Consortium Effect of *Rhizobium* Strains to Regulate Physiological and Biochemical Response in Common Bean (*Phaseolus vulgaris*) During Water Stress

By

Eticha Abdisa Bayisa

A Thesis Submitted to Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

January, 2021

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APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by Eticha Abdisa Bayisa entitled have read and evaluated his thesis entitled "Consortium effect of *Rhizobium* strains to regulate physiological and biochemical response in common bean (*Phaseolus vulgaris*) during water 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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Dr.Mulugeta Kebede _____ _____

Name of major Advisor Signature Date

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ACC	Amino Cytopropane Carboxylate
ANOVA	Analysis of variance
BNF	Biological Nitrogen Fixation
CB	Common Bean
EIAR	Ethiopian Institute of Agricultural Research
FC	Field Capacity
IAA	Indole Acetic Acid
IB	Institute of Biodiversity
MARC	Melkasa Agricultural Research Center
NCC	Nitrogen Containing Compound
PGPR	Plant growth promoting Rhizobacteria
RCBD	Randomized Complete Block Design
RDW	Root Dry Weight
ROS	Reactive Oxygen Species
ROS	Reactive Oxygen Species
RWC	Relative Water Content
SAS	Statistical Analysis System
SNF	Symbiotic Nitrogen Fixation
SOD	Superoxide Dismutase
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Common bean (Phaseolus vulgaris L.) is one of the important legume crop grown throughout the world as main dietary source of nutrient. It also plays a major role in soil fertility through biological nitrogen fixation. The major abiotic stress affecting common beans are drought stress and water stress, which cause the loss of productivity in Ethiopia and other countries. The aim of this study was to minimize the impact of water stress on common bean by using consortium effect of Rhizobium strains of Haricot Bean-429 (HB-429) and Haricot Bean of Ethiopia Agricultural legume-429 (HBEAL-429). This synergism acts as plant growth promoting rhizobacteria and expected to reduce the effect of water deficit and improve the yield. The study was carried out by using two contrasting drought tolerant (Awash-2) and drought susceptible (Nasir) common bean cultivars treated with the inoculants under greenhouse condition. Two soil water contents (80% and 40% of field capacity) for growth, physiological and biochemical adjusts were evaluated along with HB-429 or HBEAL-429 strain alone, co-inoculation of HB-429 and HBEAL-429 strains and un-inoculated seeds were grown in greenhouse condition. The experiments in greenhouse showed that co-inoculation has alleviated the plants situation under drought stress than Rhizobium strain alone and uninoculated control plants. Co-inoculation had significantly enhanced fresh and dry weight of leaves, fresh root and shoot ($p<0.05$). Then, the relative water content, fresh and dry weight of leave, root nodules number as well as proline content and chlorophyll a and b content were significantly affected ($P< 0.05$) in both varieties while a non-significant difference was observed at root nodule and root dry weight. Hence, positive effect through co-inoculation was observed in Awash-2 than in Nasir bean varieties. The present study showed that synergistic application had improved the potential to confer drought tolerance in common bean by changing various growth, physiological and biochemical parameters under drought stress conditions.

Keywords/Phrases: Consortium, drought tolerant, *Phaseolus vulgaris L.*, *Rhizobium*, strain

1. INTRODUCTION

1.1 Background of the Study

Common bean (*Phaseolus vulgaris* L.) is species of the genus *Phaseolus* belongs to family *Fabaceace* of about fifty plant species, which are all native to Latin America and Central America (Thulin,1989). Common bean is the most important legume crop and a crucial source of nutrition worldwide with high potential for direct human consumption (Sileshi, *et al*., 2014). It is grown throughout the world as main dietary source of proteins, carbohydrates, dietary fiber, and minerals (Broughten *et al*., 2003; Tharanathan and Mahadevamma, 2003; Acosta-Gallegos *et al*., 2007). Common bean also contributes for cash commodity, which serve as income employ to a large supply chain and the stems (straw) are also used as fodder for livestock (Kedir, 2017). Moreover, amino acids (22%) contained in common beans are of high dietary value (Reyes-Bastidas *et al*., 2010) and help to reduce the risks of serious human diseases (Rondini *et al*., 2012).

In Ethiopian, the common bean growing season is between 80-100 days in which the crops require various agro-ecological zones between 350-500mm of water depending on depth of soil, climate and genotype (Beebe *et al*., 2013). Many research activities have been done in Ethiopia to improve common bean varieties with respect to their yield, tolerance to different biotic and abiotic stresses (Asfaw and Blair, 2014). As a result, improved common bean varieties of Awash and Nasir were released by Ethiopian Institute of Agricultural Research (EIAR) (Shiferaw, 2004). However, research on controlling the potential of soil microbes to alleviate abiotic stress in common bean plants is still limited. So far, improvement of drought tolerant crop varieties, increase the productivity of the plant (Eisenstein, 2013).

Conventional plant breeding techniques have allowed the development of high yielding, drought tolerant crop varieties (Atkinson and Unwin, 2012). 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, 2005; Eisenstein, 2013). The approach mentioned above overlooks the ecological context of the soil environment in which the crops are grown (Morrissey *et al*., 2004).

Water stress is one of the main environmental factors which limit plant growth distribution and crop yield worldwide (Shi *et al*., 2014). Water scarcity and reductions in precipitation are

some of the major issues of climate change. Drought is a term used for water stress because of absence or inadequate rainfall and/or water supply (Toker *et al*., 2007). Hence, water is one of the most vital environmental factor regulating plant growth and development (Manivannan *et al*., 2007).

Common bean cultivars development in drought adaptation is an important strategy to minimize crop malfunction and increase food security in the face of climate change. Identification of key plant traits and mechanisms that contribute to improved drought adaptation can increase the efficiency of breeding programs through the selection of superior genotypes. However, drought tolerance is a complex trait that cannot be investigated solely, as it is influenced by many factors including morphological and physiological characteristics. Plants respond and adapt to a variety of environmental stresses in order to survive. Hence, Osmotic and drought stresses resulting from these environmental changes are causing loss of crop yields (Verma *et al*., 2016).

Most studies have focused on the effects of drought stress and drought resistance of common bean varieties on agronomic or physiological variations with less effort on the combination of these factors (Cattivelli *et al*., 2008; Kargiotidou, 2018). However, the characterization of the consortia effect of plant growth promoting Rhizobacteria (PGPR) attributes to drought tolerance using the selected varieties still remain the subject of future investigation. According to Gian *et al.,* (2009), the key factors identification and patterns of combined variability in agronomic, morphological and physiological traits under drought can draw a picture on how these factors contribute to final grain yield in a consorted way. Inoculation of seeds by *Rhizobium spp* prior to common bean planting has also been reported to be a key factor in enhancing nodulation, crop vigor and high grain yield (Figueiredo *et al.,* 2008 and Otieno *et al.,* 2009). The study conducted by Mbugua *et al.* (2009) on effects of commercial *Rhizobium* strain inoculants on yield of new dry bean showed that bean seeds inoculated with *Rhizobium* strain had higher daily germination count, crop vigor, number of seeds per pod and grain yield as compared with those of uninoculated crops. Therefore, there is a need for microbial based approaches to ameliorate drought stress effect on plants. Hence, this study was to assess the consortiatic effect of PGPR in ameliorating in common bean for advancing productivity. Focus should be given to the potential of consortia of growth promoting rhizobacteria to reduce the effect of water stress by adjusting physiological and biochemical response in common bean. The quantity of each of these variables is challenging in terms of time and resources.

1.2 Statement of the Problem

Drought is one of the most important limiting factor to common bean (*Phaseolus vulgaris L.*), disturbing its growth and productivity and, thus, contributing to significant losses in yield. Common bean is a crop that is regularly affected by drought stress to the last stages of the growing season under irrigated and rain fed conditions. The response of common bean to drought is still not adequately well characterized due to its genetic complexity and its diverse, often vague, phenotypic effects. The main statement to be tested is the combination effect of different *Rhizobium* strains on morpho-physiological traits (Polania *et al*., 2016).

In Ethiopia, it is recognized that growth and productivity of common bean is negatively affected by the increasing shortage of water resource (Asfaw and Bliar, 2014; Asfaw et al., 2012). So, water is a major limiting factor in the world agriculture. The effects of drought stress on yield and yield characteristics such as biomass, height, flowering and seed development at different growth stages of the crop is evident.

Application of bacterial as inoculants particularly 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). Therefore, it would be important to consider the combined effect of PGP rhizobacteria in ameliorating drought stress in common bean for improving productivity. It is also important to focus on the potential of consortia of growth promoting rhizobacteria by identifying physiological and biochemical attribute to improve the ability of the plant to cope with drought stress. This is for the purpose of categorizing drought tolerance indices contributing to adjustment to the moisture deficit patterns of common bean's native environment by their association with bacteria that have plant growth promoting properties. Thus, the study addressed the combination effect of different *Rhizobium* strains on physiological and biochemical traits.

1.3 Objective of the study

1.3.1 General objective

- To investigate consortium effect of *Rhizobium* strains to regulate physiological and biochemical response in common bean (*Phaseolus vulgaris*) during water stress.

1.3.2 Specific objectives

- To determine relative water content and development using two drought contrasting common bean varieties with rhizobacterial inoculation under well watered and water stress condition
- To characterize the symbiotic effect of *Rhizobia* strains for some physiological and biochemical traits.
- To analyze proline and total soluble sugar content during water stress using drought tolerant and susceptible cultivar of common bean with and without inoculation

1.4 Significance of the study

One of the major threats to common bean is drought that affect its growth and productivity and, thus, contributing to considerable losses in yield. The development of drought tolerant varieties from drought stress has, therefore, become one of the primary goals in many common bean breeding programs. Given, the regular occurrence of drought in bean growing area in Ethiopia, it would be useful to use combination of rhizobacteria inoculants that will help bean crops fight drought stress and hence produce higher yield. From the common bean's enhancement perspective, it is also important to distinguish the particular physiological and biochemical traits. This is for the purpose of identifying drought tolerance indices contributing to adaptation to the moisture deficit patterns of bean's environment by their symbiotic association with growth promoting bacteria will help to increase yield of the crop.

2. LITERATURE REVIEW

2.1 Origin, distribution and cultivation of Common Bean (*Phaseolus Vulgaris L.)*

Common bean was originated in tropical America but there are also evidences for its' multiple domestication with in Central America (Kay, 1979). The crop is now widely distributed throughout the world and is grown in all continents except Antarctic and occupies more than 90% of production areas sown to *Phaseolus* species (Arenas *et al*., 2013). Common beans are generally harvested as dry beans, harvested as dried and matured seeds, shell beans, harvested at physiological maturity before seeds are dry and green pods. It is a particularly important legume crop grown worldwide. It grows best in warm climates at temperatures of 18 to 24°C (Gebre-egziabher *et al*., 2014).

2.1.1 Genetics and botany of common bean

The common bean is an annual herbaceous dicot plant that belongs to the *Fabaceae* family. It is regarded primarily as a self-pollinating plant and it is an autogamous diploid species with a total chromosome number of 2n =22, due to floral morphology (Singh *et al*., 2010; Delgado Salinas *et al.*, 2007). Common bean is approximately 625 Mbp of haploid genome size which is one of the smallest genomes among the legume family (Broughton *et al*., 2003). There are diverse botanical varieties of the species of the genus phaseolus that vary in terms of seed and pod characteristics, agronomic features, and response to biotic and abiotic stresses, growth habit and patterns (Baeta *et al*., 2010). Crosses between the Middle American and Andean gene pools are easily accomplished. It has been noted that divergences between the two gene pools may make recovery of progeny more difficult than with crosses within the two pools, and occasionally crosses result in dwarfism or lethality (Acosta-Gallegos, Kelly and Gepts, 2007; Singh and Schwartz, 2010).

2.2 Drought stress in common bean

Drought is one of the most common abiotic stresses limiting common bean production in different parts of the world. More than 60% of the world's common bean is cultivated under non-irrigated conditions, and they also cultivated in regions where water can cause reduction in growth and nutrient uptake and drought is estimated to cause up to 80% yield losses in many regions of the world (Cuellar *et al*., 2008; Zadehbagher, 2014). Common bean

frequently suffers from drought stress towards the end of the growing season in rain-fed conditions. Ninety percent of the world's common bean is produced in areas relying upon conserved, receding soil moisture. Therefore, soil moisture is a major factor for crop productivity. Early maturing varieties of common bean have been developed to escape terminal drought (Kumar, 2012). The fluctuation of protein synthesis or degradation is one of the essential metabolic processes that may influence water stress tolerance (Najafi *et al.,* 2010). There are some evidences that plants are more tolerant to water deficit when water is with held under conditions that favor osmotic adjustment (Moinuddin and Khanna-Chopra, 2004; Talebi *et al.,* 2013). While common bean, in terms of its global harvested area, is the third most economically important grain legume after soybean (*Glycine max* (L.) Merr.), in terms of its role in direct human consumption, is the most important grain legume (Broughton *et al*., 2003).

2.3 Association of common bean with *Rhizobium* and biological nitrogen fixation

A mutualistic interaction between soil bacteria and plants of the legumes is known as *Rhizobium*-legume symbiosis. Common bean is a leguminous crop that has capability to fix biological nitrogen with the help of *Rhizobium spp*. Two species which form functional nodules when interacting with common bean roots are described as specific symbionts *Rhizobium* strains. Frequent isolation of bacteria from surface-sterilized plant roots explored a new class of Nitrogen fixing endophytes called associative symbionts, *i.e.* cyanobacteria and Azospirillum (Moore *et al*., 2006). Associative symbionts are free-living organisms that assimilate atmospheric nitrogen using a particulate oxygen sensitive nitrogenase in association with some plants (Kelly, 2004). They include a wide range of nitrogen fixing species that colonize the root surface of non-leguminous plants without formation of unique structures such as nodules (Franche *et al*., 2009).

