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RESEARCH ARTICLE

Influence of *Rhizobium* Applied in Combination with Micronutrients on Mungbean

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ABSTRACT
Legume crops are not only used as human diet but also for edifying soil fertility through biological nitrogen fixation and *Rhizobia* is well known in enhancing yield of legumes. Micronutrients are imperative module of soil fertility and they manipulate crop productivity. Therefore the application of micronutrients (Zn, Fe & Mo) along with *Rhizobium* inoculation may improve nodulation and yield of mungbean. There are scanty reports on their combined use under arid to semiarid climate of Pakistan. A pot experiment was conducted to evaluate their effect on mungbean productivity. Treatments allocated in CRD, comprised of control, *Rhizobium* inoculation alone and along with two levels (2 and 4 kg ha^{-1}) of micronutrients. The performance of *Rhizobium* alone was superior over uninoculated control in all parameters. The results showed that increasing rate of micronutrients along with *Rhizobium* inoculation significantly increased mungbean productivity except molybdenum. *Rhizobium* inoculation along with Mo @ 2 kg ha^{-1} increased plant height, root length, 100-grain weight, nodules number $plant^{-1}$ and nitrogen content in grains by 59, 67, 72, 162 and 8%, respectively, over uninoculated control whereas highest number of pods $plant^{-1}$ and grain yield was obtained along with Zn @ 4 kg ha^{-1}. Number of nodules $plant^{-1}$ was positively correlated with %N in grains and grain yield. It was concluded that micronutrients are vital for optimum activity of *Rhizobium* and plants inoculated with it enhanced mungbean productivity.

INTRODUCTION

Pulses are an important component of lucrative and sustainable farming systems. Among the grain legumes, mungbean (*Vigna radiata* L.), commonly known as green gram, is one of the important conventional pulse crop of Pakistan. Its seeds contain 24.2% protein, 1.3% fat and 60.4% carbohydrates, calcium and phosphorous are measured as 118 and 340 mg per 100 g of seeds, respectively and is rich in vitamin-A. It is also considered as a substitute of animal protein and forms a balanced diet when used with cereals (Ghildiyal, 1992). In Pakistan, mungbean is cultivated as minor crop and used as food. The area under mungbean in the country was around 141 thousand hectares with a production of 93 thousand tones in 2011 (Pakistan Economic Survey, 2012).

Owing to biotic and abiotic stresses plants display a remarkable ability to respond environmental anomalies by modifying their morphology and metabolism. An important and striking example of such flexibility concerns the symbiotic interaction between legumes and the rhizobial bacteria. It is estimated that rhizobial symbiosis with over 100 agriculturally important legumes account, at least for half of the annual amount of biological nitrogen fixation (BNF) in soil ecosystems (Peoples and Craswell, 1992). Moreover, the use of nitrogen as fertilizer has degraded large area around the world and BNF is required to replace tones of fertilizers (Burris, 1994). Therefore, BNF and legumes are particularly important both agriculturally and ecologically. Mungbean is capable of fixing atmospheric nitrogen through *Rhizobium* species living in root nodules. Inoculation of mungbean with *Rhizobium* increased plant height, leaf area, photosynthetic rate and dry matter production (Iqbal et al., 2012; Mehboob et al., 2012). The use of BNF technology in the form of *Rhizobium* inoculants in grain legumes can supplement the expensive fertilizer, particularly for improving the production of food legumes in the country.

Macro and micronutrients are required for normal growth of plants. Molybdenum application can play a vital role to increase nitrogen fixation by *Rhizobium* and for the formation of nodule (Rahman et al., 2008). Increase in flower numbers, pod set improvement and reduction in days to flowering are influenced by Mo (Prasad et al., 1998). Zinc plays a regulatory role in the intake and efficient use of water by plants. It acts as an activator of several enzymes in plants and is directly involved in the biosynthesis of growth substances such as auxin which produces more plant cells (Devlin et al., 1983). It enhances photosynthesis at early growth of plants, improves nitrogen fixation, grain protein and yields of mungbean plants (Krishna, 1995). Iron is an essential component of many enzymes. It also enters into oxidation processes that release energy from sugars and starches and reactions that convert nitrate to ammonium in the plant. Ferro-sulfur proteins play an important role in nitrogen fixation and electron movement in photosynthesis (Kadioglu, 2004). Moreover, about 70% cultivated soils of Pakistan are Zn deficient and wide spread deficiency of Fe has been reported (Imtiaz et al., 2010). Therefore, the application of micronutrients can play a vital role in increasing growth and yield of legumes and other crops may cure their deficiencies (Ali et al., 2012).

