Synergistic effect of *Mesorhizobium ciceri* and *Pseudomonas fluorescens* to adjust physiological and biochemical responses of *Kabuli* chickpea (*Cicer arietinum* L.) during drought stress

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ID: A/PR15274/10

A Thesis Submitted to Applied Biology Department Adama Science and Technology University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

School of Applied Natural Sciences

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

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Approval of Board of Examiners

We, the undersigned, members of the Board of Examiners of the final open defense by Argen Adem have read and evaluated his thesis entitled "Synergistic effect of *Mesorhizobium ciceri* and *Pseudomonas fluorescens to* adjust physiological and biochemical responses of *Kabuli* chickpea (*Cicer arietinum* L.) during drought stress" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment for the requirement of the Degree, Master of Science in Applied Biology (Biotechnology).

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To: Applied biology department

Subject: Thesis Submission

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List of Acronyms and Abbreviation

ACC	Amino Cyclopropane Carboxylate
CFU	Colony Forming Unit
CR	Congo Red
EARO	Ethiopian Agricultural Research Organization
EC	Electric Conductivity
EIAR	Ethiopian Institute of Agricultural Research
FC	Field Capacity
IAA	Indole Acetic Acid
IBC	Institute of Biodiversity Conservation
MARC	Melkassa Agricultural Research Center
NA	Nutrient Agar
OC	Organic Carbon
PGPR	Plant Growth Promoting Rhizobacteria
RCBD	Randomized Complete Block Design
ROS	Reactive Oxygen Species
RWC	Relative Water Content
TSS	Total Soluble Sugar
YEMA	Yeast Extract Mannitol Agar

Abstract

Chickpea (*Cicer arietinum* L.) is one of the most important legume plant which is being affected by drought stress. Due to drought, chickpea production has been low in Ethiopia and other chickpea producing countries. Although conventional breeding approach has enabled the production of relatively drought tolerant plants, it is time and resource consuming. Therefore, considering the application of plant growth promoting rhizobacteria is important to alleviate the effect of drought stress. Hence, the aim of this study was to reduce the impact of drought on chickpea plant by taking advantage of consortium effect of *Mesorhizobium ciceri* CP41 and *Pseudomonas fluorescens* Biotype G. Two contrasting drought tolerant (*Habru*) and susceptible (*Arerti*) *Kabuli* chickpea varieties were used. Growth, physiological and biochemical changes at two soil water contents (80% and 40% of field capacity) were analysed among *Mesorhizobium* spp. alone, *Mesorhizobium* spp. and *Pseudomonas* spp., and uninoculated seeds which were grown in greenhouse condition. *In vitro* results confirmed that these two bacteria are gram negative bacteria and are compatible to each other. Greenhouse experiments showed that co-inoculation has improved the plants condition under drought stress than *Mesorhizobium* alone and uninoculated control plants. Co-inoculation had significantly improved fresh and dry weight of leaves, root and shoot ($p<0.05$). However, non-significant difference was noted on nodules number per plant between co-inoculated and *Mesorhizobium* spp. alone inoculated in both varieties. Relative water content, proline, total soluble sugar, total chlorophyll and carotenoid content were significantly affected in both varieties. Hence positive effect through co-inoculation was observed in both *Habru* than *Arerti Kabuli* chickpea varieties. The present study demonstrated that synergistic application had better potential to confer drought tolerance in chickpea by altering various growth, physiological and biochemical parameters under drought stress conditions.

Keywords: Chickpea (*Cicer arietinum* L.), Co-inoculation, Drought stress, Rhizosphere, PGPR

1. INTRODUCTION

1.1. Background of the study

Water stress is one of the main environmental factors which limits plant growth, distribution and crop yield worldwide (Zhao *et al*., 2011; Shi *et al*., 2014). Due to insufficient, untimely and erratic rainfall in semi-arid and arid areas, most of the crops face the problem of drought at the end of the cropping season. Drought is a term used for water stress because of absence or inadequate rainfall and/or water supply (Toker *et al*., 2007). Therefore, one of the most crucial environmental factor regulating plant growth and development is water (Manivannan *et al*., 2007). Hence, drought is the main threat disturbing the life of plants and is accountable for reducing crops yield worldwide (Kavar *et al*., 2008).

Chickpea (*Cicer arietinum* L.) is legume plant of the family Fabaceae. It is one of the most important grain legume crops, grown in more than 51 countries in the world on area over 13.5 mha with an average global yield of about 967.6 Kg/ha (FAOSTAT, 2014). It is commonly grown in arid and semi-arid zones (Kumar and Abbo, 2001). Drought, cold and salinity are the major abiotic stresses affecting chickpea in order of importance (Croser *et al*., 2003). It has been estimated that 70% of the crop yield loss can be attributed to abiotic stresses, mainly drought (Bray *et al*., 2000).

In Ethiopia, chickpea is grown in different regions with different agro-ecological zones falling between 1400 to 2300m above sea level where the mean annual rainfall ranges from 700 to 2000mm (Geletu Bejiga and Million Eshete, 1996; Yadeta Anbessa and Geletu Bejiga, 2002). For several decades (1974-present time), several research activities have been done in Ethiopia to improve chickpea varieties with respect to their yield, tolerance to different biotic and abiotic stresses. As a result, improved chickpea varieties of *Kabuli* and *Desi* were released by Ethiopian Institute of Agricultural Research (EIAR) (Shiferaw Bekele and Teklewolde Hailemariam, 2007). However, research on harnessing the potential of soil microbes to alleviate abiotic stress in chickpea plants is still limited.

So far, creation of drought tolerant varieties has been the approach used to alleviate the negative effects of drought stress on crops and crop yields (Eisenstein, 2013). Conventional plant breeding techniques have allowed the development of high yielding, drought tolerant crop varieties. The drawbacks of this approach are that it is time consuming, labor intensive, may lead to the loss of other desirable traits from the host's gene pool, and that breeding confers benefits to a single crop species that are not transferrable to other crop systems (Ashraf, 2010; Eisenstein, 2013; Philippot *et al*., 2013). The approach mentioned above overlooks the ecological context of the soil environment in which the crops are grown (Morrissey *et al*., 2004). Conventional breeding considers plants as independent organisms that are solely regulated by their genetic code and cellular physiology (Barrow *et al*., 2008; Coleman-Derr and Tringe, 2014); although plant associated microbes can influence plants' response to the environmental conditions, including drought stress (Budak *et al*., 2013; Cooper *et al*., 2014). Therefore, there is a need for microbial based approaches to ameliorate drought stress effect on plants.

Beneficial rhizosphere bacteria are of two general types: those forming a symbiotic relationship with the plant and those that are free living in soil and root (Barriuso *et al*., 2005; Lugtenberg and Kamilova, 2009; Glick, 2012). The latter represent a wide range of root colonizing bacteria that have received worldwide attention because of their root colonizing ability and their capacity to produce a wide range of enzymes and metabolites that help plants tolerate biotic and abiotic stresses (Kim *et al*., 2009; Pineda *et al*., 2013; Chauhan *et al*., 2015). In addition, they have a great use when applied in consortium to alleviate plant abiotic stress and enhance plant growth and productivity under stress conditions (Jain *et al*., 2013).

Hence, it would be important to study the potential of chickpea and plant growth promoting microbes association in fighting drought stress. Also, knowledge on interrelationships among various physiological and biochemical responses to drought can offer insight for developing useful strategies to improve drought stress tolerance in chickpea. The measurement of each of these variables is demanding in terms of time and resources. Therefore, the objective of this study was to evaluate the synergistic effect of

Mesorhizobium ciceri and *Pseudomonas fluorescens* to help in drought tolerance in *Kabuli* chickpea plants by adjusting physiological and biochemical responses of the plant.

1.2. Statement of the problem

Drought is the most common abiotic stress limiting chickpea production in different parts of the world. Chickpea is a crop that is often affected by drought stress towards the end of the growing season in rain-fed conditions. This is because the world's chickpea production relies upon conserved and receding soil moisture. Although chickpea is known for its better drought tolerance than most other cool season legumes, drought does reduce productivity and can even lead to total crop failure (Turner *et al*., 2001).

In Ethiopia, it is recognized that growth and productivity of chickpea is adversely affected by the increasing shortage of water resources (Geletu Bejiga and Million Eshete, 1996; Yadeta Anbessa and Geletu Bejiga, 2002). The Ethiopian Institute of Agricultural Research (EIAR) Chickpea Research Team has focused mainly on breeding and selection of cultivars with higher yield, disease resistance and drought tolerance (Shiferaw Bekele and Teklewolde Hailemariam, 2007). However, the average yield of chickpea in Ethiopia under farmers' production condition remains less than 1.7 tons per hectare. On the other hand, the potential of the crop under improved management condition is more than 3 tons per hectare. (Legesse Dadi *et al*., 2005).

Analysis of the cost of production and market opportunities of *Desi* and *Kabuli* chickpea types in Ethiopia demonstrated that farmers using improved management would gain higher return by switching from the production of traditional *Desi* chickpea to the production of high yielding *Kabuli* chickpea type (Shiferaw Bekele and Teklewolde Hailemariam, 2007). However, The *Kabuli* chickpea type is drought susceptible unlike *Desi* chickpea type which is relatively drought tolerant (Farooq *et al*., 2018). Hence, it would be invaluable to enhance the drought tolerance of *Kabuli* type which is high yielding. Therefore, considering the potential of plant associated microbial communities for their ability to confer many of the same benefits to crop productivity and stress

resistance as have been achieved through plant breeding programs is important (Mapelli *et al*., 2013).

Application of microbial inoculants especially consortia will be one of the solutions to alleviate plant abiotic stress and enhance plant growth and productivity under stress conditions (Yang *et al*., 2009). Hence, it would be useful to use combination of bacterial inoculants that will help chickpea crop fight drought stress. From the chickpea's improvement perspective, it is also important to identify physiological and biochemical attributes. This is for the purpose of identifying drought tolerance indices contributing to adaptation to the moisture deficit patterns of chickpea's native environment by their association with bacteria that have plant growth promoting properties.

1.3. Objective of the study

1.3.1. General objective

- To evaluate the co-inoculation effect of *Mesorhizobium ciceri* and *Pseudomonas fluorescens* on physiological and biochemical responses of two drought contrasting *Kabuli* chickpea varieties (*Cicer arietinum* L.) during drought stress.

1.3.2. Specific objectives

- To characterize *Mesorhizobium ciceri* and *Pseudomonas fluorescens* for some morphological and biochemical properties and test for compatibility between the bacteria.
- To determine growth and relative water content during drought stress in two drought contrasting chickpea varieties with and without bacterial inoculation.
- To determine proline and total soluble sugar content during drought stress in two drought contrasting chickpea varieties with and without bacterial inoculation.
- To determine total chlorophyll and carotenoid content during drought stress in two drought contrasting chickpea varieties with and without bacterial inoculation.

1.4. Significance of the study

Agriculture is considered as the most vulnerable sector to climate change. Exploiting plant microbe interaction is a relevant approach to increase food production for the growing population in the current situation of climate change where drought stress has an immense impact on crops. This study showed how co-inoculation of chickpea with *Mesorhizobium* spp. and *Pseudomonas* spp. helped in amelioration of drought stress. The study also provides future implication for developing efficient microbial formulation for boosting other plants' performance under drought stress. The use of plant growth promoting microbes for drought stress alleviation can be used as a viable option for improving drought tolerance that is economically as well as ecologically sustainable.

2. LITERATURE REVIEW

2.1. Chickpea (*Cicer arietinum* L.)

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes grown and consumed worldwide. It is ranked third among the most extensively cultivated legume crop after common bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.) (FAOSTAT, 2014). There are two types of chickpea seeds: *Desi* chickpeas which have brown colored, small seeds and *Kabuli* chickpeas which have white or beige colored, large seeds (Sharma and Muehlbauer, 2007). Chickpea is an excellent source of protein, fiber, complex carbohydrates, vitamins and minerals. In combination with other pulses and cereals, it could have beneficial effects on health (Jukanti *et al.*, 2012). Thus chickpea is an important pulse crop with a diverse array of potential nutritional and health benefits.

Ethiopia is the one of the largest producer of chickpea in Africa, it nearly produced 60% of Africa's total chickpea in 2014 (FAOSTAT, 2014). Chickpea accounts about 16% of the total pulse production of the country (Menale Kassie *et al*., 2009). Chickpea is the second most important pulse crop in Ethiopia after faba bean (CSA, 2017).

2.2. Drought stress in chickpea

Drought is the most common abiotic stress limiting chickpea production in different parts of the world. Drought stress causes 40-50% decrease in chickpea yield around the world (Ahmad *et al*., 2005). Chickpea frequently suffers from drought stress towards the end of the growing season in rain-fed conditions. Ninety percent of the world's chickpea is produced in areas relying upon conserved, receding soil moisture. Therefore, soil moisture is a major factor for crop productivity (Kumar and Van Rheenen, 2000).

Early maturing varieties of chickpea have been developed to escape terminal drought (Kumar and Abbo, 2001). In chickpea, the capability of a plant to finish its life cycle before severe soil water reduction (drought escape), is associated with traits such as a deep root system, osmotic adjustment, high leaf water potential, high biomass, early flowering and maturity (Katerji *et al*., 2001).

2.3. Impact of drought stress on growth and physiological and biochemical parameters

Drought stress is primarily observed and responded by plant roots, particularly under field conditions. Therefore, length and distribution of roots plays a major role in influencing the capability of plants' to absorb water and nutrients from soil. It has been suggested that deeper root system with greater root density can be help in facilitating better extraction of soil water and to sustain optimal growth and development under water stress conditions (Lopes *et al.,* 2011). Therefore, the root system is more affected than the aerial part of the plant by water stress (Franco *et al.,* 2011).