Rhizobium spp. inoculations to leguminous crops are considered to increase nitrogen availability to the plant. However, legume cultivars respond variably to rhizobia inoculation, depending on their symbiotic compatibility with the bacterial strain and environment conditions (Heilig *et al*., 2017; Youseif *et al*., 2017). Nitrogen is one of the most important elements for plant nutrients. Therefore, it is necessary to apply reliable methods for Nitrogen determination in soils. Total nitrogen determination has been applied for geochemical, biochemical, and biogeochemical studies of this element in soils, sediments, and sedimentary

rocks. Nitrogen determination is also very important for biosphere functioning and evolution forecasting studies (Pontes *et al*., 2009).

The process that changes inert nitrogen into biologically useful ammonia is known as Biological nitrogen fixation. This process is mediated in nature only by nitrogen fixing rhizobia bacteria (*Rhizobiaceae*, alpha-*Proteobacteria*) (Sorensen and Sessitsch, 2007). Symbiotic Nitrogen Fixation (SNF) has the potential to improve soil fertility, enhancing the nitrogen (N) balance in soil and nitrogen availability to the subsequent, or accompanying crops (Triboi and Triboi-Blondel, 2014). Globally, legumes can fix about 20×10^6 Mg (1Mg = 10^6 g) of nitrogen per year to the agricultural systems (Herridge *et al*., 2008).

2.4 Drought stress tolerance mechanism mediated by rhizobacteria

The mechanism by which plants respond to drought stress needs to be explicated to grow stress tolerant plants (Amede *et al*., 2003). It is very complex process because of interaction between factors affecting and being affected by the specific stress, various physiological, biochemical and molecular phenomena resulting in altered plant growth (Choudhury *et al*., 2017). Along with water stress, it leads to physiological and biochemical changes in plants for adaptation to abiotic stress. Different types of adaptation mechanisms used by plant to tolerate drought. Beneficial effects of bacterial inoculation on plant physiological, biochemical and growth under drought stress conditions are: phytohormones biosynthesis, accumulation of protective compounds, biosynthesis of anti-oxidative enzyme and enhancement of nutrient uptake (Glick and Bernard, 2003).

Phytohormones biosynthesis: The influence of plant growth-promoting bacteria in legume plants mainly due to the synthesis of indole acetic acid (IAA) Phytohormones. They are very important in this process (Potters, 2007). Phytohormone such as IAA, gibbereline, ethylene, abscisic acid and cytokines are produced by plants, which are important for their growth and development (Imada *et al*., 2017; Liu *et al*., 2018). PGPB are also known to stimulates plant cell growth and division to become tolerant against environmental stress mediated through biosynthesis of phytohormones (Glick and Pasternak, 2003).Various plant species inoculated with IAA producing bacteria increased root growth and enhanced formation of roots (Dimkpa *et al*, 2009). Thus, it increases water and nutrient uptake, helping plants to cope with water deficit (Egamberdieva and Kucharova, 2009). Cohen *et al*. (2009) reported that the production of abscisic acid and gibberellins by *Azospirillum lipoferum* improved drought

stress in maize plants. Cellular dehydration stimulated biosynthesis of abscisic acid, an important stress hormone during water deficit condition (Kaushal and Wani, 2015).

Accumulation of protective Compounds: Another widespread characteristic among endophytic and rhizosphere bacteria is ACC deaminase activity, and regulation of ACC is a principal mechanism by which bacteria exert beneficial effects on abiotically stressed plants (Saleem *at al.*, 2007). According to Parid and Das (2005), several studies correlated accumulation of nitrogen and carbon containing compounds (NCC) which include osmolytes such as proline and sugars with drought and salt tolerance in plant. The one of the most frequently accumulating NCC includes amino acids, amides, amino acids, proteins, quaternary ammonium compounds and polyamines. Very high accumulation of proline due to increased synthesis and decrease degradation under drought stress has been documented in many plants (Szabobados and Savoure, 2005). An increased proline biosynthesis was observed for various plant species inoculated with different PGPR under abiotic stress conditions (Barka *et al*., 2006, Kohler *et al*., 2009; Sandhya *et al*., 2010).

PGPR inoculation increase proline content in plant due to up regulation of its biosynthesis pathway hence maintaining cell wall status and protecting cell membranes and proteins from injury caused by drought stress (Sandhya *et al*., 2010). Proline has been proposed to act as a compatible osmolyte and to be a way to store carbon and nitrogen. Saline and drought are known to induce oxidative stress. Several studies showed that proline may have an antioxidant activity acting as a reactive oxygen species (ROS) scavenger. 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). Proline may also function as molecular chaperones able to stabilize the structures of proteins and enhance the activity of different enzymes, and its accumulation play a role in maintenance of cytosolic pH and regulation of intracellular redox potential (Kavi Kishor and Sreenivasulu, 2014; Verbruggen and Hermans, 2008).

Biosynthesis of anti-oxidative enzyme: Production of reactive oxygen species (ROS) is a component of normal aerobic cellular metabolism of living organisms, including plants. The shortage of water could limit the yield of plants and may cause damage to plant tissues, particularly roots, through the formation of reactive oxygen species (ROS) due to high susceptibility of root meristem activity to ROS (Mahdi Dar *et al.*, 2018). Apel and Hirt,

(2004) state that, in plants reactive oxygen species (ROS) such as superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and single oxygen (O_2) are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments. ROS is capacity to cause oxidative damage to proteins, DNA, and lipids. One of the non-enzymatic molecules which have a significant part as photo-protective compounds by quenching triplet chlorophyll and singlet oxygen derived from excess light energy is carotenoid (Pogson *et al*., 2006). Carotenoids participate in energy indulgence 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).

According to Gill and Tuteja (2010), since internal O_2 concentrations are high during photosynthesis, chloroplasts are especially prone to generate activated oxygen species .Under physiological steady-state conditions, these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments (Apel & Hirt, 2004). Under normal growth conditions, the production of ROS in cells is low, whereas, during stress their rate of production is enhanced. ROS accumulation during stress results from the imbalance between production and scavenging of ROS. Major ROS scavenging mechanisms of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) enzymes. Antioxidants such as ascorbic acid and glutathione, which are found in high concentration in chloroplasts and other cellular compartments, are also crucial for plant defense against oxidative stress (Miller *et al*., 2010). For the detoxification of excess ROS in plant, the overall balance between different antioxidants is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide, and has to be tightly controlled (Mittler, 2002).

Enhancement of nutrient uptake: It has been reported by Munns and Tester (2008) that survival and productivity of crop plants exposed to environmental stresses are dependent on their ability to develop adaptive mechanisms to avoid or tolerate stress. Accumulating evidence suggests that mineral nutritional status of plants greatly affects their ability to adapt to adverse environmental conditions and in particular to abiotic stress factors. Impairment of the mineral nutrition status of plants exacerbate the adverse effects of abiotic stresses and the exogenous addition of high levels of macronutrients can alleviate the adverse effects of stress on plant growth (Endris and Mohammed, 2007; Heidari and Jamshid, 2010; Khoshgoftarmanesh *et al*., 2010).

2.5 Impact of water stress on growth, physiological and biochemical parameters

Water stress is a widespread problem throughout the world and the legumes production under water restrictive conditions is common (Munoz-Perea *et al.,* 2006). Drought has the most important impact on plant growth and development, limiting crop production throughout the world. Drought and irregular rainfall are linked to climate alter and such conditions cause a significant growth limit for many field crops, including dry beans, especially, in the semiarid Mediterranean regions (Boutraa and Sanders, 2001; Ninou *et al.*,2013). These situations greatly influence production characteristics (yield, seed quality) which in turn impact the commercial value of the end product and the farmers' income. Among grain legumes, common beans are relatively sensitive to drought stress and these findings have been confirmed by experiments in greenhouses, and in controlled conditions (Costa-France *et al*., 2000). Drought is mainly observed and responded by plant roots, mostly under field conditions. 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 rely 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, disorder the photosynthetic activity showing refuses in shoot biomass, as well as the embarrassment of photosynthetic 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 (Antolín *et al.,* 2010).

Relative water content (RWC) is an important physiological parameter in plants which is considered as an important method to measure plant water status**.** A decline in RWC indicates a loss of turgor that results in limited cell expansion and, consequentially, reduced growth in plants (Ashraf and Iram, 2005). 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 confirmed that higher RWC may help plants to contest the oxidative and osmotic stresses caused by drought stress, possibly contributing to greater productivity under stress. It has been approved 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 alterations are due to the result of detrimental effect of water deficit on vital metabolic processes as well as response of various defense mechanisms adapted by the plant under drought stress (Amede and Schubert, 2003).

Plants can partly defend 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 companionable osmolyte in drought stressed plants, which maintains redox metabolism by eliminating 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). Homayouni and Khazarian, (2014) suggested that accumulation of soluble sugars increased in stressed tissues matches to a reduction in starch concentration and is strongly correlated to the attainment of drought tolerance in plants. Drought stress causes significant harm to photosynthetic pigments, and it also leads to descent of thylakoid membranes (Kannan and Kulandaivelu, 2011).The decrease in chlorophyll content is a commonly observed change on the plant under drought stress (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, transferring the ratio in favor of chlorophyll a. A decrease in total chlorophyll with drought stress implies a lowered capacity for light harvesting.

2.6 Co-inoculation of Plant Growth Promoting Bacteria

Many studies have recently shown that inoculation with some plant growth promoting bacteria (PGPB), increases growth and yield in great number of plants including legumes (Pinlak *et al*., 2009). Co-inoculation is based on mixed inoculants, combination of microorganisms that interact synergistically, or when one organism is as functioning as 'helper' bacteria to enhance the performance of other beneficial microorganisms. In the rhizosphere the synergism between various bacterial genera such as *Rhizobium*, *Bacillus* and *Bradyrhizobium* have been demonstrated to promote plant grow and development. Co-inoculation, frequently increased growth and yield, compared to single inoculation, is provided the plants with more balanced nutrition, and improved absorption of nitrogen, phosphorus, and mineral nutrients (For recent reviews see (Bashan *et al*., 2014). A significant increase in root and shoot biomass was observed in common bean plants when co-inoculated with *Rhizobium* strain (Sindhu *et al*., 2002). Increased nodule weight, root and shoot biomass and total nitrogen of common bean plants were also reported due to co-inoculation of *Rhizobium*, *Bacillus* and *Bradyrhizobium* (Bai *et al*., 2003). According to Bai *et al*., (2003), co-inoculation with *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* increased colonization *B. japonicum* on soybean roots, nodule number and acetylene reduction assay. Elkoca *et al. (*2008) state that inoculation of *Rhizobium* with *Pseudomonas striata* or *Bacillus megaterium* combined together led to increased dry matter, grain yield and phosphorus uptake significantly over the uninoculated control in legumes.

3. MATERIALS AND METHODS

3.1 Description of the Study Area

The study was conducted at Adama Science and Technology University (ASTU) and Melkassa Agricultural Research Center (MARC). They are located in Oromia Regional State, East Shoa zone, Ethiopia. ASTU and MARC are 100 km and 115 km away from Addis Ababa, respectively. ASTU is situated between 8^0 33'43.56", latitude (N) and 39^0 17'23.28" longitude (E) with an elevation 1620 m.a.s.l whereas MARC is situated in the Central Rift Valley between 8˚24'N latitude and 39˚21' E longitude and it is located at an elevation of 1550 m above sea level (MoA, 2000).

At ASTU the mean annual rainfall of the area was 928.8 mm, with the highest mean monthly rainfall recorded in July (443.2mm) and the lowest in December (48.6mm). The mean monthly maximum temperature recorded was 30.7^{o}C in May and the mean minimum was 23^{o}C in August and maximum was 32^{o}C. Rainfall in ASTU was erratic and uneven in distribution. The soil type is dominated by Loam and clay loam soil textures (Dejene and Birhanu, 2020).

MARC has the mean annual rainfall of the area was 767.2 mm, with the highest mean monthly rainfall recorded in July (157.6 mm) and the lowest in August (161.5mm). The mean monthly maximum temperature recorded was 30.6 ^{o}C in May and the mean minimum was 26.5°C during August respectively and minimum and maximum relative humidity during the experiment was 38 % and 72 %, respectively (Fig 1) (Abebe *et al*., 2013).

According to the recent agro-ecological classification of Ethiopia (MoA, 2000), the Melkassa Hypo Calcic Regosol ecotope falls in the zone termed hot to warm semiarid lowlands. Loam and clay loam soils are the dominant textural classes (Tsion *et al.*, 2009).

3.2 Plant growth condition and sample site

The investigation of consortium effect of *Rhizobium* strains to regulate physiological and biochemical response in common bean (*Phaseolus vulgaris*) during drought stress was conducted in greenhouse environment and Nutrition Research Laboratory of MARC, and in microbiology laboratory of Adama Science and Technology University (ASTU). During the experimental study, temperature and relative humidity was measured using thermo-hydrometer measuring device. The plants growth areas with medium rainfall ranging from 350mm to 700mm (70 to 100 days) are good with a well-defined rainy season so that harvesting is done in the dry weather. An investigation of growth parameters and biochemical parameters such as proline, chlorophyll a and b content, seed color and carotenoid content as well as physiological parameters such as Relative Water Content (RWC), fresh and dry weight of leave, root nodule number and dry weight was done in green house and ASTU biology laboratory.

3.3 Source of inoculants

Commercial *Rhizobium* strain HBEAL-429 peat based carrier was obtained from Menagesha Biotech Industry P.L.C., Addis Ababa, Ethiopia. This strain has been established to increase the growth of common bean seeds under widespread ecological conditions and it was considered as a very good strain of the laboratory as far as agronomic and yield performance of common bean was concerned (Tena, *et al*., 2016), and HB-429 strain was taken from culture assortment of Holeta National Biotechnology Research center.

