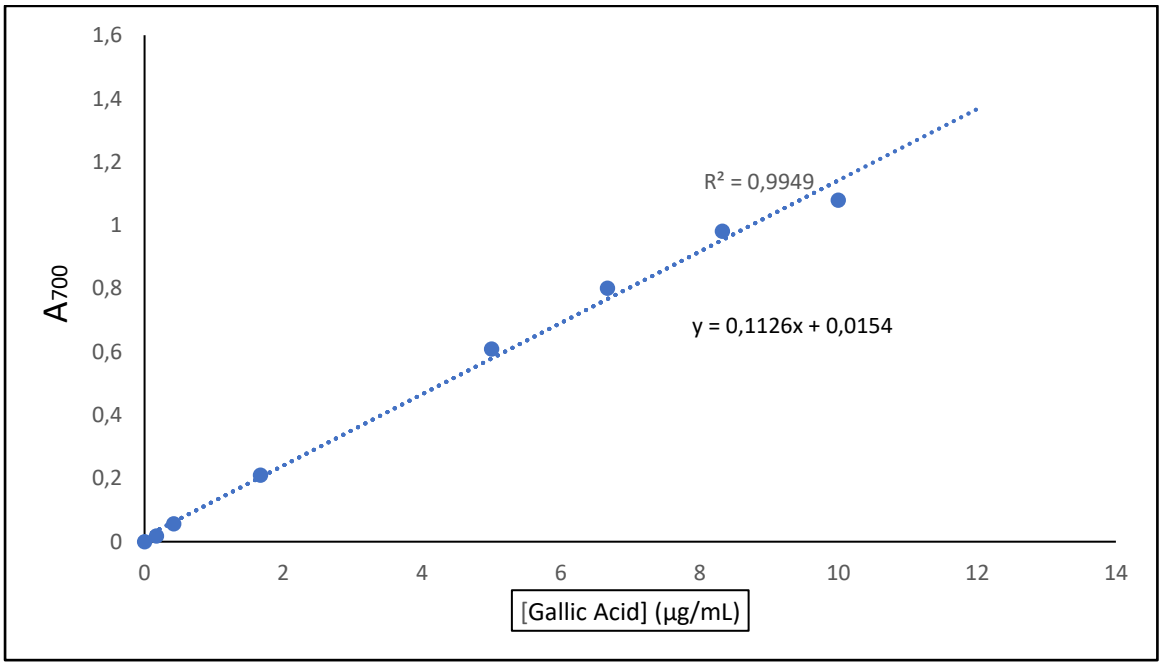

Figure S3. Gallic Acid standard curve used to determine the concentration of total phenols.

Table S1. Analysis of variance representing mean square (ms) values for the yield parameters of the six edamame cultivars under two water treatments [100% water holding capacity, (WHC) and 30% WHC].

Variable	Yield parameters		
	Cultivar (T)	**Treatment (T)**	**CxT**
100 SM	76.76***	11.42	65.39***
TPP	20.56	992.25***	17.18
TSMP	56.33***	383.64***	54.24***
TSP	335.98***	2025***	187.67**
Grand Mean	122.41	853.08	81.12

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. 100 SM = 100 seed mass, TPP = Total pods per plant, TSMP = Seed mass per plant, TSP = total seeds per plant.