Actual plant water status in leaves depends on the osmotic conditions of cells and transport of water from the shoots (Lawlor and Cornic, 2002). Drought stress can also affect shoot in which reduction in shoot and along with root dry biomass occurs. In stressed plants reduction may be due to decrease in leaf expansion and the stomata number that limit water loss through transpiration. This limits CO_2 assimilation, disrupts the photosynthetic activity showing decline in shoot biomass, as well as the inhibition of photosynthates transport to nodules (Antolín *et al.,* 2010). The reduction of nodule biomass under water deficit could be explained primarily by the decrease in the number and diameter of root hairs, or the inhibition of the emergence and elongation of these bodies and secondly, by the reduction of rhizobia growth and the initiation and development of nodules (Saadallah *et al.,* 2001; Antolín *et al.,* 2010). Under water deficit, osmotic stress suppresses enzymes of metabolic pathways involved in nitrogen fixation. These effects decrease nodule functioning and accelerate their early senescence (Mhadhbi *et al.,* 2009).

An important physiological parameter in plants which is considered as an important method to measure plant water status is relative water content (RWC). A decline in RWC indicates a loss of turgor that results in limited cell expansion and, consequentially, reduced growth in plants (Ashraf, 2010). Studies have shown that under drought stress, PGPR-treated plants maintained relatively higher RWC compared to non-treated plants,

leading to the conclusion that PGPR strains that improve survival of plants under drought stress generally increase RWC in the plants (Sandhya *et al*., 2010; Bano *et al*., 2013). These studies have also showed that higher RWC may help plants to combat the oxidative and osmotic stresses caused by drought stress, potentially contributing to greater productivity under stress.

It has been recognized that drought stress is a very important limiting factor at the initial phase of plant growth and organization. It affects both elongation and expansion growth (Kusaka *et al*., 2005, Shao *et al*., 2008). These physiological changes are due to the result of deleterious effect of water deficit on important metabolic processes as well as response of various defense mechanisms adapted by the plant under drought stress. Plants can partly protect themselves against water stress by accumulating compatible solutes which can stabilize proteins and cellular structures and are capable of maintaining cell turgor by osmotic adjustments (Mafakheri *et al*., 2010). Proline is one of the most common compatible osmolyte in drought stressed plants, which maintains redox metabolism by removing excess levels of reactive oxygen species and re-establishing cellular redox balance (Bartels and Sunkar, 2005).

Accumulation of proline has been advocated as a parameter of selection for stress tolerance (Jaleel *et al.,* 2008). Matysik *et al*. (2002) have attributed an antioxidant feature to proline, suggesting ROS scavenging activity and proline acting as a single oxygen quencher. Proline, therefore, acts both as a direct antioxidant as well as an activator of mechanisms that act as antioxidants. Another osmolyte which is important during drought stress is total soluble sugar that functions as an osmoprotectant. The hydroxyl groups of sugar substitute for water to maintain hydrophilic interactions in membranes and proteins (Homayouni and Khazarian, 2014). Increased accumulation of soluble sugars in stressed tissues corresponds to a reduction in starch concentration and is strongly correlated to the acquisition of drought tolerance in plants (Hoekstra and Buitink, 2001).

Kannan and Kulandaivelu (2011) reported that drought stress causes significant damage to photosynthetic pigments, and it also leads to deterioration of thylakoid membranes. The decrease in chlorophyll content is a commonly observed change on the plant under

drought stress (Bijanzadeh and Emam, 2010; Mafakheri *et al*., 2010; Din *et al*., 2011). Jain *et al*. (2013) in addition reported that under drought stress the reduction of chlorophyll b is greater than that of chlorophyll a, thus, transforming the ratio in favor of chlorophyll a. A decrease in total chlorophyll with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus (Mafakheri *et al*., 2010, Esfandiari *et al*., 2008).

2.4. Association of chickpea with rhizobium

Rhizobium-legume symbiosis is a mutualistic interaction between soil bacteria belonging to the α- and β-proteobacteria, together termed as rhizobia (Pini *et al*. 2011), and plants known as legumes. Chickpea being a leguminous crop has ability to fix biological nitrogen with the help of *Mesorhizobium* spp. Two species which form functional nodules when interacting with chickpea roots are described as specific microsymbionts *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* (Nandwani and Dudeja, 2009). However, recently use of molecular methods and characterization of samples from more geographical locations revealed that chickpea nodulating rhizobia belong to several species within the genus *Mesorhizobium* such as *Mesorhizobium amorphae, Mesorhizobium huakuii* and *Mesorhizobium plurifarium* may effectively nodulate chickpea (Laranjo *et al*., 2014).

For legume crops that depend on N-fixation, water stress causes a corresponding negative effect on nodulation, nodule functioning as well as N-fixation, and also changes enzymatic activities, plant growth and metabolism (Mhadhbi *et al*., 2004). Water stress may decrease persistence and the survival of rhizobia in the soil and root hair colonization as well as in infection process (Gray and Smith, 2005).

2.5. Drought stress tolerance mechanism mediated by plant growth promoting rhizobacteria

Water deficit leads to physiological and biochemical changes in plants for adaptation to abiotic stresses (Mahajan and Tuteja, 2005; Parida and Das, 2005; Des Marais and

Juenger, 2010). Beneficial effects of bacterial inoculation on growth, plant physiology, biochemistry under drought stress conditions are:

Phytohormones synthesis and modulation: The influence of bacteria in the rhizosphere of plants is largely due to the production of indole acetic acid (auxin) phytohormones (Spaepen *et al*., 2007). In addition, phytohormones such as gibberellins, ethylene, abscisic acid and cytokinins are also important for plants growth and development (Egamberdieva, 2013). Biosynthesis of phytohormone IAA by PGPRs contributes to the plant growth by playing a role in: i) cell enlargement, ii) cell division, iii) root initiation, iv) increased growth rate, v) phototropism, vi) geotropism and vii) apical dominance (Frankenberger Jr and Arshad, 1995). Hence, confer tolerance against environmental stresses mediated through the biosynthesis of phytohormones (Glick and Pasternak, 2003). Various plant species inoculated with IAA producing bacteria increased root growth and enhanced formation of lateral roots and roots hairs were observed (Dimkpa *et al*., 2009). Thus increasing water and nutrient uptake, helping plants to cope with water deficit (Egamberdieva and Kucharova, 2009).

Production of abscisic acid and gibberellins by *Azospirillum lipoferum* alleviated drought stress in maize plants (Cohen *et al*., 2009). Cellular dehydration induced biosynthesis of abscisic acid, an important stress hormone during water deficit condition (Kaushal and Wani, 2015).

Accumulation of protective compounds: several studies correlated accumulation of osmolytes such as proline and sugar with drought and salt tolerance in plants (Annapurna *et al*., 2011; Shukla *et al*., 2012; Tiwari, *et al*., 2016). Under abiotic stress conditions, increased proline biosynthesis was observed for various plant species inoculated with different PGPR (Barka *et al*., 2006; Kohler *et al*., 2009; Sandhya *et al*., 2010). In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing subcellular structures (e.g. membranes and proteins), scavenging free radicals, and buffering the cellular redox potential under stress conditions (Ashraf and Foolad, 2007).

PGPR *Pseudomonas fluorescens* strain P2 inoculation improved plant biomass, relative water content by accumulation of proline in rice plants exposed to drought stress

(Ongendra, *et al*., 2015). Ansary *et al*. (2012) also reported that inoculating maize plant with *Pseudomonas fluorescens* increased the concentrations of proline under drought stress condition. *Pseudomonas fluorescens* inoculation increased proline and total soluble sugar content in plants due to up regulation of its biosynthesis pathway hence maintaining cell water status and protecting the cell membranes and proteins from the injury caused by drought stress (Sandhya *et al*., 2010).

Biosynthesis of anti-oxidative enzymes: water deficit could limit the productivity of plants and may cause damage to plant tissues, especially roots, through the formation of reactive oxygen species (ROS) such as H_2O_2, O^{2-} and OH^-, due to high susceptibility of root meristem activity to ROS (Mahdi Dar, 2018). ROS are toxic molecules capable of causing oxidative damage to the lipids, DNA and proteins (Miller *et al*., 2010). If these molecules are not managed properly, they cause significant damage to the membranes and cause catastrophic effects on cell metabolism. The antioxidant system includes both enzymatic (e.g., superoxide dismutases, ascorbate peroxidases, and catalases) and non-enzymatic molecules (e.g., glutathione, flavonoids, carotenoids) (Mittler, 2002).

One of the non-enzymatic molecules which have a significant part as photo-protective compounds by quenching triplet chlorophyll II and singlet oxygen derived from excess light energy is carotenoid (Pogsan *et al*., 2006). Carotenoids participate in energy dissipation and can aid plant resistance against drought stress (Gunes *et al*., 2008). Drought stress can also alter the tissue concentration of chlorophyll and carotenoids (Jaleel *et al*., 2008; Kalefetoglu and Ekmekci, 2008). Farooq *et al*. (2009) stated that increased chlorophyll and carotenoid content under drought stress may be associated with leaf area reduction; it also can be a defensive response to mitigate the destructive effects of drought stress.

In order to decrease the deleterious effects of ROS, antioxidant promoting systems are required, such as PGPR application. Maize plants inoculated with five drought tolerant plant growth promoting *Pseudomonas* spp. strains namely *P. entomophila, P. stutzeri, P. putida, P. syringae, and P. montelli* subjected to drought stress showed significantly lower activity of antioxidant enzymes as compared to uninoculated plants (Sandhya *et al*.,

2010). The reduction in the antioxidant activity by inoculation with PGPR is related to less generation of free radicals hence reducing the antioxidant activity. These studies provide evidence for beneficial effect of PGPRs application in enhancing drought tolerance of plants by altering the antioxidants activity and reduction in active oxygen species under water deficit conditions (Gusain *et al*., 2015).

Enhancement of nutrients uptake: Nitrogen (N) ranks first among the major plant nutrients, yet its low availability to plants due to the high losses by emission or leaching is a limiting factor in agricultural ecosystems. Some microorganisms are capable of making atmospheric N available to plants through biological nitrogen fixation (BNF) which is of great importance (Martínez-Viveros *et al*., 2010). For agricultural sustainability advancement, an increase in the utilization of BNF as a major source of nitrogen for plants is required. The conversion of insoluble phosphate compounds (both organic and inorganic) in a form accessible to the plant is another important trait of PGPR strains. PGPR strains belonging to various genera have the ability to solubilize insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Richardson *et al*., 2009; Khan *et al*., 2009).

Many PGPR such as *Pseudomonas*, *Bacillus*, and *Rhizobium* are able to dissolve insoluble forms of phosphate (Richardson *et al*., 2009). It is commonly hypothesized that nutrient uptake is increased as a consequence of increased root surface area triggered by PGPR. However, root ion transporters are under the control of regulatory processes that adjust their activity to the plant nutritional demand (Nazoa *et al*., 2003), so that regulations of root development and ion transporter activities are antagonistically coordinated to maintain steady nutrient acquisition rate (Touraine, 2004)

2.6. Co-inoculation of plant growth promoting bacteria

Co-inoculation is based on mixed inoculants, combination of microorganisms that interact synergistically, or when microorganisms are functioning as “helper” bacteria to enhance the performance of other beneficial microorganisms. In the rhizosphere the synergism between various bacterial genera such as *Bacillus*, *Pseudomonas* and

Rhizobium has been demonstrated to promote plant growth and development. Compared to single inoculation, co-inoculation improved the absorption of nitrogen, phosphorus and mineral nutrients by plants (Figueiredo *et al*., 2010; Yadegari and Rahmani, 2010). A significant increase in root and shoot biomass was observed in chickpea plants when co-inoculated with *Mesorhizobium* spp. and *Pseudomonas* spp. (Sindhu *et al*., 2002). Increased nodule weight, root and shoot biomass and total nitrogen of chickpea plants were also reported due to co-inoculation of *Rhizobium*, *Pseudomonas* and *Bacillus* (Parmar and Dadarweal, 1999).

Combined inoculation of *Rhizobium* with *Pseudomonas striata* or *Bacillus megaterium* led to increased dry matter, grain yield and phosphorus uptake significantly over the uninoculated control in chickpea (Elkoca *et al*., 2008). Verma *et al*. (2013) reported that dual inoculation of *Mesorhizobium* spp. and *Pseudomonas aeruginosa* as PGPR were found significantly better for nodulation, plant growth and yield of chickpea over control.

Co-inoculation of ACC deaminase producing *Bacillus* isolate 23-B and *Pseudomonas* 6-P with *Mesorhizobium ciceri* for mitigation of drought stress and plant growth promotion under axenic conditions on *Cicer arietinum* varieties (*Kabuli* L-552 and *Desi* GPF-2) significantly improved germination, root and shoot length and fresh weight of chickpea seedlings under osmotic potential up to 0.4 MPa over uninoculated control. Proline content was higher in PGPR treated varieties under water stress (Sharma *et al*., 2013).

Combined application of IAA-producing *Pseudomonas* spp. and *Mesorhizobium* spp. increased nodule formation and plant dry weight compared to *Mesorhizobium* alone inoculated and uninoculated (Malik and Sindhu, 2011). Ahmad *et al*. (2011) described increased water use efficiency and relative water content by mung bean as a result of combined inoculation of *Rhizobium phaseoli* and *Pseudomonas* spp. Kumar *et al*.,(2016) reported that co-inoculation of chickpea with *Pseudomonas putida* and *Bacillus amyloliquefaciens* had reduced the impact of drought stress by significantly increasing growth parameters and defense enzymes.

3. MATERIALS AND METHODS

3.1. Study area and growth condition

Experiments during the study were done in greenhouse and Agricultural and Nutrition Research Laboratory at Melkassa Agricultural Research Center (MARC, latitude 8º24’00.0”, longitude 39º21’00.0”) Awash Melkassa, and in Microbiology Laboratory at Adama Science and Technology University (ASTU, latitude 8º33’43.56”, longitude 39º17’23.28”), Adama, Ethiopia. The plants remained in greenhouse environment at Melkassa Agricultural Research Center under direct sunlight (Figure 1). During the experiment, temperature and relative humidity were measured using thermo-hydrometer measuring device (Vici model: YCW-010). The minimum and maximum temperatures recorded were 17^0C and 36.2^0C, respectively and minimum and maximum relative humidity during the experiment were 38 % and 72 %, respectively.