3.4 Source of common bean seeds and soil substrate

Seeds of *Phaseolus vulgaris L.* varieties (Nasir and Awash-2) were obtained from MARC (Figure 1). The two common bean varieties (Nasir and Awash-2) were chosen based on their maturity time, acceptability in nutritional value, productivity and seed availability. Bean seeds were surface sterilized with 70% ethanol for 2 minutes and rinsed with distilled water.

3.5 Soil analysis

The soil from the study area was characterized before planting. Soil samples were randomly taken from the experimental field at a depth of 0 to 20cm top layer and the samples were mixed thoroughly to produce one representative combined sample of four kilogram (2kg of sand and 2kg of soil). The soil sample was air-dried and ground to pass 2 and 0.5 mm sieves and analyzed for total N, pH, organic carbon (OC), exchangeable cations and physical

properties at Melkasa Agricultural Research Center Soil Laboratory. The soil substrate was sandy loam (soil: sand in 1:1 w/v) and it was sterilized with hot air oven at 120 °C for 30 minutes and its physiological properties were analyzed. The soil pH was measured in the supernatant suspension of a 1: 2.5 w/v soil to water ratio using a standard glass electrode pH meter. The Walkley and Black (1934) method was used to determine the organic carbon (%). Total N was determined using Kjeldahl method as described by Bremner and Mulvaney (1982). Briefly, the cation exchange capacity (CEC) in cmol (+) kg-1 was measured using 1M-neutral ammonium acetate method (Jackson, 1967).

Four kilogram containing plastic pots was used to grow the common bean. The positive control plant (un-inoculated and well-watered, 80% field capacity) and water deficit treatment were received while the negative control plants (water deficit, 40% field capacity).

Figure 1: (a) Awash-2 obtained from Melkasa agricultural research center (MARC) which is a drought tolerant. **(b)** Nasir collected from MARC which is drought susceptible cultivar.

3.6 Activation and maintenance of Rhizobium species purity

According to Vincent (1984), nine milliliter of 0.85% (w/v) saline solution was mixed with One gram of carrier to make dilution of 10^{-1}-10^{-6} and 0.1ml from each dilution was placed on Yeast Extract Mannitol Agar (YEMA, LR,ALPHA CHEMIKA, India) containing 0.0025% (w/v) Congo red (CR). The components of YEMA (g/l) are: 0.5 K_2HPO4, 0.2 MgSO4, 0.1 NaCl, 10 Mannitol, 0.5 Yeast Extract and 15 Agar. All the plates were incubated at 30°C. The culture grew in 3-5 days; a single colony that did not take up the color of CR was taken and re-streaked on YEMA, since did not absorbing CR and had a distinctive character of rhizobia with only few exceptions (Somasegaran and Hoben, 2012). The *Rhizobium* strains were then characterized for some morphological and biochemical properties (tested for catalase which may indicate the bacterium's ability to indirectly promote plant growth). The purity of culture was also checked by presumptive tests were done by re-culturing the primary isolates into YEMA. Isolated colonies of *Rhizobia* were transferred on YEMA medium plates and slants containing 0.3% (w/v) $CaCO_3$ and were stored in refrigerator at 4°C for further studies (Vincent, 1984).

3.7 Biochemical and morphological verification of Rhizobium

Morphological examination was done on the basis of **s**hape, color, size, elevation, margin and texture of colonies of *Rhizobium* strain. It was determined by growing them on YEM and

nutrient broth, respectively (Vincent, 1984). Then cultures were spread on YEMA and nutrient agar plates and incubated at 28 °C for 3-5 days. After four days for isolates strain, the shape, color, size, elevation, margin and texture were noted.

3.7.1 Gram staining of the bacterial strains for identification

Gram staining was performed to check the Gram status of the isolated bacteria. It was conducted to study gram reaction and staining was carried out according to standard Gram's procedure (Somasegaran and Hoben, 2012). Gram's staining held four different reagents in the order of Crystal violet; Gram's iodine, alcohol distaining reagents, and safranin. A loop full of each isolate from broth culture was spread on a microscopic slide and a thin smear was prepared. Then the smear was gram stained and their shapes and gram reaction was observed microscopically to confirm the identity of the isolates.

3.7.2 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).

3.8 Compatibility of the strains selected for the bacterial consortium

PGPR strains were tested for their compatibility among each other was performed according to Raja *et al.* (2006). The compatibility was determined by using YEMA medium. The *Rhizobium* HB-429 strain was streaked vertically and HBEAL-429 was streaked horizontally. The plates were incubated at 30°C for 72 hours and then examined for the formation of inhibition zones around the colonies. Absence of inhibition zone shows the compatibility with relevant bacteria.

3.9 Seed inoculation with *Rhizobium* and Sowing

For inoculation, cultures of HB-429 and HBEAL-429 strain were grown for 24 hours in nutrient broth at 30 ^{0}c in an incubator shaker at 1500rpm before dilution to the required concentration of bacterial suspension (10^8 -10^9 CFU ml $^{-1}$). Cultures of strain were grown for three-six days in Yeast Extract Mannitol broth at 28 ^{o}c in shaker incubator at 1500rpm. Then, seeds were saturated in 5% of sucrose solution for the purpose of sticker. Inoculation of 1ml per pots of 1x10^9 cells/ml growth in liquid culture inoculants were applied onto the seeds

before sowing and after germination at the emergence cotyledon. For co-inoculation, HB-429 and HBEAL-429 strain were used in 1:1 ratio after thoroughly mixed.

Four common bean seeds per pot were planted for every experimental unit. Planting was done using a spacing of 40 cm between rows and 10 cm between plants. Two-third of the experimental groups was inoculated with HBEAL-429 and HB-429 alone, on another side. One-fourth of co-inoculated with HBEAL-429 and HB-429 bacteria and the rest was not inoculated at all. Water was added as necessary to maintain appropriate soil moisture throughout the length of the experiment. Tinning was done after seedlings were established and only one healthy seedling per pot was possible.

3.10 Experimental design and treatments

The experiment consisted of four adjacent blocks of 24 experimental pots for water-stressed (WS) treatment and 24 experimental pots for well-watered (WW) treatment. The experimental design was carried out using randomized complete block design (RCBD) with three replication, with two cultivars that is Nassir and Awash-2 (of one drought tolerant and one drought susceptible common bean varieties) and three inoculation (single inoculated, co-inoculated and non-inoculated), two water management system (water deficit and control) and two well watered, so they sum up 16 treatments (Table 2). Therefore, 3x16= 48 experimental units were done. The experiment was conducted from January 2020 to December 2021.

Table 1: Experimental design and treatments allocation

No.	Sample Treatments (STs)	Types of Common bean and *Rhizobium* strains
1	$ST_{1=}$ DS +HB-EAL-429 + WW	Awash-2 and Nasir
2	ST2= DS+ HB-EAL-429 +WD	HB-EAL-429 and HB-429
3	ST3=DS+ HB-429 + HB-EAL-429 + WD	
4	ST4= DS+ HB-429 + HB-EAL-429 +WW	
5	ST5=DS+WW	
6	ST6=DS+WD	
7	ST7= DS+HB-429+WW	
8	ST8= DS+HB-429+WD	
9	ST9=DT+ HBEAL-429 + WW	
10	ST10=DT+ HB-EAL-429 + WD	
11	ST11 = DT+ HB-429 + HB-EAL-429 +WW	
12	ST14= DT+ HB-429 + HB-EAL-429 +WD	
13	ST13=DT+WW	
14	ST12 =DT+WD	
15	ST15 = DT+HB -429 + WW	
16	ST16 = DT+HB- 429 + WD	

Key: Drought tolerance=DT, Drought susceptibility=DS), Sample Treatment= ST, Well watered=WW, Water deficit=WD, HB-EAL-429=Haricot Bean Ethiopian Agriculture legume-429 and HB-429= Haricot Bean-429

Note: The experimental design was complete randomized block design as well as the replication was three times throughout the experiment.

The water stress treatments were moisturized at 80% of the field capacity and it was maintained throughout the growing cycles as the control. Moisture stress 15 days after sowing was created by lowering the moisture from 80% down to 40% field capacity.

The amount of water applied was calculated as follows: First, dry weight of the soil was taken and the pot was weighed alone. Then; the soil was saturated with water. The pot with soil was weighed at field capacity; this gives wet mass of the soil with pot. Finally, weight of

pot was subtracted from wet mass of the soil with pot which gives wet mass of soil (MW), Mwp-Mp=Mw. Mw-Md= amount of water at field capacity.

Where, Md- mass of dry weight of soil, Mp-mass of pot, Mwp-mass of soil with pot.

All plants were grown under the 80% field capacity (FC) for 45 days. Then, the plants were exposed to drought stress by maintaining the water supply for 7 days resulting in a 40% decrease in soil moisture content and control plants that were well-watered. Finally, all plants were analyzed for physiological and biochemical parameters. 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. Drought stress was initiated at late flowering stage (up to 10 days after flowering). The drought stressed plots received irrigation at an interval of 12 to 14 days. The non-stressed plots received irrigations every 6 days until physiological maturity.

3.11 Experimental data collection

Plants were harvested after 45 days of sowing or planting for the experiments performed in sand with soil or sand mixture and data pertaining to nodular and vegetative characters were recorded.

3.11.1 Growth and physiological parameters evaluation

3.11.1.1 Determination of fresh and dry weight of leaves, root and shoot

The fresh weight samples were measured after it had been collected. Then samples were put in an oven for 48 hours at 70 ^{o}C. Finally, after 48 hours, the samples were weighed to determine their weight.

3.11.1.2 Root nodulation test and phenotypic characterization

Plant was excavate at depth of 40cm and carefully pulled up. The root was washed with tap water to remove any adhering soil. A number of nodules per plant were counted and values averaged to give the nodules per plant. In addition, nodules were detached from roots, oven dried at 70 ^{o}C for 24 hours, and then weighed to calculate nodule weight per plant.

3.11.1.3 Determination of relative water content

According to smart and Bingham (1974), relative water content (RWC) was measured and calculated on one fully expanded leaf per pot. The leaf from each pot was cut at the base of lamina, preserved within a paper bag and immediately brought to laboratory. The fresh weights of harvested leave (FW) were measured and then the tissue was put in a Petri dish with distilled water for 5 hours. The turgor weights (TW) of plants was measured and tissue was put into an oven for 48 hours at 70°C. Lastly, dry weight (DW) was measured. Relative water content was then calculated according to the methods of Smart and Bingham (1974), based on the following equation:

Relative water content (%) = (FW-DW) / (TW-DW) x 100 **(**Smart and Bingham, 1974)

Where, FW is the weight of freshly collected leaf, TW is the weight after rehydration (saturation) for 24 hours at room temperature and DW is the weight after drying the leaf at 70°C for 24 hours in hot air oven.

3.11.2 Biochemical parameters evaluation

3.11.2.1 Proline content

Sample preparations for analysis of proline content were measured by using the method of acid ninhydrin reagent (Bates *et al*., 1973). Briefly, the reagents used for this analysis were Acid-ninhydrin (LR,SAMIR TECH-CHEM, India) (which was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid(H_3PO_4 65%, Sigma-Aldrich, India), with agitation, until dissolved and kept cool (stored at 4°C)), extraction buffer (3% w/v) aqueous sulfosalicylic acid, which was prepared by three grams of Sulphosalicylic acid that is dissolved in 100ml of distilled water and Glacial acetic acid (LR,$C_2H_40_2$, Central Drug House, New Delhi) and toluene and Standard L-Proline solution reagent was prepared. In order to examine the amount of proline content in response to water stress as well as drought, analysis of leaf samples was done at flowering stage after water stress was induced. Proline ($C_6H_9NO_2$, NICE CHEMICALS, and India) was extracted according to the procedure of Bates *et al*. (1974), using 0.5g of leaf sample and 6 ml extraction reagents. Proline was quantified by spectrophotometer (SM-1600 Spectrophotometer, Double Beam UV/VIS Spectrophotometer, Maalab Scientific Equipment, India) at 520nm a colorimetric reaction with ninhydrin (Bates *et al*. 1974). Five hundred milligram of plant material were taken in a pestle and mortar and homogenized with 10ml of aqueous Sulphosalicylic acid (3%v/v) (Sigma- Aldrich, India). Then the homogenate was filtered through whatman No. two filter paper. The residue was re-extracted two times with of Sulphosalicylic acid (3%, v/v) and pooled. The filtrates were made up to 20ml with Sulphosalicylic acid (3%, v/v). Finally, this solution was used for estimation of proline.

Estimation: Two mililiter of extract was taken in the test tube and 2ml of Sulphosalicylic acid reagent and 2ml of Glacial acetic acid and 2ml acid-ninhydrin was added to it. The mixture was incubated for one hour at 100 ^oC in a water bath. The tube was transferred to an ice bath to terminate the reaction. Then to each test tube 4ml of toluene (LR, LOBA Chemie) was added and mixed vigorously using a test tube stirred for 20 seconds. The toluene containing the chromophore (red color) was separated from the aqueous phase with the help of separator funnel and the absorbance was measured at 520nm by spectrophotometer using toluene as a blank. The proline content was determined from a standard curve prepared with proline.

