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# Co-inoculation with *Mesorhizobium ciceri* and *Azotobacter chroococcum* for improving growth, nodulation and yield of chickpea (*Cicer arietinum* L.)

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## Abstract

*Rhizobia have the exceptional ability to form nodules on roots or stems of leguminous plants. Free living diazotrophs promote the rhizobial efficiency by altering root architecture providing more niches for nodulation and thus enhance the $N_2$-fixing ability of legumes. Field experiment was conducted to assess the co-inoculation potential of symbiotic i.e. Mesorhizobium ciceri and non-symbiotic diazotrophs i.e. Azotobacter chroococcum on the yield of chickpea. Chickpea seeds (cv. Bittle-98) were inoculated with peat-based inocula and sown following randomized complete block design with three replications. Two levels of nitrogen i.e. 30 (recommended) and 15 kg $ha^{-1}$ were applied as urea while P was applied at 60 kg $ha^{-1}$ to all the treatments as single super phosphate. Results revealed that introduction of A. chroococcum had positive impact on chickpea with and without rhizobial inoculation and the effect was more prominent when applied in combination as compared to non-inoculated control at low nitrogen level. It was observed that inoculation with M. ciceri or A. chroococcum produced significant increase in biomass and grain yield but the response was more pronounced with co-inoculation i.e. 3456 and 1772 kg $ha^{-1}$, respectively, as compared to control (2903 and 1489 kg $ha^{-1}$, respectively) at 15 kg N $ha^{-1}$. Higher nodule number $plant^{-1}$ and nodular mass was observed with co-inoculation (42 and 0.252 g $plant^{-1}$). Percent N and P content in chickpea plant were higher in the co-inoculated treatments (1.683 and 0.283%) than that of their respective controls. Similar trend was observed in grains except the rhizobial inoculation alone which produced higher N content (3.62%) than co-inoculation (3.59%). Percent N and available P in soil were also higher in the inoculated treatments. The results imply that co-inoculation with Mesorhizobium and Azotobacter could be a useful approach for improving growth, nodulation and yield of chickpea by reducing dependence on chemical fertilizers and saving ~ 50% of recommended N fertilizer. However, more comprehensive and detailed studies in different ecological zones on the farmer's field for cost effective crop production should be carried out to confirm this approach.*

**Key words:** *Mesorhizobium ciceri, Azotobacter chroococcum,* co-inoculation, nodulation, yield, chickpea

## Introduction

The commercial man-made fertilizers have played decisive role in enhancing crop yields to feed the rising population of the world. However, increasing prices of fertilizers have limited the use consequently resulting in lesser yield to feed the burgeoning population. Atmosphere embraces 79% nitrogen which is unavailable to plants. Microbial inoculants have the potential to convert this unavailable nitrogen to available form. Thus, to compensate the costly fertilizers, microbial inoculants can reduce the expense by means of biological $N_2$-fixation. *Rhizobium is* the most extensively explored microbe with $N_2$-fixing capacity on roots of more than 20, 000 species of family *Fabaceae* (Spaink *et al*., 1998). Legume inoculation with *Rhizobium* is an aged practice that has been carried out for more than a century in agricultural systems.

*Azotobacter chroococcum*, a free-living diazotroph has also been reported to produce beneficial effects on crop yield through a variety of mechanisms including biosynthesis of biologically active substances, stimulation of rhizospheric microbes, modification of nutrient uptake and ultimately boosting biological nitrogen fixation (Lakshmann, 2000; Paul *et al*., 2002; Somers *et al*., 2004).

Co-inoculation of legumes with symbiotic and free living microbes like *Azotobacter*, *Azospirillum* and *Acetobacter* has received great attention in recent years (Dashti *et al.*, 1998). Free-living diazotrophs increase the lateral roots and root hair density resulting in more infection sites for rhizobia, thus enhancing the $N_2$-fixing ability of legumes (Parmar and Dadarwal, 1999).

