Investigating the combined effects of PGPR inoculants and biochar on physiochemical responses of ground nut *(Arachis hypogaea* L.*)* under drought stress

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Thesis acceptance certificate

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DEDICATION

I dedicate my dissertation work to my family and teachers.

A special feeling of gratitude to my father

Dr. Jan Alam (Late), Mother Mrs. Dr. Jan Alam and

my loving siblings

who are the precious and loving assets of my

life.

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Tashfeen Alam

ABSTRACT

Peanut is one of the major economical legume and oilseed crop in Pakistan. It is of widely used in crop rotation and intercropping systems due to its ability to develop symbiotic association with soil bacteria for BNF. However, the impact of drought on disease, nutrition, and yield loss poses significant challenges for the peanut industry. With the world's population on the rise, water scarcity is becoming an increasing concern for agricultural activities. Exploring novel drought control methods is essential to sustain peanut production. This study explores the significant effect of use of cotton straw biochar and PGPR (Plant Growth-Promoting Rhizobacteria)-based biofertilizers as a potential remedy for drought tolerance. The experiment was conducted using a randomized complete block design in a greenhouse setting, evaluating the performance of two variable ratios of biochar in combination with PGPRs in peanut. The primary objectives were to assess whether cotton straw biochar and PGPR-based biofertilizer could improve drought tolerance in peanuts while maintaining normal growth pattern. The results of this study showed that the combination of cotton straw biochar and PGPR-based biofertilizer significantly enhanced drought tolerance in peanuts. Morphological traits were observed to be significantly enhanced in the presence of Biochar and PGPR application under drought stress. Biochemical profile of plants treated with biochar also showed lower levels of ROS accumulation in leaf tissues when drought was induced. Metabolic profile of root exudates showed significant changes in signaling molecules involved in nodulation and BNF process. To further exploit the potential benefits of this approach, future research should focus on molecular mechanisms involved in modulating nodulation process in peanut under drought stress for enhancement of plant growth as well as soil nutrient.

Keywords:

Peanut, Nodulation, Biological nitrogen fixation, organic amendments, Biofertilizers, Plant growth promoting rhizobacteria, drought, morphophysiological, antioxidant, Flavonoids, Root exudates.

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LIST OF ABBREVIATION

APX	Ascorbate peroxidase
Approx.	Approximately
BC	Biochar
BNF	Biological nitrogen fixation
CAT	Catalase
CDNB	Chloro dinitro benzene
EDTA	Ethylenediaminetetraacetic acid
FC	Field capacity
et al.	et alia
GCMS	Gas chromatography- Mass spectrometry
GSH	Glutathione
GST	Glutathione S-Transferase
i.e.,	id est means “that is”
KPK	Khyber Pakhtunkhwa
NBT	Nitro blue tetrazolium
NDMC	National Drought Monitoring Centre
PBS	Phosphate buffer
PCA	Principle component analysis
PGPR	Plant growth promoting rhizobacteria
POD	Peroxidase
RPM	Round per minute
ROS	Reactive oxygen specie
RWC	Relative water content
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
SPAD	Soil Plant Analysis Development
T3SS	Type 3 secretion system
USA	United states of America
USDA	United States Department of Agriculture
UV	Ultra violet

CHAPTER 1: INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 Arachis hypogaea L

Peanut is one of the major oilseed crop plant belonging to the legumes family. It has been given multiple names like ground nut and monkey nut while scientifically it is named *Arachis hypogaea* L. It belongs to the family **Fabaceae** and subfamily **Faboideae** or **Papilionaceae.** Peanut was the first plant of its type to be identified and described; hence, it is called the type specie of the genus *Arachis* (Bertioli et al., 2011). Commercially cultivated peanut specie is known to be originated from the multiple events of polyploidization, involving two *Arachis* species that are *A. duranensis* and *A. ipaensis* (Kochert et al., 1996; Seijo et al., 2007). Studies have shown that peanut is a native South American plant that was brought to Peru almost 500 years ago. The peanut spread from these areas to Europe, the Pacific Islands, the shores of Asia, and Africa. The introduction of the peanut was not recorded, but it eventually made its way to the colonial seaboard in what is now the southeastern United States (Hammons et al., 2016).

Currently, commercial peanut cultivation is practiced in over 40 nations, with China, the USA, Argentina, Brazil, Myanmar, India, Indonesia, Nigeria, Senegal, and Sudan serving as the primary producers. In Pakistan, it is cultivated in rain-fed (barani) as well as irrigated regions of Punjab, KPK, and Sindh. Pakistan's rain-fed lands in Rawalpindi Division saw the commercial introduction of peanuts in 1950 (Rasheed, Dawar et al. 2004). It began in the Rawalpindi Division and afterwards extended to other parts of the nation. In the districts of Rawalpindi, Chakwal, Attock, and Jhelum in the Punjab province, natural precipitation is used to cultivate 92.93% of the province's peanut harvests. In Sindh, peanut is grown through irrigation in districts of Khairpur, Ghotki, Sukhar, and Sanghar. In KPK, where most of the agricultural land is rain-fed, the primary peanut production Districts include Haripur, Karak, Hangu, Kohat, and Swabi. Kurram agency produces peanuts using irrigation.

1.1.1 Habitat of peanut

From 40°S to 40°N latitude, peanuts are grown in the geographical regions of tropical, subtropical, and warm temperate zones. It is a kharif crop, usually cultivated in the month of April which marks the beginning of summers and it needs around 3.5 to 5 months from germination to fruit ripening. It can grow in variety of habitats including xerophytic forests, grasslands, occasionally flooded places, and open portions of subtropical rainforest. Peanut grows in semiarid region with temperature ranging from 25-35°C. Different types of soils are

preferred, including rock outcrops, laterite pebble layers, heavy soils, poorly drained places, and sandy soils with good drainage (Bertioli et al., 2011). Heavy soils are necessary for the growth of peanuts, but they can also be cultivated on light, dusty, and water-poor sandy loams.

1.1.2. Morphology of peanut plant

Peanut plant is an annual, dicotyledonous plant, having herbaceous stem. It can grow up to 30 to 50 cm in height during its vegetative growth stage. According to Raunkier's classification, peanut plant is classified as therophyte with microphyllus leaves (M. Khan, 2012) Multiple branches arise from the base of the shoots , giving it a bushy appearance. Peanut possess erect as well as prostrate branching pattern (lying upon or just above the ground). Leaves of peanut plant are in the form of tetrafoliate pinnate and opposite in arrangement with each leaflet ranging from 1 to 7 cm long and 1 to 3 cm broad. Root system of the plant is in the form of extensive and well-developed taproot system with many lateral roots and nodules. It has a typical yellow colored solitary flower. Upon fertilization, petals of flower wither and results in the formation of stalk like structure called peg. This peg grows in downward direction towards the soil and pod formation takes place which contain seeds, making peanut a geocarpic plant (Krapovickas, 2017).

1.1.3. Growth and Development of Ground nut

1.1.3.1. Germination and vegetative stage

Ground nut is dicotyledonous plant with embryo enclosed in two cotyledons. Ground nut seed takes about 5 to 7 days in germination when provided with suitable conditions. After germination, cotyledons soon turn green, and seedlings are dependent on assimilates of cotyledons for 5 to 10 days. They develop tap root system from hypocotyl primarily. Lateral roots start to grow after 3 to 5 days of germination. Main stem develops from epicotyl, which is green, autotrophically active and grows upward initially in early growth stages. At the time of emergence, main stem has 4 immature leaves, hence called tetrafoliate. The leaflets have elliptical shape with a prominent midvein. During later stages of vegetative growth, it tends to grow more lateral branches. Additional branches arise from nodes on the main and lateral stems and eventually grow longer than the main stem (Prasad et al. 2010).

1.1.3.2. Reproductive stage

Ground nut cultivars typically shift from vegetative to reproductive phase in about 25 to 30 days after germination, depending upon environmental conditions. Flowers are produced in the form of spike like inflorescence with three or more flowers and continue for about 60 to 70 days of emergence. Flowers are produced on leaf axils present on primary and secondary branches. Several flowers can originate on single node. Peanut flower is a complete flower with yellow bloom and is self-pollinated (Prasad et al. 2010).

1.1.3.3. Pegging and pod formation

Fertilized ovary starts to grow specialized pointed, tube-like structure, known as a peg, which appears about 7 days after fertilization. Ovary is present at the tip of peg which later develops seed containing pod. It takes about 15 to 20 days for peg to grow fully and penetrate the soil, after which the pod begins to expand rapidly until it reaches dimensions characteristic of the cultivar. On penetrating into the soil to a depth of 4-5cm, the tip of the peg where ovary is located begins to swell and turns horizontally away from the base of the plant and develops into a pod. It is necessary for the peg to be in dark for pod formation. It takes about 3 to 4 weeks for peg to fully expend. To reach maturity and harvestable state, pods further need 60 to 80 days of development (Prasad et al. 2010).

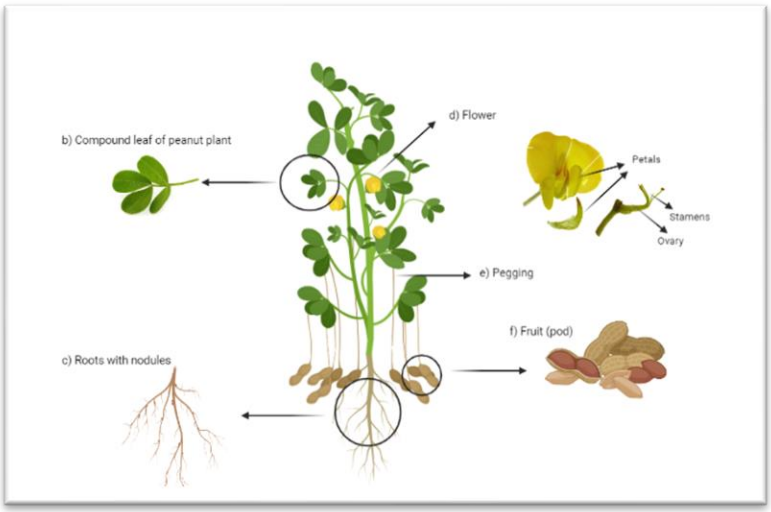

Figure 1. Morphological features of peanut plant

1.1.4. Nutritional value of peanut

Peanut has high nutritional value for it being a source of food, oil and its other medicinal properties. Being a legume, peanut is closely related to chickpeas and beans hence contain large amount of crude protein that make up about 22 to 30% of its seed. Presence of 32 different proteins is confirmed by various studies among which 18 are also identified as allergenic (Toomer, 2017). In most of the regions in world, peanut is used as primary source of edible oil because of its high content of lipids and fatty acids. Typically, peanut seed contain 44 to 56% oil content, with an average of 50% that has slightly yellow color and nutty flavor. However, lipid and fatty acid composition is substantially influenced by cultivar, seed maturity, environmental circumstances, and geographic location, flavor and quality of peanuts (Toomer, 2017). Peanut are also rich in carbohydrates with 21.51 g of carbohydrates per 100 g having starch as a major constituent while other carbohydrates include Sucrose, Fructose, Glucose, Inositol, Raffinose, Stachyose etc. (USDA, Food Composition Database, 2017). Studies has shown that variation in carbohydrate content in peanut also depends upon cultivar, maturation, and geographic location (Toomer, 2017). Peanuts also contain a decent amount of vitamin B and vitamin E which are good antioxidant agents. Studies has confirmed the presence of fair amount of macronutrient like magnesium, potassium, calcium, and phosphorus etc. in peanut seed (Toomer, 2017).

1.2. Flavonoids and Biological Nitrogen Fixation in Ground Nut

Flavonoids are chemically diverse and biologically active group of secondary metabolites, which is a sub class of polyphenols having low molecular weight. They hold significant importance as plant defense agents against various biotic and abiotic stresses. They are subdivided into flavonols, flavanols, anthocyanins, flavones, chalcones, di-hydroflavonols and di-hydrochalcones. For centuries, these secondly are of great importance for their variety of functions like anthocyanins in flowers as pollinator attractants and seed dispersal. Flavonoids, being a plant based secondary metabolite have many health benefits, including reduced risk of heart diseases, cancer and other chronic diseases. Flavonoids protect the plants from various abiotic stresses by their antioxidant ability and provide protection against UV radiations.

CHAPTER 1 INTRODUCTION

Nitrogen is one of the most essential micronutrients required for stable growth and development of plants and available atmospheric nitrogen cannot be absorbed by plants. It needs to be converted into ammonia and nitrates which can be utilized by plants. The process by which atmospheric nitrogen is converted into readily useable form is called nitrogen fixation. This process is important for growth and development of many crops, as it enhances the availability of consumable nitrogen for plant without the need of application on synthetic fertilizers. Members of legume family possess the distinct feature of biological nitrogen fixation by adopting various strategies like the Nod strategy, the T3SS (type III secretion system) strategy and the non-Nod/non-T3SS strategy (Dong & Song, 2020). Flavonoid have been studied for their positive effects of biological nitrogen fixation. For example, flavonoids such as Quercetin and Genistein have shown the ability to stimulate the growth of nitrogen fixing bacteria in soil which eventually increase the nitrogen fixation and nitrogen content in soil. It eventually increases the amount consumable nitrogen for plants. This can be particularly beneficial in agricultural practices where use of synthetic fertilizers is very limited. Flavonoids participate in different stages of the nodulation process, such as the chemo-attraction of Rhizobium in the T3SS strategy (Dong & Song, 2020). Variety of secondary metabolites are secreted by plants in the form of root exudates which serve as a chemical signal to attract soil microbes. These compounds include organic acids, amino acids and sugar while flavonoids play important role in inducing Nod gene expression leading to nodule formation in roots of legumes (Bosse et al., 2021). In legumes, isoflavonoids, a sub class of flavonoids, are majorly reported which serves as chemical signal for expression of Nod genes for the synthesis of Nod factors by rhizobia but irrelevant in chemotactic movement of rhizobia towards plant for symbiosis (Bosse et al., 2021). By working as auxin transporter inhibitors in root cells, particularly in indeterminate nodules, flavonoids play a crucial part in the organogenesis of nodules. The primary N transport form in equatorial legumes is the ureide, which is catabolized in the leaves and other sink tissues to create the amino acids and proteins required for plant development and yield (Bosse et al., 2021). Members of the legume family, which includes plants like legumes, peas, beans, and peanut, have flavonoids in their roots that aid in promoting the development of bacteria that fix nitrogen. Legumes can aid in the natural replenishment of soil nitrogen levels, which is one of the reasons they are frequently used in crop cycle systems. Overall, despite the fact that flavonoids and BNF may appear unrelated at first glance, a growing body of study is

demonstrating the significance of the connections between these two fields. Flavonoids can help to increase soil fertility and health by promoting the development of nitrogen-fixing bacteria, which can then benefit plant growth and overall agricultural output.