3.11.2.2 Total soluble sugar (TSS)

Total soluble sugar (TSS) in common bean leaf sample was determined according to Dubois *et al*. (1956) with little modifications (Tiwari *et al*., 2016). A 200 mg of fresh leaf tissues were homogenized in 5 ml of 80% methanol and were incubated in water bath at 60 ^{o}C for 30 minutes. The extract was then filtrated using filter paper into a volumetric flask and the residue was re-extracted. After incubation, 1ml of extract was mixed with 1ml of phenol (5%), (INDENTA, Chemicols-India) and 5 ml of sulfuric acid (H_2SO_4 95% v/v, Sigma-Aldrich, India) and further incubated in dark for 15 minutes. For exothermic reaction, the test tube was cooled in the air. Absorbance was then measured at 490 nm using spectrophotometer (SM-1600 Spectrophotometer, Double Beam UV/VIS Spectrophotometer) and soluble sugar was calculated from standard glucose graph. (Dubois *et al*., 1956)

$$\text{Total soluble sugar } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{Sugar value from graph } (\mu g)}{\text{Aliquot sample used}} * \frac{\text{Total volume of extract}}{\text{Weight of sample}} \text{x } 100$$

3.11.2.3 Total chlorophyll and total carotenoids contents

Photosynthetic pigments extraction and estimation with the total chlorophyll concentration were determined (Wellbum, 1994). The total chlorophyll and total carotnoids contents were determined by using Methanol (96 % v/v, LOBA Chemie). A 1g of fresh leaf materials of all the treatment were taken and homogenized with mortar and pestle using 5ml of methanol (96 % v/v). The green slurry was centrifuged at 3000 rpm for 10 minute and the supernatant was collected in test tubes. The residue pigment was re-extracted using 5ml of methanol (96 % v/v) and centrifuge as earlier. The second supernatant was added to the previously collected supernatant. The optical density was recorded at 663nm, 645nm and 470nm against 96 %(v/v) methanol as blank in UV-Vis spectrophotometer. Chlorophyll content in pigment extract and the absorbance was taken at 663 nm and 645 nm for chlorophyll a and chlorophyll b and total chlorophylls were calculated as suggested by Arnon, (1949).

Chlorophyll a: C*a* = 15.65 x A_{663} -7.340 x A_{645} (µg per ml solution)

Chlorophyll b: C*b* = 27.05 x A_{645}- 11.21 x A_{663} (µg per ml solution)

Total chlorophyll (a + b) = 8.02 x A 663 + 20.2 x A 645

x/y/z X Volume of extract X100

Total chlorophyll= (mg /100g fresh tissue) 1000 X weight of material (g)

For carotenoids the absorbance was recorded at 470 nm. It was calculated according to Wellburn (1994). Carotenoids: $C_{x+c} = 1000 \times A_{470} - 2.860 \times C_a - 129.2 \times C_b / 245$ (µg per ml solution)

Where A_{663}, A_{645} and A_{470} are the absorbance measured at 663, 645 and 470 nm, respectively. [Ca = chlorophyll a, Cb = chlorophyll b, Cx+c = chlorophyll x+c, AW= Absorbance at wavelength].

3.12 Data analysis

The common bean cultivars physiological and biochemical data were subjected to one-way analyses of variances (ANOVA) and all mean value of the parameter subjected to statistical analysis using the Statistical Package for Social Sciences (SPSS) computer software. Mean values comparison for treatments with significant effects were performed using the least significant difference (L.S.D) at 5 %(0.05) level of significant. Simple correlation analysis between traits was also carried out using the same statistical tool. Graphs were made using Microsoft Excel 2010 (Microsoft® Corp., Redmond, Washington, USA).

4. RESULTS AND DISCUSSION

4.1 Soil physico-chemical properties

Analysis of the top soil before the application of treatments shown that the soil at the experimental site was clay-loam in texture, slightly basic in reaction with a pH value of 7.69, which was in the optimum range for common bean production (Havlin *et al*., 1999). The soil substrate has physico-chemical properties with low Nitrogen (0.19 mg/g) and organic carbon content (0.11 mg/g). The cation exchange capacity (CEC) was 0.39 cmol/kg and could be rated as medium and satisfactory for agricultural crops with the use of agronomic practices (Landon, 1991).

4.2 Characterizations of common bean root nodule isolates

In this study, a colony morphology for isolates obtained from root nodules were circular in shape, white translucent colonies with entire margin and mucoid texture on Congo Red YEMA medium (Table 3) which could be a characteristic feature of *Rhizobium* isolates. Similarly, it was reported that common bean nodulating *Rhizobia* isolated from different soil samples in Ethiopian (Mulissa Jida and Fassil Assefa, 2011). Furthermore, it was reported that *Rhizobium* species is able to produce the exopolysaccharide gum of colony size, shape, border, elevation, color, mucosity and transparency feature (Somasegaran and Hoben, 2012; Liu, 2014; Muthini *et al*., 2014).

In this result, a light yellow colony with entire margin and mucoid texture were detected when the isolates had been inoculated onto nutrient agar (Table 3). In contrary to this study, Zerihun Tsegaye *et al*. (2019) reported brownish colony color on medium. It was suggested that this might have happened when different choice of media was employed for growth that is nutrient agar and glucose peptone agar. In this result, a microscopic examination for gram staining indicated that the colonies were gram negative and catalase for both isolate which could indicate properties for indirect enhancement of plant growth. It was observed that a bubble gas was formed when H_2O_2 had been added on to colonies. This strongly suggested that these isolates able to produce catalase enzyme that is able to degrade H_2O_2 in to water and O_2 gas. In line with this study, it was found that a catalase positive *Rhizobium* is one of the main reactive oxygen species (ROS) affecting plants under stress environmental condition that indirectly improve plant growth (Kumar *et al*., 2012; Wang *et al*., 2013).

Table 2: Some morphological and biochemical properties of *Rhizobiam* Strain

Morphological and Biochemical Characterization of *Rhizobium*

Rhizobium	**Shape**	**Color**	**Colony size**	**Opacity**	**margin**	**Texture**	**Gram stain**	**Catalase test**
HB-429	Circular	Light yellow	medium	White transpare	flat	Mucoid smooth	-	+
HBEAL-429	Circular	white	Medium-large	transparent	raised	muicod	-	+

4.3 Compatibility of the strains selected for the Rhizobia consortium

The compatibility of strains is critical for formulating bioinoculants that promote plant growth compatibility between two bacterial species with plant growth promoting properties could lead to formation of consortium effect against drought in plants. HB-429 and HBEAL-429 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 2). Tilak *et al*. (2006) reported that HBEAL-429 did not antagonize *Rhizobium* strain HB-429 when grown together on plates. The result of this experiment was also in line with Anandaraj and Delapierre (2010), who reported compatibility of *Rhizobium* strain with HB-429 and HBEAL-429, hence able to grow simultaneously without any inhibition in growth.

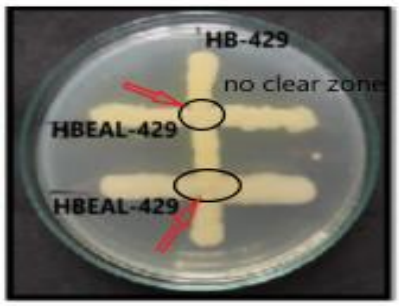

Figure 2: Compatibility test between strains of HB-429 and HBEAL-429 for the development of bacterial consortium by streak plate assay

4.4 Growth and physiological parameters improvement

Consortium inoculation had a significant improvement on growth of both drought tolerant (Awash-2) and susceptible (Nasir) varieties under water deficit condition than *Rhizobium* alone inoculated and un-inoculated plants (Figure 3). To determine the response of bacterial inoculation on leaf, root, shoot and nodule parameters of the drought tolerant and susceptible common bean varieties subjected to drought stress, plants were monitored after 14 days of water stress in both bacterial-treated and non-treated control plants.

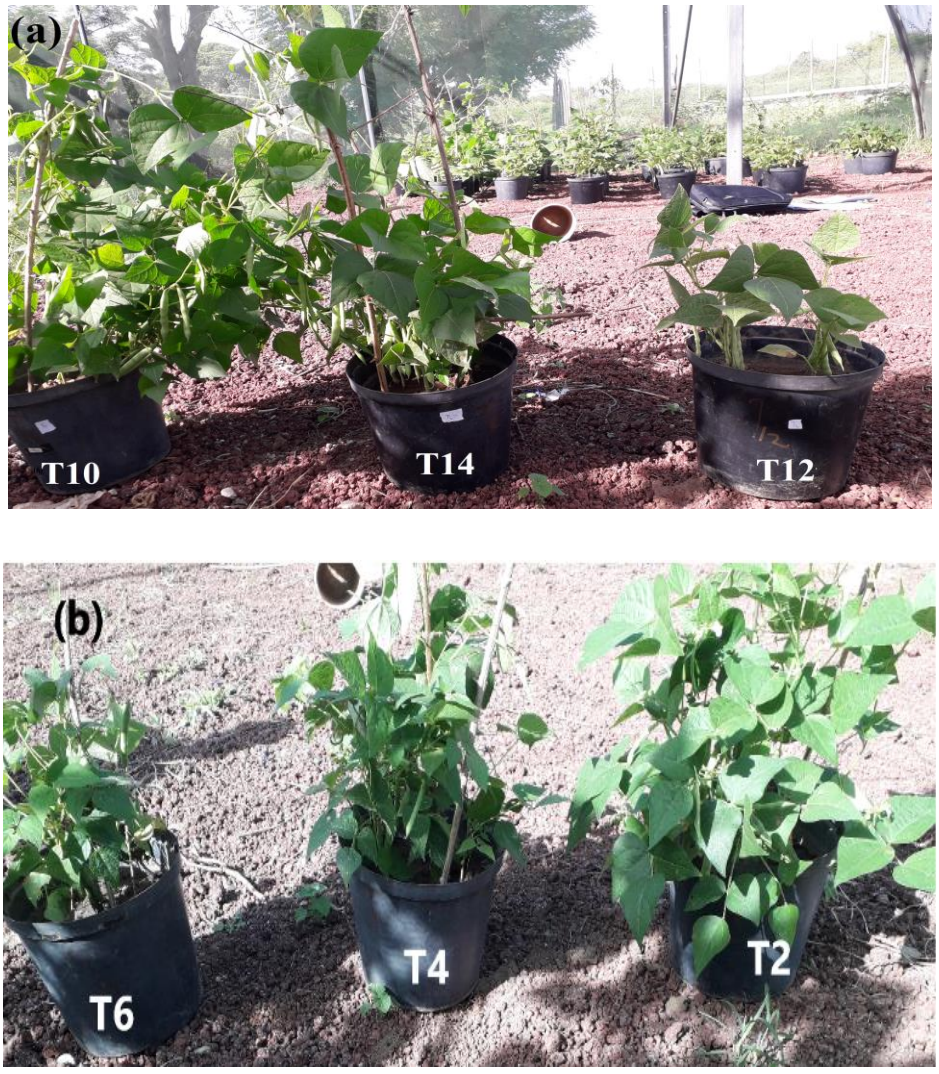

Figure 3: Different Pictures taken among *Rhizobium* alone (T2 and T10), co inoculated (T4, T14), and uninoculated bean plants (T6, T12). Drought tolerant (A) and susceptible (B) common beans varieties.

4.4.1 Fresh weight and dry weight of leaves improvement and Rhizobium inoculation

The present study exhibited high significant ($p<0.05$) variation for fresh weight of leaves (Figure 4). High fresh weight of leaves was recorded by 1.95g while low fresh weight was recorded by 0.31g. The results which recorded in drought tolerant (Awash-2) and susceptible (Nasir) significantly increase among common bean varieties inoculated with consortium of *Rhizobium* strains than un-inoculated control plants under water scarcity condition. However, *Rhizobium* strain alone inoculation only brought significant increase ($p<0.05$) in fresh weight of leaves in Awash-2 variety. Co-inoculation brought 39.7% and 70.9 % increase in fresh weight of leaves of drought tolerant and susceptible common bean plants, respectively was observed than uninoculated control plants. However, when co-inoculated plants were compared to *Rhizobium* alone, inoculated plants 19.1% and 32.4% increase in fresh weight of leaves in drought susceptible and tolerant varieties under water deficit condition respectively. Likewise, the plant fresh weight of leaves was also significantly increased by 58.9% and 98.4% under well-watered condition which was noted in Awash-2 variety than Nasir variety with *Rhizobium* alone and consortium inoculation.

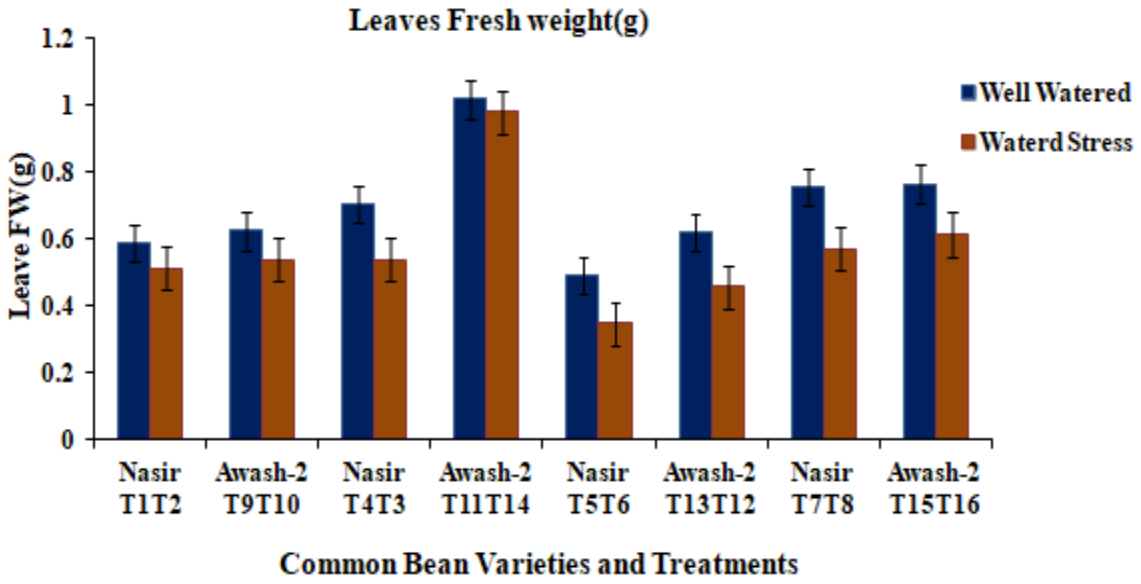

Figure 4: Leaves fresh weight of drought tolerant (Awash-2) and sensitive (Nasir) varieties under well-watered, water deficit and treatments**.**

Key: T1,T2,T7,T8,T9,T10,T15,T16 were a single isolates of inoculums; T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error.