Chickpea is the third most important legume with high protein (25-28%) and carbohydrate (57-60%) (Hulse, 1991). In Pakistan, chickpea has been reported the largest grown-legume that responds variably to inoculation (Aslam *et al*., 2000). Inconsistent response to inoculation has sometimes been attributed to the variation in bacterial

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number and competition with ineffective native population (Keatinge *et al*., 1995). Chickpea is mainly grown as a rain-fed crop, and generally on soils deprived of nutrients, hence the nutrient acquisition in chickpea must be efficient. Nutrient deficiencies in chickpea have been reported to cause yield losses of varying magnitude e.g. around 10% due to poor nodulation and hence nitrogen deficiency and 29-45% due to phosphorus (Ali *et al*., 2002).

Present study aimed to assess the co-inoculation potential of *A. chroococcum* and *M. ciceri* for improving growth and yield of chickpea at two levels of nitrogenous fertilizer.

## Materials and Methods

### *Isolation of Rhizobium and Azotobacter*

Chickpea (*Cicer arietinum* L.) root samples were collected from the Pulses Research Institute, AARI, Faisalabad. Roots were washed gently with tap water to remove the soil and nodules were separated and placed in Petri-plates. The collected nodules were surface-sterilized by momentarily dipping in 95% ethanol solution followed by dipping in 0.2% $HgCl_2$ solution for 3-5 minutes and 5-6 washings with sterilized distilled water (Russell *et al*., 1982). The surface sterilized nodules were crushed in minimal sterilized water with the help of a sterilized glass rod to get a milky suspension. The suspension was streaked out on Congo red yeast extract mannitol agar medium (CRYEMA) with the help of inoculating needle (Vincent, 1970). The rhizobial growth that did not attain the color of Congo red were picked and re-streaked persistently to obtain pure cultures. The purified rhizobial cultures were stored at 4 ± 2 $^{o}$C on slants and maintained for further experimentation.

*Azotobacter* was isolated from the rhizosphere soil of chickpea growing in the permanent layout plot at Soil Bacteriology Section, AARI, Faisalabad by preparing serial dilutions, purified and screened on Jensen agar medium (Jensen, 1953). The plates carrying Jensen agar medium (JAM) were incubated at 28 ± 2 $^{o}$C for 48 hours. The growth of *Azotobacter* was picked from each plate for purification to get pure culture. For identification, presumptive tests were carried out following standard methods as outlined in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). The pure culture was identified as *Azotobacter chroococcum*.

*M. ciceri* and *Azotobacter* isolates (four of each) were screened for their potential of auxin biosynthesis. The isolates of *M. ciceri* were grown in the (YMB) broth for 72 hours at 28 ± 2 $^{o}$C while *Azotobacter* on Jensen's broth. The auxin biosynthesis potential was determined as indole-3-acetic acid (IAA) equivalents using Salkowski's reagent as reported by Sarwar *et al*. (1992). Auxin biosynthesis of *M. ciceri* varied from 15-19 µg mL$^{-1}$ while *Azotobacter* produced 2.23-3.85 µg mL$^{-1}$ IAA equivalents. *Mezorhizobium* and *Azotobacter* isolates having the highest auxin biosynthesis potential were selected for field experiment.

### *Inoculum preparation*

Inoculum of *M. ciceri* was prepared in yeast extract mannitol (YEM) and *A. chroococcum* in Jensen medium. Both the broths were inoculated in 500 mL conical flask containing 250 mL broth and incubated at 28 ± 2 $^{o}$C under shaking at 100 rpm for three days to give an optical density of 0.5 recorded at 535 nm. Chickpea seeds were coated with slurry of the respective broth according to the treatments. Slurry was prepared by mixing 50 mL of 15% sterilized sugar solution, 100 mL broth and 500 g of sterilized peat. Control was treated with sterilized peat containing sterilized broth and sugar solution. In case of co-inoculation, slurry was prepared with both the broths in 1: 1 ratio.

