Discussion

The production of crops is severely affected by abiotic and biotic stresses. Drought stress is a major concern as it adversely affects plant growth at the physiological, biochemical, and molecular level thereby reducing yields significantly causing economic loss. The strategy of microbial inoculation in plants is accepted worldwide by researchers and farmers to increase the crop yield, without any use of harmful chemical fertilisers.

Rhizobium sp. are used as potential tool in agricultural because it has unique property of nitrogen fixation in an symbiotic association, facilitates the accessibility of nutrients to plants in stress conditions and increase the crop productivity. Some reports indicate a role of legume-*Rhizobium* symbiosis in increased tolerance to salt (Wang et al., 2016) and heavy metal stress (Hao et al., 2014), however its role in increasing drought tolerance is relatively unexplored.

In the current study, isolation and characterization of drought tolerant *Rhizobium* sp. from root nodule of *Vigna mungo* was done with an aim to increase tolerance of drought stress.

A total of twenty four nodules were collected from root nodules of plants grown in soil at two different locations (Botanical garden and KVK farm, Banasthali Vidyapith). Different bacterial strains were isolated from root nodules. Twenty four isolates with similar morphological characteristics with *Rhizobium* sp. (white, circular, convex and mucoid) were selected for nodulation test (Bergey's manual of systematic bacteriology, 2005).

In the nodulation effectiveness test, isolated bacterial strains were inoculated in plants and scored for nodulation after twelve weeks. The success of nodulation requires that the introduced strains to be highly effective in nitrogen fixation, highly competitive and well adapted to the soil (Fitouri et al., 2012). Eight isolates were able to induce effective nodules on plant roots while other the isolates induced either a few ineffective nodules or failed to induce nodules. Pink coloured nodules are the most preferred and generally indicate the effectiveness of nodulation. There are various reasons for the formation of ineffective nodules. Many non-nodulating bacteria may be internally colonized in root nodules and effect the nodule color or morphology (De Lajudie et al., 1999; Mhamdi et al., 2005; Benhizia et al., 2004; Muresu et al., 2008). Many a times, *Agrobacterium* sp. either show antagonistic activity against rhizobia and may inhibit nodulation or could

induce non specific nodulation by co-inoculation with rhizobia (Mrabet et al., 2006; Liu et al., 2010).

The environmental conditions such as drought stress may be responsible of some non specific nodulation (Romdhane et al, 2007). Drought and salinity are challenging stresses for rhizobia, because they inhibit persistence and development (Graham, 1998). Therefore selection of rhizobia strains, tolerant to drought is important for ameliorating water stress conditions in plants. In the present study, tolerance to water stress for these selected strains were tested by using PEG 6000 (-0.30, -0.51, -0.54, -0.80, -0.90 MPa) supplemented media and bacterial growth was observed by a visual inspection. An isolate selected on the basis of its growth at high concentration of PEG, was retained for all the subsequent experiments. The selection of most drought tolerant and efficient strains has the potential to contribute to the formulation of inoculants for the arid regions.

The selected bacterial strain was identified as *Rhizobium* sp. on the basis of phenotypical characterization as white in colour, circular, convex with slime / mucoid transparent appearance, 2-4 mm in diameter and did not absorb the red colour of congo dye (Bergey's manual of systematic bacteriology, 2005). The selected *Rhizobium* sp. classified as fast growing bacterial sp. on the basis of acidic reaction on medium containing BTB dye (Somasegran and Hoben, 1994). The isolated bacterial strain was confirmed as *Rhizobium pusence* by using molecular 16S rRNA sequencing.

Water potential is defined as the tendency of water to move from one area to another due to gravity, osmosis and mechanical pressure. At a water potential of -0.033 MPa, plant growth and microbial activities are optimum in soil. In the water deficit condition, the water potential is decreased in soil and at a potential of -1.5 MPa, (permanent wilting point) plants are not capable to survive as soil water is held by soil particles and cannot be taken up by the plants.

It has been described previously that *Rhizobium* strains isolated from root nodules of *Hedysarum coronarium* L. could tolerate -0.5 to -0.95 MPa osmotic potential and these strains can be used for improving the growth of these plants in a semi arid region (Fitouri et al., 2012). In present study, the isolated *Rhizobium pusence* was also found to possess drought tolerant ability as it was able to survive and produced IAA in decreasing water potential (-0.30 to -0.90 MPa). It might be possible because it was isolated from semi arid region growing *V. mungo* and a relatively better drought tolerant cultivar. According to

Kavamuraa et al., 2012, bacteria which were isolated from drought tolerant plants, are able to grow in water stress environment due to ability for EPS, IAA and ammonia production.

The isolated *Rhizobium pusence* was found to posses many plant growth promoting characteristics such as production of IAA, ammonia, HCN, siderophore and EPS. IAA production by isolated bacteria under drought stress indicate their ability to promote plant growth under abiotic stress conditions (Mayak et al., 2004; Yuwono et al., 2005; Egamberdieva et al., 2008; Belimov et al., 2009). According to Ali et al., 2014, rhizobacteria could tolerate maximum level of drought due to production of ACC deaminase enzyme which can decrease ethylene production under abiotic and biotic stress.

