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RESEARCH ARTICLE

INTERACTION EFFECT OF AM FUNGI, *RHIZOBIUM* AND DROUGHT STRESS ON CHICKPEA

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ABSTRACT

Biofertilizers are the microbial inoculam which enhanced the soil fertility and crop yield. This study was initiated with the interaction of AM Fungi, *Rhizobium* and drought stress on the growth of chickpea (*Cicer arietinum*, L.). The dual inoculation of AM fungi and *Rhizobium* showed the synergistic effect on morphological features and physiological tolerance of such legume species under green house condition with few days of drought condition. The efficiency of such biofertilizers in dual and individual applications on the quantitative yield of chickpea plant was comparatively higher than the non-inoculated control plants. This study has suggested the dual application of such biofertilizers not only enhanced the legume plant growth but also increased the soil fertility, drought tolerance and reduced the risk of application of chemical fertilizers in the agricultural field. Thus, they are generally termed as eco-friendly fertilizer and do not cause the pollution of any sort.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil habitats and form beneficial symbiosis with the roots of angiosperms and other plants (Gerdemann, 1968). They are associated with about 80% of the plant families in the world (Giovannetti and Sbrana, 1998). Most terrestrial plants associate with root colonizing mycorrhizal fungi, which improve the fitness of both the fungal and plant associates. Ubiquitous occurrence and importance of AM fungi for plant growth is now a well established fact. Distribution and abundance of AM fungi vary greatly among different sites i.e. natural and manmade ecosystems (Chaurasia, 2001 and Gianinazzi-Pearson *et al*., 1985). Natural soil offers consortium of indigenous mycorrhizal fungi and often used as source of inoculam. This can be produced on a large scale by pot culture technique. Since isolation and selection of AM species (effective for growth promotion) and rising of pure culture of these species is difficult, a suitable host is required to maintain the AM culture. The beneficial use of AM inoculam in agriculture and raising nurseries has been reported (Muthukumar *et al*., 2001; Smith and Read, 1997). The legume-*Rhizobium* symbiosis is a classic example of mutualism where rhizobia supply ammonia or amino acids (as dicarboxylic acids – malate and succinate) as a carbon and energy source.

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Different *Rhizobium* sources are *Rhizobium meliloti*, *Rhizobium trifolii*, *Rhizobium leguminosarum* and *Rhizobium phaseoli.* Soil contains native species of *Rhizobium* but all are not capable of forming nodules. Some of the strains are highly specific to certain species. These are called cross inoculation groups. For example, *Rhizobium leguminosarum* can nodulate peas only, while *R. leguminosarum* bv. *phaseoli* can nodulate french bean and other beans and *Rhizobium spp*. nodulate cow pea. Artificial inoculation helps to increase the native population of a particular species in the soil when that crop is grown. India is the largest producer and importer of the leguminous crop (Shakya *et al*., 2008). Amongst the leguminous crops, chickpea (*Cicer arietinum* L.) occupy an important position due to its nutritive values (17-23% protein) in large vegetarian population of the country (Ali and Kumar, 2006). The major chickpea producing states are Andhra Pradesh, Madhya Pradesh, Maharashtra, Rajasthan and Uttar Pradesh contributes 86% of the total population of the country.

It is interesting to note that interaction of AM fungi with other beneficial micro organisms increases the growth of plants by enhancing the uptake of minerals especially phosphate. This AM fungi confer other benefits to their host plant such as drought tolerance, protecting the host against diseases, salinity and temperature extremes, producing plant growth hormones and movement of carbohydrates from one cell to another. The wide spread presence AM symbiosis in nodulated legumes and the role of AM fungi in improving nodulation and rhizobial activity within the nodules, are both universally recognized processes (Barea *et al*., 2005b). The dual soil application of