1.3. Drought stress, a major abiotic stress

Drought is an extended time of abnormally dry weather, usually brought on by a lack of precipitation. It is a natural catastrophe that has the potential to seriously affect ecosystems, agriculture, and human populations. Anywhere in the world, there can be a drought, and they can vary in severity from slight to severe. This may result in food scarcity, crop failures, and biodiversity loss. Numerous variables, including dry weather, intense light, high and low temperatures, can cause drought. Drought stress has emerged as a significant factor limiting agricultural productivity due to the ongoing climate change. Due to water loss from drought stress, plants experience hyper osmotic stress, which ultimately causes cellular structures to disorganize, photosynthesis to slow down, ROS to accumulate, and others (Dai et al., 2019). Peanuts are typically grown in semi-arid regions that have limited water resources and are at high risk of experiencing extreme drought stress. In order to maintain the homeostasis of the plant's biological system, plant systems attempt to overcome the stress of drought through a variety of morphological, biochemical, physiological, and molecular adaptations. However, extreme drought conditions can still affect a plant's productivity and quality (Dai et al., 2019). Morphologically, decrease in shoot length, shoot dry weight, nodules count has been observed in peanut under drought stress. Physiologically, decrease in photosynthesis and increased production of hydrogen peroxide leading to protein and lipid damage in plant cell has been observed which from which plant can recovers when hydrated. Drought stress affects peanut growth and nodulation negatively. Peanut possess drought avoidance strategy by increasing soluble sugar and ABA content. Under severe drought stress, the phenolic content of peanut seeds declines on the other hand they rise in the leaves and stems. The critical variables that affect how well peanuts respond to drought stress include rate of transpiration, relative water content, leaf water potential, and leaf temperature (Furlan et al., 2012).

1.3.1. Flavonoids in drought stress

Production of ROS as a result of metabolic imbalance in plants is major physiological response under abiotic stresses like drought, heat, temperature, salinity and heavy metals

contamination etc. ROS can negatively damage the plants by causing cellular damage, which ultimately leads to cell death. Flavonoids, being a secondary metabolite are known for their antioxidant properties and are able to scavenge these reactive oxygen species to protect cells from damage which can help plants to survive and continue normal growth in drought conditions (Nakabayashi & Saito, 2015). The ability of flavonoid to mitigate oxidative and drought stress has been studies in Arabidopsis. Over accumulation of anthocyanins in Arabidopsis enhanced oxidative stress and drought tolerance and molecular studies confirmed the role of flavonoids in biotic and abiotic stress tolerance in crops (Nakabayashi et al., 2014).

1.4 Biochar and Biofertilizers as organic amendments

As discussed before, drought can cause severe damage to plant growth and development including responses like decreased water content in plant cell, reduced leaf water potential, stomatal shrinkage and loss of turgidity. Drought also show adverse effects on physiological, biochemical and molecular aspects of plant like decrease in photosynthesis and growth, decreased nutrient uptake, cellular elongation declines, enzyme action and hormone metabolism alters. In recent times, biochar has become matter of interest for its potential to mitigate drought stress. Biochar is organic material formed by the pyrolysis of organic wastes from plants and animals. It is termed as conditioner of soil which improves the soil properties making it suitable for plant growth (Gavili et al., 2019). Studies has revealed that biochar improves soil properties like pH, porosity, water holding capacity, cation exchange capacity, soil organic carbon and microbial respiration. It has been reported that drought cause adverse effects on biomass and different growth parameters of plant however, biochar has the ability to maintain normal vegetative growth in plant.(Mansoor et al., 2021a)

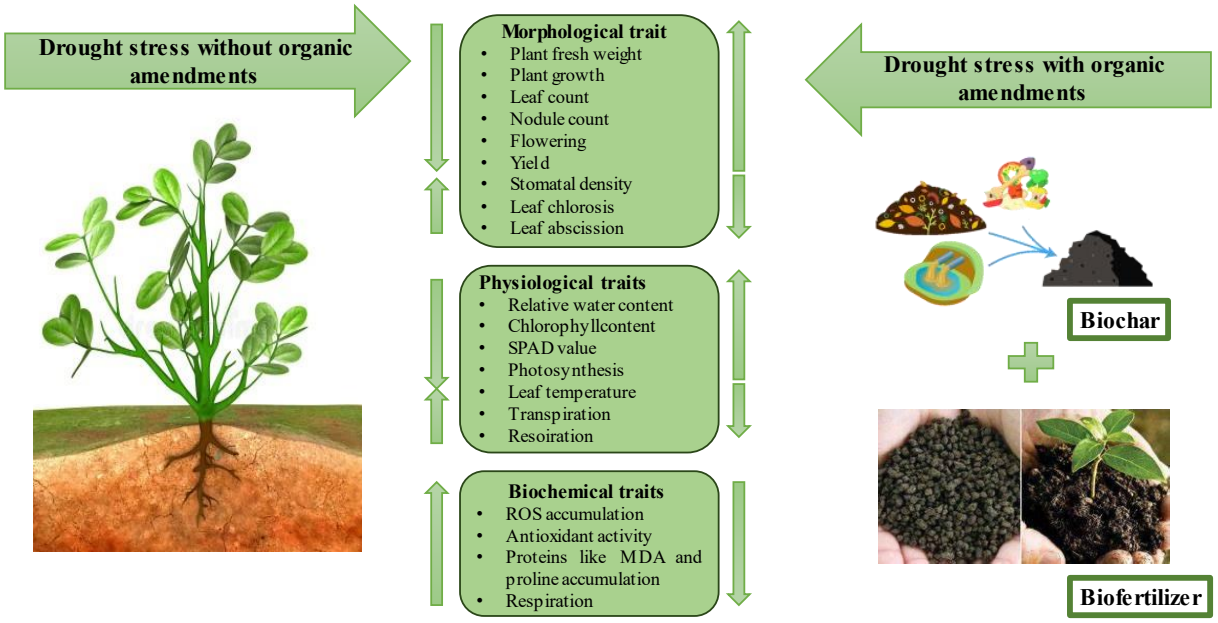

Figure 2. Role of organic amendments in mitigation of drought stress

CHAPTER 2: LITERATURE REVIEW

CHAPTER 2 LITERATURE REVIEW

2.1. Effect of drought

Plant growing in environment, either wild or cultivated, faces a variety of abiotic stresses like heat, cold, drought, salinity, heavy metal contamination in soil etc. Climate change and global warming has major impacts of agriculture and food security. Under developed and developing countries are facing drastic effects of climate change. Pakistan ranks 5th in the most vulnerable countries to climate change (Shah et al., 2023). Pakistan is facing significant fluctuation in environmental conditions in almost every region like extreme weather, irregular rainfalls pattern, and prolonged heat waves. Most of the agricultural area of Pakistan has arid geographical profile which makes it more vulnerable to abiotic stresses like water deficiency and heat. It is evident in literature in field research that climatic risks like flood, drought and extreme temperature have major role in decreasing crop productivity (Shah et al., 2023).

Plants have various adaptation strategies that play role in developing tolerance or resistance against environmental stresses. Drought is a major abiotic factor limiting plant growth and productivity. The main impact on plant growth, development, and productivity among various abiotic environmental stresses is due to photo inhibition of photosynthesis caused by an increase in ABA levels leading to stomatal closure, which avoids dehydration (Fleta-Soriano & Munné-Bosch, 2016). Legumes' responses and adaptability to drought may differ, but the end result is a lower yield because it disturbs the morphology, physiology, and growing season of the plant. The main responses a plant exhibits in response to drought stress include decreased germination rate, decreased photosynthetic activity, alteration of root and shoot development, delayed reproductive phase, an effect on reproductive organs, lesser production of viable gametes, fewer pods, and lower grain set (Nadeem et al., 2019). In case of soybean, drought affects vegetative growth of plant by decrease in root growth in dry soil, reduces leaf area, root and shoot dry matter, reduced number of nodes which eventually reduce number of pods per node. But overall growth and yield depends upon the growth stage at which plant gets exposed to drought (Tarumingkeng & Coto, 2003).

Ding et al. in 2013 elaborated the effect of drought on root structure of peanut by comparing drought resistant and susceptible varieties. They reported increased the root biomass, surface area, and volume of root systems of the two varieties in 20-40 cm soil layer, but decreased these root traits in the soil layers below 40cm. The differential responses of different varieties showed different water absorption and utilization ability under drought stress (Ding et al.,

2013). Ding et al. in 2022 stress in combination with nitrogen deficiency. The results showed decrease in pod yield of under water and N deficiency (Ding et al., 2022).

Syafriani et al. in 2022 performed a comparative study of morphophysiological responses in both cowpea and long bean plants under water deficient conditions and elaborated alteration in morphological traits of plants in terms of root and shoot fresh and dry weight, length. Biochemical profile was studied by analyzing proline deposition in plant under drought stress which increased in stressed plant in order to maintain plant turgor pressure. Stomatal narrowing was also observed in drought stressed plants (SYAFRIANI et al., 2022) In 2019, a study was conducted find out morphophysiological and biochemical responses of mung beans when subjected to drought which reported a significant decrease in RWC, protein content, leaf area, plant height, and yield traits while increase in proline content during drought stress content. The results further elaborated that vegetative phase is more susceptible to drought (Bangar et al., 2019). In 2022, morphophysiological effects of drought on sorghum were studied which reported significant decrease in plant height, leaf area, chlorophyll index and total dry matter (Ramya et al., 2022). Significant decrease in growth parameters and physiological responses under moderate and extreme drought stress were observed in various varieties of Asparagus (Namaki et al., 2022).

Drought stress alters plant physiology in terms of root architecture changes, stomatal closure, limiting photosynthesis, reduced germination rate and changes in metabolic profile of root exudates which play major role in plant-microbe interaction.

Drought also have dominant impact on microbiota of soil, which can lead to changes of microbial community compositions (Omae & Tsuda, 2022). Drought also decreases the nutrient uptake by plant. Water deficiency leads to alteration of activity of soil enzymes which may affect nutrient availability in soil (Omae & Tsuda, 2022). Fitzpatrick et al., in 2019 reported prominent impact of competition and watering on plant fitness. They observed increased plant fitness in drought conditions in the presence of soil microbes. They concluded that soil microbes can have large effects on plant fitness, depending upon the environment and individual plant genotype. Peanut possess characteristic trait of legumes of developing symbiotic relationship with soil bacteria for biological nitrogen fixation. Drought stress in reported to have adverse effect on soil micro biome. According to a study conducted by Xu et

al. in 2020, decrease in the relative abundance of Proteobacteria and Actinobacteria was observed in drought condition as compared to normal condition (Y. Xu et al., 2020).

2.2. Role of Flavonoids in drought stress

Drought is a major environmental abiotic stress that disturbs homeostasis of plant. General responses for adaptation include morphological adaptation, physiological, biochemical and molecular conformation. Plants produce thousands of different secondary metabolites which play very little to no role in normal growth and development of a plant but instead assist an organism in their interaction with other biotic and abiotic factors of environment by perform secondary functions like defense against pathogens, pests, and herbivores, response to environmental stresses, and mediating interactions between different organisms. These secondary metabolites also adds on to the economic value of plant due to their bioactive properties and their use in products like pharmaceuticals, dyes, food industry and fragrances etc. (Pang et al., 2021). Studies has shown that these secondary metabolites play major role in developing significant interaction between plant and microbiome present in soil rhizosphere (Nakabayashi & Saito, 2015). When facing certain stress, metabolic profile of plant changes which can eventually lead to changes in microbiome of soil (Pang et al., 2021). These secondary metabolites are divided in categories like alkaloids, terpenes, steroids, sterols, polyphenols, flavonoids, fatty acids an amino acid etc. depending upon their functional groups.

Polyphenols is a major category of secondary metabolites that consists of at least one phenol functional group in their structure and provide defense against various biotic and biotic stresses (Mutha et al., 2021). Flavonoids are most isolated and diverse group of polyphenols that are majorly responsible for color and fragrance in plants. They possess bioactive properties in terms of antioxidant, antiviral, antibacterial, anti-inflammatory and anti-allergic potentials. (Mutha et al., 2021). More than 8000 different flavonoids have been extracted and identified in plants (Bag et al., 2022). Flavonoids are the non-enzymatic secondary metabolites that are considered to play an important role in mitigating drought stress. They are antioxidant in nature and are capable of inducing drought tolerance by fighting off oxidative damage caused due to ROS accumulation during drought stress. This property of flavonoid has been observed in maize plant (Li et al., 2021). Level of flavonoids and expression of

flavonoid biosynthetic genes were observed at multiple time points of drought stress in *Achillea pachycephala*. Up regulation of flavonoid biosynthetic genes was observed at the beginning of drought stress due to higher ROS accumulation. Higher concentration of flavonoid and expression of relevant genes was observed in the later staged of drought (Gharibi et al., 2019). Increased level of antioxidant enzymes and enhanced biosynthesis of flavonoids was observed *Bupleurum chinense* when exposed to drought stress (L. Yang et al., 2020). Kubra et al. in 2021 performed study on flavonoid biosynthesis in multiple genotypes of peanuts under water deficit conditions and observed the increase in level of flavanols, anthocyanins and phenolic under drought condition in leaves as well as root. Molecular analysis also revealed the up regulation of flavonoid biosynthetic gene which help in coping the oxidative stress in peanut due to ROS accumulation during drought (Kubra et al., 2021). In another study on peanut by Nabi et al., in 2022, flavonoid were quantified in drought sensitive and tolerant varieties. Relatively lower concentration of flavonoids were observed in drought sensitive varieties then drought tolerant varieties, resulting in negative effects of drought stress on growth parameters and productivity of peanut (Nabi et al., 2022).

Root exudation is a major carbon sink for plants that helps in various function of plants. Exudates usually consist of variety of second metabolites of small molecular weights that perform multiple functions for plants. Exudates are composed of primary metabolite like monoacids, carbohydrates as well as secondary metabolites like flavonoid, coumarin etc. (Chai & Schachtman, 2022). Studies have shown that root exudates play an important role in survival of plants if exposed to abiotic stresses. They have critical role in coping drought stress hence various studies has been performed to find ways to enhance root exudates for better performance of plants (Chai & Schachtman, 2022). Variation in root exudates pattern was observed in maize when exposed to single or combined stresses (Tiziani et al., 2022). Effects of abiotic stress on root exudation have been studied and it was concluded that percentage composition of various secondary metabolites in root exudates changes when plant is exposed to drought. Secondary metabolites like flavonoids (acacetin), ABA, aspartic acid and amino acid (Leucine) were found in higher concentration in root exudates of *Quercus ilex* , when subjected to drought (Gargallo-Garriga et al., 2018).

Flavonoids present in root exudates are considered as important signaling molecule in process of nodulation in legumes. They are perceived as chemical signal for expression of genes

coding for NOD factors in rhizobia which initiate symbiotic infection between plant and soil microbes. Many legumes produce certain specific Flavonoids which help in nodulation (Liu & Murray, 2016). BNF in legumes is altered in legumes when exposed to drought stress, even before photosynthesis and transpiration. Drought effects BNF by reducing survival of rhizobia community, leading to downfall in mass and number of nodule formation, and eventually decreasing the activity of nitrogenase and leghemoglobin concentration in root nodules of soybean (de Freitas et al., 2022). Increased level of Naringenin, a flavanone, were observed in the exudates of peanut plant subjected to water deficient condition, which enhanced plant-microbe interaction and eventually reversing negative effects of stress (Cesari et al., 2019).