### *Field Experiment*

Field study was conducted to assess the co-inoculation with *M. ciceri* and *A. chroococcum* for improving the growth and yield of chickpea at Pulses Research Institute, AARI, Faisalabad. The surface soil was collected from the research area, air dried, thoroughly mixed, passed through 2 mm sieve and analyzed for various physico-chemical characteristics. The soil was sandy clay loam having pH 8.16; electrical conductivity, 1.43 dS m$^{-1}$; organic matter, 0.66%; total nitrogen, 0.037%; available phosphorus, 9.71 mg kg$^{-1}$. Recommended (30 kg ha$^{-1}$) and half of recommended (15 kg ha$^{-1}$) N was applied according to the treatments as urea while full dose of P (60 kg ha$^{-1}$) was applied as basal dose to all the treatments. The treatments were as under:

$T_1$: Control
$T_2$: *M. ciceri* inoculation
$T_3$: *A. chroococcum* inoculation
$T_4$: *M. ciceri* + *A. chroococcum* inoculation

All the treatments were tested at two fertilizer levels i.e. 15-60 and 30-60 kg NP ha$^{-1}$

Canal water meeting the irrigation quality criteria for crops (Ayers and Westcot, 1985) was used for irrigation. The treatments were randomized following complete block design with three replications. When the crop was blooming flowers, two plants from each plot were uprooted randomly and washed with tap water to check the nodule number plant$^{-1}$ and nodular mass. Data regarding biomass, grain yield, N and P-content in plant, 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 randomized complete block design (Steel *et al.*, 1997). The differences among treatment means were checked by applying the Duncan's multiple range tests (Duncan, 1955).

## Results and Discussion

### *Yield and nodulation*

Inoculation of chickpea with both *M. ciceri* and *A. chroococcum* significantly enhanced the biomass and grain yield over un-inoculated controls at both N levels (Table 1). The effect was more pronounced when inoculants were applied in combination. The highest biomass and grain yield (3456 and 1772 kg ha$^{-1}$, respectively) was obtained by co-inoculation at 15 kg NP ha$^{-1}$. The increases in biomass and grain yield were 19.04 and 19.0 % over un-inoculated control at half dose of N fertilizer. The biomass yield at 15 kg N ha$^{-1}$ with *M. ciceri* inoculation alone was at par with co-inoculation. Inoculation with *A. chroococcum* and *M. ciceri* alone increased the biomass and grain yield significantly over un-inoculated controls at both levels of N. *A. chroococcum* alone at both N levels compared with respective control.

In the present study, association of *Azotobacter* and *Mesorhizobium* promoted chickpea growth and yield as compared to their individual inoculations. It is highly likely that Azobacterization with auxin biosynthesis might have provided improved colonization niches through root proliferation to introduced *rhizobium* in the rhizosphere of chickpea reflecting-in better nodulation and yield. Similar to our work, various researchers have reported the synergistic effects of auxin producing plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and yield of legume crops (Tilak *et al*., 2006; Mirza *et al*., 2007). Spanik (1996) and Perret *et al*. (2000) discussed the role of signal exchange between host plant and specific rhizobial species in nodule formation. Inoculation with free-living diazotrophs increased the signal exchange between host legumes and the symbiont resulting-in more $N_2$-fixing sites and ultimately higher nutrient concentration and yield of the legume (Parmar and Dadarwal, 1999; Paul and Verma, 1999).

Co-inoculation at 15 kg N ha$^{-1}$ gave good biomass and grain yield in comparison with 30 kg N ha$^{-1}$ because of better nodulation. Legume meets its N requirement mostly through symbiosis and higher N application may not cause any economical yield increases (Marel *et al*., 1990). Increase in yield at lower N might be accredited to favor the symbiotic relationship and ultimately high available N concentration in soil. Our results contradict the finding of Paul and Verma (1999), who reported increased nodule number and weight due to *Azotobacter* inoculation but decreased with co-inoculation. This might due to variation in genotype and soil conditions. Our findings are