Figure 1: Plants growing in greenhouse under direct sunlight.

3.2. Collection of bacterial strains

Commercial *Mesorhizobium ciceri* CP41 on lignite based carrier was taken from Menagesha Biotech Industry P.L.C., Addis Ababa, Ethiopia. This strain has been proven to enhance the growth of chickpea seeds under wide ecological conditions and is considered a very good strain of the laboratory as far as agronomic and yield performance of chickpea is concerned (Wondwosen Tena, *et al*., 2016), and *Pseudomonas fluorescens* Biotype G with Indole acetic acid (IAA) producing and phosphate solubilizing ability (Zerihun Tsegaye, *et al*., 2019) was taken from culture collection of the microbiology laboratory of Institute of Biodiversity Conservation (IBC) center, Addis Ababa, Ethiopia.

3.3. Isolation and maintenance of *Mesorhizobium* spp. purity

One gram of carrier was mixed with 9 ml of 0.85% saline solution to make dilution of 10^{-1}-10^{-6} and 0.1ml from each dilution was plated on Yeast Extract Mannitol Agar (YEMA) containing 0.0025% (w/v) Congo red (CR) (Vincent, 1970). The components of YEMA g/l: 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.1 NaCl, 10 Mannitol, 0.5 Yeast Extract, 15 Agar (Vincent, 1970). All the plates were incubated at 30°C. The culture grew in 4-5 days; a single colony that did not take up the color of Congo red was taken and re-streaked on YEMA with Congo red, since not absorbing Congo red is a distinctive character of rhizobia with only few exceptions (Somasegaran and Hoben, 1994). The *Mesorhizobium* spp. was then characterized for some morphological and biochemical properties (tested for catalase and urease production properties which may indicate the bacterium's ability to indirectly promote plant growth). The purity of culture was also checked by presumptive test under microscope after Gram staining. Isolated colonies of *Mesorhizobia* were transferred on YEMA medium plates and slants containing 0.3 % (w/v) $CaCO_3$ Vincent (1970) and were stored in refrigerator at 4°C for further studies.

3.4. Maintenance and examination of *Pseudomonas* spp. purity

The obtained *Pseudomonas fluorescens* Biotype G was maintained as slant culture on nutrient agar (NA) medium containing 0.3 % (w/v) $CaCO_3$ Vincent (1970) and stored in refrigerator at 4°C for further studies. The pure culture was characterized for some

morphological and biochemical properties (tested for catalase and urease production properties which may indicate the bacterium's ability to indirectly promote plant growth) and the purity of culture was checked by presumptive test under microscope after Gram staining.

3.5. Morphological and Biochemical studies of the bacteria

Morphological study was done on the basis of shape, colour, size, elevation, margin and texture of colonies of *Mesorhizobium ciceri* CP41 and *Pseudomonas fluorescens* Biotype G by growing them on YEM and nutrient broth, respectively (Vincent, 1970). Then cultures were spread on YEMA and nutrient agar plates and incubated at 30°C. After 48 hours for *Pseudomonas* spp. and after 4 days for *Mesorhizobium* spp. the shape, colour, size, elevation, margin and texture were noted.

Gram's staining

Gram's staining was done to study gram reaction and staining was carried out according to standard Gram's procedure (Somasegaran and Hoben, 1994). Four different reagents in the order of Gentian violet, Gram's iodine, alcohol distaining reagents, and safranin were used for classifying bacteria based on their gram staining result.

Catalase Test

This test was performed to study the presence of catalase enzyme. Smear of the two bacterial cultures were made on a clean and dry glass slides, then a drop of 3% H_2O_2 was added to the smear, production of gas bubbles constituted a positive test (Anderson *et al.*, 1995).

Urease Test

Urease activity was tested by growing *Mesorhizobium* and *Pseudomonas* species on urea medium plates (20% urea aqueous solution aseptically added to urea agar base (Christensen)) containing phenol red as pH indicator. Change in color of medium from yellow to pink indicates positive test for urease production (Public Health England, 2015).

3.6. Authentication of the isolated *Mesorhizobium* strain

To check the effectiveness of the *Mesorhizobium ciceri* CP41 to form nodule, the two chickpea varieties (*Habru* and *Arerti*) were inoculated with a loop full of the isolate which did not absorb Congo red and planted in sterilized sand. The positive control was uninoculated and nitrogen fertilized with 0.05% (W/V) KNO_3 once a week, while the negative control was uninoculated and not fertilized with nitrogen. All plants were fertilized with modified Hoagland nutrient solution without nitrogen (Hoagland and Arnon, 1950) once a week. Nodule formation was seen after five weeks of growth.

3.7. Compatibility between bacterial strains

Compatibility between *Mesorhizobium ciceri* and *Pseudomonas fluorescens* was tested following the method of Fukui *et al*. (1994). The compatibility was determined using YEMA medium. The *Mesorhizobium ciceri* strain was streaked vertically and *Pseudomonas fluorescens* were streaked horizontally. The plates were incubated at 30°C for 72 hours and observed for the inhibition zone. Absence of inhibition zone indicates the compatibility with respective bacteria.

3.8. Chickpea seeds and soil substrate

For the study, two drought contrasting *Kabuli* types were used; drought tolerant (*Habru*) and susceptible (*Arerti*) chickpea varieties (Figure 2) were taken from Debre Zeit Agricultural Research Center, Bishoftu, Ethiopia. Seeds were surface sterilized with 70% ethanol for 30 seconds and with 3% Sodium hypochlorite for 2 minutes after which they were rinsed with distilled water six times. The soil substrate used was first sieved through 2mm net and sterilized at 121°C for 1 hour in hot air oven. The soil substrate had physico-chemical properties of: pH 6.95, total nitrogen (N%) 0.17, phosphorous (P mg/kg) 2.84, electrical conductivity (EC ds/m) 0.501, organic carbon (OC%) 0.22 (Table 1). Five kilogram capacity polyethene plastic bags were filled with 4kg soil substrate to grow the chickpea plants. The positive control plants (uninoculated and well-watered (80% FC)) received nitrogen containing Hoagland nutrient solution once a week, while

the negative control plants (uninoculated and water deficit (40% FC)) and inoculated plants received nitrogen free Hoagland nutrient solution (Appendix 5) once a week.

Figure 2: Drought tolerant *Habru* seeds and drought susceptible *Arerti* seeds.

Table 1: Soil physico-chemical properties used for growing the plants (done at Soil Laboratory, MARC)

Soil texture	pH(1:2.5)	EC (ds/m)	Organic carbon (%)	Available N (%)	Available P (mg/kg)
clay=18.8% Silt=13.3% Sand=67.9% Class=Sandy loam	6.95	0.501	0.22	0.17	2.84

3.9. Experimental design and treatments

The experiment was carried out using randomized complete block design (RCBD) with three replications, with two varieties (*Habru* and *Arerti*), two water regimes (water deficit and well-watered), and three inoculation treatments (singly inoculated, co-inoculated and uninoculated), making up 12 treatments (Table 2), and therefore, 36 experimental units.

All plants were grown under the 80 % field capacity (FC) for 50 days. Then the plants were divided into those which were exposed to drought stress by withholding the water supply for 7 days resulting in decrease to around 40% soil moisture content and control

plants that were well-watered. Finally, all plants were analyzed for physiological and biochemical changes.

The amount of water applied was determined following the procedure described by Million Eshete *et al*. (2005):

1. Dry weight of the soil was taken (Md).
2. The polyethene plastic bag was weighed alone (Mb).
3. Then, the soil was saturated with water.
4. Left for 24hours until no water drips from underneath.
5. The bag with soil was then weighed at field capacity; this gives wet mass of the soil with the bag used (Mwb).
6. Then, weight of pot was subtracted from wet mass of the soil with plastic bag which gave wet mass of the soil (Mw), Mwb-Mb = Mw.
7. Mw-Md= amount of water at field capacity (FC).

Md- mass of dry weight of soil, Mb- mass of plastic bag, Mwb- mass of soil with plastic bag

To maintain the soil water content near to the desired field capacity, all pots were weighed every day and water loss was replenished up to the end of the experiment. The trait measurements were made on the fully expanded leaves from the top of the plant soon after weighting the pots and replenishing water loss.

Table 2: Experimental design: treatments allocation

Kabuli chickpea Varieties	Treatments(T)
Habru (DT)	T1= DT + *M.ciceri* + WW
Arerti (DS)	T2= DT + *M.ciceri* + WD
	T3= DT+ *M.ciceri* + *P.fluorescens*+ WW
	T4= DT + *M.ciceri* + *P.fluorescens*+ WD
	T5= DT + WW
	T6= DT + WD
	T7= DS + *M.ciceri* + WW
	T8= DS + *M.ciceri* + WD
	T9= DS + *M.ciceri* + *P.fluorescens*+ WW
	T10= DS + *M.ciceri* + *P.fluorescens*+ WD
	T11= DS + WW
	T12= DS + WD
Replication: were three times **Design**: Randomized complete block design	

Keys: DT= Drought tolerant, DS= Drought susceptible, T= treatments, WW= well-watered (80% moisture content), WD= water deficit (40% moisture content), *M. ciceri*= *Mesorhizobium ciceri* CP41, *P. fluorescens*= *Pseudomonas fluorescens* Biotype

3.10. Seed inoculation and sowing

For inoculation, culture of *Pseudomonas fluorescens* was grown for 48hours in nutrient broth media at 28±2^{o}C in an orbital shaker at 120 rpm. Culture of *Mesorhizobium ciceri* was grown for five days in yeast extract mannitol (YEM) broth at 28±2^{o}C on orbital shaker at 120 rpm till turbid (Appendix 2, II). The suspension was then centrifuged in sterile plastic tubes (10 ml) at 2000 rpm for 20 min. The pellets were re-suspended in normal saline (0.85% w/v of NaCl) solution to give a final concentration of 10^{8}-10^{9} CFU mL^{-1}. Culture density (CFU mL^{-1}) was checked by plate count after serial dilution method in triplicate and using OD 600(≈ 0.6). Seeds were first soaked in 10% sucrose

solution for sticker purpose and then inoculated with relevant bacterial cell suspension (10^{8}-10^{9} CFU mL^{-1}) by dipping them for 3 hours in sterile test tubes with the bacterial cultures before sowing. For co-inoculation, both bacterial cultures were used in 1:1 ratio after thoroughly mixed with vortex mixer. Sterility was maintained by doing the entire process in the Laminar Airflow Cabinet (Appendix 1). All inoculated seeds were air dried in a shade before sowing.

Three seeds per pot were sowed. To reduce contamination uninoculated seeds were first sowed. Rearrangement of pots was done every other day to give a random distribution of growth conditions in the greenhouse during the experimental period. The patterns for rearrangement were generated by writing a small script (Appendix 4) on Python 3.7.2 (Python software foundation, 2019).

3.10.1. Growth and physiological parameters evaluation

3.10.1.1. Determination of fresh and dry weight of leaves, root, shoot and nodule

Plant samples were collected and fresh weights of leaves (which were also used for relative water content determination), root, shoot, and nodule were measured. Nodule number was also counted from the roots of the chickpea plants. Then samples were put in an oven for 24 hours at 70^{o}C. After 24 hours, samples were weighed to determine their dry weight.

3.10.1.2. Determination of relative water content (RWC)

Relative water content was measured according to Smart and Bingham (1974). Fresh weights of harvested leaves (FW) were measured and then put in a Petri dish with distilled water for 5 hours. The turgor weight (TW) of leaves was measured and then put into an oven using paper bag for 24 hours at 70^{o}C (Appendix 3, II). Lastly, dry weights (DW) were measured. Relative water content was calculated with the following formula.

- Relative water content (%) = (FW-DW) / (TW-DW) x 100

  FW= fresh weight, DW=dry weight, TW= turgor weight

3.10.2. Biochemical parameters evaluation

3.10.2.1. Proline content

Proline content was determined by using acid ninhydrin reagent by the method of Bates *et al*, (1973). Fresh plant sample (0.25 g) was homogenized with 10 ml of 3 % aqueous sulfosalicylic acid and the homogenate was filtered using Whatman No.2 filter paper. The filtrate was used for proline concentration determination. Two milliliter of the filtrate was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube and allowed to react for 1 hour in a water bath at 100 ^{o}C. It was then cooled, producing brownish color (Appendix 3, I). The reaction mixture was extracted with 4 ml of toluene, mixed vigorously in a test tube and stirred for 15-20 seconds until separate layers were formed. The content from each tube was transferred to funnel. Chromophore or upper toluene layer containing the color complex due to proline ninhydrin reaction was separated from aqueous phase to other test tube and warmed at room temperature and absorbance was read at 520 nm by spectrophotometer (Genesys 10 UV, Thermo Electron Corporation, USA). The proline concentration was determined from standard curve constructed from known concentration of L-proline (Figure 3) and expressed in micro-mole (µmol) of proline per g fresh weight of leave.

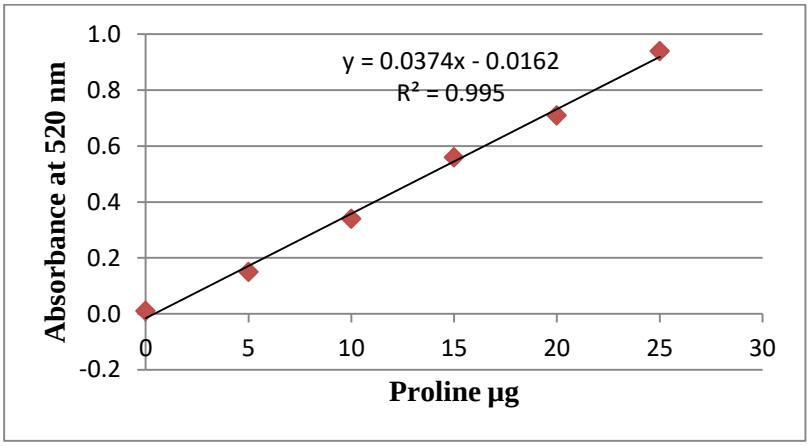

Figure 3: Standard curve prepared from L-Proline (µg) and read at 520 absorbance

3.10.2.2. Total soluble sugar (TSS)

Total soluble sugar in chickpea leaf samples was determined according to Dubois *et al*. (1956) with some modifications (Tiwari, *et al*., 2016). About 200 mg of fresh leaf tissues were homogenized in 5 ml of 80% methanol and was incubated in water bath at

70 ^{o}C for 30 min. After incubation, 1ml of extract was mixed with 1 ml of 5% phenol and 5 ml of 95% H_2SO_4 and further incubated in dark for 15 min. Absorbance was then measured at 490 nm using spectrophotometer (Genesys 10 UV, Thermo Electron Corporation, USA). Results were then compared with standard curve constructed using glucose (Figure 4). TSS concentration was then expressed in milligram (mg) per g fresh weight of leave.