Rhizobium sp. also promotes plant growth by indirect mechanism as may also act as biocontrol agents. The isolated drought tolerant *Rhizobium pusence* from *V. mungo* showed antifungal activity against *Fusarium*. It also produces siderophore, HCN and ammonia.

Many rhizobacteria are able to produce HCN, siderophore, antifungal metabolites and antibiotics (Bhattacharya and Jha, 2012). *Rhizobium pusence* can inhibit the growth of phytopathogenic fungi like *Fusarium oxysporum* due to the production of siderophore, (Buonassisi et al., 1986; Chao, 1990; Arfaoui et al., 2006; Kucuk, 2013). Siderophore bind to available form of iron ($Fe3^{+}$) and make it available to plants and unavailable to pathogens, thus protecting the plant from pathogens and thereby promote plant growth (Wani and Khan, 2013). On the other hand, HCN is toxic and forms strong bonding with iron of cytochrome oxidase and inhibit the electron transport chain in cellular respiration. HCN producing bacteria can protect themselves from HCN as it posses many enzyme system like rhodenase which convert the cyanide into thiocynate to detoxify HCN (Cipollone et al., 2007). The production of siderophore and HCN or interaction of these two with other metabolites might be responsible for the antifungal activity in many bacterial strains (Ahmad et al., 2008).

Production of ammonia also plays an important role in biocontrol. Ammonia is a volatile compound that was produced by many rhizobacteria (Brimecombe et al., 2001). Minaxi et al., (2012) reported that isolated PGPR is able to promote growth and yield of cowpea, because PGPR have ability to act as biocontrol agent through ammonia production.

Besides having antifungal activities and producing siderophore, HCN and ammonia, the *Rhizobium pusence* isolated for *V. mungo* was found to be resistant to ampicillin and chloramphenicol and was sensitive towards to erythromycin, gentamycin and streptomycin. Antibiotic resistant bacterial strains have selective advantage over other microbes as it makes them more competitive in soil and successful in establishment of symbiosis (de Oliveira Longatti et al., 2013). The antibiotic resistant bacteria that can produce antibiotic as well, act as good biocontrol agent (Trieu-cuot et al., 1987a). In previous studies, antibiotic resistance has been reported in *Rhizobium* and *Bradyrhizobium* especially to chloramphenicol, amoxicillin, and broadly ampicillin andvancomycin (Ahmad et al., 2001). According to de Oliveira Longatti et al., 2013, most *Rhizobium pusence* resistant to chloramphenicol and ampicillin are sensitive towards kanamycin and gentamycin.

Phosphorus solubilisation is another plant growth promoting trait of the isolated *Rhizobium pusence* with about 200 % phosphate solubilisation ability on pikovskaya's agar plates and solubilising upto 80 mg / l phosphorus in pikovskaya's broth by 8^{th} day of inoculation.

Pallavi and Gupta (2013) suggested that phosphate solubilisation is depends on sufficient amount of energy available to microbes for formation of organic acids by using different carbon sources in the media. It was observed that during the growth of the isolated *Rhizobium* sp. in culture in the present study, the pH of medium decreased gradually over time. Lowering in pH of culture media by production of acid might be a basic requirement of phosphate solubilisation by the bacterial strain; as a result phosphate solubilisation occurred after 1 week of incubation. It is possible that the isolated *Rhizobium pusence* produced acid during the growth because it was able to utilize carbon source and produced acid by fermentation. This was confirmed by a change in colour of phenol red which turned yellow colour during the biochemical tests. Our result agree with that of Kasturibai and Raju, 1980, who reported that bacterial isolates from soil, produce acid by utilization of different carbon source in medium. Phosphate solubilisation increases with lowering of pH due to production of acid by the particular strain in media (Rashid et al., 2004; Minaxi et al., 2012). The time taken by various bacterial strains to solubilise P in the culture media is reported to vary between 7-14 days (Illmer and Schinner,1995; Seshadri et al., 2000; Kim et al., 1998; Delvasto.et.al,2008.

Phosphorus is an essential nutrient in soil and is found in two forms as organic and inorganic. Both forms are found in insoluble form. Sharma et al., (2011) suggested that phosphorus solubilising bacteria play a role in phosphorus nutrition by enhancing its availability to plants through release from inorganic and organic forms by solubilisation and mineralization. Plants can use the phosphorus in the form of HPO_4^{2-} and $H_2PO_4^-$. Bacterial strains like *Pseudomonas*, *Bacillus, Rhizobium, Achromobacter, Agrobacterium Micrococcus, Aerobacter, Flavobacterium Mesorhizobium*, *Erwinia* etc. have the ability to solubilise insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, and rock phosphate (Goldstein 1986; Rodriguez and Fraga 1999; Rodriguez et al., 2006; Ahmad et al., 2008; Jadhav, 2013). While some species of *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* are not able to solubilise P-Ca, *Mesorhizobium mediterraneum* strains effectively solubilise P-Ca in solid medium (Peix et al., 2001a). *Sinorhizobium* is reported to be better for phosphate solubilisation in liquid medium than *Rhizobium* and *Agrobacterium* sp. (Kumar and Ram, 2014). This indicates that rhizobial strains exhibit much variation in phosphate solubilisation and may be related to host and environmental factors.