**Table 1. Effect of *M. ciceri* and *A. chroococcum* inoculation on yield and nodulation of chickpea**

(Average of 3 repeats)

| Treatment | Biomass Yield (kg ha$^{-1}$) | | Grain Yield (kg ha$^{-1}$) | |
|---|---|---|---|---|
| | 15 kg N ha$^{-1}$ | 30 kg N ha$^{-1}$ | 15 kg N ha$^{-1}$ | 30 kg N ha$^{-1}$ |
| Control | 2903 d* | 3068 c | 1489 f | 1573 e |
| *M. ciceri* inoculation | 3420 a | 3307 b | 1737 ab | 1696 c |
| *A. chroococcum* inoculation | 3136 c | 3147 c | 1608 de | 1614 d |
| *M. ciceri* + *A. chroococcum* | 3456 a | 3372 ab | 1772 a | 1729 bc |
| LSD | 103.6 | | 35.95 | |
| | **Nodule Number plant$^{-1}$** | | **Nodular mass (g plant$^{-1}$)** | |
| Control | 17 d* | 20 d | 0.222 c* | 0.222 c |
| *M. ciceri* inoculation | 28 c | 33 b | 0.242 b | 0.246 ab |
| *A. chroococcum* inoculation | 18 d | 21 d | 0.221 c | 0.222 c |
| *M. ciceri* + *A. chroococcum* | 42 a | 38 a | 0.252 a | 0.249 ab |
| LSD | 3.688 | | 0.00959 | |

*Means sharing the same letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test

Co-inoculation also exhibited significantly higher nodule number and mass (42 and 0.252 g plant$^{-1}$) at half N level (Table 1), the increase in nodule number and mass were 147 and 13.5% over un-inoculated control, respectively. *Mezorhizobium ciceri* inoculation alone gave 64 and 9% increase in nodule number and mass over un-inoculated control at half N level, respectively. Statistically similar nodule number and mass were observed with *A.*

corroborated with the Parmar and Dadarwal (1999), Bai *et al*. (2002) and Mirza *et al*. (2007) who reported significantly increased yield and nodulation of legumes due to co-inoculation.

## Plant and grain N, P-contents

Data regarding NP contents of chickpea plant and grains are presented in Table 2. Co-inoculation produced highest N-content in plant (1.678, 1.683%) at half and full N application. Similarly, the highest P-content in plant (0.283%) was observed with co-inoculation followed by 0.272% with rhizobial inoculation at higher N application.

**Table 2. Effect of *M. ciceri* and *A. chroococcum* inoculation on plant and grain analysis of chickpea**

(Average of 3 repeats)

| Treatment | Plant N (%) | | Plant P (%) | |
|---|---|---|---|---|
| | 15 kg N $ha^{-1}$ | 30 kg N $ha^{-1}$ | 15 kg N $ha^{-1}$ | 30 kg N $ha^{-1}$ |
| Control | 1.572 c* | 1.581 c | 0.223 e* | 0.242 de |
| *M. ciceri* inoculation | 1.665 ab | 1.667 ab | 0.257 bcd | 0.272 ab |
| *A. chroococcum* inoculation | 1.647 b | 1.654 b | 0.234 ef | 0.250 cd |
| *M. ciceri* + *A. chroococcum* | 1.678 a | 1.683 a | 0.258 bc | 0.283 a |
| LSD | 0.0215 | | 0.0157 | |
| | Grain N (%) | | Grain P (%) | |
| Control | 3.51 e* | 3.53 de | 0.252 d* | 0.267 cd |
| *M. ciceri* inoculation | 3.60 a | 3.62 a | 0.314 ab | 0.331 a |
| *A. chroococcum* inoculation | 3.53 de | 3.54 cd | 0.278 cd | 0.294 bc |
| *M. ciceri* + *A. chroococcum* | 3.57 bc | 3.59 ab | 0.326 a | 0.338 a |
| LSD | 0.027 | | 0.030 | |

*Means sharing the same letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test

*Mezorhizobium ciceri* inoculation registered highest N in grains (3.60 and 3.62%) which was at par with co-inoculation at both N application rates. Co-inoculation produced highest P-content in grains i.e. 0.338% that differed non-significantly from rhizobial inoculation at full dose of N fertilizer. Enhanced N-P content of plant due to co-inoculation of free living diazotrophs and rhizobia has been reported by various researchers (Lata and Tilak, 2000; Garcia *et al*., 2004). Because of $N_2$-fixing ability of legumes, only small amount of N fertilizer is applied as starter to let the symbionts work efficiently. In present study, increase in N concentration in plant at low fertilizer (15 kg N $ha^{-1}$) might be attributed to high available N in soil due to biological $N_2$-fixation. Interactive effect of rhizobia with rhizobacteria enhanced the acquisition of nutrients and other parameters as compared to un-inoculated ones that might be due to production of growth hormones and higher root mass for better uptake of nutrients (Yuming *et al*., 2003; Barea *et al*., 2005).

