



## Field evaluation of multistrain biofertilizer for improving the productivity of different mungbean genotypes

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### Abstract

Mungbean can successfully be grown in the small slots present in the existing cropping systems that may increase the farmer income and can also restore soil fertility. The inoculation of crop plants with bacterial inoculants has the potential to increase crop productivity even under different soil and climatic conditions. A field experiment was conducted to evaluate the effectiveness of multi-strain biofertilizer prepared through combined use of *Rhizobium phaseoli* and *Pseudomonas fluorescens* and *Bacillus subtilis* for enhancing the growth, nodulation and productivity of ten mungbean genotypes under field conditions, and effect of inoculation on total bacterial DNA (population) in soil. The experiment was laid out in randomized complete block design (RCBD) with factorial arrangements and three replications. Results revealed that inoculation with multistrain biofertilizer increased the nodule numbers, growth, and yield under different mungbean genotypes when compared with their respective uninoculated control. The genotypes showed different productive potentials either with or without inoculation under field conditions. The genotype NCM 2015 yielded more but inoculation was more effective with genotypes NM 17, NM 19 and NCM-252-10 under field conditions of Bahawalpur. Results of 16S rRNA analysis showed a higher number of gene copies in the rhizosphere of inoculated plants of all mungbean genotypes than those of uninoculated plants. Maximum total bacterial population was observed in the rhizosphere of inoculated plants in NM 11 that was significantly better than un-inoculated control plants of the same mungbean line but non-significant when compared with other lines under inoculation. It is concluded that the use of multistrain biofertilizer prepared through combined use of *Rhizobium* and PGPR strains containing ACC-deaminase could be an effective approach to improve growth, nodulation and yield of mungbean genotypes. The response of different genotypes to the inoculation varied significantly. So, research for the development of inoculum for different advanced genotypes should be continued and more emphasis should be deployed to develop biofertilizers with efficient strains to use them under different climate and soil conditions.

**Keywords:** mungbean, intensification, profitability, sustainability, biofertilizers

### Introduction

The small landholder farmers in Pakistan grow only major crops such as cotton, rice, wheat and sugarcane and they have negligible place for minor crops including pulses in the cropping systems. This may be due to lack of interest and awareness regarding new technologies and supporting crops. The incorporation of legume crops in the cropping system may increase the profitability and can also restore soil fertility. Mung bean is one of the important pulse crops grown in the world. It is a cheap source of high quality nutrition including 24.2% protein, 1.3% fat, 60.4% carbohydrate, 4% minerals and 3% vitamins (Afzal *et al.*, 2008). Apart from its dietary role, it is also important from soil fertility point of view due to its ability to form

symbiotic relation with *Rhizobium* that have the ability of fixing atmospheric nitrogen (Achakzai *et al.*, 2012). It is a warm season crop that can easily fit into the existing cropping systems without disturbing them. Diversification of local production systems through inclusion of mungbean can be profitable for farmers and can improve soil fertility. Nitrogen is one of the most limiting plant nutrients that decrease crop productivity. Despite of its availability in air (more than 79%), it is not available to plants directly. However, it can only be utilized and fixed by soil microbes including *Rhizobium* making it available not only to plants but also for entire ecosystem (Ahmad *et al.*, 2013a).

In addition to *Rhizobium*, soil inhabiting plant growth promoting rhizobacteria (PGPR) are also associated with

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mung bean and play a significant role in plant growth and development. These bacteria play a vital role in plant growth and their presence ensures normal functioning of several rhizospheric and plant metabolic processes (Nadeem *et al.*, 2014). These PGPR colonize the rhizosphere and promote plant growth by various direct or indirect mechanisms including nitrogen fixation, metabolites production (plant growth regulators etc.), nutrient solubilization, mineralization of organic matter, pathogen control as well as play a significant role in induction of stress tolerance in plants (Ahmad *et al.*, 2013b; Nadeem *et al.*, 2014).

However, under intensive field conditions, the activity of Rhizobium and PGPR is poor, that not only affect mung bean production but also soil health (Ashraf *et al.*, 2003). Several reports indicated that the combined Rhizobium and PGPR significantly enhance the plant growth and rhizospheric activity, resulting in significant improvement in yield and productivity of legume crops (Anjum *et al.*, 2006; Qureshi *et al.*, 2013; Ahmad *et al.*, 2013a; 2014). Therefore, present study was conducted to evaluate the effectiveness of multistrain biofertilizer prepared through combined use of Rhizobium, *Pseudomonas fluorescens* and

and effect of inoculation on total bacterial DNA (population) in soil.