Leaves dry weight showed high significant ($p<0.05$) variation under water deficit condition (Figure 5). High dry weight (0.39g) of Leaves was recorded for with consortium inoculation under water deficit condition while low (0.04g) DW was recorded for drought tolerant and susceptible common bean varieties when compared with uninoculated control plants. However, no significant difference ($p>0.05$) was observed in dry weight of leaves between co inculcated and *Rhizobium* alone inoculated drought tolerant and susceptible varieties under water scarcity condition.

The results were reliable with the work of Lobata *et al*. (2013) who reported an increase in leaf biomass under drought stress in common bean with corresponding 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 a single isolates of *Rhizobium* inoculated and non-inoculated control plants under well-watered and water deficit conditions, though dry weight was improved by inoculation better in Awash-2 variety than Nasir variety under well-watered condition.

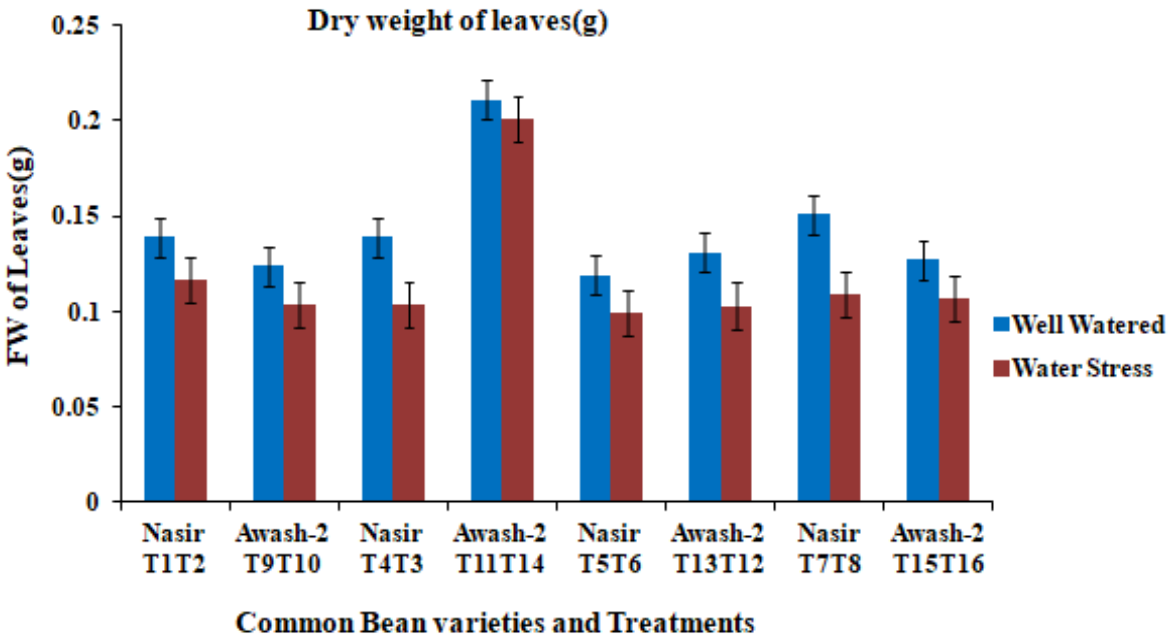

Figure 5: Dry weight of leaves of drought tolerant (Awsh-2) and sensitive (Nasir) varieties under well-watered, water deficit and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were a single isolates of inoculums; T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error.

4.4.2 Fresh and dry weight of root and shoot

Drought stress affects several features of legume growth and development, including germination, root and shoot development. Syndicate inoculation of seeds of *P.vulgaris* varieties with *Rhizobium* strain had a significant positive effect on root and shoot fresh and dry weights compared with *Rhizobium* only inoculated and non-inoculated common bean plants under well watered and water deficit conditions.

Root fresh weight significantly ($p<0.05$) increased during co-inoculation by 55.3% and 59% in drought susceptible and tolerant common bean varieties, respectively than uninoculated control plants under water deficit (40% FC), (Figure 6). However, significant improvement when co-inoculation compared to *Rhizobium* single-handedly inoculated was found in drought tolerant (Awash-2) variety only, while non-significant ($p>0.05$) difference from that of *Rhizobium* only inoculated was observed in drought susceptible Nasir diversity.

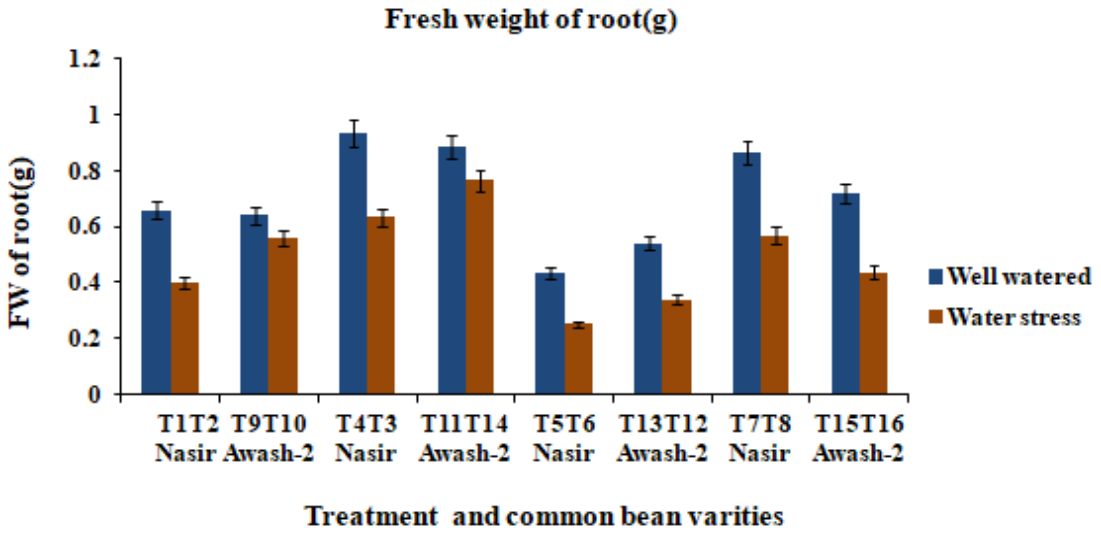

Figure 6: Root fresh weight of drought tolerant (Awsh-2) and sensitive (*Nasir*) varieties under well-watered, water deficit, and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were single *Rhizobium* inoculated , T3,T4,T11,T14 were non- inoculated control plants and T5,T6,T13,T12 were co-inoculated. Each bar represented the mean of three replicate values along with standard error.

Dry weight of root increased significantly ($p<0.05$) due to co inoculation in both drought tolerant and susceptible common bean varieties, than non-inoculated control plants. On the other, co-inoculation brought significant variation ($p<0.05$) than *Rhizobium* only inoculated plants in both varieties (Figure 7).

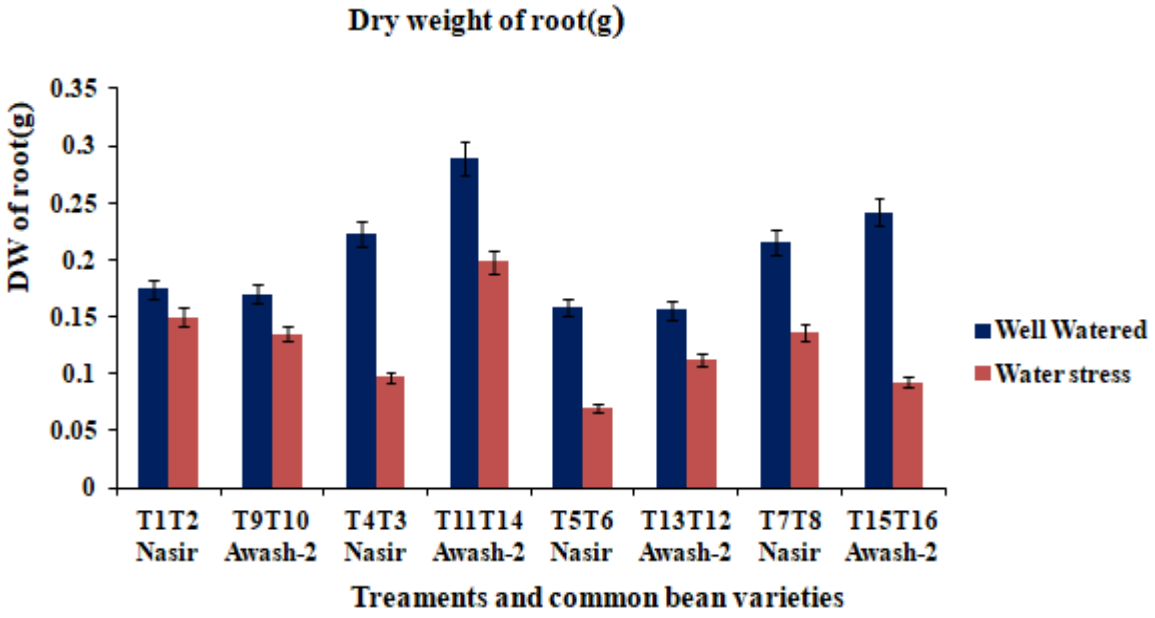

Figure 7: Dry weight root of drought tolerant (Awsh-2) and sensitive (*Nasir*) varieties under well-watered, water deficit and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were *Rhizobium* alone inoculated, T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error.

Consortium co-inoculation with *Rhizobium* strain increased fresh weight of shoot significantly ($p<0.05$) by 41.2% and 47.3% in drought susceptible and tolerant common bean plants than uninoculated control groups respectively (Figure 8**).** However, significant improvement when co-inoculation, non-significant ($p>0.05$) difference from that of *Rhizobium* only inoculated and uninoculated was observed in both diversity. The finding of the present study was contradictory with Karnwal and Kumar, (2012) who reported enhancement in shoot and root biomass due to inoculation of common bean by plant growth promoting rhizobacteria.

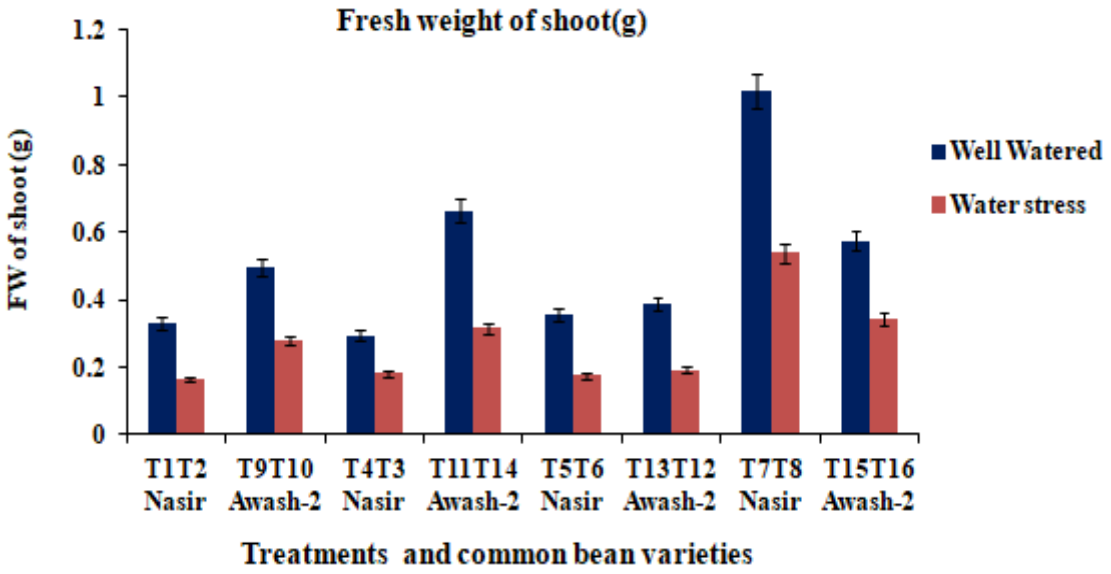

Figure 8: Fresh weight of shoot (g) of drought tolerant (Awsh-2) and sensitive (Nasir) varieties under well-watered, water deficit, and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were *Rhizobium alone* inoculated, T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were uninoculated control plants**.** Each bar represented the mean of three replicate values along with standard error.

Dry weight of shoot co-inoculation brought significant improvement ($p<0.05$) by 29.4 to 85.6 % in both drought susceptible and tolerant common bean plants than non-inoculated control groups under water deficit and well-watered conditions (Figure 9). An increase in growth of shoots and roots due to increased uptake of nutrients and hence increase in dry weight because of IAA production was reported by Malik *et al*. (2006) in common bean. Similarly increase in the plant dry weight of common bean seeds were noticed when inoculated with Rhizobum strain *(*Sindhu *et al*., 2011). The increase in dry weight of shoot could be for biologically fixed nitrogen (Fatima *et al*., 2008). Ahmad and Kibret, (2014) also reported an increase in dry weight of shoot in common bean plants ranged from 19 to 81% over the control. Similar results were noted by Timmusk *et al*. (2014) who reported bacterial induced alterations in root architecture may lead to an increase in total root surface area, and thus lead to enhanced water and nutrient uptake, with positive effects on plant growth as a whole under water deficit environment.