Rhizobium and VAM showed synergistic effect on all mung bean cultivars. Among form cultivars tested variety “Vaibhav” was found host responsive to root nodulation, growth parameters and grain yield (Manke *et al*., 2008). *Rhizobium* inoculation is a promising fertilizer because it is cheap, easy to handle and improves chickpea plant growth and seed quality under pot experiment (Nishita and Joshi, 2010). The result revealed that, the bacterized seeds showed 14.06% in total length, 10.83% in total weight and 9.0% on germination over control in pot experiment. Biofertilizers are used to reduce the use of chemical fertilizers, enhance soil fertility and yield crops in agriculture. Such biofertilizers confer other benefits to their host against diseases, salinity and different environmental stresses - water, radiation, drought, wind and pressure, sound, magnetic, electrical and biotic. Among these, drought stress is of particular importance, the former showed water deficit which refers to situation in which plant water potential and turgor are reduced to interfere the normal functioning of plants and the later showed homeostasis disruption in water potential and ion distribution. This has lead to research into drought tolerance with the aim of improving crop plants inoculated with biofertilizers.

This study was initiated to know the interaction of biofertilizers (*Rhizobium* and AM Fungi) and drought stress on the growth of chickpea (*Cicer arietinum*, L.). Such work was an ecofriendly beneficial to all in some way with the following objectives:

- To study the morphological changes, nutrients uptake and physiological tolerance in biofertilizer inoculated chickpea plants under green house condition with few days period of drought stress.
- To know the per cent infection of microbial fertilizer (AM Fungi) on chickpea plants and the number of nodule development (i.e. *Rhizobium* infection) per plant.

MATERIALS AND METHODS

Legume plant grown under green house condition

Seeds of chickpea (*Cicer arietinum* L.) was surface sterilized with 0.1% mercuric chloride for 5 min and washed with sterile water repeatedly. Sterile garden soil was used to fill the earthen pots (20 cm height; 25 cm diameter). About 3 kg of sterile soil were taken in each earthen pot. Ten seeds were sown in each pot. After germination, the seedlings were thinned out to 6 in each pot. All experimental plants were maintained in the green house under conditions of broad day light. Sterile tap water was used to water the plants.

The pots were assigned for the following treatments in chickpea plants

C - Control (without biofertilizer treatment)
T1 - *Rhizobium* treated
T2 - AM fungi treated
T3 - *Rhizobium* and AM fungi treated

Inoculation with AM fungi

The selective AM fungal inoculam was mass cultured (Plate:1) in the host maize plants in sterile soil under potted conditions. Five gram of soil inoculam with AM fungal spores and sporocarps, and infected root bits were spread over the lower layer of soil (1 kg) in each AM labeled pots. Then 2 kg of soil was layered over the inoculam before sowing.

Inoculation with *Rhizobium*

The selective *Rhizobium* inoculam was mass cultured (Palte:1) under laboratory conditions. The carrier based inoculam was mixed thoroughly to form inoculam slurry and was mixed with seeds properly. Then such seeds were shade dried and the *Rhizobium* (10^5 to 10^6 cells) coated seeds were sown in *Rhizobium* labeled pots.

Induction of Stress

Drought stress was given by withholding water supply from 31st day to 35th day for 5 days.

Determination of growth

The legume plant vegetative growth was measured for the following growth parameters at regular interval of 15 days.

Determination of fresh and dry matter

The plant materials were cut into bits and weighed. Then they were and dried in an oven at 90°C until the weight became constant.

Shoot and root length determination

The shoot and root lengths of the plants were measured using a meter-scale.

Determination of root nodule number

The number of root nodules per plant were collected and counted in all bio-inoculated legume plants.

Assessment of AM fungal infection

The root materials of AM treated legume plants were cleared and stained using the improved procedure of Phillips and Hayman (1970).

Chlorophyll estimation

The chlorophyll content of leaf tissue was estimated by the method of Arnon (1949).

Carotenoid estimation

The carotenoid content of the leaf tissue was estimated by Ridley’s (1977) method.

Proline estimation

The Free proline content in the leaves of chickpea was determined by the method of Bates *et al*., (1973).

Total nitrogen estimation

The total nitrogen content of the dried biomass of chickpea was estimated (Umbreit *et al*., 1972) by micro kjeldahl method.

Total phosphorus estimation

The total phosphorus content of the dried biomass of chickpea was estimated (Bartlett, 1959) by micro kjeldahl method.