Most PGPR which were able to solubilise the phosphorus show other PGPR activities like ammonia, siderophore, HCN production and antifungal activity as well (Viruel et al., 2011).

The *Rhizobium pusence* isolated from *V. mungo* was optimised for growth and IAA production in media containing different supplements such as carbon, nitrogen and tryptophan etc. In the present work optimum growth of isolated *Rhizobium pusence* was obtained at pH 7. The optimum pH for *Rhizobium* sp. is reported to be pH 7-7.2 under culture conditions (Mandal et al., 2007, Sahasrabudhe, 2011).

The isolated *Rhizobium pusence* could be grown in all of the five carbon sources supplemented media but ideal growth was obtained in mannitol supplemented medium (1.5 %) at 48 h. Log phase of growth was obtained at 18 h to 36 h. Graham and Parker, 1964, reported that mostly fast growing bacteria are being able to utilize wide range of carbon sources than slow growing bacteria. Mostly, fast growing isolates utilized carbon source and produced acid in medium while slow growing isolates showed either alkali or neutral reaction (Kumar and Ram, 2012).

In the present study, IAA production was optimized in media containing tryptophan (2 mg / ml) and different carbon source like dextrose, fructose, mannitol, myoinositol and sucrose respectively. Highest IAA production was obtained in mannitol supplemented medium (1.5 %) followed by dextrose at 48 h. IAA production by bacterial isolate may be dependent on the growth conditions of media.

In previous reports, several *Rhizobium* strains are reported to have maximum IAA production in mannitol supplemented media (Sridevi et al., 2008; Sahasrabudhe, 2011; Kumar and Ram 2012; Ghosh et al., 2015). Some *Rhizobium* strains prefer media containing glucose for optimum IAA production (Datta and Basu 2000; Mandal et al., 2007). Other reports suggest that ribose, mannose, sorbitol can also be used as carbon source for IAA production and optimum IAA production by *Rhizobium* sp (Bhattacharya and Basu, 1992). In some studies, the optimum media for growth and IAA production of the same *Rhizobium* strain differ as observed by Roy and Basu (1989) where ideal bacterial growth was obtained in sucrose supplemented medium but IAA production was maximum in maltose (1%) supplemented medium. Decline in IAA production is generally observed towards the end of the stationary phase which might be due to release of IAA degrading enzymes like IAA oxidase and peroxidase (William and Singer, 1990).

These studies suggested that carbon source of media is a determining factor for growth and IAA production and carbon utilization depend on species of isolated *Rhizobium*.

After optimization of carbon source in media, different nitrogen sources were studied for growth. The maximum growth was observed in KNO_3 supplemented medium (0.1 %) followed by $NaNO_3$, NH_4Cl and $NH_4(SO_4)_2$ at 48 h of inoculation. Vincent, (1974) reported that *Rhizobium* sp. could utilize a wide range of nitrogen sources for growth.

In the present study, IAA production was also optimized in media containing tryptophan (2 mg / ml) and different nitrogen source. Similar to growth, IAA production was also highest in KNO_3 supplemented medium followed by $NaNO_3$. Our findings corroborate with Mandal et al., (2007) who reported high amount of IAA production by *Rhizobium sp.* in KNO_3 (0.1%) supplemented medium. According to Jordan 1984, nitrogen source might be responsible for increased IAA production. Nitrogen source is very prominent variable which mostly affect the growth and yield of IAA (Shokri and Emtiazi, 2010). Additionally L-tryptophan is reported to be the preferred isomer for IAA production (Datta and Basu., 1997, 2000).

In the present study, growth and IAA production of the isolated *Rhizobium* spwas studied in media containing different concentration of L-tryptophan (0.5, 1, 1.5, 2, 2.5, 3 µg/ ml), optimal carbon source, i.e., mannitol (1.5 %) and KNO_3 (0.1 %) as optimal nitrogen source. Growth was found best at 2 mg/ ml concentration of tryptophan followed by 2.5 and 1.5 mg / ml. Both high and low tryptophan was inhibitory for growth and IAA production. Our findings corroborate with other workers who reported growth and IAA production by *Rhizobium* sp. isolated from root nodules of *Phaseolus mungo* (Ghosh et al., 2008) and *Vigna mungo* (Chakarborty et al., 2010) are optimum in 2 mg / ml of L-tryptophan. However many reports suggested that 2.5 mg / ml concentration of L-tryptophan was optimum for IAA production from *Rhizobium* sp. isolated from nodules of *Dalbergia lanceolaria* (Ghosh and Basu, 2002) and 3 mg / ml concentration was optimum for *Rhizobium* sp. isolated from root nodules of *Roystonia regia* (Basu and Ghosh 2001). These studies indicate that IAA production by different species of *Rhizobium* depend on the utilization of different concentrations of L-tryptophan.