It indicates that selection of effective combination of micronutrients with *Rhizobia* for leguminous crop is a critical aspect. Therefore pot study was conducted to assess the effect of *Rhizobium* inoculation on nodulation and yield of mungbean, and to determine which level of micronutrients (Zn, Mo and Fe) along with *Rhizobium* can improve mungbean productivity.

MATERIALS AND METHODS

Pot experiment was conducted to evaluate the effect of *Rhizobium* in combination with micro-nutrients on mungbean productivity, in glasshouse of the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad during kharif season using mungbean variety AZARI-2006.

Status of soil

The prior to run experiment a composite soil sample was taken and determined basic physico-chemical properties following standard protocols; saturation percentage 34.57%; ECe 1.46 dS m^{-1}; pH 8.1; organic matter 0.71%; total nitrogen 0.045%; available phosphorus 8.73 $mg.kg^{-1}$; extractable potassium 175 mg kg^{-1} and textural class sandy loam (sand 50.5%, silt 35% & clay 14.5%).

Isolation of rhizobia

Mungbean plant uprooted and brought to laboratory in polythene bags. The non-rhizosphere soil was removed from plant roots by gentle shaking while rhizospheric soil adhered to roots was removed by dipping and gentle shaking in sterilized water under aseptic conditions in laminar air flow hood to avoid contamination. The nodules from mungbean roots were removed and placed in petri plate. Surface-sterilization of nodules were carried out by dipping momentarily in 95% ethanol and 0.2% $HgCl_2$ solution for 2-3 minutes followed by 6 washings with sterilized distilled water. Surface-sterilized nodules were crushed in sterilized test tube with glass rod which was also sterilized. The suspension obtained was used to inoculate yeast manitol agar (YMA) media and pure cultures were obtained by further streaking 3-4 times on fresh media. Fast growing colonies of bacteria were selected and stored in slants at -20°C for subsequent use.

Preparation of inoculum and seed treatment

The inoculum for the pot trial was prepared by using yeast extract manitol (YEM) broth media. It was incubated at 28±1°C for three days. The inoculum containing 10^8-10^9 colony forming units mL^{-1} (achieved by dilution on the basis of optical density) of selected rhizobial strain was mixed into sterilized peat. For inoculation, seed dressing was carried out with inoculated peat mixed with 15% sugar solution. Autoclaved peat and sugar mixture was used to treat seeds for control treatment.

Pot trial

Plastic pots of 6 cm diameter and 12 cm depth were used in experiment. Each pot aligned from inside with polythene sheet to avoid loss of water and nutrients. Each pot filled with 8 kg soil (sieved through 2 mm sieve) and irrigated with water two days before sowing to attain suitable conditions for seed sowing and germination. Basal doses of NPK fertilizers as Urea, single super phosphate (SSP) and sulphate of potash (SOP) were applied at 25: 60: 60 kg ha^{-1}, respectively, in soil two days before sowing. Full doses of PK were applied before sowing while N was applied in two splits (half dose before sowing and remaining at blooming stage). *Rhizobium* inoculation alone and along with two levels (2 and 4 Kg ha^{-1}) of micro-nutrients (Zn as $ZnSO_4.7H_2O$, Fe as $FeSO_4.7H_2O$, and Mo as Na_2MoO_4) was applied in a completely randomized design and replicated six times. Three seeds were sown in each pot which was thinned to one plant after germination. All the agronomic practices like weeding; irrigation and plant protection measures were performed as and when necessary. Data regarding nodulation was recorded at flowering stage using three replicates while yield at maturity from left over replicates.

Chemical analysis

Plant samples were oven dried in oven at 70 °C for 48 hours and ground on rotary mil (model Polymix PX-MFC, Switzerland). The ground material (0.1 g) was digested with sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) according to the method of Wolf (1982). Grain nitrogen was determined by Kjeldahl method.

Statistical analysis

Analysis of variance was performed according to Steel et al. (1997). Treatment means were compared by Duncan multiple range test (DMR) at 5% level of significance (Duncan, 1955) using MSTAT-C software, 1.3v. Simple correlation between different parameters of mungbean was determined using the same software.