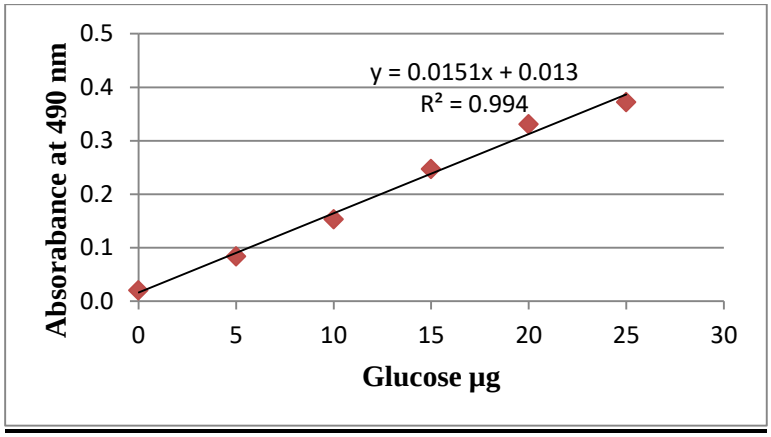

Figure 4: Standard curve prepared from L-Glucose (µg) and read at 490 absorbance

3.10.2.3. Total chlorophyll and carotenoids contents

Photosynthetic pigments were extracted and estimated according to the formulae of Lichtentaler and Wellburn (1985) slightly modified by Dere *et al*. (1998). A hundred mg of fresh leaf material from all the treatments were homogenized with mortar and pestle using 5ml of 100% (v/v) acetone. The green slurry was filtered through filter paper and centrifuged at 2500 rpm for 10 minutes. The supernatant was taken in test tubes. The optical density was recorded at 662 nm, 645 nm and 470 nm against 100% (v/v) acetone as blank in spectrophotometer (Genesys 10 UV, Thermo Electron Corporation, USA). Chlorophyll and carotenoids contents were expressed in mg per g fresh weight of leave.

Chlorophyll a, chlorophyll b and total carotenoid levels present in the pigment extract were determined employing the following formulae:

$Chl.a = 11.75A_{662} - 2.350\ A_{645}$

$Chl.b = 18.61\ A_{645} - 3.960\ A_{662}$

$Cx+c = 1000\ A_{470} - 2.270\ Chl.a - 81.4\ Chl.b/227$

Total chlorophyll= (Chl.a + Chl.b)

[Chl.a = chlorophyll a; Chl.b = chlorophyll b; Cx+c = total carotenoid; AW = Absorbance at W nm wavelength]

3.11. Data analysis

All mean values of the parameters were subjected to statistical analysis using two-way ANOVA with the Statistical Package for Social Sciences (SPSS) software, version 22 (IBM® Corp., Armonk, NY, USA) and compared with least significant difference (L.S.D.) at 5 % level of significance. Graphs were made using Microsoft Excel 2010 (Microsoft® Corp., Redmond, Washington, USA).

4. RESULTS AND DISCUSSION

Drought stress is one of the most common adverse environmental condition that reduce crop production and productivity globally. Improving drought stress tolerance of crop plants through traditional breeding could be one of the approach for enhanced agricultural productivity. However, since this approach is long drawn, labor and cost intensive, the use of plant growth promoting microbes for improving stress tolerance of crop plants will be significant (Nautiyal *et al*., 2013). The present study demonstrated the synergistic co-inoculation effect of *Mesorhizobium ciceri* CP41 and *Pseudomonas fluorescens* Biotype G in promoting growth as well as drought stress alleviation in drought tolerant (*Habru*) and drought susceptible (*Arerti*) *Kabuli* chickpea varieties.

4.1. Bacterial properties

Morphological characters of the *Mesorhizobium ciceri* CP41 isolate was circular shaped, white translucent colonies with entire margin and mucoid texture on Congo Red YEMA medium (Table 3). Mulissa Jida and Fassil Assefa (2012) reported similar findings from chickpea nodulating rhizobia isolated from different Ethiopian soils. Gauri *et al*. (2012) and Kamble *et al*. (2006) described *Mesorhizobium* spp. on the basis of their growth characteristics such as colony shape, colour and texture. On the other hand, *Pseudomonas fluorescens* Biotype G produced circular shaped, light yellow colonies with entire margin and mucoid texture when grown on nutrient agar (Table 3). Zerihun Tsegaye *et al*. (2019) reported similar findings except for brownish colony color. This might have happened when different choice of media was employed for growth i.e. nutrient agar and Glucose peptone agar.

Microscopic observation for both bacteria confirmed that they are gram negative bacteria with pink color. Catalase test and urease test for both species were positive which could indicate properties for indirect enhancement of plant growth (Appendix 2). Catalase is important to degrade H_2O_2 which is one of the main reactive oxygen species (ROS) affecting plants under stress; hence, bacterial cultures positive for catalase test could indirectly enhance plant growth (Kumar *et al*., 2012; Wang *et al*., 2013). Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide,

hence, urease is involved in nitrogen remobilization helping plants in their N uptake (Krajewska, 2009).

Table 3: Some morphological and biochemical properties of *Mesorhizobium* spp. and *Pseudomonas* spp.

Morphological properties and Biochemical properties									
Bacteria	Colony shape	Color	Size	Elevation	Margin	Texture	Gram stain	Catalase test	Urease test
M. ciceri	Circular	White translucent	Medium-large	Raised	Entire	Mucoid	-	+	+
P. fluorescens	Circular	Light yellow	medium	Raised	Entire	Mucoid	-	+++*	+

* +++ more bubbles

4.2. Authentication result

The isolated *Mesorhizobium ciceri* CP41 which did not absorb Congo red was effective in forming nodule in both *Habru* and *Arerti* varieties hence authenticated as root nodule bacteria (Figure 5). This strain was reported to form nodule in many chickpea varieties including *Habru* and *Arerti* (Ibsa Aliyi, 2013; Wondwosen Tena *et al*., 2016; Endalkachew Wolde-meskela *et al*., 2018).

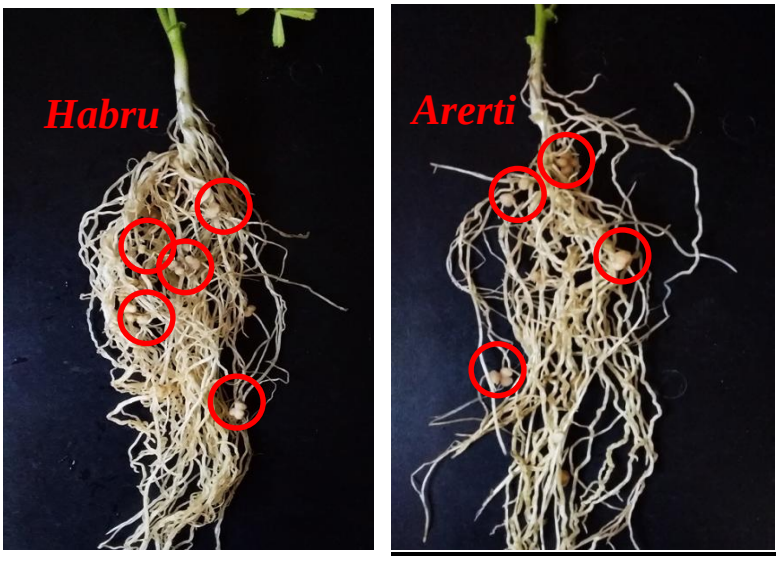

Figure 5: Authentication result showing nodules formed by *Mesorhizobium ciceri*

4.3. Compatibility result

Compatibility between two bacterial species with plant growth promoting properties could lead to formation of synergistic effect against drought in plants. *Mesorhizobium ciceri* CP41 and *Pseudomonas fluorescens* Biotype G cultures were tested for their compatibility under *in vitro* conditions. The result indicated that there was no inhibition zone and there was overgrowth of bacterial cultures suggesting that they are compatible with each other (Figure 6). Tilak *et al*. (2006) reported that *Pseudomonas fluorescens* did not antagonize *Rhizobium* strain AR-2-2 k when grown together on plates. The result of this experiment was also in line with Anandaraj and Delapierre (2010), who reported compatibility of *Rhizobium* spp. with *Pseudomonas fluorescens* and *Bacillus megaterium*, hence able to grow simultaneously without any inhibition in growth.

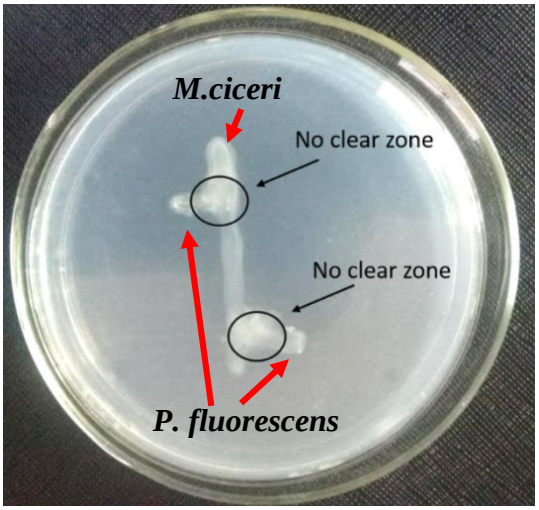

Figure 6: Compatibility between *Mesorhizobium ciceri* (Vertical) and *Pseudomonas fluorescens* (Horizontals) showing no clear zone hence compatible

4.4. Growth and physiological parameters

Consortium inoculation had a significant improvement on growth of both drought tolerant (*Habru*) and susceptible (*Arerti*) varieties under water deficit condition than *Meso*rhizobium alone inoculated and uninoculated plants (Figure 7). To determine the response of bacterial inoculation on leaf, root, shoot and nodule parameters of the drought tolerant and susceptible chickpea varieties subjected to drought stress, plants

were monitored after 7 days of water stress in both bacterial-treated and non-treated control plants.

Figure 7: Pictures taken showing difference among *Mesorhizobium* alone inoculated (T2 and T8), co-inoculated (T4, T10), and uninoculated chickpea plants (T6, T12). Drought tolerant (*Habru*, photo A), and susceptible (*Arerti*, photo B) chickpea varieties under water deficit condition.

4.4.1. Fresh weight and dry weight of leaves

The results demonstrated that significant increase in fresh weight of leaves were recorded in drought tolerant (*Habru*, $p<0.01$) and susceptible (*Arerti*, $p<0.05$) varieties inoculated with consortium of *Mesorhizobium ciceri* and *Pseudomonas fluorescens* than uninoculated control plants under water deficit condition (Figure 8). However, *Mesorhizobium* alone inoculation only brought significant increase ($p<0.01$) in fresh weight of leaves in *Habru* variety. Co-inoculation brought 64.9 % and 61% increase in fresh weight of leaves of drought tolerant and susceptible chickpea plants, respectively was observed than uninoculated control plants. However, when co-inoculated plants were compared to *Mesorhizobium* alone inoculated plants 17.3% and 29% increase in fresh weight of leaves in drought tolerant and susceptible varieties, respectively was observed under water deficit condition. Similarly, significant increase in fresh weight of leaves under well-watered condition was noted in *Habru* variety than *Arerti* variety with consortium inoculation and *Mesorhizobium* alone.

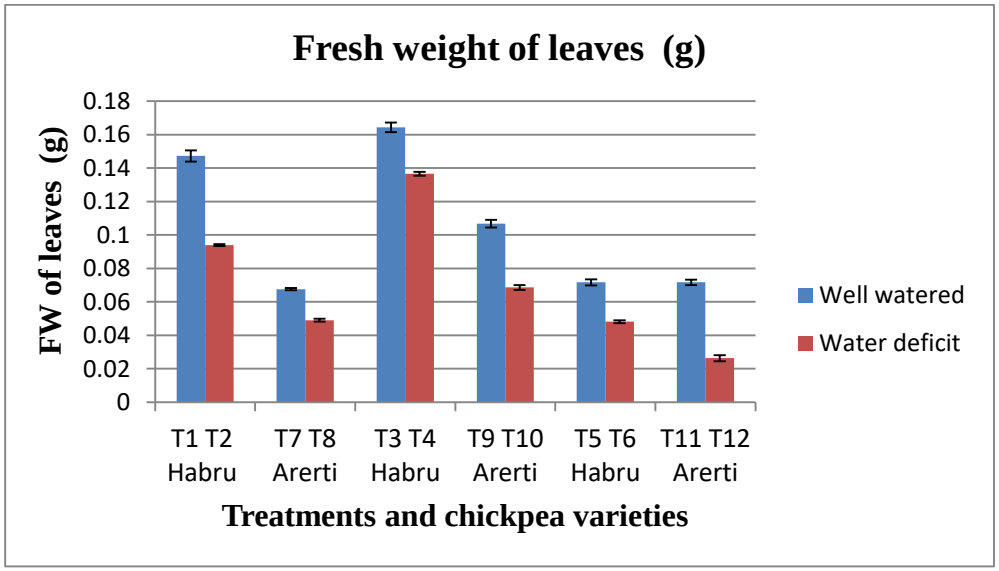

Figure 8: Mean fresh weight of leaves (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium alone* inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

Consortium inoculation increased dry weight of leaves significantly ($p<0.05$) under water deficit condition by 43% and 44.2 % in drought tolerant and susceptible chickpea varieties, respectively than uninoculated control plants (Figure 9). However, non-significant difference ($p>0.05$) was observed in dry weight of leaves between co-inculcated and *Mesorhizobium* alone inoculated drought tolerant and susceptible varieties under water deficit condition.

