# PHYSIOLOGICAL MECHANISMS IN DROUGHT TOLERANT *Digitaria exilis* L.

**BY**

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. **ABSTRACT**

Drought-induced stress in plants is associated with excessive production of radicals resulting in oxidative damage. *Digitaria exilis* (DE) locally known as Acha is a drought tolerant food crop cultivated mainly in semiarid areas of West Africa. However, there is paucity of information on the radical scavenging mechanisms underlying its drought tolerance. Therefore, this study was aimed at investigating the physiological mechanisms that account for drought tolerance in various accessions of *D. exilis*.

Three DE accessions (Dinat Iburua-DI, Jakah Iburua-$JI_1$ and Jiw Iburua-$JI_2$) and two DE accessions (NG/11/JD/061 and NG/11/JD/062) were obtained from National Cereal Research Institute, Badeggi and National Centre for Genetic Resources and Biotechnology, Ibadan, respectively. Using *in vitro* techniques, Murashige and Skoog medium was supplemented with varied concentrations of poly- ethylene glycol 6000 to create osmotic conditions of -9.29, -13.93, -20.13, -26.32, -32.51 and 0MPa (control) in a completely randomised design. Seeds were inoculated and growth parameters (number of germinated seeds, leaf length, root and shoot weights) were measured after four weeks. In screenhouse experiments, plastic pots filled with sterilised top-soil (2kg each) were arranged factorially 5×3×4. The seeds were planted and subjected to 0, 4, 8 and 12-day water stress. Chlorophyll contents, free radicals (Hydroxyl ($OH^-$) and 2, 2-Diphenyl 1-picryhydrazl hydrate (DPPH)), Catalase, Superoxide Dismutase (SOD), Anthocyanin and proline were determined following standard procedures. Nanodrop lite spectrophotometry was used to quantify the plant DNA. The drought tolerant index of 4.00-5.00 (drought tolerant), 2.00-3.99 (mild-drought tolerant) and below 2.00 (drought susceptible) were calculated using standard formula. Leaves were examined for anatomical features. Data were subjected to descriptive statistics and ANOVA at $\alpha_{0.05}$ was used to determine the best parameter.

The number of germinated seeds (5.00±0.61), leaf length (40.00±4.95cm) and fresh shoot weights (0.80±0.18g) of $JI_1$ were significantly different from other accessions in the *in vitro* experiments. Accession $JI_1$ had highest total chlorophyll (298.60µg/ml), Catalase (3.63units/mg protein), SOD (1.70units/mg protein) and proline (0.05mg/ml) compared with

others. Proline concentration in $JI_1$ was significantly higher (LSD 0.009) than other accessions; suggesting that $JI_1$ exhibited high antioxidant enzymes which were osmoprotected by accumulation of proline. Scavenged radicals were high in $JI_1$: 80% $OH^-$ and 78% DPPH compared to other accessions. In screenhouse experiment, DNA concentration (96mg/µl) and dry root weight (2.25g) were highest in DI which indicates well-developed deep and prolific root systems. The NG/11/JD/061 had the highest anthocyanin content (1114.65µg/ml) and low lipid peroxidation (0.0000069M), which lowered the osmotic potential of the leaves. The $JI_1$ was drought tolerant (4.32); NG/11/JD/061 (3.93) and DI (2.12) were mildly drought tolerant, while $JI_2$ (0.77) and NG/11/JD/062 (1.75) were susceptible to drought stress. Leaves of $JI_1$ (113-175µm) and DI (132µm) had intercellular spaces which indicate rapid flow of gaseous exchange. All drought stressed accessions revealed turgid bulliform cells which indicate reduce transpirational loss.

*Digitaria exilis* accession Jakah Iburua had the most osmolyte which stabilised the activities of catalase and superoxide dismutase against hydroxyl radicals generated during oxidative stress.

**Keywords:** Antioxidant enzymes, *D. exilis*, Free radicals, Leaf anatomy, Physiological parameters

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## CERTIFICATION

This is to certify that this research was carried out by Oyinade Aderoju, DEDEKE (Matric No. 172131) under the my supervision as part of the requirements for the award of Doctor of Philosophy (Ph.D.) in the Department of Botany, Faculty of Science, University of Ibadan.

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Prof. O. Osonubi Date

(Supervisor)

..... .....

Dr. (Mrs) Idayat T. Gbadamosi Date

(Co-supervisor)

## DEDICATION

I dedicate this research accomplishment to the Almighty God. I praise God for being the pillar behind my success and for giving me the unimaginable confidence needed for the successful completion of this programme. He is my father and my provider. Indeed, He is awesome, gracious, mighty and wonderful in all His ways.

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# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background to the Study

Food and basic needs for human survival are obtained from plants. Plant biodiversity provide important raw materials in the production of food, medicines, fibres, rubber and other products (Janovska *et al*., 2012). Biodiversity of plants is increasingly declining and being threatened by the effects of modernisation, agricultural practices and environmental factors (Janovska *et al.,* 2012). Consequently, food security is adversely affected in many developing economies of the world. Approximately, about 10,000 plant species were identified as food for mankind globally (Fowler and Hodgkin, 2004). However, only about 150 of these species feed most of the world's population, and just 12 species of this number providing about 80% of global dietary requirements (Fowler and Hodgkin, 2004).

*Digitaria exilis*is phylogenetically located in the family Poaceae, commonly called Fonio millet. *D. exilis* is an essential under-utilized local cereal cultivated in arid zone of West-Africa. It is an important species grown in the wild and domesticated in the northern part of West-Africa. *D. exilis* is a very small-sized grain that can help to improve nutrient contents of food. This plant has the potential to combat food security challenges, as well as surrogate rural growth and aid prolonged utilization of land. Adoukonou-Sagbadja *et al.* (2007) therefore reported as an essential crop because of fast maturity, high nutrients composition and the potential to attain reproduction stage within a short duration.

Furthermore*, Digitaria exilis* is nutritious, medicinal and of economic importance. It is also used in preparation of Africa delicacy (Gwete soup) which is used in treating of diabetes (Adoukonou-Sagbadja *et al.,* 2007). It survives well and gives optimum yields in an area of prevailing and

disturbed climatic conditions. One of the things that make *D. exilis* sought for as grain is its nutrient composition. Nevertheless, protein was found to be much higher in other grain cereals like *Pennisetum glaucum*, *Zea mays* and *Sorghum bicolor*(Adoukonou-Sagbadja *et al.,* 2007). Methionine is an important amino acid fortified with sulphur, and *D. exilis* stores double the amount of Methionine found in *Zea mays* or *Pennisetum glaucum* and triple when differentiated with the *Oryza sativa* (Adoukonou-Sagbadja *et al.,* 2007). During hulling, converged mineral nutrients in the husks were lost during the processing. Linoleic and oleic acid are unsaturated fatty acid left in the decorticated grain. Also, saturated fat called palmitic acid is found in its grain. Del *LourdesMoreno et al. (2014*) revealed the positive effect of *D. exilis* on people living with diabetic complications.

The nutritional value of decorticated and whitening *D. exilis* is about 1470kJ and 1430kJ per 100grams respectively. The nutrient composition is very high with great taste value. In addition, it has been identified as a good source of fibres and phytonutrients (Adoukonou-Sagbadja *et al.,* 2006). It contains methionine and cysteine as mentioned above with great importance to the wellbeing of humans. However, *Triticum aestivum*, *Orzya sativaZea mays* and some cereals plants are deficient in these two amino acids (Seignobos and Tourneux, 2002). Thus, Dansi *et al.,* (2010) reported an advance from the level of amino acid to macromolecules. *D. exilis* has a greater protein, carbohydrate and fibre in comparison with other grain cereals. Hence, the nutrient benefits from *D. exilis* outweigh the benefits from other correspondent grain plants. *D. exilis* has ability to germinate easily in numerous soil textures and structures (Jideani, 2000). The plant demands little soil nutrient with few scattered rains. It is suitable for a marginal land, growing adequately in a poor soil (Jideani, 1990). It is a dependable crop, especially where rainfall is short and unassertive.

Drought detrimentally affects the morphology and reproduction of plants. Water deficit, is widely prevalent than other stresses limiting growth and productivity of crops. Substantial numbers of African countries were classified as water stressed countries, and the rate of increase is progressing due to population growth (Davis, 2005). Water stresses have directly contributed to the degradation of watersheds by changing land-use practices, siltation of river basins as well as reduction of plant biodiversity (Davis, 2005). Many African countries are considered drought

stress prone and are largely classified as arid to semi-arid areas (Davis, 2005). Thus, in an effort to prevent further decrease in agricultural productivity in water stressed regions, identifying stress tolerant crops becomes a feasible and efficient strategy.

Cellular metabolisms in plants usually generate by-product called Reactive Oxygen Species (ROS). Numerous abiotic stresses like drought generate ROS resulting in the oxidation injury and eventually causing death of plant cell organelles. In spite of their fatal act, ROS featured as second messengers for different cells mechanisms such as tolerance of plant to abiotic stresses. Conditions that will make ROS to serve either as a signaling factor or inflicting injury rely on the formation rate of radicals and its scavengers. The structure of radicals generated during stress depends on the activities and existence of various antioxidants in the tissues of plants (Sharma *et al.,* 2011).

Srivastava and Dubey (2011) reported that environmental stress like drought can principally lead to ROS formation in plants because of the interruption of the cell homeostasis. Higher concentrations of free radicals are exceedingly detrimental to plants. When the production of radicals in plant tissues exceeds its protective mechanisms, the tissues are said to experience oxidative injury. Mishra *et al.* (2011) revealed that an increase in ROS formation could constitute damage to the cellular organelles by increasing Malondialdehyde (MDA) contents, causing injuries to proteins and DNA, hindering enzymes activities, development of Programmed Cell Death (PCD) and finally causing damage to the cellular organelles. Because of the functioning of the ROS and its character, cells need to importantly influence and monitor the degree of ROS generation in other to reduce further damage in form of oxidation death. The roles of antioxidant systems in detoxifying excessive ROS are well-structured and effective in plant. Noctor and Foyer (1998) noted that enzymatic and non- enzymatic antioxidants presented themselves as powerful scavengers of radicals.

Stress application under *In vitro* culture is a technique that helps to downplay the effect of climatic variation as a result of its nutrient constrained situation and uniformity. The straightforwardness of the manipulation qualifies substantial number of plants cultivation under different stress treatments within a restricted area for a particular duration. Simulation of water stress in an *in vitro* condition comprises of easy paths to research the role of drought stress on the

plants during plant regeneration (Kaufmann and Eckard, 1971). Applying osmotic stress during regeneration phase was found to be one of the most efficient methods required for selection of tolerant plants because the capacity of crops to escape drought stress at its juvenile stage would undoubtedly permit adult plants to survive water stress situations during growth and flowering periods (Kaufmann and Eckard, 1971). Thus, ability to screen for drought tolerant plant is granted. The poly-ethylene glycol molecules are employed to imitate drought, which also helps to minimize water potential and simulate soil drying. Plants grown on soil and PEG solution show resemblance in their water potential. The largest PEG molecules like PEG 6000 are essential for imitating soil drying (Kaufmann and Eckard, 1971).

## 1.2 Statement of Problem

Water is a major part of cells that displays an essential functioning in the survival of floras and faunas, including microscopic life. A basic role of water in life has to do with motion of molecules within and between cells and tissues. Plants are firm and they are often confronted by challenging environmental factors. Accordingly, they depend greatly on provision of water from the soil for their growth and fruiting. Quite number of abiotic factors related to plant-water showed negative consequences on the overall development of crops such as reduced root absorption, germination and metabolic activities leading to a decreased growth and even death in harsh environmental conditions. Drought stress tolerance is quantitatively polygenic in nature, therefore making it challenging to understand the major mechanisms controlling its advancement and improvement. In this regard, having a clearer understanding of plants tolerance to stress and how to enhance tolerance to stress in plants becomes important, particularly in the face of the potential implications of a constantly expanding global population for food production and food security.

## 1.3 Justification

Adaptation, response and survival of plants subjected to drought are influenced by various morph-physiological processes, as well as by molecular and biochemical mechanisms. For a plant to be described as a drought tolerant, it must exhibit abilities to grow, flower and fruit optimally under severe stress. Water stress influences the interaction of water at level of cell,

tissue and organ, consequently affecting definite as well as indefinite reactions and ultimately inflicting damages to the plants. For crops to withstand stress, tolerant plants have to trigger its protective mechanisms against water limitation. Selection of drought stress tolerant could probably be achieved through the understanding of interactive force that connect the operative physiological, biochemical, anatomical and gene regulatory network mechanisms in plants. Crucially, there is currently a paucity of information on behaviour of *D. exilis* response to drought. Therefore, understanding the major mechanisms of drought tolerance of *D. exilis* at different levels would be of immense benefits to the crop scientists and plant breeders. This research sets out to fill the identified vacuum.

## 1.4 Objectives

i. To identify drought tolerant accession of *D. exilis*

ii. To understand mechanisms underlying water tolerance using biochemical and physiological methods

iii. To identify the drought stress responsive radicals and scavengers.

# CHAPTER TWO

# 2.0 LITERATURE REVIEW

## 2.1 Description of Genus *Digitaria*

Clayton and Renvoize (1986) have described the genus *Digitaria* as a plant native to the Tropics and Sub-Tropics regions. Genera *Digitaria* is the biggest of all in the family poaceae. It belongs to the biggest genera which compose of approximately three hundred and twenty five annual and perennial species. *D. exilis* is an orphan African cereal crop cultivated in arid zone of West-Africa (Abrouk *et al.,* 2020). *Digitaria* has a broad diversity that has been attributed not only to its ancestry but also serves as evolutionary process to become distinct species. There is a wide change in morphological features between and within species. Although scarcely paniculate species is also observed, *Digitaria* species are predominantly $C_4$ plants with digitate inflorescences (Abrouk *et al.,* 2020).

Some of *Digitaria* were grown for forage purposes, while others are grown as grain. Earliest *Digitaria* are grown in the semiarid region of West-Africa. Since pre-historic time, various food products from *Digitaria* are treated as primary food for many people with relatively small income. Fonio is an optimal food for people living with diabetic problem (Moreno *et al.,* 2014). Distinguishing features of *D. exilis* includes their physiologically and jointed spikelet and inflorescence characters. About 300,000 ha are dedicated annually to the growing of *D. exilis*. This is not surprising, as *D. exilis* millets are generally regarded as one of the most appropriate grain crops for optimal yield. Nevertheless, Adoukonou- Sagbadja *et al.* (2006) observed that *D. exilis* productivity is poor and extremely sensitive to environmental changes.

## 2.2 Physiological activities of plant under water stress

When relative humidity is low, desiccation of the atmosphere and soil and soil surface evaporation in the daytime is inevitable. Absence of precipitation implies dryness for both the atmosphere and soil, with the soil in particular losing moisture to evaporation during daytime. Larcher (1995) noted that while drying of soil is generally slow, reduced relative humidity can be accelerated. It is in this context that plants require adequate mechanisms both in their roots and leave to resist or tolerate drought stress. As Assmann *et al.* (2000) observed that plants shut their stomata without delay on recognizing high vapour pressure of the leaves. Assmann *et al.* (2000) noted further that it takes several minutes for this response to be completed. Whether the process involving the closure of stomata is subjected to Abscisic acid production or not still remains undefined (Assmann *et al.,* 2000).

In Abscisic acid synthetic pathway, four enzymes are known and functionally localized as signalling factor in the leaves (Koiwai *et al.,* 2004). Decrease in epidermal turgidity and water potential of the leaf is as a result of exposure of leaves to environmental dryness. Similarly, it was observed that the location of dryness and production of Abscisic acid (ABA) signals were viewed to be either near or in the guard cells (Pantin *et al.,* 2013). It is clear that while evaporation reduced the water potential of soil, it also alleviates the salinity of soil. Besides water deficit, osmotic stress has been noted to influence heat stress is an advancement stress on the leaves and root (Koiwai *et al.,* 2004). High solute concentration in the root surfaces surrounding or elevated osmotic pressure in the root denotes water deficit of the soil environ. However, it is essential to note that water sensor has not been identified in plant (Shinozaki and Shinozaki, 2005).

Koiwai *et al.* (2004) have observed that the root tips or parenchyma or vascular tissues secrete ABA from carotenoid under water or salinity stress. Commenting on how ABA synthesized in the root, Sauter *et al.* (2002) noted that the entry is done via the xylem vessels in a detachable shape or coupled with glucose, thereafter, it was transferred to the leaves. It must however be noted that what remains unclear and undetermined is the formation of conjugate in the cytosol of the cortex. Nevertheless, it is generally believed that the conjugated form of ABA is appropriate in saving plants from roots to leaves. This is because the detachable shape is capable of possible

avoidance of tissues from the low pH xylem sap to the high tissues environment. However, Sauter *et al.* (2002) further observed that cumulative sum of ABA increased significantly in some crops during water and salinity stress. Dietz *et al.* (2000) observed that hydrolysis of Abscisic acid conjugated to a detachable shape by β-D-glucosidase in the apoplastic extension. They further noted an induced stomatal closure in the guard cell with the aid of signalling system (Dietz *et al., 2000*). More stomata per unit area were related to the enlarged leaves guard cells under well irrigated condition and reduced dense stomata when subjected to limited water (Shatil-Cohen *et al.,* 2011). It is instructive to note that smaller stomata are advantageous, and this is because stomata space can be reduced in a short time span after the guard cells receives signal from abscisic acid. Interestingly, there is an uncertain closure of stomata in some plants, despite the addition of high concentrations of abscisic acid. However, this is in variance with Loewenstein and Pallardy (1998) who revealed that the field-grown plant has their transpiration rate drastically reduced with a stomata restriction under a severe water stress. Consistent with the realization that stomata blockage was not achieved at the additional 300μM ABA in *Citrullus lanatus* plants, Yokota *et al.* (2002) have therefore suggested a likely substitute to drought impetus from the roots to leaves.

### 2.3 Germination Inhibition during Water Stress

The place of seed germination in plants cannot be discounted. In fact, the seed germination phase displays vital metabolic processes in the existence of plants. It is in this view that Guo *et al.* (2013) reported that reduced water potential is a limiting component hindering germination of seed during water stress. According to Guo *et al.* (2013), wheat germination was hindered as the PEG concentration increased. Abdoli and Saeidi (2012) found that reduction in availability of water has affected all germination properties, except for germination period. According to Abdoli and Saeidi (2012), water stress deficiency has lesser effect on wheat Sivand landrace that has weighty biomass and grain with excellent germination properties. Similarly, Edward and Wright (2008) reported decrease in yield indices such as grain number and size in water stressed wheat plants at pre-anthesis stage. To further underline the importance of water during the seed germination phase, constant phase like germination and seedling growth are critical, though impacts of drought could be manifested at any period of the existence of plant.

A study by Jajarmi (2009) indicated a notable reduction in the radical length above -6 bars during drought. The study also showed that cultivar keraceharvand had the root length of 63.58 mm which was the longest. In addition, he found that cultivar Azar revealed a significant plumule length compared with other cultivars. Cultivar Gv 3-20 recorded the highest germination percentage of 78% as against cultivar Arvand, which had the lowest germination percentage of 36%. Perhaps it is worth noting that the germination percentage and velocity will decrease as water stress go beyond above -12bars. Jajarmi (2009) observed a significant high germination velocity in cultivars Gv 3-20 and tabaci, while cultivar tabaci showed a significant high coefficient of velocity of germination. It is however important to note that cultivars with tolerant features showed no impressive reduction up to -3 bars (Jajarmi, 2009; Basu *et al.,* 2016).

## 2.4 Productivity Responses of Plant to Water Stress

Water deficit is a critical factor responsible for reduction in plant yield, Lerner (1999) defined water stress as a factor that operates outside the usual rate of homeostatic regulate. Similarly, Oerke *et al.* (1994) reported that approximately 42% of the crop yield is lost due to numerous ecological factors annually.

Liu *et al.* (2004) showed high amount of glucose absorbed in drought susceptible variety Longchun 8139-2 during water stress, especially the severe water stressed plants. Furthermore, same study suggested lower quantity of glucose wrapped in water in drought-resistant spring-wheat Dingxi 24 under water stress conditions. Considering the report given by Liu *et al.* (2004), wheat cultivar Longchun 8139-2 had a significant high leaf number (11.29), leaf weight (2.701g) and carbohydrate (4.03 mg) under well-water. Thus, it was concluded that drought show a notable consequences on the wheat biomass (David *et al.,* 2018). Notably, Liu *et al.* (2004) showed that there were variation in response of drought susceptive wheat than tolerant varieties in terms of leaf weight, carbohydrate, bioenergetics and biomass. In term of yield, varieties with high potential however produce less compare to low yield cultivars (Gao *et al.,* 2017). However, high-yield cultivars appear to have adapted better to water stress. A crossover of approximate 2–3 t/ha for most grain crops cultivated was recorded under water deficit situations. Beyond the differences in their yield potential, intrinsic variance occurs in all the tested landraces thus, drought tolerance can be adduced as reason for a crossover exhibited under

varying water stress. Accordingly, Blum (2005) noted that the tested cultivars had no tolerant traits however; the cultivars differ in their produce because they were exposed briefly to stress adaptation.

Falaki *et al.* (2009) reported that wheat variety Ster/TR had a significant grain and spike number while varieties Seri/Buc/weaver/PFau showed the least grain and spike number under water stress. Falaki *et al.* (2009) reported that wheat variety Ster/TR had a significant grain and spike number while varieties Seri/Buc/weaver/PFau showed the least grain and spike number under water stress. In addition, Falaki *et al.* (2009) noted that cultivar HD2206 was significantly high in grain weight/spike whereas, gain yield of cultivar Seri/Buc/weaver/PFau were the sensitive to drought. There was a variant in grain weight of all the cultivars tested under water stress. The variations in yield contents were linked to the genetic composition of the wheat cultivars. Furthermore, there was an inverse interaction between the post anthesis water deficiency and yield component of the cultivars.

## 2.5 Relation between Cuticle Conductance and Water Stress

It is clear that plants strive to shield itself against transpiration by shutting their stomata under severe drought stress. It is also worth noting that transpiration occurs mainly in the plant through opened stomata and cuticles (Boyer, 2015). Kerstiens (1996) observed that there were differences in water transfer through cuticle which varies from species to species. Riederer and Schreiber (2001) reported a very low conductance value in the cuticle of Vanilla plants. Although Kerstiens (1996) opined that there were no sufficient evidence regarding the mutual relationship between cuticle conductance and drought tolerant, Riederer and Schreiber (2001) were of the opinion that water filled pores of molecular size could add to cuticle transpiration.

## 2.6 Photosynthetic Systems of a Water Stressed Plants

Pfannschmidt (2003) affirmed that photosynthesis occur in the chloroplasts containing organized thylakoid membrane that bear all other photosynthetic apparatus components, thereby supplying structural features for optimum light harvesting. It was noted that during photosynthesis, oxygen produced in the chloroplasts move through photosystem consequently leading to the generation of $O^{2-}$ as electrons were accepted. Accordingly, Pfannschmidt (2003) has identified chloroplast as one of the principal location of ROS generation. They developed at various zones like triplet chlorophyll and ETC in PI and II. Formation of free radicals in Arabidopsis chloroplast was boosted due to numerous stresses like drought, salinity etc. It is normal in this sense that electrons were directed to NADP then moved to photosystem centres where excitation occurred (Zhao *et al.,* 2020). Consequently, NADP was reduced to NADPH. The electron penetrates the Calvin cycle where the final electron acceptor reduces carbon (IV) oxide. Accordingly, oxygen was reduced to superoxide through Mehler reaction as the portion of the electron flow, turning ferredoxin to oxygenin condition to burden ETC. It was observed that the acceptor site of the ETC in photosystem II also supplies zones for generation of superoxide through electron leakage. Buchert and Forreiter (2010) noted that $^1O_2$ were principally generated in photosystem II when subjected to reduced light intensity which were the natural by-product of photosynthesis. According to Buchert and Forreiter (2010), as stromal membrane reached the external, superoxideimmediately was reduced to hydrogen peroxide using CuZn-SOD. It was therefore reported that ROS is primarily generated in the chloroplasts. Researches, as noted by Mur *et al.* (2008), have related ROS generation in plant chloroplast as hypersensitive responses of plant. In this context, Gray *et al.* (2002) has related transmission and circulation of wound induced PCD in maize tissue to ROS generation within the chloroplast. Excessive light energy trapped caused elevation in ROS production. The activities of antioxidant might as well increase along with severe drought stres (Wang *et al.,* 2018).

Hu *et al.* (2008) have meanwhile related an elevation in defensive processes in thermal dissipation, optional respiratory route and free radicals scavenging processes in the chloroplast and mitochondria to reduced temperature. Diaz-Vivancos *et al.* (2008) also posited that electron disruption transportation in pea leaves were responsible for instability in antioxidant systems and

high ROS formation in chloroplast thus, denoting chloroplast as a major site of oxidative stress during disease advancement (Tisarum *et al.,* 2019).

Perhaps it is important to point out that carotenoid achieve primarily two roles in photosynthetic organisms. Foremost, it's a light collector pigments that stretch its spectrum making it accessible for utility in the photosynthetic mechanisms. In playing this role, light was gripped by carotenoids within the wavelength of 450-570 nm and beyond the chlorophyll molecules; energy flow was seized in the chlorophylls (Zhao *et al.,* 2020). Following the role of carotenoids, photosynthesis mechanisms utilize photo-protective approach against abiotic stress. Accordingly, Reddy *et al.* (2004) detected superoxide in chloroplasts of water stressed wheat. In this context, Tuteja (2008) observed that carotenoid prevent the formation of singlet oxygen, which is a remarkable strong oxidant that is sufficiently potent to produce dying of the organisms by scavenging triplet status of the chlorophyll as they rises. Chlorophyll and carotenoid contents were decreased when subjected to drought. The speed of decline in a drought-sensitive landrace was much faster than in a drought resistant landrace (Tisarum *et al.,* 2019).

Chlorophyll contents could be elevated during drought situations. Reddy *et al.* (2004) observed that photosynthesis could be responsible for the metabolic changes during drought, which show a great complication than stomatal limitation thus, resulting to major decline in photosynthetic pigment of sunflower. Significant decrease in chlorophyll contents and cumulative pigmentation were observed in drought stressed plants. Similar trend was recorded in water stressed sunflower and Agropyroncultivars (Sawhney and Singh, 2002).

According to Holton and Cornish (1995), Anthocyanins are pH dependant and water-soluble pigments, and they associate themselves to the lineage of flavonoids with a range of colours. As noted in Holton and Cornish (1995), glucosides (three benzene rings) and attached groups helps to create distinctions between several kinds of anthocyanins. For example, Holton and Cornish (1995) reported that pelargonidin-3-glucoside possesses one hydroxyl, while cyanin-3-glucoside and delphinidin-3-glucoside had two and three OH groups respectively. Anthocyanins are localized in the epidermis, vacuole and mesophyll of plants (Chalker-Scott, 1999). Hatier and Gould (2008) noted that anthocyanin were involved in protecting plants against abiotic stress like radiation, herbivores, water deficit and cold, and they also aid in the attraction of pollinators.

Physiological functioning of anthocyanins as an antioxidant, osmotic regulation, and photo-protectants against irradiation was reported by Close and Beadle (2003). According to Close and Beadle (2003), anthocyanins were cumulated in a young tissue and in autumnal senescing leaves of deciduous species during abiotic stress.

## 2.7 Anatomical Responses of Plant Tissues to Water Stress

As already reported by Venora and Calcagno (1991), leaf anatomical characters are regarded as an indicator of stress influence. The epidermal size and stomata count decreases as water stress increases. Meanwhile, Aberentthy *et al.* (1998) revealed solitary stomata on abaxail of native *Festuca novae*. Drought tolerant and susceptible wheat genotypes behaved inversely in term of stomata frequency when exposed to varying water stress conditions. Though xerophytic plants generally have thick cuticles, nevertheless, there was a further elevation in maize cuticle thickness during drought stress. On the contrary, drought tolerant genotypes of cotton showed thin epidermal layer. While Rojas *et al.* (1983) observed that drought tolerant sugar cane showed wider epidermis size whereas; epidermal size was reduced in water stressed *Lolium perenne*. However, increased trichomes number in water stressed *Lolium perenne* was observed. Decrease in leaf thickness was linked directly to drought stress. Nevertheless, Venora and Calcagno (1991) observed high thickness of leaf blade in *Glycine* spp. and durum wheat which resemble and represent high degree of xeromorphic features. It was noted that the wheat mesophylls were sensitive to drought than the bundle sheath. More so, winter drought sensitive wheat had wider vascular bundles while a drought tolerant variety had a larger sclerenchyma (David *et al.,* 2017).

## 2.8 Mechanisms of Drought Acclamation and Tolerant

Plants have exploited countless processes to defend photosynthetic components against numerous abiotic stresses. With a view to abate the injurious consequence of stress at the cellular level, plants tend to modify their metabolisms to survive the stress. Although there are numerous mechanisms which plants engage in coping against dehydration, it is also worth noting that plants differ in their ability to survive dehydration. According to Hoekstra *et al.* (2001), two drought tolerances were identified according to their water relation: moderate and tolerance to dryness. Drought tolerant plants are described by Hoekstra *et al.* (2001) as plants that range from steady dehydration to availability of moisture content owing to absent of massive cytoplasmic water deficit.

As conceptualized by Hoekstra *et al.* (2001), drought tolerance includes potential of cells to recover uninterruptedly. The early phase of stress evident the alteration in gene expression arrangement. And some of these changes promote and lengthen the defense of plants against stress. It has been reported that there was an accumulation of compatible solutes during water deficit (Bohnert and Shen, 1999; Mkhabela *et al.,* 2019). It was noted further that many of the compatible solutes are osmolytes which uses osmotic regulation approach. By implication and inference from the observations by Bohnert and Shen (1999), it is therefore clear that "compatible solutes" may be responsible for defending of antioxidant enzyme, membrane shapes and inhibiting the formation of ROS.

Ruban and Horton (1995) have observed chlorophyll fluorescence quenching (qN) as a foremost process engaged by floras to intercept or to reduce injury done to the photosynthetic products, noting that there was a surplus light energy distribution in form of heat energy in photosystem II. As stated by Deltoro *et al.* (1998), drought influenced production of Zeaxanthin and Anteratxanthin, which mediated photo-defensive apparatus in drought sensitive *Frullania dilatata*. Therefore, low relative water content resulted to $CO_2$ fixation and decrease in ATP consumption thus, the operative electron flow generates acidifies thylakoid lumen that stimulates Zx and Ax synthesis. Deltoro *et al.* (1998) have also proposed that the photo-protective system culminate to differences in energy from the reaction centres. However, xanthophyll cycles perform an important or peculiar function in the distribution and absorption of light energy.

According to Tambussi *et al.* (2002), zeaxanthin and anteraxanthin content and non-photochemical fluorescence quenching (qN) notably increase after exposure to mild drought. However, Tambussi *et al.* (2002) observed no similarities between increase in xanthophyll and elevated qN at severe drought. In addition, Tambussi *et al.* (2002) observed that β-carotene has a notable increase during harsh water stress, implying an elevation in antioxidant protective. The distribution of excite energy as an effective protective mechanism under water deficit at the leaf which differs from photosynthetic carbon metabolism (Chaves *et al.,* 2002; Sun *et al.,* 2013).

Studies have identified other mechanisms of energy dissipation. For example, Lichtenthaler (1996) has found that the energy was distributed through ATP and NADPH in shut stomata. According to Lichtenthaler (1996), apart from metabolic functions done by ATP and NADPH, they are also involved in mechanisms of drought tolerance and they defend plants against drought and photo-oxidative injury. Most of these stress indicators generate a definite similar impact on plants, with each indicator having its own unique impacts. It should also be noted that the usual attacks of most environmental stress factors are on the membrane structure, which are involved in sustaining life processes. Hence, membranous processes activities were attacked during the stress.

As noted in Vranova *et al.* (2002), there are links between AOS with aerobic life. Stresses like light, pollution stress and heavy metal are specified to elevate the production of AOS which resulted to injuries done on the membrane organelles and relative cell functioning (Mittler *et al.*, 2004). In the same context, Foyer and Harbinson(1994) noted that anti-oxidative defence done by enzymatic and non-enzymatic played a vital task in stabilizing and obstructing oxidative impairment. However, Foyer and Harbinson(1994) further reported that the production and effectiveness of the anti-oxidative structures is related to phylotaxy and genetic composition. In spite of the close relationship between active oxygen species and aerobic life nevertheless, formation, functions, relevance in signalling facts and their inhibiting act are obviously not expounded.

### 2.8.1 Morphological mechanisms

It is clear that alteration at whole-plant, tissue, physiological and molecular stage is an attribute of drought tolerance plant. Thus, evidence of an individual or a union of innate alterations define the efficiency of plant to maintain itself during drought stress. Below is an account of the several physiological processes under limited water supply.

#### 2.8.1.1 Escape

An abridge in life cycle or growing season is an essential drought adaptation that permit plants to escape from drought, as plants are able to propagate ahead the environment dryness period. Araus *et al.* (2002) reported that abridging reproductive chain could be an essential character linked to drought adaptation which can result to drought escape. As reported by Dingkuhn and Asch (1999), the crop duration and environment were influenced by plant genotype which was responsible for the plant efficiency to avoid climatic stresses. Siddique *et al.* (2003) noted that to obtain high seed yield in plant, it is important to equalize plant growth duration to soil moisture availability. Plant escape drought takes place when phenology phenomenon is equated to duration of obtainable soil moisture; the escape happens in plants with short growing season and predominates at extreme drought stress (Araus *et al.*, 2002; Salehi-Lisar *et al.,* 2016). Based on an observation of coffee field-grown clones, DaMatta (2004) reported that shedding of leaf occurred in systemic manner from the oldest to youngest in reaction to drought. The degree of dropping of leaves was directly connected to the susceptibility of cultivars to drought (DaMatta, 2004). Kumar and Abbo (2001) observed the flowering duration as a principal attribute of adjustment of crops to its environment, especially when their growth period is restrained by extreme drought and heat. In effect, Kumar and Abbo (2001) therefore proposed that developing cultivars with short life-cycle is an operative plan for reducing productive loss from extreme drought because quick maturity could assists the plants to escape the stress injuries. However, Turner *et al.* (2001) reported that yield is associated to crop season when subjected to an ideal propagation treatments, and any notable decline in the crop duration beneath the ideal could strain its yield.

### 2.8.1.2 Avoidance

Kavar *et al.* (2007) have observed that mechanisms of avoidance of drought stress is by reducing water lost through stomata adjustment against transpiration, sustaining water uptake through the development of broad and abundant rooting system. As reported in Subbarao *et al.* (1995). During severe drought season, the only character that promotes plant growth and production is the rooting characteristics which are biomass, length, density and depth. Similarly, Kavar *et al.* (2007) found that extensive rooting system promote movement of water from appreciable depths. Ludlow and Muchow (1990) reported that waxy coating or powdery bloom on the surface of the leaves contributed to the sustainability of water potential and thereby representing an excellent character of drought tolerant plants. Ludlow and Muchow (1990) noted further variation in powdery blooming of the leaves surfaces in wheat plants. Consequently, elevation in water use efficiency did not influence the drought indices such as harvest index. It is clear that the temperature of waxy coated leaves surface is cooler (0.7 $^{o}$C) with reduced aging processes than non-waxy coated. Therefore, six hours reduction in leaf temperature (0.5 $^{o}$C) per day will be adequate to prolong the grain-filling duration. Nevertheless, the variation in waxy coated leaves surfaces is relatively affecting yield indices during drought stress (Yang *et al.,* 2020).

### 2.8.1.3 Phenotypic flexibility

It is well acknowledged that plant adapted their biomass and morphological traits to the unveiling ecological conditions though, growth of plants are always sensitive to water stress. Schuppler *et al.* (1998) noted plants responses to drought using reduced leaves area and number which is a way of reducing plant water-use at the detriment of decline yield. Root expansion, growth and density are the major reactions of plants to water deficit because roots are primary pathway through which plants obtain water from soil (Kavar *et al.*, 2007). Reductions of leaves are attributes related to xeric environ which has been long established. According to Ball *et al.* (1994), xeromorphic plants tolerate drought, though their vegetative yield are comparatively reduced. It can also be gleaned from Ball *et al.* (1994) that xeric plants are associated with the

presence of fine hair on the surface of leaves. This is an essential feature protecting leaves from extreme heat.