RESULTS AND DISCUSSION

The vegetative growth of mungbean was improved by the application of Mo at the rate of 2 kg ha^{-1} in combination with inoculation of *Rhizobium*. Similar trend was observed in above ground (plant height) as well as in underground part (root length) of the plant (Table 1). The plant height of mungbean achieved with different treatments ranged between 17 and 27 cm. The maximum increased plant height 59 and 35% over uninoculated control and *Rhizobium* inoculation, respectively, was observed in treatment received Mo at the rate of 2 kg ha^{-1} and *Rhizobium*. The highest plant height (27 cm) was statistically at par with T_4 (25.6 cm) and T_8 (25.3 cm) and significant over all other treatments. The lowest value 17 cm was recorded in uninoculated control. Plant received 2 kg Mo ha^{-1} along with *Rhizobium* inoculation gave higher root length which was 67 and 43% higher over uninoculated control and *Rhizobium* inoculation, respectively. *Rhizobium* inoculation alone gave 15 and 17% higher plant height and root length respectively, which was significantly differing from uninoculated control. Rhizobia are well known to affect plant growth and development by multifarious mechanisms such as N_2 fixation, production of plant growth regulators (PGRs), improved mineral uptake and suppression of plant diseases (Kennedy et al., 2004; Patten and Glick, 1996). The sole inoculation of *Rhizobium* increased vegetative growth of mungbean over uninoculated control indicates contribution of bacteria in nitrogen fixation by developing nodules on mungbean roots. Thus chemical and biologically fixed nitrogen is the most dominating factor influencing the plant height. The significant increase in plant growth by *Rhizobium* inoculation have been reported (Karaca and Uyanöz, 2012; Iqbal et al., 2012; Bhuiyan et al. 2008; Togay et al., 2008).

Rhizobium inoculation alone and in combination with micronutrients had a significant effect on number of pods plant^{-1} and 100-grain weight compared to uninoculated control (Table 1). In case of uninoculated control, average number of pods plant^{-1} was 9, which was 19% less than inoculated plant. The highest number of pods plant^{-1} was recorded in treatment received *Rhizobium* inoculation plus Zn at the rate of 4 kg ha^{-1}. It enhanced maximally the number of pods plant^{-1} up to 39% over *Rhizobium* inoculation alone and 65% over control. Number of pods plant^{-1} increased with increasing Zn and Fe levels (2 to 4 kg ha^{-1}) with the highest mean value of 14 and 13 pods plant^{-1}, respectively. The application of Mo at the rate of 2 kg ha^{-1} increased the number of pods and it was reduced with further addition of Mo (4 kg ha^{-1}). Control had the lowest mean value for 100-grain weight which was recorded 4.05 g plant^{-1}. *Rhizobium* inoculation alone significantly increased 100-grain weight which was 26% higher compared to uninoculated control. The maximum increase in 100-grain weight was caused by *Rhizobium* inoculation along with the application of Mo at the rate of 2 kg ha^{-1} which produced 72 and 36% increase over uninoculated control and *Rhizobium* inoculation alone, respectively. Seed yield of mungbean was significantly influenced by application of different levels of micronutrients along with *Rhizobium* inoculation (Fig. 1). The highest mean value (5.64 g plant^{-1}) for grain yield was found in T_4 which caused an increase of 132% over uninoculated control and 71% over *Rhizobium* inoculation alone. As for as the effect of sole *Rhizobium* inoculation on grain yield plant^{-1} was concerned, there was a significant increase (36%) compared to uninoculated control.

The beneficial effect of zinc fertilizer on yield and yield contributing parameters could be attributed to its vital role in the function of enzymes for the biological

Table 1: Effect of *Rhizobium* inoculation in combination with micronutrients on mungbean

Treatments	Plant height (cm)	Root length (cm)	No. of Pods plant^{-1}	100-grain weight (g)	No. of nodules plant^{-1}
Control	17.0^{e}	12.0^{e}	8.67^{f}	4.05^{f}	8.00^{e}
Rhizobium	20.0^{d}	14.0^{d}	10.33^{e}	5.11^{e}	11.00^{d}
Rhizobium + Zn 2 kg ha^{-1}	24.0^{bc}	16.0^{c}	11.67^{cd}	6.04^{c}	14.67^{c}
Rhizobium + Zn 4 kg ha^{-1}	25.6^{ab}	19.0^{ab}	14.33^{a}	6.79^{ab}	19.67^{ab}
Rhizobium + Mo 2 kg ha^{-1}	27.0^{a}	20.0^{a}	12.67^{bc}	6.95^{a}	21.00^{a}
Rhizobium + Mo 4 kg ha^{-1}	23.3^{c}	17.6^{bc}	11.00^{de}	6.20^{c}	19.00^{b}
Rhizobium + Fe 2 kg ha^{-1}	23.0^{c}	17.0^{c}	11.67^{cd}	5.76^{d}	15.00^{c}
Rhizobium + Fe 4 kg ha^{-1}	25.3^{ab}	19.3^{ab}	13.00^{b}	6.57^{b}	19.00^{b}
CV (%)	4.42	6.20	6.45	2.82	7.32