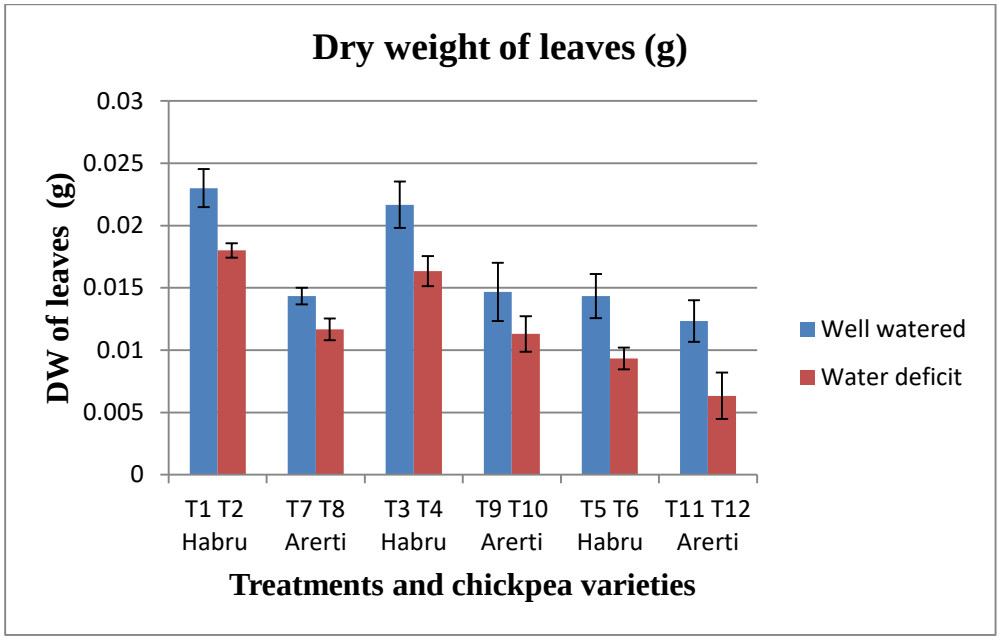

Figure 9 Mean dry weight of leaves (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

The results were consistent with the work of Lobata *et al*. (2013) who reported an increase in leaf biomass under drought stress in common bean with contrasting drought tolerance ability, which were inoculated with plant growth promoting microbes. Hence, the results showed co-inoculation had increased the leaf biomass in both varieties under drought stress than *Mesorhizobium* alone inoculated and uninoculated control plants under well-watered and water deficit conditions, though dry weight was improved by inoculation better in *Habru* variety than *Arerti* variety under well-watered condition.

4.4.2. Fresh and dry weight of root and shoot

Drought affects several aspects of legume growth and development, including germination, root and shoot development. Consortium inoculation of chickpea seeds with *Mesorhizobium* spp. and *Pseudomonas* spp. had a significant positive effect on root and shoot fresh and dry weights compared with *Meso*rhizobium alone inoculated and uninoculated chickpea plants under well watered and water deficit conditions.

Under water deficit (40% FC), co-inoculation increased root fresh weight significantly ($p<0.01$) by 36% and 52% in drought tolerant and susceptible chickpea varieties, respectively than uninoculated control plants (Figure 10). However, significant improvement when co-inoculation compared to *Meso*rhizobium alone inoculated was found in drought tolerant (*Habru*) variety only, while non-significant ($p>0.05$) difference from that of *Mesorhizobium* alone inoculated was observed in drought susceptible *Arerti* variety.

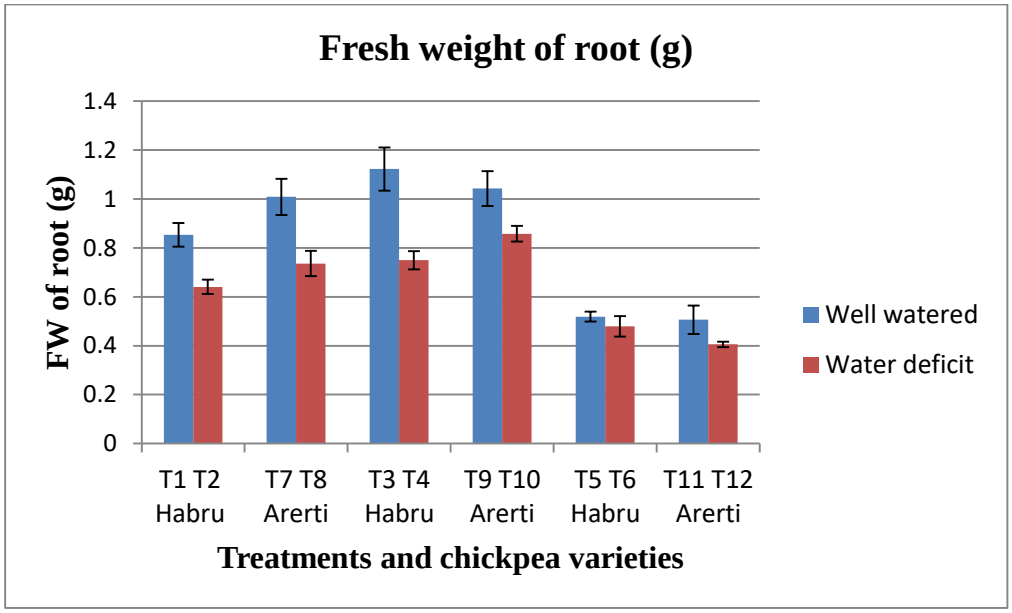

Figure 10: Mean fresh weight of root (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

On the other hand, dry weight of root increased significantly ($p<0.01$) due to co-inoculation in both drought tolerant and susceptible chickpea varieties, than uninoculated control plants. However, Co-inoculation didn't bring significant difference ($p>0.05$) than *Mesorhizobium* alone inoculated plants in both varieties (Figure 11).

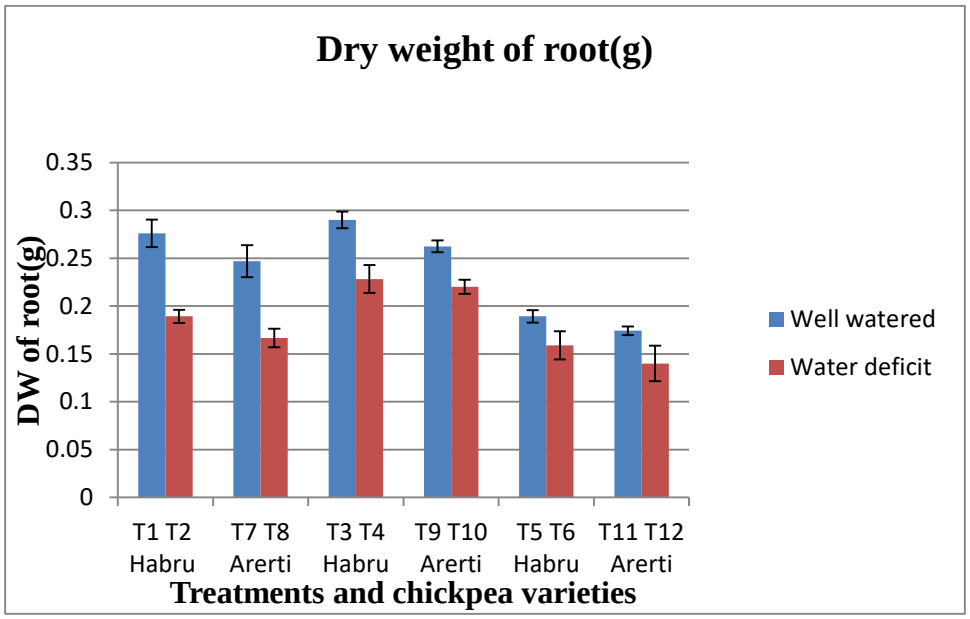

Figure 11: Mean dry weight of root (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

Co-inoculation with *Mesorhizobium* spp. and *Pseudomonas* spp. increased fresh weight of shoot significantly ($p<0.01$) by 33.2% and 39.2% in drought tolerant and susceptible chickpea plants, respectively than uninoculated control groups (Figure 12). Results are in consistent with Karnwal and Kumar, (2012) who reported enhancement in shoot and root biomass due to inoculation of chickpea by plant growth promoting rhizobacteria.

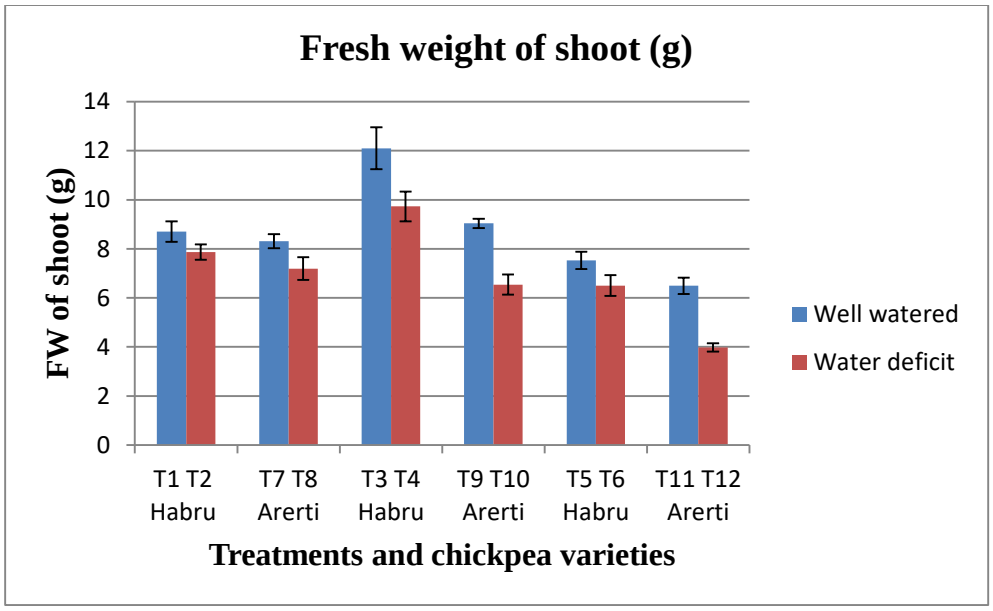

Figure 12: Mean fresh weight of shoot (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

Co-inoculation brought Significant improvement ($p<0.05$) in dry weight of shoot in both drought tolerant and susceptible chickpea plants than uninoculated control groups under water deficit and well-watered conditions (Figure 13). An increase in growth of shoot and root due to increased uptake of nutrients and hence increase in dry weight because of IAA production was reported by Malik *et al*. (2006) in chickpea. Similarly, Malik and Sindhu (2011) reported that chickpea seeds inoculated with *Mesorhizobium spp.* increased the plant dry weight. The increase in dry weight of shoot could be for biologically fixed N_2 (Fatima *et al*., 2008). Similar results were reported by Ahmad *et al*. (2011) who reported an increase in dry weight of shoot in chickpea plants ranged from 33 to 100% over the control. Timmusk *et al*. (2014) reported that bacterial induced alterations in root architecture may lead to an increase in total root surface area, and consequently lead to improved water and nutrient uptake, with positive effects on plant growth as a whole under water deficit environment.

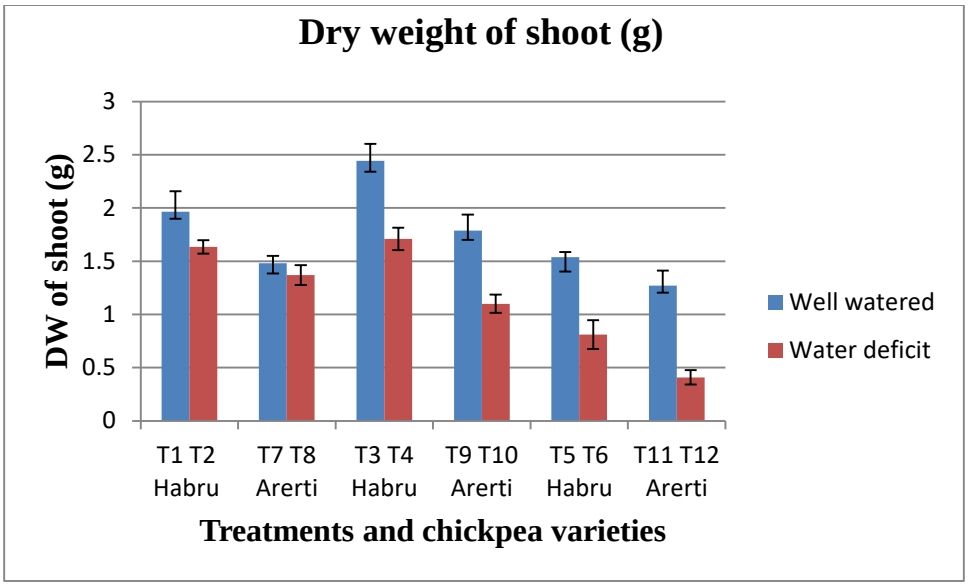

Figure 13: Mean dry weight of shoot (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

4.4.3. Number of nodules per plant (NNPP), nodule fresh and dry weight

No significant difference ($p>0.05$) was observed in the number of root nodules in both co-inoculated and *Mesorhizobium* alone inoculated varieties of drought tolerant and susceptible varieties on exposure to drought stress (Figure 14). Highest nodules number per plant was 34 and 32 in drought tolerant *Habru* and drought susceptible *Arerti* variety, respectively per sample.