Total potassium estimation

The total potassium content of the dried biomass of chickpea was estimated by Flame photometer method.

Estimation of proteins

The total protein content of fresh leaf tissue and dried seed was estimated by Lowry's method (Lowry *et al*., 1951).

Statistical Analysis

The data collected in this study was subjected to analysis of variance (ANOVA) and means comparison has done using Duncan's multiple range test (DMRT) (Little and hills, 1978).

RESULT AND DISCUSSION

Legumes are consumed as a source of human food and animal feed. Their importance as food lies primarily in their high protein content. Its grain protein is the natural supplement to cereal grain protein. They also provide fat and carbohydrates. Moreover, legumes are high in bone building minerals and vitamins essential for good health (Porres *et al*., 2003). Biofertilizers are inputs containing microorganisms which are capable of mobilizing nutritive elements from non-usable form to usable form through biological processes; they include mainly the nitrogen fixing, phosphate solubilizing and plant growth promoting microorganisms (Goel *et al*., 1999). Sharma *et al*. (2005) addressing the effect of legume rhizo deposition on bacterial communities, showed a distinct plant-dependent rhizosphere effect on the distribution of different bacterial groups present in legume rhizosphere. In the present study, the dual mixture of two biofertilizers (*Rhizobium* and AM fungi) was significantly ($P<0.05$) more effective than single species inoculam. The fresh and dry weight of the chickpea was gradually increased from 1.04±0.02g, 0.29 ±0.01g to 5.03±0.01g, 2.37±0.03g in *Rhizobium* with AM fungi inoculated plants when compared with control and individual inoculated plants (Table 1).

Lin *et al*. (1993) have investigated that the mycorrhizal inoculation with *Rhizobium trifolii* on *Trifolium repens* significantly increases the dry weight of shoots and roots, nodulation, nitrogen fixation, total nutrient uptake, final dry matter and phosphorus absorption. Drought stress is one of the major abiotic stresses limiting the productivity of crops in agriculture worldwide (Bohnert *et al*., 1995). It is also a significant yield-limiting factor in chickpea production as the major chickpea growing areas are in arid and semi-arid zones and about 90% world's chickpea is grown under rain fed conditions (Kumar and Abbo, 2001). Chickpea showed the mechanisms for overcoming this condition. The present study showed that the mild drought stress in chickpea plants had little effect on fresh and dry matter yield of both individual and combined inoculation of *Rhizobium* and AM fungi treated legume plants and control plants. However, the dry matter yield during the recovery period (35 to 45 days) was remarkable in the mycorrhizal plants. These results were positively related to the inoculation effect of single and dual inoculation with *Gigaspora rosea*, *Glomus intraradices* with *Gigaspora rosea* and *Glomus etunicatum* with *Glomus intraradices* on the growth and nutrients uptake (NPK) on *Medicago sativa*. It showed the significant increase in dry weight of shoot and root (Khan *et al*., 2008).

(a)

(b)

Plate 1. Mass cultured AM fungi and *Rhizobium*

The present investigation reported that the shoot and root length of the control and all biofertilizers treated chickpea plants increased progressively with age. Significant ($P<0.05$) increase in shoot and root length was found in *Rhizobium* with AM fungi inoculated as compared to the other treated chickpea plants (Table: 1 and Plate: 2). The response of arbuscular mycorrhizal fungi and *Rhizobium* inoculation on the growth and chlorophyll content of *Vigna unguiculata* (L) Walp Va. Pusa 151 was investigated (Arumugam *et al*., 2010) and recorded as a significant ($p<0.05$) increase over control in

Interaction effect of AM fungi and Rhizobium and drought stress on the shoot length, root length, fresh weight and dry weight of chickpea plant