As noted in Sandquist and Ehleringer (2003), though pubescent leaves decrease leaf temperatures and transpiration, the character exist with variation in inter and intra specific. Sandquist and Ehleringer (2003) found an increase in production of leaves pubescence which boosted light reflectance and reduced transpiration by increasing the resistivity of the epidermal layer to water flow from the surface of the leaves during heat and radiation stress. Sandquist and Ehleringer (2003) reported further that though water deficit promotes production of hair appendages on adaxial and abaxial of wheat leaves, no notable effect was observed on the boundary layer resistance. Nerd and Neumann (2004) observed 4% reduction in moisture content and 0.25 MPa water potential of plants under water stress. Nerd and Neumann (2004) reported a constant supply of food by phloem and stem succulent to store water as mechanisms that are used by the stems of *Hylocereus undatus* to survive and sustain their growth during drought. Moreover, Nerd and Neumann (2004) reported that though encircled phloem of the stems hinders the production, the production of sucrose-based nectar exudates were preserved during drought. It is in this context clear that roots proliferation were vitals organ necessary for plant adjustment to water deficit. As Nguyen *et al.* (1997) rightly maintained that if plants tolerance ability is to sustain leaf area and growth under a prevailing drought, then the principal foundation of variant becomes visible. Thus, the root system architecture sustained more favourable plants under the water conditions. And as noted in Nguyen *et al.* (1997), root proliferation, thickness and extension permit water availability at high depth of soil. These features, Nguyen *et al.* (1997) noted, are important determinants of drought tolerance in upland rice. Drawing on Nguyen *et al.* (1997), it can be suggested that it is the root structure and distribution that influence the most proficient plan of water uptake during growing season.

The role of root proliferation and structure in drought tolerant tea, onion and cotton has already been noted. Subbarao *et al.* (1995) have therefore advocated the choice of using broad and deep rooting system to elevate yield of legume crops which could maintain the ability to store water when subjected to drought. Semi-dwarfing genes were found to decrease the height of plants into greater root biomass at anthesis because of elevated root thickness by excessive assimilation

(Miralles *et al.*, 1997). Hence, the advantages of higher absorption ready for root proliferation were not indicated. Therefore, variation in adaptiveness of root proliferation and extension has been attributed to desiccation of soil (Liu *et al.,* 2004).

As noted in Farooq *et al.* (2009), numerous ways through which plants may escape drought stress has been recorded. One observed way of doing this is for plants to cut short their growth duration. According to Farooq *et al.* (2009), maintained elevated water potential, reduced transpiration and enhanced water uptake could help the plants to escape drought. Also, Farooq *et al.* (2009) found reduction of leaves surfaces either by leaf dropping or formation of smaller leaves as another strategy engaged by plants to escape drought.

## 2.9 Reactive Oxygen Species, Sites of Production, and Their Effects

Reactive oxygens species play a central role in plant signalling and regulate diverse cellular processes. Studies have shown strategies how ROS mechanisms controlling ROS biogenesis and signaling in plant immunity (Qi *et al.,* 2017). Very reactive ions generated from oxygen during stress are called free radicals. Record has proved that approximately 1.0% $O_2$ respired by plants is channelled to ROS formation (Faize *et al.,* 2011) in numerous cells compartment like chloroplasts, mitochondria, peroxisomes. Free radicals carried out two-folded function, both as destructive and productive species based on the degree of production in plants. High production of free radical harm biomolecules, while low concentration play a role of second messenger in intracellular signalling, which rapidly mediate numerous reactions in plant cells. Frequent ROS are $^1O_2$, $O^{2-}$, $OH^-$ and $H_2O_2$ (Qi *et al.,* 2017).

Apel and Hirt (2004) observed that oxygen molecules in their ground state are not toxic though two unpaired electrons possesses collateral spin that makes it paramagnetic thus, it is not involved in reactions with organic molecules except if initiated. Integration of adequate energy necessary to undo the revolution on one of the unpaired electrons and sequential monovalent reduction are processes required for oxygen activation. At first, singlet oxygen is produced while at end, oxygen is step wisely converted to superoxide, hydroxyl and hydrogen peroxide. Electrons in bi-radical state of $O_2$ have collateral spinning. Adequate energy uptake will cause

the reversal of one of the unpaired electron resulting to the production of $^1O_2$ having two electrons in inverse rotation.

Apel and Hirt, (2004) showed that the active electron surpasses the spin constraint. Therefore,singlet oxygen can be engaged in reactions which required concurrent relocation of the two electrons. In addition, very reactive singlet oxygen was formed throughtriplet chlorophyll in the antenna complex and in the reaction core of PII (Krieger-Liszkay, 2005). In the antenna, inadequate energy released during photosynthesis can result to the production of chlorophyll (Chl) triplet state in the antenna, whereas in the reaction centre it is produced through charge recombination of the light-induced charge pair (Krieger-Liszkay, 2005). The Chl triplet state can react with $^3O_2$ to release dangerous singlet oxygen

Furthermore, closing of stomata decreases existence and accessibility of carbon (iv) oxide during drought which determine the generation of singlet oxygen. Life duration of singlet oxygen in tissues is approximately three micro-second or less. Foyer and Harbinson, (1994) reported that diffusion of singlet oxygen is in a speed of 100nm and it can be activated in polar solvent for four micro seconds and hundred micro seconds in non-polar solvents. Singlet oxygen is very reactive with most of the organelles at its diffusion site, which reduces lipid, protein and DNA in the plant (Wagner *et al.,* 2004).

### 2.9.1 Production of Reactive Oxygen Species and its Location

Free radical generations are found at various sites in the chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum and cell walls. Usually, reactive oxygen species develop inevitable discharges of electrons ($e^-$) from the electron carriage services of chloroplast, plasma membrane and mitochondrion. Free radicals may also appear to be the result of numerous metabolisms confined in various cell chambers.

#### 2.9.1.1 Chloroplasts

Chloroplastic ROS production is tightly associated with light-dependent photosynthetic reactions, and elevated ROS production serves as a marker of changing internal or external status that require the adjustment of metabolism. Chloroplast serves as a source of ROS and signal

transduction that mediate between the plant and environ interactions.Free radicals are generated from numerous sites in the chloroplast and in sundry patterns. The main origins of ROS include chloroplast ETC in P I and II. Drought restrained carbon (iv) oxide fixation in plants as well as the conjunction of two or more stresses increases the generation reactive oxygen species (Gupta and Igamberdiev, 2015). During an accustomed ambient, electron moves from the tensed photosystems sites to nicotinamide adenine dinucleotide phosphate, which is further broken down to NADPH, in entry to the Calvin cycle and the last electron acceptor (carbon (iv) oxide) was deteriorated. When the electron transport centres is overburdened as a result of low nicotinamide adenine dinucleotide phosphate allotted during abiotic stress, electrons leaks from iron-sulphur protein to oxygen, which further breaks down to superoxide *(*Elstner, 1991).

It is worth noting that electrons can possibly move to oxygen from the iron-sulphur protein aggregated in the electron transport centres of photosystem I. Primary and secondary acceptor Quinone a and b are found in the side of electron transport centre of photosystem II. The movement of electron from its location to oxygen enhances the formation of superoxide. The production of superoxideby reduced oxygen is a cost-hindering pace. As soon as superoxide is produced, reactive oxygen species are produced forcefully. Addition of hydrogen ion to superoxide may form hydroperoxyl on the inside lumen or it can be broken down by enzyme superoxide dismutate or automatically to hydrogen peroxide on the outside stroma (Cleland and Grace, 1999).

### 2.9.1. 2 Mitochondria

Mitochondria have ability to generate reactive oxygen species in numerous locations of electron transport centers. Continuous conversion of oxygen to superoxide occurs inside the flavoprotein zone of nicotinamide adenine dinucleotide phosphate dehydrogenase section of the respiration pathway in the mitochondria (Turrens, 2003). As soon as reduced nicotinamide adenine dinucleotide phosphate connect to the substrates, complex I (Ubiquinone oxidoreductase) are impeded and electron moves in an opposite path from complex II (Succunate dehydrogenase) to I. This mechanism to a large extent has revealed elevation in formation of reactive oxygen species at Ubiquinone oxidoreductase complex, which is controlled by hydrolysis of Adenosine triphosphate (Mittova *et al.,* 2015). Cytochrome c-oxidoreductase section or cytochrome $b_{c1}$ or

complex III of the electron transport centres generates superoxidefrom $O_2$. It is opined that complete reduction of ubiquinone contributes an electron to cytochrome $b_{c1}$ thereby; leaving an unsteady, and reduced semiquinone ions that is conducive for flow of electron to oxygen and thus leading to formation of superoxide (Murphy, 2009). In plants, during usual aerobic situations, electron transport centres and Adenosine triphosphate syntheses are firmly conjugated; nevertheless, series of abiotic stress elements resulted to restraint and reduction of its constitutes, resulting to excessive moderation of electron transporters and, thus resulting to free radical formation. Numerous enzymes manifested in the matrix of mitochondrion which can generate ROS. Some of them generated reactive oxygen species without deviation, for instance enzyme aconitase and 1-galactono- $\gamma$ lactone dehydrogenase supplies electrons to the electron transport centre (Rasmusson *et al.,* 2008). The major radicals formed from the reduction of monovalence in the ETC are singlet oxygen. It is transformed readily either by the superoxide dismutase 2, mitochondrial (SOD2) or ascorbate peroxidase into a moderately, stable and membrane-penetrable hydrogen peroxide. Free radical $H_2O_2$ can later be transformed to intensely functional hydroxyl.

#### 2.9.1.3 Endoplasmic Reticulum

Reduced nicotinamide adenine dinucleotide phosphate is reliant on electron movement which entails enzyme cytochrome P450 (CPYs) that generates superoxide in endoplasmic reticulum. The first to react is the organic substrate with CPYs thereafter, it was reduced by enzyme flavoprotein to produce an intermediate ions cytochrome $P450R^-$ (Mittler, 2002). The $^3O_2$ can easily react with the cytochrome $P450R^-$ individually, with each having one unpaired electron. Cytochrome $b_{c1}$ reduced oxygenated complex (Cyt P450-$ROO^-$) or sometimes be degraded liberating superoxide.

#### 2.9.1.4. Peroxisomes

Intracellular hydrogen peroxide generation due to their oxidative type of metabolism has its primary production site in the peroxisomes. Fatty acid $\beta$-oxidation, enzymatic reaction of flavin oxidases, glycolate oxidase reaction, and disproportionation of superoxideions are major metabolic activities necessary for the formation of $H_2O_2$ (Baker and Graham, 2002). Generation

of $H_2O_2$ in the peroxisomes during photorespiration is through the oxidation of glycolate by enzyme glycolate oxidase. Peroxisomes generate superoxideas an output of theirusual processes similar to mitochondria and chloroplasts. Two locations of superoxide have been figured out in the peroxisomes of *Pisum sativum* leave and cotyledons of *Citrullus lanatus*. The first location is found in the matrix, which activated the oxidation of xanthine to uric acid to generate superoxideand second location is the membranes where electron transport centre is made of enzyme flavoprotein, NADPH and Cytochrome $b_{c1}$. The generation of superoxide radicals rely basically on three peroxisomal membrane polypeptides (PMPs) with distinct molar mass of 18, 29, and 32 kDa. The molecular masses of 18- and 32-kDa PMPs utilized NADPH as electron donor for formation of superoxideradical. As described by L´opez-Huertas *et al.* (1999), the 29-kDa PMP further demonstrated a visible reliant on reduced nicotinamide adenine dinucleotide phosphate which is capable to reduce cyt c along with NADPH as electron donor. The PMP with molecular mass 18-kDa was the primary origin of $O_3^{2-}$ which was suggested as the cytochrome that was owned by b-type category. The 32-kDa PMPs was perhaps correlated to monodehyroascorbate reductaseand the third superoxideproducing polypeptide, and 29-kDa probably may associate with peroxisomal reduced nicotinamide adenine dinucleotide phosphate and cyt P450 reductase. The superoxide generated was eventually transformed into hydrogen peroxide by superoxide dismutase SOD (Baker and Paudyal, 2014).

#### 2.9.1.5 Plasma Membranes

Oxidoreductases that transfer electron majorly at the plasma membranes resulted into the formation of ROS at the site (Heyno *et al.,* 2011). The NADPH interceded in the formation of superoxidein plasma membranes. It was inferred that the membrane of *Glycine max* formed superoxide, which could be ascribed to the function of two enzymes namely reduced nicotinamide adenine dinucleotide phosphate oxidase and quinone reductase. A force flow of electrons from cytoplasmic NADPH to oxygen and finally to $O_3^{2-}$ is done by reduced nicotinamide adenine dinucleotide phosphate oxidase (Siddique *et al.,* 2014). Furthermore, superoxide is dismutated to $H_2O_2$ involuntarily or through the activity of SOD. The NADPH has been suggested by Kwak *et al.* (2003) to play a notable role in the production as well as cumulation of ROS in plants stressed.

#### 2.9.1.6 Cell Walls

The active locations for the production of ROS are in the cell walls. There is a direct correlation between the functions of the cell wall and peroxidase during production of hydrogen peroxide. Peroxidase co-related with secluded cell walls, which fasten the rate of $H_2O_2$ formationin the company of NAPDH with response that were energized by various monophenols. The NAPDH was exclusively generated by MDA. Production of $O_3$ in the cell wall was implicated by diamine oxidases through the use of diamine or polyamines to reduce quinone which later auto-oxidizes to produce $H_2O_2$ (Elstner, 1991).

#### 2.9.1.7 Apoplast

Enzymes found at the cell wall have shown to be accountable for formation of reactive oxygen species at the apoplast. Related cell wall enzymes called oxalate oxidase liberates hydrogen peroxide and carbon (iv) oxide from $C_2H_2O_4$. Lane (2002) reported that enzyme cumulates $H_2O_2$ at the apoplast. Enzyme amine oxidase may show a notable function in shielding the responses that occurred in the apoplast due to abiotic stress primarily through hydrogen peroxide generation (Kimura *et al.,* 2017). Enzymes amine oxidase was used to fasten the oxidative removal of amino from polyamines using coenzyme falvin adenine dinucleotise. Hydroxylproduction at the apoplast relies totally or partly on peroxidase situated in the cell wall (Heyno *et al.,* 2011).

### 2.9.2 Reactive Oxygen Species Chemistry

Halliwell (2006) reported that molecular oxygen were brought into our immediate environ through the process of photosynthesis and thus, free radicals were generated during cellular metabolisms. Free radical oxygen molecule has two harmed electrons with common spin quantum number. As a result, oxygen is compelled to take electrons systematically and thus producing reactive oxygen species which can destroy organelles. Navrot *et al.* (2007) noted in a study that free radicals were produced uninterrupted due to numerous metabolic processes that were found in the peroxisomes, chloroplasts and mitochondria under stress. It is worth noting that during photosynthesis, oxygen produced received electrons via photosystem to form $O^{2-}$.

Foyer and Noctor(2005) found that the reactive oxygen species were inhibited by numerous anti-oxidative protective mechanisms under steady state conditions.

The stability between formation and scavenging of free radicals can be distorted by environmental stresses. Bhattachrjee (2005) observed an increase in intracellular free radicals which brought a sudden disturbance and lead to notable injury to the cellular structures. Bhattachrjee (2005) also noted that estimated 1e2% oxygen dissipation induced the formation of free radicals in plant tissues. It is important to note that $O^{2-}$ brings about production of $H_2O_2$, $OH^-$ and other free ROS(s) under several conditions. Free radicals are extremely reactive and harmful, producing impairment to cell organelles which finally lead to cellular death. Cumulation of reactive oxygen species is due to numerous abiotic stresses which have continuously reduced agricultural produce globally.

Foyer *et al.* (2005) reported that cell functioning is directly affected by free radicals which eventually destroy nucleic acids, oxidizing proteins which are the genesis of lipid peroxidation. Gratao *et al.* (2005) reported that for radicals to act as a destroyer, defensive or an indication factors, it must depend on the equilibrium between free radicals generation and its inhibitory ability. It is important to note that free radicals can destroy cells as well as creating gene expression responses. The effect elicited on cells was highly determined by numerous factors. The subcellular site for production of reactive oxygen species may be particularly crucial for an extreme free radicals, owing to it fast solubility reaction with cell molecules. Mittler *et al.* (2004) observed that free radicals influenced by stress are hindered by enzymatic (superoxide peroxidase, Ascorbic peroxidase, Glutathione peroxidase, GST, and Catalase) and non-enzymatic (ASH, free Glutathione, a-tocopherol, carotenoids and flavonoids) antioxidant systems.

Chen and Dickman(2005) noted that proline can be considered as non-enzymatic antioxidants required for scavenging the effects of free radicals. The improvement of antioxidant enzymes at *in vivo* can help to boost tolerance against plant stress by detoxifying cells for survival. It was shown that free radicals affect the signal transduction cycles and expression of gene number. This, as noted in Dalton *et al.* (1999), is a suggestion that cells have developed means to utilize reactive oxygen species as a biological stimuli and signals that engage and control numerous

genomics reactions. Accordingly, it is certain that plants involved in formation of reactive oxygen species to regulate numerous unique physiological mechanisms such as environmental stress, pathogen defense and systemic signalling. Initially considered by-products from aerobic metabolism, reactive oxygen species (ROS) have emerged as major regulatory molecules in plants and their roles in early signaling events initiated by cellular metabolic perturbation and environmental stimuli are now established (Waszczak *et al.,* 2018).

The major origin of free radicals in plant are chloroplast, mitochondria or peroxisomes which are accompanied with great oxidizing metabolic process or excessive speed of electron flow. Green plants are particularly at the danger of oxidative injury, which is largely attributed to bio-energetic lifestyle and excessive photosensitizers. The appearance of $O_2$ was involved in the respiratory processes and operative energy production which utilized $O_2$ as the last electron acceptor thus, resulting to production of ROS (Temple *et al.*, 2005). Scandalios (2005) noted that in spite of non- reactive ability of atmospheric oxygen, it produces free radicals which include superoxide, $OH^-$, $^1O_2$, $H_2O_2$ etc. Instructive to note that at reduced pH, dismutation of superoxide radical cannot be escaped, resulting to one superoxide radical by releasing additional electron to another superoxide radical. This is also accompanied with addition of proton thus, resulting in the formation of $H_2O_2$.

In addition, $O^{2-}$ added proton to produce $HO^{2-}$. Further reactions can take place in through closeness of transition metals, which includes Copper and Iron. The further reaction can occur via the Habere Weiss process or the Fenton mechanisms that releases a highly reactive hydroxyl radical. Surprisingly, $O^{2-}$ radical can as well react with another very powerful indicator like nitric oxide radical which releases peroxynitrite ($OONO^-$).

#### 2.9.2.1 Superoxide radicals ($O^{2-}$)

Free radicals are persistently formed by incomplete diminish of oxygen or transfer of energy to them. The thylakoid membrane-bound primarily accept electron at PI, which is the major production of superoxide radical. The production of ROS must in this regard be understood as an unavoidable effect of aerobic respiration. Four electrons were transported during the reaction of terminal cytochrome oxidase and alternative oxidase with oxygen, water was liberated as end

product. It is clear that oxygen however occasionally react with cytochrome oxidase system (Khaleghi *et al.,* 2019). Here, one electron is moved and consequently superoxide radicals were produced. Halliwell (2006) observed that superoxide radical is commonly the foremost free radicals produced in the tissues of plant, with approximately 1e2% of oxygen dissipation resulting into production of superoxide radicals. The superoxide generated during reduction of oxygen is done in the ETC's of chloroplast during the electron transportation. In photosystem I, oxygen is reduced to superoxide which takes place in the ETC. According to Halliwell (2006), production of superoxide may be activated to generate radicals like $OH^-$, $^1O^2$. High production of these reactive ROS like hydroxyl and singlet oxygencan result to lipid peroxidation and cell weakening. Production of strong oxidizing $HO_2^-$ from the protonation of superoxide invaded the PUFA in a negatively charged membrane surfaces.

Scarpeci *et al.* (2008) suggested that superoxide radical produced in the chloroplast resulted into stimulation of gene signalling pathways. A work by Gambarova and Gins (2008) that studied $C_3$ and $C_4$ photosynthetic pathways under salinity in *Amaranthus* revealed detoxification of superoxide by superoxide dismutase and antioxidant Amarathine, and thereby leading to reduction in the degree of MDA. Also, Gambarova and Gins (2008) reported an equivalent interaction between superoxide dismutase and amaranthine during salinity of *Amaranthus* leaves.

#### 2.9.2.2 Singlet oxygen ($^1O_2$)

Singlet oxygen is an uncommon radical, it's the first excited electronic state of $O_2$ due to lack of electron transfer connection to the oxygen. Deficit energy consumption results to production of chlorophyll triplet state during photosynthesis thus, the triplet state reacts with $^3O_2$ to generate $^1O_2$. Many studies revealed that generation of $^1O_2$ has caused great injuries to the entire photosynthetic machinery (photosystem I and II). In addition, environmental stresses resulted to stomata closure and reduced intercellular carbon dioxide in the chloroplast hence, generation of singlet oxygen was encouraged. The life span of $^1O_2$ as reported by Hatz *et al.* (2007) takes about three minutes in cell and it only takes a fraction of singlet oxygen to diffuse over a large miles of 100nm. The solubility of $^1O_2$ in water takes about four minutes and hundred minutes in polar solvent. It is clear from Krieger-Liszkay *et al.* (2008) that $^1O_2$ has a broad scope of molecules responsible for the reduction of organelles, and that it is also an essential species liable for

inducing light lost in photosystem II and eventual destruction of cell. As already observed in Maisch *et al.* (2007), photosensitized bacteria can produce singlet oxygen upon exposure to light hence oxidation of cells result to the death of bacteria. Triantaphylides *et al.* (2008) reported an optimal generation of $^1O_2$ which was accountable for above 80% lipid peroxidation in the leaves of *Arabidopsis*. As indicated by Krieger-Liszkay *et al.* (2008), establishment of non-enzymes antioxidant are effective scavengers of singlet oxygen, though singlet oxygen look after the up-regulation of genes that are liable in the molecular guard adverse to photo-oxidative stress.

#### 2.9.2.3 Hydrogen peroxide Radical

Univalent diminution of $^1O_2$ formed hydrogen peroxide. Radical $H_2O_2$ is averagely reactive with long life span unlike free radicals like $OH^-$, $^1O_2$ and $O^{2-}$ with short life span (Bhattachrjee, 2005). Also, it was claimed that enormous hydrogen peroxide has resulted to continuous event of oxidation death in plant cell. During this process, hydrogen peroxide may stop the functioning of antioxidants accountable for the oxidizing of thiol groups. High hydrogen peroxide was generated in AtCLH1 silenced plants *E. carotovora* under an elevated light intensity (Kariola *et al.,* 2005). Hydrogen peroxide ($H_2O_2$) displayed double role of signalling molecule which prompt plants to response during stress at a reduced concentration (Quan *et al.,* 2008). Hydrogen peroxide $H_2O_2$ was widely responsible for the regulation of processes like senescence, photo-respiration and photosynthesis, closing of the stomata and cell reproduction, growth and development (Noctor and Foyer, 1998). The capacities of $H_2O_2$ radical to act as a signalling molecules may be due to its prolonged half span along with its fast porosity over membranous cells (Quan *et al.,* 2008).

### 2.9.3 Role of Reactive Oxygen Species as Messengers

Reactive oxygen species has been required as second messenger in the intracellular signalling at concentration below average, which influences numerous plant reactions in plant cells, and this includes PCD, closing of stomata, development of tolerance to environmental stresses and gravitropism (Cheng and Song, 2006). Plants can detect, convert and transfer reactive oxygen species signal into suitable cells reactions assisted by some redox-susceptible proteins, protein phosphorylation, gene expression and calcium mobilization. Reactive oxygen species can be

detected immediately also by vital signalling proteins like a tyrosine phosphatase via oxidation of preserved cysteine residues. Various mechanisms were controlled by ROS such as signalling of protein phosphatases, protein kinases and transcription factor which transfuse with the pathways of other signal factors creating a division of the signalling factors that powers the reaction downstream for the free radicals (Miller *et al.,* 2008). The firmness, lifespan and volume of the reactive oxygen species signalling pool relies on the equipose between the generated oxidant and disposal by the antioxidant.

Using mutant deficit in vital reactive oxygen species inhibiting enzymes, Miller *et al.* (2008) distinguished a signalling track that is triggered in cells in reactions to ROS cumulation. Interestingly, series of vital roles includes different Zn finger proteins and WRKY transcription factors, which are also key modulators of environmental stress reactions. Free radicals are viewed by Yan *et al. (*2007) as a second messenger in abscisic transduction chains found in the guard cells. Abscisic acid-induced hydrogen peroxide is an important indicator intermediating the closing of stomata to the decreased transpiration through a functional calcium-permeable passages in the cell membrane. Jannat *et al.* (2011) noted that abscisic acid generated cytosol hydrogen peroxide, which triggered abscisic acid (ABA) signals for stomatal closure, while integral enlargement of hydrogen peroxide did not lead into the closure of stomata. The ROS functions as signalling factor in response to root gravitropism. Unsymmetrical motion of auxin observed by Joo *et al.* (2001) triggered gravity for sixty minutes and auxin animates free radicals production to intercept gravitropism. In addition, antioxidant such as *N*-acetylcysteine, ascorbic acid, and Trolox scavenged ROS that hindered root gravitropism. Free radicals were suggested to have caused reduction in seed dormancy. Abscisic signalling was high in response to low production of ROS causing grains dormancy in barley under control situations.

Abscisic synthesis and signalling was not significantly affected by exogenous $H_2O_2$, instead Gibberellin (GA) signalling responds significantly to trigger modification in balancing of hormone that facilitates vegetative growth (Bahin *et al.,* 2011). Also, ROS initiated by GA had played an important role in PCD found in barley aleurone cell. It was further noted that protoplast treated with GA are sensitive to both endogenous and exogenous functional $H_2O_2$ than protoplasm treated with ABA which suggested that radicals are elements responsible for the

controlling of hormone that resulted to cellular damage track in barley aleurone cells. An intricate controlled mechanism was developed in plants to intercede environmental stress reactions based on the biosynthesis of ROS, activities of the scavengers and signalling factors (Nanda *et al.,* 2010). The ROS function as a second messenger, as stated by Orozco-C´ardenas *et al.* (2001), to help to develop defensive genomes in *Lycopercium* against wounding. Free radicals are generated near the walls of the vascular bundles of *Lycopersicum* thereafter, hydrogen peroxide produced during wounding serve as second messenger to activate defensive genomes in the mesophyll cells rather than genomes in the vascular tissues. Response of plant to abiotic stress also depends on Lignin biosynthesis in plant. Genetic system activated lignin biosynthesis in response to injury caused on the cell-wall through an active relationship between Jasmonic acid and free radicals (Denness *et al.,* 2011).

## 2.10 The Cell Chemistry of Reactive Oxygen Species

### 2.10.1 The activities of lipid peroxidation

Garg and Manchanda (2009) defined lipid peroxidation as a detrimental mechanism that happens in an organism. During peroxidation, polyunsaturated precursors produced hydrocarbon sections like ketones, malondialdehyde (MDA) etc. Free radical reached above acceptable limit during cell and organ membranes peroxidation, thereby affecting both cell functioning and therefore intensifying the oxidation formed by the lipid-derived radicals.

The beginning, progress and end are procedures required in lipid peroxidation. Complexing is the first stage that involves metals like copper and iron (Fam and Morrow, 2003). Hydroxyl radical has majorly accounted for the start of peroxidation by extracting hydrogen atom from an acyl chain of PUFA residue though; superoxide and hydrogen peroxide are also efficient enough to begin the reactions. The ROO- is produced from addition of oxygen to fatty acid located at lipid radical carbon centre in an aerobic environ (Simova-Stoilova *et al.,* 2006). Thereafter, development of peroxidation chain is further carried out by hydrogen atom extraction from the adjacent polyunsaturated fatty acid side chain using $ROO^{-}$. Fam and Morrow (2003) revealed that the resultant of the lipid peroxidation can be further degraded into numerous radicals such as lipid alkoxyl radicals, aldehydes, alkanes, lipid epoxides etc. Distinct initiations have shown

ability to produce several peroxides. The ultimate impact of this peroxidation is to decline the membrane fluidity, suitable and simple for phospholipid to interchange between the two halves of bilayers thereby elevating the discharge of the membrane that ideally cannot pass through a particular route without inflicting an injury on the membrane proteins, receptors and enzymes.

Polyunsaturated fatty acids are sensitive to strike singlet oxygen and hydroxyl radicals resulting to mixtures of lipid hydroperoxides. Elevated polyunsaturated fatty acid are developed to decline in fluidity of the membrane, elevated discharge and afflicted minor injury to the membrane protein (Moller and Jensen, 2007). Numerous aldehydes like 4-hydroxy-2- nonenal and MDA, $OH^-$ and keto fatty acids are generated from polyunsaturated fatty acid peroxidation. The decomposition of the aldehyde products generated a conjugation between the DNA and protein. The generation of aldehydes in the mitochondria could be responsible for sterility in maize due a restorer gene (Moller and Jensen, 2007).

Production of ROS made crops vulnerable to numerous stresses, which could lead to high lipid peroxidation. Variation in the production of peroxidation in the two cultivars (Begunbitchi and Lunishree) of *Oryza sativa is* directly related to elevated free radical scavenging ability which reacted with a large effective defensive processes against salinity stress (Khan and Panda, 2008). A remarkable high MDA content was observed by Kukreja *et al.* (2005) in *Cicer arietinum* root when subjected to salinity. Similar trend such as high lipid peroxidation, membrane damage, formation of $H_2O_2$ and hydroxyl radicals were observed in relation to water stressed *Phaleolus vulgaris* plants (Simova-Stoilova *et al.,* 2006). Simova-Stoilova *et al.* (2010) noticed a membrane impairment and oxidation injury in lipid which were more obvious in susceptible cultivars of wheat plants. Similarly, elevated MDA contents were recorded in *Glycyrrhiza uralensis* Fisch seedling during salinity stress (Pan *et al.,* 2006). Also, lipid peroxidation was observed to be an essential biochemical processes that is used to screen tomato plant under water stress (Sanchez-Rodriguez *et al.,* 2010).

### 2.10.2 The Reaction of DNA towards Water Deficits

Tuteja *et al.* (2009) described plant genomics stability to be unwavering, though DNA might be disturbed and destroyed when exposed to severe genotoxic stress. Injuries done to DNA resulted

in the generation of radicals such as $OH^{-1}$, $O_2$ and NO. This type of DNA injury is known as spontaneous DNA impairment. Elevation in generation of free radicals might harm the cell structure, protein, lipid and nucleic acids (Valko *et al.,* 2006). Radical $OH^-$ is most reactive, thus, it is responsible for all injuries afflicted on DNA components thereby, destroying purines and pyrimidines bases (Halliwell and Gutteridge, 1999). Wiseman and Halliwell (1996) reported that single oxygen mainly strike guanine while hydrogen peroxide and oxygen don't react. Tuteja *et al.* (2001) reported that free radicals has ability to damage cell macromolecules like DNA through base deletion, pyrimidine dimers, cross-links, strand breaks and base modification. Eventually the growth of plant was retard due to diminished protein synthesis, damaged cellular membrane and photosynthetic apparatus (Britt, 1999). Furthermore, Cooke *et al.* (2003) reported that damaged molecular DNA could either result into damage or initiation of transcription, transduction path and genetic inconstancy.

## 2.11 Compatible Solutes and Drought Stress

As a method of permitting stress, a broad diversity of organisms secrete and assemble small molecules compound called compatible solutes which are referred to as cell osmolytes or osmo-defendant. Osmolytes are broadly dissoluble at high concentrations without suppression of other cell constitutes. The plant osmolytes comprises of proline, citrullin, fructan, fructose, pinitol, sucrose, glycine-betaine, trehalose, 3-dimethylsulfonopropionate and mannitol. Of these, glycine betaine were secreted in xerophytic and halophytic plants, while Kawasaki *et al.* (2000) observed that citrulline was collected in leaves of drought stressed wild *Citrulllus lanatus*.

### 2.11.1 Functions of Compatible Solutes

It is important to state that the processes which osmolytes engaged to defend cells components are quiet veiled in several occasion. Chen and Murata (2002) however suggested that production of plant osmolytes help plants to generate resistance to numerous environmental stresses. Water solubility features of an osmolytes acts as an alternate for liberation of water molecules from leaves, it is clear that osmolyte promote tolerance to stress in plant through their functioning as osmo-regulators. It is pertinent to note that osmolytes could also serve as ROS scavengers or thermos-stabilizers in some cases (Akashi *et al.,* 2001). Cumulating proline is a common reaction of plant to water deficit. Exposure of plant to drought resulted into high concentration

of proline, thereby, increases the total concentration of responsive amino acids that are responsible to fight oxidative stress in plants (Lum *et al.,* 2014).

Increased osmolytes concentration can elevate cell osmotic potential. Moreover, turgidity, moisture content of cells and protection against transpiration were ascribed to its high solubility when exposed to water stress (Hoeskstra *et al.*, 2001). Osmolytes might also be substituted for water molecules, as observed by Hoeskstra *et al.* (2001), around the plants organelles like DNA, proteins and membranes when subjected to drought owing to their exceptional solubility characteristics. Hoeskstra *et al.* (2001) observed that decrease in cell water could lead to elevation of concentration of charged small molecules, thus causing a disruption of macromolecules. In effect, Hoeskstra *et al.* (2001) noted that osmolytes might stop interrelationships that exist between the radicals generated and cell composition through substitution by molecules of water and ultimately stabilizing the cells during stress.

Studies by Nomura *et al.* (1998) have shown the facts that osmolytes steady the production and functioning of enzymes. For instance, Sakamoto and Murata (2002) found that osmolyte proline and glycine betaine can protect enzymes RuBisCO activity from inhibition while high concentration of sodium chloride suppressed its activity. Glycine betaine has also been shown in Sakamoto and Murata (2002) to steady PSII super-complex in the existence of increased concentrations of Sodium chloride. However, Trehalose exercises its role at low concentrations than osmolytes. *Myrothamnus flabellifolius* generally called resurrection plants assemble trehalose to elevate the protein's thermos-stability nature. Meanwhile, Garg *et al.* (2002) reported that resistivity to numerous stresses is presented at reduced concentrations in plant cells. Water stress creates cell membrane injury and escape of ions from plant cells. Similarly, Hincha *et al.* (2000) observed that fructans are also osmolyte that are capable of maintaining phosphatidylcholine liposomes during cold stress.

Closure of stomata helps to prevent transpiration as the influx of carbondioxide move into the leaves during water stress. Therefore, the solar radiant cannot be utilized for $CO_2$ fixation. Alternatively, it was employed for the production of AOS molecules in the chloroplasts. Hong *et al.* (2000) observed decrease in formation of radicals as proline accumulation increased in Tobacco. Reaction of proline in scavenging hydroxyl radicals is lowered when compared to

activities of citrulline and mannitol. Akashi *et al.* (2001) reported that citrulline can rightly degrade all hydroxyl radicals at its production centres.

### 2.11.2 Biosynthesis of Compatible Solutes

Nuccio *et al.* (1999) instructively noted an accumulation of metabolites which were achieved by aided synthesis or suppressed decomposition, or both. Osmolytes medium are made up of metabolites composing of principal metabolic pathway, though instability of metabolites for

synthesis of osmolytes has been greatly managed.

Hare *et al.* (1999) revealed production of proline from glutamate using P5C synthetase (P5CS) and P5C reductase (P5CR). Kishor *et al.* (1995) reported that Arabidopsis mutant over-expressed gene responsible for accumulated high proline and therefore the reaction was accelerated by P5C synthetase. Deuschle *et al.* (2001) has shown that decomposition of proline was accelerated by series of reactions using enzymes proline dehydrogenase and P5C dehydrogenase which were determined by an aggregate of proline in the mitochondria. Thus, all genes responsible for the production and decomposition of proline are up-controlled. The accumulation of proline, as it is clear in Deuschle *et al.* (2001), is strictly regulated and accomplished when the rate of production predominate the decomposition.