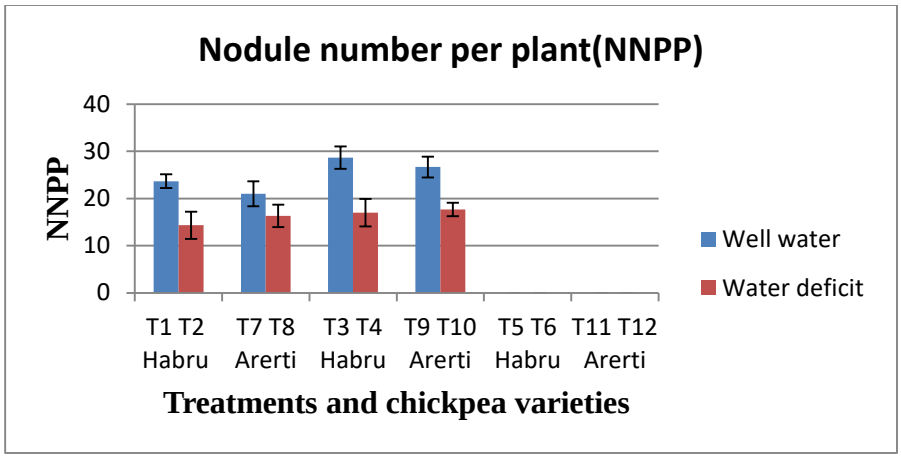

Figure 14: Mean nodule number per plant (NNPP) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated, T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

Nodule fresh weight increased significantly ($p=0.02$) by 25.7% in drought tolerant and ($p<0.01$) by 13% in drought susceptible varieties (Figure 15). On the other hand, dry weight of nodules did not show statistically significant difference ($p>0.05$) in both varieties (Figure 16). Nodulation was not observed in uninoculated control as soil was sterilized.

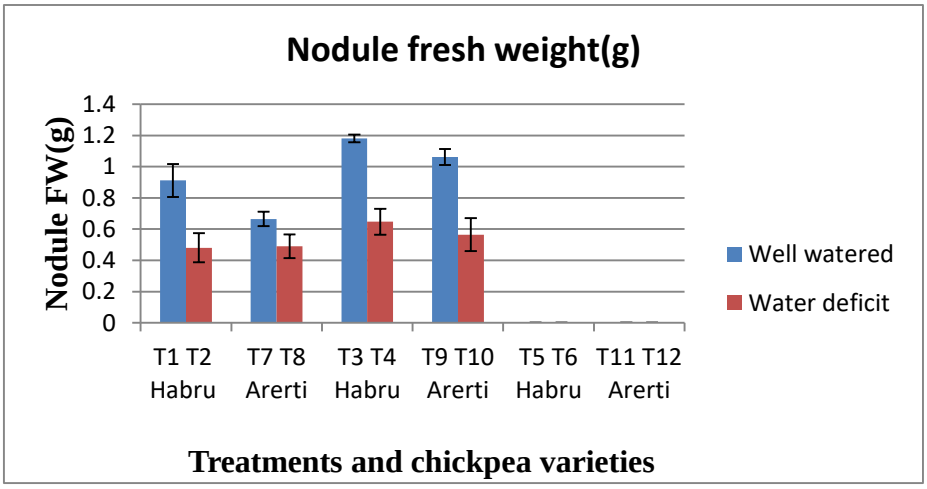

Figure 15: Mean nodule fresh weight (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-

inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

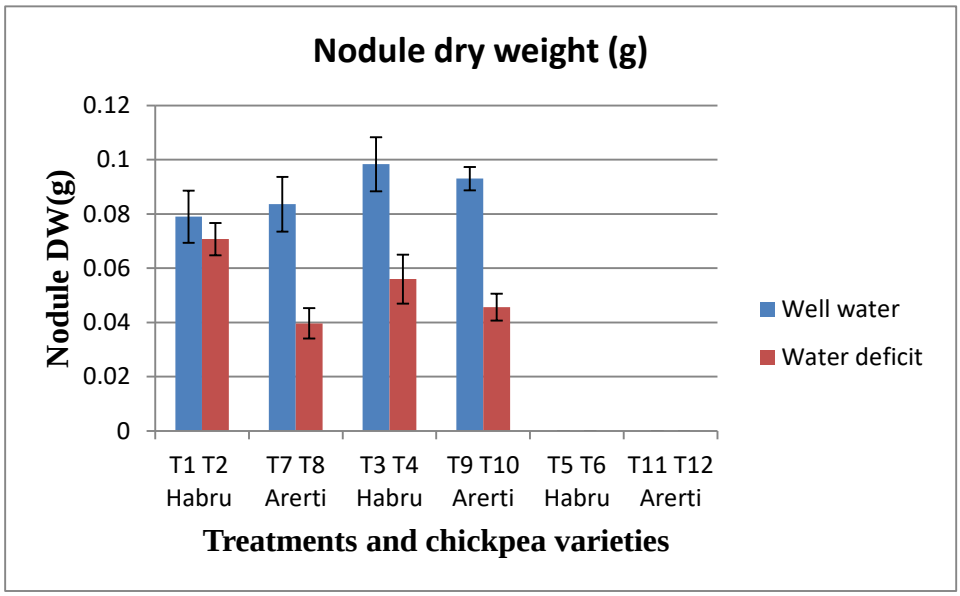

Figure 16: Mean nodule dry weight (g) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

Low soil moisture during growth of chickpea resulting in decrease in nodule formation was reported by Gan *et al*. (2005). The reduction in nodule mass may be due to decreased number of nodules which is attributed to the effect of drought on the process of nodulation and the activity of nitrogenase enzyme. The reduction in nodule dry weight was also noted by Kumar *et al*. (2010) in different chickpea genotypes under drought conditions.

Enhancement of nodulation and yield in chickpea by *Mesorhizobium* spp. as compared to control has been reported by several researchers (Bhuiyan *et al*., 2008, Ibsa Aliyi 2013; Verma *et al*., 2013). However, in this study the number of nodules was less than that reported by Wondwosen Tena *et al*. (2016) and Ibsa Aliyi (2013) who used the same strain of *Mesorhizobium* spp. to inoculate chickpea seeds, and this might be because of the very high temperature of the greenhouse at the day time. Inoculation of seeds with *Rhizobium* spp. increased nodulation, yield and growth parameters and N

uptake in chickpea (Rudresh *et al.,* 2005). However, enhancement in mere number of nodules cannot be used as criteria for effective nodulation as total number of nodules include both effective and ineffective nodules present on the root system.

4.4.4. Relative water content (RWC)

Relative water content (RWC) is reliable parameter for quantifying plant drought stress response and as an indicator of plant water status (Siddique *et al*., 2000; Yan *et al*., 2016). In this study, drought stress decreased RWC in both drought tolerant and susceptible chickpea varieties. However, co-inoculation of the varieties has helped the plants to increase in their relative water content under water deficit condition than that of uninoculated control plants (Figure 17).

Two-way ANOVA for relative water content in uninoculated, *Mesorhizobium* alone inoculated and co-inoculated chickpea varieties of both drought tolerant and susceptible varieties showed that there was significant change in relative water content ($p<0.01$). Post hoc comparison using the least significant difference (L.S.D.) test indicated that the mean RWC score for co-inoculation was significantly improved ($p<0.01$) than uninoculated in both varieties, also from that of *Mesorhizobium* alone inoculated was significant in drought tolerant and susceptible varieties ($p<0.01$) and ($p=0.028$), respectively under water deficit condition.

Taken together, these results suggested that inoculation really do have an improvement on relative water content in both drought tolerant (*Habru*) and susceptible (*Arerti*) chickpea varieties. Specifically the results suggest that under water deficit condition co-inoculated drought tolerant chickpea variety increased RWC by 18% and 22.2% over *Mesorhizobium* alone inoculated and uninoculated control, respectively. While co-inoculated drought susceptible chickpea variety increased RWC by 20.5% and 36.8% over *Mesorhizobium* alone inoculated and uninoculated control, respectively. The results are in consistent with Sandhya *et al*., (2010), Bano *et al*., (2013), Tiwari *et al*. (2016), who reported higher RWC in plants under drought stress due to inoculation. The tolerance or sensitivity of chickpea to drought is related to its capability to maintain good leaf water status (Krouma, 2010). Talebi *et al*. (2013) also indicated that cultivars

which have high RWC are more resistant to drought stress. This work's results showed that *Habru* variety is better than *Arerti* variety in preserving water under drought stress and consortium inoculation increased relative water content in both varieties under drought stress than uninoculated control.

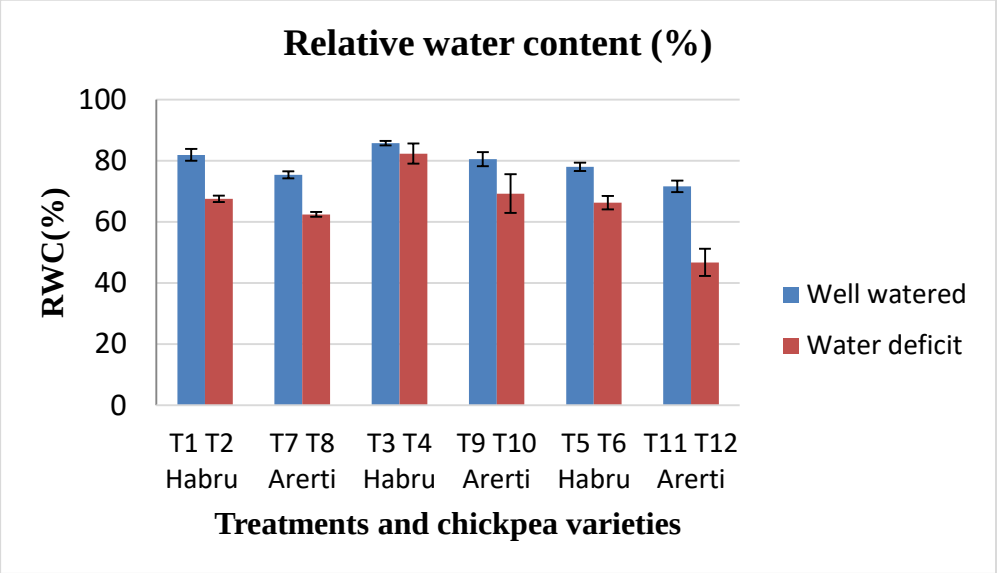

Figure 17: Mean relative water content (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Meso*-alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

4.5. Biochemical parameters

4.5.1. Proline content

Proline accumulation is believed to play an adaptive role in plant stress tolerance (Verbruggen and Hermans, 2008) and has been advocated as a parameter of selection for stress tolerance (Jaleel *et al*., 2008). Significant increase in proline content ($p<0.01$) in susceptible *Arerti* variety and non-significant difference in drought tolerant *Habru* variety ($p>0.05$) under water stress condition as compared to the control condition in both inoculated and uninoculated conditions were observed as shown in (Figure 18). However, highest value was observed in *Habru* variety (in single sample) which was co-inoculated and under drought stress (17.9 µmol/g) and lowest was observed in Arerti variety (1.76 µmol/g) under control condition. Increase in proline under drought stress

due to inoculation has been reported in different plants (Gusain *et al*., 2015; Kabbadj *et al*., 2017).

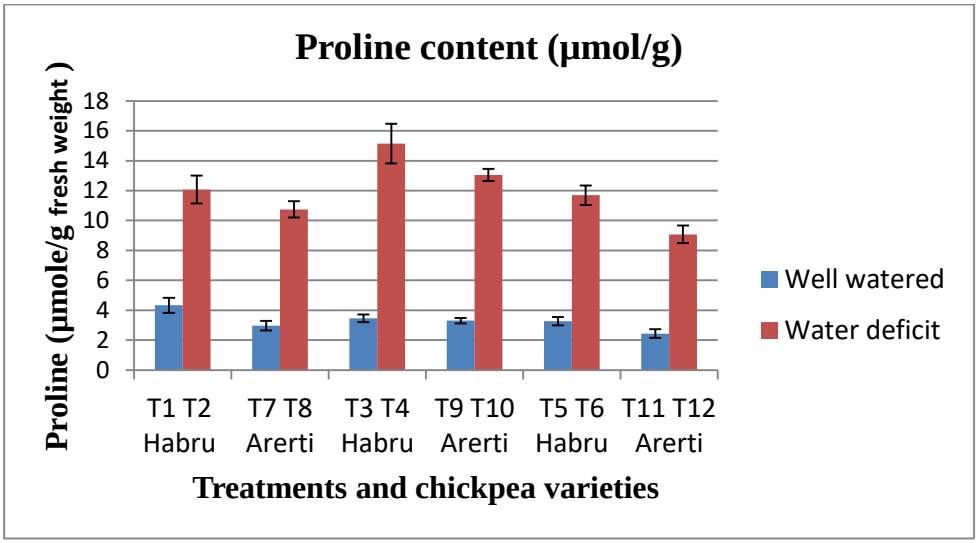

Figure 18: Mean proline content (µmole/g fresh weight) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Meso*-alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

The accumulation of osmolytes such as proline during stress is well documented (Vendruscolo *et al*., 2007; Lum *et al*., 2014) and, it is known for a long time that the concentration of proline increases in a large variety of plants under drought stress. In addition to its role as osmolyte, proline may also protect protein structure and membranes from damage, and reduce enzyme denaturation (Lhout *et al*., 2001). Islam *et al*. (2009) reported that, proline is an important osmolyte that activate an alternative detoxification pathway to protect plants under drought stress from the effect of reactive oxygen species (ROS). The accumulation of more proline from this study implies that the consortium inoculation has helped both varieties to accumulate higher proline hence better tolerance to drought stress.

4.5.2. Total soluble sugar (TSS)

Inoculation had significant effect on drought tolerant *Harbu* ($p=0.037$) and drought susceptible *Arerti* varieties ($p< 0.01$) on total soluble sugar under well-watered and

water deficit conditions. Total soluble sugar in well-watered control condition for uninoculated plants was found low in comparison to the water stress condition while, co-inoculated and *Mesorhizobium* alone inoculated plants showed decrease in TSS under drought stress than their well-watered control counterpart (Figure 19). Maximum sugar content (in single sample) was observed in *Habru* (50.45 mg/g fresh weight) and minimum was observed in *Arerti* (24.79 mg/g fresh weight).