Treatments	Growth parameter	15D	30D	35D	50D
C	Shoot length (cm/plant)	$7.40^{a}\pm0.16$	$16.70^{a}\pm0.26$	$17.22^{a}\pm0.19$	$23.80^{a}\pm0.16$
	Root length (cm/plant)	$3.20^{a}\pm0.16$	$8.14^{a}\pm0.11$	$8.32^{a}\pm0.08$	$11.40^{a}\pm0.16$
	Fresh weight(g/plant)	$0.55^{a}\pm0.01$	$2.06^{a}\pm0.01$	$2.11^{a}\pm0.05$	$3.53^{a}\pm0.01$
	Dry weight(g/plant)	$0.11^{a}\pm0.01$	$0.25^{a}\pm0.01$	$0.26^{a}\pm0.11$	$1.04^{a}\pm0.02$
T1	Shoot length(cm/plant)	$7.80^{b}\pm0.16$	$17.74^{b}\pm0.24$	$18.48^{b}\pm0.38$	$25.92^{b}\pm0.19$
	Root length (cm/plant)	$3.68^{b}\pm0.08$	$9.26^{b}\pm0.17$	$9.78^{b}\pm0.26$	$12.46^{b}\pm0.21$
	Fresh weight(g/plant)	$0.62^{b}\pm0.02$	$2.13^{b}\pm0.02$	$2.14^{a}\pm0.01$	$3.77^{b}\pm0.02$
	Dry weight(g/plant)	$0.12^{b}\pm0.01$	$0.29^{b}\pm0.01$	$0.29^{b}\pm0.01$	$1.39^{b}\pm0.02$
T2	Shoot length (cm/plant)	$9.80^{c}\pm0.16$	$24.64^{c}\pm0.40$	$26.46^{c}\pm0.30$	$33.42^{c}\pm0.27$
	Root length (cm/plant)	$4.32^{c}\pm0.13$	$11.16^{c}\pm0.15$	$11.60^{c}\pm0.19$	$15.80^{c}\pm0.16$
	Fresh weight(g/plant)	$0.63^{b}\pm0.01$	$2.35^{c}\pm0.01$	$2.38^{b}\pm0.02$	$4.83^{c}\pm0.02$
	Dry weight(g/plant)	$0.14^{c}\pm0.01$	$1.10^{c}\pm0.01$	$1.13^{c}\pm0.11$	$1.97^{c}\pm0.03$
T3	Shoot length (cm/plant)	$10.10^{d}\pm0.16$	$25.84^{d}\pm0.18$	$27.82^{d}\pm0.19$	$35.92^{d}\pm0.08$
	Root length (cm/plant)	$4.64^{d}\pm0.17$	$11.50^{d}\pm0.22$	$12.08^{d}\pm0.22$	$18.04^{d}\pm0.17$
	Fresh weight(g/plant)	$1.04^{c}\pm0.02$	$2.51^{d}\pm0.02$	$2.56^{c}\pm0.02$	$5.03^{d}\pm0.01$
	Dry weight(g/plant)	$0.29^{d}\pm0.01$	$1.15^{d}\pm0.01$	$1.17^{d}\pm0.01$	$2.37^{d}\pm0.03$

Values are mean of five replicates ± SD The mean difference is significant at the 0.05

D- Days; Drought stress period - 31 to 35 Days

C- Control T1-*Rhizobium* T2-AM fungi T3- AM fungi+ *Rhizobium*

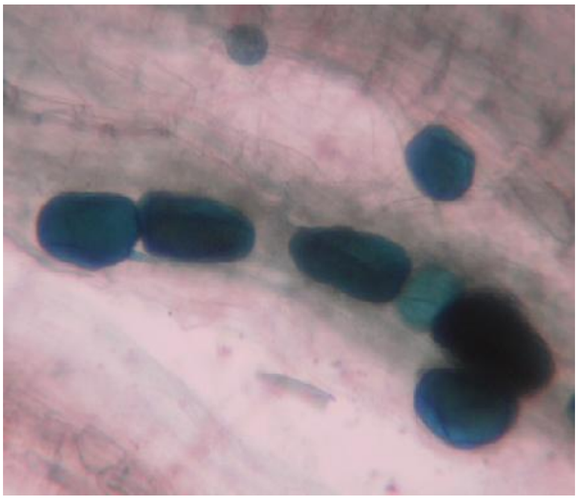
(a)

(**b**)

