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GROWTH AND YIELD RESPONSE OF CHICKPEA TO CO-INOCULATION WITH *MESORHIZOBIUM CICERI* AND *BACILLUS MEGATERIUM*

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ABSTRACT

Among rhizosphere microflora, a subset is beneficial to plant growth through N_2-fixation, P-solubilization and hormone production. A pot experiment was conducted to evaluate the effect of co-inoculation with *Mesorhizobium ciceri* and *Bacillus megaterium* on growth and yield of chickpea at different fertilizer levels (0-15, 15-30, 30-60 kg NP ha^{-1}). Results revealed that *Mesorhizobium* and *Bacillus* significantly increased the yield of chickpea yield as compared to control. However, co-inoculation of *M. ciceri* and *B. megaterium* further enhanced pod yield in comparison with uninoculated controls. Co-inoculation produced 25.77 g pot^{-1} pod and 29.07 g pot^{-1} straw yield whereas rhizobial inoculation produced 24.77 g pot^{-1} pod yield and 28.57 g pot^{-1} straw yield at full fertilizer level. Similarly, co-inoculation produced higher root fresh weight (181.4 g), root length (58.4 cm) and number of nodules per pot (61) as compared to uninoculated (94.7 g, 52.1 cm and 14) control. *Mesorhizobium* and *Bacillus* inoculation also increased the NP contents in chickpea straw and grain but this effect was more with co-inoculation. Inoculation significantly enhanced post harvest soil N and P. Results suggested that co-inoculation (*M. ciceri* and *B. megaterium*) could be an effective approach at recommended NP fertilizer than single strain inoculation; however, more extensive field studies are needed in different ecologies to reinforce this approach.

Keywords: Co-inoculation, *Mesorhizobium*, *Bacillus*, nodulation, yield, chickpea

INTRODUCTION

World population has been doubled during the last five decades i.e. from 1960's and a synchronized doubling of food production (Vance, 2001). Synthetic fertilizers helped in boosting crop yields to nourish the increasing population of the world. Use of mineral nitrogen has amplified more or less nine times and P-fertilizers more than four times (Vance, 2001). Use of microbial inoculants to prevail over the ecological problems resulting from the loss of plant nutrients and enhancing nutrient use efficiency / nutrient availability can provide sustainable solution for agriculture system. Legume inoculation is an old practice that has been adopted for more than a century in agricultural systems (Brockwell and Bottomley, 1995).

Legume-*rhizobium* symbiosis depends on the specificity of plant and bacterial species because of chemical signaling that resulted in formation of specialized structures i.e. nodules in which the bacteria are hosted and reduced atmospheric nitrogen into ammonium (Rao and Cooper, 1994; Bai *et al*., 2002). It is established and studied fact that world supply of organic nitrogen is met via the symbiosis between root nodulating bacteria and leguminous host plants (Postgate, 1998).

Nitrogen and phosphorus are the most limiting nutrients for plant growth (Schachtman *et al*., 1998). However, soil may contain enormous amounts of nutrients but mostly they are unavailable to plants. Almost 75–90% of added P-fertilizer is precipitated by metal cation complexes (Stevenson, 1986) like calcium (Lindsay *et al*., 1989) in calcareous soils like Pakistan (Hinsinger, 2001). Further, it has also been speculated that the amount of total phosphorus has been increased to such an extent in arable soils that are sufficient to sustain utmost crop yields worldwide for about 100 years (Goldstein, 1986; Goldstein *et al*., 1993). This situation has certainly brought the subject of mineral phosphate solubilization to the vanguard and reliance on costly mineral fertilizers has been lessened in future.

Plant growth promoting rhizobacteria (PGPR) are responsible to mediate the soil processes such as decomposition, nutrient mobilization, mineralization, solubilization, nitrogen fixation and growth hormone production (Dobbelaere *et al*., 2003; Khan *et al*., 2003). PGPR having the P-solubilizing capacity are called as phosphate solubilizing microorganisms (PSM) or phosphate solubilizing bacteria (PSB) have been reported to increase P-concentration by converting insoluble forms to soluble ones through the production of organic acids (Maliha *et al*., 2004) and hence increased the crop yields (Zaidi 1999; Gull *et al*., 2004). Inoculation of soil with P-solubilizing bacteria is a promising approach that may alleviate the deficiency of phosphorus (Cakmakci, 2005). This bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species and nutritional status of soil (Hoflich *et al*., 1995). Species of the genus *Bacillus*, *Pseudomonas*, *Aspergillus* and *Penicillium* have been identified by many workers as P-solubilizers (Seshadri *et al*., 2004; Wakelin *et al*., 2004).