2.3. Impacts of Biochar use as an organic amendment

Biochar is a carbon rich organic material that is obtained from pyrolysis of the agricultural residue and livestock wastes in the presence of minimal or no oxygen. It can also be prepared from municipal solid wastes as well as from sludge, solid waste generated during waste water treatment (Wang & Wang, 2019). Biochar has unique feature of stable structure, large surface area, adsorption ability, cation exchange capacity and high carbon content which gives it a great importance in field of agriculture. It provide an important source for remediation, decontamination and carbon sequestration (Wang & Wang, 2019). Biochar act as a conditioner of soil by enhancing water holding capacity and cation exchange capacity of soil, maintaining pH of soil, resulting in stabilizing the soil medium for plant growth (Mansoor et al., 2021b). Recent studies has shown enhanced growth and development of plants in water deficit conditions when soil is amended with biochar. Biochar enhances soil water content results in growth, photosynthetic rate and productivity. Biochar also helps in improving xylem water potential, chlorophyll content and stomatal conductance (Mansoor et al., 2021b)

Biochar has the ability to enhance plant growth, development and productivity. Biochar can significantly improve peanut biomass, morphological traits, physiological performance in terms of photosynthesis and nitrogen fixation, resulting in more yield and better-quality product. It can also increase soil nitrogen that is left behind by legumes. This also makes it an important strategy in crop rotation practices (C.-Y. Xu et al., 2015). Studies have shown that biochar can improve soil properties by enhancing nutrient availability in soil and prevent it leaching hence also play an important role in retention of nutrients in soil. While the capacity

of biochar to improve soil quality rather depends upon the source of biochar from which it is made and production conditions (Igalavithana et al., 2016). Performance of biochar in terms of improving plant growth and stabilizing soil conditions is due to its large surface area, porous nature and cation exchange capacity (Xie et al., 2016).

In last 20 years, Biochar has become focus of interest in field of research to mitigate drastic damage caused by various abiotic stresses. Recent studies have demonstrated effective use of biochar in reversing the negative effects of abiotic stresses of plants morphological and physiological traits. Impact of biochar in drought conditions was tested on *Chenopodium quinoa* Willd in 2011 by using three different proportions of biochar. Increase in growth parameters of plant treated with biochar and drought tolerance was observed as compared to stressed plant without biochar (Kammann et al., 2011). Similar results were observed in *Brassica compestris* when treated with poultry manure biochar in presence of heavy metal contamination and drought (Fiaz et al., 2014). Biochar enhances soil properties while gypsum is known to improve plant productivity and nutrient availability in soil. Application of biochar alone was observed to be more effective in providing resistance against drought as compared to combine application of biochar and gypsum (Batool et al., 2015). Effect of biochar on wheat under drought stress was analyzed at different growth stages and was revealed to be capable of mitigating adverse damage caused by drought (Haider et al., 2020).

Biochar is not only useful for drought stress, but it has also shown remediation abilities against other abiotic stresses like heavy metals, salinity etc. It has proven to improve plant growth and development under saline conditions in sorghum (Ibrahim et al., 2021). When subjected to dual stress of limited water and salinity, biochar has shown positive results in mitigating both independent and combined damage caused by drought and salinity stress in quinoa (A. Yang et al., 2020). Biochar has been used for remediation of contaminated soil. It is found to be more effective in reducing heavy metal content in soil and its uptake by plant, when compared to other organic amendments like compost (Irfan et al., 2021). Biochar has been found effective in decreasing cadmium accumulation in roots in pepper but somehow ineffective when cadmium is present in very high concentration (D. Xu et al., 2016). Similar results were observed in rapeseed (*Brassica napus* L.) when exposed to heavy metal stress. Biochar application showed significant effects of morphological traits of plants including root and shoot length, fresh and dry weight. Enhanced antioxidant enzymatic activity was also

observed (Kamran et al., 2020).

2.4. Role of plant growth promoting rhizobacteria

Plant growth promoting rhizobacteria or shortly PGPRs are soil bacteria present in soil rhizosphere where they interact with plant through metabolites released in the form of root exudates. These microbes build symbiotic association with plant hence improve plant growth and development. These bacteria perform functions like nitrogen fixation, decomposition on organic matter for nutrient recycling, bioremediation of contaminated soil and helps plant in both biotic and abiotic stress conditions. Various studies has shown that PGPRs have the ability to mitigate drought stress in many different ways giving significant results in terms of stable plant growth (Ahluwalia et al., 2021). Manipulation of below ground microbial community has shown significant effects on plant growth in stress conditions. Multiple PGPR strain were observed to have significant impact on growth and antioxidant enzyme status in at different levels of drought stress in *Oryza sativa* (Rice) showing their potential in improving drought tolerance (Gusain et al., 2015). Application of PGPRs in *Cicer arietinum* L. enhanced lipid peroxidation, proline content in leaves and alteration in metabolic profile of plants which can reduce damage caused by drought (N. Khan et al., 2019). Up regulation of stress responsive genes in *Capsicum annuum* L. (pepper) was observed when inoculated with *Bacillus licheniformis* K11 in drought stress conditions (Lim & Kim, 2013).

Use of PGPRs in combination with other organic amendments has also been widely studied in various crops which has shown significant results. ACC deaminase produced by bacteria is helpful in dissociating ACC which cause lesser production of stress induced ethylene. Combine use of AAC deaminase producing bacteria and biochar was found to be effective in improving plant growth in *Zea mays* (Maize) under mild and severe drought stress (Danish et al., 2020). Similar results were observed in wheat. Co-application of ACC deaminase producing PGPRs and timber waste biochar enhanced photosynthesis, chlorophyll content, transpiration rate and grain weight (Danish & Zafar-ul-Hye, 2019). Biochar in combination with Bacillus sp. Showed maximum increase in photosynthesis and enzymatic activity while combine application of Biochar and *P. phytofirmans* showed increase in grain yield, when applied on Soybean in drought condition (Nawaz et al., 2022).

Recently, bio fertilizers are becoming topic of interest in field on agriculture. Bio fertilizers are microbe-based preparations containing beneficial strains of rhizobacteria which converts

soil nutrients to consumable form for plant. In Pakistan, Bizote MAX is commercially available PGPR based bio fertilizer that is produced and distributed by PARC, Pakistan (Khan et al., 2017). Root dipping method was found to be more effective than seed coating for enhanced growth and yield in rice (ULLAH et al., 2017). Improved productivity was observed in pea plant in terms of pod length pod yield, number of nodules, dry root biomass and shoot dry biomass when treated with Biozote-N combined with compost (Panazai et al., 2019).

Biozote combined with finely grounded biochar showed enhanced plant height, leaf area and leaf area index, biological yield, and grain weight in maize crop in legume-cereal crop rotation system. Increase in soil fertility was also observed at residual level (Ali et al., 2022).

2.5. Objective of study

The aim of this study was to investigate the effects of varying ratio of biochar along with use of biofertilizer against drought stress.

The specific objectives of this study were:

- To investigate the impacts of biochar and PGPR based biofertilizer on morphological growth pattern of selected variety of peanut in severe drought stress.
- To evaluate the impacts of organic amendment treatments on the physiological and biochemical profile of peanut plant under drought conditions.
- Unravel the alteration of metabolomic profile of root exudates due to presence of biochar and PGPR based biofertilizer to assess nodulation in Peanut under drought stress.

CHAPTER 3: MATERIAL AND METHODS

3.1. Glass house experiment

3.1.1. Soil and seed collection

Fresh seeds of six different varieties of *Arachis hypogaea* were collected for experimental purposes from oilseed research department of National Agriculture Research Center (NARC) Islamabad. Sandy loamy soil was obtained from the research fields designated for peanut cultivation and growth.

3.1.2. Variety screening and germination rate

Seed of each variety, 20 seeds per variety including Pothowar, Bari 2011, BARD-92, BARD-479, Golden and NARC-2019 were surface sterilized by soaking them in 70% ethanol for 1 minute, followed by soaking in 5% sodium hypochlorite (bleach) for 3 to 5 minutes. After sterilization, seeds were washed with distill water multiple time to remove any residues of chemicals used and were air dried in safety cabinet. Sterilized seeds were then aligned on filter paper, placed in a germination box wrapped in aluminum foil to minimize light penetration and shifted to dark place with temperature of 25°C-28°C for 5 days. Germination rate of each variety was calculated by dividing number of healthy seedlings having same growth rate by total number of seeds used in the test and multiply by hundred. NARC 2019 was selected for experiments as it had highest germination rate.

Table 1 Calculated germination rate of different varieties of peanut

S. No	Variety	Total seeds used	Germinated seedling	Germination rate
1	**Pothowar**	20	15	75%
2	**BADR-479**	20	13	65%
3	**BARD-92**	20	16	80%
4	**Golden**	20	16	80%
5	**Bari-2011**	20	15	75%
6	**NARC-2019**	20	19	95%

3.1.2. Soil preparation and pot experiment

Six different treatments were designed for pot experiment characterized with variable ratios and combinations of biochar and PGPRs. Three different type of soil was prepared for experiment, i.e., only soil, soil treated with biochar at two different W/W ratios i.e., 1% and 2% of the total weight of soil. Cotton straw biochar was used for the experiment. 96 Pots were

filled with untreated soil, soil containing 1% of biochar and soil containing 2% of biochar. Each pot contained 1kg soil. All the pots were watered for two weeks before seedlings transplantation for biochar to absorb as much water as it can. For plantation, seeds were divided into two batches, one batch of seeds was sown uncoated in all three types of soil, while second batch was coated with Biozote max, a biofertilizer containing PGPRs obtained from NARC, Islamabad. The seeds were wetted with a sugar solution, coated with PGPRs, and air-dried before being sown in soil.

3.1.3. Water holding capacity of soil

For the measurement of water holding capacity, three pots were filled with 1kg of soil, one pot for control group or untreated soil, two pots with soil containing biochar of different ratio. Water was added to all three pots till it start dripping from the bottom of the pot. Pots were placed on inverted sieve for 4 hours till the water stopped dripping. Pots were weighted with electrical balance and was named as W1. Then soil from each pot was oven dried at 60ºC for 48hrs, weighted again and named as W2. 100% Field capacity of each type of soil was calculate by formula given as under:

$$\mathrm{WHC} = \frac{\mathrm{W1} - \mathrm{W2}}{\mathrm{W1}} \times 100$$

Where,

W1= weight saturated soil (soil +water)

W2= weight of dried soil

W1-W2= maximum amount of water soil can hold.

Table 2 Water holding capacity/Field capacity or soil

Soil type	W1	W2	100% FC	70% FC	30%FC
Control group	1250 g	1000 g	250 ml	175 ml	75 ml
1% biochar	1261 g	1000 g	261 ml	182.7 ml	78.3ml
2% biochar	1269 g	1000g	269 ml	188.3 ml	80.7 ml

3.1.4. Plant growth, stress induction and sampling

Six different treatment consisting varying ratio of biochar and PGPR coated seeds were designed. Each treatment group consist of 12 replicates. Three seed per pot were sown. After 10 days of sowing, the process of thinning was conducted, resulting in the removal of excess

seedlings and leaving only one seedling per plant that exhibited consistent growth. Optimum growth conditions of 16/8 h of light and dark period, temperature of 25-32ºC and up to 60% humidity were strictly maintained and monitored. All plants were grown on 70% FC for two weeks. Drought stress was induced as explained by Kubra et al. (2021) Six treatments were subdivided into two groups, having 6 replicates each. One group was designated as the control group, consisting of plants growing under normal conditions (70% FC), while the other group was referred to as the treated group, in which drought stress was induced. The induction of drought stress was achieved by reducing watering to 30% of FC. The weight of the pots was monitored daily for a duration of 14 days to track changes in weight. On the 14th day, three randomly selected replicates from the stress period were harvested for data collection and sampling. These replicates were used for morphological, physiological and biochemical assessment of plants in drought conditions. The remaining three replicates were subjected to rewatering, bringing the moisture level up to 70% of FC, and were then harvested after an additional 7-day duration for further assessment. Leaf and root samples were promptly frozen in liquid nitrogen and subsequently stored at -80°C for laboratory testing. Experiment plan is explained in detail in Table 3.

Table 3 Experiment Plan

Treatment	Control group (70% fc)	Treated group (30% fc)
T1	Sole peanut plant	Peanut plant + Stress
T2	Peanut plant + 1% BC	Peanut plant + 1% BC+ Stress
T3	Peanut plant+2% BC	Peanut plant + 2% BC+ Stress
T4	Peanut plant + PGPRs	Peanut plant + PGPRs+ Stress
T5	Peanut plant + 1% BC+PGPR	Peanut plant + 1% BC+PGPR+ Stress
T6	Peanut plant + 2% BC+PGPR	Peanut plant + 2% BC+PGPR+ Stress

Figure 3 Glass house experiement

3.2. Morphological traits analysis

On 14th day of stress, morphological data was collected. Number of leaves were counted manually. Shoot and root length was measured in cm with measuring tape. Fresh weight of shoots and roots was measured with electrical weighting balance having LC of 1mg. After uprooting the plants. Roots were washed thoroughly with deionized water to remove soil and fresh weight and root length was measured. Number of nodules were counted manually in roots. For dry weight of plants, fresh roots and shoots were oven dried at 60ºC for 24 hours and values were taken by electrical weighting balance. Leaf area was measured by using Image j software. Images of leaves were taken by Canon. Canoscan LiDE 120 and leaf area, median and mode were measured using software.

3.3. Physiological traits analysis

3.3.1. SPAD value

SPAD value is a measurement used to estimate the chlorophyll content in plant leaves. It is a non-destructive and rapid method that provides an indirect assessment of chlorophyll levels in plants. The correlation between chlorophyll content and SPAD value has been described by Ling et al. (2011). SPAD value was measured by using SPAD 502 meter. Fully expanded third leaf from the top was used for observations. Three reading per replicate were noted for analysis.

3.3.2. Relative water content

Relative water content is the measure of water status or hydration level of a plant tissue. Fully expended third leaf from the top was used to determine RWC as described by Smart & Bingham, (1974). Leaf was plucked, edges were slightly cut to remove cuticle layer to make it easy to absorb water. Fresh weight of leaf tissue was noted. Leaf tissues were submerged in distill water for 24 hours. Turgid weight was noted after surface drying with absorbent paper towel. Leaf tissue were then oven dried at 70ºC for another 24 hours and dry weight was determined. RWC was calculated by using following formula:

$$\text{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

3.4. Biochemical assays

3.4.1. Superoxide dismutase (SOD) enzyme activity

SOD activity was measured by following the method explained by Elavarthi & Martin (2010). 0.5 g of fresh leaf sample was grinded into fine powder in liquid nitrogen and homogenized in 8ml PBS buffer. Sample was centrifuged at 8,000 rpm for 30 minutes at 4ºC. Supernatant was collected in a separate tube and stored on ice. Reaction mixture was prepared using 1ml PBS buffer, 33µl EDTA, and methionine, NBT, and Riboflavin, 66µl each. 50µl of crude extract was added to reaction mixture and absorbance was measured at 540nm through spectrophotometer. Activity was calculated using the formula:

$$SOD = \frac{(Ack - Ae) \times V}{0.5 \times Ae \times W \times vt}$$

Where Ack= OD value for the control tube under light conditions (at 4000 lux for 20 minutes)

Ae = OD value on the spectrophotometer

V= Total volume of the buffer solution used to extract the enzyme

W= Fresh weight of the sample

vt = Amount of enzyme extract used in reaction solution to test SOD

3.4.2. Peroxidase (POD) enzyme Activity

Peroxidase activity was measured by following the method explained by Elavarthi & Martin (2010). 0.5 g of fresh leaf sample was grinded into fine powder in liquid nitrogen and homogenized in 8ml PBS buffer. Sample was centrifuged at 8,000 rpm for 30 minutes at 4ºC. Supernatant was collected in a separate tube and stored on ice. Reaction mixture was prepared using 2.7ml PBS buffer, 100µl Guaicol, and 100µl 30% hydrogen peroxides (H_2O_2). It was taken as a blank. 50 µl of enzyme extract was added to reaction mixture and absorbance was measured at 270 nm. POD activity was calculated by formula:

$$POD\ Activity = \frac{O.D \times V/vt}{E \times \mathrm{W}}$$

Where O. D= Absorbance of sample at 270nm

V= Volume of PBS buffer used for enzyme extraction

vt=Volume of extract used

W= weight of fresh leaf sample used for extraction

E= Activity constant that is 26.6 mM/cm.