## 2.12 Antioxidant Defensive Mechanism of Scavenging Reactive Oxygen Species

Generation of ROS can increase when exposed to severe ecological conditions, which include high temperature, drought stress, pollutants stress (air pollutant and heavy metal), nutrient stress or salinity. Cellular structures like chloroplast, mitochondria and peroxisomes used antioxidant protectant mechanisms to shield away from toxic oxygen intermediates. The formation of cell antioxidant tools is an important defence of plant against numerous stresses. The non-enzymatic antioxidants activities viz., glutathione, ascorbate and enzymatic antioxidant activities protected the rice shoots and roots oxidative stress. Enhancement in activities of the overall antioxidant enzymes under drought stress reflects their functions in the adaptation process (Nahar *et al*., 2018).

### 2.12.1 The components of antioxidant defence

#### 2.12.1.1 Superoxide dismutase (SOD)

Several studies have shown that metallic-enzyme SOD was found to be the most powerful enzymatic antioxidant situated within the cell which is present everywhere in all organisms and its smaller divisions. The numerous ecological stresses frequently resulted to high generation of radicals; this is well established in the literature. It is also acknowledged that SOD is crucial for any plant to tolerate stress because it supplies protection to counter the harmful impacts of increased free radicals (Apel and Hirt, 2004; Khaleghi *et al.,* 2019).

There was an acceleration of dismutation as oxygen was removed, the first oxygen was reduced to $H_2O_2$ while the second oxygen was oxidized to $O_2$ (Edwards *et al.,* 1990). By removing $O^{2-}$, Edwards *et al*. (1990) reported that SOD reduces the danger of hydroxyl generation using metal catalysis called HabereWeiss-type reaction. In addition, Edwards *et al* (1990) noted that SOD display principal function in protection against oxidative injury done in aerobic organisms. Also, elevated SOD activity was recorded in plants as recorded by Apel and Hirt (2004) under numerous environmental stress like drought and heavy-metal stress. Accordingly, elevated superoxide dismutase activity is frequently related with the tolerant ability of plants against abiotic stresses. Superoxide dismutase can be subordinately employed as an indicator standard for evaluating tolerance in plants. Excessive synthesis of SOD improved plant tolerance to survive oxidative stress (Stevens *et al.*, 2008).

#### 2.12.1.2 Catalases (CAT)

It is worth noting that catalase has four structural subunits having greater possibility to catalysed $H_2O_2$ to $H_2O$and $O_2$ instantly (Garg and Manchanda, 2009). Garg and Manchanda, (2009) noted that catalase are expedient for reactive oxygen species removal during abiotic stress, thus, it recorded the highest overturn compared to other enzymes. Moreover, catalase rapidly changes lots of $H_2O_2$ into water within one mins. Also, catalase is crucial in the elimination of $H_2O_2$ production inside the peroxisomes by oxidases. Catalase isozymes have related their uniqueness for $H_2O_2$, which is being broadly studied in plants. However, weak response was recorded against

organic peroxides. Catalase presents no cellular reducing equivalent, though plants contain numerous enzymes responsible for hydrogen peroxide decomposition. More so, catalase reportedly has a quick overturn compared to APX with greater affinity for hydrogen peroxide. Yin *et al.* (2010) observed that $H_2O_2$ was mainly formed in the peroxisomes. Process of photo-respiratory oxidation and β-oxidation of fatty acids, catalase inhibited hydrogen peroxide radicals produced in this organelle.

Hydrogen peroxide radicals have been included in various abiotic stress condition with a recurrent facts that catalase occur in plant cytosol, chloroplast, and mitochondria. Eltayeb *et al.* (2006) reported that hydrogen peroxide is decomposed by catalase requiring energy when the cells are stressed, leading ultimately to the production of hydrogen peroxide through catabolic metabolism. Based on the concentration, duration and kind of stress, abiotic stress has caused improvement or lessens activity of catalase (Dixon *et al.* 2010). As noted in Noctor *et al.* (2002), reduced catalase activity was produced more in sensitive plant to paraquat, salinity and ozone but not for cold stress. According to Noctor *et al.* (2002), in genetically engineered *Nicotiana tabacum* (10% wild-type), catalase cumulate glutathione disulphide and reduced Ascorbic acid four times, showing that catalase is an important enzyme needed for sustaining and stabilizing oxidation and reducing reaction. Dixon *et al.* (2010) noted that an overexpression of CAT gene brought about an enhancement in the tolerant ability of *Nicotiana tabacum* that was transferred from *Brassica juncea.*

#### 2.12.1.3 Ascorbate peroxidase (APx)

It's been widely reported in the literature thatAPx inhibited$H_2O_2$ in water and GS-ASH cycle by making use of ASH as an electron donor. The family ascorbate peroxidase comprise of minimum of five distinct isoforms, namely: those generated in the thylakoid (tAPx); those formed in glyoxisome membrane (gmAPx); those produced in stromal chloroplast mainly soluble forms (sAPx); those formed in the cytosolic (cAPx) as described by Noctor and Foyer (1998). Ascorbate peroxidase has a greater choice for hydrogen peroxide than catalase and peroxidase and they therefore display a crucial function in manipulation of free radicals during water deficit (Khaleghi *et al.,* 2019).

High Ascorbate peroxidase was recorded in reaction to environmental stresses like high salt concentration, water stress, heat stress, heavy-metal stress and UV irradiation (Tuteja *et al.*, 2010). Similarly, Smirnoff (2005) reported that an elevation in expression of Ascorbate peroxidase generated in Pea cytosolic improved acclimation to drought and tolerance in Pea and genetic modified tomato plants against oxidative damages done by cold as well as salinity. Also, Athar *et al.* (2008) reported elevated expressed Ascorbate peroxidase gene to the high tolerant ability of *Nicotiana tabacum* and *Arabidopsis* against oxidative stress.

#### 2.12.1.4 Glutathione reductase (GR)

Glutathione reductaseis a flavonoids based-protein oxidoreductase that develops in cellular organisms (Romero-Puertas *et al.*, 2006). Hence, it's an enzyme with a capacity to be involved in Glutathione-ascorbate cycle and it plays an important function in the protection mechanism of free radicals by maintaining the decline state of GR. Creissen *et al.* (1994) reported that Glutathione reductase is principally developed in chloroplast, while small amount is formed in the mitochondria and cytosol. Glutathione reductase, as indicated in Chalapathi Rao and Reddy (2008), accelerates the reduction of free Glutathione and is therefore engaged in numerous mechanisms of metabolic activities as well as anti-oxidation processes in plants where Glutathione reductase accelerates NADP dependently on reaction with disulphide bond.

Several authors have observed an elevation in Glutathione reductase due to abiotic stress. Pietrini et *al.* (2003) observed association between the tolerance to oxidative damage and activity of Glutathione reductase and therefore maintained that injury done by application of paraquat and as well as hydrogen peroxide radical could quicken Glutathione reductase de novo synthesis, which occur likely at translation stage by pre-existing mRNA. Metwally *et al.* (2005) noted that antisense-mediate reduction of *Lycoersicum* chloroplast. Glutathione reductase increased its susceptibility to stress. An elevation in ascorbic acid due to high production of Glutathione reductase in the leaves of *Nicotiana tabacum* and *Salicoideae populus* plants was recorded, and it was observed that this elevation resulted to improved plants tolerance to oxidative damage (Srivastava *et al.*, 2005). Overexpression of one constituent of anti-oxidative protective system

may modify the ability of the pathway owing to complex radicals detoxification mechanisms (Fini *et al.,* 2013).

Creissen *et al.* (1999) observed an overexpression co-operation of antioxidant enzymes in genetically modified plants which show significant interactive impact on the stress. Verbruggen and Hermans (2008) demonstrated that concurrent indication of APx and Copper/Zinc SOD genes in the chloroplasts of *Nicotiana tabacum* increases tolerance of the plant to herbicide called viologen paraquat ($MV^{2+}$) stress. Similarly, Molina *et al.* (2008) noted that an increase in tolerance to several environmental stresses has broaden a concurrent overexpression of enzymes SOD and APx genes produced in the chloroplast, as well as SOD and CAT produced in cytosol as reported by Creissen *et al.* (1999).

Furthermore, concurrent generation of several antioxidant enzymes, which include Cu-Zn SOD, APx and Dehydroascorbate reductase, were formed in the chloroplasts (Wagner *et al.*, 2004). The alliance expressions have demonstrated to be more productive than an individual expression in producing genetically modified plants with an increase in tolerance rate to several stresses. Therefore, to accomplish tolerance to several abiotic stresses, genetically modified plants need to increase many than one antioxidant enzymes expression.

#### 2.12.1.5 Guaiacol Peroxidase (GPx)

Guaiacol peroxidase is an iron compound accommodating protein majorly present in living organisms. GPx prefer to oxidize aromatic electron donor like Guaiacol and Pyragallol than Hydrogen peroxide radicals. Sappl *et al.* (2004) noted that a Guaiacol peroxidase enzyme possesses four stable disulfide bonds with two structural calciumions. Frova (2003) observed that several enzymes with same functions but different structure of Guaiacol peroxidase in plant confined in vacuoles, cell wall and cytosol. Accordingly, Edwards *et al.* (2000) reported that Guaiacol peroxidase is identified with numerous essential biosynthesis phenomenon like lignified cell wall, decomposition of indole acetic acid, biological production of ethylene, healing of wounds, and protection against ecological stresses.

Guaiacol peroxidase is widely accepted as stress "enzyme." In this context, Dixit *et al.* (2001) reported that Guaiacol peroxidase can be used as a successful scavenger of reactive mediate kinds of oxygen and peroxy radicals under stressed conditions. As noted in Hong *et al.* (2007), numerous abiotic stresses have been proven to increase Guaiacol peroxidase activities. Moons *et al.* (2003) linked elevated Guaiacol peroxidase activity to oxidative damage done by metallic toxicity situations and they therefore proposed its possibility as biomarkers for sub-lethal metallic toxicity in plants. Similarly, Jogeswar *et al.* (2006) also noted higher defense of salt-tolerant *Carthamus tinctorius* plants against oxidative damage hence, as well as acceleration of an effective production of defined isoenzymes through an elevated GPX activity.

#### 2.12.1.6 Dehydroascorbate Reductase (DHAR)

Smirnoff (2000) reported that dehydroascorbate reductase performs a crucial function in maintaining reduced ascorbic acid, given that it accelerated the decrease of Dehydroascorbate to ascorbic using a free GH as a reducing agent. Quite a lot of dehydroascorbate often generates oxidized Ascorbic acid in leaves and other tissues, nevertheless the likelihood of the enzyme and non-enzyme to reproduce ascorbic acid is straight from MDHA (Khaleghi *et al.,* 2019). Dehydroascorbate is a temporary chemical that can be broken down by water permanently to 2, 3-diketogulonic acid. Studies, including those conducted by Jimenez *et al.* (1998), also reported an overexpression of dehydroascorbate reductase in *Nicotiana tabacum* leaves, *Zea mays*, and *Solanum tuberosum* and high ascorbic acid composition. Therefore, it was recommended that dehydroascorbate reductase perform a crucial function in regulating the size of Ascorbic acid.

Dehydroascorbate reductase is a monomer of sulfhydryl enzyme located in seeds that are dry, roots and etiolated shoots. Previous study has revealed chloroplast and non-chloroplast as an origin of purified dehydroascorbate reductase in several species of plants (Xiang *et al.*, 2001). Spinach leaves and potato tuber (Mullineaux and Rausch, 2005) have been identified as one of the several plant species in which DHAR has been purified. High dehydroascorbate reductase activities in plants was associated with the presence of abiotic stress, which includes water stress, metal toxicity, and cold (Rausch and Wachter, 2005). As shown in Meyer (2008), *Lotus. japonicus* was more tolerant than legumes due to consistent up-regulation of the gene encoding dehydroascorbate reductase in cytosolic. The upward requirement of dehydroascorbate reductase

was linked to ascorbic acid reuse in the apoplast. Briviba *et al.* (1997) noted AtDHAR1 of Arabidopsis cytosolic overexpression in genetically modified *Solanum tuberosum* and attendant increased in tolerant ability to water stress, salinity and herbicide.

## 2.13 Non-enzymatic Components of the Antioxidant System

Constituent of non-enzymatic anti-oxidative defensivesystem built up the inner cellular redox. Antioxidants associated with various cells, as described by De Pinto and De Gara (2004). Apart from engaging in key functions like protection and as cofactors, the defensive system also affects regulating activities of cellular elongation and mitosis, aging to cellular death.

### 2.13.1 **Ascorbate** (AsA)

Ascorbate is an antioxidant with a small molar mass which performs crucial protection function in opposition to oxidative stress, which gave a higher degree of ROS. Ascorbate is strong and has capacity to provide electrons in response to number of antioxidants. Ascorbate displays an important function in many metabolism (growth and differentiation) activities in plants. Ascorbate is produced through uronic acid intermediates, such as D-galacturonic acid (Wheeler *et al.,* 1998). Ascorbate was formed from oxidation of L-galactono-1, 4-lactone by L galactono-1, 4-lactone dehydrogenase enzyme. In the mitochondria, synthesis of Ascorbate is done by L-galactono-$\gamma$-lactone dehydrogenase. And thereafter it migrated to other cellular parts through facilitated diffusion (Nahar *et al.,* 2018).

Plant cell types, organelles and apoplast are one of the photosynthetic tissues that revealed surplus ascorbate (Shao *et al.,* 2008). Approximately 90% of ascorbate was restrained in cytoplasm which is distinct from other soluble antioxidants that carried a significant part away to the apoplast. External oxidants are firstly shielded by Apoplastic ascobate which are prospectively detrimental to the cells. Ascorbate majorly shielded macromolecules from oxidative damage. In standard situations, ascorbate exists and also functions as coenzyme of ascorbate oxidoreductase therefore, supporting degeneration of excessive excited energy (Barnes *et al.,* 2002). Ascorbate provided membrane shield through the reaction of superoxide, hydrogen peroxide and transformed $\alpha$-tocopherol from tocopheroxyl ions by conserving enzymatic

activities with transitional metals (Khan *et al.,* 2020**)**. Ascorbate possesses primary function in elimination of hydrogen peroxide through AsA-GSH cycle. Ascorbate undergoes process of oxidation in two successive steps that starts with the formation of monodehydroascorbate MDHA and afterwards dehydroascorbate DHA is produced. During Ascorbate-glutathione cycle, APx makes used of two molecules of ascorbate to degrade hydrogen peroxide to the production of MDHA along with formation of water. Monodehydroascorbate is a short-life spanned free radical that can be dismutated into dehydroascorbate and Ascorbate or degraded to Ascorbate by reducing nicotinamide adenine dinucleotide phosphate dependent enzyme monodehydroascorbate reductase. Tatarate and oxalate is formed (at pH >6.0) from unstable disintegration of Dehydroascorbate. In order to escape this, speedy degradation of DHA to ascorbate was carried out by enzyme DHA reductase utilizing degradation alternative from glutathione. Radyuk *et al.* (2009) observed that ascorbate level is modified in reaction to diverse stresses.

The level of ascorbate produced as explained by Chaves *et al.* (2002) during stress is a function of equilibrium between the speed and its synthesis ability. Conversion of GDP-D-mannose to GDP-L-galactose was assisted by an overexpression of enzymes Mannose-GDP 3-5-epimerase (GME), which indicates an important pathway in bio-synthesis of ascorbate in tolerating abiotic stress. Overexpression of GME genomics family, as explained by Zhang *et al.* (2011), brought about further cumulation of ascorbate and enhanced tolerance in *Lycopersicum esculentum* to environmental stresses. Also, overexpression of *Fragaria* D-galacturonic acid reductase results in cumulation of ascorbate and improved its potato tolerant ability to stress (Wang *et al.,* 2010).

### 2.13.2 Glutathione (GSH)

Glutathione is a vital thiol with a low molar mass that displays a crucial function in protecting intracellular cells against induced free radicals causing oxidative impairment. Cytosol, chloroplasts, endoplasmic reticulum, vacuoles, and mitochondria are the primary site of glutathione production (Foyer *et al.,* 1997). Cytosol and chloroplasts sections produce glutathione as an isoform of glutamate cysteine ligase and glutathione synthetase. Preservation of cell redox state relies on the equilibrium nature of glutathione and glutathione disulfide (GSSG).

As a result of its degradation potential, GSH, as explained by Foyer *et al.* (1997), shows important functioning on several metabolism processes like cell growth, control of sulfate transport signal transduction, metabolites and enzymatic control, phytochelatins for metal chelation, detoxification of xenobiotics, and the expression of the stress sensitive genes. Glutathione is an ROS scavenger that can react with $O^{2-}$, $HO^{-}$ and $H_2O_2$ and shielded macromolecules.

Enzymes DHAR is responsible for the degradation of oxidized AsA that was recycled by glutathione. Enzyme DHA can further be degraded through the process of non-enzymatic mechanism using glutathione (pH>7, >1mM) (Nahar *et al.,* 2018). For this mechanism to occur in the plant's chloroplast, the concentration of GSH must be as high as 5mM, pH approximately 8 and light must also be present. The process of production through *de novo* synthesis using NADPH as a cofactor and conservation of degraded GSH pool has an important significance to the cell. The GSH functions as a stress marker in antioxidant defensive system. Under a prevailing drought stress, *Malus* tree first experienced very little oxidation of GSH pool thereafter, the concentration were later heightened. Moderate stress resulted into more oxidized GSH pool due to low concentration of GSH, thus leading to system degradation and also resulting into varying ratio in concentration of GSH/GSSG (Roychoudhury *et al.,* 2012). Indian *Brassica nigra* showed overexpression of gamma–glutamycysteine synthetase, which reduced the responsiveness of the plants to cadmium stress eventually improve tolerant level of plant against chloroacetanilide herbicides. Degradation of glutathione in transgenic Solanum tuberosum significantly improved the defensive system against oxidation damage (Eltayeb *et al.*, 2010).

### 2.13.3 Carotenoids

Carotenoids are components of lipophilic antioxidants that are capable of detoxifying different types of radicals (Young, 1991). They are found in plants and also in microbes. Plants take up light within the wavelength of 400 and 550nm of the visible light, and thereafter transferring the acquired energy directly to Chlorophyll. Suppression of oxidation impairment as a result of scavenging $^1O_2$ in order to extinguish triplet sensitizer ($3Chl^*$) and agitated chlorophyll ($Chl^*$) molecule so as to reduce generation of singlet oxygen that shielded the photo system (Mibei *et*

*al.,* 2017). They also function as forerunners to signalling factors, swhich affect growth of plants and its response to environmental stress (Li *et al.,* 2008). Chemical specificity of carotenoid is associated to its ability to inhibit, stop or reduce the formation of triplet chlorophyll. Carotenoids permits free uptake of energy from agitated ions and degenerate excessive as heat energy. Suitable adaptability of *Saccahrium officinale* to saline soil is related to high accumulation of carotenoids (Gomathi and Rakkiyapan*,* 2011).

### 2.13.4 Phenolic Compounds

Metabolites with antioxidants properties are called phenolics. Grace and Logan, (2000) discovered its bountiful presence in plant tissues. The aromatic ring with $HO^-$ or OCH3 substituents is responsible for their antioxidant actions and biological processes (Sarker and Oba, 2018). Effectiveness of Polyphenol is more than the familiar ascorbic acid and $\alpha$-tocopherol. They show ability to chelate transition metallic ions thereby inhibiting ROS and reducing level of MDA content by confining lipid peroxidation radicals. Arora *et al.* (2000) observed that polyphenols can change lipid filling sequence and reduce membranes fluidity. Thus, the variation could obstruct the movement of ROS and restricts peroxidation. Flavonoids and phenyl propanoids scavenged hydrogen peroxide radicals through the process of oxidation of peroxidase. It was observed that free radicals could also operate as a signalling factor in $Cu^{2+}$ stress (Janas *et al.,* 2009).

## 2.14 Oxidative Stress Tolerance

Although water is important for the survival of the floras and faunas, molecular oxygen also challenges organisms through their formations of ROS. Elevation in the synthesis of radicals were recorded during stress like salinity, drought etc. Importantly, formations of radicals are liable to oxidation injuries. It is clear from the literature that harmful by-products produced during the reaction of free radical with the plants lipids and proteins resulted into plant death (Gao *et al.,* 2013). In this context, production of lipid peroxides due to oxidative stress can lead to cell injury damages. Appearance of the genomics accountable for biosynthesis and modification of antioxidants pathway are controlled by distinct function of antioxidant enzymes or non-enzymes. This was done to promote the tolerance of plant against oxidative damage through metabolic modification.

As observed in Slooten *et al.* (1995), genetically modified *Nicotiana tabacum, Medicago sativa*, *Solanum tuberosum* and *Gossypium* showed an overexpression of superoxide dismutase in the plant's chloroplast due to the high tolerant ability against oxidation stress. An extreme formation of SOD in the mitochondria and cytosol of *Medicago sativa* and *Solanum tuberosum* respectively were recorded (McKersie *et al.,* 1997). Transgenic *Nicotiana tabacum* was modified due to overproduction of Manganese Superoxide dismutase in chloroplast, which in turn supplies defence to plant that lack protection from Manganese, thus interceding oxidative damage. The tripeptide glutathione detoxifies surplus $H_2O_2$ during oxidative stress, which a principal cell anti-oxidant. Increase in oxidative stress tolerance of the plants was controlled by GSH (Krishnamurthy and Rathinasabapathi, 2013). Noctor and Foyer (1998) observed that genetically modified plants that overproduce GR had high GSSH, which promote the plant tolerance to oxidation injuries. Unsaturated fatty acid hydroxide was displaced by enzyme GPX in the cell membranes during oxidation stress. As reported in Yoshimura *et al.* (2004), genetically modified *Nicotiana tabacum* overexpressed *Chlamydomonas* GPx in the chloroplast or the cytosol, and thereby improving the integrity of *Nicotiana tabacum* membrane, which ultimately promoted its tolerance.

## 2.15 Excessive production of ROSunder Drought

Under a normal vegetative growth of plant, generation of radicals are generally low. Nevertheless, reaction to abiotic stresses extremely reduced the generation of ROS causing disruption of the standard balance of most reactive ROS in the intracellular environ. The impact of drought stress on production of ROS is explained as follows.

### 2.15.1 Drought

Drought induces closing of stomata due to reduction in carbon dioxide ($CO_2$) entry resulting into damaged photo system, as well as disparity in the captured and utilization of light energy. Hence, cause alteration in the chloroplast photochemistry and consequently, lead to overproduction of ROS (Hasanuzzaman *et al.,* 2020). In the course of drought stress, responsive oxygen species formation is elevated in different ways. Suppression of $CO_2$ in-take changes in Photosystem activities and photosynthetic carrier ability, as explained by Asada (1999), resulted into high formation of free radicals throughthe chloroplast Mehler reaction. Closing of stomata is as a

result of limitation in fixation of carbondioxide, which consequently resulted in degraded $NADP^+$ formation via the Calvin cycle. Lack of electron acceptor due to excessive depletion of photosynthetic ETC consequently caused higher discharge of electrons to the oxygen. Biehler and Fock (1996) further noted that the discharge of electrons in *Triticum* is 50% significantly higher in plant subjected to drought than control plants. According to Foyer and Noctor (2000), the activities of photosystems are suppressed in the tissues of plant because of disequilibrium between capturing of light and its uses during water stress. Release of extra light energy in the photosystem II core and antenna caused formation of free radical that is prospectively harmful during water stress. Furthermore, photo-respiratory track was upgraded primarily when ribose 1, 5 bisphosphate oxygenated at the highest because of restraint in $CO_2$ fixation (Zhao *et al.,* 2020). Foyer and Noctor (2000) have projected that photorespiration produce over 70% of total hydrogen peroxide generated under drought. Singlet oxygenstarts a chain reaction resulting to the generation of lots of harmful ROS finally, causing damage beyond the beginning of the reaction products.

Generation of hydroxyl in the thylakoids has been considered as one of the most important threat around the chloroplast through the process of reducing hydrogen peroxide which was catalysed and assisted by iron and SOD and AsA respectively. Enhanced generation of radicals resulted into oxidation death of growing plants (Kapoor *et al.,* 2015). Elevated MDA contents and reduced chlorophylls, some antioxidants, soluble protein and thiols were attributed to an excessive production of singlet oxygen in rice seedlings under drought (Boo and Jung, 1999). Similarly, Campos *et al.* (2019) reported high MDA content under along with high production of hydrogen radical under severe water stress. Thus, decrease in yield owing to drought at the reproductive stage was observed consequently, resulted into decrease in number of filled spikelets per panicle though, spikelet number per panicle were not affected (Guo *et al.,* 2016). In order to fight against hazard generated by radicals, plants must develop numerous scavenging enzymes and metabolites. Enhanced antioxidant capacity was attributed to drought tolerant genotypes than susceptible genotypes. In comparison to the genotype of *Triticum* HD 2329 that is sensitive to drought, genotype C 306 displayed elevation in the activities of APx, CAT, AsA content and declined $H_2O_2$ and lipid peroxidation as attributes of drought tolerance. A research conducted by Maize Genotype Giza 2 was relatively tolerant to drought in contrast to drought

sensitive genotype Trihybrid 321 due to reduced production of $H_2O_2$ because of low MDA contents experienced as a result of high production of SOD, CAT, and POX activities (Moussa and Abdel-Aziz, 2008). As noted by Wang *et al.* (2012), antioxidant enzymes, AsA and GSH were relatively high in *M. prunifolia* compared to *M. hupehensis* under water stress. Ascorbic peroxidase functions as a crucial element of plant defensive system during water stress.

### 2.16 Plant Root Adaptation during Drought Stress

Plant exhibits well-developed root/ shoot dry matter ratio in response to drought. Which influence high root density against leaf area. Modification in root structure and anatomy are also other adaptive strategies and trait to withstand drought. Research had shown that root grown in an arid environ penetrate far in the soil to resist prolong duration of harsh water deficit. It was further revealed that adaptive strategies depend on variety/ accessions. Three root structures were therefore described for perennial species. We have shallow and deeper roots not more 20cm, secondly, shallow adventitious roots have their roots align to each other, growing into a few metres deep, fashioned for rapid water uptake after short precipitations. The third rooting structure was featured with very long taproots accompanied by several lateral roots around one meter long (De Micco and Aronne, 2012). The taproot is structured to reach deep down the soil where zone of saturation exist permanently, thus preventing seasonal fluctuations of water availability. More so, shallow and deep rooting structures can exist together in semiarid environs. Distribution of root architectures in the soil depend primarily more on water availability at the superficial layers than at the deeper layers (Blum, 1996). The Xeromorphic characteristic nature of roots depends on the character connected to control of water uptake and development of water storage tissues. Also, Eissenstat (1992) revealed the presence of reduced roots diameter under drought, which is regarded as a strategy to increase surface absorption and assist fast move of mineral nutrients. Furthermore, specialized tissues like a well–established suberized exodermis and rhizodermis with thickened outer cell walls can also be developed to control water loss. Suberized layers of cells found at the root perimeter showed that the strategy was used to control inverse water influx from the root back to the soil during severe drought stress (Hose et al., 2001). Similarly, North and Nobel (1995) reported that limitation imposed on the root radial

hydraulic by suberized layers were high during the root development under drought (North and Nobel, 1995). Upon movement of water from epidermis and exodermis to the cortical parenchyma, there are number of reduced cortical layers which were regarded as an added adaptive advantage during drought stress. Rapid outspread water movement were sustained due to reduced pathway between the stele and soil. However, Robards *et al.* (1979) reported the presence of cortical cell ruptures during reverse water influx results to the development of cortical lacunae that discontinue the outspread water move back to the soil. Also, the numerous wide cracks found on the outside of the cortical tissues can further open pathway for quick move of water in cactus during drought stress (North and Nobel, 1996). Lately, Zhu *et al.* (2010) reported that presence of cortical lacunae has been shown to be beneficial during severe drought stress because it help to reduce root metabolism. Though, Striker *et al.* (2007) revealed that the development of cortical lacunae could incapacitate root strength thereby increasing its water sensitivity to expand and contract cycles shifts. Mostajeran and Rahimi-Eichi (2008) also revealed that the weaken roots mechanical strength can be rectified by increased lignified tissues as accompanied with thicker cells walls.

Furthermore, reversal of reduced root parenchyma during drought stress could occur if there are increased suberized cell walls (Peña-Valdivia *et al.,* 2010). Also, formation of endodermis with thick cell walls and the development of additional suberized walls around the stele could help to prevent the drying of meristematic tissues. North and Nobel (1996) reported high number of endodermis cell along with casparian strip during drought stress in cactus. The mechanism followed the variant of anatomical alteration such as development of high suberized phellem that is responsible for decrease water permissibility into the cortex (Schönherr and Ziegler, 1980). Steudle (2000) also showed that apoplast barrier played a vital role in regulating flow of water which relies on the developmental stages in the root. At the entry of water to the stele, effective transportation occurred immediately although the plant to maintain interrupted flow of water to the shoot. Importantly, water transportation and hydraulic protection of the vascular tissues in water deficit regions are crucial in plant survival. However, drought stress always accompanies an increase in formation of xylem vessel cavitation which is connected to the structure of xylem in the root.

# CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Planting Materials

Accessions of *D. exilis* namely: Didat Iburua (DI), Jakah Iburua ($JI_1$), Jiw Iburua ($JI_2$), NG/JD/06/11/062 (NG062) and NG/JD/06/11/061 (NG061) were collected in the study. The first three accessions DI, $JI_1$ and $JI_2$ were collected from NCRI, Badeggi, Niger State. The other two seeds NG061 and NG062 were collected from NACRAB, Ibadan, Nigeria (Passport information: Collected in Bogera Local Government Area, Baunchi State, June, 2011).

## 3.2 *In vitro* Techniques

### 3.2.1 Disinfection of Plant Materials

Collected seeds were washed and rinsed with detergent and distilled water respectively. After which sterilization was done for 5mins using ethanol (70 %) and cleansed twice. Thereafter, 5% chlorex (Sodium Hypochlorite) with a few drop of tween 20 (to ease the surface tension of the seed) were added to surface sterilize the seeds for 20 minutes inside the laminar airflow. The seeds were rinsed thrice with distilled water. Prior to inoculation, laminar airflow was switched on, swapped and sprayed with absolute ethanol. Glassware such as jars, canistals containing Petri-dishes, blade holder were all sterilized in an autoclaved at 121°C, 1.5atmfor the duration of 30 minutes.

### 3.2.2 Preparation of Media

Murashige and skoog media (MS) of 1.25L were prepared by addition of stock 1 (60.00ml), stock 2 (6.00ml), sucrose (36.00g), inositol (0.12g), vitamins (6.00ml), Na EDTA.$2H_2O$ (0.04476g) and Ferrous sulphate (0.0278g ) to 600ml deionized water placed on magnetic stirrer. Osmotic conditions of -9.29, -13.93, -20.13, -26.32, -32.51 and 0MPa (control) represented by A, B, C, D, E and F were created by addition of 30g/l, 45g/l, 65g/l, 85g/l and 105g/l and 0g/l Poly ethylene glycol PEG to the MS media. The mixture was make up to 1.25 litres after addition of 200ml deionized water. Phytagel (0.46g) was included after which the pH of the media was normalized using hydrochloric acid to 5.7± 0.3. Five millilitres (5ml) were dispensed into autoclaved jars after melting for 15mins in an oven.

#### 3.2.2.1 Component of the Stock Solutions

The stock solution I was a mixture of macronutrients and stock ii was a mixture of micronutrients (Table 2.1 and 2.2).

### 3.2.3 Seeds Inoculation

Sealed autoclaved jars were placed in the growth room after the inoculation of five (5) sterilized seeds on the media inside the laminar airflow.

### 3.2.4 *In vitro* Plant Assessment

After four weeks, the following were determined: shoot weight; number of seed germinated; and number of leaves.

**Table 3.1: Composition Stock Solution I (Macro nutrient)**

| S/N | Composition | g/Litre | g/500ml |
|---|---|---|---|
| 1 | $NH_4NO_3$ | 33g | 16.5g |
| 2 | $KNO_3$ | 38g | 19g |
| 3 | $CaCl_2.2H_2O$ | 8.8g | 4.4 |
| 4 | $MgSO_4.7H_20$ | 7.4g | 3.7 |
| 5 | $KH_2PO_4$ | 3.4 | 1.7 |

**Table 3:2: Composition of Stock II Solution (Micro Nutrients)**

| S/N | Composition | g/Litre |
|---|---|---|
| 1 | KI | 0.16 |
| 2 | $H_3BO_3$ | 1.24 |
| 3 | $MnSO_4$. 4H2O | 4.46 |
| 4 | $ZnSO_4$. 7H2O | 1.72 |
| 5 | $Na_2MOO_4.2H_2O$ | 0.005 |
| 6 | $CuSO_4.5H_2O$ | 0.005 |
| 7 | $CoCl_2$. $6H_2O$ | 0.005 |

### 3.3 Screen-house Experiment

Top soils were used for the screen-house experiment. Soils of about 0-5 cm depth were collected in Ado-Ekiti, sieved and sterilized to kill microorganisms and then mixed together to obtain homogenous soil. Five kilogram (5 kg) of the soil was filled into 60 plastic pots, with seeds (5) planted and monitored for 12 weeks. The soil was watered to field moisture capacity. The experiment was factorially (5×3×4) arranged with three replications each. Experimental design was put in a complete randomized design and thinned to five plants per pots. Three (3) weeks after planting, the plants were subjected to control (CNT), 4 days, 8 days and 12 days water stress. Experiment was terminated at the end of 12 weeks.

#### 3.3.1 Plant Growth and Yield Assessment in Screen House

Plants were monitored for a period of 12 weeks, and plant morphology parameters were measured based on number of leaves, plant height, number of spike, spike and peduncle length.

#### 3.3.2 Chlorophyll Determination

Eleven (11) weeks after planting, mature leaves were collected for chlorophyll estimation. Approximately 75% ethanol was used to extract chlorophyll content. Thereafter, absorbance of 663nm and 645nm were done using UV- Visible Spectrophotometer Model LT-290, Labtronics India. Chlorophyll quantities were calculated using Lichtentaler and Wellburn, (1985) formula. Formula used in calculation of chlorophyll contents is stated as follows.

$Chlorophyll_A = 15.65A_{663nm} - 7.340A_{645nm}$

$Chlorophyll_B = 27.05A_{645nm} - 11.21A_{663nm}$

(Lichtentaler and Wellburn, 1985)

### 3.4 Drought Indexes

These were calculated according to the following methods:

1. Drought Tolerance Index (DTI):

DTI= (Dpi x Dsi)/Dp2

(Fernandez, 1992);

2. Mean Productivity (MP):

MP= (Dpi+Dsi)/2

Where:

Dsi= yield of accession in stress condition,

Dpi= yield of accession in normal condition

Ds= sum of yield mean in stress condition,

Dp= sum of yield mean in normal condition

### 3.5 Proline Determination

Potassium phosphate ($K_3PO_4$) buffer (50 mM, pH 7.8) were uniformly emulsify with leaves samples at 4 $^oC$. The buffer solution was thereafter mixed with disodium EDTA (1 mM) and polyvinylipid peroxidationlypyrrolidone (2% w/v). Afterwards, centrifugation was done at 13,000g for forty minutes. The supernatants were collected using Whatman no 2 filter paper and uniformly stirred with 10ml of sulfosalicylic acid (3%) at 4 °C (Bates *et al.,* 1973). The filtrate collected (2ml) were emulsified with acid-ninhydrin (2ml) and glacial acetic acid (2ml). Incubation was done at 100$^oC$ for an hour. Extraction was done using Toluene solution (4ml). Absorbance was taken at 520 nm after separation of chromophore containing toluene from the hydrated phase. Toluene was used as the blank. The proline concentration was expressed in mg/ml

### 3.6 Determination of Ascorbate Peroxidase (APx)

Plant extract (0.1 ml) mixed with $K_3PO_4$ buffer (50mM, pH 7.0) was added to 0.5 mM AsA and $H_2O_2$ (0.1 mM). Distilled water was added up to 1 ml. The plant absorbance extract were taken at 290 nm and APx was expressed in mmol $ml^{-1}$ ascorbate oxidized $min^{-1}$. The absorption coefficient was 2.8 $mM^{-1}$ $cm^{-1}$ (Nakano and Asada, 1981).