Co-inoculation with P-solubilizing bacteria and *Rhizobium* stimulated plant growth more profoundly than their separate inoculations (Perveen *et al*., 2002; Zaidi *et al*., 2003). Positive interaction of *Rhizobium* with P-solubilizing sp. of *Bacillus* has translated into significant yield increases of legumes (Toro *et al*., 1998).

Present study was designed to evaluate the co-inoculation effect of N_2-fixing and P-solubilizing bacteria (*Mesorhizobium* and *Bacillus* sp.) on growth and yield of chickpea.

MATERIALS AND METHODS

Isolation of Mesorhizobium and Bacillus: Chickpea (*Cicer arietinum* L.) nodulated roots were collected from the research area of Soil Bacteriology Section, Ayub Agricultural Research Institute (AARI), Faisalabad. Roots were washed gently with tap water to remove the soil; nodules were separated and placed in Petri-plates. The nodules were surface-sterilized by dipping momentarily in 95% ethanol followed by 0.2% $HgCl_2$ solution for 3-5 minutes and 5-6 washings with sterilized water (Russell *et al*., 1982). The nodules were crushed in a minimal volume of sterilized water with the help of a sterilized glass rod to obtain a suspension. The suspension with the help of an inoculating needle was streaked out on congo red yeast extract mannitol agar medium (Vincent, 1970). The prolific single colonies were picked and re-streaked on fresh prepared plates to obtain pure cultures. The purified rhizobial cultures were stored at 5 ± 1^{o}C on slants and maintained for further experimentation.

Bacillus was isolated by dilution plate technique from the rhizosphere soil of chickpea growing at Soil Bacteriology Section AARI, Faisalabad. For the isolation of *Bacillus,* rhizosphere soil suspension was subjected to heat shock at 80^{o}C for 30 minutes in an oven (Claus, 1964) and on cooling inoculated the selective medium (Nautiyal, 1999). Plates carrying selective medium were incubated at 28 ± 2^{o}C for seven days. The growth of *Bacillus* was purified and screened out on the selected medium. From each plate, the growth was picked and sub-cultured frequently to get a pure culture. After preliminary screening, standard methods [Gram (+), Catalase (+), starch hydrolysis (-) and citrate utilization (+)] as outlined in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and host plant infectivity leaded to predict the isolates as *Mezorhizobium ciceri* and *Bacillus megaterium*.

Determination of auxin biosynthesis: Chickpea *Rhizobium* (*M. ciceri*) and *Bacillus* isolates (four of each) coded as "Rh_1, Rh_2, Rh_3 and Rh_4 and B_1, B_2, B_3 and B_4" were screened for their auxin biosynthesis potential. The isolates of chickpea *Rhizobium* were cultured on the YMA broth for 72 hours and *Bacillus* on Pikovskaya's broth. The auxin biosynthesis potential was determined as Indole-3-acetic acid (IAA) equivalents using Salkowski's reagent (2 mL of 0.5M $FeCl_3$ +98 mL of 35% $HClO_4$) as described by Sarwar *et al*. (1992). *Mesorhizobium ciceri* and *Bacillus* isolates showing the highest auxin biosynthesis were selected for the study.

Phosphate solubilization of isolates: The solubilization capacity of *Bacillus* isolates B_1, B_2, B_3 and B_4 were checked on the Pikovskaya's medium containing (g L^{-1}): glucose 10, Ca_3 $(PO_4)_2$ 5.0, $(NH_4)_2SO_4$ 0.5, NaCl 0.2, $MgSO_4$. $7H_2O$ 0.1, KCl 0.2, yeast extract 0.5, $MnSO_4.H_2O$ 0.002, and $FeSO_4$. $7H_2O$ 0.002, agar 17 and the pH was adjusted to 7 before autoclaving (Pikovskaya, 1948). These four isolates were capable to solubilize insoluble phosphates in the Pikovskaya's medium by forming the halos. The growth and solubilization diameter were determined after incubation at 28 ± 2 ^{o}C for seven days. On the bases of diameter of clearing halo zones, solubilzation efficiency (SE) and solubilization index (SI) (Gaur, 1990; Nguyen *et al*., 1992; Vazquez *et al*., 2000) were calculated using the following formulae.

$$SE = \frac{\text{solubilization diameter}}{\text{Growth diameter}} \times 100$$

$$SI = \frac{\text{colony diameter + halozone diameter}}{\text{Colony diameter}}$$

Auxin biosynthesis of *Mesorhizobium* ranged from 13.9-20.8 ppm whereas *Bacillus* isolates from 2.1-3.0 ppm. Isolates Rh_3 and B_3 showed highest biosynthesis potential and phosphate solubilization were selected for experimentation.