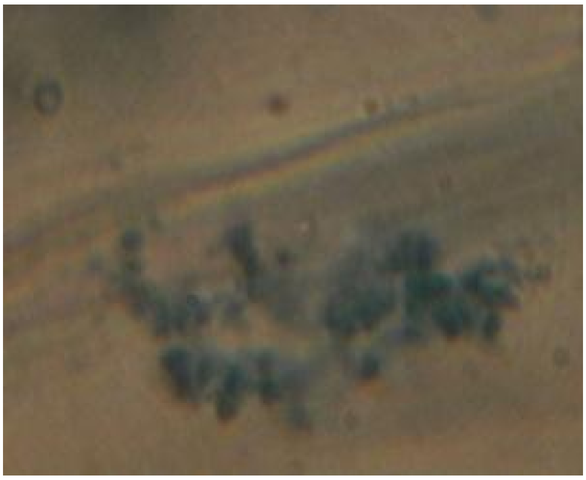
Plate: 2 Interaction efect of AM fungi & *Rhizobium* and drought stress on chickpea plant (a and b)

root length (45.6 cm), shoot length (12.2 cm), dry weight of root (0.4 g) and shoot (1.8 g), total number of nodules (39.6 nos.), dry weight of nodules (0.5 g), percentage of mycorrhizal infection (96.6%), chlorophyll *a* (0.83 mg/g fr.wt), chlorophyll *b* (1.19 mg/g fr.wt) and total chlorophyll (2.24 mg/g fr.wt) in dual inoculated (AM Fungi and *Rhizobium*) plants than plants with individual inoculation. The number of root nodules was higher in *Rhizobium* with AM fungi treated chickpea (15±2.0 in numbers.) plants than the AM fungi and *Rhizobium* alone treated and control plants. The biofertilizers (*Rhizobium* and AM fungi) inoculated legumes showed normal growth period under drought stress. The wide spread presence of the AM symbiosis in nodulated legumes and the role of AM fungi in improving nodulation and rhizobial activity within the nodules, are both universally recognized processes (Barea *et al*., 2005b). Colonization of a legume by AMF can increase the number of nodules (Giri and Mukerji, 2004; Rabie and Almadini, 2005; Garg and Manchanda, 2008). This may indicate a positive influence of AMF on legume-nitrogen fixing bacterial symbiosis. The present study revealed that the AM fungal colonization in roots of chickpea was maximum as 95.20±1.92 in *Rhizobium* with AM fungi inoculated plants and was minimum (88.00±2.92) in AM fungi treated chickpea plants. Drought stress does not affect the percent of AM infection in both single and dual inoculation with *Rhizobium* treated plants. The presence of oval, round or irregularly lobed vesicles occurring between or inside cortical cells, attached to hyphae and containing oil globule was a sign of AM fungal infection in chickpea plant roots.

These vesicles were act as storage structures. The presence of arbuscules in the infected roots are intended to serve as two way channels for transport of nutrients, more particularly carbohydrates structures known as appressoria connect AM fungal ramifications inside roots with the mycelium of the fungus outside the root and serve as absorbing elements from soil to roots (Plate:3). In the present study, the AM fungi either alone or in combination with *Rhizobium*, caused about significant ($P<0.05$) difference in chlorophyll *a*, *b* and total chlorophyll content (73.60±0.30µg; 34.40±0.37µg and 104.00±0.50µg) was noticed at 50th day period of chickpea. But these contents were reduced during drought and salt stress condition in all plants. However on rewatering, the biofertilizers inoculated legumes recovered fast in comparison with the control plants (Fig: 1). Such results were previously reported by Ommen *et al*., 1999; and Manivannan *et al*., 2007. The decrease in chlorophyll under drought stress is mainly the result of damage to chloroplasts caused by active oxygen species (Simirnoff, 1995). The carotenoid contents of chickpea showed minimum increase in biofertilizers treated plants compared to control and was observed maximum during drought stress period.