The TSS content of inoculated chickpea plants showed significant decline during drought stress as compared to uninoculated plants. This may be due to the bacteria used induced systemic tolerance response of chickpea plants since its inoculation may have stimulated root growth by IAA production, biofilm formation and conservation of soil moisture which may have resulted in enhanced root growth and nutrient uptake thereby improving plant health under stress condition. The result of this study were in line with Tiwari *et al*. (2016) who reported decrease in total soluble sugar content of chickpea plants under drought stress which were inoculated with *Pseudomonas putida*. Total soluble sugar as osmolyte materials increases flows of water into cells and maintain turgor of cells through adjusting water content (Rasaei *et al*., 2013).

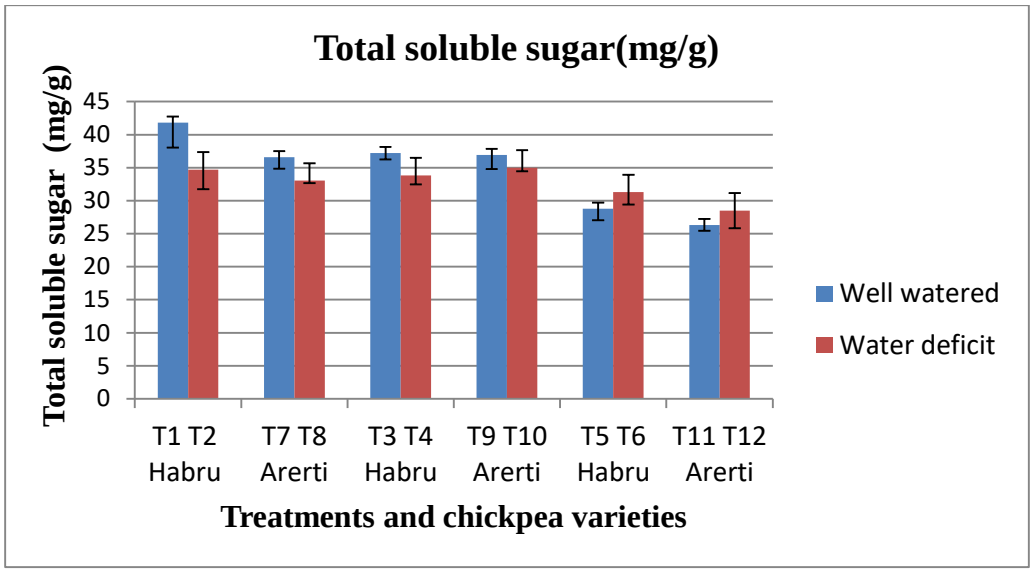

Figure 19: Mean total soluble sugar (mg/g fresh weight) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated,

T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

4.5.3. Total chlorophyll and carotenoid content

Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers. Drought tolerant *Habru* and susceptible *Arerti* variety were significantly improved by co-inoculation of *Mesorhizobium and Pseudomonas* spp. ($p<0.05$) than uninoculated controls, but no significant difference from that of *Mesorhizobium* alone inoculated control plants ($p>0.05$) on the content of total chlorophyll (Figure 20).

Results were well in accordance with studies of Bejandi *et al*. (2012) who also reported that seed inoculation with *Rhizobium* has significant effect on chlorophyll content in chickpea. The increase in chlorophyll content of leaves could be attributed to increased plant nutrition and photosynthesis. Phosphorous solubilization by plant growth promoting rhizobacteria in chickpea has shown to have an important role in energy transfer and photosynthesis, thus contributing towards chlorophyll content (Aouani *et al*., 2001).

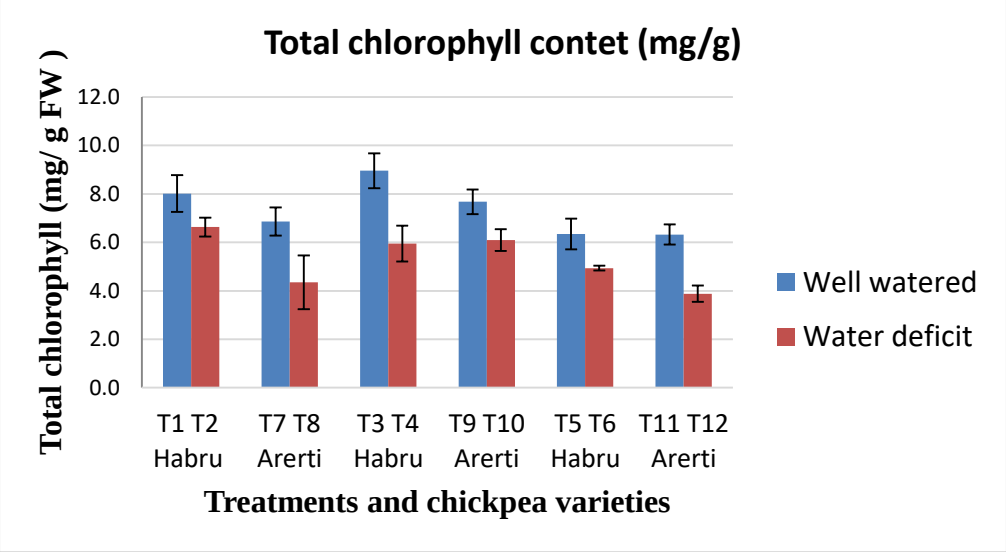

Figure 20: Mean total chlorophyll content (mg/g fresh weight) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated ,

T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

Similarly, drought tolerant *Habru* and susceptible *Arerti* varieies were significantly affected by co-inoculation of *Mesorhizobium* spp. and *Pseudomonas* spp. ($p<0.05$) than uninoculated controls, but co-inoculation didn't have significant difference from that of *Mesorhizobium* alone inoculated control plants ($p>0.05$) on the content of carotenoid (Figure 21).

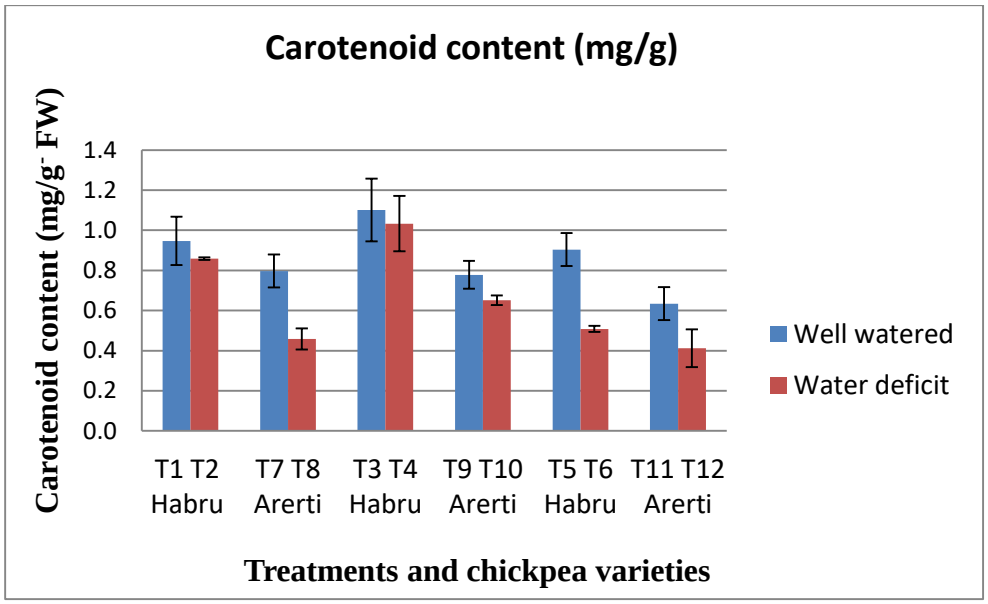

Figure 21: Mean carotenoid content (mg/g fresh weight) (±SEm) of drought tolerant (*Habru*) and sensitive (*Arerti*) varieties under well-watered, water deficit, and different inoculation treatments. T1,T2,T7,T8 were *Mesorhizobium* alone inoculated , T3,T4,T9,T10 were co-inoculated and T5,T6,T11,T12 were uninoculated control plants. Data are average of three biological replicates along with standard error bars.

The contents of total chlorophyll and carotenoids under water deficit condition had been found to reduce. However, the parameters were enhanced in inoculated plants than control plants. The enhanced chlorophyll and carotenoid content may increase the photosynthetic efficiency of inoculated chickpea plants and thus maybe important for tolerance to drought stress. The results were in consistent with Gusain *et al*. (2015) who reported enhanced chlorophyll and carotenoid content hence better drought tolerance in drought tolerant and susceptible rice varieties inoculated with different plant growth promoting rhizobacteria.

Chlorophyll content is often measured in plants in order to assess the impact of environmental stress, as the changes in pigment levels are linked to visual symptoms of plant illness and photosynthetic productivity. Carotenoid pigments protect chlorophylls from photo-oxidative destruction and its accumulation in plants subjected to drought can act in osmoregulation and results in high relative water content and also carotenoid participate in energy dissipation, hence better drought tolerance ability by plants (Gunes *et al*., 2008).

5. CONCLUSION AND RECOMMENDATIONS

In this study, plant soil microbe interaction was investigated by exploiting *Mesorhizobium ciceri* CP41 *and Pseudomonas fluorescens* Biotype G in ameliorating drought stress in drought tolerant (*Habru*) and susceptible (*Arerti*) *Kabuli* chickpea varieties. Drought stress significantly affected the growth and development of both chickpea varieties by affecting fresh weight and dry weight of leaves, root, shoot and nodule. Reduced RWC, total chlorophyll content and carotenoid, and enhanced osmolytes like proline and total soluble sugar were also observed. Conversely drought induced symptoms of the above parameters in chickpea were significantly improved in the presence of the plant growth promoting microbe inoculation. More positive effect was observed when inoculation was done in consortium. Interestingly the bacteria not only improved the growth of tolerant chickpea variety (*Habru*) but also satisfactorily improved the drought stress ameliorating ability of relatively drought sensitive chickpea variety (*Arerti*), indicating its greater potential in enhancing agricultural productivity of this economically important legume. The results thus set up an essential step towards understanding the physiological and biochemical basis of the plant growth promoting bacteria mediated drought stress response and adaptation in chickpea. Thus applicability of plant growth promoting microbes in drought stress amelioration at field level should be worked out.

Based on the study the following recommendations were made:

- Understanding the diversity of plant bacterial associations and their role in plant development is necessary if these associations are to be manipulated to increase crop production.
- The selection of co-inoculant PGPR strains having multiple mechanisms of actions should be studied to develop it as bio-inoculant for drought affected areas.
- The identification and evaluation of more efficient combinations of PGPR and rhizobia, which enable inoculated legume plants to behave in a more competitive way and remediate soils when established in contaminated fields, and discovering the mechanisms underlying their success is important.

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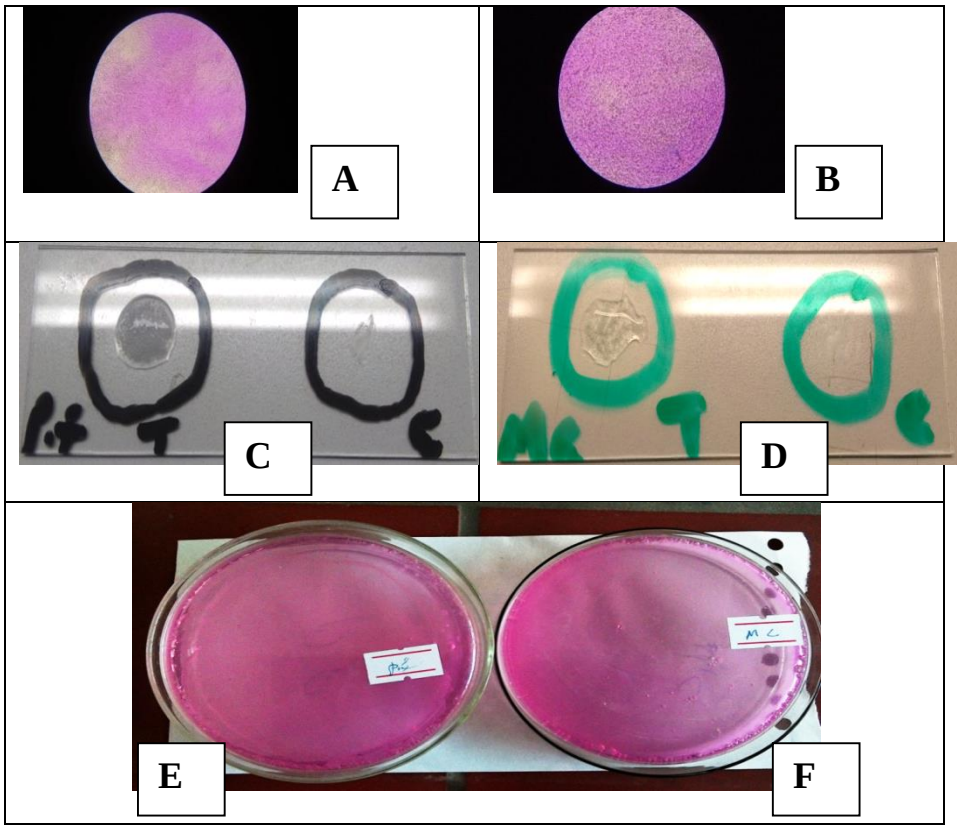
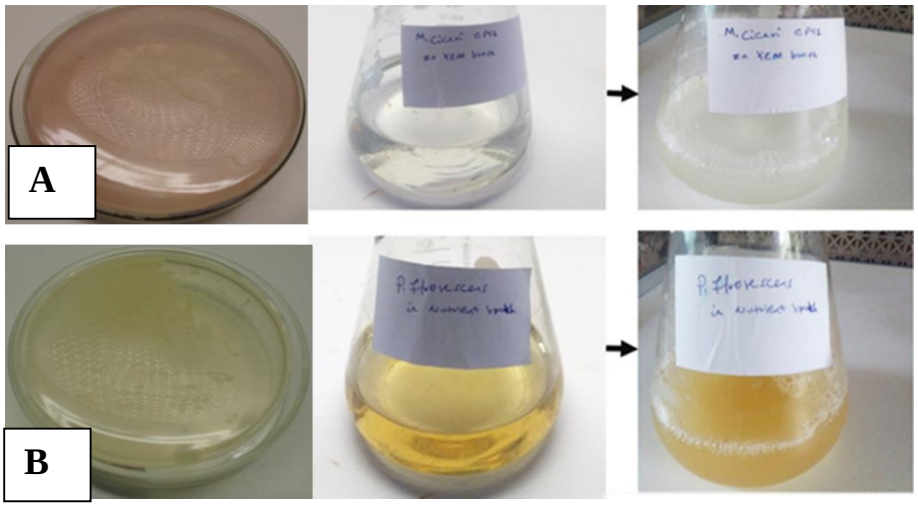
7. APPENDICES