IAA production by *Rhizobium* sp. also depends on the pH of medium. In the present studies IAA production by *Rhizobium sp.* was found to maximum at pH 7. The results are in close conformity of those described in the literature, IAA production by *Rhizobium* sp. was found optimum at pH 7 (Sahasrabudhe, 2011) and pH 7.2 (Mandal et al., 2007).

The IAA produced by the isolated *Rhizobium* sp. was confirmed by comparison with authentic Indole acetic acid as standard on TLC plate. IAA spots were visualized in ethyl acetate: isopropanol: ammonium hydroxide (45:35:20) solvent system and by using ehmann's spraying reagent.

According to Satyanandam et al., (2013), effect of different supplements on growth and IAA production can vary according to utilization of supplements in media by different species of *Rhizobium*. In earlier reports, production of secondary metabolites occurred in the stationary phase but IAA production was observed during the symbiont growth, it can be concluded that bacteria produce IAA along with growth which is necessary for the growth of bacteria as well as root nodule maintenance/ formation (Ghosh et al., 2008). IAA production varied prominently among different species and also affected by culture conditions, availability of substrate in medium and growth stage, reported earlier by Vijila, 2000. Mandal et al., (2009) reported that the amount of IAA was more in nodulated roots than non-nodulated roots which indicate that high IAA production in the

nodulated roots is induced by the symbiont (*Rhizobium* sp.). Moreover all the supplements which are necessary for IAA production may be available for bacteria within the nodules like tryptophan pool which may contribute to produce higher level of IAA for the benefit of the host plant (Ghosh et al., 2008). Along with nitrogen as first line symbiosis, IAA content in nodule and supply to the host may be considered as second line symbiosis between root nodule and *Rhizobium* sp., as reported by Ghosh et al, 2008. According to Tsavkelova et al., 2006, the phytohormone IAA is directly correlated with plant growth promotion. Furthermore, *Rhizobium* sp. isolated from *Vigna mungo* has ability to produce IAA that has a role in nodulation (Mandal et al, 2007).

In the present study, the isolated *Rhizobium pusence* was also be able to produce IAA under osmotic stress condition, which might have a promising consequence to promote plant growth in the semi arid region.

Along with IAA other phytohormones such as ABA, GA and jasmonic acid is produced by endophytic bacterial strains (Piccoli et al, 2011). *Bradyrhizobium* induced increase in the phytohormones i.e. higher ratio of GA and IAA relative to ABA is reported to increase drought tolerance in chickpea plants (Bano et al., 2010). Moreover under drought stress, co-inoculation with *Rhizobium* and two *Paenibacillus polymyxa* strains induced hormonal changes and promote plant growth (Figueiredo et al., 2008).

Several reports have suggested that the balance of phytohormones, particularly that between auxin and cytokinin, is part of the nodulation stimulus, but it is not obvious a priori in which direction the balance is shifted. The appropriate IAA/cytokinin balance is essential for plant development, however not all effects of cytokinins and auxins in plant development are dependant on this balance (Pospisilova, 2003).

Bacteria also have the unique property of EPS production for survival in water deficit conditions. A correlation was observed by Hartel and Alexandre (1986), between EPS production by *Bradyrhizobium* sp. and increased drought tolerance. Moreover EPS provide protection from various biotic and abiotic stresses like desiccation through increase water retention and regulate diffusion of carbon source (Chenu and Roberson,1996), predation and the effects of antibiotics (Donot et al., 2012).

In the present study, the isolated *Rhizobium pusence* was able to produce EPS in culture media at the stationary phase of growth. The EPS was extracted from media supplemented with different carbon sources like dextrose, fructose, mannitol, myoinositol

and sucrose (1%). The EPS production was optimum in mannitol supplemented medium after 54 h of incubation. The viscosity was also observed to be maximum in mannitol supplemented medium. EPS was not obtained from control due where there was no carbon source. Hence carbon source might be an important factor for EPS production in media.

Similar observations were described in previous reports, for *Rhizobium* sp. isolated from the root nodules of *Vigna mungo* (Mandal et al., 2007), *Rhizobium ciceri* (Kucuk and Kivanc, 2009), and *Rhizobium* strains (Kumar et al., 2014) showed maximum growth and EPS production in mannitol supplemented medium. However EPS extracted from media containing glucose also gave maximum EPS production by *Rhizobium sp*. from *Crotalaria saltiana* (Mukherjee et al., 2011), media supplemented with xylose showed maximum EPS production by *Rhizobial* sp. from *C. cajan* (Fernandes, 2011) and sucrose supplemented media also resulted in maximum EPS production by *Rhizobium* sp. from *Phaseolus mungo* (Ghosh et al., 2011). Overall EPS production in media depends on carbon source utilization by the isolated species of *Rhizobium*.