(a)

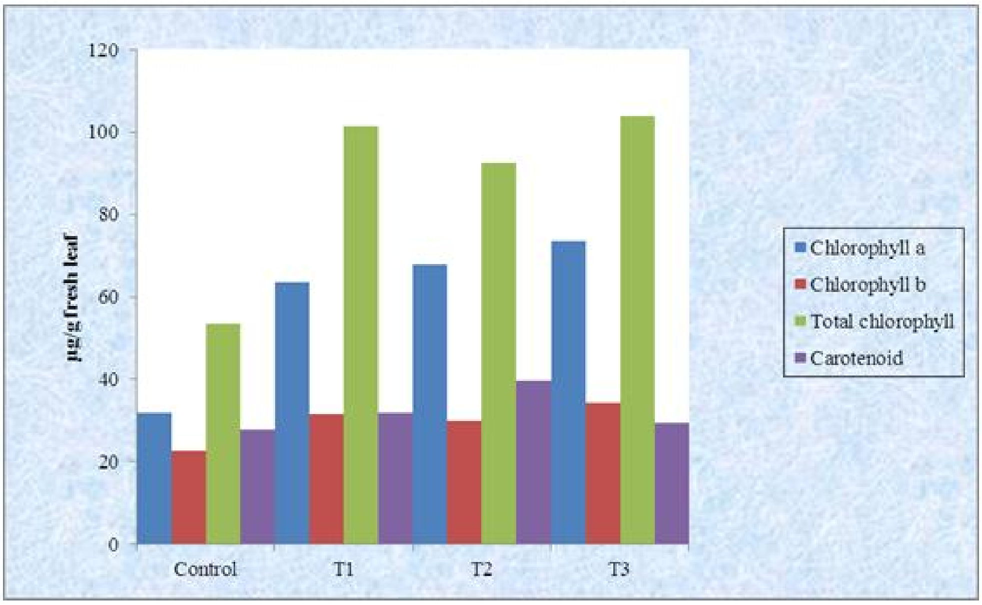


(b)

Plate 3. Vesicular and arbuscular mycorrhizal infections in root tissues of chickpea (a and b)

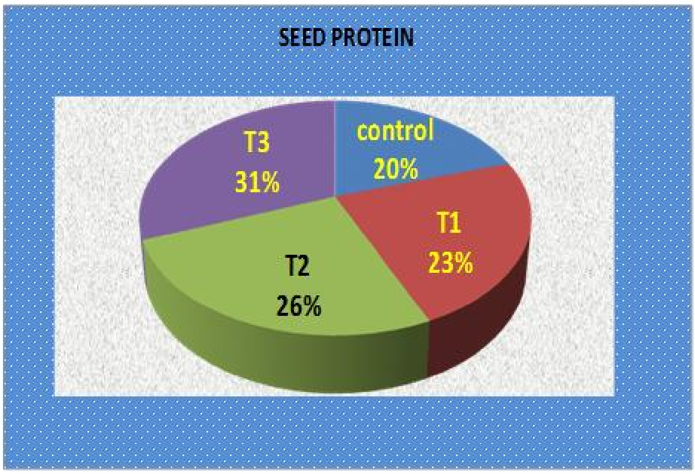
During recovery period, the carotenoid content declined progressively in both control and biofertilizers inoculated drought stressed plants (Fig: 1). Plants can partly protect themselves against mild drought stress by accumulating osmolytes. Proline is one of the most common compatible osmolytes in drought stressed plants. For, example, the proline content increased under drought stress in Pea (Sanchez *et al.*, 1998; Alexieva *et al*., 2001). Proline accumulation might also be a part of the stress signal influencing adaptive responses (Maggio *et al*., 2002). Its metabolism in plants, however, has mainly been studied in response to osmotic stress (Verbruggen and Hermans, 2008). It induced accumulation of soluble sugars and proline in two maize varieties was studied (Mohammadkhani and Heidari, 2008). Proline accumulation may also be part of the stress signal influencing adaptive responses (Maggiio *et al*., 2002). This accumulation has been advocated as a parameter of selection for stress tolerance (Yancy *et al*., 1982; Jaleel *et al*., 2007). The effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars was studied (Mafakheri *et al*., 2010).