### 3.7 Determination of Catalase (CAT)

Phosphate ($PO_4^{3-}$) buffer (0.1M, pH 7.4) were mixed with 50µl of the plant extract in a cuvette thereafter, $H_2O_2$ (500 µl of 20 mM) was added. Spectrophotometer was used to record the extract abundance at 240 nm for one minute. Catalase was expressed in units/mg protein. The molar extinction coefficient of hydrogen peroxideused was 43.6 M $cm^{-1}$ following below formula (Aebi, 1984).

Calculation:

$$\text{Units/ml} = \frac{\Delta A/\text{min} \times d \times 1}{V \times 0.0436}$$

d = dilution of original sample for Catalase Reaction

V = Sample volume in Catalase Reaction (ml)

$0.0436 = \varepsilon^{mM}$ for hydrogen peroxide

1 = Total reaction volume

### 3.8 Determination of Superoxide dismutase (SOD)

Tris-hydrochloric acid buffer solution (pH 8.2, 75 mM) were combined with the plant extract (50 µl). The resultant mixture was stirred with EDTA (30 mM). Thereafter, pyrogallol (2 mM) was added to the overall mixture. Spectrophotometer was used to record the extract absorbance at 420 nm for 3 mins (Mccord and Fridovich, 1969). Superoxide activity is expressed in units/mg protein.

Calculation:

Increase in absorbance per minutes $=\frac{A3-A0}{2.5}$

Where $A_0$ = absorbance of the extract after 30s

$A_3$ = absorbance of the extract after 150s

% inhibition = 100 – 100x (increase in absorbance for substrate/increase in absorbance for blank)

### 3.9 Determination of 2, 2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) Assay

Ascorbic acid (Standard) and varying concentrations (1 ml) of the plant extracts were emulsified with 1ml of 0.3mM DPPH in methanol. Reaction mixtures were stirred and incubated in the dark for thirty minutes. Spectrophotometer was used to record the extract absorbance at 517 nm (Brand-Williams *et al.,* 1995).

Percent of inhibition = $[(A_{blank}-A_{sample})/A_{blank}] \times 100$

Where:

$A_{blank=}$ Absorbance of the control

$A_{sample}$ = Absorbance of the tested extract.

### 3.10 Determination of Nitric Oxide (NO) Radical

About 0.1ml of 10, 5, 2.5, 1.25, 0.625, 0.3125mg/ml of plant extracts were mixed with sodium nitroprusside (0.9ml, 2.5mM) in saline $PO_4^{3-}$buffer thereafter, incubation was done for 150mins in the presence of illumination. After incubation, 0.5ml of 1% sulphanilamide in 5% phosphoric acid was added. The mixture was incubated in the dark for 10mins, and then 0.5ml N-1-napthylethylenediamine dihydrochloride (0.1%) was added. Spectrophotometer was used to record extract absorbance at 546nm (Marcocci *et al.,* 1994).

### 3.11 Determination of Hydroxyl ($OH^-$) Radical

Deoxyribose (3.0 mM) was mixed with 0.1 mM EDTA, 2 mM $H_2O_2$, 0.1 mM AsA, $FeCl_3.6H_2O$ (0.1 mM) in $PO_4^{3-}$ buffer (10 mM) at pH 7.4 and different concentration of plant extracts ranged from 50-350 µg/ml. Incubation was done at 37 $^oC$ for one hour. After which thiobarbutric acid TBA (1.0 ml of 1 % (w/v)) in hydrochloric acid (0.25 N) and Trichloroacetic acid TCA (1.0 ml of 10 % (w/v)) were added. The mixture was placed and heated in 100 $^oC$ water bath for twenty minutes. Spectrophotometer was used to record the extract absorbance at 532 nm against the blank (Halliwell, 1990).

The percentage inhibition was calculated using the expression:

$$\text{Percentage Scavenged} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

### 3.12 Determination of Hydrogen Peroxide ($H_2O_2$) Radical

Plant extract (20 g/ml) was emulsified with 40 mM $H_2O_2$ developed in $PO_4^{3-}$ buffer (50mM) at pH 7.4 in distilled water. Spectrophotometer was used to record the absorbance for ten minutes at 230nm against the blank ($PO_4^{3-}$ buffer), and ascorbic acid was used as a positive control(Ruch *et al.*, 1989).

$$\% \text{ Scavenged } (H_2O_2) = (A0 - A1 \div A0) \times 100$$

Where:

A0 = Absorbance of blank;

A1 = Absorbance of the plant extract. .

### 3.13 Malondialdehyde Determination (MDA)

Potassium phosphate buffer (0.5 ml of 0.1 M) at pH 8.0 was mixed with plant extract (0.5 ml). The resultants were added to TCA (0.5 ml of 24%). Thereafter, incubation was done at $22^{O}C$ for 10 minutes. After which it was centrifuge at 2000rpm for 20 mins. About 1ml of the filtrates was mixed with TBA (0.25ml of 0.33%) in 20% acetic acid (Ohkawa *et al.,* 1979). The resultants were placed and boiled in 95 $^{o}C$ water bathe for 1 hour. Spectrophotometer was used to record absorbance at 532nm. (Extinction coefficient of MDA, ( ε ) = 1.53 x 105 $M^{-1}$ $cm^{-1}$).

### 3.14 Determination of Total Anthocyanin Content

About 125ml of Potassium chloride buffer solution (0.2N) was mixed with HCl (385 ml of 0.2N) and distilled water (490 ml). Alteration of buffer solution to pH 1.0 was done using HCl (0.2N). Sodium acetate buffer solution (440ml of 1.0M) at pH 4.5 and HCL (200ml of 1.0M) in distilled water (360ml) were prepared. After which, adjustment of the mixture pH to 4.5 using hydrochloric acid (1.0M) were done. Plant extraction of about 0.5ml was mixed with 12.5ml buffer solutions of pH 1.0 and 4.5 buffers each. The incubation was done in the dark for 2hours (Wrolstad *et al.,* 2005). Ultraviolet spectrophotometer was used to record the reaction absorbance at 512 and 700nm. Changes in absorbance (ΔA) at the two pH (s) 1.0 and pH 4.5 were estimated as follows

$\Delta A = (A_{512nm}\, pH_{1.0} - A_{700nm} pH_{1.0)} - (A_{512nm} pH_{4.5} - A_{700nm}\, pH_{4.5})$.

$$\text{Total anthocyanin} = \frac{(\Delta A \times MW) \times DF \times 1000}{\varepsilon \times 0.1 \times 1}$$

Where,

MW = Molecular weight of cyanin 3-glucoside (449.2g/L)

DF = dilution factor to express the extract on per gram of plant samples

ε= molar absorbance coefficient of cyanin 3-glucoside ($26900 M^{-1}cm^{-1}$)

0.1= the conversion factor for per 1000 grams to 100 grams basis

### 3.15 Anatomical Studies

Transverse sections of fresh leaves and roots tissues were cut into sections using microtome under different treatments. Plant samples were embedded in parafin wax before sectioning on a sliding microtome. Twenty micrometer (20µm) thick sections were rinsed in distilled water, stained with safranin O for two minutes. Thereafter, stained sectioned were rinsed in distilled water until it became colourless. After which, stained sections were dehydrated by washing in high concentration of ethanol (Wahid *et al.*, 1998). Clove oil was added to the slide for one hour to remove the ethanol. Canada balsam was added and tissue paper was used to drain the oil outflow. Cover slips were placed gently to avoid air bubbles formed on the slide. Anatomical parameters were viewed and measured using ocular micrometre mounted on photographic microscope.

### 3.16DNA Extraction Procedure

**(Zymos Plant Genomic DNA Purification Mini prep Kit Protocol)**

- Leaf tissue of 100mg were weighed and ground with mortar and pestle after which, distilled water (95 µl) was added. Incubation was done for 60 minutes at 55 $^{o}$C and shake in a water bathe after the addition of Protinase K (50µl). Precipitation solution (130µl) was adduced and mixed by inverting the tubes individually 3 times. Centrifugation (10,000rpm) for 5 minutes was done. Supernatants (500µl) were collected and transferred into a micro-centrifuge tube. Plant gDNA (400µl) solution and 96% ethanol (400µl) were added and mixed. Half of the prepared mixture 600µl was transferred to the spin column thereafter; centrifugation was done for 1minute at 6,000 × 7,000g. Flow-through solutions were discarded. Centrifugation (6,000 × 7,000g ) was applied to the mixture on the same column for 1 minute
- The mixture was centrifuge (8,000 × 10,000g) for 1 minute after the addition of wash buffer I (500µl) to the column. The flow-through was throw away after which the columns were

returned into the collection tube. Centrifugation (20,000 × 14,000g) was done for 3 minutes after the wash buffer II (500µl) were adduced to column. The flow-through inside the collection tube was discarded after transporting the column into a sterile micro-centrifuge tube (1.5ml). Elution Buffer (100µL) was added to the centre of the column after which incubation (room temperature) and centrifugation (8,000 × g (10,000 rpm)) were done for 5 and 1 minute respectively. The second elution was performed using 100µL Elution Buffer in the same elution tube and the genomic DNA was separated.

### 3.16.1 DNA Quantification using Spectrophotometry

The DNA quantity was determined using Nanodrop Lite spectrophotometer by Thermo. Concentration and purity were taken at absorbance 260nm:280nm

### 3.16.2 DNA Quality Confirmation

One gram of agarose was melted into Agarose Tris-Acetic acid buffer (100ml) and microwaved for 5 mins. Ethidium bromide (7.0 µl) was included and mixed after cooling for some period. Wells were made using comb after casting the gel on the supplied tray. Cooling of the gel was done for 20 mins (room temperature) on a flat surface. The gel was placed into an electrophoretic unit containing 1X TAE buffer. The Loading were separately done into wells of 5µL 1kb ladder, DNA samples (5 µL) + 2 µL 6x Loading Buffer using micropipette followed thereafter**.** The gel was run for 50 minutes at 150v**.** The gel was then exposed to UV light and photographed using the gel imaging equipment and gel analysis software. The intact band of genomic DNA was observed on the gel aided by the intercalating dye.

## 3.17 Statistical Analysis

Analysis of variance (ANOVA) which involved the use of Duncan Multiple Test (DMRT) and Least Significant difference LSD were used to separate the mean of biochemical and molecular results. Histograms and line graphs with standard error bars were used on plant growth, weight and vessel diameters. Regression and principal component analysis (PCA) were used to determine the effect of drought stress on biochemical and physiological properties of different accessions of *D. exilis*

# CHAPTER FOUR

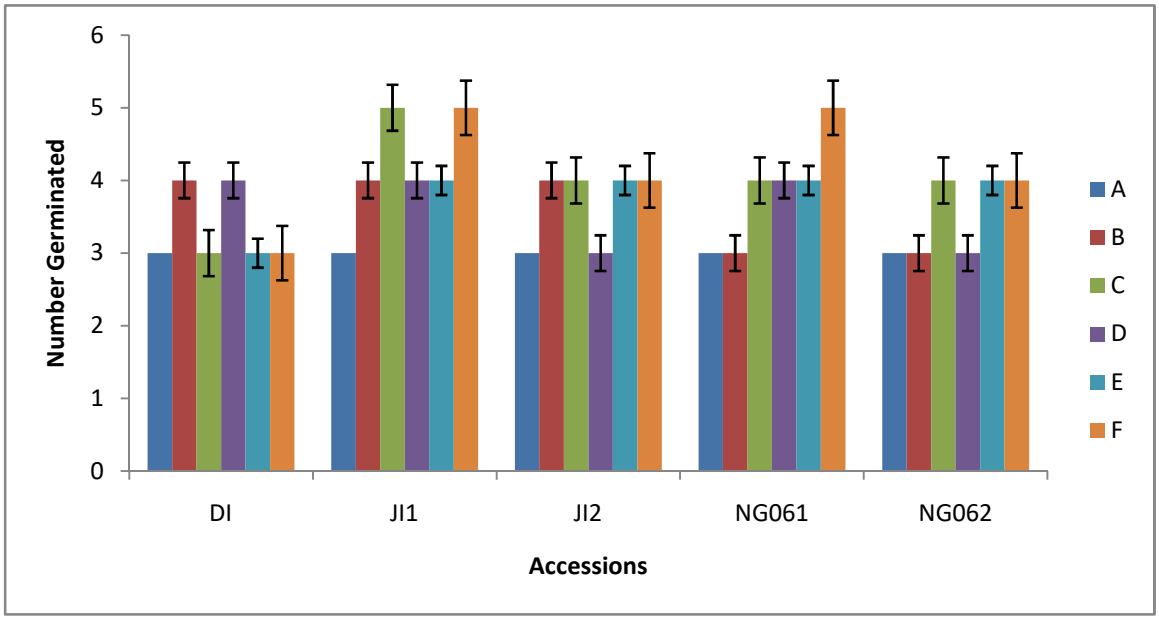
# 4.0 RESULTS

## 4.1 Plant Growth under Osmotic Stress

Accession $JI_1$ had a relatively high number of germinated seeds (100%) i.e. five germinated seeds out of six seeds at C and F osmotic potential (Fig 4.2). All other accessions showed no specific pattern in their number of germinated seeds. However, their germination percentages range from 60-80% (Fig 4.2).

Accession DI at the first four levels of osmotic conditions and NG062 at D level of osmotic stress showed the highest number of leaves (NL) than the F level (control) and all other accessions. Number of leaves of osmotic stressed plants and the control in accessions $JI_1$, $JI_2$, NG061 and NG062 showed no significant variation (Fig 4.2).

Also, leaves length of osmotic stressed $JI_2$, DI, NG061 and NG062 showed no significant difference with their controls except for accession $JI_1$ with the highest leaves length of 40cm (Plate 4.1, 4.2 & 4.3) (Fig 4.1). Despite the osmotic stress, the growth and shoot weight (SHW) of *D. exilis* were significantly higher than the control in $JI_1$ and DI (Plate 4.1, 4.2 & 4.3) (fig 4.1). Nevertheless, accessions $JI_2$, NG061 and NG062 displayed no notable variations between the osmotic stressed plants and control plants (Fig 4.1). More so, highest shoot weight was recorded in accession $JI_1$ at E level of osmotic potential and $JI_2$ showed consistently lowest value in all osmotic stressed plants (Fig 4.1).



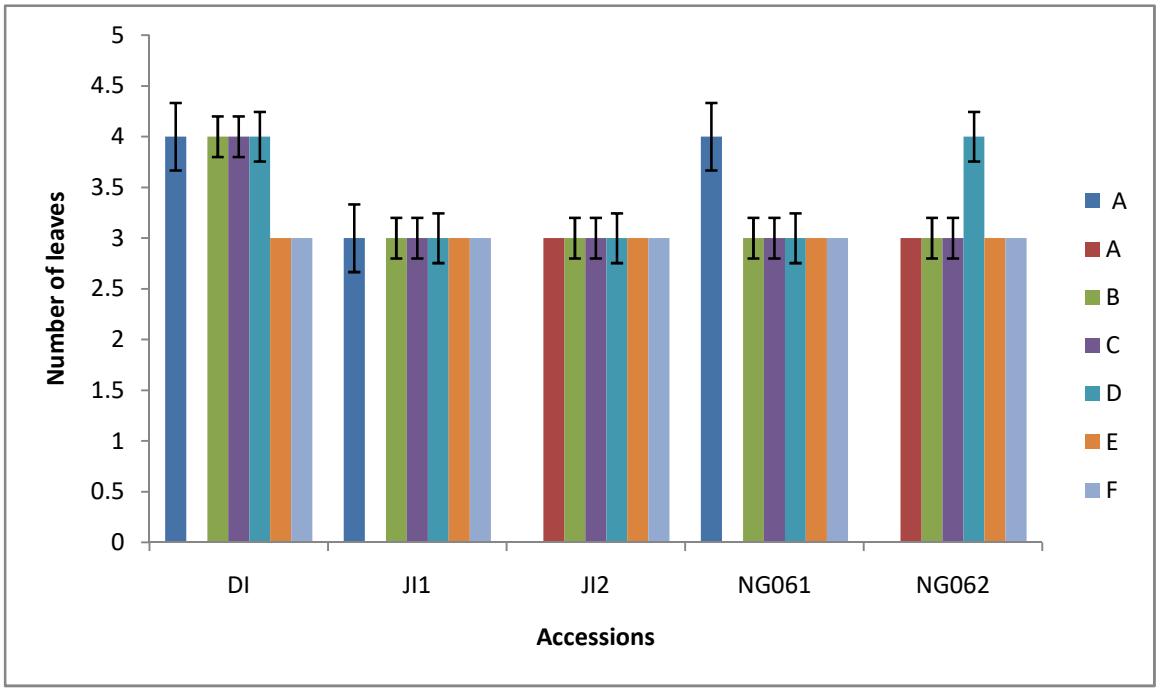




Figure 4.1: Number of Germinated Seeds (NG) and Number of Leaves (NL) of *D. exilis* under osmotic stress

Where: A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa (Control), Error Bar means Standard Error

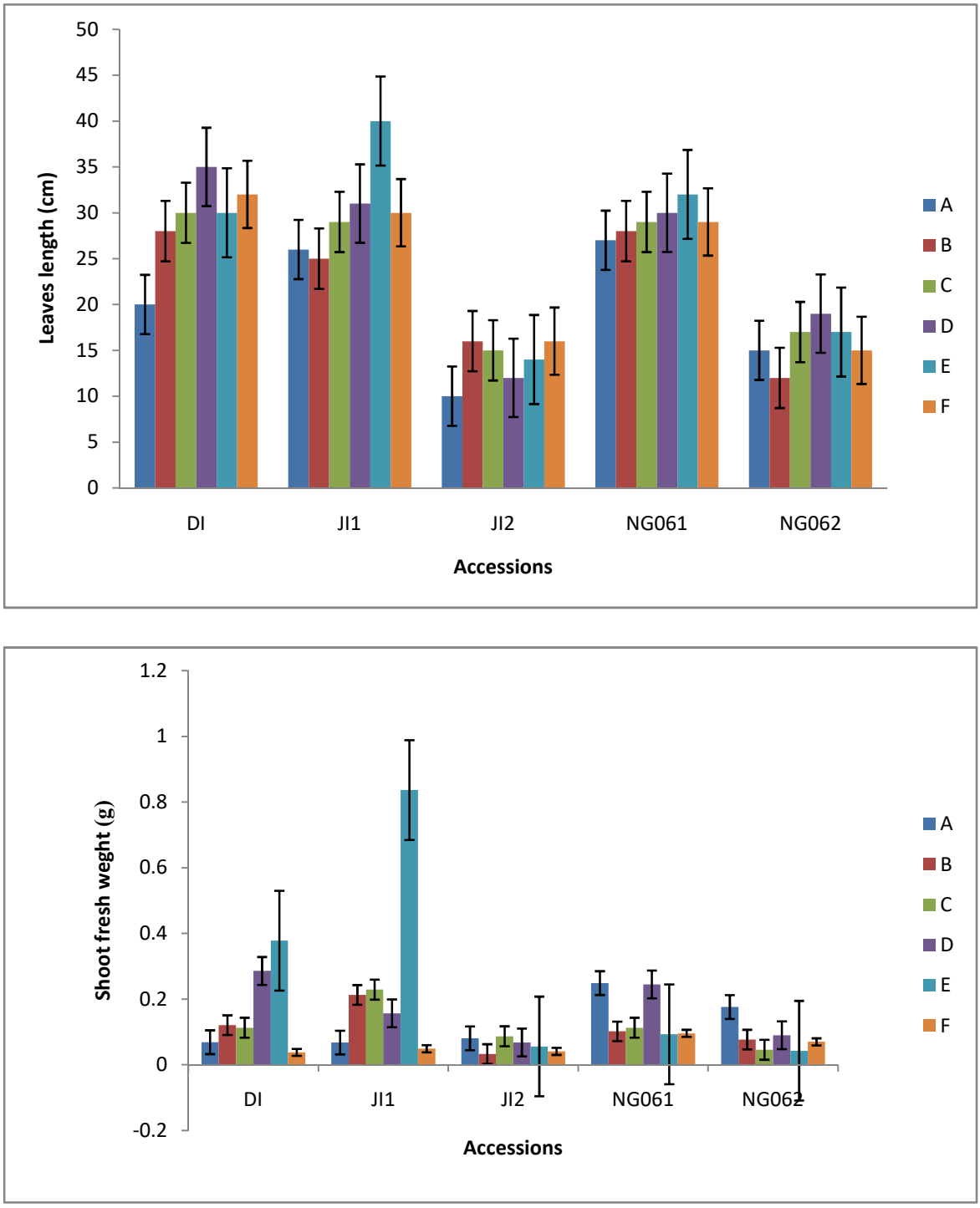



Figure 4.2: Leaves length and Shoot fresh weight of *D. exilis* when subjected osmotic stress

Where: A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa (Control), Error Bar means Standard Error

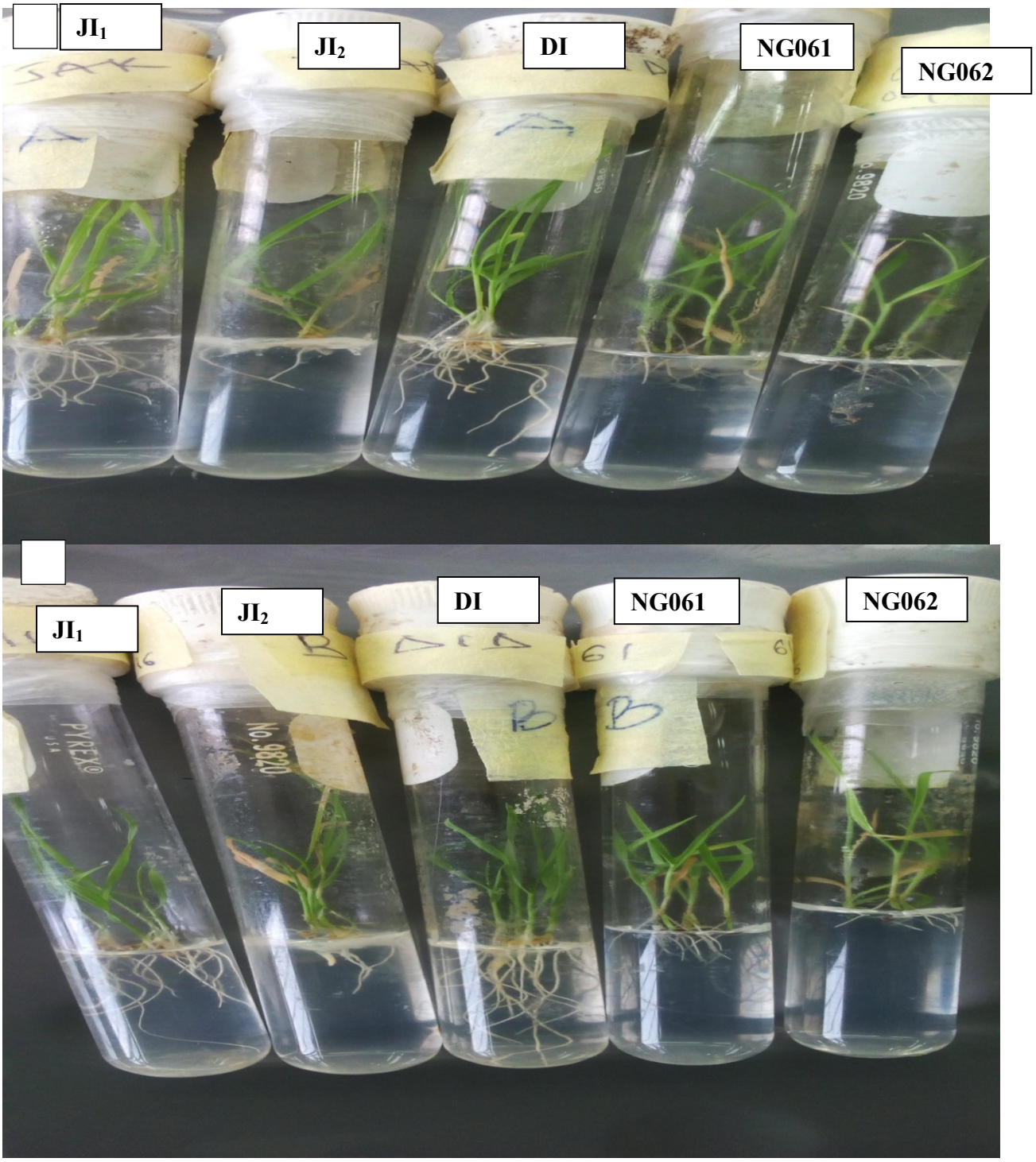



**Plate 4.1: *In vitro* Growth of *D. exilis* at A and B levels of Osmotic Stress**

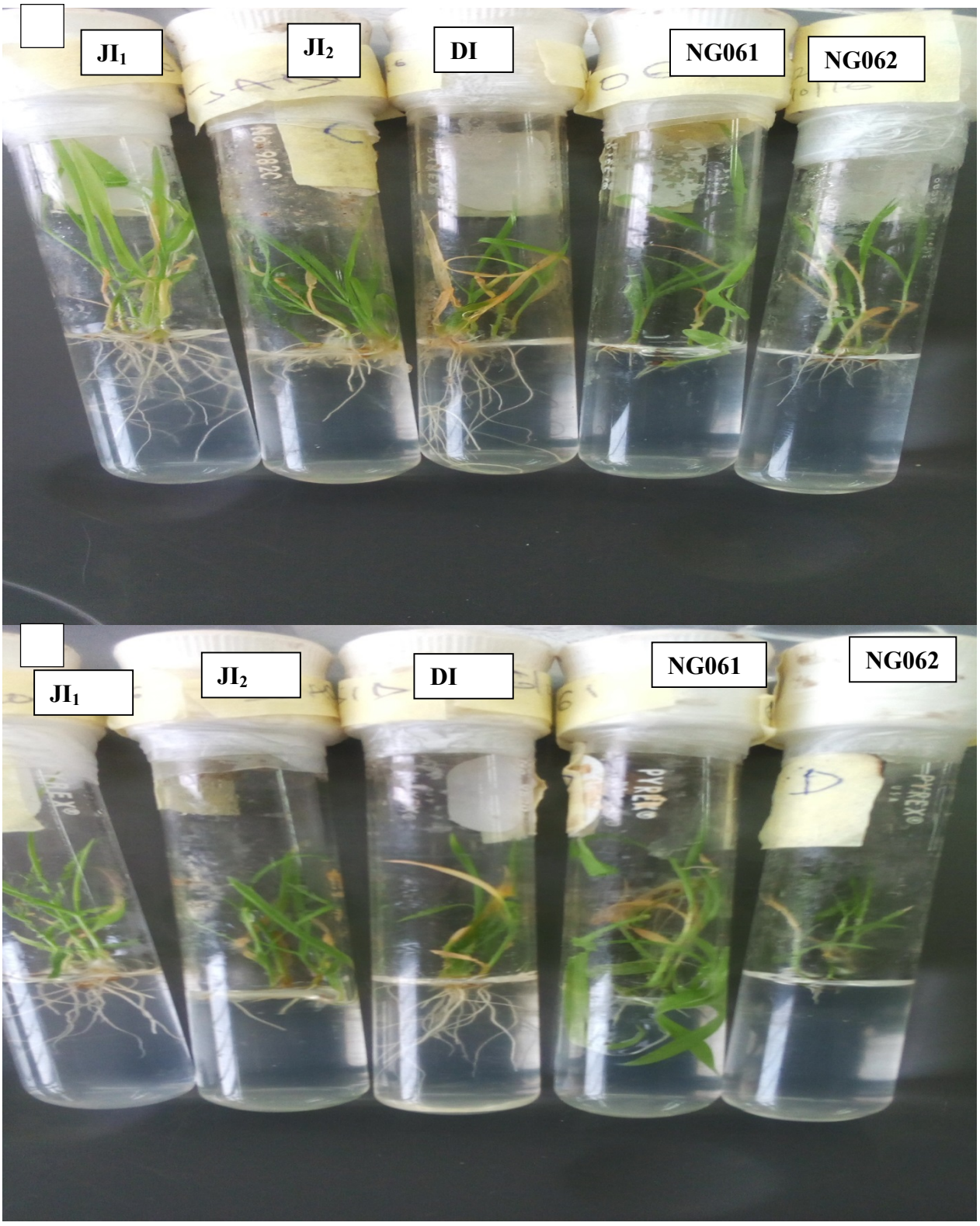



**Plate 4.2: *In vitro* Growth of *D. exilis* at C and D levels of Osmotic Stress**

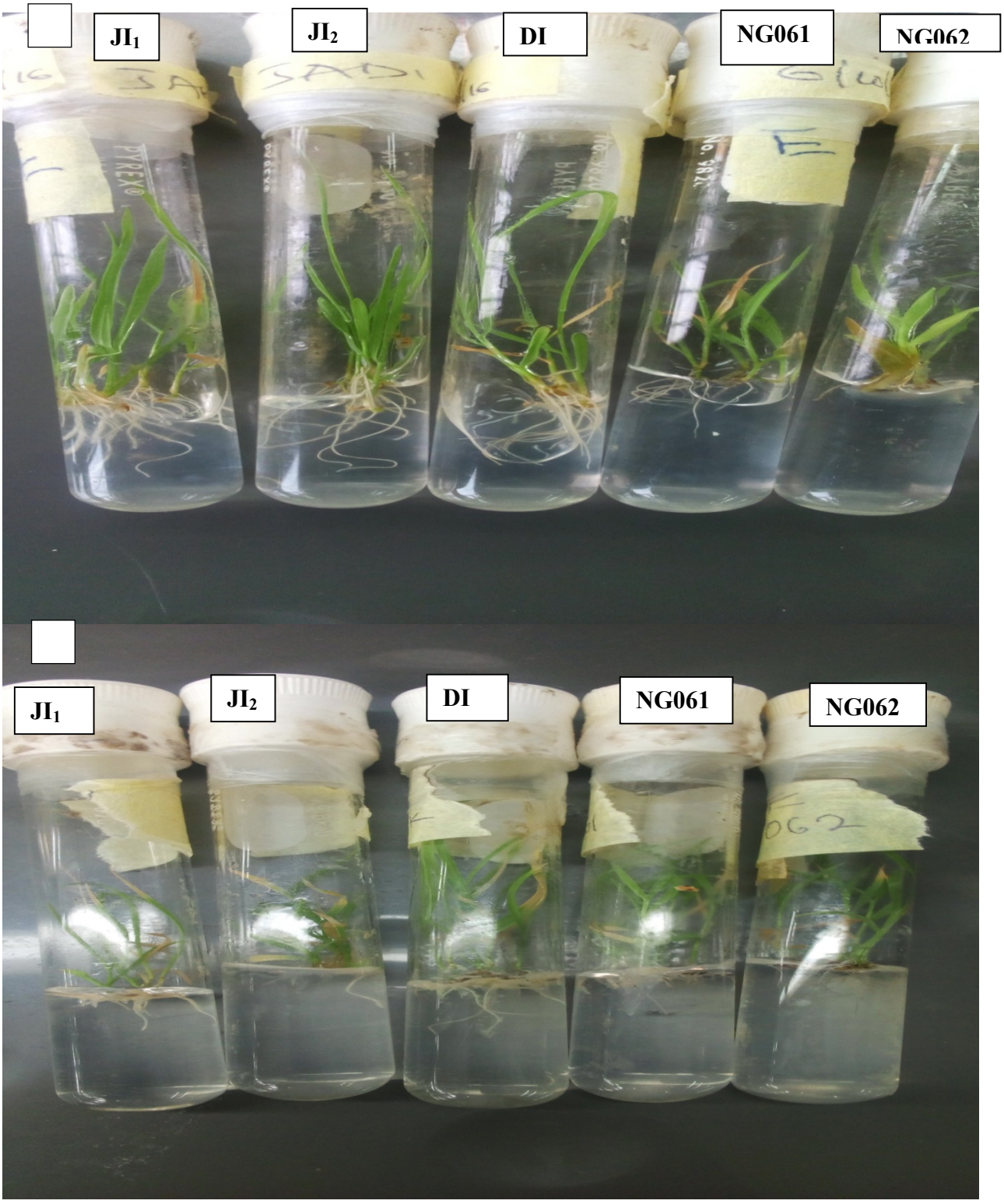



**Plate 4.3: *In vitro* Growth of *D. exilis* at E and F levels of Osmotic Stress**

## 4.2 Drought Tolerance Index (DTI) of DE

From table 4.1 below, accessions $JI_1$ and NG061 had the highest value of DTI, and DI had an average value of DTI, while $JI_2$ and NG062 had significantly lower value. Thus, higher DTI connote greater tolerance. Furthermore, mean productivity (MP) revealed that accessions $JI_1$, DI and NG061 were significantly high while $JI_2$ and NG062 had the lowest value (Table 4.1).

## 4.3 Chlorophyll Contents of *D. exilis* under Osmotic Stress

The chlorophyll contents (A, B and total chlorophyll) of accessions $JI_2$ and NG062 were decreased significantly (Table 4.2). Although, chlorophyll content showed no significant variation in some of the accessions, except for DI and $JI_1$ which showed higher level of chlorophyll contents (A, B and total chlorophyll) at D level of osmotic stress.

## 4.4 Proline Contents of *D. exilis* under Osmotic Stress

Except for A level of osmotic stress in $JI_1$ accession, the proline contents of DI, $JI_1$ and $JI_2$ accessions were similar and lower than the other two accessions (NG061 and NG062) (Table 4.3). In accessions DI, $JI_1$ and $JI_2$, the proline contents in their control plants were lower than other treatments and control plants of NG061 and NG062. In NG061, the proline contents of all the accessions were similar though lower than the proline contents in its control plants. While in NG062, the proline contents of B, D and control levels of osmotic stress treatments were higher than other treatments (Table 4.3). Accessions DI and $JI_2$ showed no significant variation in their proline levels. Accession $JI_1$ had the highest and $JI_2$ had the lowest proline level at all levels of osmotic potential (Table 4.3).

**Table 4.1: Values of drought tolerance index (DTI) based on the shoot weight under osmotic stress**

| Accessions | DTI | MP |
|---|---|---|
| NG061 | 3.93a | 0.20a |
| NG062 | 1.75bc | 0.08b |
| $JI_1$ | 4.32a | 0.17a |
| DI | 2.12b | 0.12a |
| $JI_2$ | 0.77d | 0.05b |

Figures with identical alphabets in each column are not significantly different at DMRT ($P<0.05$).