The total carbohydrate and protein content was determined in EPS extracted from media containing different carbon source. The total carbohydrate and protein were highest in EPS extracted from media supplemented with mannitol. Similar result was earlier obtained by Mandal et al., 2007.

A composition analysis of EPS extracted from different carbon source was done by using FTIR (Table 5.1). The FTIR study of EPS reveals a hydroxyl group between 3600-3400 cm^{-1}. Primary and secondary amine group were observed at 1662, 1540 cm^{-1}, and C=O stretching vibration of carboxylic acid at 1700 cm^{-1} revealed the presence of protein in EPS. The carbohydrate C-O-C ring (1300- 1000 cm^{-1}) (Jiao et al., 2010) was observed at 1075 cm^{-1} and a peak between 900-1000 cm^{-1} suggests the presence of glycoside bond of polysaccharide (Bosch et al., 2006). A peak was observed at 1459 cm^{-1} indicate the presence of alkenes bend (C-H) (1465- 1450 cm^{-1}) In earlier reports similar band of hydroxyl group at 3,600-3,000 cm^{-1} and glycoside bond (850–1,200 cm^{-1}) of the polysaccharides were observed in EPS secreted by *Rhizobium* sp. isolated from *V. mungo* (Mandal et al., 2007). Another report has been showed that a band observed at 3343 cm^{-1} (hydroxyl), 1651 cm^{-1} (carboxyl) , 1071 cm^{-1} (cyclic C—O) suggested the presence of glucuronic acid, mannuronic acid and O-acetyl ester in EPS (Patil et al., 2009).

Bramchari and Dubey (2006) have been suggested EPS composed from carbohydrate and protein.

The extracted EPS was highly compact and collapsed in nature as observed by the FESEM study. In a previous FESEM study of EPS from *Lactobacillus*, flake like highly compact structures have been observed (Yadav et al., 2011).

Table 5.1

Frequency (cm^{-1})	**Functional groups**	**Reference**
3600-3200	O–H str of hydroxyl	(Mandal et al., 2007), (Tapia et al., 2009)), (Patil et al., 2010), (Yadav et al., 2011)
1640-1450	Amide I, >C=O str and C–N bending of protein and peptides amide	(Naumann 2000), (Bosch et al., 2006), (Tapia et al., 2009), (Jiao et al., 2010),
1627-1615	>C=O str (asym) COO−	(Navarini et al., 1997), (Batsoulis et al., 2004), (Bosch et al., 2006), (Tapia et al., 2009),
1540	Amide II, N–H bending, C–N str of proteins and peptides	(Beech et al., 1999), (Naumann 2000), (Jiao et al., 2010),
1745–1730	>C=O str of alkyl esters, carboxylic acid	(Navarini et al., 1997),(Abd and Ismail, 2012)
1200-900	C–OH str modes and C–O–C, C–O ring vibrations of carbohydrates (oligo, polysaccharides, and alginate)	(Synytsya et al. 2003), (Bosch et al., 2006), (Patil et al., 2010), (Jiao et al., 2010),
900-800	Glycosidic linkage type “anomeric region”	(Synytsya et al., 2003), (Bosch et al., 2006), (Mandal et al., 2007),

TLC of hydrolyzed EPS extracted from media containing different carbon source showed glucose and fructose monomers. It is previously reported that EPS composition included monosugar like glucose, glucuronic acid, galactose, fructose (Mody et al., 1990; Mukherjee et al., 2011; Abd and Ismail, 2012) and mannose, arabinose, xylose were found in polysaccharide secreted from *Rhizobium* (Ghosh et al., 2011). Datta and Basu,

(1999) also observed that glucose, mannitol and fructose were present in EPS produced by *Rhizobium* strains.

In the present study, antioxidant properties of EPS extracted from media containing different carbon source were also determined. EPS extracted from media supplemented with fructose and mannitol, showed highest DPPH scavenging activity. Reducing and total antioxidant activity was also highest in EPS extracted from media supplemented with fructose and mannitol. These results revealed that EPS extracted from mannitol and fructose supplemented media were potent antioxidant.

It is previously reported that polysaccharides act as free radical scavengers or as antioxidant for the prevention of oxidative damage caused by reactive oxygen species (Zhang et al., 2004). It has been suggested that bacteria and fungi produce polysaccharide that act as antioxidant (Kodali and Sen, 2008; Pan and Mei, 2010). Sun et al., (2012) reported that EPS from *Pleurotus eryngii* SI-02 had DPPH radical scavenging activity and reducing power higher than synthetic antioxidant butylated hydroxytoluene while EPS from *Lysinibacillus fusiformis* and *Bacillus subtilis* showed ideal DPPH radical scavenging activity (Mahendran et al., 2013; Razack et al., 2014). Polak-Barecka et al., (2013) reported that EPS secreted by *Lactobacillus rhamnosus* in media containing galactose, lactose and sucrose showed highest antioxidant activities. It is suggested earlier that antioxidant property may be influenced by the arrangement of monosaccharide and glycosidic linkage and conformation of the polysaccharide (Kumar et al., 2007). EPS produced by *Sinorhizobium meliloti,* is reported to protect symbiotic nitrogen fixation from H_2O_2 (Lehman and Long, 2013).