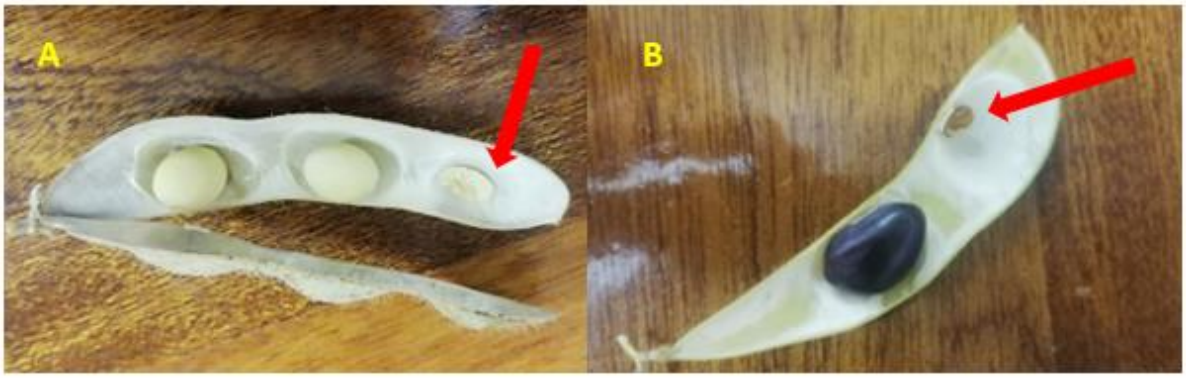

Figure S4. Empty pods in UVE17 (A) and UVE8 (B). Initially, the cultivars attempt to produce a certain (full) number of seeds, but due to water shortage at the pod filling stage, they abort the formation of some seeds (especially the seed at the end of the pod), resulting in empty pods, this reduces the total number of seeds produced per plant.

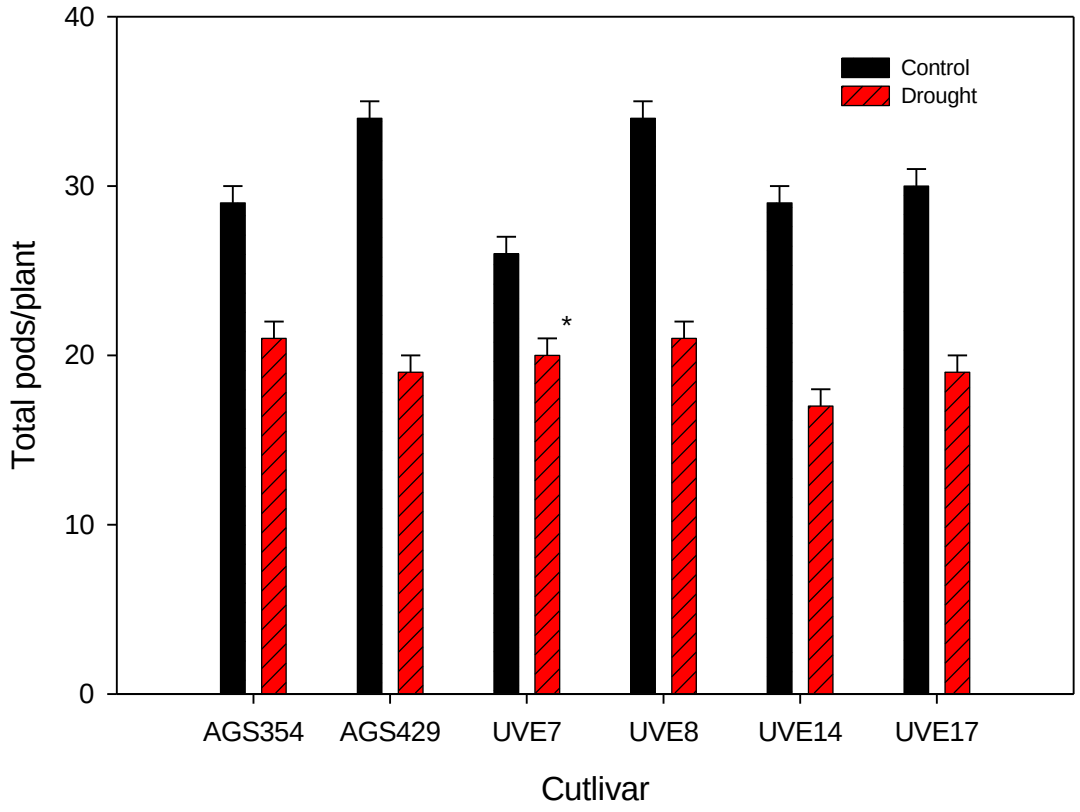

Figure S5. The total pods per plant of the six edamame cultivars under drought stress. Letters represent differences or similarities in 100 seed mass between cultivars. Asterisks represent a significant increase/ decrease under drought stress. Values represent means ± SD (n = five biological replications).