The figures in a column having same letter (s) do not differ significantly at P≤0.05

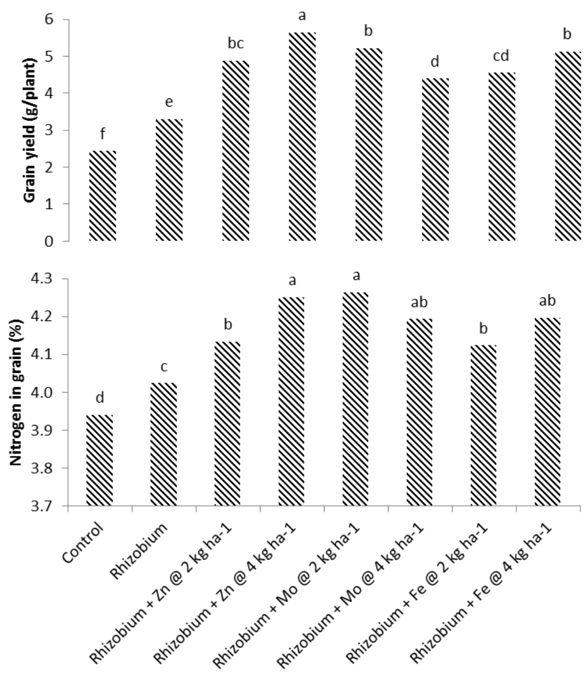

Fig. 1: Effect of *Rhizobium* in combination with micronutrients on grain yield and nitrogen contents of mungbean. (Similar letter (s) don't differ significantly at P≤0.05)

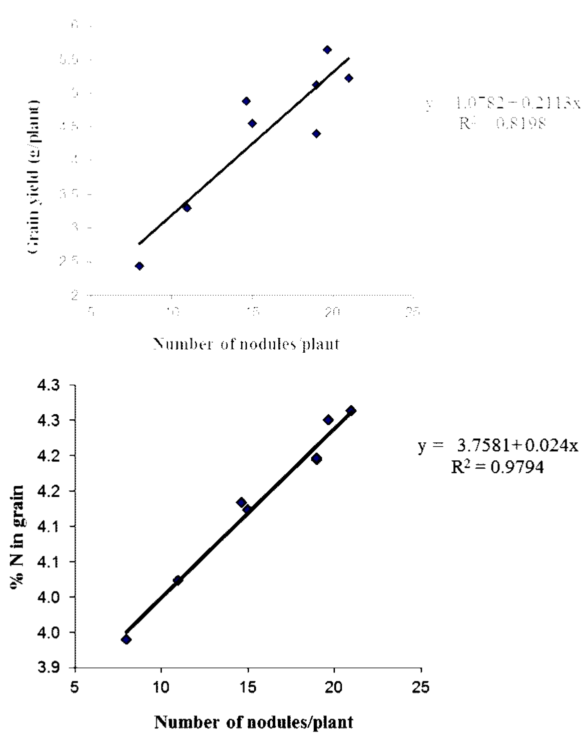

Fig. 2: Relationship between number of nodules plant^{-1} with grain yield and grain nitrogen contents of mungbean

processes in plants which lead to increase in yield components. It is involved in several enzyme systems and required in the synthesis of auxin from the amino acid tryptophan, the compound that regulates the plant's growth rate and development. It assists the utilization of phosphorus and nitrogen in plants. It also plays a regulatory role in the intake and efficient use of water by plants. The increase in 100-grain weight showed that micronutrients along with *Rhizobium* inoculation are necessary for healthier and robust seeds in mungbean. The results are in agreement with the findings of Rabbani et al. (2005) who found that the number of pods plant^{-1}, 100-grain weight and grain yield was increased with *Rhizobium* inoculation in combination with micronutrients. It has been also reported that inoculation with *Rhizobium* enhanced grain yield and number of pods plant^{-1} (Karaca and Uyanöz, 2012; Iqbal et al., 2012; Ahmed et al., 2007; Raza et al., 2004). Furthermore, Gobarah et al. (2006) reported that the different zinc levels had significant effect on groundnut growth, yield and its components. The results are in line with the findings of Arshad and Mahmood (2010) Chowdhury et al. (1998) who reported that the combination of bio-fertilizers and micro-nutrients significantly increased crop yield over control.