3.4.3. Catalase (CAT) enzyme Activity

Peroxidase activity was measured by following the method explained by Elavarthi & Martin (2010). 0.5 g of fresh leaf sample was grinded into fine powder in liquid nitrogen and homogenized in 8ml PBS buffer. Sample was centrifuged at 8,000 rpm for 30 minutes at 4ºC. Supernatant was collected in a separate tube and stored on ice. Reaction mixture was prepared by combining 2.8ml PBS buffer and 100µl 30% hydrogen peroxides (H_2O_2). It was taken as blank. 100µl enzyme extract was combined with reaction mixture and absorbance was measured ay wavelength of 240nm. CAT activity was calculated by formula:

$$CAT\ Activity = \frac{O.D\ \times V/vt}{E \times \mathrm{W}}$$

Where O. D= Absorbance of sample at 240nm

V= Volume of PBS buffer used for enzyme extraction

vt=Volume of extract used

W= weight of fresh leaf sample used for extraction

E= Activity constant that is 39.4mM/cm.

3.4.4. Ascorbate peroxidase (APX) Enzyme activity

Ascorbate Peroxidase activity was measured by following the method explained by Elavarthi & Martin (2010). 0.5 g of fresh leaf sample was grinded into fine powder in liquid nitrogen and homogenized in 8ml PBS buffer. Sample was centrifuged at 8,000 rpm for 30 minutes at 4ºC. Supernatant was collected in a separate tube and stored on ice. Reaction mixture was prepared by combining 2.8ml PBS buffer, 100µl of Ascorbic acid (ASA), and 100µl 30% hydrogen peroxides (H_2O_2). It was taken as blank. 100µl enzyme extract was combined with reaction mixture and absorbance was measured ay wavelength of 290nm. CAT activity was calculated by formula:

$$APX\ Activity = \frac{O.D\ \times V/vt}{E \times \mathrm{W}}$$

Where O. D= Absorbance of sample at 290nm

V= Volume of PBS buffer used for enzyme extraction

vt=Volume of extract used

W= weight of fresh leaf sample used for extraction

E= Activity constant that is 2.8mM/cm

3.4.5. Glutathione transferase (GST) Activity

Activity of glutathione transferase was measured by following the method of (Chen & Zhang, 2016). Crude enzyme extract was prepared in 100Mm PBS buffer. Reaction mixture was prepared by combining 0.4ml of 5Mm of GSH and 0.8ml of 1.5Mm of CDNB. 50 µl of crude enzyme extract was combined with reaction mixture and absorbance was measured at 340nm at every 15 seconds for 2 minutes through UV spectrophotometer. Activity of GST was calculated using the following formula:

$$GST\ Activity = (\Delta A - \Delta Ack) \times \frac{\frac{V}{Vt}}{t}/Cp$$

Where ΔA= Change in absorbance value of sample during every 15 sec

ΔAck= Change in absorbance of control every 15 sec

V= Total volume of crude enzyme extract

Vt= Volume of extract used in test

t= Reaction time

Cp= Crude protein concentration in mg/ml.

3.4.6. Malondialdehyde (MDA) quantification

MDA accumulation due to lipid peroxidation was measured as described by Senthilkumar et al., 2021. Crude protein extract was prepared by grinding frozen leaf sample in liquid nitrogen

and homogenize it in 0.1% of TCA. Sample was centrifuged at 12.000 rpm for 15 minutes; supernatant was transferred into new tube and was mixed with 4ml of 20% TCA containing 0.67% of TBA. Mixture was boiled at 95ºC for 15 minutes and immediately cooled on ice bath for 10 minutes to stop the reaction. Mixture was again centrifuged at 10,000 rpm for 5 minutes. Supernatant was collected and absorbance was measured at 532 and 600 nm through UV spectrophotometer. Concentration of MDA was calculated by following formula:

$$MDA\ conc. = A532 - A600) \times Vt \times (V/Vt)/\varepsilon \times 1000/Cp$$

Where A_{532}= Absorbance at 532nm

A_{600}=Absorbance at 600nm

Vt= volume of extract used in test

V=Total volume of extracts

ε = Extinction coefficient (1.53 $Mm^{-1}cm^{-1}$) and

Cp= Crude protein concentration

3.5. Scanning electron microscopy (SEM) and energy-dispersive X−ray spectrometry

SEM was performed to unravel the stomatal behavior of plant in variable condition. Leaf sample fixation was performed as stated by Talbot & White, 2013. Fresh, fully expanded leaf from plants were collected and suspended in 100% methanol for 10 minutes followed by shifting into absolute ethanol for 30 minutes. Leaves were then subjected to air drying at room temperature. For observation through SEM, gold coating was performed. Images were taken at magnification ranging from 1000x to 5000x magnification.

For elemental composition of plant tissues, energy-dispersive X−ray (EDX) spectrometry (model JSM 6490A; JEOL Ltd) was used. For analysis, voltage of 15 kV and current of 12 μA was applied.

3.6. Root exudates metabolites profile

To evaluate the effect of organic amendment on nodulation in peanut, GCMS of root exudates was performed. For collection of exudates, methods described by Ankati & Podile, (2019) and

Gargallo-Garriga et al. (2018) were followed. Plants were grown in pots for 2 weeks in normal conditions. Drought stress was induced for 14 days. After 14 days, plants from both control group and stressed group were uprooted. Roots were thoroughly washed with distill water to remove any soil and other impurities and immersed in tubes containing 50 ml of sterile distill water for 48 hours at room temperature. Collected exudates were centrifuged at 8000 rpm for 15 minutes to removes any impurities, filtered through 0.22µM Syringe filter and lyophilized. Lyophilized samples were dissolved in 80% methanol and incubated on ice for 2 hrs. The samples were subjected to centrifugation at 8200 × g for 5 minutes at a temperature of 4 ℃. Following centrifugation, the supernatant was freeze-dried and subsequently dissolved in 70% methanol for GC-MS/MS analysis.

3.6. Statistical Analysis

All the data collected was statistically analyzed using R Studio Software, version 4.2.3. Means and standard deviations of the data was calculated. Two-way ANOVA was used to find significance of the data while p values were calculated by Tukey's HSD post-hoc test. Data was plotted in bar plots through R studio. For Pearson correlation test, corrplot package was used.

CHAPTER 4: RESULTS

CHAPTER 4 RESULTS

4.1. Co-application of biochar and PGPRs enhances morphological growth of peanut plant in drought conditions

4.1.1. Leaf count

Number of leaves were counted manually for each replicate of all treatments namely T1 (Soil only), T2 (BC 1%), T3 (BC 2%), T4 (PGPRs), T5 (BC 1% + PGPRs) and T6 (BC2% + PGPRs) in both control and experimental group at two different growth stages i.e., 14 days drought stage, followed by 7 days of rewatering. Two-way ANOVA and Tukey's HSD post hoc test was used for normalization and calculation of p value for significance. Their mean value, standard deviations and p value were calculated by R studio.

Significant increase of 31.25%, 43.75%, 31.25%, 62.5% and 78.12% in number of leaves was observed respectively in T2 (BC 1%), T3 (BC 2%), T4 (PGPRs), T5 (BC 1% + PGPRs) and T6 (BC2% + PGPRs) as compared to T1 (soil only). In drought conditions, 31.25% of decrease in leaf count was observed in the absences of organic amendment whereas in the presence of biochar and PGPRs, this decline in leaf count seemed to be recovered by 77.29%, 90.9%, 100%, 54.56% and 81.84% in T2, T3, T4, T5 and T6 respectively, making their leaf count almost similar to their respective amendments in control group (Figure 2). On rewatering, significant increase in number of leaves was observed due to emergence of young leaves in previously stressed plants when stress was relieved indicating capability of speedy recovery of plant species from stress conditions.

In both control group and experimental group, T5 and T6 shows the most significant results in both growth stages reflecting their capability in enhancing growth of plant even in stress condition leading to the conclusion that they help the plant in coping with water deficit conditions.

4.1.2. Leaf Area

Pictorials of leaves from each replicate were taken through scanner and leaf areas was measured using ImageJ software at two different growth stages i.e., 14 days drought stage, followed by 7 days of rewatering. Two-way ANOVA and Tukey's HSD post hoc test was used for normalization and calculation of p value for significance. Their mean value, standard deviations and p value were calculated by R studio.

In 4-week-old plant, non-significant difference leaf area was observed in control group. In experimental group, leaf area decreased by 27.58% in plant of same age suffering for 14 days drought conditions. On the other hand, recovery of 8.13%, 19.72%, 21.95%, 23.2% and 63.47% in leaf area was observed in T2, T3, T4, T5 and T6 respectively when compared to control of experimental group.

On 2nd growth stage i.e., 7 days of rewatering, non-significant difference was observed in both control and experimental group. Hence it can be concluded that, leaf area remains unaffected when treatments are under control conditions but can be effective in maintain normal growth in drought.

4.1.3. Plant height

Height of plants were measured measuring tape for each replicate of all in both control and experimental group at two different growth stages i.e., 14 days drought stage, followed by 7 days of rewatering. Two-way ANOVA and Tukey's HSD post hoc test was used for normalization and calculation of p value for significance. Their mean value, standard deviations and p value were calculated by R studio.

In control group, none of the treatment showed significant enhancement in plant height. When subjected to 14 days drought conditions, 39.3% decrease in plant height was observed in the absences of organic amendment whereas in the presence of biochar and PGPRs, this decrease in plant height was observed to be recovered by 38.63%, 36.6%, 48.13%, 43.45% and 33.49% in T2, T3, T4, T5 and T6 respectively. Similar pattern of plant heights was observed on 2nd stage i.e., 7 days of rewatering. We can conclude that various combinations of Biochar and PGPRs has no effect on plant height but it helps plant in maintaining normal growth in water deficient conditions.

4.1.4. Shoot fresh and dry weight

Fresh and dry weight of shoots were measured using electrical balance with L.C of I mg at two different growth stages i.e., 14 days drought stage, followed by 7 days of rewatering. Their mean value, standard deviations and p value were calculated by R studio. Significance was determined by Two-way ANOVA and Tukey's HSD post-hoc test.

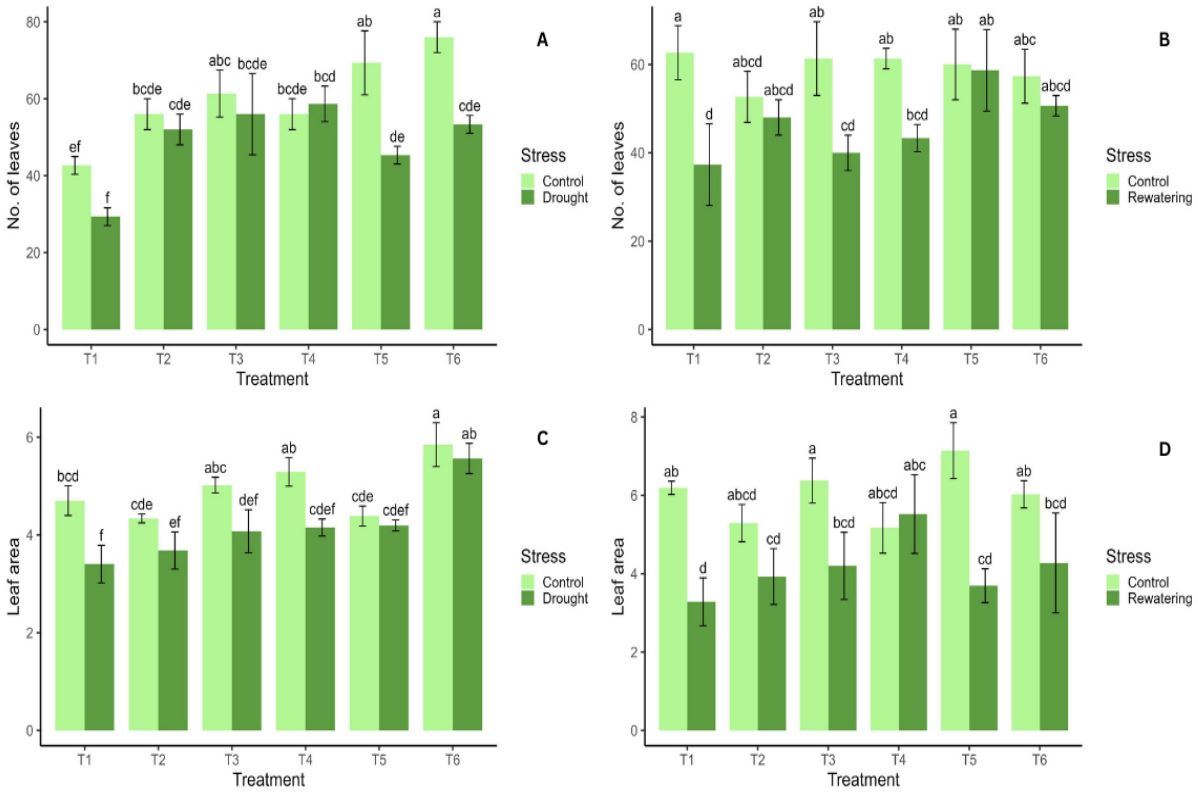

Figure 4. Effect of biochar and PGPRs application on leaves in two growth stages, A (Drought) and B (Rewatering). Data showing means and standard deviation of three biological replications (n=3), followed by Two-way ANOVA and Tukey's HSD post-hoc test to find significance. Treatments sharing different letterings are significantly different at $p < 0.05$.

In 4-week-old plant, maximum enhancement in fresh weight of shoots was observed in T3 and T6, 30% and 51% respectively, in control group. On the other hand, plant of same age suffering for 14 days drought conditions, almost 60% decrease was observed which seemed to be recovered in the presence of 2% biochar and PGPRs by 68% and 76% in T3 and T5 respectively. Similar pattern was observed on 2nd stage i.e., 7 days of rewatering. Fresh and dry weight of plants were significant in T1 of both control and experimental group representing the compromised shoot growth under drought stress, while insignificance was observed in rest of the treatments in both control and experimental group representing the normal shoot growth.

Graphical representation of dry weight in figure also shows the average increase in dry matter in T3 and T6 as compared to T1 in control group. A decrease of 59% in drought stressed plant was observed to be recovered 100% in T3 and T6, maintaining the normal growth of plant when compared with T1 of both control group and treated group. It concluded that drought decreases the growth rate of peanut plant which can be recovered by application of various combinations of Biochar and PGPRs in appropriate ratio.