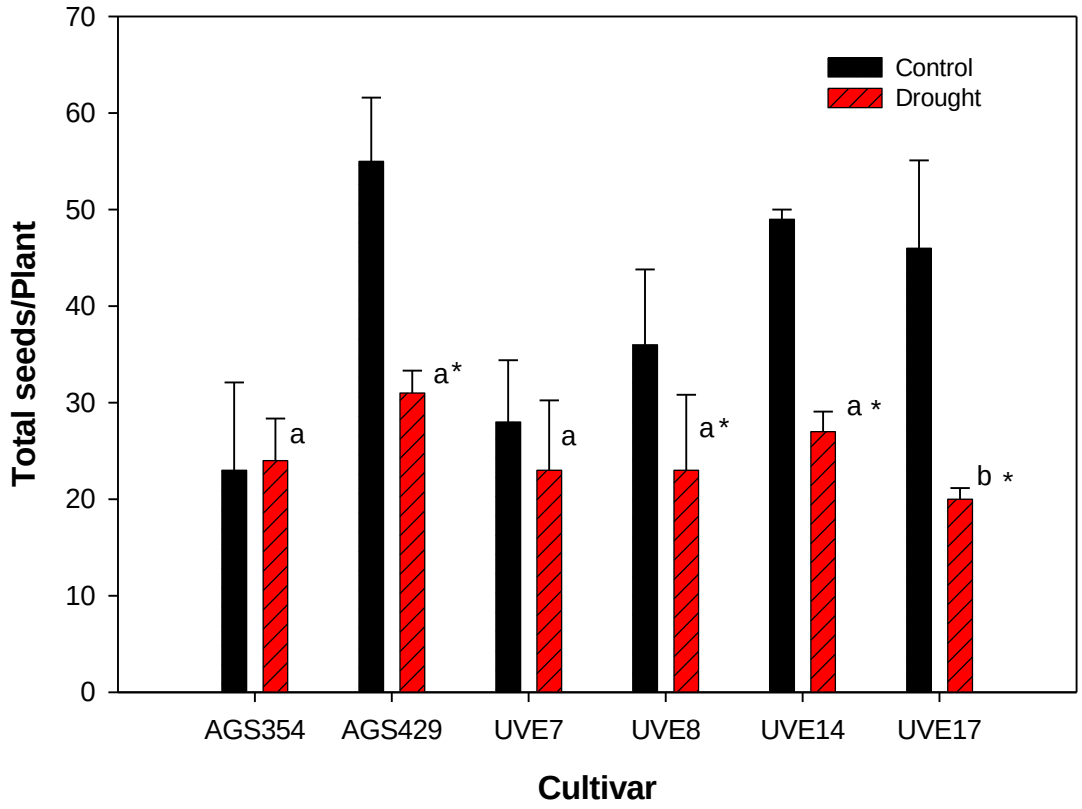

Figure S6. The total seeds per plant of the six edamame cultivars under drought stress. Letters represent differences or similarities in 100 seed mass between cultivars. Asterisks represent a significant increase/ decrease under drought stress. Values represent means ± SD (n = five biological replications).