In the present investigation the proline content in the control and biofertilizers inoculated chickpea plant was minimum at all stages of the plant. After drought stress period, its level declined sharply in both control and biofertilizers inoculated plants. This proline accumulation in both legume plants was stimulated by the biofertilizers under mild drought conditions. Further, the diffusion of proline after rehydration of legumes might be taken to indicate that proline served as a storage compound during stress (Fig: 3). The leaf protein content was increased gradually in chickpea of both control and biofertilizers treated plants as on plant age. This content was declined during the drought stress. After its recovery, it was observed maximum (8.82±0.07; 8.96±0.04 mg) as in dual inoculation of *Rhizobium* and AM fungi treated plants compared to control (3.68±0.04; 4.78±0.05 mg) plants. After harvesting, the seed protein content of chickpea was estimated and it was significantly higher (<31%) in *Rhizobium* and AM fungi treated Plant than the control (20%).



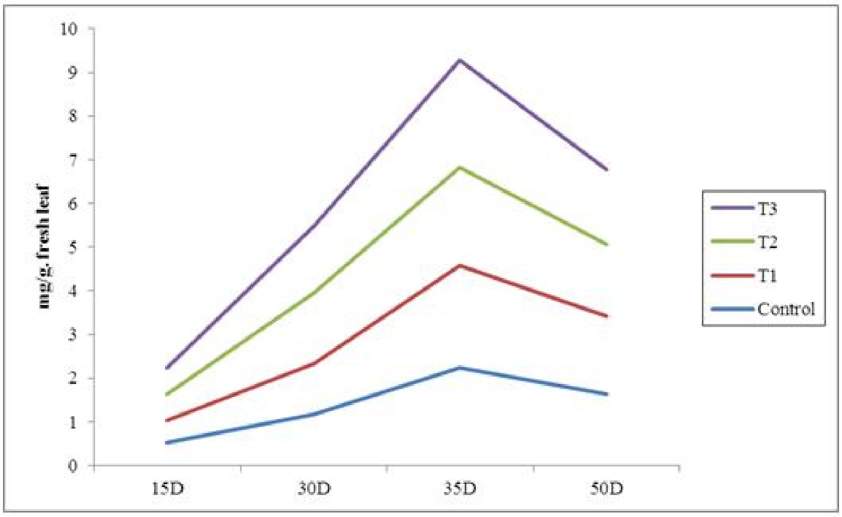
C- Control T1-*Rhizobium* T2-AM fungi T3- AM fungi+ *Rhizobium*

Fig. 1. Interaction effect of AM fungi & *Rhizobium* and drought stress on the chlorophyll



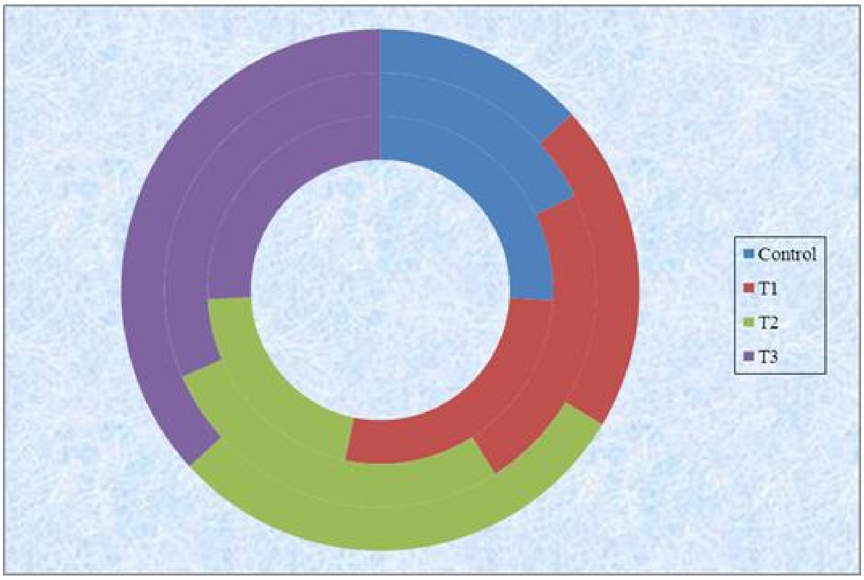
C- Control T1-*Rhizobium* T2-AM fungi T3- AM fungi+ *Rhizobium*