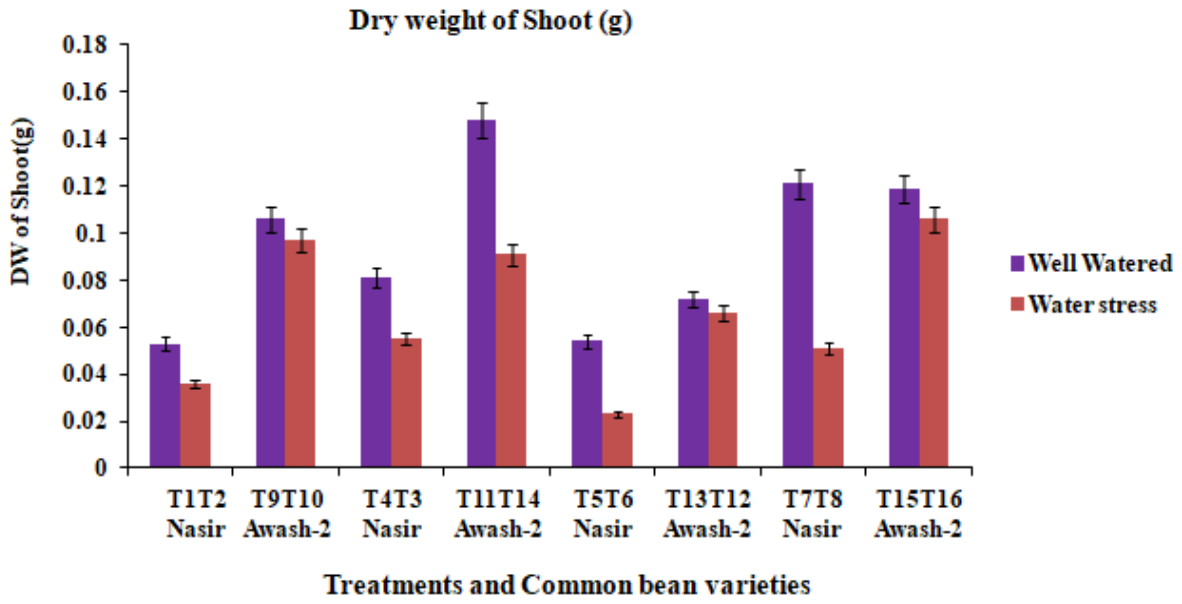

Figure 9: Dry weight of shoot (g) of drought tolerant (Awsh-2) and sensitive (Nasir) varieties under well-watered, water deficit and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were *Rhizobium* alone inoculated, T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error.

4.4.3 Improvement of number of nodules per plant (NNPP) in fresh weight

The number of root nodules was significant difference ($p<0.05$) demonstrated in both co-inoculated and *Rhizobium* alone inoculated varieties of drought tolerant and susceptible varieties on exposure to drought stress (Figure 10). The highest nodules number per plant was 61 and 51 in drought tolerant Awash and drought susceptible Nasir variety, respectively per sample of plant. Similarly, nodule fresh weight increased significantly ($p<0.05$) by 62% in drought tolerant and ($p<0.03$) by 51.6% in drought susceptible varieties (Figure 10). There was few nodulation observed in un-inoculated control as soil was sterilized.

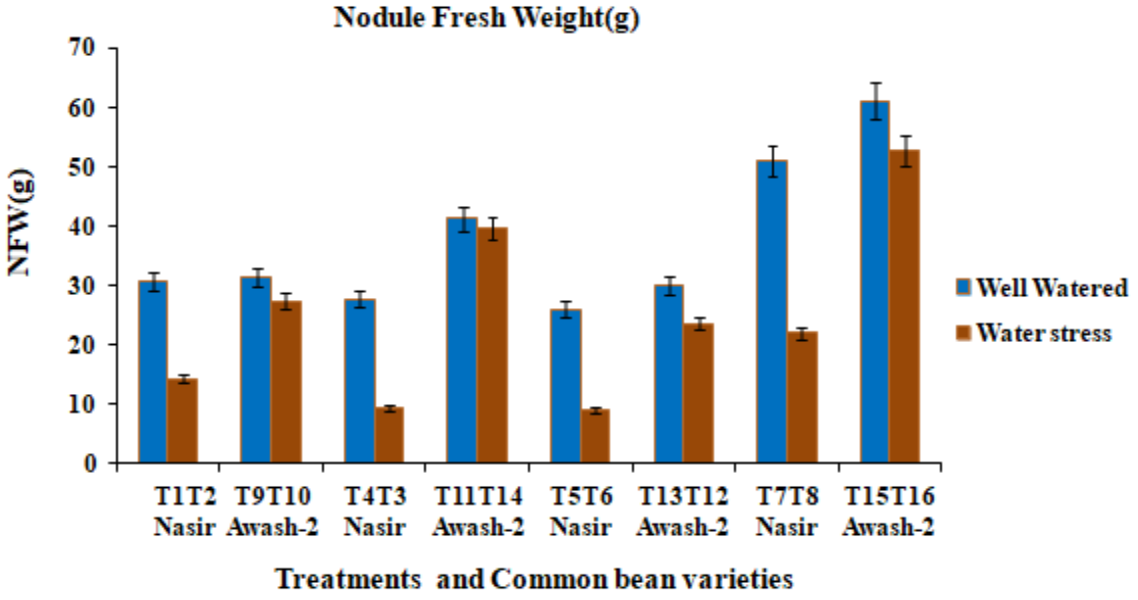

Figure 10: Nodule fresh weight (g) of drought tolerant (Awsh-2) and drought susceptible (Nasir) varieties under well-watered, water deficit and treatments.

Key: T1,T2,T7,T8,T9,T10,T11,T14 were *Rhizobium* alone inoculated, T3,T4,T15,T16 were co- inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error.

4.4.4 Relative water content (RWC)

In this study, drought stress reduced RWC in both drought tolerant and susceptible common bean varieties. Conversely, 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 11). Relative water content (RWC) is one of an indicator of plant water status and it is a consistent and less error prone parameter for enumerating plant drought stress response (Siddique *et al*., 2000).

Relative water content in uninoculated, *Rhizobium* alone inoculated and co-inoculated *Phaseolus vulgaris* varieties of both drought tolerant and susceptible varieties demonstrated that there was significant alteration in relative water content ($p<0.05$). Post hoc evaluation using the least significant difference (L.S.D.) test indicated that the mean RWC score for co-inoculation was significantly improved ($p<0.05$) than non-inoculated in both varieties, also from that of *Rhizobium* alone inoculated was significant in drought tolerant ($p<0.05$) and susceptible varieties and ($p<0.41$) under water deficit condition respectively. In use mutually, these results recommended that inoculation really have an enhancement on relative water content in both drought tolerant (Awash-2) and susceptible (Nasi*r*) common bean varieties. Plant growth regulators, had significantly enhanced the Relative Water Content when applied alone or in combination. Specifically the results propose that under water deficit condition co inoculated drought tolerant common bean variety enhanced RWC by 87.35% and 69.2% over *Rhizobium* alone inoculated and uninoculated control, respectively. While co-inoculated drought susceptible common bean variety increased RWC by 80.8% and 70.9% over Rhizobium alone inoculated and uninoculated control, respectively. The findings of the present study was consistent with Sandhya *et al*., (2010), Bano *et al*., (2013) and Tiwari *et al*. (2016), who reported higher RWC in plants under drought stress due to inoculation. The tolerance or sensitivity of common bean to drought is related to its capability to maintain good leaf water status (Krouma, 2010). Common bean cultivars which have high RWC are more resistant to drought stress (Talebi *et al*. 2013). The findings of this study demonstrated that awash variety is better than Nasir variety in conserving water under drought stress and firm inoculation increased relative water content in both varieties under drought stress than non-inoculated control.

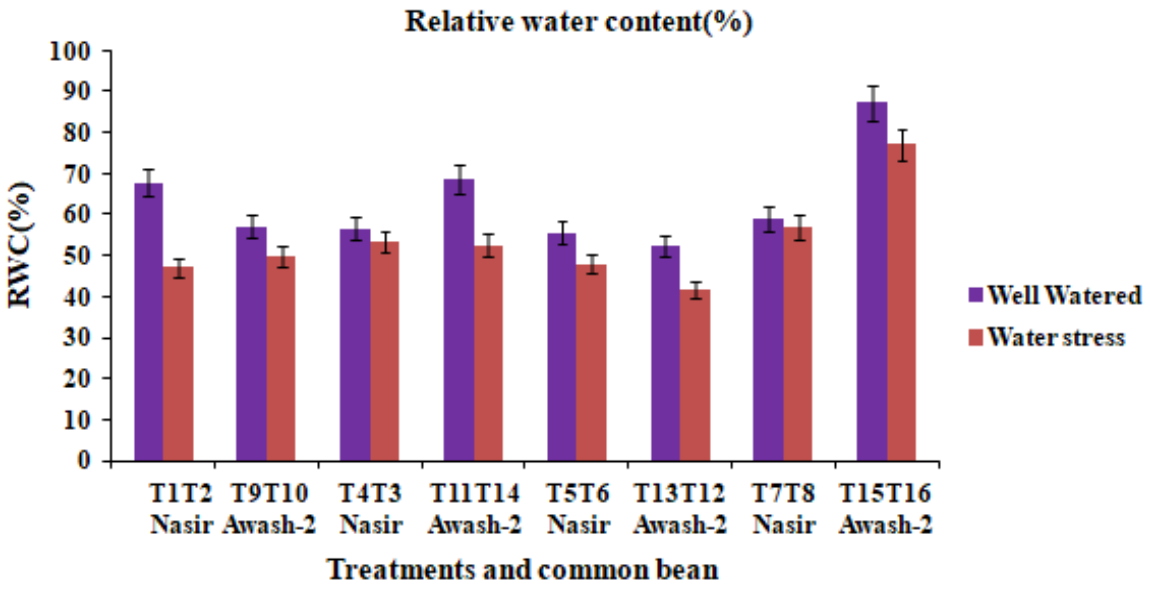

Figure 11: Relative water content (%) of Awsh-2 and Nasir varieties under well-watered, water deficit and treatments**.**

Key: T1,T2,T7,T8,T9,T10,T15,T16 were *Rhizobium* alone inoculated, T3,T4,T11,T14 were co- inoculated and T5,T6, T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error.

4.5 Biochemical parameters

4.5.1 Proline content

The quantitative estimation was made by comparing the optical density (OD) values from a standard curve prepared from proline. The proline concentration was determined from standard curve constructed from known concentration of L-proline (Figure 12) and the results were expressed in micro-gram (µg) of proline per g fresh weight of leave.

$$\text{Proline content} = \frac{[(\mu g\ \text{proline}^{-ml} * \frac{ml\ \text{toluene}}{115.5 \mu g^{-}\mu mole}]}{[\frac{g\ \text{sample}}{s}]}$$

Where, 115.5 is the molecular weight of proline

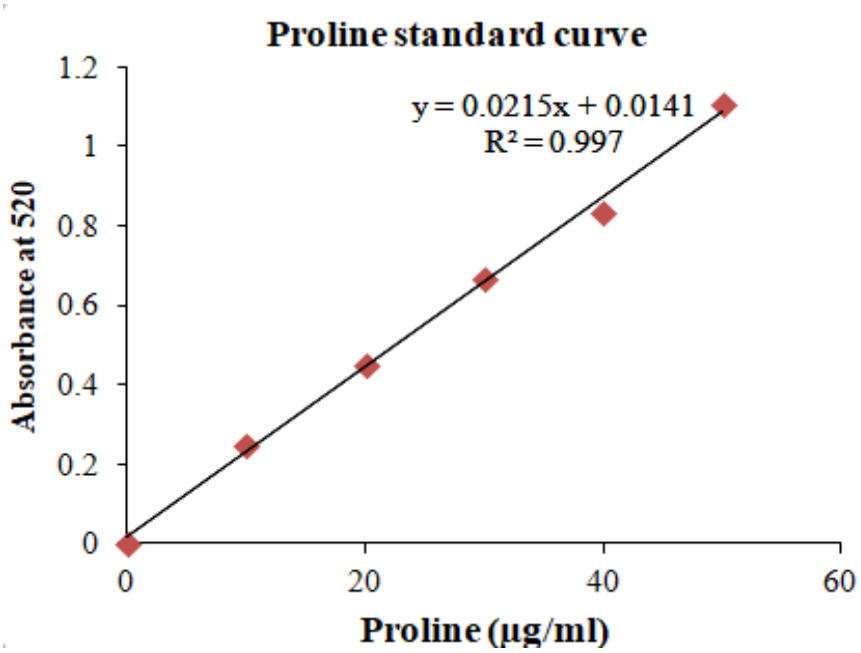

Figure 12: Standard curve prepared from L-Proline (µg) and read at 520 absorbance

The significant increase in proline content ($p<0.04$) were observed in Nasir and Awash-2 variety ($p<0.05$) under water deficit condition as compared to the control condition in inoculated and uninoculated conditions (Figure 13). However, the maximum value was recorded in Awash-2 variety (in single sample) which was co inoculated and under drought stress (16.5 µmol/g) and lowest was observed in Nasir variety (1.04 µmol/g) under control condition. Proline concentration varied from 5% under normal condition to 80% under stressed condition in many plants (Szabados and Savoure, 2009). There was significant interaction between stress levels and variety in proline content. Accumulation of proline has been established as a parameter of choice for stress tolerance (Jaleel *et al*. 2007) and it is supposed to play an adaptive role in plant stress tolerance (Verbruggen and Hermans, 2008; Hayat *et al.*, 2012). Rhizobial inoculation significantly enhanced proline content of *P. vulgaris* (*L.)* under drought stress which has been reported in different plants (Ashraf and Foolad, 2007; Tatar and Gevrek, 2008).