Seed inoculation with *Rhizobium* obviously increased nodules number compared to uninoculated control (Table 1). The lowest nodules number (8) was found in control. *Rhizobium* inoculation along with the application of Mo at the rate of 2 kg ha^{-1} produced maximum number of nodules plant^{-1} which was 163 and 91% higher over uninoculated control and *Rhizobium* inoculation alone, respectively. Inoculation with *Rhizobium* alone also increased the number of nodules plant^{-1} significantly compared to uninoculated control. Nitrogen content of mungbean grains was significantly increased by the application of micronutrients in combination with *Rhizobium* inoculation (Fig. 1). *Rhizobium* inoculation alone was found significant over control which caused 3% increase in nitrogen content of grains. *Rhizobium* inoculation along with the application of Mo at the rate of 2 kg ha^{-1} caused an increase of 9% over control and over *Rhizobium* alone it was 6%. Present study showed that number of nodules plant^{-1} had positive relation with grain yield of mungbean and nitrogen concentration in grains (Fig. 2). It exhibited the importance of nodules for the improvement of nitrogen contents in grains resulting enhanced seed yield and quality. Positive correlation was found in all the parameters under study (Table 3). Number of nodules plant^{-1} showed strong association with plant height, root length, 100-grain weight, grain yield and nitrogen content. It elucidated that the increased in root length improved nodulation of mungbean which assist in fixation of atmospheric nitrogen through nitrogenase activity of *Rhizobium*. On

Table 2: Pearson correlation between different parameters of mungbean

	Plant Height	No. of pods	Grain yield	100-grain Weight	Root Length	Percent Nitrogen
No. of pods	0.79*	---	---	---	---	---
Grain yield	0.92*	0.88*	---	---	---	---
100-grain weight	0.93*	0.89*	0.94*	---	---	---
Root length	0.89*	0.82*	0.86*	0.93*	---	---
Percent Nitrogen	0.91*	0.76*	0.92*	0.91*	0.83*	---
No. of nodules	0.90*	0.79*	0.87*	0.87*	0.93*	0.90*

*Asterisk shows significant relationship between parameters at $P \leq 0.05$, n=24

the other hand plant growth and yield increased owing to nodulation. Grain yield increased parallel to plant height and nitrogen content in grain indicated the role of nitrogen either biological or chemical in inducing yield improvement.

Number of nodules showed positive linear relation with grain yield and %N of mungbean. The maximum value of coefficient of determination (R^2) between number of nodules and %N (R^2=0.98) showed their strong relationship. It implied that plants inoculated with *Rhizobium* fixed atmospheric nitrogen in higher proportion than uninoculated palnts thus their tissue N contents was increased. There was a weak association between grain yield and number of nodules (R^2=0.82) compared to %N (R^2=0.98). It elucidates the importance of applying micronutrients along with *Rhizobium* in improving BNF. The improvement in nodule formation may be due to molybdenum which is required by rhizobia for the fixation of atmospheric nitrogen in legumes. It is a constituent of nitrate reductase, nitrogenase and is required for both synthesis and activity of the enzymes. In nitrate reductase it is bound to a unique compound named molybdenum cofactor- Moco, which binds to diverse apoproteins. One of the two nitrogenase cofactors, MoFe-cofactor is related to the binding site and involved in the reduction of N_2 from the atmosphere (Orme-Jonson, 1985). Similar work was done by Paudyal et al. (2007) who reported that higher number of nodules was obtained with *Rhizobium* inoculation along with Mo. Inoculation of mungbean with *Bradyrhizobium* sp. (*Phaseolus*) increased number of nodules by 254% (Provorov et al., 1998). That may be as a result of the beneficial effect of micronutrients used on the biofertilizers as mentioned by Amara (1998) who found that spraying with micronutrients resulted in a large increase in total microbial counts in the rhizosphere and increased nodule number. The increase in N contents of mungbean grains may be due to the beneficial effect of micronutrients on *Rhizobium* intensification. Soil micro-organisms have the ability of mobilizing the unavailable forms of nutrient elements to available forms and have successfully been used to increase crop productivity (Fardous et al., 2010; Ibrahim et al., 2010). The microbial inoculants showed the highest values of nitrogen content in the seeds of legumes (Iqbal et al., 2012). Similarly, Mehboob et al. (2012) reported increase in maize yield with *Rhizobium* application.

Conclusions

Micronutrients applied in combination with *Rhizobium* increased growth and yield of mungbean. Seed inoculation with *Rhizobium* improved all the indices of mungbean. Lower level of molybdenum and all the levels of zinc and iron along with *Rhizobium* inoculation considered beneficial in boosting mungbean productivity. It was found that integrated use of micronutrients and *Rhizobium* inoculation increased NPK contents of mungbean. Positive linear relation and strong correlation of number of nodules plant^{-1} with studied parameters elucidate the role of *Rhizobium* inoculation and micronutrients application in improving nitrogen fixation which ultimately enhanced yield of mungbean.

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