Inoculum preparation: Inoculum of chickpea *Rhizobium* was prepared in yeast extract mannitol (YEM) medium and *Bacillus* in selective medium (Nautiyal, 1999). Both the media were inoculated in 500 mL conical flasks containing 150 mL medium and incubated at 28 ± 2 ^{o}C under shaking at 100 rpm for three days to give an optical density of 0.5. Peat as carrier was sterilized at 121 ^{o}C and 15 psi pressure for one hour and inoculated with broth cultures of *M. ciceri* and *B. megaterium* in 1:1 by volume of the same OD (Seed-slurry ratio (1.25-1.00) was used for inoculation whereas slurry was prepared by mixing 30 mL pure culture (10^7-10^8 CFU mL^{-1}) with 15 mL sugar 10% sugar solution in 50 g carrier.

Pot Experiment: Pot study was conducted in medium textured soil having pH 7.5, EC 1.6 d Sm^{-1}, N 0.029% and available P 9.6 mg kg^{-1} at Soil Bacteriology Section, AARI, Faisalabad. Three fertilizer (NP) levels viz. 0-15, 15-30 and 30-60 kg NP ha^{-1} were applied before sowing as per treatment i.e. un-inoculated, *Mesorhizobium ciceri*, *Bacillus megaterium* and combination of *Mesorhizobium ciceri* and *Bacillus megaterium* (1:1). The experiment was laid out in completely randomized design (CRD) having four replications and twelve treatment (Table 2).

At flowering, plants were up-rooted for determination of number of nodule and nodular mass, root length and root mass were checked. Data regarding pod yield, plant dry matter, N P-content in plant and grains and post harvest soil N and available P were recorded. Nitrogen was determined according to Kjeldhal method (Bremner and Mulvany, 1982) while phosphorus by modified Olsen method (Olsen and Sommers, 1982). Data were subjected to statistical analysis by following CRD using standard procedures (Steel *et al*., 1997). The differences among the treatment means were compared by applying the Duncan's multiple range tests (DMR) (Duncan, 1955).

RESULTS AND DISCUSSION

Yield parameters and grain NP-content: Co-inoculation of chickpea with *M. ciceri* and *B. megaterium* significantly increased the pod and straw yield (Table 2) while the effect was more prominent when they were used in combination as compared to uninoculated control. Highest pod yield was produced with co-inoculation treatment (25.77 g pot^{-1}) followed by rhizobial inoculation at 30-60 kg NP ha^{-1}. Similarly, co-inoculation produced higher straw 29.07 g pot^{-1} followed by rhizobial inoculation i.e. 28.57 g pot^{-1} as compared to control i.e. 26.7 g pot^{-1}. Percent increase in pod yield by co-inoculation was 20.1, 18.3 and 10.5% while with rhizobial inoculation alone 11.8, 13.6 and 6.17% at fertilizer levels 0-15, 15-30, 30-60 kg NP ha^{-1}, respectively. Like wise increase in straw yield with co-inoculation was 15.5, 11.2 and 8.75% while with rhizobial inoculation alone 10.6, 6.85 and 6.88 % at all fertilizer levels, respectively.

Data regarding NP-content in grains are presented in Table 2. Co-inoculation produced highest N-content in chickpea grains i.e. 4.32, 4.36 and 4.41% at fertilizer levels 0-15, 15-30 and 30-60 kg NP ha^{-1}, respectively that differed non-significantly from rhizobial inoculation alone. Similarly, the highest P-content was observed with co-inoculation (0.40%) followed by rhizobial inoculation at higher NP-level.

Plant and Soil NP content: Data regarding NP-content in chickpea straw, post harvest soil N and available P are summarized in Table 3. Rhizobial inoculation yielded highest N-content in chickpea straw (1.95%) followed by co-inoculation at recommended fertilizer level (30-60 kg NP ha^{-1}). *Bacillus* inoculation also enhanced the level of N in chickpea straw compared to uninoculated control. The highest P-content was observed in co-inoculation (0.34%) followed by *Bacillus* inoculation (0.33%) at full NP level.