### Inoculum preparation

The bacterial cultures were prepared in 250 mL Erlenmeyer flasks. For this purpose, after preparation of culture media, the flasks were inoculated respective bacterial strains. The liquid cultures were incubated for 72 hours at 30 °C with continuous shaking at 100 rpm in shaking incubator (Model SI9R-2, Shellab-USA). After incubation for three days, the bacterial cells were harvested by centrifugation at 9000 rpm and 22 °C for 20 minutes (Model: UNIVERSAL 320R, Hettich – Germany). The supernatant was discarded and the pellets were re-suspended in sterilized distilled water. This washing procedure was repeated and the pellets were dissolved in sterilized distilled water to get uniform cell density (OD = 0.45; cell count  $10^8$  cfu mL<sup>-1</sup>).

### Seed inoculation and field experiment

For seed inoculation, pre-pelleted and re-suspended broth cultures of PGPR strains i.e. Mk20 (*Pseudomonas fluorescens*) and Y16 (*Bacillus subtilis*) and *Rhizobium*

**Table 1: Physico-chemical characteristics of the soil**

Parameter	Unit	Value
EC <sub>e</sub>	dS m <sup>-1</sup>	2.1
pH	---	7.9
Organic matter	%	0.47
Total Nitrogen	%	0.062
Available Phosphorus	mg kg <sup>-1</sup>	3.0
Extractable Potassium	mg kg <sup>-1</sup>	77
Saturation percentage	%	34
Textural class	---	Sandy loam

*Bacillus Subtilis* (PGPR) for enhancing the growth, nodulation efficiency and productivity of different mung bean genotypes under field conditions.

## Materials and Methods

### Field experiment

A field experiment was conducted at research area of the Department of Soil Science, Islamia University of Bahawalpur by using 10 genotypes/accessions. These genotypes were taken from the gene pool maintained at Crop Sciences Research Institute, NARC, Islamabad-Pakistan. The objective of the experiment was to evaluate the effectiveness of multi-strain biofertilizer prepared through combined use of *Rhizobium phaseoli* and *Pseudomonas fluorescens* and *Bacillus subtilis* for enhancing the growth, nodulation efficiency and productivity of different genotypes under field conditions

i.e. M9 (*Rhizobium phaseoli*) with uniform cell density were mixed in equal proportion and vortexed for 5 minutes to ensure homogenized cell density of different bacterial strains for preparation of slurry. The slurry was prepared with broth culture, sterilized peat, and sugar solution (10%) in 4:5:1 ratio as a carrier. In the case of un-inoculated control, seeds were coated with the sterilized (autoclaved) peat treated with sterilized broth.

The sowing was done on April 04, 2016 and harvested on July 16, 2016. The experiment was laid out in Randomized Complete Block Design (RCBD) with factorial arrangements and three replications. Before sowing, a composite soil sample was collected from experimental site and analyzed for physico-chemical properties (Table 1) by following the protocols as described by Ryan *et al.* (2001).



Recommended dose of N, P, K fertilizers (20: 60: 60 kg ha<sup>-1</sup>) were applied as urea, diammonium phosphate and sulphate of potash, respectively. All fertilizers were applied as basal dose at the time of sowing. The crop was irrigated with canal water and all agronomic practices were performed as per requirement of the crop. The data regarding nodulation were recorded at flowering while growth and yield parameters were taken at harvest upon maturity.

### Analysis of bacterial community variation

For the analysis of total bacterial DNA, five best performing mungbean lines were selected. Rhizosphere soil samples were collected after 50 days of sowing. Five plants were uprooted, and non-adhering soil was removed by shaking. The soil adhering to plant roots was collected by scrapping for use as rhizospheric soil. Composite sample was prepared by thorough mixing of soil collected from five plants. Soil samples were collected (50 g) and placed individually into plastic bags, transported to the laboratory and freeze-dried. The samples were transported to the Institute of Agricultural Sciences, University of Hohenheim, Germany, and freeze-dried until further analysis.

Total genomic DNA for bacteria was extracted using a commercially available DNA extraction kit (FastDNA® Spin for Soil Kit, MP Biomedicals, Solon, Ohio, USA). Extracted DNA was quantified photometrically using Nanodrop (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) and purified DNA was stored at -20 °C.

For qPCR analysis of 16S rRNA gene, 25 µL PCR cocktails were prepared containing 9.0 µL aqua dest, 12.5 µL 1X SYBR green master mix (Applied Biosystems, Foster City, CA, USA), 0.50 µL of T4 gene 32 protein (500 µg mL<sup>-1</sup>, MP Biomedicals), 0.4 µM of each oligonucleotide and 5 ng of template DNA. The PCR reactions were run on StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Reaction was started with initial denaturation at 95°C temperature for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 35 s and elongation at 72°C for 45 s. Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer dimers or other artifacts. The DNA samples were processed in triplicate reactions, while standard curves were generated using duplicate 10-fold dilutions of isolated plasmid DNA (Rasche *et al.*, 2011; Musyoki *et al.*, 2016). Automated analysis of PCR

amplicon quality and quantity was performed with StepOne™ software version 2.2 (Applied Biosystems).