4.1.4. Root length

Drought stress has immediate effect on root growth. Response of plant varies from specie to specie, including decrease in length while increase in branching and lateral growth, increase in root depth etc. Root length was measured by measuring tape. Mean, standard deviation and p value were calculated using R studio. Two-way ANOVA and Tukey's HSD post hoc test was used to determine significance.

In figure, it is shown that root length in T1 of experimental group has decreased by 40% as compared to T1 of control group, while in T2, T3, T4, T5 and T6, pattern of root length was found to be non-significant to each other but significant as compared to T1 of experimental group i.e., the drought stress. In 2nd growth stage, effect of amendments was found to be less significant reflecting the ability of rapid recovery of plant

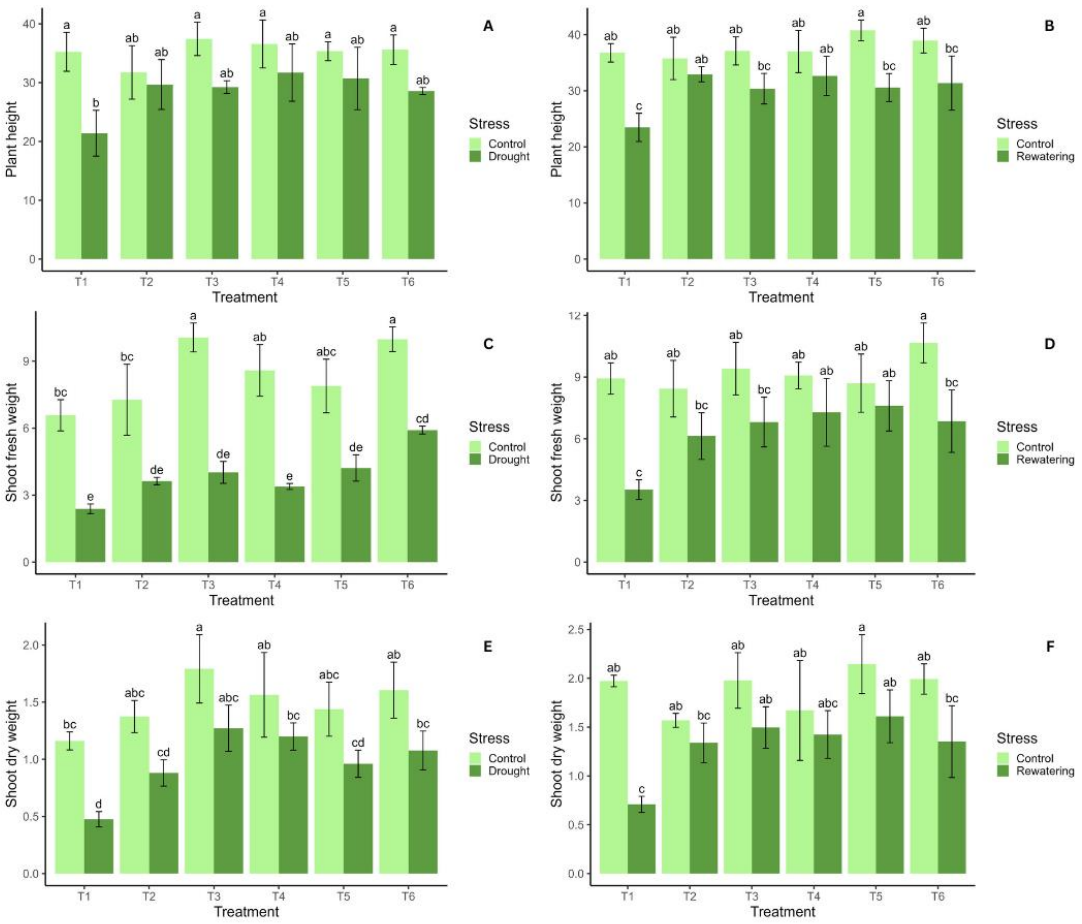

Figure 5. Effect of biochar and PGPRs application on shoot growth in two growth stages, i.e. Drought and rewatering. Data showing means and standard deviation of three biological replications (n=3), followed by Two-way ANOVA and Tukey's HSD post-hoc test to find significance. Treatments sharing different letterings are significantly different at $p < 0.05$.

4.1.5. Root fresh and dry weight

Fresh and dry weight of roots were measured using electrical balance with L.C of I mg at two different growth stages i.e., 14 days drought stage, followed by 7 days of rewatering. Two-way ANOVA and Tukey's HSD post hoc test was used for normalization and calculation of p value for significance. Their mean value, standard deviations and p value were calculated by R studio.

In 4-week-old plant, maximum enhancement in fresh weight of shoots was observed in T3 and T6 in control group. On the other hand, plant of same age suffering for 14 days drought conditions, almost 60% decrease was observed which seemed to be recovered in the presence of biochar and PGPRs by 100% in T5 and T6 as compared to T1 of control group. T3, T4 and T6 were observed to be effective in enhancing dry weight of plant as compared to control. In experimental group, non-significant difference was observed in all treatments, reflecting the capability of plant to maintain steady growth pattern.

Similar pattern was observed on 2nd stage i.e., 7 days of rewatering. T3 and T6 were found effective under control conditions showing maximum increase in fresh weight while in experimental group, T2, T3, T4, T5 and T6 were found to be nonsignificant as compared to T1 of control group showing their normal pattern of root growth. T3, T4, T5 and T6 were found effective in improving dry weight of roots in control group while in experimental group, difference in different treatments were non-significant.

4.1.6. Number of nodules

Number of nodules were counted at two different growth stages i.e., 14 days drought stage, followed by 7 days of rewatering. Two-way ANOVA and Tukey's HSD post hoc test was used for normalization and calculation of p value for significance. Their mean value, standard deviations and p value were calculated by R studio.

In 4-week-old plant, non-significant difference in number of nodules was observed in control group. In experimental group, plant of same age suffering for 14 days drought conditions, almost 60% decrease was observed. On the other hand. T4, T5 and T6, treatments containing PGPRs were observed to be effective in enhancing nodulation resulting in similar numbers to that of control group

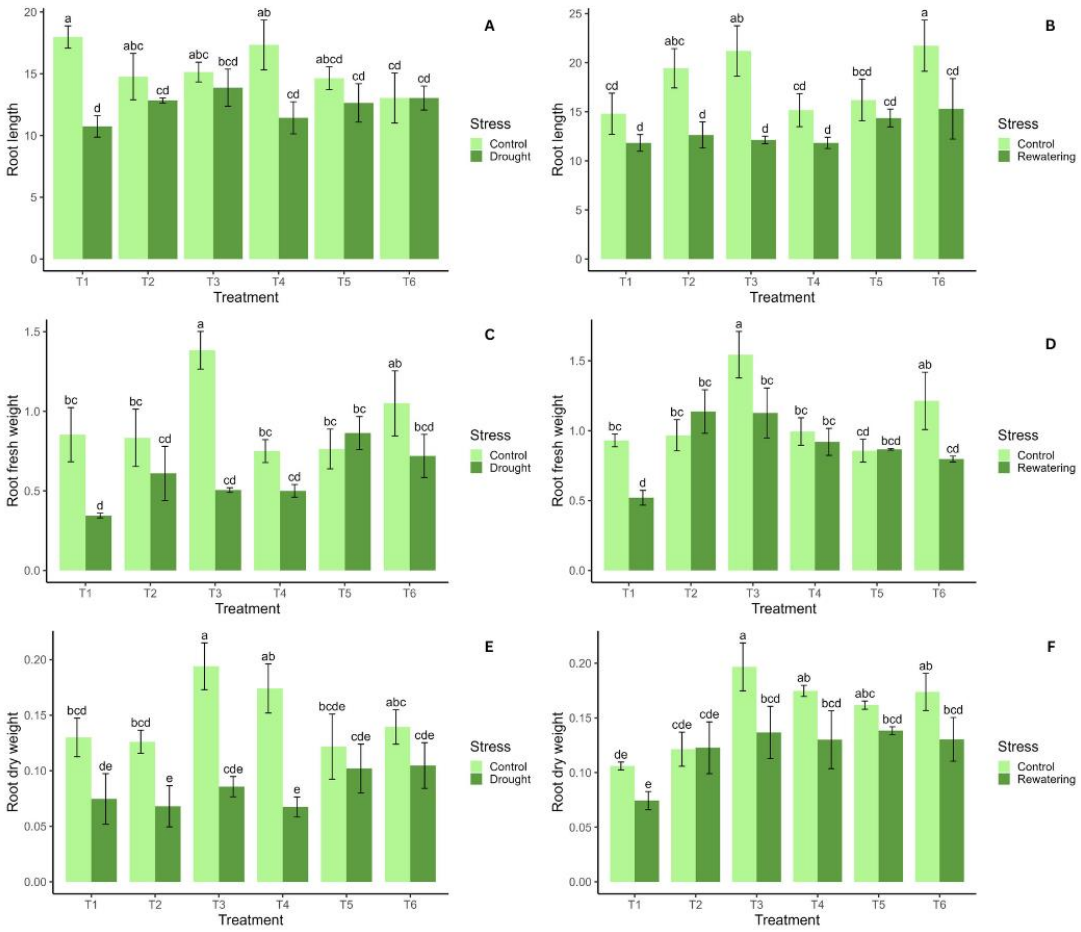

Figure 6. Effect of biochar and PGPRs application on root growth in two growth stages, i.e. Drought and rewatering. Data showing means and standard deviation of three biological replications (n=3), followed by Two-way ANOVA and Tukey's HSD post-hoc test to find significance. Treatments sharing different letterings are significantly different at $p < 0.05$.

Similar pattern was observed on 2nd stage i.e., 7 days of rewatering. T3, T5 and T6 were found effective under control conditions showing maximum increase in nodulation while in experimental group, T4, T5 and T6 were found to be significant as compared to T1of both control and experimental group. Hence it can be concluded that even though natural ability of nodulation in the selected plant variety is efficient, it is affected in drought but can be recovered when treated with PGPRs.

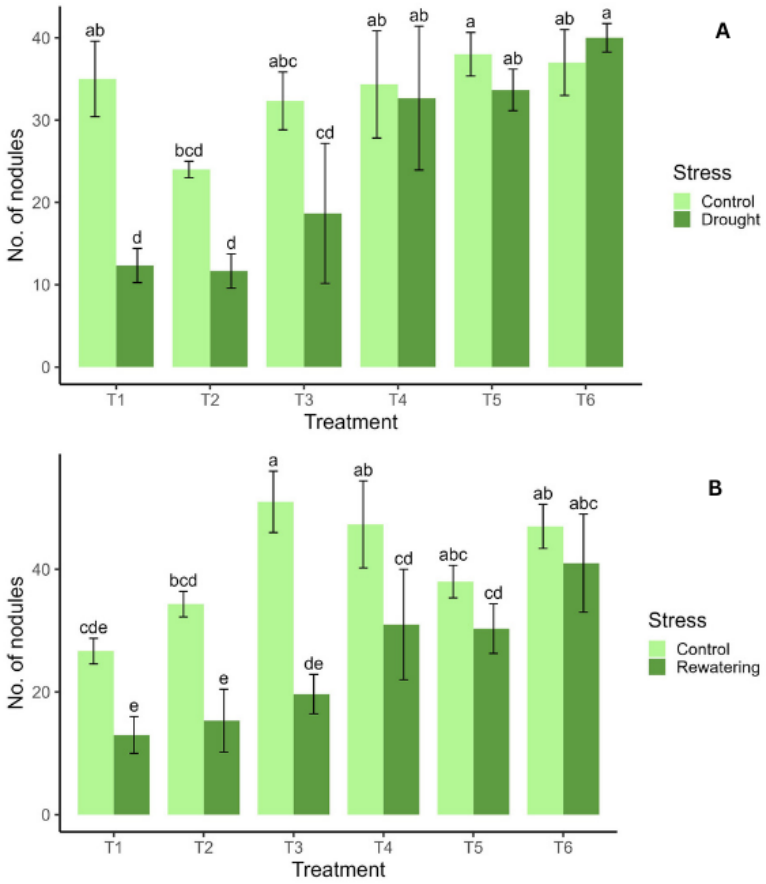

Figure 7. Effect of biochar and PGPRs application on root growth in two growth stages, i.e. Drought and rewatering. Data showing means and standard deviation of three biological replications (n=3), followed by Two-way ANOVA and Tukey's HSD post-hoc test to find significance. Treatments sharing different letterings are significantly different at $p < 0.05$.

4.1.7. SEM imaging

SEM was performed to analyze stomatal behavior under drought stress. Stomata were observed at different resolutions ranging from 1000x to 5000x and multiple images of different regions were taken. Widely open stomata were observed in control group, while they were tightly closed when subjected to 14 days drought stress. Stomata were relatively smaller in size showing drought resistance and adaptation properties of the variety.

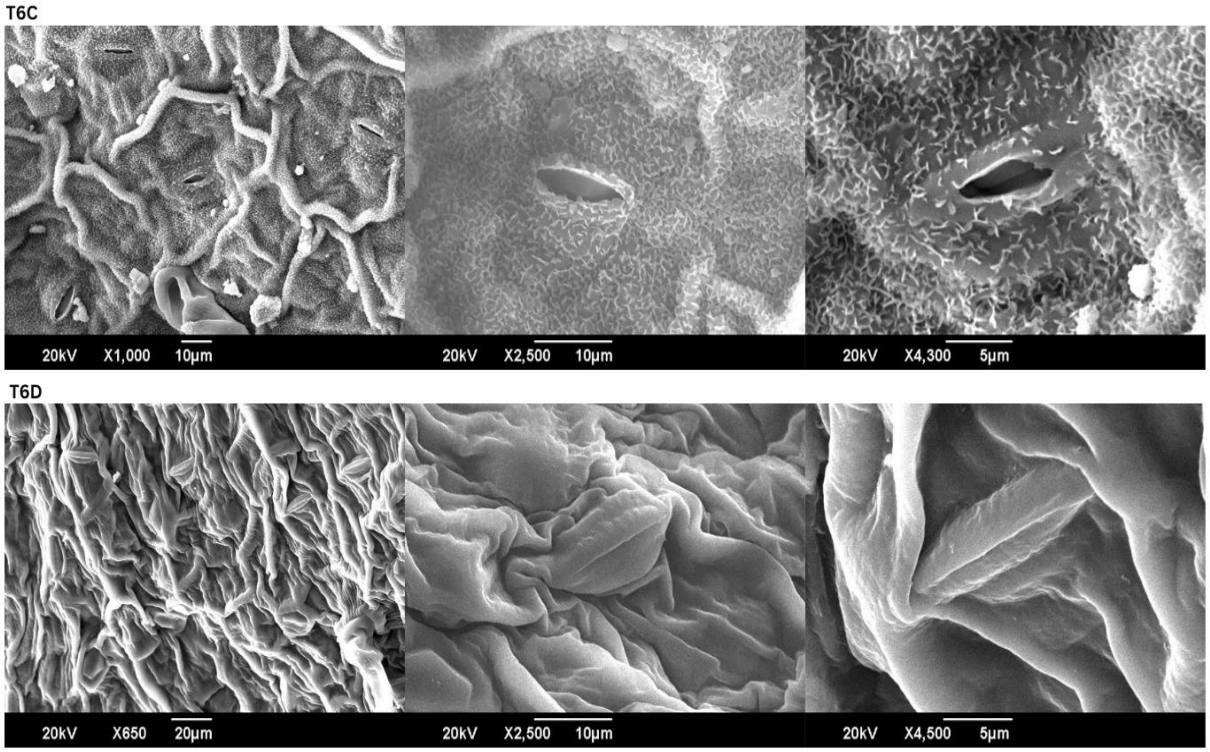

Figure 8 SEM images of leaf stomata of drought stressed plant under variable magnification and resolution

4.2. Physiological traits

4.2.1. Relative water content

Relative water content is one of the major drought stress indicating physiological trait as it is reduced in prolonged drought. Data was collected on two growth stages, i.e., 4 weeks old plants suffering from 14 days (2 weeks) of drought, followed by 1 week of rewatering. In 4-week-old plant, significant difference in RWC was observed between control and experimental group. 60% decrease in RWC of leaves was observed in drought stressed plants

which was recovered by 66%, 27.8%, 30.2%, and 31.7% in T2, T3, T5 and T6 respectively when compared to control of experimental group.