*Where: DTI= Drought tolerance index, MP= Mean Productivity*

4-5= drought tolerant

2-4= mild tolerant

Below 2 = drought susceptible

**Table 4.2: Chlorophyll Contents (µg/ml) of *D. exilis* under varied osmotic stress**

| Accessions | $\Psi_S$ | $Chl_a$ | $Chl_b$ | T. Chlorophyll |
|---|---|---|---|---|
| NG061 | A | 14.93e | 34.97cd | 49.90d |
| NG061 | B | 32.91ab | 77.14b | 110.04b |
| NG061 | C | 42.12a | 107.91a | 150.04a |
| NG061 | D | 19.17d | 28.27e | 47.43d |
| NG061 | E | 19.13d | 29.47de | 48.6d |
| NG061 | F | 24.24c | 39.93c | 64.17c |
| | | | | |
| NG062 | A | 20.30b | 46.06b | 66.36bc |
| NG062 | B | 20.42b | 24.16d | 44.58d |
| NG062 | C | 7.24d | 13.89e | 21.13e |
| NG062 | D | 32.82a | 81.12a | 113.94a |
| NG062 | E | 16.97c | 41.40bc | 58.37c |
| NG062 | F | 30.12a | 48.35b | 78.47b |
| | | | | |
| $JI_1$ | A | 25.72c | 78.16b | 103.88b |
| $JI_1$ | B | 23.84c | 55.71bc | 79.54c |
| $JI_1$ | C | 25.79c | 41.35c | 67.13d |
| $JI_1$ | D | 87.54a | 211.07a | 298.6a |
| $JI_1$ | E | 16.54d | 17.87e | 34.41e |
| $JI_1$ | F | 34.20b | 38.39cd | 72.6cd |
| | | | | |
| DI | A | 18.86de | 47.14c | 65.99c |
| DI | B | 29.21c | 66.8b | 96.01b |
| DI | C | 37.93a | 78.41ab | 116.34ab |
| DI | D | 33.65b | 87.89a | 121.54a |
| DI | E | 19.77d | 20.04d | 39.8d |
| DI | F | 33.44b | 65.21b | 98.65b |
| | | | | |
| $JI_2$ | A | 24.12ab | 58.29ab | 82.41b |
| $JI_2$ | B | 28.34ab | 42.55b | 70.89bc |
| $JI_2$ | C | 31.63a | 78.30a | 109.94a |
| $JI_2$ | D | 15.38c | 37.65bc | 53.03d |
| $JI_2$ | E | 18.13b | 42.85b | 60.99c |
| $JI_2$ | F | 34.00a | 67.82a | 101.82a |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). Where, $\Psi_S$ = Osmotic potential, A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa (Control), $Chl_a$= Chlorophyll a, $Chl_b$= Chlorophyll b, T. Chlorophyll= Total chlorophyll

**Table 4.3: Proline Contents of *D. exilis* under Varied Osmotic Stress**

| ACCESSIONS | $\Psi_S$ | PROLINE(mg/ml) |
|---|---|---|
| NG061 | A | 0.044b |
| | B | 0.038c |
| | C | 0.043b |
| | D | 0.034d |
| | E | 0.038c |
| | F | 0.054a |
| NG062 | A | 0.016e |
| | B | 0.036a |
| | C | 0.018e |
| | D | 0.030c |
| | E | 0.020d |
| | F | 0.036b |
| $JI_1$ | A | 0.052a |
| | B | 0.019b |
| | C | 0.013c |
| | D | 0.014c |
| | E | 0.015c |
| | F | 0.009d |
| DI | A | 0.014d |
| | B | 0.023ab |
| | C | 0.025a |
| | D | 0.015d |
| | E | 0.020b |
| | F | 0.017c |
| $JI_2$ | A | 0.014a |
| | B | 0.013a |
| | C | 0.014a |
| | D | 0.009d |
| | E | 0.010cd |
| | F | 0.010c |
| | LSD (0.05) | 0.009 |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). Where, $\Psi_S$ = Osmotic potential, A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa (Control)

## 4.5 Antioxidant Enzymes of *D. exilis* under Different Osmotic Stress

### 4.5.1 Ascorbate peroxide (APx)

Ascorbate peroxidase was significantly higher in $JI_1$ accession as the level of osmotic stress increased (Table 4.4). Enzyme activity APx in accessions $JI_2$ and NG062 were lowered and among the treatment used, no significant variation was observed. In all the accessions, the APx in the control plant was consistently low except for NG061 control plants, which had the highest APx in all the accessions. It is important to point out that the highest proline and APx contents were found in the control plants of NG061 (Table 4.3 and 4.4). Under osmotic stress, $JI_1$ accession recorded the highest APx activities (0.016 units/mg protein) at E level of stress (Table 4.4).

### 4.5.2 Superoxide dismutase (SOD)

Superoxide dismutase activities were high in accessions $JI_1$, $JI_2$, NG061 and NG062 when osmotic stress was induced (Table 4.4). Accessions $JI_2$ showed a significant elevation in SOD under osmotic-stress than the control. For accession DI, the SOD activities in control plants were either higher or comparable with the osmotic-stressed plants. When osmotic stress was induced (A-E), accession NG062 had the lowest SOD (0.054 units/mg protein) activities at D level of stress while $JI_1$ had the highest SOD (1.659 units/mg protein) at same level of stress. Accession NG061 showed no significant variation when compared with the control plants (Table 4.4).

### 4.5.3 Catalase (CAT)

Accessions $JI_1$ and NG062 had the highest catalase at A level of osmotic stress (Table 4.4). Accession $JI_1$ showed significant elevation CAT activities than its control, whereas in $JI_2$ a significant reduction in CAT was recorded in osmotic stressed plants when compared to control. There was no consistent trend in the CAT activities of all the accessions whether osmotic stressed or not. Accession $JI_2$ had the lowest level (0.222units/mg protein) of CAT. Activities of CAT enzyme were similar in accession DI in contrast to the control plants (Table 4.4).

**Table 4.4: Enzymatic Activities of *D. exilis* under different Osmotic stress**

| ACCESSIONS | $\Psi_S$ | APx (mmol/ml/min) | SOD (units/mg protein) | CAT (units/mg protein) |
|---|---|---|---|---|
| NG061 | A | 0.012b | 1.373a | 2.155a |
| | B | 0.003f | 1.129b | 0.451d |
| | C | 0.007d | 1.324a | 1.937b |
| | D | 0.010c | 1.361a | 0.682c |
| | E | 0.005e | ND | ND |
| | F | 0.021a | 1.341a | 1.608c |
| NG062 | A | 0.002c | 1.412a | 3.301a |
| | B | 0.004a | 1.406a | 0.657f |
| | C | 0.005a | 1.119b | 1.528d |
| | D | 0.003b | 0.054e | 2.005c |
| | E | 0.004b | 0.589d | 1.003e |
| | F | 0.004b | 0.765c | 2.649b |
| $JI_1$ | A | 0.010b | 1.634a | 3.628a |
| | B | 0.005e | 1.536b | 1.477d |
| | C | 0.008c | 1.560b | 2.330c |
| | D | 0.006d | 1.659a | 2.344c |
| | E | 0.016a | 1.651a | 2.710b |
| | F | 0.003f | 1.648a | 1.146e |
| DI | A | 0.002e | 0.874b | 2.190a |
| | B | 0.003d | 0.723c | 1.745b |
| | C | 0.005c | 0.810b | 2.341a |
| | D | 0.014a | 0.852b | 1.801b |
| | E | 0.002e | 1.615a | 1.201c |
| | F | 0.006b | 1.476a | 1.745b |
| $JI_2$ | A | 0.002d | 1.500ab | 0.801d |
| | B | 0.003c | 1.563a | 0.375e |
| | C | 0.004b | 1.556a | 0.222f |
| | D | 0.005a | 1.395ab | 1.246c |
| | E | 0.005a | 1.392b | 1.948b |
| | F | 0.002d | 0.770c | 2.328a |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$).

Where, $\Psi_S$ = Osmotic potential, A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa (Control). APx= Ascorbate peroxidase, CAT= Catalase, SOD= Superoxide Dismutase

### 4.6 Lipid Peroxidation of *D. exilis* under Osmotic Stress

Accession $JI_1$ showed a reduced lipid peroxidation throughout the osmotic stress conditions except for control plants (Table 4.5). There was a sharp decline in the lipid peroxidation in DI accession from A to D levels of osmotic t stress. Accessions $JI_2$ and DI showed a significant increase in lipid peroxidation than control plants. Accession $JI_1$ had the lowest level of lipid peroxidation (3.79E-07 M) while $JI_2$ had the highest at C and D levels of osmotic stress (Table 4.5).

### 4.7 Reactive Oxygen Species Scavenged in *D. exilis* under Drought Stress

#### 4.7.1 Hydroxyl ($OH^-$)

Control plants (F level of osmotic stress) inhibited $OH^-$ than osmotic stress plants in NG061 and $JI_2$. Accessions $JI_1$, DI and NG062 displayed notable increase in percentage inhibition of $OH^-$ than the control plants. During osmotic stress, accession $JI_1$ had 80 % inhibition of $OH^-$ which was significantly higher at E level. Lowest inhibition percentage was recorded in accessions $JI_2$ and NG061 at the same E level of osmotic stress (Table 4.6).

#### 4.7.2 Hydrogen Peroxide ($H_2O_2$)

There was no consistent relationship in the inhibition of $H_2O_2$ in all the levels of the osmotic stress between the accessions (Table 4.6). In osmotic stressed plants, accession DI showed the highest level of $H_2O_2$ inhibition (62%) at F level of osmotic stress.

#### 4.7.3 Nitric Oxide (NO)

The percentage inhibition of NO in $JI_1$ accession was relatively constant with high values when the osmotic stress was imposed. With respect to their control plants, there were significant decrease in percentage inhibition of NO in osmotic stressed DI, NG062 and NG061. Highest percentage inhibition (57%) of NO was observed in NG061 at F level of osmotic stress (Table 4.6). In all the accessions, plants at F level (control) of osmotic stress inhibited NO higher than the other levels except for accession $JI_1$ and $JI_2$ which didn't follow the pattern (Table 4.6).

### 4.7.4 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH)

Osmotic stressed plants significantly inhibited DPPH radicals higher than plants at F level of osmotic stress (control) except for NG061. Accession $JI_1$ significantly increase percentage inhibition of accession $JI_1$ that ranged from 10-78% significantly greater than the control at all the levels of osmotic stress (Table 4.6). There was a significant decline in percentage inhibition for accessions NG061 and NG062. There was no difference in DPPH among the accessions of all levels of osmotic stress except for $JI_1$ accession (Table 4.6).

## 4.8 Relationships between Assays

Ascorbate Peroxidase was positively correlated with all assays except for MDA and NO. Catalase was positively correlated with APx, CAT, proline and OH. Superoxide dismutase was positively correlated with APx, MDA and NO. Proline was positively correlated with APx, CAT and $H_2O_2$. The MDA was positively correlated with SOD. The NO acid radical was positively related to superoxide dismutase. Hydroxyl radical was positively correlated with APx and CAT. Hydrogen peroxide was positively correlated with APx and proline (Table 4.7)

**Table 4.5: Malondialdehyde (MDA) Contents in *D. exilis* under Varied Osmotic Stress**

| ACCESSION | $\Psi_S$ | MDA (M) |
|---|---|---|
| NG061 | A | 7.84E-07b |
| | B | 8.99E-07a |
| | C | 8.39E-07ab |
| | D | 8.65E-07c |
| | E | 8.74E-07a |
| | F | 8.42E-07ab |
| NG062 | A | 5.96E-07b |
| | B | 5.96E-07b |
| | C | 5.47E-07c |
| | D | 6.59E-07a |
| | E | 6.54E-07a |
| | F | 4.74E-07d |
| $JI_1$ | A | 5.07E-07c |
| | B | 4.58E-07e |
| | C | 3.79E-07d |
| | D | 6.18E-07b |
| | E | 5.86E-07b |
| | F | 7.80E-07a |
| DI | A | 1.58E-06a |
| | B | 1.25E-06b |
| | C | 7.76E-07cd |
| | D | 7.52E-07d |
| | E | 8.50E-07c |
| | F | 6.66E-07e |
| $JI_2$ | A | 1.41E-06c |
| | B | 9.04E-07d |
| | C | 2.03E-06a |
| | D | 1.81E-06b |
| | E | 6.94E-07e |
| | F | 5.56E-07f |
| | LSD (0.05) | $3.98 \times 10^{-7}$ |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). Where, $\Psi_S$ = Osmotic potential, A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa(Control)

**Table 4.6: Inhibitions Percentage (%) of Free Radicals under Varied Osmotic Stress**

| Accessions | $\Psi_S$ | $OH^-$ | $H_2O_2$ | NO | DPPH |
|---|---|---|---|---|---|
| NG061 | A | 52.61b | 35.67c | 17.50e | 21.99e |
| | B | 55.02b | 31.58d | 27.13c | 24.81d |
| | C | 53.61b | 45.61b | 21.76d | 21.75e |
| | D | 46.59c | 44.44b | 25.37c | 28.63c |
| | E | 40.36d | 50.88a | 33.15b | 40.07b |
| | F | 62.27a | 53.85a | 57.64a | 63.59a |
| NG062 | A | 49.60a | 38.60d | 38.24b | 26.11b |
| | B | 41.77b | 45.61c | 34.17c | 15.99d |
| | C | 50.20a | 45.61c | 42.13ab | 18.21c |
| | D | 51.00a | 43.86c | 41.20b | 37.11a |
| | E | 52.81a | 50.88b | 39.35b | 16.91cd |
| | F | 42.31b | 60.07a | 45.72a | 27.06b |
| $JI_1$ | A | 63.86b | ND | 13.15e | 19.33d |
| | B | 65.26b | 54.39a | 20.00d | 20.93d |
| | C | 46.18d | 59.65ab | 12.04f | 78.29a |
| | D | 53.61c | 19.30d | 50.65a | 49.13c |
| | E | 80.22a | 36.84c | 29.54c | 56.07b |
| | F | 62.25b | 51.28b | 43.78b | 10.17e |
| DI | A | 59.84a | 45.61b | 16.57cd | 16.91d |
| | B | 62.45a | 28.65e | 13.06e | 26.89b |
| | C | 60.04a | 36.84c | 17.59c | 30.28a |
| | D | 56.63a | 32 .16d | 15.83d | 31.15a |
| | E | 60.24a | 36.84c | 26.39b | 18.31d |
| | F | 41.76b | 62.27a | 31.81a | 24.15c |
| $JI_2$ | A | 36.55e | 31.58c | 41.02b | 23.69c |
| | B | 43.98d | 50.88a | 38.61bc | 14.34d |
| | C | 56.83b | 41.52b | 31.02d | 29.99a |
| | D | 46.59c | 40.35b | 31.20d | 26.55b |
| | E | 40.36d | 53.22a | 49.72a | 31.44a |
| | F | 62.27a | 52.38a | 36.87c | 26.58b |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). Where, $\Psi_S$ = Osmotic potential, ND= Not Determined, A= -9.29MPa, B = -13.93 MPa, C= -20.13 MPa, D= -26.32 MPa, E= -32.51 MPa and F= 0MPa (Control)

**Table 4.7: Correlation between Assays**

| | APx | CAT | SOD | PROLINE | MDA | NO | $OH^-$ | $H_2O_2$ |
|---|---|---|---|---|---|---|---|---|
| Ascorbate Peroxidase | 1.00 | 0.15 | 0.16 | 0.49 | -0.24 | -0.05 | 0.25 | 0.02 |
| Catalase | 0.15 | 1.00 | -0.07 | 0.04 | -0.37 | -0.13 | 0.27 | -0.03 |
| Superoxide Peroxidase | 0.16 | -0.07 | 1.00 | -0.12 | 0.04 | 0.01 | -0.04 | -0.02 |
| Proline | 0.48 | 0.04 | -0.12 | 1.00 | -0.26 | -0.06 | -0.03 | 0.05 |
| Malondialdehyde | -0.24 | -0.38 | 0.04 | -0.25 | 1.00 | -0.09 | -0.04 | -0.29 |
| Nitric Acid | -0.05 | -0.13 | 0.01 | -0.06 | -0.09 | 1.00 | .-0.31 | 0.18 |
| Hydroxyl | 0.25 | 0.27 | -0.04 | -0.03 | -0.04 | -0.31 | 1.00 | -0.24 |
| Hydrogen Peroxide | 0.02 | -0.00 | -0.02 | 0.05 | -0.28 | 0.18 | -0.24 | 1.00 |

Where, APx- Ascorbate peroxidase, CAT- Catalase, SOD- Superoxide dismutase, MDA-Malondialdehyde, NO-Nitric oxide, $OH^-$ - Hydroxyl, $H_2O_2$- Hydrogen peroxide

## 4.9 Plant Growth at Screen-House

Number of leaves in drought stressed plants was significantly higher than CNT in accessions NG061, $JI_1$ and DI (Fig 4.3) at 5 weeks after planting (WAP). At 4 days drought stress, number of leaves in accessions NG061 and $JI_1$ were significantly high. At 8 days level of drought stress, accessions NG061 were significantly higher than other accessions. At 12days, number of leaves of CNT plants had no variation among the accessions. In all the treatments, accessions NG061, $JI_1$ and DI showed no significant difference in their plant height. Plant height of accession NG062 was significantly low when drought stresses were imposed (Fig 4.3). Leaves length of accession NG061 and NG062 were significantly lower compared to all other accessions irrespective of level of drought stress. The leaves length of accessions NG061 and $JI_1$ increased as the drought stress increased progressively. There were no significant variations between leaves length of accessions $JI_1$ and DI (Fig 4.3). Tillers were first observed and recorded highest at four (4) days drought stress. Number of tillers produced in accession DI and NG062 at 4, 8 and 12 days drought stress were not significantly different from each other (Fig 4.3).

At 7 WAP, Leaves length of accessions NG061 and NG062 were significantly lower than accessions $JI_1$ and DI (Fig 4.4). There was no significant variation between leaves length of plants under CNT and drought stressed. Number of tillers in $JI_1$ increased at 8 days drought stress. Number of tillers produced in accession $JI_1$ at 8 days drought stress was not significantly different from number of tillers produced by NG062 at 8 days, 12 days and CNT. Similar number tillers were observed in accession DI as drought stress increased. Accession NG061 had the highest number of tillers at 8days drought stress (Fig 4.4). Number of leaves in accessions DI and $JI_1$ were not significantly different except for plants at 8 days drought stress in $JI_1$ accession. Similarly, number of leaves of NG061 and NG062 were not significantly different from each other except for plants at 8days which also had the overall highest number of leaves (Fig 4.4). There were no significant variations in plant height of accessions DI, NG062, $JI_1$ and NG061 except for CNT and 8days drought stress in accessions $JI_1$ and NG061 respectively which also had the highest plant height (Fig 4.4).

At 11 WAP, accession NG061 had the highest number of leaves and plant height though with low values of leaves length (Fig 4.5). Number of leaves and plant height were significantly high

in 8days drought stress than other drought stress. Accession $JI_1$ had the highest leaves length though there was no significant difference with that of accession DI (Fig 4.5). Accession NG061 produced highest number of tillers at 4 and 8 days drought stress than CNT and 12 days (Fig 4.5). Number of tillers remains the same in accessions $JI_1$ and NG062 when drought stress was induced (Fig 4.5).

### 4.10 Inflorescence Traits of *D. exilis* under Drought Stress

Inflorescences were noticed at 9 WAP in accessions NG061. Inflorescences parameters such as number of spike, peduncle length, spike length and flag length were significantly higher at 8days drought stress than other treatments (Fig 4.6). Accession NG061 produced the highest significant number of inflorescence parameters in all the treatments. Drought stress induced production of spikes than CNT (Fig 4.6). Accession DI produced no inflorescences in all the treatments. Accession $JI_1$ only produced inflorescences at 8 days drought stress. Accession NG062 didn't produce inflorescences at CNT (Fig 4.6).

### 4.11 Plant Weight and Root Structure of *D. exilis* under Drought Stress

The weight of *D. exilis* under drought stress is presented in table 4.8 and fig 4.7. There was no specific pattern in fresh weight of the *D. exilis* when introduced to different drought stress (Table 4.8). Accession DI had the highest fresh root (3.40 g) and shoot (9.70g) at 8 days drought stress and lowest was found in accession NG061 at 4days (0.29g and 0.8g fresh root and shoot respectively) drought stress. Fresh root and shoot weight of accession $JI_1$ decreased as the drought stress increase (Table 4.8). Dry root of accession $JI_1$ showed constant dry weight across the drought stress. Accession DI had a significant high dry root and dry shoot value than other accessions at 8days (Fig 4.7). Accession NG061 had the lowest dry root and shoot weight at 4days drought stress. Accession JI1 and DI had an increase in lateral roots at 4days drought stress whereas, long tap roots were observed at 12days drought stress in accessions JI and NGO61 (Fig 4.8).

### 4.12 Molecular Properties of *D. exilis* under Drought stress (Screen-house Experiment)

DNA concentration of *D. exilis* ranged from 21.5- 95.7mg/µl. Accession DI had a significantly high DNA concentration of 95.70mg/µl at 12 days drought stress, followed by accession $JI_1$ at 8 days with 67.50mg/µl, and accession NG062 had the lowest (21.5 mg/µl) concentration of DNA (Table 4.9). Accessions NG061, $JI_1$ and DI showed positive effect of drought stress on DNA concentrations except DI accession with negative regression value (Table 4.10). The concentration of DNA was high in drought stressed plants than CNT except for accession NG062. The purity of DNA showed a significant variation in drought stressed plants and CNT plants of all the accessions. The purity of DNA of *D. exilis* ranged from 1.76-1.84. Accession $JI_1$ and NG062 had the highest purity level at 4days and CNT respectively, while accession NG062 at 4 days had the lowest purity level of DNA (Table 4.9).

DNA electrophoretic of four accessions of *D. exilis* under drought stress is presented in figure 4.8. The smaller molecules of DNA move faster than the larger molecules of DNA, as they move from the negatively charged pole to the positively charged one. Accessions $JI_1$, DI and NG062 presented nine bands at 1000Kb at both CNT and drought stress. Six (6) good and sharp bands out of twelve (6/12) were present under drought stressed plants while three (3) good and quality bands out of four (3/4) were presented under CNT. Bands were presented in accessions DI and NG062 under CNT, 8 days and 12 days drought stress while Accession $JI_1$ presented bands at the same loci under CNT, 4days and 12 days drought stress (Fig. 4.9).

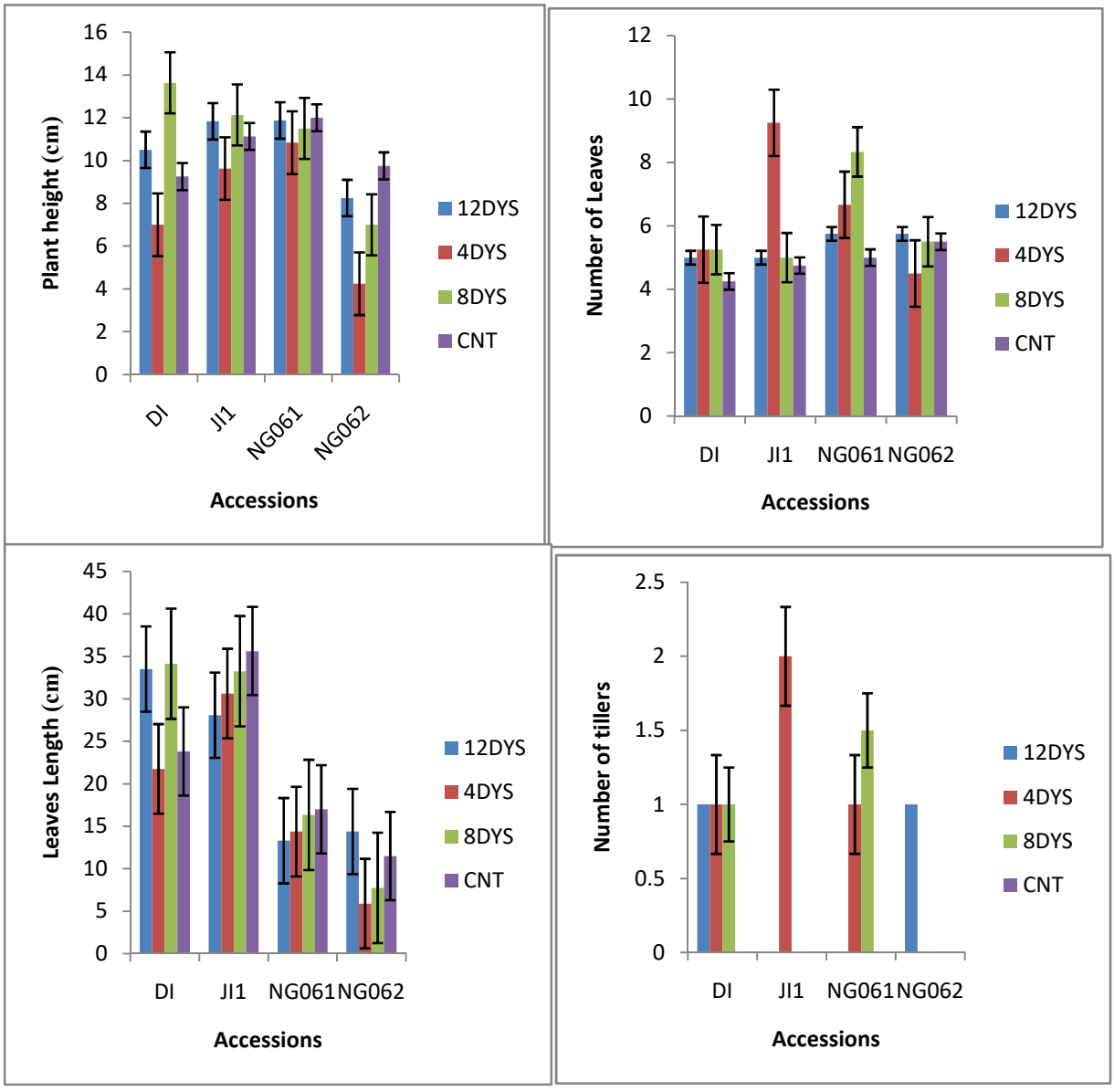



Figure 4.3: Plant Growth of *D. exilis* under drought stress at 5WAP

Where, Bars means Error bars with Standard Error, 12 DYS= 12 Days, 8 DYS= 8 Days, 4DYS = 4 Days Drought Stress & CNT= Control

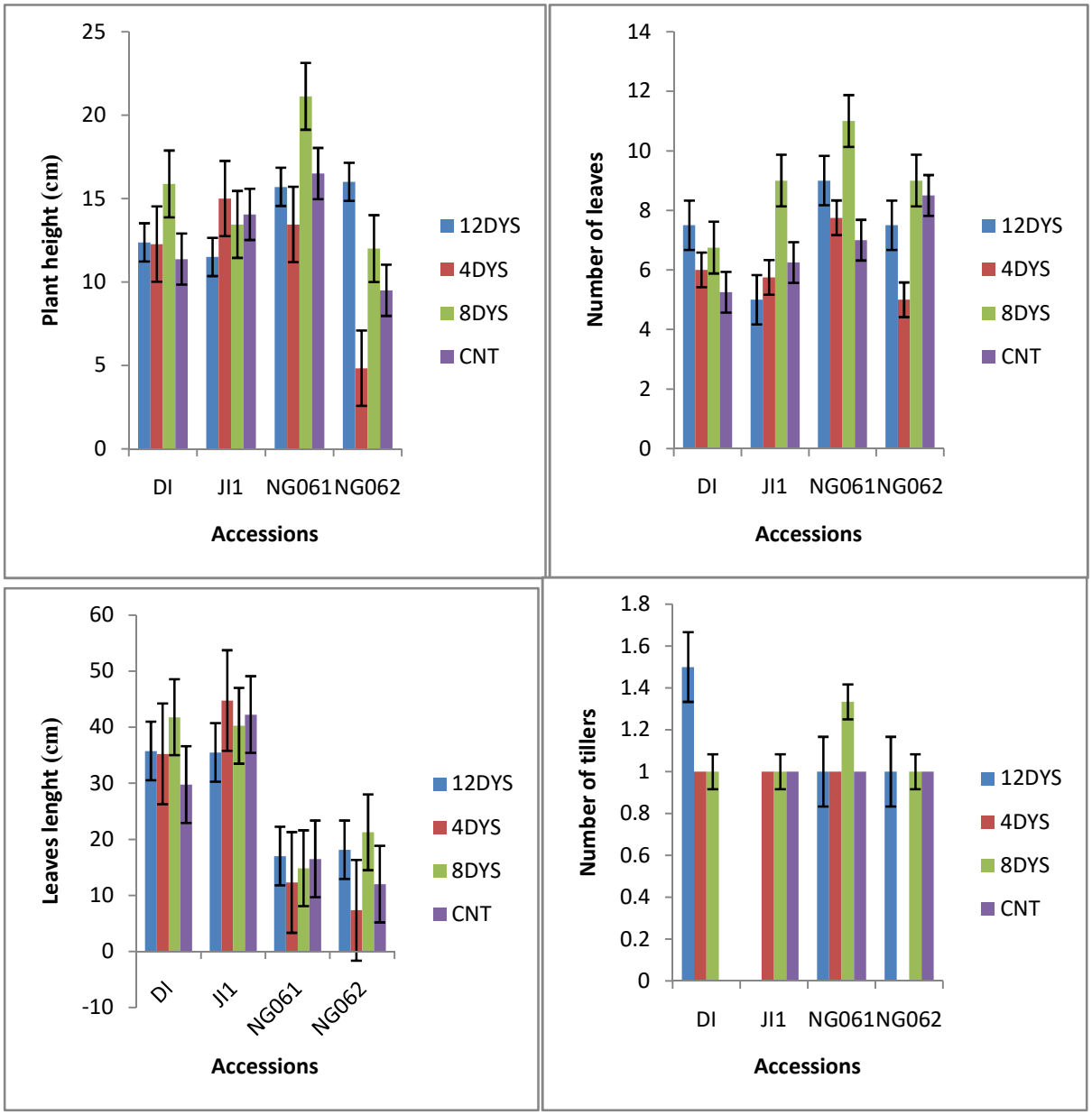



Figure 4.4: Plant Growth of *D. exilis* under drought stress at 7WAP

Where, Bars means Error bars with Standard Error, 12 DYS= 12 Days, 8 DYS= 8 Days, 4DYS = 4 Days Drought Stress & CNT= Control

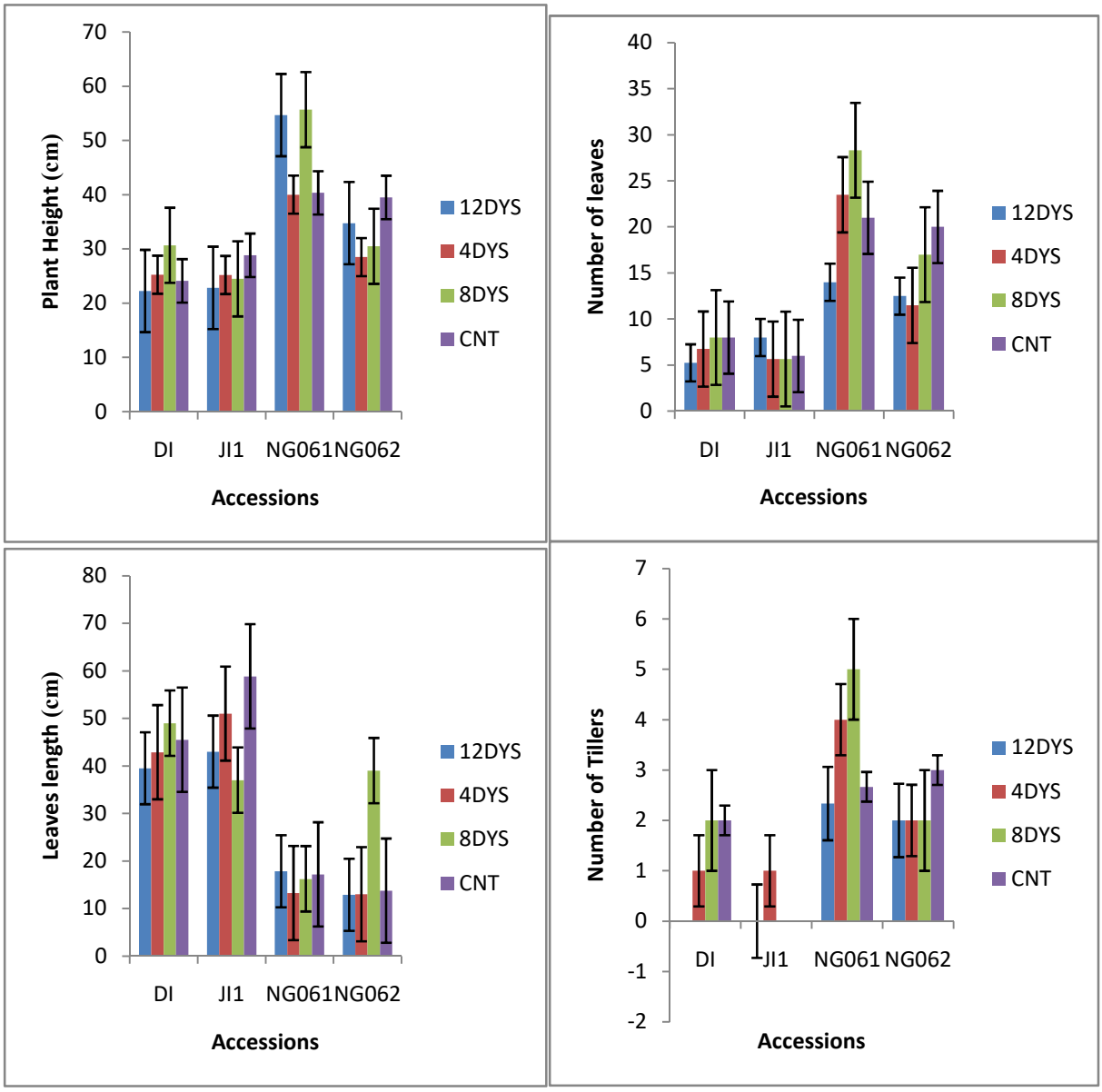



Figure 4.5: Plant Growth of *D. exilis* under drought stress at 11WAP

Where, Bars means Error bars with Standard Error, 12 DYS= 12 Days, 8 DYS= 8 Days, 4DYS = 4 Days Drought Stress & CNT= Control

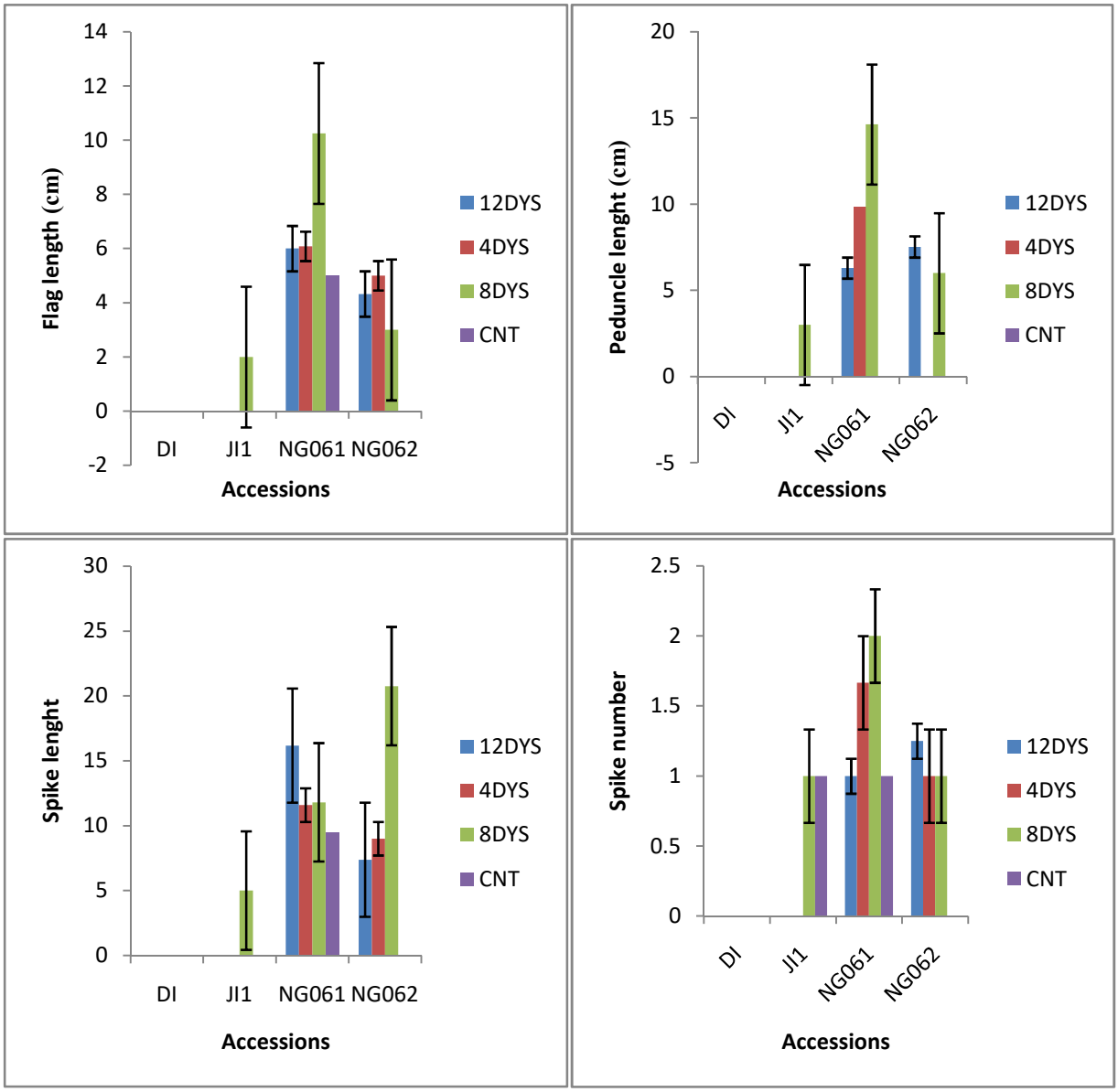



Figure 4.6: Inflorescences Part of *D. exilis* under drought stress at 11WAP

Where, Bars means Error bars with Standard Error, 12 DYS= 12 Days, 8 DYS= 8 Days, 4DYS = 4 Days Drought Stress & CNT= Control

**Table 4.8: Fresh weight of *D. exilis* under Drought Stress**

| Accessions | Drought Levels | Root Fresh Weight (g) | Shoot Fresh Weight (g) | Total Fresh Weight (g) |
|---|---|---|---|---|
| NG061 | CNT | 0.40b | 1.55b | 1.90b |
| | 4 DAYS | 0.29bc | 0.81c | 1.10c |
| | 8 DAYS | 1.01a | 2.39a | 3.40a |
| | 12 DAYS | 0.35b | 1.50b | 1.90b |
| NG062 | CNT | 0.50b | 1.40b | 1.90c |
| | 4 DAYS | 1.02a | 1.98a | 3.00a |
| | 8 DAYS | 0.45b | 0.85c | 1.30d |
| | 12 DAYS | 1.04a | 1.56b | 2.60b |
| $JI_1$ | CNT | 1.50a | 2.50a | 4.00a |
| | 4 DAYS | 1.25b | 2.15b | 3.40b |
| | 8 DAYS | 1.01b | 2.19b | 3.20b |
| | 12 DAYS | 0.83c | 1.36c | 2.19c |
| DI | CNT | 2.01b | 5.49b | 7.70b |
| | 4 DAYS | 1.72c | 3.38c | 5.10c |
| | 8 DAYS | 3.40a | 9.70a | 13.10a |
| | 12 DAYS | 1.41d | 3.09c | 4.50d |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$).