### Statistical analysis

The data were analyzed using Analysis of Variance techniques in Statistix 8.1® software and means were compared using LSD at 5% level of probability to see the difference among treatment means (Montgomery, 2013).



**Picture 1: Comparison in number and size of nodules in inoculated (left) and un-inoculated (right) plants of genotype NCM 2013**

### Results

Results showed that inoculation was effective in improving the nodulation (Picture 1). Genotypes also showed variable response to inoculation, but this variation was non-significant in most of the cases (Table 2). Maximum nodules (17.1) were observed in inoculated plants of NCM 2013 followed by NM 11 of inoculated treatment with 16.2 nodules plant<sup>-1</sup>. Minimum nodules were observed in un-inoculated treatment of NM 16. Under inoculated treatment, minimum nodules were observed in plants of NCM 2015 that were non-significantly less than all other genotypes except NM 11 and NCM 2013. Results regarding the effect of inoculation on nodules fresh and dry weight of different mungbean genotypes (Table 2) showed that maximum nodule fresh weight (2.1 g plant<sup>-1</sup>) due to inoculation was observed in NM 17 while maximum nodule dry weight (0.108 g plant<sup>-1</sup>) was recorded in the inoculated plants of NCM 2015. A significant effect of inoculation on nodule fresh and dry weight was observed in most of the varieties as compared to their respective un-inoculated controls.

The results (Table 3) depicted that different mungbean genotypes showed variation in their plant height both with and without inoculation. Under un-inoculated conditions,

**Table 2: Effect of inoculation with multistrain biofertilizer on nodulation in different mungbean genotypes (average of 3 replicates)**

Genotype	Number of nodules plant <sup>-1</sup>		Nodules fresh weight (g plant <sup>-1</sup> )		Nodules dry weight (g plant <sup>-1</sup> )	
	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated
NM 11	8.3 c-f	16.2 ab	0.44 d-g	1.73 a-c	0.042 de	0.090 ab
AZRI 06	9.7 b-f	12.8 a-d	0.57 d-g	1.16 a-f	0.062 cd	0.085 a-c
NM 16	3.3 f	10.6 a-e	0.49 d-g	1.93 ab	0.048 de	0.101 ab
NM 17	12.7 a-d	11.3 a-e	0.30 eg	2.01 a	0.032 ef	0.099 ab
NCM 2015	9.3 b-f	8.8 c-f	0.65 d-g	1.87 ab	0.077 bc	0.108 a
NM 19	7.0 d-f	13.8 a-d	0.17 g	1.31 a-d	0.016 f	0.090 ab
NM 18	9.0 c-f	14.5 a-c	0.50 d-g	1.79 a-c	0.083 bc	0.089 ab
NM 06	7.1 d-f	14.7 a-c	0.24 fg	1.19 a-e	0.016 f	0.079 bc
NCM-252-10	6.9 d-f	13.0 a-d	0.86 c-g	1.03 b-g	0.079 bc	0.083 bc
NCM 2013	4.7 ef	17.1 a	0.56 d-g	1.66 a-c	0.065 cd	0.089 ab
LSD (p≤0.05)	7.0355		0.9433		0.239	

Means sharing the same letter(s) do not differ significantly at  $p \leq 0.05$ .

**Table 3: Effect of inoculation with multistrain biofertilizer on plant height (cm), and shoot fresh and dry weights (g plant<sup>-1</sup>) in different mungbean genotypes (average of 3 replicates)**

Genotype	Plant height (cm)		Shoot dry weight (g plant <sup>-1</sup> )		Root dry weight (g plant <sup>-1</sup> )	
	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated
NM 11	47.40 a-f	49.84 a-d	9.65 a-c	10.02 ab	0.60 e	0.99 a
AZRI 06	46.07 a-f	46.87 a-f	6.48 d-f	8.29 b-d	0.38 f	0.87 ab
NM 16	39.97 ef	47.29 a-f	6.46 d-f	6.53 d-f	0.61 de	0.84 a-c
NM 17	43.64 b-f	44.07 a-f	5.33 f	5.67 ef	0.38 f	0.82 a-d
NCM 2015	46.50 a-f	47.93 a-f	11.57 a	11.91 a	0.93 ab	1.02 a
NM 19	39.20 f	41.99 d-f	5.37 f	6.76 d-f	0.65 c-e	0.73 b-e
NM 18	45.31 a-f	53.25 a	8.45 b-d	8.74 b-d	0.73 b-e	0.92 ab
NM 06	43.07 c-f	48.95 a-e	7.65 b-f	7.28 c-f	0.86 ab	0.86 ab
NCM-252-10	39.10 f	52.59 ab	8.05 b-e	8.48 b-d	0.96 a	0.92 ab
NCM 2013	41.91 d-f	51.75 a-c	7.81 b-e	8.04 b-e	0.87 ab	1.00 a
LSD (p≤0.05)	9.3191		2.3958		0.2083	

Means sharing the same letter(s) do not differ significantly at  $p \leq 0.05$ .

the maximum plant height was observed by the genotype NM 11 that was non-significant with all other genotypes under un-inoculated conditions. The inoculation gave significant improvement in plant height of NCM-252-10 and NCM 2013 with respect to their un-inoculated controls. While maximum plant height (53.25 cm) was observed in NM 18 which was non-significant as compared to un-inoculated treatment of NM18. All other genotypes gave

non-significant improvement in plant height due to inoculation.