These studies gave the evidence that EPS from microorganisms had great potential of antioxidant activity and it depends on substrate which was used in EPS culture.

Rhizobacteria diversity and community are very responsive towards a very little change in soil water and these changes may alter the functional potential of microbial population and provokes a clear osmotic stress response including the production of compatible solutes that increase intracellular C demand. Subsequently, a microbial population emerges with a greater capacity for extracellular enzyme production targeting macromolecular carbon (Bouskill et al., 2016).

In the present study, the plant growth promoting traits like IAA, HCN, ammonia and siderophore production, phosphate solubilisation, antifungal activities and EPS produced by the isolated *Rhizobium pusence* may contribute to survive under drought stress environment and can increase drought tolerance for plant survival in arid or semi arid regions. Moreover it has been reported if a microbe shows traits like ammonia production, IAA production, phosphate solubilisation, siderophore production in vitro, it reveals its plant growth promoting ability in vivo (El-Deeb et al., 2012,2013; George et al., 2013).

In present work the isolated *Rhizobium pusence* was inoculated in *V. mungo* plants and non-inoculated plants served as control. Dry weight, fresh weight, length of root and shoot were measured at 30 DAS. The plants inoculated with the isolated *Rhizobium pusence* showed increased root and shoot length, fresh and dry weight of root and shoot. More leaf area and effective nodules were also observed in inoculated plants.

It is similar to the previous reports that the root and shoot length, dry and fresh weight of root and shoot increase on inoculation br *Rhizobium* (Hossain and Solaiman, 2004; Solaiman and Rabbani, 2004). Moreover, the highest number of nodules obtained from pea plants inoculated with *Rhizobium* strain (Talukdar et al., 2008) and inoculation with *B. japonicum* showed effective nodulation in adzuki bean (Delic et al., 2010). According to Minaxi et al., (2012) *Bacillus* sp. treated cowpea plants had more leaf area than untreated, chemical fertilizer and biofertilizer treated. Leaf area is also reported to increase with inoculation of PGPR like *Psuedomonas*, *Azospirillium* and *Azotobacter* strains (Martinez-Toledo et al., 1988; Siddiqui and Shaukat, 2002).

Total phosphorus and nitrogen content was also observed to be higher in root and shoot of inoculated plants than non inoculated plants. Similarly, it has been described earlier that N, P content increased after the inoculation of *Rhizobium* strain (Talukder et al., 2008), *Bacillus* (Biswas et al., 2000; Minaxi et al, 2012) and other PGPR inoculation in *V. unguiculata* (Linu et al., 2009) and *P. vulgaris* (Collavino et al., 2010).

The present study indicates that the isolated *Rhizobium pusence* shows plant growth promoting traits and it might be helpful to tolerate water stress and plant growth under water deficit conditions. Drought is major concern because it is limiting to crop productivity worldwide (Kim et al, 2012). The isolated *Rhizobium pusence* could tolerate

higher level of drought and can resist in water stress conditions. We further examined the role of the isolated *Rhizobium pusence* on water stress alleviation in the host plant.

The *Rhizobium sp* inoculated plants was observed to have increased chlorophyll content, proline content and highe activity of ROS scavenging enzymes in *V. mungo* plants under water deficit condition. It is reported that plant photosynthetic pigment and photosynthetic system can undergo severe damage under the drought stress (Ashraf and Harris, 2013). In the present study, chlorophyll a, chlorophyll b and total chlorophyll content were present in higher amount in inoculated plants than non-inoculated plants. However under water deficit conditions, chlorophyll content decreased in inoculated plants but it was still more than that of the non-inoculated plants. This study agree with with other researchers who conclude that chlorophyll content under drought stress increase in PGPR inoculated plants (Kang et al., 2014; Sharma and Saikia, 2014; Gusain et al., 2015). Moreover, according to Gururani et al, (2013) photosynthetic performance increased under abiotic stress when *Solanum tuberosum* was inoculated with *Bacillus* sp. Thus, plants inoculated with the *Rhizobium pusence* with increased the chlorophyll content may be responsible for increasing the photosynthetic efficiency which support to plants to survived in drought stress condition.

In general, ROS increases due to abiotic stress conditions at the cellular level. It has been suggested that superoxide radical (O_2^-), hydrogen peroxide and hydrogen radical are ROS that cause lipid peroxidation of membranes (Sgherri et al., 2000; Hemavathi et al., 2010). Malondialdehyde (MDA) is a secondary breakdown product of lipid peroxidation and lipid peroxidation is calculated in the form of MDA.