Inoculations alone (*Rhizobium* or *Bacillus*) or in combination produced higher soil N and available P at all levels as compared to control (Table 3). The highest soil N was observed in case of co-inoculation i.e. 0.044% followed by *mesorhizobium* at 30-60 kg NP ha^{-1}. However, maximum increase in soil N (20.4% more over un-inoculated control) was recorded by co-inoculation followed by rhizobial inoculation alone at 30-60 kg NP ha^{-1}. Co-inoculation exhibited maximum available P (13.72 mg kg^{-1}) that differed non-significantly from *Bacillus* inoculation at recommended fertilizer level.

Root parameters: Root length, root mass, number of nodules per pot and nodular mass as affected by bacterial inoculations are presented in Figure 1, 2, 3 and 4. Inoculations either alone or in combination enhanced the root length and mass as compared to non-inoculated control. Maximum root length and mass were obtained in combined inoculation treatment i.e. 66.0 cm and 188.4 g pot^{-1} followed by rhizobial inoculation alone at full fertilizer level (Figure 1 and 2).

Co-inoculation showed increase (3.6, 4.7 and 4.3 fold) in nodule number pot^{-1} whereas rhizobial inoculation gave 2.6, 4.2 and 3.4 fold increase at fertilizer levels 0-15, 15-30 and 30-60 kg NP ha^{-1} over un-inoculated control, respectively. Similarly, increase in nodular mass by co-inoculation was 1.6, 1.5 and 1.57 fold at all fertilizer levels, respectively as compared to uninoculated controls. Free living *Bacillus* also enhanced nodule number per pot and nodular mass alone at all fertilizer levels.

In addition to nitrogen fixation, synthesis of plant growth regulators (PGRs) (auxins) by *Rhizobium* sp. is considered among plausible mechanisms in promoting legume plant growth (Zahir et al., 2004; Mirza *et al*., 2007). Combined use of microorganisms having P-solubilizing capacity and producing PGR's is gaining importance as an effective approach for enhancing yield of crops (Zaidi *et al*., 2003; Zaidi *et al*., 2004).

Mesorhizobium and *Bacillus* sp. were isolated from chickpea nodules and the rhizosphere, respectively. Isolates were characterized for their auxin biosynthesis in the absence of precursor. In laboratory study, all the isolates produced auxin (expressed as IAA equivalents) but with variable degree (see Table 1). Microbial production of auxins and their role in plant growth promotion have been reported and reviewed by many researchers (Sarwar *et al*., 1992; Sarwar and Kremer, 1995; Frankenberger and Arshad, 1995; Zahir *et al*., 2004).

Strains of *M. ciceri* and *Bacillus* were evaluated for their growth promotion at different N-P levels viz. 0-15, 15-30 and 30-60 kg ha^{-1} under wire house conditions. Fertilizer levels were also tested separately. In present study, significant increases in root growth, nodulation, yield and nutrient uptake were observed when both the *Mesorhizobium* and *Bacillus* inoculation were combined with fertilizer levels over un-inoculated control. However, *Mesorhizobium* inoculation proved to be more effective in improving growth and yield of chickpea compared with *Bacillus* inoculation at full NP level. The positive effects of inoculum on plant growth and development observed in case of *Mesorhizobium* in this study were found fertilizer rate–dependent. These findings are supported by the work of previous researchers who elucidated the effect of bacterial sp. on the growth and development of various legumes (Zaidi, 1999; Zaidi *et al*., 2003; Rajasekhar and Reddy, 2000; Mirza *et al*., 2007). Huang and Erickson (2007) treated legume seeds with *Rhizobium* spp. and observed improved seedling growth, nodulation and root-shoot biomass. Similarly, Yuming *et al*. (2003) reported the synergistic effect of *Bacillus* inoculation on crop plants.

Table 1. Some important features of isolates tested during the investigation

Isolates	IAA equivalents (µg mL^{-1})	Solubilization Efficiency (SE)	Solubilization Index (SI)
Rh_1	14.4	-	-
Rh_2	13.9	-	-
Rh_3	20.8	-	-
Rh_4	16.9	-	-
BS_1	2.1	260.0	3.6
BS_2	2.6	233.3	3.3
BS_3	3.1	266.7	3.7
BS_4	2.4	241.7	3.4

In the present study, combined inoculation of *Mesorhizobium* and PGPR (*Bacillus* sp.) was more pronounced and further increased the yield and nodulation of chickpea at full dose of NP fertilizers compared to uninoculated control. Reason behind the improvement in number of nodules and consequently biomass and yield due to combined inoculation might be the increase in root length and growth, thus providing more number of active sites/niches for nodulation by the rhizobial strains. Increase in nodulation and yield components of legume crops following inoculation with N_2-fixing and P-solubilizing microbes have also reported by other researcher (Garcia *et al*., 2004; Gupta, 2004). Results of this study contradicted with the findings of Paul and Verma (1999) who observed increased nodule number and mass due to free-living diazotrophic inoculation but found decreased with co-inoculation. Dashti *et al*. (1998) and Dubey (1996) reported that increased nodule number and weight in two soybean cultivars as a result of co-inoculation with *B. japonicum* and PGPR as compared to inoculation of the *B. japonicum* alone.