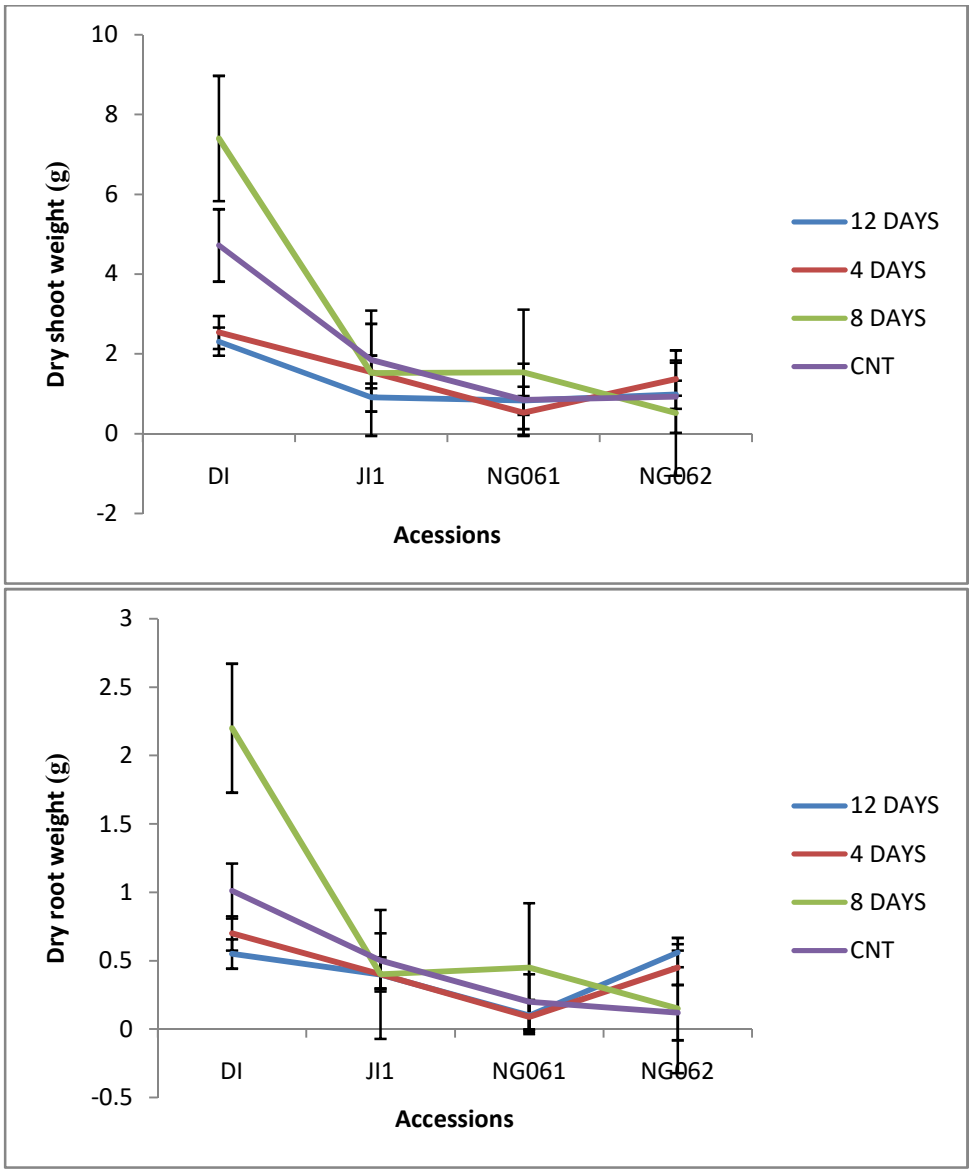



**Figure 4.7: Shoot and Root Dry Weight of the *D. exilis* under Drought Stress**

Where, Bars means Error bars with Standard Error, Drought Levels = 12, 4, 8 Days & CNT


**Figure 4.8: Dry Root Structure at different levels of drought stress**

**Where, A= CNT, B= 4 Days, C= 8 Days and D= 12 Days Drought Stress, LR: Lateral Root, TR: Tap root; $JI_1$ =1, DI =2, NG061=3, NG062 =4**

**Table 4.9: DNA Quantification of *D. exilis* under drought stress**

| ACCESSIONS | DROUGHT LEVELS | CONC. (mg/µl) | PURITY LEVEL |
|---|---|---|---|
| NG061 | CNT | 31.00d | 1.80ab |
| NG061 | 4DAYS | 35.50c | 1.81a |
| NG061 | 8DAYS | 54.10a | 1.80ab |
| NG061 | 12DAYS | 44.70b | 1.76c |
| | | | |
| NG062 | CNT | 44.70a | 1.84a |
| NG062 | 4DAYS | 21.50d | 1.76bc |
| NG062 | 8DAYS | 28.80b | 1.78b |
| NG062 | 12DAYS | 27.40c | 1.78b |
| | | | |
| $JI_1$ | CNT | 40.20d | 1.80ab |
| $JI_1$ | 4DAYS | 48.00c | 1.84a |
| $JI_1$ | 8DAYS | 67.50a | 1.79bc |
| $JI_1$ | 12DAYS | 55.80b | 1.79bc |
| | | | |
| DI | CNT | 40.20c | 1.80a |
| DI | 4DAYS | 58.90b | 1.79ab |
| DI | 8DAYS | 39.50d | 1.79ab |
| DI | 12DAYS | 95.70a | 1.77bc |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). Where, Conc= Concentration

**Table 4.10: Regression analysis showing effect of drought stress on DNA concentration of *D. exilis***

| ACCESSIONS | R-value | P-value |
|---|---|---|
| NG061 | 0.75 | 0.25 |
| NG062 | -0.58 | 0.42 |
| $JI_1$ | 0.73 | 0.27 |
| DI | 0.72 | 0.28 |

The significance differences were determined by the 95% confidence intervals.

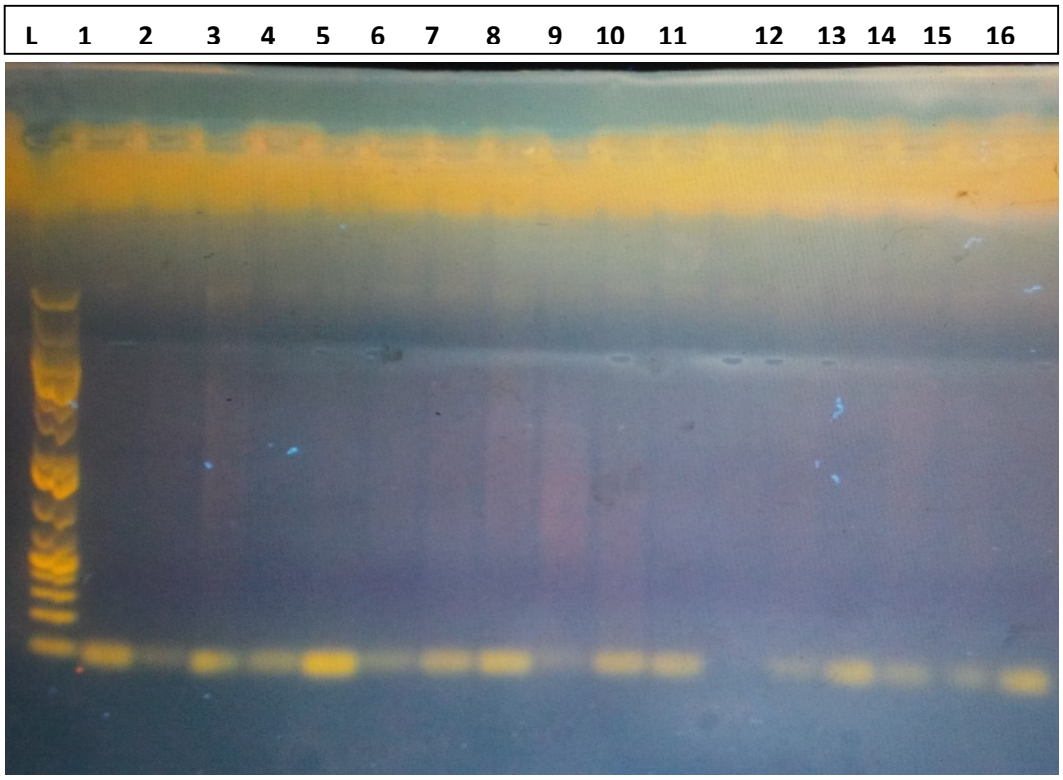



**Figure 4.9: DNA Bands of *D. exilis* under Drought Stress**

Where, 1=$JI_1$-4DAYS, 2=NG061-12DAYS, 3=DI-12DAYS, 4=DI-4DAY5, 5=DI-CNT, 6=NG062-4DAYS, 7= $JI_1$-12DAYS, 8= NG062-8DAYS, 9=DI-8DAYS, 10=NG062-12DAYS, 11=NG061-4DAYS, 12=$JI_1$-8DAYS, 13= $JI_1$-CNT, 14=NG061-8DAYS, 15= NG061-CNT, 16=NG062-CNT

Sharp bands showed good quality of DNA while a smeared band indicates that the DNA was degraded by a protein during the extraction process

## 4.13 Biochemical Analysis of *D. exilis* Planted in Screenhouse

### 4.13.1 Chlorophyll Contents under Drought Stress

Drought-stressed plants had high significant chlorophyll 'a' than CNT while CNT plants had high significant chlorophyll 'b' than drought-stressed plants (Table 4.11). Accession $JI_1$ had the highest chlorophyll 'a' of about 15.90 µg/ml at 4days drought stress, accession DI had the highest chlorophyll 'b' (25.07 µg/ml) under CNT. Lowest chlorophyll 'a' was found in accession DI in CNT plants and lowest chlorophyll 'b' was found in $JI_1$ at 4 days in drought stressed plants (Table 4.11).

### 4.13.2 Pigment Contents under Drought Stress

The CNT plants had the highest total chlorophyll contents than drought stressed plants in all the accessions (Table 4.12). Accession $JI_1$ had the highest anthocyanin content among all the accessions in CNT plants and all drought stressed except for NG061 at 12 days level of drought stress. Although, there was stability of total chlorophylls in accessions, the latter had greater total chlorophyll contents than the former (NG062). Chlorophyll content of accession NG061 showed a positive regression while other accessions showed a negative effect though total chlorophyll content of accession $J1_I$ was significant ($P<0.05$). Total anthocyanin estimated in all accessions showed negative regression effect under drought stress (Table 4.13).

### 4.13.3 Activities of Proline when Subjected to Drought

Control plants showed a low accumulation of proline content during drought stress as observed in all accessions (Table 4.14). Accession $JI_1$ (0.10 Mm) had the highest proline contents followed by DI (0.08 mM) at 8 days drought stress. Accession NG062 had the lowest proline contents (0.01 mM) under CNT (Table 4.14). Although, proline contents of all the accessions showed positive regression during drought stress (Table 4:15).

**Table 4.11: Chlorophyll Contents of *D. exilis* under Drought Stress**

| ACCESSIONS | DROUGHT LEVEL | CHLOROPHYLL a (µg/ml) | CHLOROPHYLL b (µg/ml) |
|---|---|---|---|
| NG061 | CNT | 8.20d | 22.63a |
| | 4 DAYS | 13.79c | 13.17b |
| | 8 DAYS | 15.44ab | 10.43cd |
| | 12 DAYS | 15.55a | 10.49c |
| NG062 | CNT | 8.29cd | 13.28a |
| | 4 DAYS | 8.46a | 13.04ab |
| | 8 DAYS | 8.34ab | 12.42d |
| | 12 DAYS | 8.31bc | 13.07bc |
| $JI_1$ | CNT | 15.56cd | 11.02a |
| | 4 DAYS | 15.90a | 10.23cd |
| | 8 DAYS | 15.59bc | 10.59b |
| | 12 DAYS | 15.79ab | 10.37bc |
| DI | CNT | 6.79d | 25.07a |
| | 4 DAYS | 7.94c | 13.92b |
| | 8 DAYS | 15.29ab | 10.88c |
| | 12 DAYS | 15.31a | 10.72cd |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$).

**Table 4.12: Pigment Contents of *D. exilis* under Drought Stress**

| ACCESSIONS | DROUGHT LEVELS | TOTAL ANTHOCYANIN (µg/ml) | TOTAL CHLOROPHYLL (µg/ml) |
|---|---|---|---|
| NG061 | CNT | 171.16b | 30.84a |
| | 4 DAYS | 60.53c | 26.96b |
| | 8 DAYS | 56.36d | 26.13cd |
| | 12 DAYS | 1114.65a | 26.31bc |
| NG062 | CNT | 144.03b | 21.83a |
| | 4 DAYS | 8.35d | 21.75ab |
| | 8 DAYS | 150.29a | 21.01cd |
| | 12 DAYS | 54.27c | 21.56bc |
| $JI_1$ | CNT | 594.90a | 27.57a |
| | 4 DAYS | 390.34b | 26.39b |
| | 8 DAYS | 379.90c | 26.39b |
| | 12 DAYS | 223.35d | 26.39b |
| DI | CNT | 100.19b | 32.29a |
| | 4 DAYS | 96.02c | 22.12d |
| | 8 DAYS | 144.03a | 26.44b |
| | 12 DAYS | 45.92d | 26.30bc |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$).

**Table 4.13: Pigment Regression of *D. exilis* Accessions under drought stress**

| ACCESSIONS | TOTAL CHLOROPHYLL | | TOTAL ANTHOCYANIN | |
|---|---|---|---|---|
| | R-value | P-value | R-value | P-value |
| NG061 | 0.71 | 0.29 | -0.84 | 0.16 |
| NG062 | -0.24 | 0.76 | -0.54 | 0.46 |
| $JI_1$ | -0.95 | 0.05* | -0.77 | 0.23 |
| DI | -0.37 | 0.63 | -0.42 | 0.58 |

The significance differences were determined by the 95% confidence intervals.

**Table 4.14: Proline Contents of *D. exilis* under drought stress**

| ACCESSIONS | DROUGHT LEVELS | PROLINE (mM) |
|---|---|---|
| NG061 | CNT | 0.05cd |
| | 4 DAYS | 0.075a |
| | 8 DAYS | 0.06bc |
| | 12 DAYS | 0.07ab |
| NG062 | CNT | 0.01d |
| | 4 DAYS | 0.04b |
| | 8 DAYS | 0.03c |
| | 12 DAYS | 0.05a |
| $JI_1$ | CNT | 0.02cd |
| | 4 DAYS | 0.10a |
| | 8 DAYS | 0.03c |
| | 12 DAYS | 0.05b |
| DI | CNT | 0.03d |
| | 4 DAYS | 0.06b |
| | 8 DAYS | 0.08a |
| | 12 DAYS | 0.04c |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$).

**Table 4.15: Proline Regression of *D. exilis* under drought stress**

| ACCESSIONS | R-value | P-value |
|---|---|---|
| NG061 | 0.52 | 0.48 |
| NG062 | 0.83 | 0.17 |
| $JI_1$ | 0.07 | 0.93 |
| DI | 0.29 | 0.71 |

The significance differences were determined by the 95% confidence intervals.

### 4.13.4 Antioxidant Enzymes under Drought Stress

Accession $JI_1$ produced the highest value of CAT at the highest level of drought stress among its own treatments (Table 4.16). All other accessions have their highest values at either CNT treatments (NG061, NG062 and DI) or at 4 days level of drought stress (NG061 and DI). Enzyme APx were high at 4 days level of drought stress in accessions NG061, NG062 and $JI_1$ (Table 4.16).The control plants had the highest SOD in all the accessions.Catalase was significantly high during drought stress in $JI_1$ though, all the accessions showed positive regression except for NG061.It is important to note that APX and SOD showed negative regression for all the accessions during the drought stress, though accession DI was significantly higher than other accessions (Table 4:17).

### 4.13.5 Lipid Peroxidation under Drought Stress

Low lipid peroxidation was observed in CNT plants in contrast with plants subjected to drought. Accession $JI_1$ (4.31373E-05 M) at 12 days drought stress showed the highest lipid peroxidation followed by NG062 (3.63137E-05 M) at 4days drought stress. Lowest lipid peroxidation was found in accession DI (1.96078E-06 M) under CNT plants (Table 4.18). Drought stress has showed positive effect on the Lipid peroxidation of all the accessions (Table 4.19).

## 4.14 Principal Component Analysis (PCA) of Physiological Biochemical Markers of *D. exilis*

Eight PCs were identified with cumulative variance of 100%. PC1 was positively correlated with root and shoot weight, which accounted for 29.20% of the total variation. PC2 was positively correlated with catalase, which accounted for 25.90% of the total variation. Ascorbate peroxidase (APx) was positively correlated and whereas total chlorophyll was negatively correlated with PC3, which accounted for 17.30% of the total variation. Proline content was negatively correlated with PC4, which accounted for 14.70% of the total variation (Table 4.20). Accessions were plotted closer and furthest to the vector line in figure 4.10. The relationship between D. *exilis* physiological and biochemical traits are illustrated using principal component bi-plots in figure 4.11. High correlation of traits was indicated by smaller angles between dimension vectors. Accessions DI and $J_I$ at 4 and 12days were grouped to the same vector based on higher

values of root and shoot weight, proline and catalase. Control plants of DI and $J_1$ were grouped along with high value of Anthocyanin and SOD. Accessions NG061 and NG062 were grouped along with high value of APx and total chlorophyll (Figure 4.10 and 4.11).

### 4.15Scoring of Osmotic Tolerant Accessions

According to table 4.21 below, the osmotic tolerance of $JI_1$ was the highest with 85%, followed by NG061 with 65%, DI with 55%, NG062 had 48% and $JI_2$ had 47%. The osmotic tolerant of *D. exilis* ranged from 85%-47%.

### 4.16 Scoring of Drought Tolerant Accessions

Scoring of drought tolerant accessions using biochemical and molecular parameters is showed in Table 4.22. Drought tolerant level of *D. exilis* ranged from 75-45%. Accession $JI_1$ had the highest tolerant level of 75% while NG062 had the lowest drought tolerant level of 45%. Accession $JI_2$ has been screened out *in vitro* using osmotic stress.

### 4.17 Root and Mid Rib Anatomical Structure of *D. exilis* under Drought Stress

Vessels diameter ranged from 20.4 mμ (CNT) - 84.49mμ (12 days drought stress). At the root, 8 days drought stress produced significant high value of vessels diameter in all accessions except for accession NG062 (Fig 4.12). Highest vessel diameter of 84.49mμ was recorded in accession NG062 and lowest was found in the CNT of the same accession (Plate 4.9). Small and well compacted mesophyll at mid rib of drought stressed plant (Plate 4.8 b, d, f & h) while CNT plants had large mesophyll cells (Plate 4.8 a, c, e & g). Also, bulliform cells were observed under the epidermis which was more prominent and turgid in drought stressed plant than the CNT plants (Plate 4.8). Intercellular spaces were absent in all treatments except for accessions $JI_1$ at 8days and 4days drought stress and DI at 12 days drought stress. The size ranged from 113 μm - 175 μm (Table 4.23). Significant high value of vessels diameter was observed at the mid-rib of accessions $JI_1$, DI and NG061 at 12 days drought stress. Accession $JI_1$ had a significantly high vessels diameter at the mid-rib than other accessions (Fig 4.13).

**Table 4.16: Antioxidant Enzymes of *D. exilis* under Drought Stress**

| ACCESSIONS | DROUGHT LEVELS | APx (unit/ml) | CAT (unit/ml) | SOD (unit/ml) |
|---|---|---|---|---|
| | CNT | 4.43E-06c | 0.24cd | 1.63a |
| | 4 DAYS | 7.31E-06a | 0.36a | 1.21d |
| | 8 DAYS | 6.15E-06b | 0.27b | 1.61b |
| NG061 | 12 DAYS | 8.89E-07d | 0.25bc | 1.28c |
| | CNT | 5.22E-06b | 0.23c | 1.70a |
| | 4 DAYS | 6.30E-06a | 0.36ab | 1.04c |
| | 8 DAYS | 2.61E-06d | 0.34b | 1.66b |
| NG062 | 12 DAYS | 3.69E-06c | 0.37a | 1.01d |
| | CNT | 3.02E-07c | 0.26c | 1.71a |
| | 4 DAYS | 5.47E-06a | 0.32b | 1.54c |
| | 8 DAYS | 1.69E-06b | 0.36b | 1.65b |
| $JI_1$ | 12 DAYS | 2.22E-07d | 0.42a | 1.05d |
| | CNT | 4.56E-06a | 0.31bc | 1.65a |
| | 4 DAYS | 3.68E-06b | 0.40a | 1.33d |
| | 8 DAYS | 1.64E-06c | 0.37ba | 1.63b |
| DI | 12 DAYS | 1.57E-06d | 0.33b | 1.38c |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). APx- Ascorbate peroxidase, CAT- Catalase, SOD- Superoxide dismutase

**Table 4.17: Antioxidants Regression of *D exilis* under Drought Stress**

| ACCESSIONS | APx | | CAT | | SOD | |
|---|---|---|---|---|---|---|
| | R-value | P-value | R-value | P-value | R-value | P-value |
| NG061 | -0.54 | 0.46 | -0.14 | 0.86 | -0.39 | 0.62 |
| NG062 | -0.66 | 0.34 | 0.80 | 0.20 | -0.49 | 0.51 |
| $JI_1$ | -0.21 | 0.79 | 0.99 | 0.003* | -0.80 | 0.20 |
| DI | -0.95 | 0.05* | 0.09 | 0.90 | -0.39 | 0.60 |

The significance differences were determined by the 95% confidence intervals.

**Table 4.18: Lipid peroxidation of *D. exilis* under Drought Stress**

| ACCESSIONS | DROUGHT LEVELS | LIPID PEROXIDATION **(M)** |
|---|---|---|
| NG061 | CNT | 5.82E-06d |
| | 4 Days | 6.86E-06c |
| | 8 Days | 8.56E-06b |
| | 12 Days | 1.51E-05a |
| NG062 | CNT | 6.95E-06d |
| | 4 Days | 3.63E-05a |
| | 8 Days | 1.65E-05c |
| | 12 Days | 1.98E-05b |
| $JI_1$ | CNT | 9.83E-06d |
| | 4 Days | 2.14E-05c |
| | 8 Days | 2.71E-05b |
| | 12 Days | 4.31E-05a |
| DI | CNT | 1.96E-06d |
| | 4 Days | 7.66E-06c |
| | 8 Days | 8.16E-06b |
| | 12 Days | 1.10E-05a |

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$).

**Table 4.19: Regression Analysis of Lipid peroxidation of *D. exilis* under drought stress**

| ACCESSIONS | R-value | P-Value |
|---|---|---|
| NG061 | 0.91 | 0.08 |
| NG062 | 0.20 | 0.80 |
| $JI_1$ | 0.98 | 0.02* |
| DI | 0.94 | 0.06 |

The significance differences were determined by the 95% confidence intervals.

**Table 4.20: Principal component analysis explained physiological and biochemical variances in *D. exilis* under drought stress**

| Variables | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 |
|---|---|---|---|---|---|---|---|---|
| T-Chl | -0.05 | -0.42 | **-0.60** | -0.21 | 0.26 | 0.14 | **-0.58** | 0.01 |
| Anthocyanin | 0.28 | -0.34 | 0.07 | -0.40 | **-0.80** | -0.01 | -0.07 | -0.05 |
| Proline | 0.16 | 0.24 | -0.20 | **-0.76** | 0.29 | -0.31 | 0.35 | -0.03 |
| APx | -0.20 | 0.20 | **0.64** | -0.41 | 0.15 | 0.20 | **-0.53** | -0.02 |
| CAT | 0.13 | **0.59** | -0.24 | 0.14 | -0.26 | -0.49 | -0.49 | 0.10 |
| SOD | 0.23 | -0.49 | 0.36 | 0.13 | 0.28 | **-0.69** | -0.12 | 0.01 |
| Root weight | **0.62** | 0.13 | 0.023 | 0.11 | 0.15 | 0.22 | -0.10 | -0.71 |
| Shoot weight | **0.63** | 0.05 | 0.07 | 0.03 | 0.15 | 0.29 | -0.02 | 0.70 |
| Explained variance Eigenvalue | 2.34 | 2.07 | 1.38 | 1.18 | 0.60 | 0.28 | 0.11 | 0.04 |
| Proportion of the total variance (%) | 29.20 | 25.90 | 17.30 | 14.70 | 7.50 | 3.50 | 1.40 | 5.00 |
| Cumulative variance (%) | 29.20 | 55.10 | 72.40 | 87.10 | 94.60 | 98.10 | 99.50 | 100 |

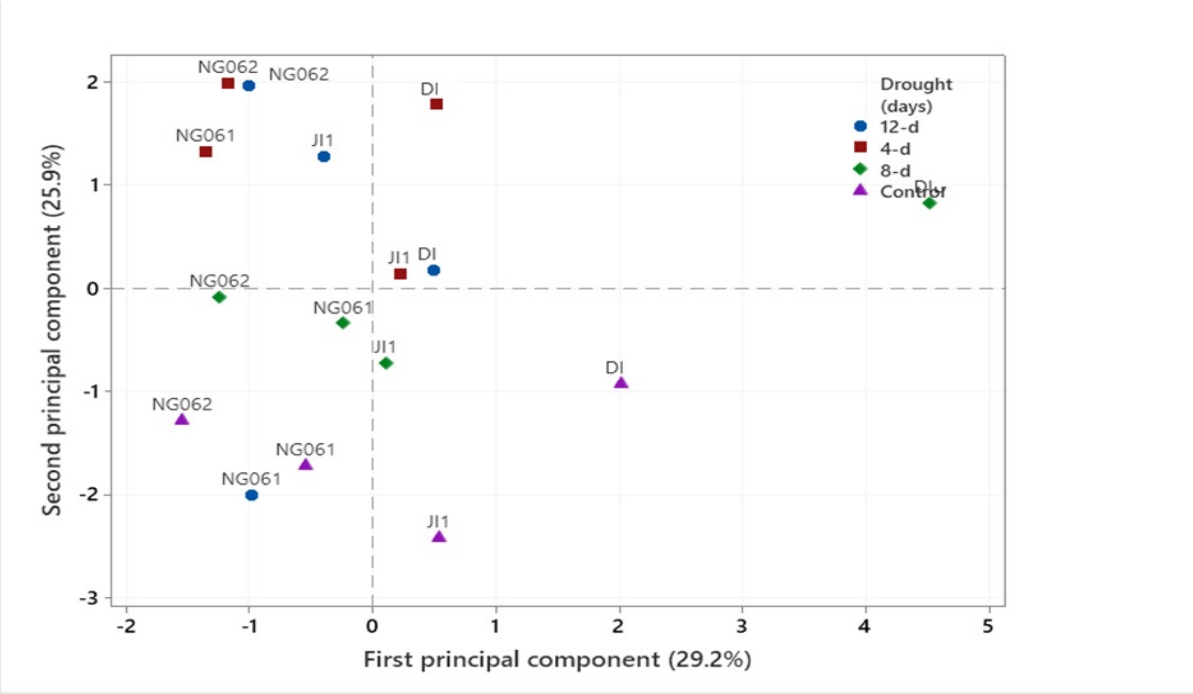



Figure 4.10: Principal Component Analysis (PCA) of *D. exilis* Accessions under Drought Stress

Where, 12-d = 12 Days, 8-d= 8 Days, 4-d= 4 Days Drought Level, Control = CNT

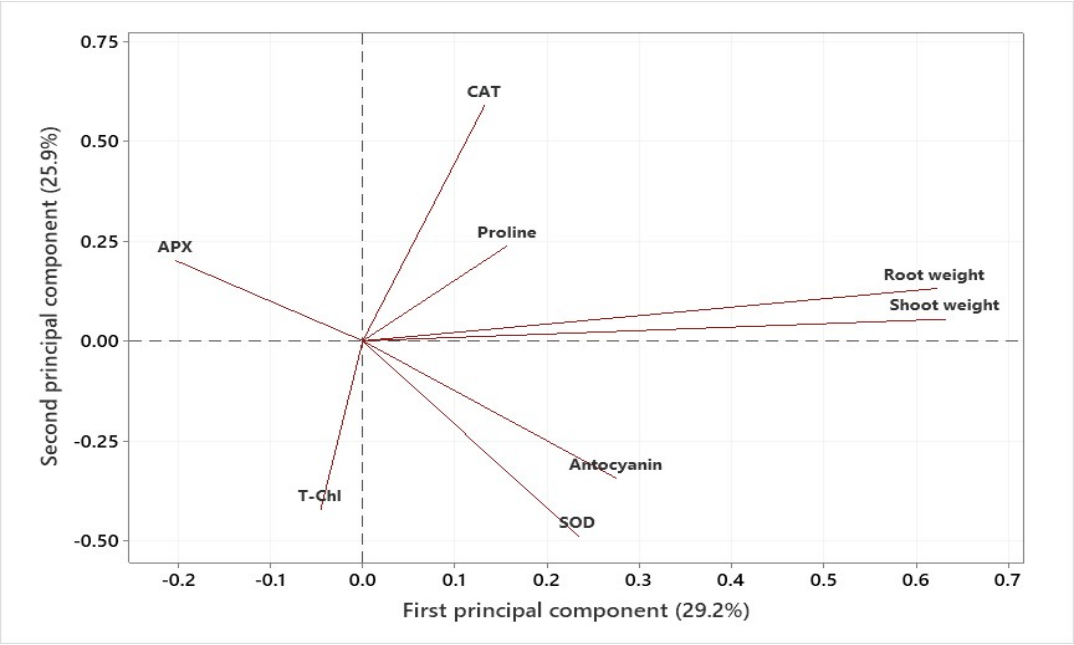



Figure 4.11: Principal Component Biplot (PCA) showing *D. exilis*Physiology and Biochemical Markers of *D. exilis* under Drought Stress

**Table 4.21: Percentage Osmotic Tolerant Scoring**

| ACCESSION | Chl a | Chl b | T. Chl | Prol | APx | SOD | CAT | MDA | $OH^-$ | $H_2O_2$ | NO | DPPH | % Osmotic TOL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NG061 | 3 | 3 | 3 | 5 | 4 | 3 | 2 | 4 | 3 | 3 | 2 | 4 | 65 |
| NG062 | 1 | 1 | 1 | 4 | 1 | 1 | 4 | 3 | 2 | 5 | 5 | 1 | 48 |
| $JI_1$ | 5 | 5 | 5 | 3 | 5 | 4 | 5 | 5 | 5 | 1 | 3 | 5 | 85 |
| DI | 4 | 4 | 4 | 2 | 3 | 2 | 3 | 2 | 4 | 2 | 1 | 2 | 55 |
| $JI_2$ | 2 | 2 | 2 | 1 | 2 | 5 | 1 | 1 | 1 | 4 | 4 | 3 | 47 |

Where, $Chl_a$- Chlorophyll a, $Chl_b$- Chlorophyll b, T. Chl- Total Chlorophyll a, Prol- Proline, APx- Ascorbate peroxidase, CAT- Catalase, SOD- Superoxide Dismutase, MDA- Malondialdehyde, NO-Nitric oxide, $OH^-$ - Hydroxyl, $H_2O_2$- Hydrogen peroxide, DPPH- 2,2-Diphenyl 1- picrylhdrazyl hydrate, % Osmotic Tol= % Osmotic tolerant

**Table 4.22: Percentage Drought Tolerant Scoring**

| ACCESSION | PROL | Chl a | Chl b | T. Chl | ANTHO | SOD | APx | CAT | MDA | DNA | DNA Purity | % DRGT TOL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NG061 | 1 | 3 | 2 | 3 | 4 | 2 | 3 | 1 | 3 | 2 | 3 | 61.00 |
| NG062 | 2 | 1 | 4 | 1 | 1 | 1 | 4 | 2 | 2 | 1 | 1 | 45.00 |
| $JI_1$ | 4 | 4 | 1 | 4 | 3 | 3 | 2 | 4 | 1 | 3 | 4 | 75.00 |
| DI | 3 | 2 | 3 | 2 | 2 | 4 | 1 | 4 | 4 | 4 | 2 | 70.45 |

Where, Prol- Proline, $Chl_a$- Chlorophyll a, $Chl_b$- Chlorophyll b, T. Chl- Total Antho- Anthocyanin, SOD- Superoxide Dismutase, APx- Ascorbate peroxidase, CAT- Catalase, MDA- Malondialdehyde, % Drgt Tol= % Drought tolerant

**Table 4.23: Presence of Intercellular Spaces in Drought Stress Plants**

| Accessions | Drought Stres | Size of Intercellular Spaces (μm) |
|---|---|---|
| $JI_1$ | 4DAYS | 113 |
| $JI_1$ | 8DAYS | 175 |
| DI | 12DAYS | 132 |