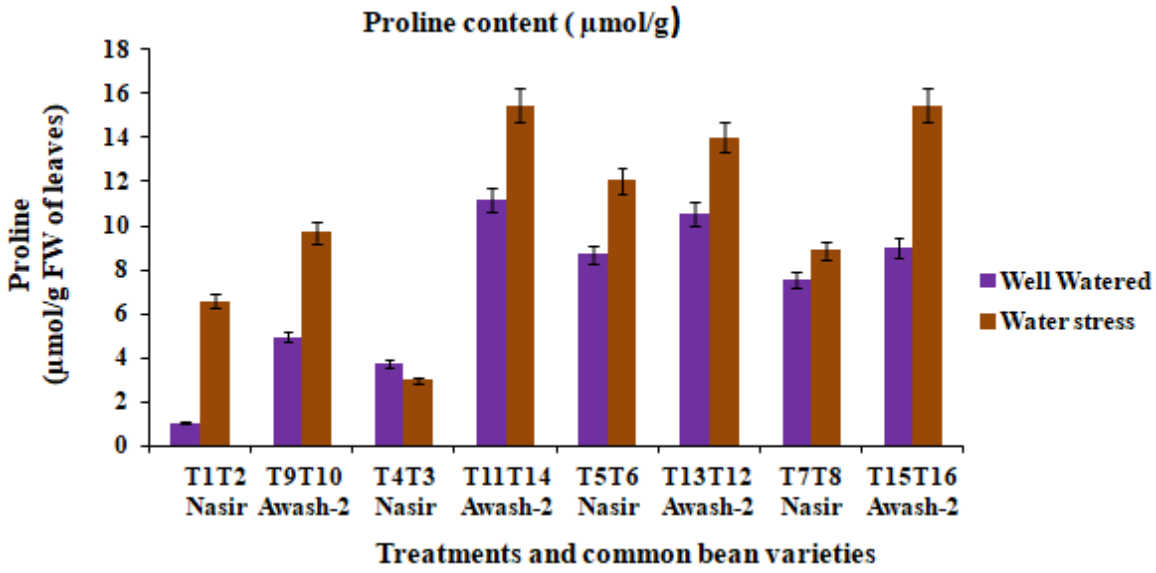

Figure 13: Proline content of drought tolerant (Awsh-2) and sensitive (Nasir) varieties under well-watered, water deficit and treatments.

4.5.2 Total soluble sugar

Total soluble sugar (TSS) had significance difference with inoculation effect on drought tolerant Awash-2 ($p < 0.048$) and drought susceptible Nasir varieties ($p < 0.03$) on total soluble sugar under well-watered and water deficit conditions. The results were then compared with standard curve constructed using glucose (Figure 14). Total soluble sugar (TSS) concentration was then expressed in milligram (mg) per g fresh weight of leave.

$$\text{Total soluble sugar } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{Sugar value from graph } (\mu g)}{\text{Aliquot sample used}} * \frac{\text{Total volume of extract}}{\text{Weight of sample}} \text{x } 100$$

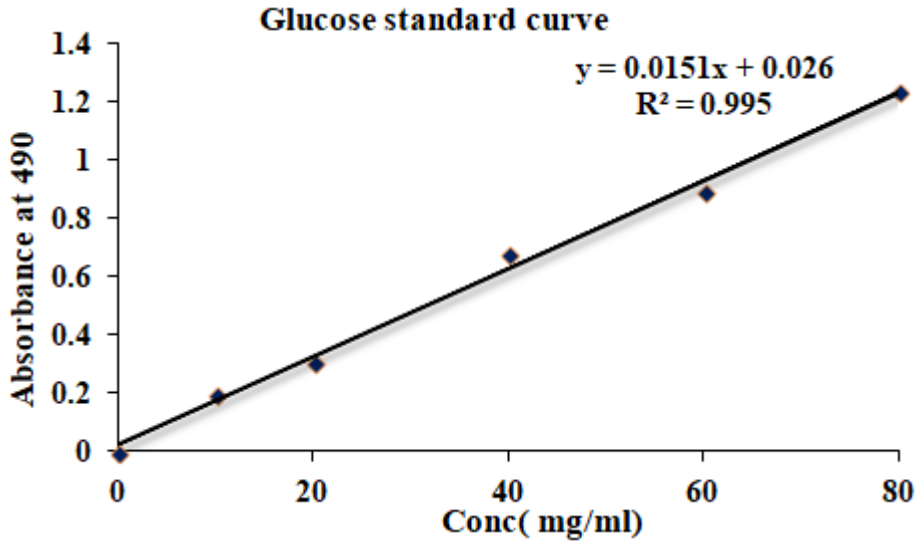

Figure 14: Standard curve prepared from L-Glucose (µg) and read at 490 Abs

Total soluble sugar in well-watered control condition from un-inoculated plants was found low in comparison to the water stress condition while, co-inoculated and *Rhizobium* alone inoculated plants showed decrease in TSS under drought stress than their well-watered corresponding item (Figure 15). Greatest sugar content (in single sample) was observed in Awash-2 (76.9 mg/g fresh weight) and minimum was observed in Nasir (27.4 mg/g fresh weight). The TSS content of inoculated common bean plants showed significant increase during drought stress as compared to uninoculated plants. This may be due to the bacteria used induced systemic tolerance response of common bean 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 common bean

plants under drought stress which were inoculated with HB-429. According to Rasaei *et al*., (2013) reported total soluble sugar as osmolyte materials increases flows of water into cells and maintain turgor of cells through adjusting water content.

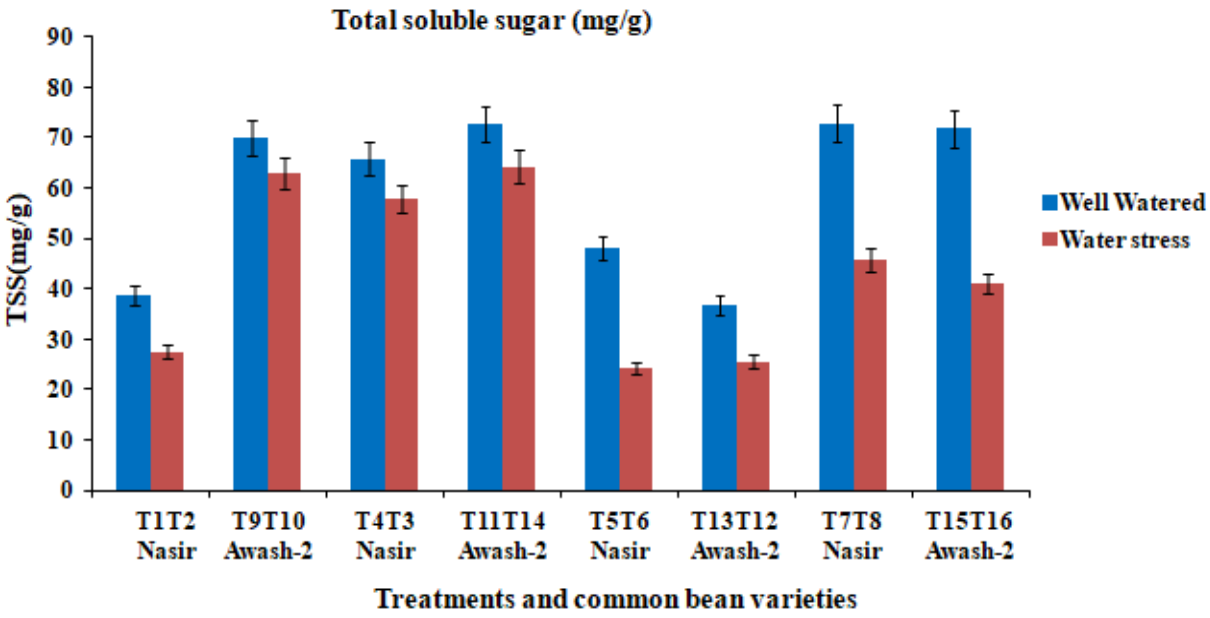

Figure 15: Total soluble sugar of drought tolerant (Awsh-2) and sensitive (Nasir) varieties under well-watered, water deficit and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were *Rhizobium* alone inoculated, T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error

4.5.3 Total chlorophyll and carotenoid content

Drought tolerant Awash-2 and susceptible Nasir variety were significantly improved by co-inoculation of HB-429 and HBEAL-429 strains ($p<0.05$) than uninoculated controls, but no significant difference from that of *Rhizobium* strain alone inoculated control plants ($p>0.05$) on the content of total chlorophyll (Table 3). Single and dual inoculations of *Rhizobium* strains showed variations in their effect to enhance the common bean tolerance to drought (Figure 16).

The finding of the present study was parallel with studies of Samavat *et al*., (2012) who have reported that seed inoculation with *Rhizobium* strain treatment has significant effect on leaves chlorophyll content in common bean, as compared to control. The increase in chlorophyll content of leaves could be qualified to increased plant nutrition and photosynthesis. Phosphorous solubilization by plant growth promoting rhizobacteria in common bean has shown to have an important role in energy transfer and photosynthesis, thus contributing towards chlorophyll content (Aouani *et al*., 2001).

The contents of total chlorophyll (chl.a + chl.b) 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 common bean plants and thus maybe important for tolerance to drought stress. This study was in consistent with Gusain *et al*. (2015) who reported enhanced chlorophyll and carotenoid content hence better drought tolerance in drought tolerant and susceptible common bean varieties inoculated with different plant growth promoting rhizobacteria.

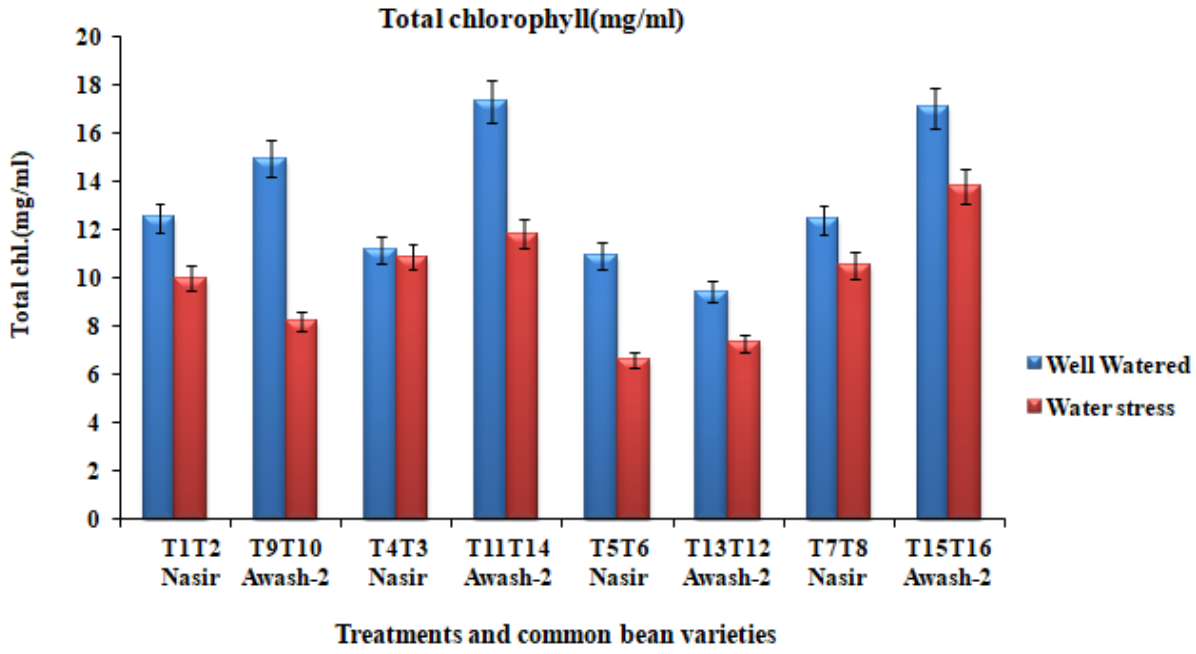

Figure 16: Total chlorophyll of Awsh-2 and Nasir varieties under well-watered, water deficit and treatments.

Key: T1,T2,T7,T8,T9,T10,T15,T16 were *Rhizobium* alone inoculated, T3,T4,T11,T14 were co inoculated and T5,T6,T13,T12 were non-inoculated control plants. Each bar represented the mean of three replicate values along with standard error

Table 3: Estimation of Chlorophyll (chl.a, chl.b) and carotene content in leaves, root and shoot treated using Methanol

Ts	Cultivars	O.D (nm)			Chl.a		Chl.b		Total Chl.		Carotene	
		663	645	470	ww	ws	ww	ws	ww	ws	ww	ws
T1T2	Nasir	1.89, 1.48	0.84, 0.68	2.59,2.53	23.71	18.09	1.25	1.88	12.48	9.99	9.66	9.09
T9T10	Awash-2	1.13, 2.09	0.45,1.0	1.14,2.28	25.74	15.35	0.69	4.52	14.94	8.21	6.69	4.39
T4T3	Nasir	1.8, 0.9	0.75,0.51	2.34,2.25	21.11	20.48	1.26	1.23	11.17	10.87	8.66	8.29
T11T14	Awash-2	2.24, 1.24	0.52,0.97	1.4,2.54	29.54	24.91	4.67	1.09	17.29	11.81	8.01	6.91
T5T6	Nasir	1.54, 1.2	0.71,0.49	2.69,1.9	30.86	21.15	5.00	2.07	17.93	11.61	7.31	5.88
T12T13	Awash-2	1.76, 2.15	0.72,0.52	1.96,2.33	23.71	25.96	0.93	0.93	13.45	12.32	8.74	7.24
T7T8	Nasir	1.84, 1,63	0.85,0.64	2.18,1.8	22.51	20.85	0.21	0.69	12.41	10.53	7.06	6.62
T15T16	Awash-2	2.29, 2.5	1.38,1.6	2.49,2.34	31.02	26.53	3.06	1.07	17.04	13.80	8.88	7.08

WW= Well Watered, WS= Water Stress, OD=Optical Density, Ts= Treatments

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The study revealed that water deficit significantly affects the growth and development of both common bean varieties by affecting fresh weight and dry weight of leaves, root, shoot and nodule. Plant associated beneficial microbe was investigated by developing *Rhizobium* HB-429 and HB-EAL429 in improving drought stress in drought tolerant (Awash-2) and susceptible (Nasi*r*) common bean varieties in the present study. Reduced RWC, total chlorophyll content and carotenoid, and enhanced osmolytes like proline and total soluble sugar were also observed. Conversely, drought induced indicators of the above parameters in common bean were significantly improved in the presence of the plant growth promoting microbe inoculation. More positive effect was observed when inoculation was done in synergistically. Interestingly the bacteria not only improved the growth of tolerant common bean variety (Awash-2) but also satisfactorily improved the drought stress ameliorating ability of relatively drought sensitive common bean variety (Nasir), indicating its greater potential in enhancing agricultural productivity of this economically important legume. The finding of the present study point out essential steps towards understanding the physiological and biochemical basis of the plant growth promoting bacteria mediated drought stress response and adaptation in common bean plants. Thus, applicability of plant growth promoting microbes in drought stress amelioration at field level should be worked out.