In the present study lower lipid peroxidation was observed in inoculated plants than the non-inoculated plants under water stress conditions. It has been described earlier, that PGPR inoculation in plants is responsible to reduce membrane potential due to changes in proton efflux activities and changes in phospholipid content (Bashan et al., 1992). Moreover lipid destruction occurs due to more phosphatdylcholine and less amount of phosphatidylethanolamine in cell membrane in water deficit plants and it was prevented by inoculation of drought tolerant PGPR (Sueldo et al, 1996). Thus inoculation of plants with the isolated *Rhizobium pusence* exhibit lower a level of MDA content in stressed and non stressed conditions than non inoculated plants indicating a lower accumulation of ROS and membrane damage in the inoculated plants.

H_2O_2 level increase in drought stress condition, viz. superoxide ion formation by electron transport chain which dismutase and cause the increased level of H_2O_2 in chloroplast and mitochondria under water deficit condition (Elstner, 1991) and increased level of H_2O_2 can be react with superoxide radical which is responsible for formation of reactive hydroxyl radical (Prousek, 2007). These increase level of H_2O_2 promote the destruction of cell protein, peroxidation of membrane lipids and plant cell death (Jaw and Ching, 1998).

The present study revealed that under drought stress, H_2O_2 content increased in inoculated plants but the rate of increase was higher in non inoculated plants. It indicated that the *Rhizobium* sp. might be having an efficient antioxidant system that can maintain a steady level of H_2O_2 and increase the potential of plant to better survive under drought stress. *Rhizobium* sp. can alleviate water deficit conditions by decreasing the H_2O_2 content of plant. However, H_2O_2 (ROS) content was initially higher in inoculated plants than non inoculated plants which suggest that H_2O_2 might be involved in early signally or defence response during *Rhizobium* legume symbiosis.

It has been described previously that in the early stages of *Rhizobium* legume symbiosis, oxidation of nitro blue tetrazolium (NBT) occurred in infection threads which suggested that O_2^- is produced during the infection process and have a role in symbiosis (Santos et al., 2001; Ramu et al., 2002). Moreover, ROS (H_2O_2) production depends on the nod factors which are produced during the symbiosis. Ramu et al., 2002, suggested that mutant bacteria which are unable to produce nod factors inoculated in *Medicago truncatula* plants show no ROS production. Moreover, H_2O_2 steady level is necessary for effective nodulation; mutant *S meliloti* over expressing catalase which decline the H_2O_2 content from the steady level showed delayed nodulation phenotype than wild type strain, suggested the role of H_2O_2 in the early stages of symbiosis (Jamet et al., 2007). Other studies revealed that H_2O_2 (ROS) observed in cortical cells of *M. truncatula* roots after inoculation with *Sinorhizobium* (Peleg-Grossman et al., 2007) and a increase level of intracellular ROS at the tip of growing root of *Phaseolus vulgaris* has been observed after the treatment of Nod factors (Cardenas et al., 2008).

It is previously reported that ROS have not been observed in rhizobia which is present in infection thread, showed the rhizobia have an enzymatic antioxidant system that is essential for development of symbiosis (Santos et al., 2001; Rubio et al., 2004) and *S. meliloti* have two superoxide dismutases and three catalase which are able to scavenge

H_2O_2 (Herouart et al., 1996; Ardissone et al., 2004). Moslemi et al, 2011, suggested that PGPR inoculation in plants served as scavenger of ROS and sustain the lower level of H_2O_2. Moreover, PGPR inoculated rice show lower H_2O_2 content under drought condition (Gusain et al., 2015). Thus, PGPR inoculation in plant maintain the level of H_2O_2 in plants and protect the destruction of cell protein and membrane lipids under water deficit conditions.

The production of different osmolytes such as glycine betaine as well as L-amino acids and their D-isomers enhanced the survival capability of bacteria under the stressed environment (Roberson and Firestone 1992; Shahjee et al. 2002). Furthermore D'Souza-Ault et al., 1993, reported that N-acetylglutaminylglutamineamide and glycine betaine are cytoplasmic osmoprotectants, that accumulated inside the bacterial cell in response of stressed environment. Proline is amino acid which helps microbial cell to protect under water stress environment (Sarma and Saika, 2014).

In the present study, proline content increased in both inoculated and non-inoculated plants but a higher amount of proline content was observed in plants inoculated with the isolated *Rhizobium pusence* in response to drought stress indicate improved stress response of inoculated plants in terms of proline content. Proline content higher inoculated than non-inoculated plants under non stressed conditions. Similarly in earlier reports, high proline content was also detected in absence of abiotic stress in pepper inoculated with *Arthrobacter and Baccilus,* indicate that bacterial inoculation may cause biotic stress on plants (Sziderics *et al.* 2007). Moreover increased level of proline biosynthesis is noticed in abiotically stressed plants inoculated with beneficial bacteria such as *Burkholderia* (Barka *et al.* 2006), as well as *Arthrobacter* and *Bacillus* (Sziderics *et al.* 2007). According to Kohler et al. (2008), PGPR- mediated drought stress alleviation can be observed by the process of proline accumulation. Proline accumulation helps to maintain the osmotic potential in plant cells and protect the macromolecules adjustment in cell membrane under the drought stress conditions (Thapa et al., 2011). Proline accumulation provides a mechanism for better plant survival during periods of drought stress (Ashraf et al. 2003). Also, this amino acid can also serve as a source of nitrogen and carbon for the plant.