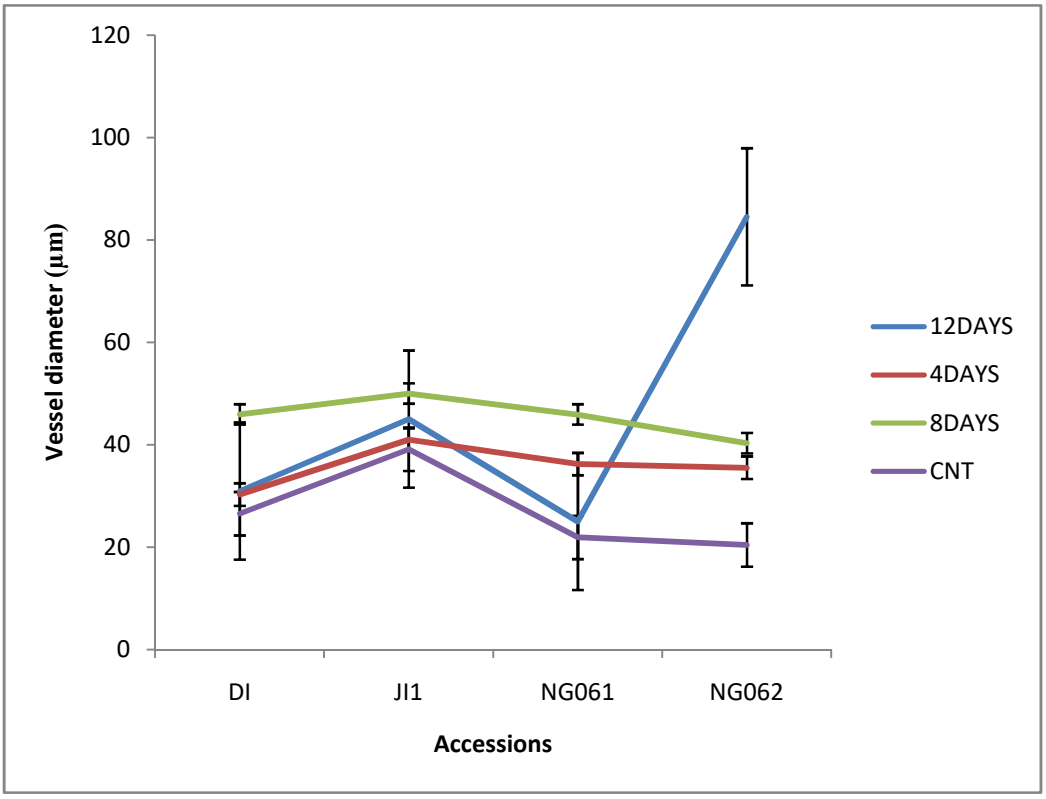



**Figure 4.12: Vessel diameters at the Root of *D. exilis* under Drought Stress**

Where, Bars means Error bars with Standard Error, Drought Stress Levels = 12, 4, 8 Days & CNT

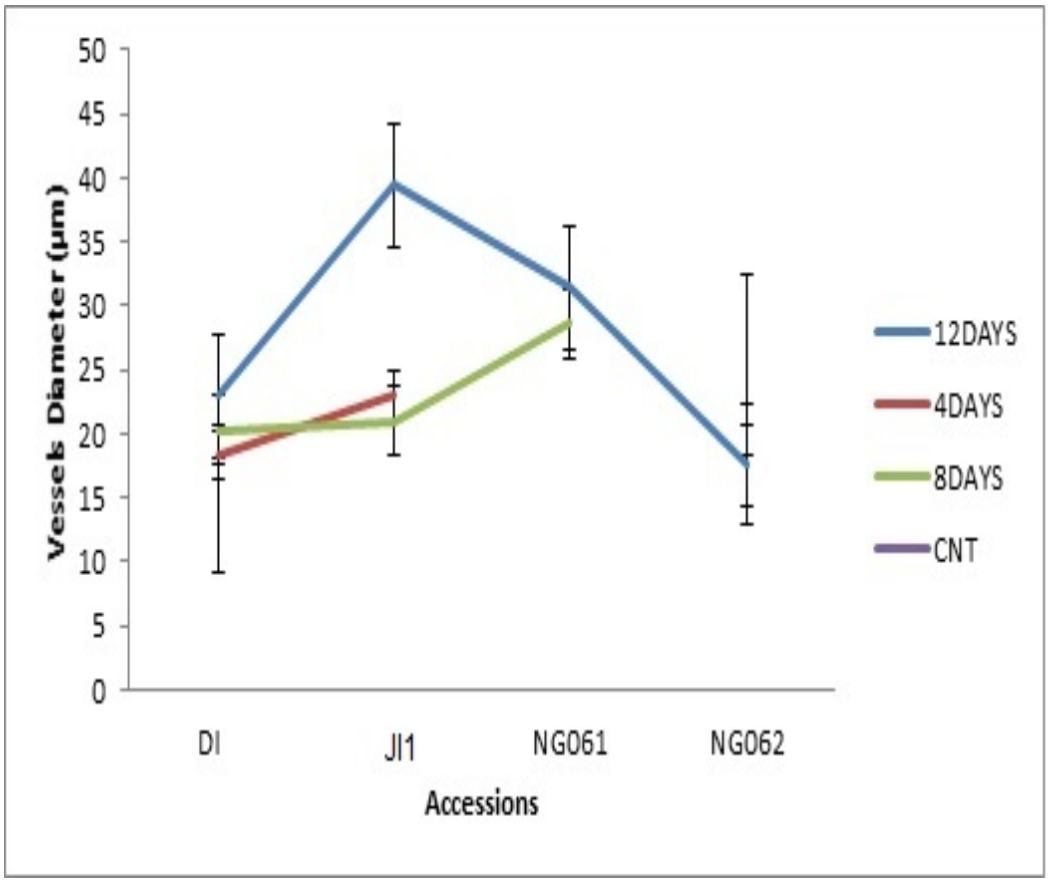



**Figure 4.13: Vessel diameters at the mid-rib of *D. exilis* under Drought Stress**

Where, Bars means Error bars with Standard Error, Drought Stress Levels = 12, 4, 8 Days & CNT

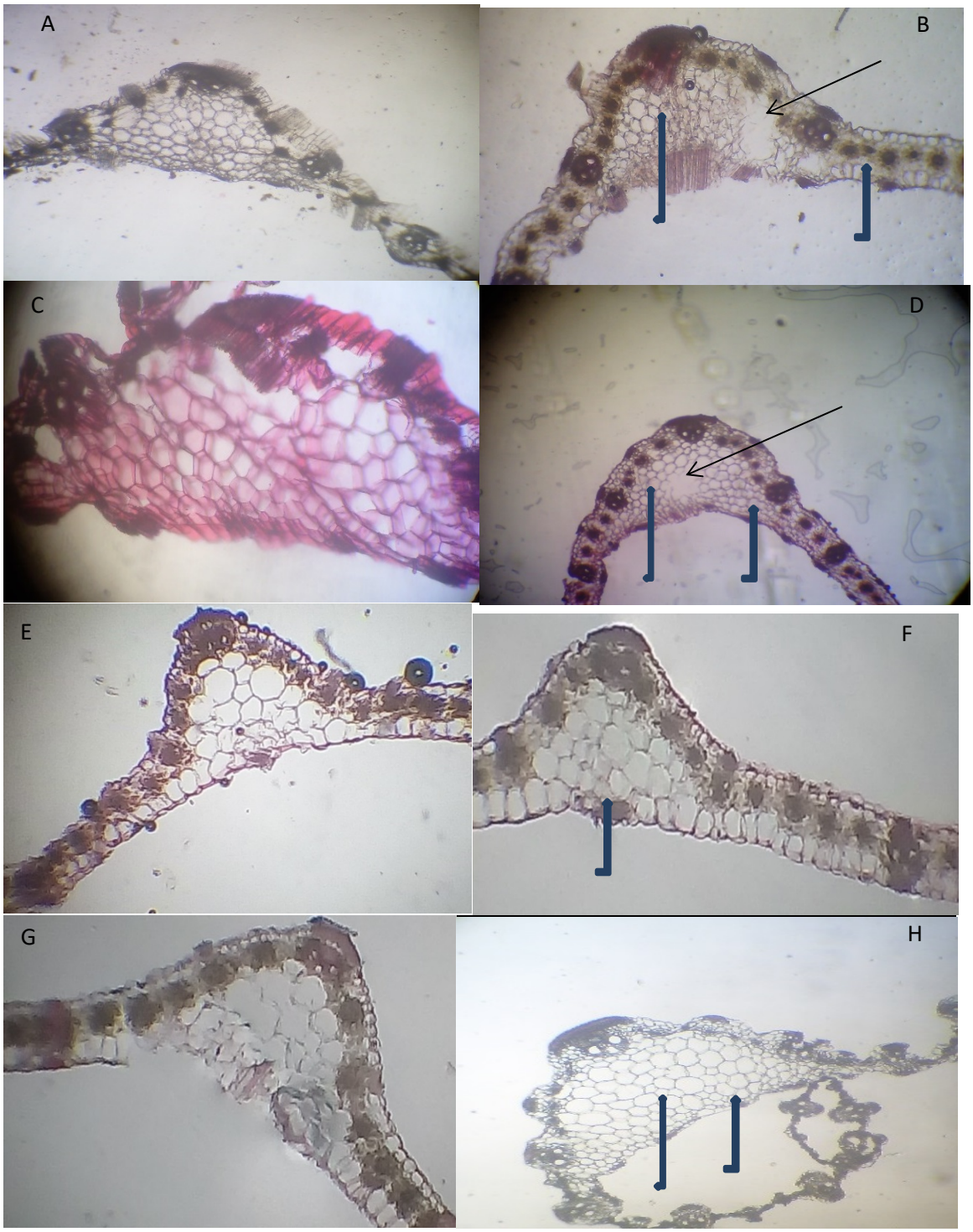



**Plate 4.8: Anatomical structure of the Leaf Mid Rib of *D. exilis* under Drought Stress**
Accessions DI= A & B, $JI_1$ = C & D, NG061 = E & F, NG062 = G &H. Unstressed Plants = A, C, E & G; Stressed Plants: B, D, F & H. Left up arrow = Small compacted mesophyll, Bent up arrow = bulliform cell, Thin arrow = Intercellular spaces.

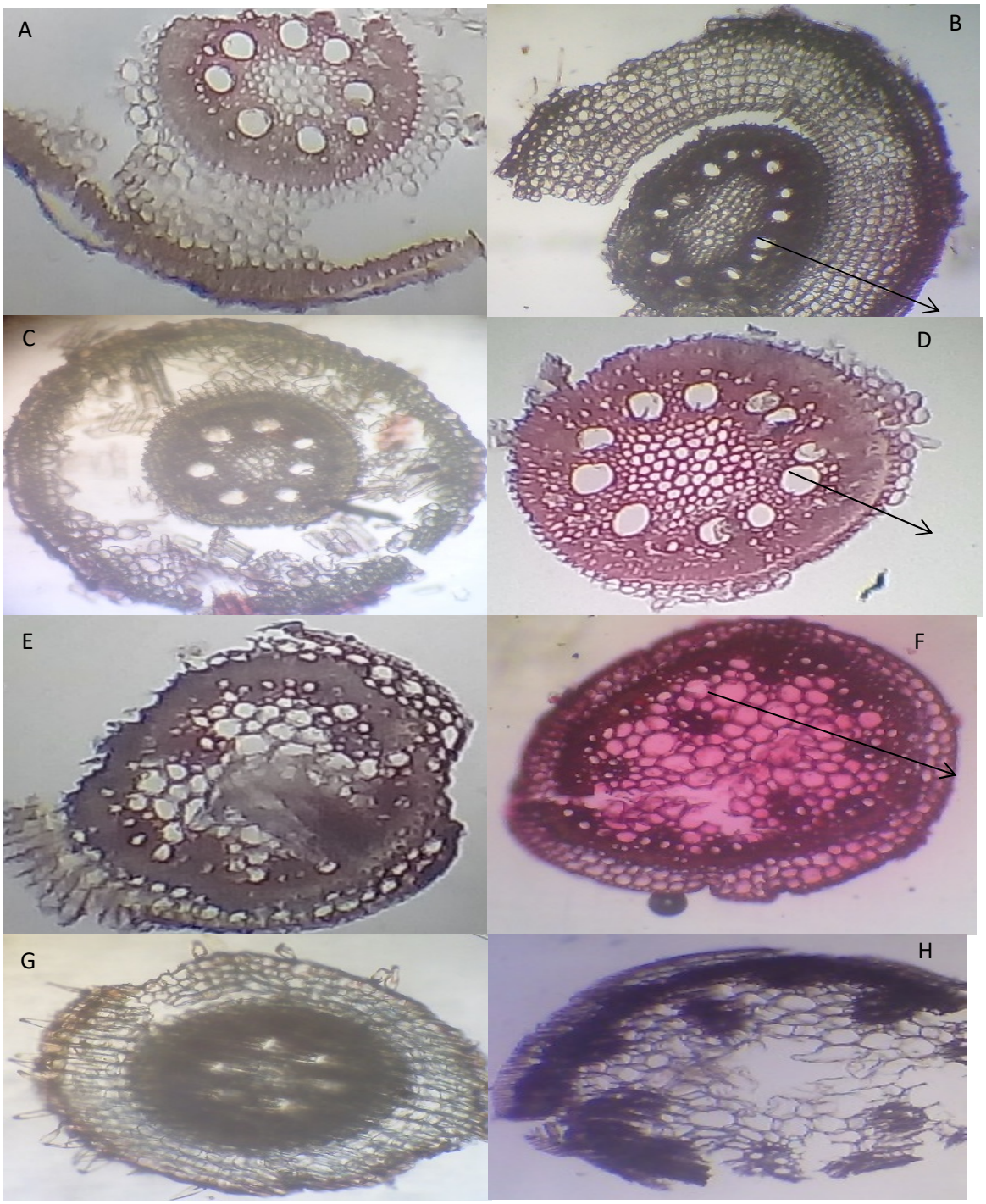



**Plate 4.9: Root anatomical structure of *D. exilis* under Drought Stress**.

Accessions DI= A & B, $JI_1$ = C & D, NG061 = E & F, NG062 = G &H. Unstressed Plants = A, C, E & G; Stressed Plants: B, D, F & H. Thin arrow = Vascular bundles

# CHAPTER FIVE

## 5.0 DISCUSSION

Sinhababu and Kar (2003) define poly-ethylene glycol PEG as a non-ionic, water-binding polymer with impervious prolong chain, perfect for simulating drought. Sinhababu and Kar, (2003) regarded application of PEG-6000 in an *in vitro* as an alternative method to monitor and contrast responses of plants against water deficit. Although, plants under drought suffer osmotic shock, their metabolism reactions to such shocks are suggestive of their relative traits of drought tolerance at different metabolic state (Reddy *et al.,* 2004).

During drought stress, number of leaves and tillers, shoots weight, plant height and leaf lengths observed in the screenhouse and *in vitro* were higher than control plants. The inflorescences growths were rapid in drought stressed plants than their control counterparts. Vegetative and flowering growth of accession NG061 was enhanced during drought stress. On the contrary, accessions DI and NG062 produced no tillers at control. The growths of *D. exilis* are sensitive to drought stress. The capability of seedling to survive and withstand several abiotic factors relies on the strength and its genetic makeup (Moles and Westoby, 2004). Plants with ability to pull out sufficient water from the whole soil profile structures can show a better resistance to drought. Observed root weight of accession DI was the highest while $JI_1$ possessed relative constant high values of root weight during drought stress. The survival ability of accessions DI and $JI_1$ at 4 and 12 days was also linked to the deep, large and prolific roots developed by the two accessions during drought stress. The presence of prolific deep root structure enhanced water absorption in DI and $JI_1$ compared to shallow-rooted NG062, NG061 and $JI_2$ except for NG061 at 12days drought stress that developed long taproot with very few lateral roots. The root architecture of accessions DI and $JI_1$ were changed in conformity with drought conditions (Yamauchi *et al.,* 1996). The deep taproot of $JI_1$ at 4 and 12 days drought stress were accompanied with many lateral roots which were structured to reach deep down the soil where zone of saturation exist permanently, thus preventing seasonal fluctuations of water availability. Moreover, drought stress instigated wide morpho-anatomical traits which are regulated

depending on the intensity of the drought stress and varied per different accessions (Peña-Valdivia *et al.,* 2010). Wang and Yamauchi (2006) reported that plants with greater rooting depth; proliferation and high density are considered as drought avoidance traits. Thus, genotypes with more root growth are considered in a drought environments. This provides better transport of water and nutrient required to explore deeper soil horizon in search of larger water volumes (Padilla *et al*.,2007). The functionality and the structure of the roots system can improve the effectiveness of plant hydraulic to release water to the leaves (Vilagrosa, *et al.,* 2012). In addition, drought tolerant index and mean productivity of accessions NG061 and $JI_1$ was high during the osmotic stress. Accession $JI_2$ remains low in all growth and biomass parameters determined *in vitro* and in screen house. Tolerant accessions always demonstrated high biomass than the susceptible accessions under prevailing drought stress (Khan *et al.,* 2019). Overtime, plant developed changes in morpho-functional traits that led to alteration root hydraulic conductance which is strategy for controlling transpiration (Trubat *et al.,* 2006).

Photosynthetic pigments played an important role in fixation of carbon; they were involved in capturing solar energy (Farooq *et al.,* 2012). Chlorophyll b was significantly reduced in drought stressed plant than the control plants, which contributed to the low total chlorophyll recorded in drought stressed accessions. Chlorophyll b content in control plants were therefore sustained by activities of APx and CAT. Drought stress severely affects Chlorophyll b contents in *Tagetes erecta* (Asrar and Elhindi, 2011). Similarly, water stress diminished the photosynthetic rate with declining photochemical efficiency (Fv/Fm) of Chlorophyll fluorescence in primary leaves of kidney beans (Miyashita *et al.,* 2005). On the contrary, chlorophyll a was exceptionally high in drought-stressed plants owing to the high production of total anthocyanin, SOD and proline. This suggests that tolerant accessions could demonstrate higher chlorophyll content than the susceptible accessions hence; the susceptible accessions showed a damaged photosystem due to drought than the tolerant accessions (Khan *et al.,* 2019**)**. Also, it was observed that chlorophyll a and b showed an inverse relation to each other when subjected to drought. Furthermore, there was an enhanced up regulation of anthocyanin contents in NG061 and $JI_1$ during drought stress. The induced production of total anthocyanin and proline could be responsible for protection of the chlorophyll content in NG061 and $JI_1$ accessions. The high and consistent value of total anthocyanin and SOD observed in these two accessions must have shielded chlorophyll A from further degradation during drought stress.This indicated that anthocyanin protects chlorophyll a from excessive light intensity as a result of their

capability to absorb light fall between 400nm and 600nm wavelength. High production of anthocyanin helps to stabilize water potential, this is a specific mechanism adopted by drought tolerant plants (Tahkokorpi, 2010). There was a direct association between accumulation of anthocyanins and drought tolerant. Induction of anthocyanin during water stress tendered multitude roles which include radical scavengers, photo-defendants, and signalling factors (Kovinich *et al.,* 2015).

The control plants of accession DI had an improved chlorophyll contents due to high chlorophyll b. This was in line with record of Manivannan *et al.* (2007) which presented higher total chlorophyll in *Helianthus annum* without drought stress. Chlorophyll b is a main component of the chlorophyll, considering its positive interrelationship with the photosynthetic rate. Low chlorophyll contents were observed in $JI_2$ and NG062 accessions under osmotic stress.Chlorophyll damaged during drought stress account for the inactive photosynthesis. In the present study, decline in chlorophyll contents has been considered as a distinctive indication of oxidative damage thus, may cause photo-oxidation and disintegration of chlorophyll.

Furthermore, Zhang and Kirkham (1996) reported a reduction in chlorophyll contents due to loss of chloroplast membranes under water deficit which resulted to an extreme enlargement, deformation of the lamellae vesiculation alongside with high MDA contents. Decline in chlorophyll contents can directly be responsible for the limitation on photosynthetic apparatus and therefore causing a reduction in the primary productivity. Mesophyll cells revealed low chlorophyll contents with a decrease in quantity lost from the bundle sheath cells during drought stress. Observation on *in vitro* plants showed variation in chlorophyll contents of all the accessions under different level of osmotic stress. Zhang and Kirkham (1996) reported similar stability in chlorophyll under prevailing water deficit.

Osmotic adjustment is the key adaptation of plants at the cellular level, it minimize the effects of drought-induced damage in crop plants (Blum 2005) and helps plants under drought in two ways, namely: (1) it helps maintain leaf turgor which improve stomatal conductance for efficient intake of $CO_2$ (Kiani *et al.,* 2007); and (2) it promotes the root's ability to uptake more water (Chimenti *et al.,* 2006). Accumulation of proline was high in all the accessions during drought and osmotic stress than their control plants. Accessions $JI_1$ and NG061 revealed higher accumulation of proline during the

drought stress. Maintaining higher water potentials was one of the methods adopted by drought tolerant plants due to proline accumulation. Hence, Kumar *et al.* (2003) attributed this osmoregulation to a permits given to water movement from the immediate environ was assisted by proline accumulation. Consequently, water potential of accessions $JI_1$ and NG061 were increased, reducing the instant death of plant during drought stress. Accumulation of solutes stimulated water in and assisted their cell to maintain turgidity as a result of reduced osmotic potential of the cell in accessions $JI_1$ and NG061. The accession $JI_1$ and NG061exhibited dehydration-avoidance mechanism by increasing tissue water potential through accumulation of proline, assisted by several adaptive anatomical features involved in the reduction of water loss and optimisation of water transport. *Digitaria exilis* involved in water minimization as a water-saving plants and accession $JI_1$ are water expenders because they depend on osmotic adjustment to avert desiccation. The drought tolerance approaches developed in accession $JI_1$ enable it to withstand low water potential posed by drought through adaptive characters with an association with proline accumulation and formation of compact mesophyll cells. Morgan (1990) revealed that plant cytoplasm stored in solutes assisted plants in osmotic regulation and thus, preserve the balancing of cell water thereby lessen the injurious outcomes of water stress. Despite the reduced leaves water potential experienced during drought stress, rate of photosynthesis (chlorophyll content ) were preserved owing to their turgidity conservation and hence, huge growth were recorded in drought-stressed accessions ($JI_1$ and NG061) than their unstressed counterparts. Taiz and Zeiger (2006) reported that an osmotic regulation showed significant characteristics in detaining dehydration impairment in water deficit environ through progressive sustenance of cell turgidity and metabolisms.Accumulation of free proline assisted plant to survive in prevailing water deficit, and thereby allowing movement of water from the environment through the process of osmoregulation (Jalil *et al.*, 2007).

Variation in activities of SOD, CAT and APx were related to different levels of drought/osmotic stress. Accession $JI_1$ showed high activity of SOD in both osmotic and drought stress due to the accumulation of proline. Activities of enzyme SOD remain constant which might either cause retardation in the generation of radicals or an elevation in the estimation of radicals (Basu *et al.,* 2010). Drought and osmotic stressed plants had high CAT than their control plants. Although NG062 had the highest CAT under osmotic stress nevertheless, accession $JI_1$ had good and consistent high value of CAT under a harsh drought or osmotic stress. Furthermore, APx activities were significantly noted under water stressed accession NG061. This has being found to display a

main innate function of inhibiting radicals and protecting cells in plants. Ascorbate peroxidase (APx) has shown high affinity for hydrogen peroxide than catalase and this may play a searchable function in controlling reactive oxygen species. An increase in APx activities was expressed during introduction of drought stress conditions. On a contrary, accessions $JI_2$ and NG062 revealed a reduction in APx activity.

Accession $JI_1$ recorded high activities of CAT under drought stress.Accession $JI_1$ turn on its defensive mechanisms by up regulating scavenging enzymes CAT; this played an important role in oxidative stress tolerance. Garg and Manchanda, (2009) reported that catalase enzymes during stress essentially detoxify ROS implicitly by reducing $H_2O_2$ to $H_2O$ and $O_2$. The role of catalase in the disintegration of $H_2O_2$ by oxidases in peroxisomes cannot be over emphasized. Therefore, CAT ability to degrade $H_2O_2$ to non-reactive oxygen in accessions DI and $JI_1$ could have been responsible for their drought tolerant level. While CAT activity was reduced in osmotic susceptible $JI_2$ and NG062

Accession $JI_1$ showed high SOD activities under drought and osmotic stresses, which played a crucial function in destroying radicals, it also work as a stimulants to disintegrate $O^{2-}$ into $H_2O_2$ which were elucidated by catalase and other antioxidant enzymes. High SOD was preferred to be an essential enzyme for plants to be called stress tolerant. Removal of $O^{2-}$ lessen the hazardous effect of hydroxyl radical generated in $JI_1$ through metal catalyst reaction. This reaction, speed up conversion rate to ten thousand times than unpremeditated dismutation (Edwards *et al.,* 1990). Apparently accession $JI_1$ developed steadiness as assisted by compatible solute accumulation. This further helped in stability of antioxidant enzymes, lipid biosynthesis and modification during the stress response. Compatible solutes have been proven to improve the membrane stability during stress (Kumar *et al.*, 2003). The structures of plant membranes are modified by drought stress to lessen the degree of unsaturated lipids in the *D. exilis*.

*D. exilis* cultivated during drought stress had high MDA as against low MDA observed in an osmotic stressed plant which was assisted by SOD. This was agreed by Sofo *et al*. (2004) who observed a notable high lipid peroxidation in water-stressed *Olea europaea* trees and *Coffea canephora*. Lipid peroxidation is recognized as injurious operation in *D. exilis* and it was observed that CAT enzyme and total anthocyanin aided the average reduction of MDA content observed in

accession $JI_1$ and DI at 4 and 8 days drought stress respectively. Degree of lipid peroxidation during stress is measured by membrane damage which at times features as a single unit.

Water stressed *Phaleolus vulgaris* showed the highest MDA content with a high $H_2O_2$ and $OH^-$ concentration consequently, causing membrane damage (Simova-Stoilova *et al*., 2010). Susceptible varieties of wheat plants had shown pronounced impaired membrane and high MDA contents when subjected to drought (Simova-Stoilova *et al*., 2010). Similarly, elevated lipid peroxidation in water stressed *Glycyrrhiza uralensis* Fisch was recorded (Pan *et al*., 2006). Especially, $OH^-$ was the main highly reactive ROS known. This hydroxyl $OH^-$ is greatly accountable for interceding $O_2$ toxicity *in vivo (*Scarpeci *et al.,* 2008). Hydroxyl possibly react with organelles and factually all the cell composition because of reduced activities of antioxidant enzymes that could responsible for the exclusion of these radical. Surplus generation of hydroxyl ($OH^-$) eventually resulted in cell death. However, low lipid peroxidation was observed in accession DI and $JI_1$ during drought stress and osmotic stress respectively than control plants.

Ability to scavenge $H_2O_2$ and NO in accessions NG061, NG062, DI and $JI_2$ decreased drastically in osmotic stressed plants compared to the control plants. The CNT plants of NG061 and DI inhibited 58% NO and 62% $H_2O_2$ respectively. Superoxide dismutase aided in the scavenging of NO in osmotic stressed plant while APx and proline were responsible for the inhibition of $H_2O_2$.Continued photosynthetic light reactions during drought stress under limited intercellular $CO_2$ concentration results in the accumulation of reduced photosynthetic electron transport components. This potentially reduced molecular oxygen, resulting in the production of ROS and further inflicting severe damage to the photosynthetic apparatus (Lawlor and Cornic, 2002). However, accession $JI_1$ had capacity to inhibit 80% $OH^-$ than the control plants. This could be linked to synergistic force between activities of APX and proline. More so, increased activities of SOD and CAT in $JI_1$ might also be responsible for the scavenging activities of $OH^-$ radicals. Stabilization of APx, CAT and SOD by an osmolyte proline helped in the removal of superoxide ions which later oxidized to $OH^-$ and to $H_2O_2$, finally to water and oxygen. Oxidation damage was greatly reduced in drought stressed plants; this was linked to upregulated enzyme SOD. Ascorbate peroxidases also reinforced the effectiveness of detoxification of hydroxyl radicalin drought stressed plants. Elevation of transcript expression of APx genes in *Vigna unguiculata* served as a proof of proportionality between APx expression and plant tolerant to drought (Chaves *et al.,* 2003).

Increase in proline accumulation found in accession $JI_1$ under drought permitted high functioning of antioxidant enzymes. This confirmed that accumulated proline in accession $JI_1$ assisted in adjustment of osmotic condition. Consequently, water absorption balance was maintained during severe stress conditions (Chaves *et al.,* 2003). Many plant species also showed conjunction between accumulations of proline and drought tolerance. Drought tolerant landraces wheat, mulberry and olive had a significant high proline accumulation than sensitive cultivars (Reddy *et al.,* 2004). Similarly, Elsheery and Cao, (2008) revealed that two *Magnifera* landraces showed greater accumulations of proline. However, low proline content in drought-stressed accessions $JI_2$ and NG062 could have been responsible for their susceptibility to both high osmotic potential and water deficit.

Accession $JI_1$ scavenged approximately 78% of DPPH radical than the control plants while, other accessions showed significant decreased in ability to scavenged DPPH. Radical DPPH possessed the capacity to give hydrogen atom to free anionic $O^{2-}$ in other to end ROS reactions. This DPPH engaged in ROS scavengers through chain disconnection processes, which otherwise act as a determinant factor of non-enzymatic antioxidant (Benard and Runner, 2004). Elevation recorded in DPPH-scavengers activity was accorded to the tolerance ability of seedling under stress (Kang and Saltveit, 2002). Similarly, there was an interrelationship between the stress shock inducing stress tolerance and increased DPPH radical scavenging activities in rice (Kang and Saltveit, 2002).

Despite the drought stress, accessions DI and $JI_1$ had the highest concentration of DNA i.e. their DNA remains intact and were not destroyed during the drought stress. The DNA of *D. exilis* subjected to drought stress remains unimpaired and higher than the control plants in $JI_1$. During water stress, Plant DNA get injured when exposed to severe abiotic stress though, plant genes were relatively stable (Tuteja *et al.,* 2009). Although reactive oxygen species generally destroy DNA, count of processes occurred in n the nucleus and mitochondria to recover injured DNA (Anjum *et al.,* 2011). Greater amount of DNA in accessions $JI_1$ and DI was disclosed with the presence of bands at 1000kb on gel electrophoresis. The existence of bands in accessions $JI_1$ and NG062 were also correlated to the level of their purities. Although the concentration of DNA under drought stressed plant was higher than the control nevertheless, control plants present more bands (quality) at 1000kb than the drought stressed plant. Enzymes CAT and proline could be factors responsible for stability of DNA, which directly mop up free radicals produced during drought stress in accessions

$JI_1$ and DI. Accession NG061 presented low DNA concentration compared to the control plants which suggested that formation of radicals was sufficient to impel injury to the cellular organelles like DNA. Britt (1999) linked affected plant growth and development to damaged DNA consequently, decreased protein synthesis, destroyed cell membrane and photosynthetic proteins. Elevated levels of ROS have ability to destroy cellular composition (Valko *et al.,* 2006). Most effective $OH^-$ radicals damaged cell contents of DNA, destroying purines and pyrimidine bases (Halliwell, and Gutteridge, 1999). Also, high MDA content could attack the nucleic acid and change the symmetry of cellular proteins which were responsible for the oxidative damage in plant tissues (Farheen and Mansoor, 2020).

Anatomically, vessel size of *D. exilis* varies with drought levels. Wider vessels diameters were observed in root of drought stressed plants. Also, accession NG062 had the widest root vessels diameter at 12 days drought stress. In contrary, Plavcová and Hacke (2012) and Worbes (1999) reported decreased vessel diameter during drought and larger in well-watered plants. Sevanto *et al.,* (2005) further linked modification in vessel diameter to soil water content on the top sub-stratum. Accession $JI_1$ had the widest vessels diameter at the mid-rid of the leaves. Water deficit significantly brought about decrease in Predawn xylem diameter and recovery of the xylem size when it started to rain (Offenhalter *et al.,* 2001). The wider vessels at the mid rib, intercellular spaces and small mesophyll cells suggested high vapour pressure (high humidity) inside the leaves. This further suggested effective flow of water within the leaves (Hilarie and Graves, 1998). This facilitated the process of photosynthesis despite the infliction of drought stress. Although mesophylls of the control plants were significantly wide with no intercellular spaces, mesophylls cells of $JI_1$ and DI at 12 and 4days drought stress respectively were small and well compacted. This increased leaf stiffness, although limited gaseous exchange could minimize water loss and strengthen high leaf tissue density (Niinemets, 2001). At the same time, $JI_1$ and D1 at 8 and 12days respectively also showed another dehydration–avoidance strategy due to presence of intercellular spaces that ranged from 113µm-175µm in size. It was also observed that this accessions at this same level of drought stress (8 and 12days) with presence of intercellular spaces presented highest concentration of DNA. Consequently, damage caused to lipid was reduced and concentration of chlorophyll A was enhanced in accession DI at 12 days drought stress. The thick cell-walled mesophylls developed intercellular spaces in $JI_1$ and D1 which facilitated uninterrupted active photosynthesis during the

drought. High mesophyll thickness enhances the photosynthetic capacity if it is accompanied by high chloroplasts and intercellular spaces in areas exposed near the surface (Oguchi *et al.,* 2005).

In addition, turgid bulliforms cells were observed at the mid rib of drought stressed accessions NG061, $JI_1$ and DI at 12 days drought stressed while bulliform cells of other accessions showed loss of turgidity. Presence of turgid bulliform cells in drought stressed plants focused on the strategy to withstand water shortage and avoidance of drying out of tissues. This suggested an adaptive direct link between the leaf gaseous exchange ability and hydraulic effectiveness at the shoot and root parts, in addition with the whole plant (Brodribb, 2010). Accessions $JI_1$ and DI shared different root adaptive structures and anatomical adaptation which allowed their survival during drought. The accumulation of proline and anthocyanin could have been responsible for ease movement of water and hydration of mesophylls in accessions $JI_1$ and DI hence, DNA were further protected from oxidative effect of free radicals generated during the drought stress.

# CHAPTER SIX

## 6.0 SUMMARY AND CONCLUSION

The study revealed that accession $JI_1$ had the highest chlorophyll a, CAT and SOD activities and proline under osmotic stress with relatively high values of DNA in drought stress. Also, chlorophyll a, total anthocyanin, SOD, CAT and proline levels of accession $JI_1$ were shown to be responsible for scavenging radicals and defending the plant against oxidative stress. Furthermore, the showed that $JI_1$ study inhibited 80% $OH^-$(reactive oxidizing agent) at E level of osmotic stress and 78% DPPH radicals than the control. Anatomical results further showed wider vessels diameter at the mid-rid of accession $JI_1$, which facilitated movement of water and dissolved salts and contributed to the resistant ability of the accession $JI_1$. Thus, accession $JI_1$ could be viewed as drought tolerant accession.

Accession DI invested more in root and shoot weight with lots of lateral roots for fast absorption and penetration down the soil. This explains the low lipid peroxidation and high DNA concentration that were observed under drought stress. The presence of intercellular spaces and wide vessels at the mid rib leaves and high concentration of DNA in drought stressed accessions $JI_1$ and DI suggested that these accessions were trying to avoid the drought conditions. A well-developed tugid bulliform cells found in drought stressed plants which ultimately checked for water loss through the leaves surface also complemented the strategy. It could therefore be inferred that accessions $JI_1$ and DI were fully involved in water minimization mechanism called dehydration avoidance.

Accession NG061 accumulated higher anthocyanin with averagely high APx, which displayed a crucial function in defending plant against drought stress. Accordingly, accession NG061 produced highest plant height, number of leaves, tillers and spike and peduncle length during drought stress. Accession NG061 accumulated higher anthocyanin with averagely high APx which displayed a crucial function in defending plant against drought stress. Consequently, accessions DI and NG061are overall mildly resistant to drought stress. In contrast, accessions NG062 and $JI_2$ showed

low shoot weight, reduced chlorophyll a, decreased in CAT, SOD activities, low proline accumulation and destroyed DNA. They are in this regard highly susceptible to drought stress.

Furthermore, the following interactions were revealed during drought stress: an inverse relationship between chlorophyll a and b; APx promoted the activities of chlorophyll b; and synergistic effort was observed between total anthocyanin and proline and therefore preventing the decomposition of chlorophyll a.