Fig. 2. Interaction effect of AM fungi and *Rhizobium* and drought stress on the seed protein content (µg/g seed) of chickpea



C- Control T1-*Rhizobium* T2-AM fungi T3- AM fungi+ *Rhizobium*

Fig. 3. Interaction effect of AM fungi & *Rhizobium* and drought stress on the proline content (mg/g. fresh leaaves) of chickpea



C- Control T1-*Rhizobium* T2-AM fungi T3- AM fungi+ *Rhizobium*

Fig. 4. Interaction effect of AM fungi & *Rhizobium* and drought stress on the nitrogen, phosphorus and potassium content (mg/g.dry weight) of chickpea

But in *Rhizobium* and AM fungi the protein content was 23% and 26% (Fig: 2). Generally, in legumes the AM fungi increased nodulation and nitrogen fixation as a consequence of improved phosphorus nutrition (Athar, 2005). Many researchers have reported enhancement of phosphate uptake and growth of leguminous plants by vesicular arbuscular mycorrhizal fungi (Ezawa *et al*.,2000; Arihara and Karasawa,2000; Meshram *et al*., 2000; Joner, 2000; Mayoral, 2000; Guriqbal *et al*., 2001; Mamtha *et al*., 2002; Atimanav and Adholeya, 2002). The present investigation revealed that the chickpea plant's total nitrogen, phosphorus and potassium contents were higher in biofertilizers inoculated plants than the control, at all stages of growth. Drought stress did not affect the nitrogen accumulation in *Rhizobium* and AM fungi at both individual and dual inoculated plants.

There was an increase in total phosphorus and potassium content was found in the mycorrhizal plants followed by *Rhizobium* inoculated plants even during the drought stress period (Fig: 4). Morte *et al*. (2000) have explained that mycorrhizal plants have accumulated more potassium in shoots and roots than control plants under both normal irrigation and drought – stress conditions. The main advantage of mycorrhiza is its greater soil exploration and increasing uptake of nitrogen, phosphorus, potassium, zinc, copper, sulphur, iron, calcium, magnesium and manganese supply to the host roots (Li *et al*., 1991; Champawat and Pathak, 1993; Marschner and Dell, 1994; Smith *et al*., 1994; Malik, 2000). The *Glomus etunicatum* inoculated maize plants in sandy loam soil, under water stressed conditions, absorbed more phosphorus than non-mycorrhizal plants (Muller and Hofwer, 1991). To the well-known positive impacts of AM fungi on plant yields such as a better survival rate of colonized plants, the maintenance of plant biodiversity, the improvement of soil microflora (Boer *et al*., 2005), the resistance to biotic (BØdker *et al*., 2002; Dalpe, 2005) and abiotic environmental stresses (Evelin *et al*., 2009; Neumann and George, 2009), the improvement of soil structure and reduction of pesticide use (Strack *et al*., 2003).

Conclusion

Arbuscular mycorrhizal fungi are ubiquitous in soil habitats and form beneficial symbiosis with the roots of angiosperms and other plants. Most terrestrial plants associate with root colonizing mycorrhizal fungi, which improve the fitness of both the fungal and plant associates. *Rhizobium* is a symbiotic nitrogen fixing bacteria applied as biofertilizers in agricultural crop improvement and management. They played a major role in the plant metabolism and crop productivity. The present study experimentally reported that the dual inoculation of AM fungi and *Rhizobium* could help the growth and yield of chickpea plants. The results from this study showed that AM fungal symbiosis could protect such legume plants against the mild drought stress. It could be confirmed that the mycorrhizal plants were resistant to mild drought stress than control plants. Crop plants with dual symbiotic association posses both nutritional and ecological advantages to compensate nutrient deficient situation, and the establishment of these association can be improved by inoculation with its mutualistic partners *Rhizobium* and arbuscular mycorrhizal fungi. This study suggested the both biofertilizer (AM fungi and *Rhizobium*) application in agricultural fields of economically important crops can yield higher quantity and quality of seeds and also these plants showed mild drought tolerance when compare to non-biofertilizer inoculated crops. They were considered as environment friendly fertilizers and do not cause any kind of pollution for a generation to come.

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