Appendix 1: Pictures from laboratory work at ASTU microbiology laboratory and agricultural and nutrition laboratory at MARC

Appendix 2: Some pictures taken for bacterial properties and before inoculation

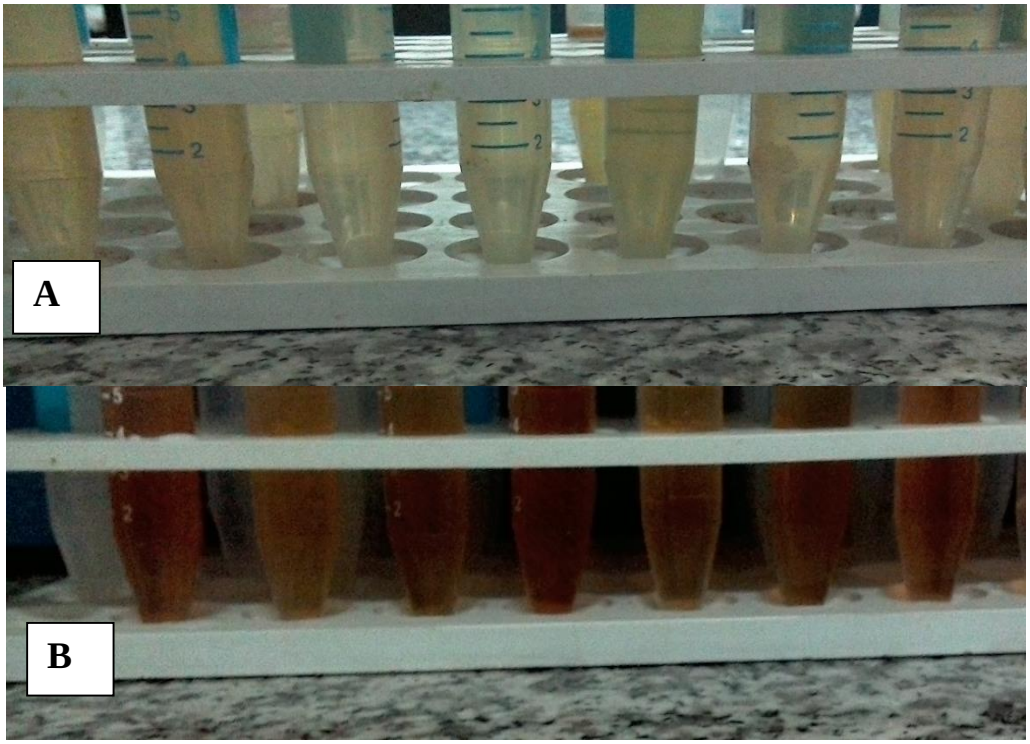


I: some morphological and biochemical tests, A and B showing gram staining (under 40X magnification) for *Pseudomonas fluorescens* and *Mesorhizobium ciceri* respectively. C and D show catalase test and E and F show urease test result for *Pseudomonas fluorescens* and, *Mesorhizobium ciceri*, respectively.

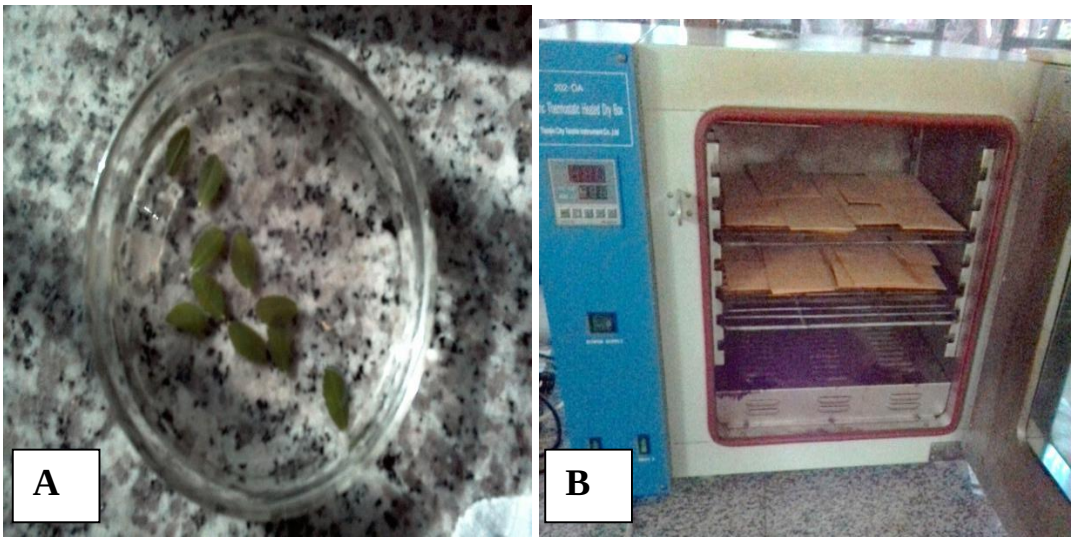


II: Turbid bacterial culture before inoculation. A, shows of *Mesorhizobium ciceri* culture and B shows *Pseudomonas fluorescens* culture.

Appendix 3: Some pictures from experimental procedures



I: Brownish color (B) during proline content determination after preparation (A) was warmed at 100^0C.



II: Leaves soaked in distilled water for turgid weight determination for relative water content (A), samples put in paper bag for dry weight determination (B).

Appendix 4: Script used to generate random position for the plants in the greenhouse

```
import collections
import random
cont = []
ls = []
for i in range(1, 13):
    for j in range(1, 4):
        s = f'T{i}R{j}'
        ls.append(s)
random.shuffle(ls)
random.shuffle(ls)
random.shuffle(ls)
cont.append(ls)
for k in range(27):
    new_ls = collections.deque(ls)
    new_ls.rotate(1)
    cont.append(new_ls)
    ls = new_ls
for k in range(1):
    random.shuffle(cont)
    filename = f'rand_position{k}.txt'
    with open(filename, 'w') as f:
        days = 1
        for i in cont:
            f.write('Day {:02d} - '.format(days))
            ix = 1
            for j in i:
                f.write('{:8}'.format(j))
                if ix == 12 or ix == 24:
                    f.write('\n\n\t ')
                ix += 1
            ix = 1
            s = '-'
            f.write(f'\n\n{s*105}\n\n')
            days += 2
```

Appendix 5: Modified Hoagland nutrient composition without nitrogen

Ingredients	**milligrams/liter**
Potassium phosphate monobasic	136.03
Potassium chloride	372.70
Calcium chloride.$2H_2O$	554.90
Magnesium sulphate	240.33
Manganese chloride.$4H_2O$	1.81
Boric acid	2.86
Copper chloride	0.045
Zinc chloride	0.11
EDTA ferric monosodium salt	33.00
TOTAL	**1.341 gm/liter**

Appendix 6: All raw data for plant growth, physiological and biochemical parameters

T	Leave FW(g)	Leave DW(g)	Root FW(g)	Root DW(g)	Shoot FW (g)	Shoot DW(g)	NNPP	NFW(g)	NDW(g)	RWC(%)	Proline (µmole/g)	TSS (mg/g)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)	Total carotenoid (mg/g)
T1R1	0.123	0.021	0.783	0.233	9.710	2.420	27	0.672	0.063	82.927	4.530	50.453	5.405	4.4147	9.820	0.654
T1R2	0.128	0.012	0.970	0.319	8.100	1.630	21	0.945	0.072	75.714	3.295	40.107	4.9303	2.56125	7.492	1.129
T1R3	0.191	0.026	0.807	0.276	8.301	1.840	23	1.117	0.102	85.492	5.167	34.855	4.01145	2.71056	6.722	1.058
T2R1	0.082	0.017	0.620	0.187	7.700	1.770	21	0.702	0.066	67.708	11.532	41.270	4.12425	2.44091	6.565	0.868
T2R2	0.122	0.019	0.711	0.211	7.300	1.500	13	0.417	0.085	69.595	14.265	28.881	4.9303	2.56125	7.492	0.865
T2R3	0.078	0.018	0.590	0.170	8.600	1.630	9	0.322	0.061	65.217	10.408	33.943	3.4169	2.42985	5.847	0.843
T3R1	0.143	0.015	0.920	0.263	10.200	2.170	34	1.182	0.115	83.893	4.044	36.616	5.2781	2.39058	7.669	1.442
T3R2	0.160	0.023	1.156	0.292	12.300	2.700	28	1.230	0.096	85.625	3.407	39.604	5.34155	3.22446	8.566	1.081
T3R3	0.190	0.024	1.691	0.315	13.804	3.120	24	1.130	0.084	87.368	2.920	35.484	5.29925	5.34124	10.640	0.781
T4R1	0.170	0.018	0.832	0.263	9.270	1.900	12	0.448	0.043	79.167	15.276	36.774	4.26055	2.34152	6.602	0.884
T4R2	0.113	0.017	0.675	0.179	8.740	1.468	24	0.788	0.078	77.419	17.896	31.082	2.8623	1.29823	4.161	1.369
T4R3	0.127	0.014	0.741	0.243	11.180	1.760	15	0.705	0.047	90.400	12.280	33.629	4.7047	2.38783	7.093	0.846
T5R1	0.059	0.015	0.520	0.210	7.502	1.621	0	0.000	0.000	80.000	3.070	24.792	3.572	2.71612	6.288	0.880
T5R2	0.076	0.017	0.550	0.187	8.300	1.570	0	0.000	0.000	74.684	3.931	29.572	4.04905	0.98736	5.036	1.319
T5R3	0.080	0.011	0.467	0.171	6.800	1.420	0	0.000	0.000	79.310	2.808	32.025	4.10075	3.607	7.708	0.511
T6R1	0.043	0.009	0.417	0.135	7.520	0.800	0	0.000	0.000	58.621	11.532	35.170	3.03855	1.95155	4.990	0.508
T6R2	0.048	0.008	0.580	0.132	6.210	1.100	0	0.000	0.000	66.998	13.141	27.308	3.2665	1.83909	5.106	0.541
T6R3	0.053	0.011	0.440	0.210	5.780	0.530	0	0.000	0.000	66.561	10.408	31.396	2.9375	1.77179	4.709	0.476
T7R1	0.062	0.013	1.034	0.304	8.916	1.560	16	0.574	0.052	75.385	3.182	33.126	4.1125	1.82124	5.934	0.993
T7R2	0.072	0.015	1.150	0.228	7.700	1.310	20	0.652	0.066	72.692	3.519	36.113	4.8175	3.00908	7.827	0.736
T7R3	0.069	0.015	0.841	0.209	8.310	1.570	27	0.768	0.095	76.056	2.209	59.384	5.3345	2.92393	8.258	0.660
T8R1	0.051	0.010	0.727	0.192	6.250	1.150	21	0.672	0.036	57.746	11.644	33.881	3.8493	2.6583	6.508	0.555
T8R2	0.041	0.012	0.850	0.173	8.230	1.540	17	0.432	0.047	58.000	9.435	32.969	1.42175	0.41174	1.833	0.336

T8R3	0.055	0.013	0.631	0.135	7.110	1.720	11	0.366	0.046	60.781	11.157	32.245	2.66725	2.04105	4.708	0.484
T9R1	0.134	0.019	1.320	0.278	8.682	1.500	23	0.936	0.083	85.821	85.821	31.962	4.540	2.978	7.518	0.940
T9R2	0.098	0.014	1.243	0.267	9.479	2.130	25	1.120	0.101	79.434	79.434	38.000	3.927	2.757	6.684	0.655
T9R3	0.088	0.011	0.945	0.242	8.960	1.730	32	1.131	0.095	76.238	73.047	40.830	4.030	4.789	8.819	0.740
T10R1	0.061	0.009	0.781	0.198	6.800	1.200	15	0.485	0.044	74.286	12.243	33.629	3.35815	2.0042	5.362	0.605
T10R2	0.078	0.014	0.910	0.242	7.260	1.210	21	0.819	0.057	60.415	13.965	35.799	3.2665	2.10636	5.373	0.644
T10R3	0.067	0.011	0.881	0.221	5.570	0.890	17	0.391	0.036	87.363	12.917	35.579	3.7647	3.23917	7.004	0.704
T11R1	0.082	0.014	0.571	0.187	5.800	1.200	0	0.000	0.000	70.833	2.583	28.566	2.1667	3.24319	5.410	0.445
T11R2	0.043	0.009	0.731	0.176	7.200	1.600	0	0.000	0.000	68.000	2.965	25.421	3.84225	2.53595	6.378	0.788
T11R3	0.090	0.014	0.490	0.160	6.484	1.011	0	0.000	0.000	76.000	1.760	25.013	3.2242	3.95587	7.180	0.668
T12R1	0.019	0.005	0.282	0.090	4.371	0.400	0	0.000	0.000	42.424	10.446	25.736	2.21135	1.55325	3.765	0.434
T12R2	0.033	0.010	0.591	0.200	3.920	0.270	0	0.000	0.000	40.351	8.050	24.792	2.0821	1.15136	3.233	0.425
T12R3	0.027	0.004	0.473	0.130	3.653	0.555	0	0.000	0.000	57.500	8.724	34.950	2.57325	2.07273	4.646	0.376

Keys: T= treatments, FW= fresh weight, DW=dry weight, NNPP= nodule number per plant, NFW=nodule fresh weight, NDW= nodule dry weight, RWC= relative water content, TSS= total soluble sugar