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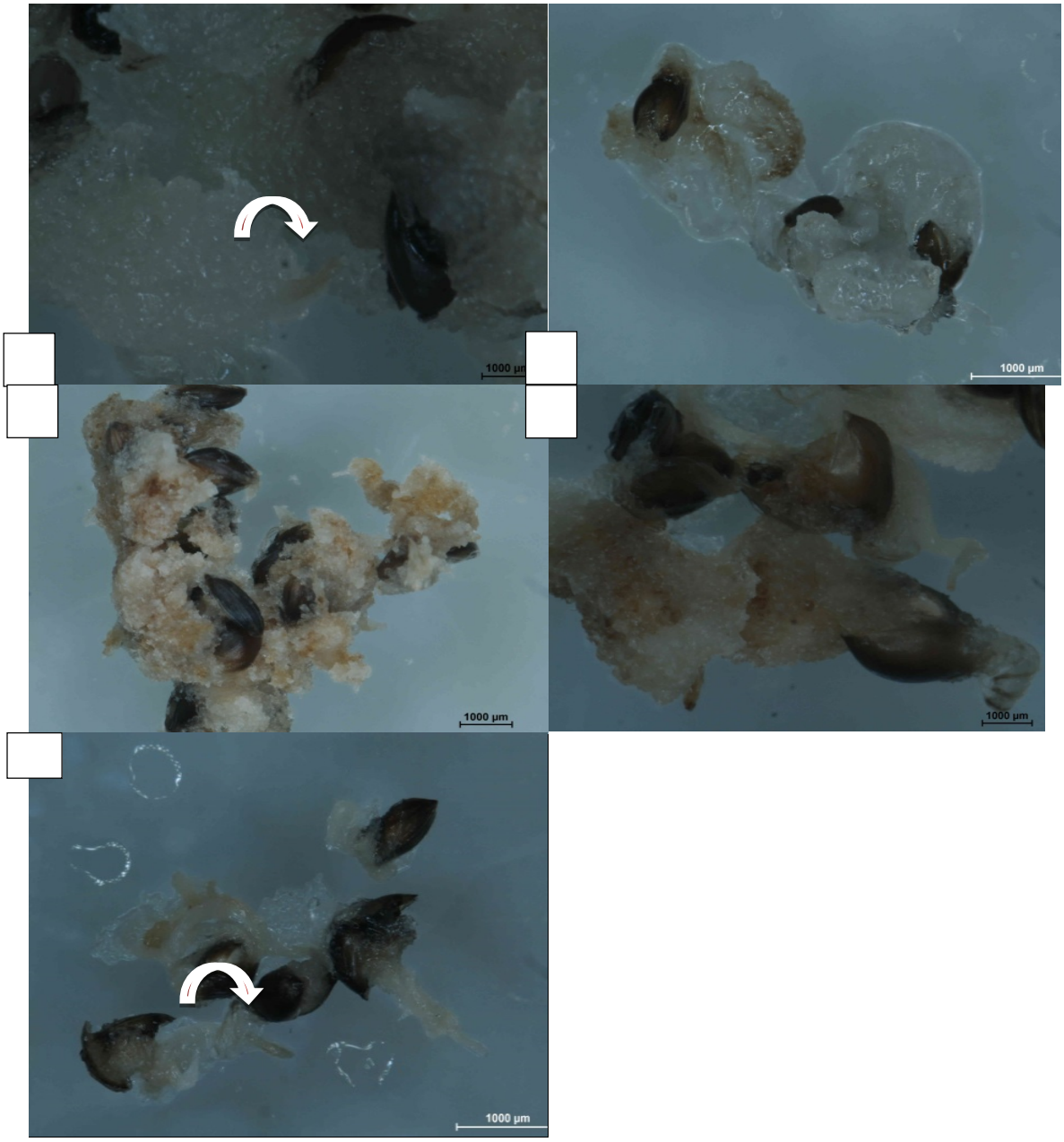
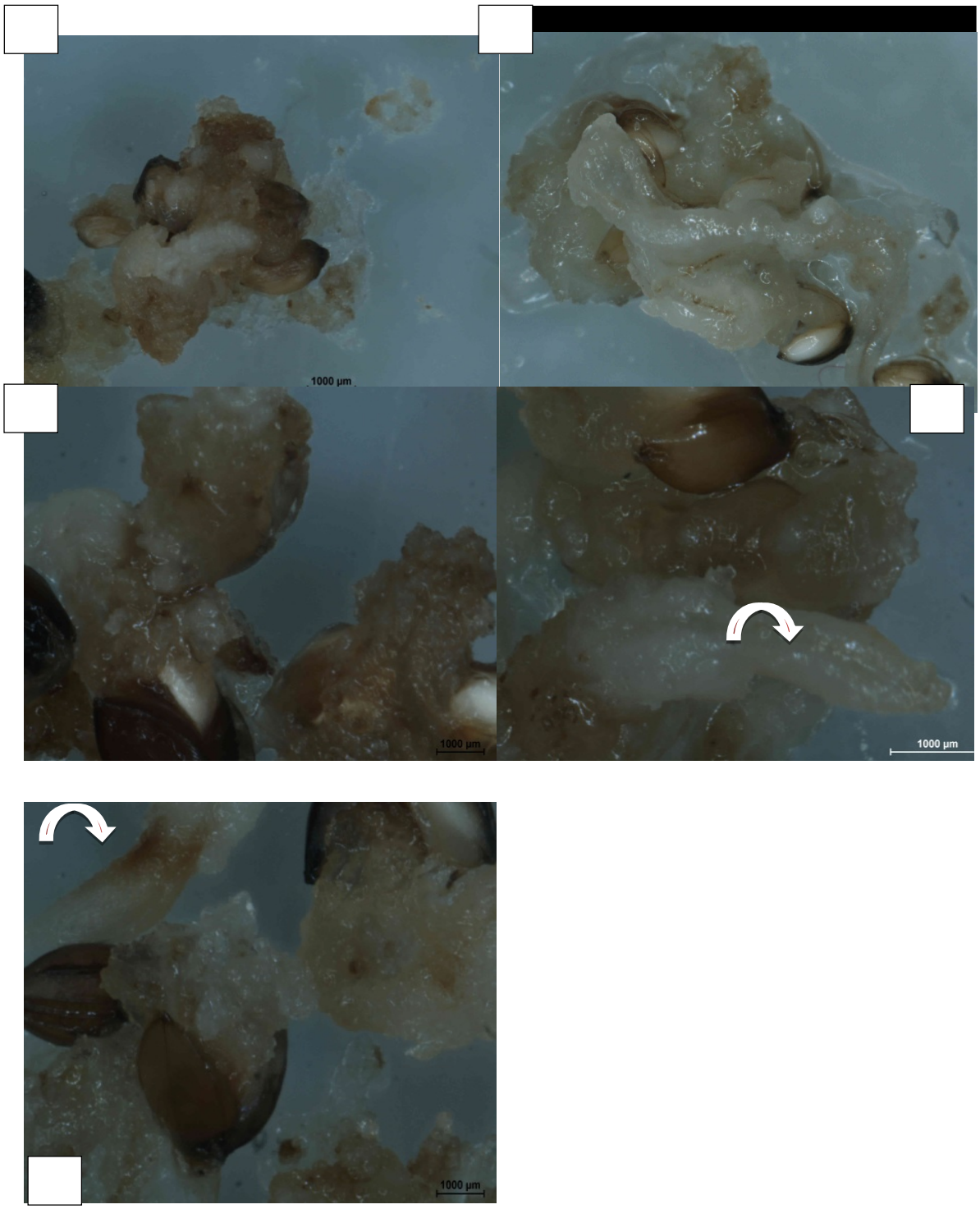
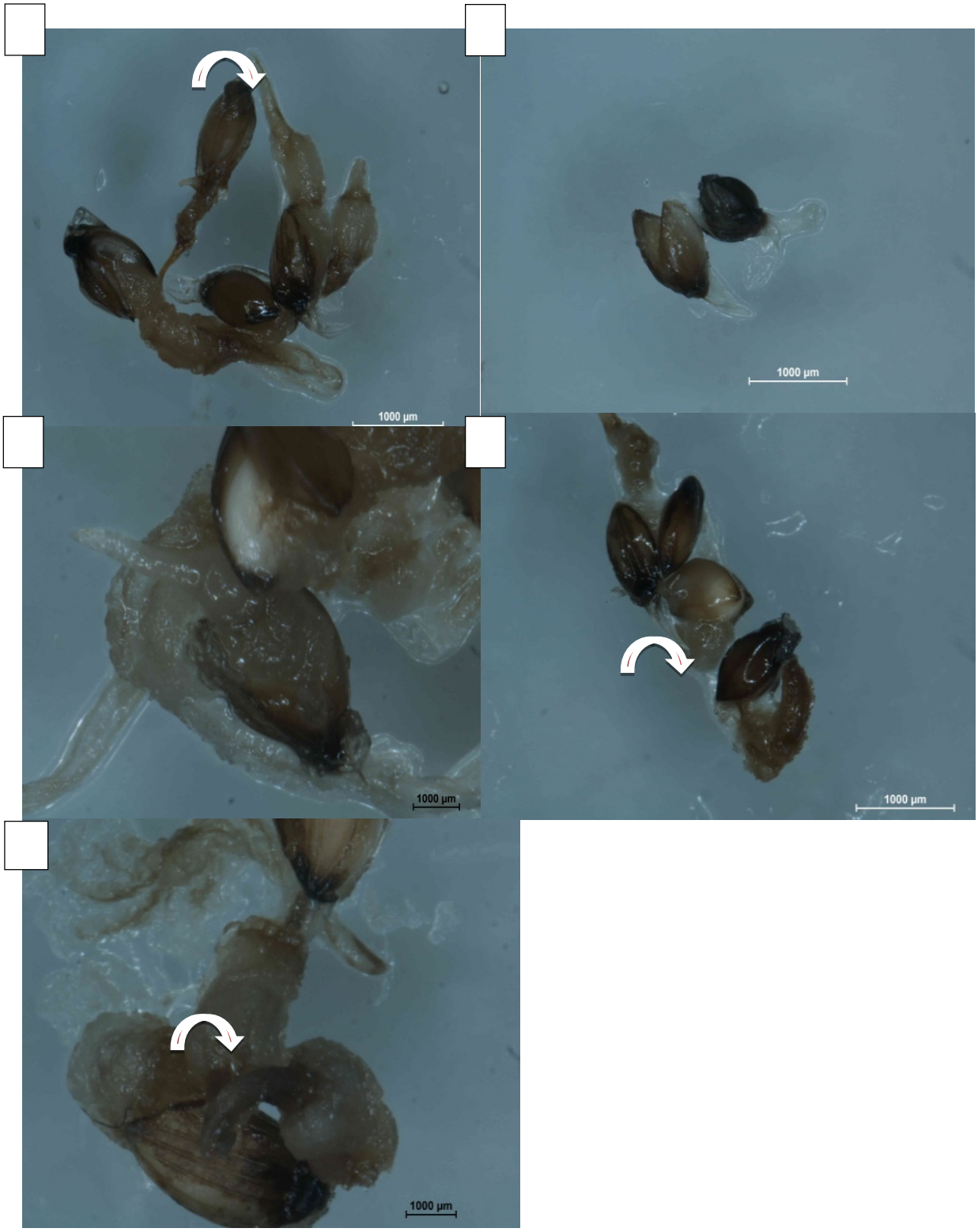
# APPENDICES



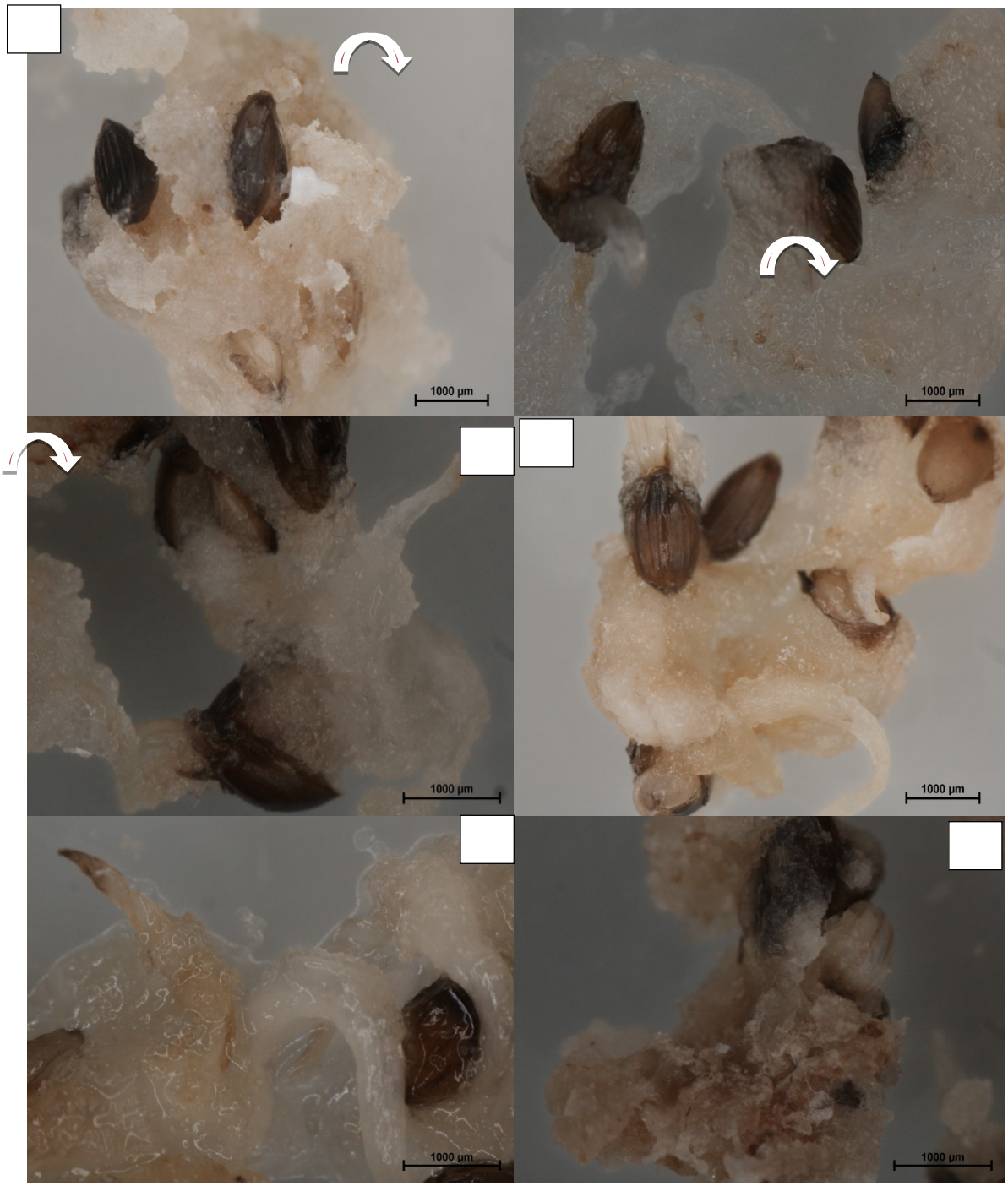

**Appendix 1: Callus Image of Accession NG062 under Osmotic Stress**



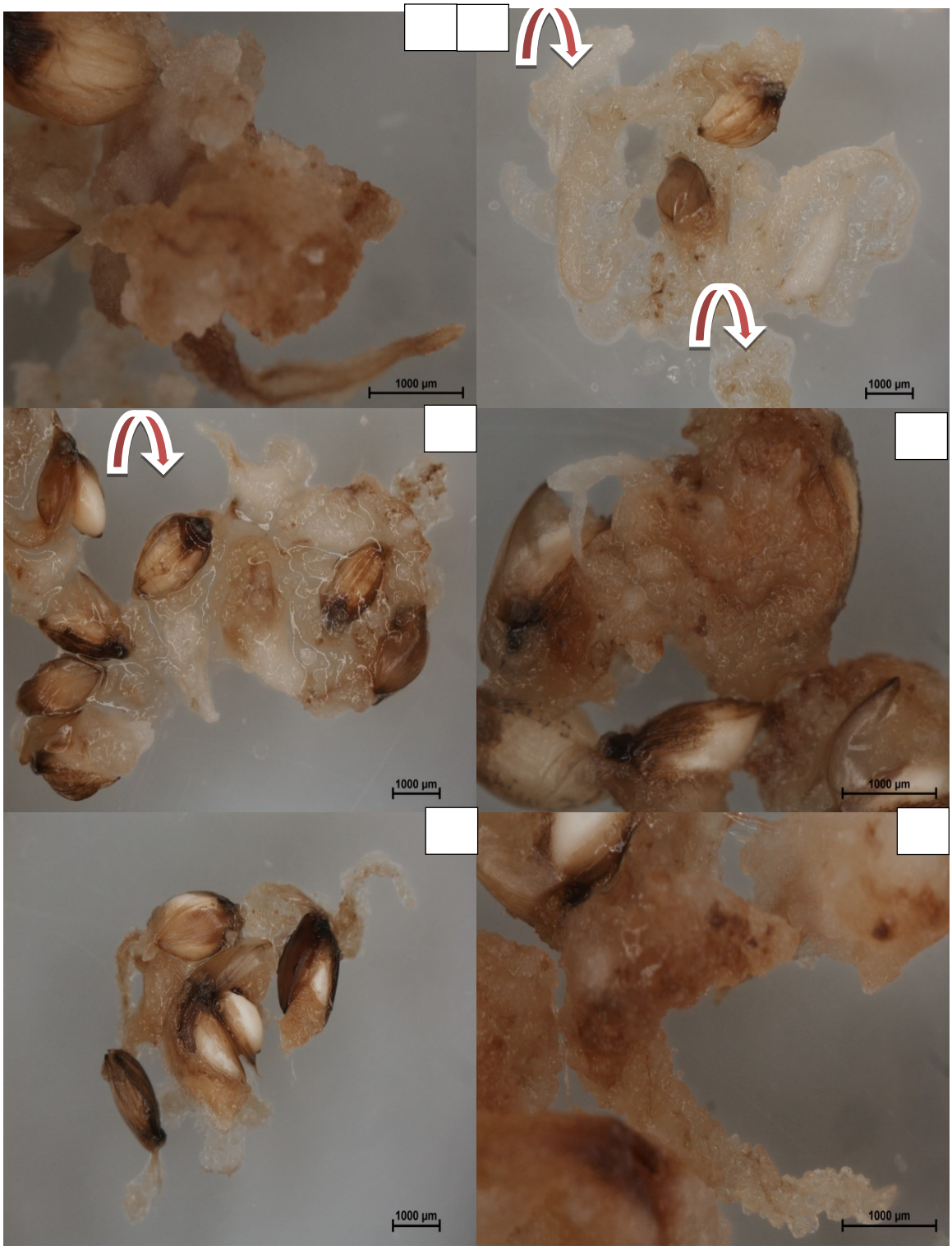

**Appendix 2: Callus Image of Accession DI under Osmotic Stress**




**Appendix 3: Callus Image of Accession $JI_2$ under different Osmotic Potential**




**Appendix 4: Callus Image of Accession NG061 under Osmotic Stress**




**Appendix 5: Callus Image of Accession $JI_1$ under Osmotic Stress**

**Appendix 6: Absorbance of Anthocyanin at pH 1.0**

| Accessions & Treatments | 700nm | | Accessions & Treatments | 512nm | |
|---|---|---|---|---|---|
| NG061( 4days) | 0.024 | 0.027 | NG061( 4days) | 0.040 | 0.042 |
| NG061 (8days) | 0.029 | 0.034 | NG061 (8days) | 0.057 | 0.060 |
| NG061(12days) | 0.120 | 0.150 | NG061 (12days) | 0.496 | 0.504 |
| NG061 (CNT) | 0.424 | 0.420 | NG061 (CNT) | 0.478 | 0.482 |
| NG062 (4days) | 0.151 | 0.155 | NG062 (4days) | 0.157 | 0.160 |
| NG062 (8days) | 0.032 | 0.036 | NG062 (8days) | 0.059 | 0.064 |
| NG062(12days) | 0.225 | 0.228 | NG062(12 days) | 0.239 | 0.245 |
| NG062 (CNT) | 0.050 | 0.058 | NG062 (CNT) | 0.120 | 0.128 |
| DI (4days) | 0.033 | 0.040 | DI (4days) | 0.135 | 0.140 |
| DI (8days) | 0.026 | 0.030 | DI (8days) | 0.201 | 0.210 |
| DI (12days) | 0.104 | 0.110 | DI (12days) | 0.235 | 0.240 |
| DI (CNT) | 0.034 | 0.038 | DI (CNT) | 0.195 | 0.201 |
| $JI_1$ (4days) | 0.015 | 0.018 | $JI_1$ (4days) | 0.026 | 0.029 |
| $JI_1$ (8days) | 0.157 | 0.160 | $JI_1$ (8days) | 0.231 | 0.237 |
| $JI_1$ (12days) | 0.053 | 0.050 | $JI_1$ (12days) | 0.089 | 0.095 |
| $JI_1$ (CNT) | 0.048 | 0.052 | $JI_1$ (CNT) | 0.082 | 0.090 |

**Appendix 7: Absorbance of Anthocyanin at pH 4.5**

| Accessions & Treatments | 700nm | | Accessions & Treatments | 512nm | |
|---|---|---|---|---|---|
| NG061( 4days) | 0.029 | 0.032 | NG061( 4days) | 0.032 | 0.031 |
| NG061 (8days) | 0.184 | 0.187 | NG061 (8days) | 0.197 | 0.201 |
| NG061 (12days) | 0.390 | 0.405 | NG061(12days) | 0.491 | 0.500 |
| NG061 (CNT) | 0.045 | 0.049 | NG061 (CNT) | 0.06 | 0.068 |
| NG062 (4days) | 0.102 | 0.110 | NG062 (4days) | 0.106 | 0.113 |
| NG062 (8days) | 0.029 | 0.037 | NG062 (8days) | 0.021 | 0.028 |
| NG062 (12 days) | 0.093 | 0.102 | NG062(12days) | 0.097 | 0.103 |
| NG062 (CNT) | 0.204 | 0.208 | NG062 (CNT) | 0.239 | 0.244 |
| DI (4days) | 0.034 | 0.039 | DI (4days) | 0.041 | 0.047 |
| DI (8days) | 0.050 | 0.070 | DI (8days) | 0.144 | 0.149 |
| DI (12days) | 0.036 | 0.041 | DI (12days) | 0.113 | 0.118 |
| DI (CNT) | 0.055 | 0.059 | DI (CNT) | 0.07 | 0.083 |
| $JI_1$ (4days) | 0.039 | 0.045 | $JI_1$ (4days) | 0.027 | 0.033 |
| $JI_1$ (8days) | 0.054 | 0.059 | $JI_1$ (8days) | 0.093 | 0.102 |
| $JI_1$ (12days) | 0.100 | 0.109 | $JI_1$ (12days) | 0.124 | 0.144 |
| $JI_1$ (CNT) | 0.031 | 0.046 | $JI_1$ (CNT) | 0.039 | 0.062 |

**Appendix 8: Absorbance f Chlorophyll at Different Wavelength**

| Accessions & Treatments | 470nm | | | 644nm | | | 662nm | | |
|---|---|---|---|---|---|---|---|---|---|
| NG061( 4days) | 1.754 | 1.771 | 1.769 | 1.472 | 1.457 | 1.479 | 1.479 | 1.472 | 1.452 |
| NG061 (8days) | 1.739 | 1.785 | 1.756 | 0.871 | 0.874 | 0.856 | 1.452 | 1.467 | 1.468 |
| NG061 (12days) | 1.758 | 1.783 | 1.766 | 0.874 | 0.877 | 0.867 | 1.476 | 1.486 | 1.456 |
| NG061 (CNT) | 1.773 | 1.771 | 1.771 | 0.850 | 0.866 | 0.860 | 0.876 | 0.857 | 0.875 |
| NG062 (4days) | 1.779 | 1.775 | 1.752 | 0.856 | 0.852 | 0.863 | 0.880 | 0.895 | 0.895 |
| NG062 (8days) | 1.739 | 1.644 | 1.750 | 0.846 | 0.803 | 0.818 | 0.889 | 0.876 | 0.851 |
| NG062 (12 days) | 1.771 | 1.802 | 1.786 | 0.848 | 0.850 | 0.864 | 0.884 | 0.857 | 0.873 |
| NG062 (CNT) | 1.777 | 1.778 | 1.820 | 0.860 | 0.866 | 0.873 | 0.880 | 0.862 | 0.892 |
| DI (4days) | 1.005 | 0.962 | 0.938 | 0.887 | 0.886 | 0.897 | 0.864 | | 0.847 |
| DI (8days) | 1.764 | 1.792 | 1.826 | 0.873 | 0.897 | 0.892 | 1.456 | 1.459 | 1.449 |
| DI (12days) | 1.802 | 1.817 | 1.809 | 0.893 | 0.867 | 0.879 | 1.440 | 1.454 | 1.470 |
| DI (CNT) | 0.991 | 1.012 | 0.993 | 1.449 | 1.445 | 1.456 | 0.872 | 0.868 | 0.934 |
| $JI_1$ (4days) | 0.985 | 0.979 | 1.005 | 0.867 | 0.866 | 0.865 | 1.485 | 1.503 | 1.511 |
| $JI_1$ (8days) | 0.963 | 0.991 | 1.003 | 0.866 | 0.889 | 0.880 | 1.483 | 1.481 | 1.468 |
| $JI_1$ (12days) | 1.013 | 0.996 | 0.992 | 0.872 | 0.858 | 0.884 | 1.498 | 1.501 | 1.477 |
| $JI_1$ (CNT) | 1.007 | 0.961 | 1.016 | 0.873 | 0.889 | 0.868 | 1.487 | 1.472 | 1.464 |

**Appendix 9: Absorbance Of Lipid Peroxidation (MDA)**

| ACCESSIONS | TREATMENTS | ABS 1 | ABS 2 | ABS 3 |
|---|---|---|---|---|
| | 4 Days | 0.104 | 0.087 | 0.102 |
| | 8 Days | 0.100 | 0.106 | 0.106 |
| | 12 Days | 0.139 | 0.137 | 0.143 |
| NG061 | CNT | 0.094 | 0.082 | 0.085 |
| | 4 Days | 0.108 | 0.110 | 0.117 |
| | 8 Days | 0.082 | 0.091 | 0.078 |
| | 12 Days | 0.204 | 0.167 | 0.205 |
| NG062 | CNT | 0.090 | 0.083 | 0.093 |
| | 4 Days | 0.424 | 0.531 | 0.434 |
| | 8 Days | 0.220 | 0.194 | 0.219 |
| | 12 Days | 0.246 | 0.258 | 0.255 |
| DI | CNT | 0.125 | 0.124 | 0.127 |
| | 4 Days | 0.261 | 0.282 | 0.274 |
| | 8 Days | 0.350 | 0.341 | 0.344 |
| | 12 Days | 0.546 | 0.560 | 0.544 |
| $JI_1$ | CNT | 0.263 | 0.216 | 0.271 |

**Appendix 10: Absorbance of Ascorbate Peroxidase (APx) inAccession NG061**

| NG061 (4days) | | NG061 (8days) | | NG061 (12days) | | NG061 (CNT) | |
|---|---|---|---|---|---|---|---|
| 1.974 | 1.932 | 2.148 | 2.171 | 2.412 | 2.423 | 2.217 | 2.224 |
| 1.957 | 1.909 | 2.103 | 2.133 | 2.434 | 2.451 | 2.200 | 2.184 |
| 1.933 | 1.896 | 2.083 | 2.111 | 2.418 | 2.446 | 2.190 | 2.175 |
| 1.932 | 1.858 | 2.070 | 2.101 | 2.412 | 2.446 | 2.175 | 2.159 |
| 1.932 | 1.857 | 2.073 | 2.106 | 2.418 | 2.440 | 2.175 | 2.162 |
| 1.926 | 1.886 | 2.073 | 2.106 | 2.402 | 2.451 | 2.181 | 2.172 |
| 1.926 | 1.861 | 2.075 | 2.103 | 2.418 | 2.429 | 2.178 | 2.165 |
| 1.926 | 1.856 | 2.073 | 2.106 | 2.412 | 2.434 | 2.178 | 2.159 |
| 1.925 | 1.861 | 2.073 | 2.101 | 2.412 | 2.434 | 2.181 | 2.162 |

**Appendix 11: Absorbance of Ascorbate Peroxidase (APx) inAccession NG062**

| NG062 (4days) | | NG062 (8days) | | NG062 (12days) | | NG062 (CNT) | |
|---|---|---|---|---|---|---|---|
| 2.078 | 2.051 | 1.526 | 1.457 | 2.200 | 2.217 | 2.145 | 2.068 |
| 2.005 | 2.020 | 1.502 | 1.450 | 2.184 | 2.181 | 2.122 | 2.042 |
| 1.980 | 2.001 | 1.490 | 1.449 | 2.159 | 2.175 | 2.111 | 2.020 |
| 1.967 | 2.003 | 1.481 | 1.443 | 2.150 | 2.178 | 2.090 | 2.003 |
| 1.961 | 2.011 | 1.481 | 1.442 | 2.136 | 2.181 | 2.085 | 2.004 |
| 1.978 | 1.998 | 1.484 | 1.446 | 2.150 | 2.190 | 2.103 | 2.015 |
| 1.970 | 1.986 | 1.481 | 1.444 | 2.148 | 2.181 | 2.101 | 2.009 |
| 1.963 | 2.007 | 1.481 | 1.446 | 2.142 | 2.178 | 2.090 | 2.007 |
| 1.967 | 1.984 | 1.482 | 1.443 | 2.153 | 2.181 | 2.098 | 2.005 |

**Appendix 12: Absorbance of Ascorbate Peroxidase (APx) inAccession $JI_1$**

| $JI_1$ (4 days) | | $JI_1$ (8days) | | $JI_1$ (12days) | | $JI_1$ (CNT) | |
|---|---|---|---|---|---|---|---|
| 2.457 | 2.440 | 2.463 | 2.469 | 1.665 | 1.666 | 1.663 | 1.658 |
| 2.452 | 2.452 | 2.469 | 2.469 | 1.664 | 1.669 | 1.664 | 1.651 |
| 2.407 | 2.434 | 2.463 | 2.463 | 1.663 | 1.666 | 1.660 | 1.657 |
| 2.402 | 2.429 | 2.457 | 2.463 | 1.663 | 1.666 | 1.663 | 1.659 |
| 2.396 | 2.429 | 2.469 | 2.463 | 1.663 | 1.665 | 1.662 | 1.659 |
| 2.396 | 2.423 | 2.457 | 2.457 | 1.662 | 1.667 | 1.659 | 1.656 |
| 2.391 | 2.429 | 2.463 | 2.452 | 1.662 | 1.667 | 1.662 | 1.659 |
| 2.391 | 2.412 | 2.463 | 2.452 | 1.662 | 1.667 | 1.661 | 1.657 |
| 2.391 | 2.423 | 2.457 | 2.446 | 1.661 | 1.666 | 1.656 | 1.653 |

**Appendix 13: Absorbance of Ascorbate Peroxidase (APx) inAccession DI**

| DI (4days) | | DI (8days) | | DI (12days) | | DI (CNT) | |
|---|---|---|---|---|---|---|---|
| 2.321 | 2.295 | 2.434 | 2.407 | 2.407 | 2.440 | 2.190 | 2.327 |
| 2.352 | 2.321 | 2.446 | 2.412 | 2.429 | 2.451 | 2.227 | 2.335 |
| 2.304 | 2.279 | 2.434 | 2.407 | 2.396 | 2.440 | 2.172 | 2.315 |
| 2.300 | 2.283 | 2.407 | 2.402 | 2.391 | 2.429 | 2.159 | 2.307 |
| 2.304 | 2.283 | 2.440 | 2.396 | 2.391 | 2.429 | 2.159 | 2.309 |
| 2.300 | 2.279 | 2.412 | 2.412 | 2.391 | 2.429 | 2.153 | 2.307 |
| 2.295 | 2.283 | 2.428 | 2.402 | 2.391 | 2.423 | 2.156 | 2.309 |
| 2.300 | 2.279 | 2.412 | 2.402 | 2.391 | 2.423 | 2.156 | 2.306 |
| 2.295 | 2.279 | 2.412 | 2.412 | 2.418 | 2.440 | 2.156 | 2.305 |

**Appendix 14: Absorbance of Catalase in NG061**

| NG061 (12days) | | NG061 (CNT) | | NG062 (4days) | |
|---|---|---|---|---|---|
| 2.173 | 2.231 | 1.859 | 1.831 | 0.989 | 1.207 |
| 2.168 | 2.221 | 1.858 | 1.833 | 0.989 | 1.209 |
| 2.172 | 2.229 | 1.863 | 1.830 | 0.988 | 1.206 |
| 2.168 | 2.233 | 1.855 | 1.831 | 0.985 | 1.208 |
| 2.172 | 2.227 | 1.862 | 1.829 | 0.987 | 1.204 |
| 2.156 | 2.223 | 1.854 | 1.822 | 0.972 | 1.179 |
| 2.161 | 2.225 | 1.855 | 1.831 | 0.981 | 1.210 |
| 2.136 | 2.217 | 1.839 | 1.825 | 0.970 | 1.172 |
| 2.161 | 2.223 | 1.851 | 1.830 | 0.977 | 1.186 |

**Appendix 15: Absorbance of Catalase in NG062**

| NG062 (8days) | | NG062 (12days) | | NG062 (CNT) | |
|---|---|---|---|---|---|
| 0.587 | 0.726 | 1.777 | 1.79 | 1.452 | 1.401 |
| 0.588 | 0.726 | 1.771 | 1.794 | 1.454 | 1.403 |
| 0.585 | 0.725 | 1.769 | 1.785 | 1.449 | 1.400 |
| 0.583 | 0.721 | 1.785 | 1.788 | 1.443 | 1.394 |
| 0.584 | 0.725 | 1.782 | 1.788 | 1.445 | 1.398 |
| 0.579 | 0.719 | 1.749 | 1.763 | 1.434 | 1.380 |
| 0.582 | 0.720 | 1.786 | 1.783 | 1.439 | 1.392 |
| 0.574 | 0.719 | 1.745 | 1.744 | 1.444 | 1.366 |
| 0.581 | 0.720 | 1.768 | 1.773 | 1.435 | 1.388 |

**Appendix 16: Absorbance of Catalase in $JI_1$**

| $JI_1$ (12days) | | $JI_1$ (CNT) | | DI (4days) | |
|---|---|---|---|---|---|
| 2.356 | 1.978 | 2.211 | 2.112 | 1.254 | 1.507 |
| 2.351 | 1.971 | 2.209 | 2.110 | 1.252 | 1.500 |
| 2.348 | 1.975 | 2.207 | 2.110 | 1.253 | 1.503 |
| 2.348 | 1.961 | 2.194 | 2.107 | 1.249 | 1.488 |
| 2.345 | 1.969 | 2.205 | 2.109 | 1.252 | 1.498 |
| 2.345 | 1.965 | 2.201 | 2.109 | 1.250 | 1.493 |
| 2.343 | 1.953 | 2.192 | 2.104 | 1.247 | 1.483 |
| 2.335 | 1.948 | 2.190 | 2.103 | 1.245 | 1.475 |
| 2.315 | 1.958 | 2.196 | 2.092 | 1.220 | 1.470 |

**Appendix 17: Absorbance of Catalase in DI**

| DI (8days) | | DI (12days) | | DI (CNT) | |
|---|---|---|---|---|---|
| 2.014 | 2.097 | 2.092 | 2.059 | 1.423 | 1.454 |
| 2.008 | 2.089 | 2.092 | 2.057 | 1.421 | 1.444 |
| 2.007 | 2.082 | 2.085 | 2.055 | 1.418 | 1.443 |
| 2.007 | 2.094 | 2.084 | 2.051 | 1.415 | 1.443 |
| 2.006 | 2.085 | 2.083 | 2.051 | 1.424 | 1.442 |
| 2.005 | 2.086 | 2.078 | 2.048 | 1.411 | 1.442 |
| 2.003 | 2.094 | 2.077 | 2.044 | 1.401 | 1.442 |
| 2.001 | 2.089 | 2.074 | 2.040 | 1.383 | 1.442 |
| 1.999 | 2.092 | 2.070 | 2.039 | 1.400 | 1.441 |