On 2nd growth stage i.e., 7 days of rewatering, T1 in both control and experimental group had significant difference while T2, T3, T4, T5 and T6 were non-significant in both control and experimental group. Hence it can be concluded that, addition of Biochar to soil by ratio of both 1% and 2% are effective in maintain water content during stress as well as speedy recovery when stress is relieved.

4.2.2. SPAD value

In plant physiology, SPAD value represent relative quantity of photosynthetic pigment, photosynthetic activity and plant health. SPAD value for each treatment at two time points was measured using chlorophyll meter SPAD-502 plus. Data was analyzed and plotted through R studio.

After two weeks of drought stress, significant difference between control and experimental group was observed. Variation in SPAD value in response to stress was found to be more significant. Biochar and PGPR treatment showed less significant effect on photosynthetic activity under control condition while more significant difference was observed in experimental group. At stage of rewatering, completely non-significant variation was observed in all the treatments of both control and experimental group.

We can conclude that although photosynthetic activity of plant seemed to be unaffected by biochar and PGPRs, its enhancement in drought conditions shows hyperaccumulation of chlorophyll as compensation strategy reflecting the stress resistance or tolerance ability of the selected variety.

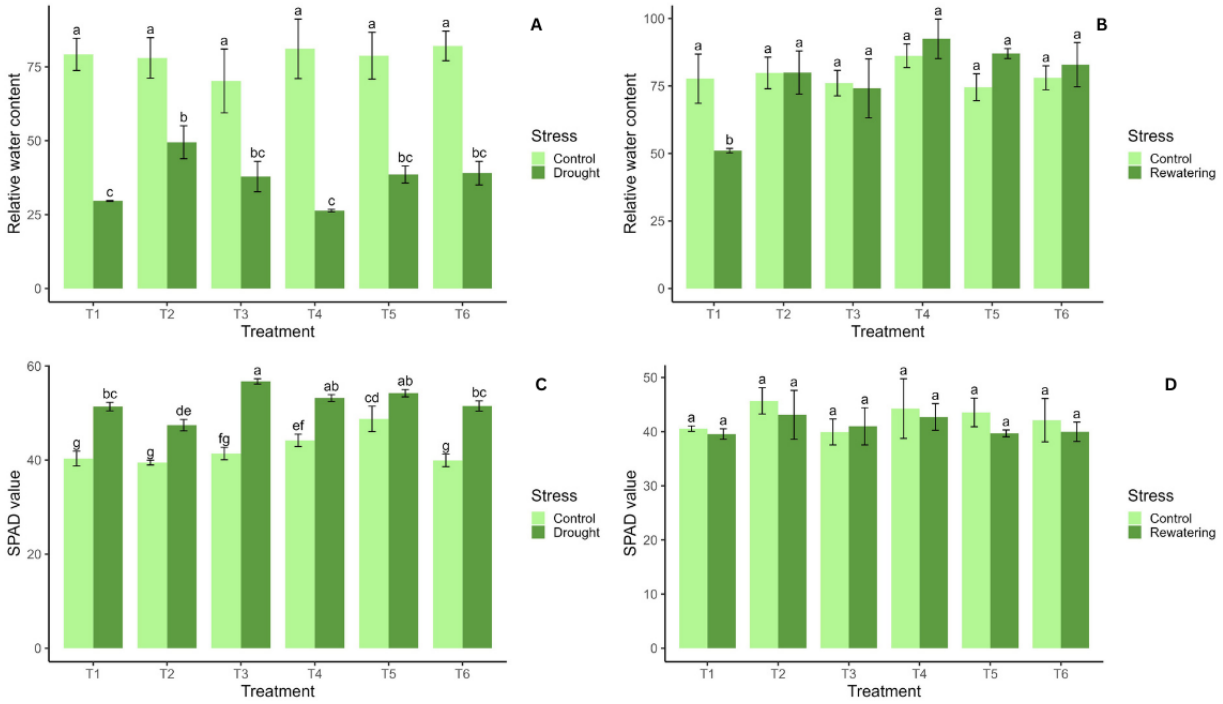

Figure 9. Effect of biochar and PGPRs application on physiological traits in two growth stages, i.e. Drought and rewatering. Data showing means and standard deviation of three biological replications (n=3), followed by Two-way ANOVA and Tukey's HSD post-hoc test to find significance. Treatments sharing different letterings are significantly different at $p < 0.05$.

4.3. Pre-treatment of soil with biochar and seeds with Biofertilizers decreases ROS accumulation resulting in lower antioxidant activity in plant

Activity of SOD, POD, CAT, APX and GST was measured and tests were performed to quantify MDA accumulation. Significance of data was analyzed using Two-way ANOVA combined with the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$) using R studio.

The activity of antioxidant enzymes including SOD, POD, CAT, APX and GST were observed to be increasing in the presence of drought stress while their activity was downregulated in the presence of biochar. The SOD value was found to be increasing under drought stress. In control group, a gradual decrease in levels of SOD was observed in T2 and T3 plants treated with biochar which increases with addition of PGPRs in T4, when compared to T1. In experimental group, highest levels of SOD activity were observed in T1 of experimental group. 30% increase in SOD activity experimental group was observed as compared to negative control of T1 which gradually decreased by 17%, 12%, 21%, 23% and 27% in T2, T3, T4, T5 and T6 respectively. Results showed less ROS accumulation in presence of BC and PGPRs under drought stress due to which activity of enzymes was found to be similar to negative control.

The POD activity was found to be increasing under drought stress. In control group, non-significant difference was observed in all the treatments. In experimental group, the levels of POD were observed to increase slightly (9.41%) in T1 as compared to negative control. POD activity in T2. T3, T4, T5 and T6 were found to be significant, compared to positive control while non-significant to control group representing minimum to no change in its concentration. Hence it can be concluded that in the presence of BC and PGPRs, drought induced ROS accumulation is reduced.

The upregulated activity on CAT was observed under drought stress. In control group, difference in catalase activity was non-significant in all the treatments. In experimental group, the levels of enzyme were observed to be increased significantly (128.9%) in T1, which decreased by biochar application in T2 (22.06%) and T3 (31.03%). PGPRs were also observed to be a factor stimulating CAT activity in T4, which was equal to that of positive control T1. T6 showed 16% decrease in catalase activity in comparison to positive control. It

can be concluded that BC has the ability to lower drought induced stress level on plant and maintaining its normal growth and development.

An increasing trend was observed in the enzymatic activity of APX under drought stress. In control group, APX activity remained unaffected in presence of biochar but increase be 105.4% in T4 in treatments involving PGPRs. On the other hand, in experimental group, its activity was observed to be increased by several folds in T1 (284.5%), which were downregulated by 18.14%, 34, 57%, 20.86%, 23.4% and 27.9% in T2, T3, T5 and T6 respectively due to the presence of BC.

Highest value of GST was observed in the presence of drought stress. Lowest values of enzymes were observed when plants were treated with 2% of biochar that are T3 and T6 in both control and experimental group. Difference between T2, T3, T4, T5 and T6 was found to be non-significant but were significant as compared to T1 of experimental group.

Accumulation of MDA due to membrane lipids peroxidation in stress condition was also measured through spectrophotometry. Results showed increased production of MDA in drought stress conditions (154.3%) as compared to T1 of control group which decreased significantly by 65.69%, 72.41%, 46.2%. 42.75% and 26.89% in T2, T3, T4, T5 and T6 respectively, when plant was treated with biochar.

From analysis of antioxidant activity status of peanut, it can be concluded that biochar has positive role in lowering the mitigating drought stress. A progressive increase in their activity reflects the higher level of stress. Biochar limits drought stress induced oxidative damage which is reflected in lower values of enzymatic activity. Our results coincide with other studies that shows similar pattern of variation in antioxidant activity in *Phragmites karka*,,*Tritecum aestivum* and *Vigna unguiculata* (Abideen, Z., et al. 2020; Zulfiqar , B., et al. 2022; Farooq et al. 2021).

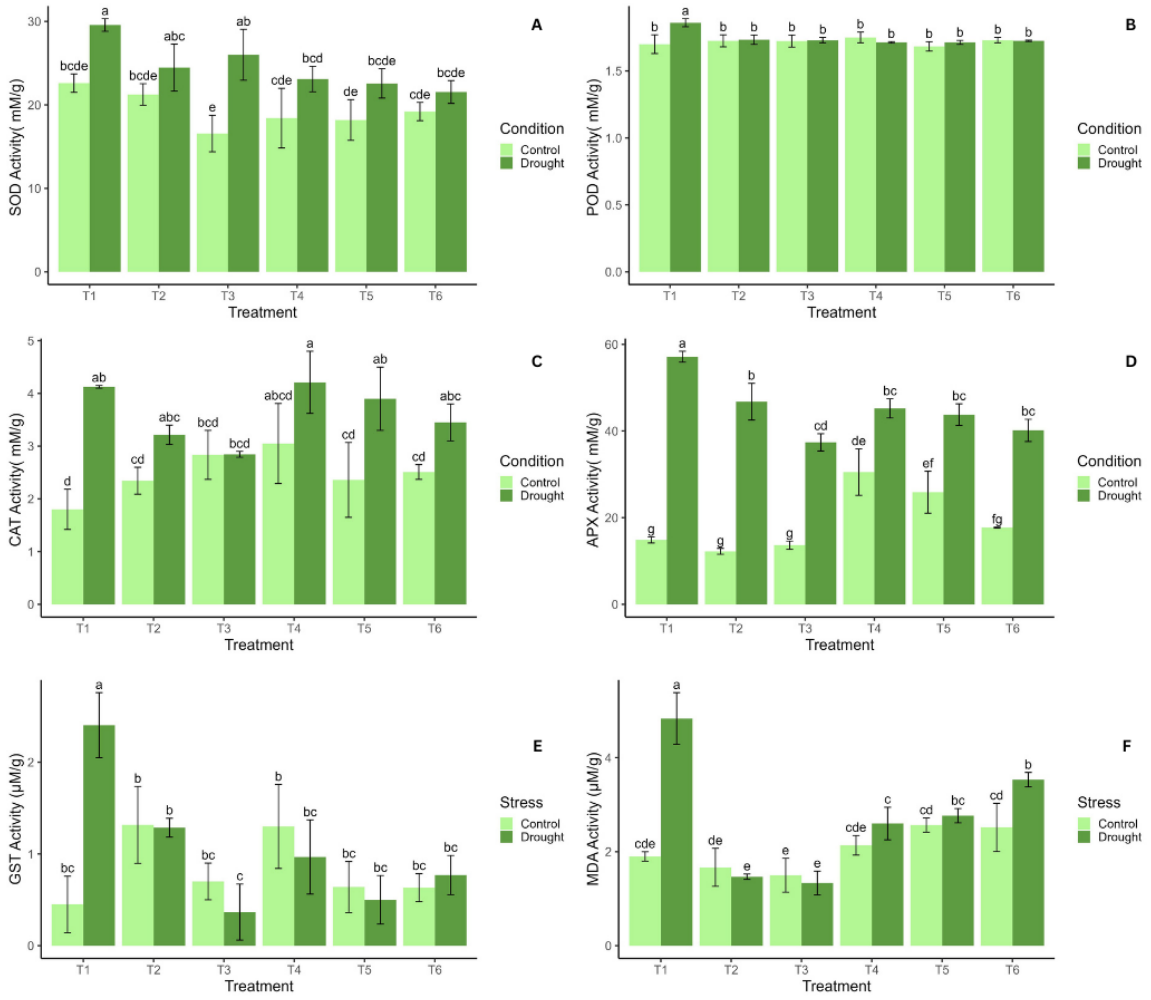

Figure 10. Effect of biochar and PGPRs application on biochemical profile of antioxident activities in control and drought conditions Data showing means and standard deviation of three biological replications (n=3), followed by Two-way ANOVA and Tukey's HSD post-hoc test to find significance. Treatments sharing different letterings are significantly different at $p < 0.05$

4.4. Pearson correlation coefficient

Pearson correlation analysis was performed to show relationship between morphological, physiological and biochemical profile of peanut in drought conditions. Morphological traits were observed to show significantly positive correlation among them when treated with biochar and PGPRs treatments in the presence of drought stress, while they show negative correlation with biochemical profile of plants which represent lower accumulation of ROS, lower antioxidant enzymatic activity and mitigation of drought stress. Antioxidant parameters showed positive correlation among them. RWC showed positive correlation with morphological traits and negative correlation with biochemical profile of plant while SPAD value showed negative correlation with plant morphology and positive correlation with biochemical profile of plant in drought condition.

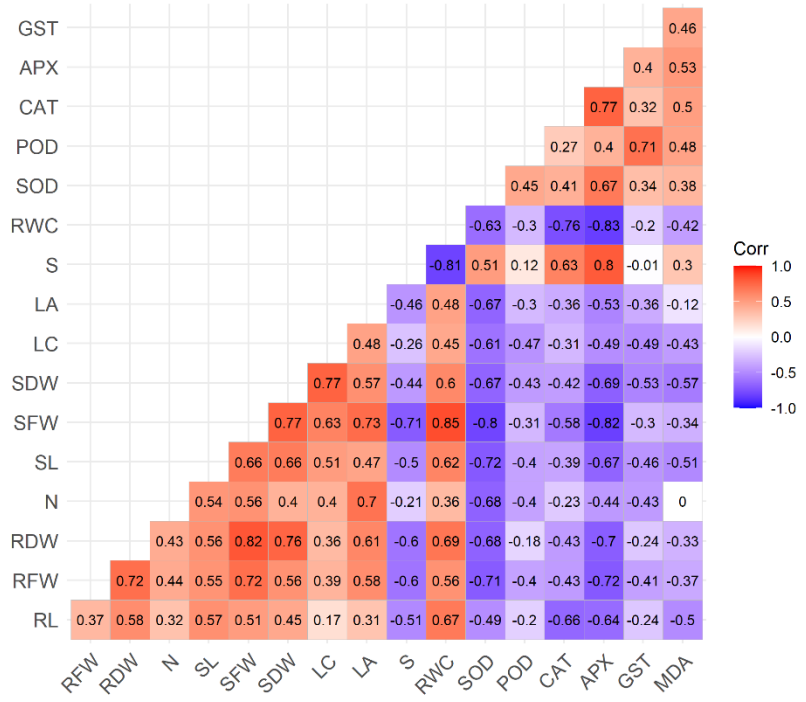

Figure 11. Pearson correlation between morphological, physiological and biochemical attributes of peanut in the presence of treatments containing variable combinations of BC and PGPRs under drought stress. The intensity of color shows the strength of correlation. The red color indicates the positive and the blue color shows a negative correlation.