Table 2. Co-inoculation effect on yield and grain NP-content

Treatments	Pod Yield (g pot^{-1})	Straw Yield (g pot^{-1})	Grain N-content (%)	Grain P-content (%)
T_1: 0-15 kg NP ha^{-1}	16.57^{j}	22.17^{h}	4.077^{g}	0.30^{h}
T_2: 15-30 kg NP ha^{-1}	19.13^{g}	24.80^{f}	4.170^{ef}	0.32^{g}
T_3: 30-60 kg NP ha^{-1}	23.33^{c}	26.73^{d}	4.247^{cd}	0.34^{ef}
T_4: T_1+ Rhizobial inoculation	18.53^{h}	24.53^{f}	4.207^{de}	0.33^{fg}
T_5: T_2+ Rhizobial inoculation	21.73^{e}	26.50^{d}	4.260^{c}	0.35^{def}
T_6: T_3+ Rhizobial inoculation	24.77^{b}	28.57^{b}	4.357^{b}	0.37^{bc}
T_7: T_1+ *Bacillus* inoculation	17.90^{i}	23.90^{g}	4.137^{f}	0.33^{fg}
T_8: T_2+ *Bacillus* inoculation	19.63^{f}	24.80^{f}	4.243^{cd}	0.36^{cde}
T_9: T_3+ *Bacillus* inoculation	21.30^{e}	26.40^{d}	4.337^{b}	0.38^{b}
T_{10}: T_1+ Co-inoculation†	19.90^{f}	25.60^{e}	4.317^{b}	0.36^{bcd}
T_{11}: T_2+ Co-inoculation	22.63^{d}	27.57^{c}	4.360^{ab}	0.37^{bc}
T_{12}: T_3+ Co-inoculation	25.77^{a}	29.07^{a}	4.410^{a}	0.40^{a}
LSD:	0.474	0.409	0.0533	0.0169

†*Rhizobium* + *Bacillus* inoculation in 1:1 v/v of the same OD
*Means sharing similar letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test

Co-inoculation also improved the plant and grain N, P concentrations in present study compared with uninoculated control. *Bacillus* (with highest SE and SI capability) along with *Mesorhizobium* exhibiting higher N and P contents in plants and grains might be due to increased nutrient concentration in the root zones of plants. Plants might also be owed to proliferated roots and better microbe plant interaction in the rhizosphere. Yuming *et al*. (2003) reported that interaction of auxin producing microbes increased the root length and mass thus enhanced the NP concentration in plants. Similarly, Khan *et al*. (2006) reported that dual inoculation of N_2-fixing *Rhizobium* and P-solubilizing *Bacillus* upshot more soil N and the available P by lowering the soil pH and producing organic acids.

Present study clearly depicted that co-inoculation effect of *M. ciceri* and *B. megaterium* influenced positively the growth and yield of chickpea.

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Inoculation with either microbe enhanced the yield and nutrient contents in chickpea but their interactive effect was more prominent. More extensive and exhaustive field studies must be carried out to reinforce this approach.

Table 3. Co-inoculation effect on Plant NP-content and post harvest soil analysis