5.2 Recommendations

Based on the findings of the present study, the following recommendations are made for future consideration:

- Optimizing growth condition and increased self-life of PGPR products, tolerate adverse environmental condition and cost effective PGPR products for use of agricultural farmer will be recommended.
- Understanding the diversity of plant legume *Rhizobium* bacteria interactions and their role in plant development is essential if these associations are to be manipulated to increase crop production as well as further work is required on molecular identification of gene to manage physiological and biochemical traits.
- Since this experimental plants were grew under controlled condition, the consistency of observed parameters should be studied under field and different environmental condition to solve the problem in water stress. Thus, it is of paramount significance to select and recommend cultivars for the area.
- Among the growth parameters, the present findings revealed that the total chlorophyll and plant dry weight of root were not significantly affected ($p > 0.05$) by water stress among the varieties that it will recommended to reinstate with other traits to see their effects of inoculation towards water stress.

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7. APPENDICES

Appendix 1: some images took from experimental procedures

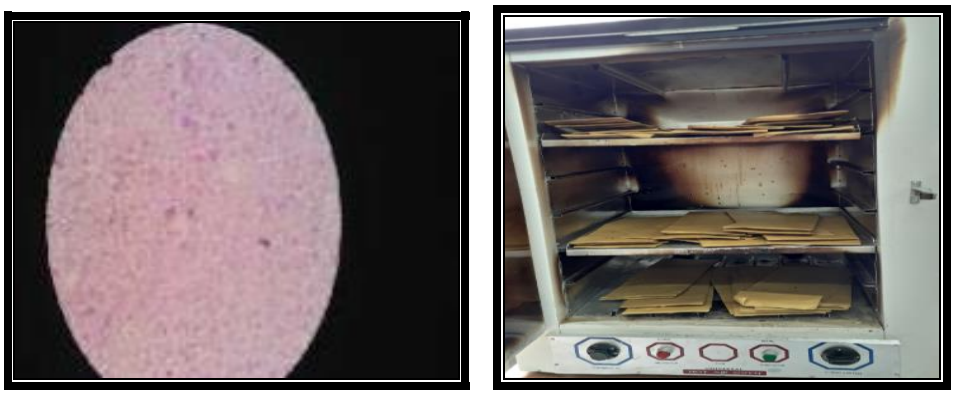
I. Experiment during proline content determination before and after preparation which was warmed at 100 ^{0}C in water bath.

II: Leaves soaked in distilled water for turgid weight and relative water content determination on petri plate.

Appendix 2: Some images took during bacterial characterization before inoculation

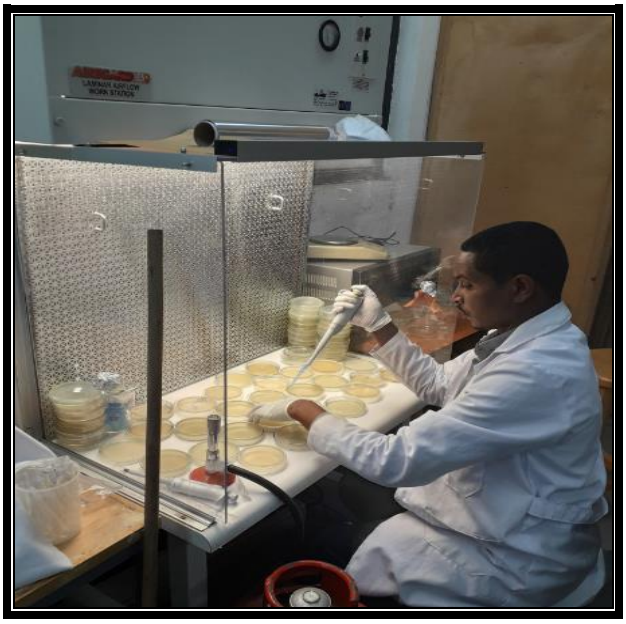
A. Showing Congo-red absorption B. Showing no Congo-red absorption

I. Growth response of *Rhizobium* isolates on YEMA medium containing Congo red



Gram staining reaction (under 1000X magnification) (A) and samples in dry air oven containing paper bag for dry weight determination (B)

Appendix 3: Enumerating of rhizobia strains on YEMA and YEMB for inoculants



Inoculating of bacterial samples by spreadind protocol in biological safty cabinet (hood) and culture in incubator.

Appendix 4: Image of common bean growing in greenhouse condition

Research activity done on common bean plants growing condition in greenhouse

Appendix 5: YEMA for rhizobial strains plate counting media

Nutrients	**Gram per liter**	**Micronutrients**	**Milligram per liter**
K_2HPO_4	0.5	H_3BO_3	1.0
NaCl	0.2	$ZnSO_4$	1.0
$CaSO_4 \cdot 2H_2O$	0.1	$CuSO_4 \cdot 5H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.2	$MnCl_2 \cdot 4H_2O$	0.5
Mannitol	10.0	$Na_2MoO_4 \cdot 2H_2O$	0.1
Yeast extract	2.0	Fe-EDTA	10.0
Agar	15.0		
Congo red	10	Congo red	0.025

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

Tnts	Leave FW(g)	Leave DW(g)	Root FW(g)	Root DW(g)	Shoot FW(g)	Shoot DW(g)	NFW (g)	RWC (%)	Proline (µmole/g)	TSS (mg/g)	Chl. a (mg/g)	Chl. b (mg/g)	Total chl.(mg/g)	Carotene
T1R1	0.479	0.107	0.243	0.083	0.053	0.009	8	66.844	5.012	43.74	18.43	1.667	20.097	8.904
T1R2	0.405	0.101	0.116	0.032	0.021	0.001	11	68.776	8.534	38.547	25.97	0.861	26.831	9.692
T1R3	0.66	0.143	0.833	0.348	0.619	0.099	24	87.521	4.005	34.326	26.72	1.235	27.956	10.381
T2R1	0.478	0.106	0.635	0.165	0.063	0.012	18	36.143	7.159	28.881	23.71	0.909	24.619	10.02
T2R2	0.621	0.163	0.818	0.241	0.155	0.021	5	60.02	2.991	21.291	13.152	2.086	15.161	7.982
T2R3	0.66	0.149	0.526	0.044	0.28	0.126	69	45.327	0.07	31.978	17.421	2.637	20.058	9.26
T3R1	0.334	0.092	0.509	0.133	0.132	0.022	13	52.688	0.056	49.735	28.612	1.016	29.628	9.33
T3R2	0.616	0.159	0.569	0.18	0.445	0.07	19	67.904	2.407	69.201	20.716	0.787	21.503	9.02
T3R3	0.395	0.06	0.395	0.048	0.306	0.152	51	49.097	0.398	54.635	13.966	1.873	15.839	7.63
T4R1	0.296	0.053	0.297	0.051	0.064	0.012	2	24.745	8.379	56.424	14.753	0.982	15.735	9.59
T4R2	0.441	0.108	0.523	0.107	0.155	0.021	6	55.746	2.701	55.231	25.818	2.671	28.489	7.27
T4R3	0.474	0.145	0.482	0.044	0.334	0.133	4	79.8	3.254	85.695	20.885	0.136	21.021	8.01
T5R1	0.33	0.04	0.563	0.183	0.279	0.037	12	43.177	11.377	68.543	30.699	5.53	36.229	7.73
T5R2	0.576	0.128	0.464	0.12	0.294	0.024	10	87.984	15.741	69.338	30.921	4.765	35.636	6.39
T5R3	0.732	0.129	0.926	0.207	0.256	0.029	17	45.572	9.049	60.927	30.959	4.715	35.674	3.52
T6R1	0.647	0.235	0.901	0.305	0.372	0.058	12	69.864	3.629	65.298	18.417	5.735	24.194	5.74
T6R2	0.721	0.121	1.035	0.358	0.599	0.194	35	60.075	6.315	99.867	27.365	0.268	27.572	6.89

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Tnts	Leave FW(g)	Leave DW(g)	Root FW(g)	Root DW(g)	Shoot FW(g)	Shoot DW(g)	NF (g)	RWC (%)	Proline (µmole/g)	TSS (mg/g)	Chl. a (mg/g)	Chl. b (mg/g)	Total chl.(mg/g)	Carotene
T6R3	0.709	0.15	0.866	0.11	0.696	0.271	55	69.543	16.115	52.781	18.845	0.207	10.052	9.31
T7R1	0.699	0.114	0.675	0.142	0.126	0.034	14	48.84	3.254	42.119	18.026	0.967	18.993	8.26
T7R2	0.416	0.098	0.485	0.111	0.238	0.013	23	47.342	3.694	66.821	18.851	-1.1	17.751	4.75
T7R3	0.597	0.115	0.547	0.155	0.351	0.104	29	80.617	4.654	40.245	30.662	7.021	37.683	8.18
T8R1	0.788	0.171	0.761	0.08	0.444	0.19	52	49.2	9.912	76.623	20.926	0.825	21.175	6.79
T8R2	0.731	0.166	0.56	0.212	1.346	0.137	26	73.974	8.3	63.589	18.616	-1.045	17.071	5.59
T8R3	0.745	0.115	1.265	0.356	1.467	0.217	75	47.736	4.361	90.496	23.001	0.851	23.852	7.48
T9R1	0.499	0.124	0.939	0.134	0.352	0.174	21	45.272	6.721	96.622	10.055	0.491	10.546	3.18
T9R2	0.423	0.095	0.56	0.188	0.231	0.064	32	36.152	4.605	61.391	16.024	0.648	16.672	4.89
T9R3	0.668	0.15	0.175	0.083	0.254	0.049	35	90.14	3.547	45.695	21.128	0.934	22.062	5.09
T10R1	0.454	0.105	0.35	0.132	0.08	0.016	38	41.174	8.809	51.854	22.438	0.774	23.212	6.92
T10R2	0.456	0.068	0.667	0.167	0.348	0.058	13	54.684	12.712	37.881	29.893	12.401	42.294	5.55
T10R3	0.675	0.138	0.899	0.211	0.456	0.245	34	54.17	7.551	99.305	23.719	0.384	24.103	7.61
T11R1	1.175	0.253	0.744	0.278	0.234	0.096	43	46.6	11.979	33.17	26.677	1.681	28.358	9.01
T11R2	0.955	0.188	0.883	0.32	0.982	0.328	29	69.273	6.054	13.617	20.038	0.869	20.907	6.25
T11R3	0.813	0.191	0.665	0.27	0.163	0.021	52	90.029	16.375	25.894	20.908	0.712	21.62	8.79
T12R1	0.313	0.089	0.984	0.271	0.432	0.067	19	45.923	9.016	59.801	21.924	1.343	23.267	6.26
T12R2	0.946	0.144	0.783	0.201	0.361	0.12	30	65.349	12.761	93.112	29.847	1.073	30.92	5.41

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Tnts	Leave FW(g)	Leave DW(g)	Root FW(g)	Root DW(g)	Shoot FW(g)	Shoot DW(g)	NF W(g)	RWC (%)	Proline (µmole/g)	TSS (mg/g)	Chl. a (mg/g)	Chl. b (mg/g)	Total chl. (mg/g)	Carotene (mg/ml)
T12R3	0.712	0.126	1.057	0.296	0.068	0.012	47	78.462	18.833	71.258	19.368	0.381	19.749	10.06
T13R1	0.591	0.109	0.873	0.28	0.648	0.094	4	43.498	20.884	72.317	30.569	2.249	32.818	9.8
T13R2	0.491	0.087	0.682	0.054	0.341	0.221	3	56.234	11.996	92.185	17.602	0.143	17.745	7.72
T13R3	1.371	0.283	0.663	0.094	0.774	0.232	64	67.451	9.196	50.123	27.417	0.398	27.815	8.69
T14R1	0.588	0.088	0.447	0.031	0.22	0.125	28	41.934	9.879	36.358	32.161	4.211	36.372	9.32
T14R2	0.516	0.131	0.329	0.178	0.215	0.027	49	83.941	12.566	52.715	29.781	2.241	32.022	6.55
T14R3	1.95	0.397	1.484	0.404	0.221	0.03	42	31.762	11.133	55.563	27.783	11.124	16.663	4.87
T15R1	0.533	0.135	0.394	0.138	0.304	0.052	22	64.213	7.991	17.152	20.152	3.998	24.998	9.9
T15R2	0.629	0.1	0.507	0.13	0.459	0.234	96	76.876	7.871	45.761	28.289	2.862	31.151	10.28
T15R3	0.598	0.142	0.406	0.011	0.27	0.071	40	91.763	11.052	30.132	18.437	2.323	20.76	6.48
T16R1	0.359	0.07	0.586	0.199	0.432	0.201	68	78.504	24.01	79.801	26.188	2.131	28.32	6.24
T16R2	0.664	0.118	0.675	0.246	0.363	0.055	70	83.854	13.119	69.271	25.949	4.415	30.364	9.61
T16R3	0.815	0.134	0.887	0.281	0.334	0.061	45	99.7	9.296	81.19	27.442	5.512	32.954	5.39

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