4.5. Elemental profile of plant tissues

The elemental composition of the tissues was performed using energy-dispersive X−ray (EDX) spectrometry (model JSM 6490A; JEOL Ltd). For analysis, voltage of 15 kV and current of 12 μA was applied. The analysis of dried leaf samples showed percentage composition of selected macro and micronutrient in samples (see table 4). EDX results show less effect on total carbon of plant tissues but show increase in total nitrogen and oxygen content in plant when treated with biochar and PGPRs in both control and drought conditions. Increased amount of other micro and macronutrient was also observed in T6 in both control and drought conditions.

Table 4 Percentage composition of micro and macronutrients in leaf tissues

Element	T1C		T6C		T6D	
	Weight %	Atomic %	Weight %	Atomic %	Weight %	Atomic %
C	57.3	64.9	53.8	62.3	49.6	57.9
N	3.7	3.6	3.7	3.7	4.2	4.2
O	35.7	30.4	36.8	31.9	41.0	35.9
Na	0.1	0.1	0.4	0.2	0.4	0.3
Mg	0.3	0.2	0.6	0.3	0.7	0.4
Al	0.1	0.1	0.1	0.1	0.1	0.1
K	0.3	0.1	0.9	0.3	0.7	0.3
Ca	1.6	0.5	2.5	0.9	2.4	0.8
Mg	0.0	0.0	0.1	0.0	0.1	0.0
Cu	0.4	0.1	0.9	0.2	0.6	0.1
Zn	0.4	0.1	0.2	0.0	0.1	0.0

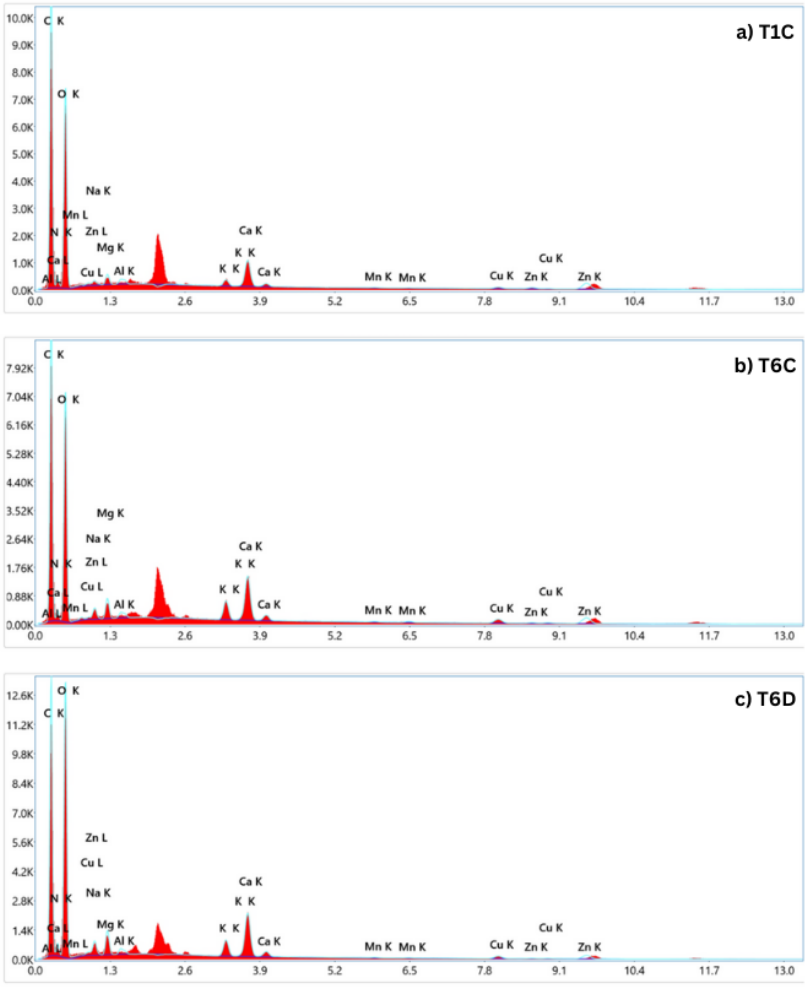

Figure 12. EDX (Energy-dispersive X−ray spectrometry) graphs for elemental analysis on leaf tissues. (a) negative control group (NC), (b) T6 consisting biochar and PGPRs in control conditions and (c) T6 in drought conditions

4.6. Metabolic profile of root exudates

Lyophilized samples of root exudates were subjected to GC/MS using methanol as solvent. 361 compounds were detected in negative control (T1C), 343 in positive control (T1D), 359 in T6C and 349 in T6D were detected. 56 were identified and classified into targeted chemical classes for analysis. 32 were present in positive control, 24 in negative control, 17 in T6C and 14 in T6D. Among amino acid, alanine was found in all treatments. Its concentration increased in drought conditions which were observed to be decreased in the presence of biochar and PGPR treatment. In drought conditions, alanine was also found on oligopeptide form, attaches to another amino acid. Variation in type and quantity of amino acids present in each treatment was observed. Similar pattern was observed in case of flavonoids and sugars. Among flavonoids, chalcones were found in high concentration in both positive control as well as T6 combined with drought signifying role of flavonoids in stress tolerance.

4.6.1. Principle component analysis of roots metabolites

To reveal the metabolic changes in root exudates by application of BC and PGPRs under the drought stress, quantitative and qualitative metabolites analysis of root exudates in four different conditions was carried out i.e. positive control, negative control, combination of 2% BC and PGPRs in both control and drought conditions. An obvious chromatographic sample groups revealed that the retention time was reproducible and stable, implying that the metabolomic analysis was reliable. The metabolite profiles showed a significant difference in all treatments.

Based on the PCA and Heatmap analysis results, a separation of samples under control and drought with and without application of organic amendment in roots was observed. The samples of different treatments were separated by the first principal component (PC1) and the second principal component (PC2). Each number in the loading plot represented a variable. Metabolites contributing to the separation between different treatments could be identified due to the most of the variables that were away from the coordinate centre. The samples of control (T1C) and drought stress (T1D) with or without organic amendment (T6C and T6D) were clearly separated by PC1, whereas PC2 represents the presence and absence of metabolites. Flavonoids (Chalcones) were found in both T1D and T6D showing significant role as antioxidant in stress conditions. The contribution of metabolites in roots exudation of peanut for PC1 and PC2 came from a number of metabolites with the most discrimination

power dominated by sugars and their intermediate metabolites (Erythritol, 1,6-Anhydro-.alpha.-d-Galactofuranose, D-Galactose, diethyl mercaptal pentaacetate, 2,7-Anhydro-l-galacto-Heptulofuranose, Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)- pyrimidin), amino acids (Alanine, Asparagine, cystine, cycloserine and leucine), flavonoids and their dreivatives (Dihydrofuranno(3,2-g)chromanone, Chalcones, 3-Phenyl-2H-chromene and 2,6-Lutidine 3,5-dichloro-4-dodecylthio) and organic acids (e.g. glycolic acid, salicylic acid, Octynoic acid, Acetic acid, Hexenoi acid etc).

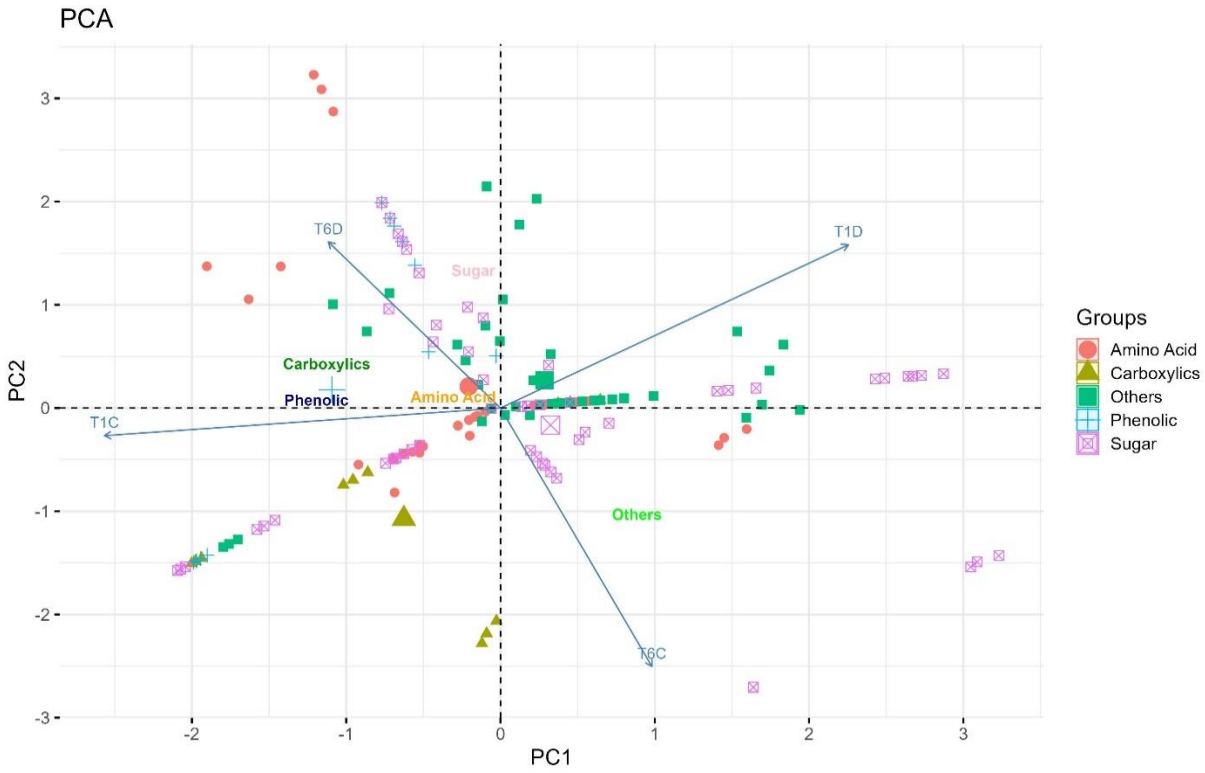

Figure 13 Principle component analysis, PC1 and PC2 of metabolites in root exudates of peanut under control and drought conditions with and without application of BC and PGPRs

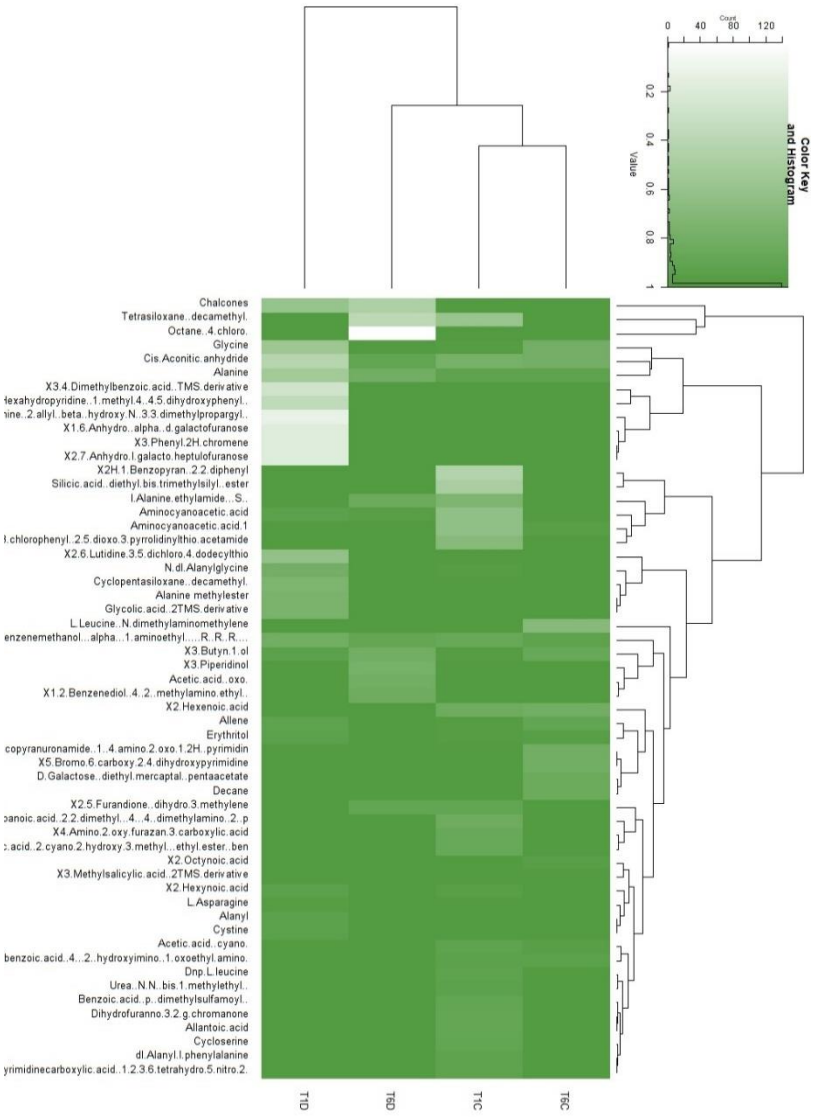

Figure 14 Heatmap of metabolites in root exudates of peanut under control and drought conditions with and without application of BC and PGPRs, T1C (Negative control), T1D (Positive control), T6C(2% BC and PGPRs without stress) and T6D (2% BC and PGPRs with stress)

CHAPTER 5: DISCUSSION

CHAPTER 5 DISCUSSION

5.1. Discussion

Peanut is one of the major oilseed crops all across the world due to its rich content of proteins and edible oils. Due to emerging concerns of climate change and global warming, crops are increasingly encountering a range of abiotic stresses with drought being particularly significant and pressing concern. Drought has emerged as prominent stress limiting growth and productivity of crops. It alone causes more loss in crop production globally then pathogen (Benaffari et al. 2022). In stress condition, levels of antioxidant enzymatic activity increase in plant to overcome ROS induced damage during stress.

Even though peanut is resilient to drought, still suffers from damage due to water deficiency like reduced photosynthetic activity, membrane damage due to lipid peroxidation, change in lipid content of seed etc. (Vasanthaiah & Kambiranda, 2011). Use of biochar and PGPRs has shown significant effect on enhancement of plant growth and yield, particularly in drought conditions (Lalay et al., 2022; Nafees et al., 2022). These two agricultural practices have gained huge importance due to their potential to improve crop productivity and resilience in water deficient conditions.

Biochar is a type of organic carbon formed by the thermal decomposition of dead organic matter in a low or oxygen free environment, which is known as pyrolysis. Biochar has shown positive impact on plant growth as well as soil properties in terms of conditioning the soil, composting and enrichment of biochar can also help in improving its nutritional values well as it assists slow release of nutrients, minimizing the leaching (Mansoor et al., 2021; Schulz et al., 2013). PGPRs are group of beneficial bacteria that colonize in soil and plant root system promoting its growth and development through different interaction mechanisms. They enhance root architecture and nutrient uptake efficiency of plants.