Treatments	Plant N-content (%)	Plant P-content (%)	At Harvest N-content (%)	At Harvest Avail. P ($mg\ kg^{-1}$)
T_1: 0-15 kg NP ha^{-1}	1.59^{i*}	0.25^{f}	0.0333^{e}	9.52^{e}
T_2: 15-30 kg NP ha^{-1}	1.62^{hi}	0.27^{ef}	0.0343^{e}	10.57^{de}
T_3: 30-60 kg NP ha^{-1}	1.66^{gh}	0.30^{cd}	0.0363^{de}	11.62^{cd}
T_4: T_1 + Rhizobial inoculation	1.87^{b}	0.27^{ef}	0.0360^{de}	10.04^{e}
T_5: T_2 + Rhizobial inoculation	1.91^{ab}	0.30^{cd}	0.0397^{abcd}	11.62^{cd}
T_6: T_3 + Rhizobial inoculation	1.95^{a}	0.32^{abc}	0.0423^{ab}	13.20^{ab}
T_7: T_1 + *Bacillus* inoculation	1.68^{fg}	0.29^{de}	0.0343^{e}	11.62^{cd}
T_8: T_2 + *Bacillus* inoculation	1.70^{efg}	0.30^{bcd}	0.0353^{de}	12.15^{bc}
T_9: T_3 + *Bacillus* inoculation	1.71^{ef}	0.33^{ab}	0.0370^{cde}	13.72^{a}
T_{10}: T_1 + Co-inoculation†	1.74^{de}	0.32^{abc}	0.0383^{bcde}	12.15^{bc}
T_{11}: T_2 + Co-inoculation	1.78^{cd}	0.33^{ab}	0.0417^{abc}	13.20^{ab}
T_{12}: T_3 + Co-inoculation	1.80^{c}	0.34^{a}	0.0437^{a}	13.72^{a}
LSD:	0.0533	0.0238	0.005	1.172

†*Rhizobium* + *Bacillus* inoculation in 1:1 v/v of the same OD
*Means sharing similar letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test

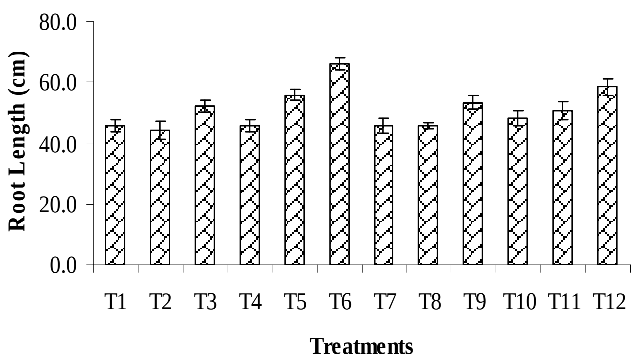

Figure 1. Co-inoculation effect on chickpea root length.

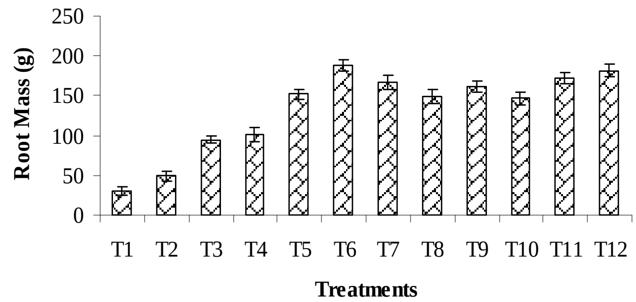

Figure 2. Co-inoculation effect on chickpea root mass.

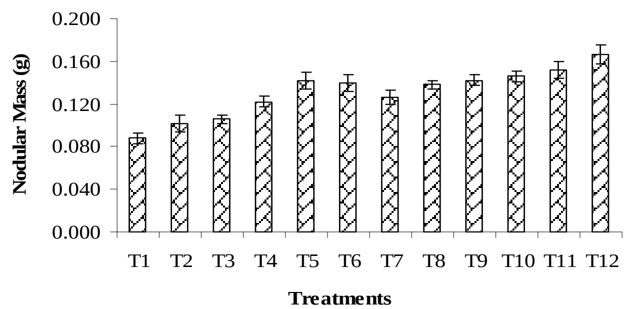

Figure 3. Co-inoculation effect on chickpea nodular mass.

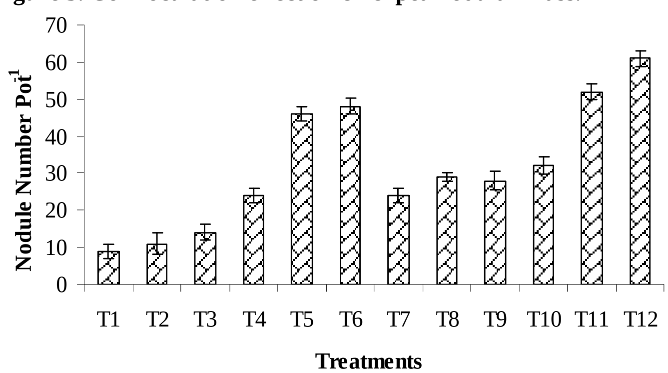

Figure 4. Co-inoculation effect on chickpea nodule number per pot.

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