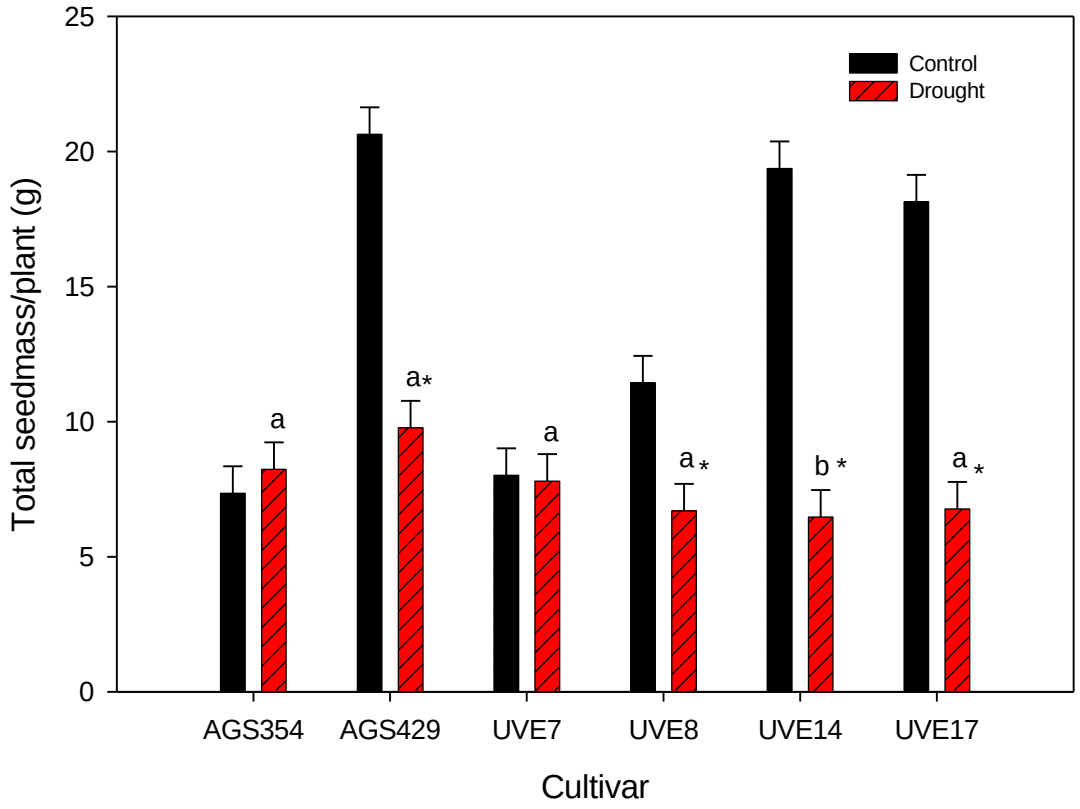

Figure S7. The total seed mass per plant of the six edamame cultivars under drought stress. Letters represent differences or similarities in 100 seed mass between cultivars. Asterisks represent a significant increase/ decrease under drought stress. Values represent means ± SD (n= five biological replications).

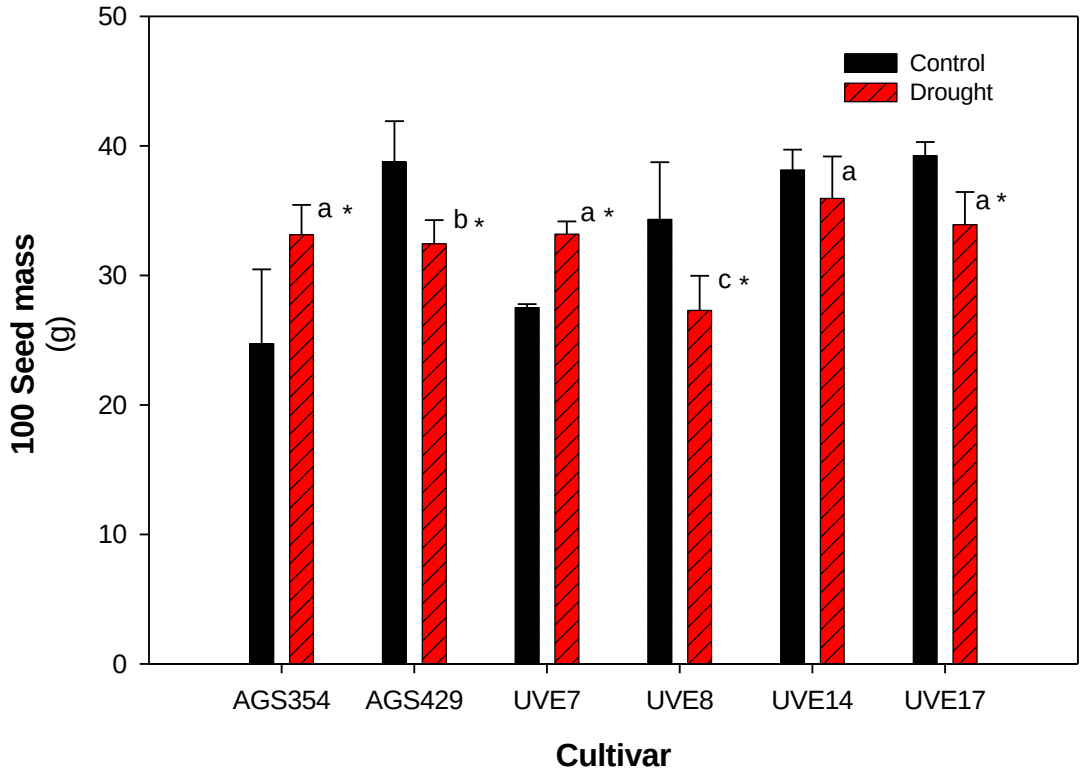

Figure S8. The 100 seed mass of the six edamame cultivars under drought stress. Letters represent differences or similarities in 100 seed mass between cultivars. Asterisks represent a significant increase/ decrease under drought stress. Values represent means ± SD (n= five biological replications).

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