Furthermore, they also help in elevating antioxidant activity in plants which play a crucial role in mitigating stress induced damage. In this study, decrease in growth of peanut in drought state was observed to be recovered in the presence of biochar and PGPRs treatment. 2% biochar was found to be more effective for water retention in soil. However, excessive use of biochar is also reported to be potential risk factor in weeds outbreak (Safaei et al., 2018).

Use of PGPRs along with biochar has shown synergistic effects in enhancing plant growth in other leguminous plants. In present studies, compromised growth pattern and elevated enzymatic activity was observed in the water deficient conditions which was reversed up to

50 to 60% when seeds were treated with PGPRs and soil with biochar in 2% W/W ratio. Fresh and dry biomass are one of the major indices reflecting the response of plant toward drought. In present study, Significant difference was observed in fresh weight and dry weight. Pour-Aboughadareh et al. (2019) reported that shoot fresh and dry weight was highly reduced by drought stress as compared to control which supports our results (Pour-Aboughadareh et al., 2019).

Leaf RWC and SPAD value are another important physiological feature that is widely used to define a plant's sensitivity to tissue and cell dehydration (Ullah et al., 2021). Several studies have reported that the minimum reduction of RWC under water deficit stress indicates stress resistance (Hussain et al., 2019, Pour-Aboughadareh et al., 2019). In present study, decrease in RWC at drought stage was observed which was recovered up to 30% when soil was pretreated with biochar. This difference was found to be non-significant at rewatering stage showing the ability of recovery of physiological functioning in selected peanut variety. On the other hand, SPAD value was observed to be increased in drought stress conditions. Several studies have reported increase in chlorophyll content and photosynthetic activity during stress condition as strategy of stress tolerance of the specie variety (Ullah et al., 2021).

Antioxidant enzyme activity was also observed to be upregulated when experienced stress. Lower levels of enzyme activity in plants treated with combination of two amendments in drought condition indicates lower stress experienced by plant. Similar results were observed in wheat when grown in soil treated with 2% biochar and *B. amyloliquefaciens* inoculum (Danish et al., 2019). Enhancement in physiological traits, growth and productivity was attained in maize by co-application of biochar with ACC-deaminase producing rhizobacteria (Danish et al., 2020). Enhancement in plant nitrogen content on co-application of biochar and PGPRs has been reported in French beans (Saxena et al., 2013). Combination of these amendments are also reported to have significant role in mitigating drought stress in *Quercus brantii* (Heydari et al., 2023).

While suffering from drought stress, plant experience reduced growth and productivity and in current times, drought is one of the major concerns in the field of agriculture. Co-application of Plant growth promoting bacteria along with biochar has shown its potential in sustainable management of drought (Ullah et al., 2021). Significant increase in yield of pea and quinoa

was observed when soil was amended with compost under water deficient conditions (Hirich et al.,2014).

Soil rhizosphere provide medium for interaction between soil biota and plant. Benefits of organic amendments like biochar and PGPRs on plant growth are well documented however their role in alteration of metabolic interaction through secondary metabolites in soil rhizosphere are still unexplored (Grover et al., 2021). Plants release variety of secondary metabolites through their root system which serves as major signaling molecules for interaction between microbes and plant system. Release of exudates is sensitive to drought but exact pattern of changes due to stress conditions is unknown (Canarini et al. 2016). However, this leads to irreversible negative impact on soil microbiota resulting in reduced symbiotic interactions (Gargallo-Garriga et al., 2018).

In addition to stabilizing physical and chemical properties of soil, organic amendments play another important role of shaping soil microbial communities (Pantelides et al., 2023)). Our results suggested that drought changes the composition and quantity of root exudates resulting in alteration in interaction between plant and microbes. Drought alters metabolomic profile of exudates which may allow interaction with microbes present in soil reversing the damage caused to plant by restrictive water condition (Cesari et al., 2019). Increase in saccharides secretion in drought condition is useful in maintain osmotic potential in drought stress which is previously observed in maize (*Zea mays*) (Bornø et al. 2022) (*Hordeum vulgare*) (Calvo et al. 2017), sunflower (*Helianthus annuus*), and soybean (*Glycine max*) (Canarini et al. 2016).

CHAPTER 6: CONCLUSION

6.1. Conclusion

The combination of PGPRs and, Biochar helps in plant growth and assists in dealing with water deficient conditions. Solitary application of biochar as well as in combination with PGPRs showed significant results in stabilizing growth condition due to its water retention properties maintaining normal growth pattern of plant while reducing stress level. Experiment showed 30 to 60% decrease in morphological growth traits under drought stress which was observed to be reversed on treatment of variable ratios of biochar and in combination with PGPRs. Activation of the different antioxidant enzymes has been noticed (SOD, POD, CAT, APX, GST and MDA) under stress conditions. Antioxidant enzyme activity was higher in drought conditions which reduced in presence of biochar. Detection of antioxidant activity confirms the activation of ROS species and it decline indicated modulation of stress condition and decrease in ROS accumulation.

On the other hand, Application of PGPRs containing biofertilizer showed positive effects on plant-microbe interactions by enhancing microbial diversity. Drastic decrease in these interactions due to drought have been reported in several studies. Use of biochar in combination with biofertilizers is helpful in enhancing microbial diversity in rhizosphere, even under drought condition, leading stable growth and development of plant.

Here we have investigated the consequence of drought in peanut plant and helped to overcome it with biochar, and PGPRs based biofertilizer consortium. These combinations boost the morpho-physiological aspect of peanut plant. Further studies are needed to understand the underlined mechanism of stress tolerance in plants and metabolome assisted plant microbe interaction pattern to improve stress tolerance.

7. References

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APPENDICES

Appendix 1. Metabolic profile of targeted metabolites detected in root exudates in GC/MS

Table 5 List of Compounds detected in root exudates of peanut through GC/MS

S No.	Compounds	T1C		T1D		T6C		T6D	
		Ret. time	Peak area%	Ret. time	Peak area%	Ret. time	Peak area%	Ret. Time	Peak area%
Amino Acids and derivatives									
1	Alanine	8.28	0.09	27.657	0.67	5.742	0.08	16.081	0.29
2	dl-Alanyl-dl-valine	-	-	15.857	0.08	-	-	-	-
3	l-Alanine, N-(1-oxopentyl)-, methyl ester	-	-	26.441	0.32	-	-	-	-
4	Glycine, N-(dithiocarboxy)-N-methyl	8.174	0.03	26.477	0.61	16.085	0.24	-	-
5	L-Leucine, N-dimethylaminomethylene	-	-	-	-	9.65	0.38	-	-
6	L-Asparagine	-	-	9.042	0.02	-	-	-	-
7	Cystine	-	-	10.075	0.08	-	-	-	-
8	N-dl-Alanylglycine	8.417	0.01	16.705	0.27	-	-	-	-
9	Cycloserine	12.779	0.11	-	-	-	--	-	-
10	Dnp-L-leucine	15.358	0.09	-	-	-	-	-	-
11	dl-Alanyl-l-phenylalanine	14.575	0.1	-	-	-	-	-	--
12	l-Alanine ethylamide, (S)-	20.392	0.32	-	-	-	-	20.114	0.2
	Flavonoids and its derivatives								
13	Dihydrofuranno(3,2-g)chromanone	14.172	0.13	-	-	-	-	-	-
14	Chalcones	-	-	30.65	0.55	-	-	31.311	0.74
15	3-Phenyl-2H-chromene	-	-	30.392	1.44	-	-	-	-
16	2,6-Lutidine 3,5-dichloro-4-dodecylthio	-	-	26.551	0.52	-	-	-	-

	Organic acids								
17	3,4-Dimethylbenzoic acid, TMS derivative	-	-	30.764	1.16	-	-	-	-
18	2-Hexenoic acid	6.099	0.2	-	-	9.333	0.22	-	-
19	benzoic acid, 4-[[2-(hydroxyimino)-1-oxoethyl]amino]	8.661	0.06	-	-	25.618	0.1	-	-
20	Allantoic acid	8.977	0.13	-	-	-	-	-	-
21	Benzoic acid, p-(dimethylsulfamoyl)-	10.844	0.12	-	-	-	-	-	-
22	propanoic acid, 2,2-dimethyl-, 4-[[4-(dimethylamino)-2-(p	11.818	0.22	-	-	-	-	-	-
23	Aminocyanoacetic acid	11.917	0.49	9.208	0.08	-	-	11.336	0.03
24	Acetic acid, cyano-	8.797	0.09	-	-	5.683	0.03	-	-
25	4-Amino-2-oxy-furazan-3-carboxylic acid	20.612	0.17	-	-	-	-	-	-
26	4-Pyrimidinecarboxylic acid, 1,2,3,6-tetrahydro-5-nitro-2,	19.816	0.1	-	-	-	-	-	-
27	Aminocyanoacetic acid	11.917	0.49	-	-	6.867	0.02	-	-
28	2-Hexynoic acid	22.342	0.05	20.052	0.06	-	-	-	-
29	2-Octynoic acid	-	-	-	-	5.687	0.03	-	-
30	Valeric acid, 2-cyano-2-hydroxy-3-methyl-, ethyl ester, ben	20.233	0.22	-	-	-	-	-	-
31	Cis-Aconitic anhydride	15.577	0.27	27.933	0.83	15.283	0.25	24.392	0.12
32	Acetic acid, oxo-	-	-	-	-	-	-	11.633	0.24
	Sugars and derivatives								
33	Erythritol	5.98	0.03	10.692	0.07	25.4	0.04	-	-
34	1,6-Anhydro-.alpha.-d-galactofuranose	-	-	28.242	1.39	-	-	-	-
35	D-Galactose, diethyl	-	-	-	-	20.377	0.18	-	-

	mercaptal, pentaacetate								
36	2,7-Anhydro-l-galacto-heptulofuranose	-	-	28.246	1.39	-	-	-	-
37	Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidin	-	-	-	-	16.13	0.21	-	-
38	Urea, N,N'-bis(1-methylethyl)-	8.9	0.08	-	-	-	-	-	-
39	3-Piperidinol	-	-	-	-	-	-	12.167	0.29
40	1,2-Benzenedio l, 4-[2-(methylamino)ethyl]-	-	-	-	-	-	-	15.957	0.22
41	Allene	6.184	0.02	5.975	0.1	6.462	0.1	6.983	-
42	5-Bromo-6-carboxy-2,4-dihydroxypyrimidine	-	-	-	-	16.13	0.21	-	-
43	Octane, 4-chloro-	-	-	-	-	-	-	26.706	4.82
44	Decane	6.1	-	-	-	6.096	0.17	-	-
45	Glycolic acid, 2TMS derivative	-	-	27.158	0.32	-	-	-	-
46	Hexahydropyridine, 1-methyl-4-[4,5-dihydroxyphenyl]-	-	-	28.547	0.94	-	-	-	-
47	3-Butyn-1-ol	6.223	0.01	17.334	0.06	7.065	0.15	5.725	0.25
48	Benzenemethanol, .alpha.-(1-aminoethyl)-, [R-(R*,R*)]-	18.361	0.16	22.405	0.24	5.739	0.08	20.44	0.16
49	3-Phenoxypropylamine, 2-allyl-.beta.-hydroxy-N-[3.3-dimethylpropargyl]-	-		28.375	1.61	-	-	-	-
50	N-(4-Chlorophenyl)-2-[1-(3-chlorophenyl)-2,5-	20.274	0.4	-	-	-	-	-	-

	dioxo-3-pyrrolidinylthio]acetamide								
51	Cyclopentasiloxane, decamethyl-	-	-	9.325	0.35	-	-	-	-
52	Silicic acid, diethyl bis(trimethylsilyl) ester	28.011	0.7	-	-	-	-	-	-
53	2H-1-Benzopyran, 2,2-diphenyl	29.947	0.78	-	-	-	-	-	-
54	2,5-Furandione, dihydro-3-methylene	16,494	0.11	-	-	-	-	9.297	0.13
55	Tetrasiloxane, decamethyl-	30.879	0.55	-	-	-	-	28.357	0.92

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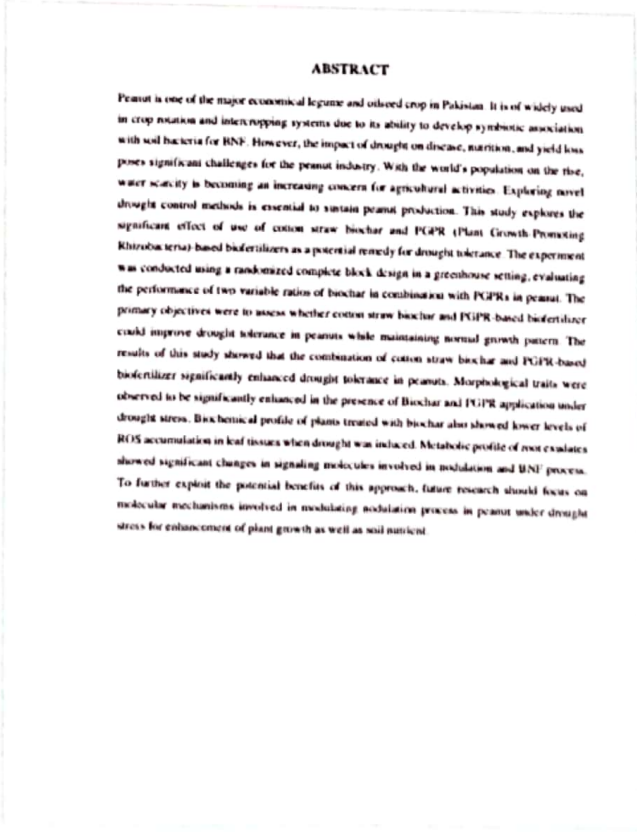

ABSTRACT

Peanut is one of the major economical legume and oilseed crop in Pakistan. It is of widely used in crop rotation and intercropping systems due to its ability to develop symbiotic association with soil bacteria for BNF. However, the impact of drought on disease, nutrition, and yield loss poses significant challenges for the peanut industry. With the world's population on the rise, water scarcity is becoming an increasing concern for agricultural activities. Exploring novel drought control methods is essential to sustain peanut production. This study explores the significant effect of use of cotton straw biochar and PGPR (Plant Growth Promoting Rhizobacteria)-based biofertilizers as a potential remedy for drought tolerance. The experiment was conducted using a randomized complete block design in a greenhouse setting, evaluating the performance of two variable ratios of biochar in combination with PGPRs in peanut. The primary objectives were to assess whether cotton straw biochar and PGPR-based biofertilizer could improve drought tolerance in peanuts while maintaining normal growth pattern. The results of this study showed that the combination of cotton straw biochar and PGPR-based biofertilizer significantly enhanced drought tolerance in peanuts. Morphological traits were observed to be significantly enhanced in the presence of Biochar and PGPR application under drought stress. Biochemical profile of plants treated with biochar also showed lower levels of ROS accumulation in leaf tissues when drought was induced. Metabolic profile of root exudates showed significant changes in signaling molecules involved in nodulation and BNF process. To further exploit the potential benefits of this approach, future research should focus on molecular mechanisms involved in modulating nodulation process in peanut under drought stress for enhancement of plant growth as well as soil nutrient.

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