Phenolics are the major compound which produced by the plants as a response to abiotic and biotic stress. Phenolics are also involved in defence mechanism and act as signalling

molecules (Mandal et al., 2010). In the present study, higher phenolic content is recorded in inoculated plants as compared to non inoculated plants that might have a protective role from oxidative burst under water stress conditions. The enzyme activity of PAL was noticed higher in inoculated plants and exhibits an increasing trend also in water deficit condition. Inoculation with PGPR induces the synthesis of PAL activity which is directly proportional to synthesis of phenolic compounds (Basha et al., 2006; Martins et al., 2013).

It has been reported earlier, phenolics like 4-O-β-glucosides of p-hydroxybenzoic, protocatechuic, vanillic acids, gallic acid and its methyl ester extracted from nodules of soybean act as antioxidants (Moran et al., 1997). Furthermore, flavonoids like phenolic acids may have antioxidant properties that protect the cell from the oxidative damage (Rice-Evans, 2001).

Drought impairs the electron transport system leading to the formation of reactive oxygen species that accumulate and can damage the photosynthetic apparatus. Plants have no immune system to protect themselves from oxidative damage but they have tools in form of antioxidant enzymes to overcome the stress and could be surviving in stress environment. Under stress, ROS is accumulated in plants and removed by enzymatic system thus positive correlation occurred between enzymatic activities and stressful environment (Gong et al., 2005; Koussevitzky et al., 2008). Bacterial inoculation in plants shows antioxidant protection and adaptation to drought under arid conditions (Armada et al., 2014).

The influence of isolated drought tolerant *Rhizobium pusence* on the enzyme system of *V. mungo* in response to water deficient condition was determined by using CAT, POD, SOD and PAL activities. Initially CAT and POD activity was higher in non inoculated plants than inoculated plants but under water stress *Rhizobium pusence* stimulated both enzyme activities of the inoculated plants. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. Under drought stress SOD activity increased in both inoculated and non-inoculated plants but higher amount of SOD activity was observed in non inoculated plants. It is previously reported that SOD activity was significantly less in inoculated plants than non inoculated plants under water deficit condition accompanied by enhanced H_2O_2 scavenging enzymes such as CAT and POD activities that have great importance to survive with oxidative stress during stress

conditions (Kohler et al., 2008). The increase in the activity of H_2O_2 scavenging enzymes such as CAT and POD in inoculated plants, that alter the H_2O_2 homeostasis under stress environment which led to increased plant growth. The triggering effect of PGPRs on genes encoding for antioxidant enzymes may be responsible for increasing the activities of ROS scavenging enzymes in inoculated plants (Gururani et al., 2013).

CAT, POD and SOD activities were increased after the inoculation with the isolated *Rhizobium* sp under drought stress which is similar to the previous findings (Saravanakumar et al., 2011; Gururani et al., 2013; Sharma and Saikia, 2014). According to Islam et al., 2016 ROS scavenging enzymes like CAT, POD activities was superior over non inoculated and less activity of SOD enzyme was observed in inoculated mungbean plants under stress. It is reported by Kohler et al. 2008, that CAT production was observed in lettuce (*L. sativa* L.) under drought stress, when co-inoculated with *P. mendocina*, and arbuscular mycorrhizal fungi (*G. intraradices* or *Glomus mosseae*) and CAT, POD enzyme activities were increased in inoculated *Cucumis sativus* with *Burkholderia* sp., *Acinetobactor* sp. and *Promicromonospora* sp under drought stress (Kang et al., 2014). Furthermore, CAT, SOD enzyme activities were increased by inoculation of *Bacillus thuringiensis* under drought stress (Timmusk et al., 2014). *Bacillus* sp. possesses SOD which is involved in the alleviation of oxidative stress (Wang et al., 2007). However Armada et al. (2014) suggested that SOD activity was maintained without any change in inoculated *L. dentata* and *S. officinalis* plants, which indicate that SOD enzyme play a non significant role in defence against the oxidative stress during drought, but changes in CAT, APX activities as a result of inoculation suggests bacterial inoculation provide defence to plants survival in drought stress.

Thus, the use of bacteria to control drought stress in plants is an important and sustainable strategy. Several bacterial traits have been suggested to be involved in conferring drought tolerance to treated plants including the production of EPS, volatile organic compounds and IAA. Thus, the overall the present study has established that the isolated *Rhizobium pusence* from root nodules of *Vigna mungo* growing in semi arid region can efficiently alleviate drought stress condition through its plant growth promoting traits and increased the level of antioxidant enzymes, phenolics, and cell osmolytes like proline. The microbial strain has capability to survive under drought stress and promote plant growth under water deficit condition which is a good criterion to select the beneficial bacterial strain for use as future prospective in agriculture.