## Post harvest soil analysis

Data on post harvest soil analysis revealed that inoculation (*Mezorhizobium* and *Azotobacter*) enhanced soil N and available P at both N application rates as compared to un-inoculated control (Table 3). Significantly higher soil N i.e. 0.052 % was observed with rhizobial inoculation at full dose of N fertilizer followed by co-inoculation with *Mezorhizobium* and *Azotobacter*. Data on available P given in Table 3 depicted that maximum P-content (12.32 mg $kg^{-1}$) was observed by combined inoculation of *M. ciceri* with *A. chroococcum* at full dose of N fertilizer. Results revealed that inoculations significantly reduced the soil pH as compared to un-inoculated ones. However, non-significant response of soil EC was observed with inoculations. Percent increase in the post harvest soil N with co-inoculation was 16% while with *rhizobium* it was 14% higher compared to un-inoculated control at 15 kg N $ha^{-1}$. Like wise, co-inoculation gave maximum increase of available P-content (54%) followed by 18% increase with *M. ciceri* inoculation alone compared to un-inoculated control at half dose of N fertilizer. Co-inoculation resulted in more $N_2$-fixation and P-solubilization by lowering the soil pH and producing organic acids (Gupta *et al*., 1998; Khan *et al*., 2006). Production of organic acids resulted in acidification of the microbial cell and its surroundings (Alagwadi and Gaur, 1988; Khan *et al*., 2006). Barea *et al*. (2005) demonstrated that the interactive effect of rhizobia and rhizobacteria mediated the soil processes and thus enhanced availability of nutrients. Previously, many researchers reported an increase in the percent N and available P contents with the co-inoculation (Gupta *et al*., 1998; Suneja *et al*., 2007). The release of protons during $N_2$-fixation by *Rhizobium* and the production of organic acids reduced the soil pH which very likely might be the possible mechanism for reduction in pH (Khan *et al*., 2006).

Present study depicted the effect of co-inoculation with *M. ciceri* and *A. chroococcum* on the growth and yield of chickpea. *M. ciceri* and *A. chroococcum* inoculation alone enhanced the yield parameters of chickpea but the effect was more pronounced with their combined application and reduced dependence on chemical fertilizers and saved ~ 50 % of recommended N fertilizer. However, more comprehensive and detailed studies in different ecological zones on the farmer's field for sustainable crop production should be carried out to confirm this approach.

**Table 3. Effect of *M. ciceri* and *A. chroococcum* inoculation on post harvest soil analysis**

**(Average of 3 repeats)**

| Treatment | N in soil (%) | | Available P (mg kg$^{-1}$) | |
|---|---|---|---|---|
| | 15 kg N ha$^{-1}$ | 30 kg N ha$^{-1}$ | 15 kg N ha$^{-1}$ | 30 kg N ha$^{-1}$ |
| Control | 0.043 c* | 0.046 bc | 7.77 e | 8.47 de |
| *M. ciceri* inoculation | 0.049 ab | 0.052 a | 9.17 cde | 10.22 bcd |
| *A. chroococcum* inoculation | 0.047 abc | 0.046 bc | 10.57 abc | 11.62 ab |
| *M. ciceri* + *A. chroococcum* | 0.050 ab | 0.051 ab | 11.97 ab | 12.32 a |
| LSD | 0.0055 | | 1.857 | |
| | pH | | EC (d Sm$^{-1}$) | |
| Control | 8.12 a | 8.15 a | 1.419 | 1.426 |
| *M. ciceri* inoculation | 8.08 b | 8.06 b | 1.430 | 1.425 |
| *A. chroococcum* inoculation | 7.97 c | 7.98 c | 1.429 | 1.428 |
| *M. ciceri* + *A. chroococcum* | 7.96 c | 7.97 c | 1.427 | 1.423 |
| LSD | 0.0327 | | NS | |

*Means sharing the same letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test

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