Data regarding the effect of multi-strain biofertilizer on shoot dry weight (Table 3) showed that shoot biomass was improved due to inoculation in all the mungbean genotypes, maximum shoot dry weight was observed in NCM 2015 in inoculated plants that was 11.91 g plant<sup>-1</sup>. However,





maximum improvement in shoot dry weight was observed in AZRI 06 that was 28% higher than uninoculated control

was non-significant with un-inoculated control plants. Maximum improvement in grain yield due to inoculation

**Table 4: Effect of inoculation with multistrain biofertilizer on 1000-grain weight (g) and grain yield (t ha<sup>-1</sup>) of different mungbean genotypes (average of 3 replicates)**

Genotype	1000-grain weight (g)		Grain yield (t ha <sup>-1</sup> )	
	Un-inoculated	Inoculated	Un-inoculated	Inoculated
NM 11	61.00 a-c	67.23 a-c	1.20 c	1.28 bc
AZRI 06	59.11 bc	60.58 a-c	0.98 d	1.03 d
NM 16	62.70 a-c	56.65 c	1.17 c	1.23 c
NM 17	61.50 a-c	64.06 a-c	0.84 ef	1.03 d
NCM 2015	63.45 a-c	72.92 a	1.35 ab	1.39 a
NM 19	62.09 a-c	71.02 ab	0.80 f	0.94 de
NM 18	56.31 c	60.33 a-c	0.75 fg	0.84 ef
NM 06	63.34 a-c	57.48 c	0.68 g	0.77 fg
NCM-252-10	54.71 c	56.36 c	0.81 f	0.97 d
NCM 2013	55.06 c	62.07 a-c	0.95 de	1.05 d
LSD ( $p \leq 0.05$ )	12.784		0.1090	

Means sharing the same letter(s) do not differ significantly at  $p \leq 0.05$ .

**Table 5: Effect of inoculation with multistrain biofertilizer on total bacterial DNA analyzed through 16S qPCR in the rhizosphere of different mungbean genotypes (average of 3 replicates)**

Variety	Total bacterial DNA (million copies g <sup>-1</sup> dry soil)	
	Un-inoculated	Inoculated
NM 11	1379.6 bc	3481.8 a
NM 16	1643.3 a-c	2015.0 a-c
NM 17	577.7 c	2441.6 a-c
NCM 2015	608.9 bc	2596.9 ab
NCM 2013	800.4 bc	2022.1 a-c

Means sharing the same letter(s) do not differ significantly at  $p \leq 0.05$ ; Standard Error for Comparison = 950.13; Critical Value for Comparison = 1996.1

plants. The improvement in shoot dry weight due to inoculation was non-significant in all the genotypes. A significant improvement in root dry weight was observed due to use of multi-strain biofertilizer in most of the mungbean genotypes. Maximum improvement in root dry weight was observed in AZRI 06 followed by NM 17, NM 11 and NM 16 where significant difference in root dry weight of inoculated and uninoculated plants was observed.

Results (Table 4) depicted that inoculation improved the 1000-grain weight and grain yield of mungbean but this improvement was non-significant in most of the cases. Maximum 1000-grain weight and grain yield was observed by the genotype NCM 2015 in the inoculated treatment that

was observed by the genotype NM 17 where 23% increase in grain yield over the un-inoculated control plants was observed. Inoculation gave significant improvement in grain yield of the genotypes NM 17, NM 19 and NCM-252-10 as compared to un-inoculated control. All other genotypes showed non-significant improvement. However, the improvement in 1000-grain weight due to inoculation was non-significant in all the genotypes.

The results (Table 5) showed that inoculation with multi-strain biofertilizer has improved the indigenous bacterial population in the rhizosphere of different mungbean lines as observed by the abundance of 16S rRNA gene (total bacteria). In general, a higher number of gene



copies were determined in the rhizosphere of inoculated plants of all mungbean genotypes than those of uninoculated plants. Maximum number of copies of 16S rRNA gene (total bacteria) was observed in the rhizosphere of inoculated plants in NM 11 that was significantly better than un-inoculated control plants of the same mungbean line but non-significant when compared with other lines under inoculation. The inoculation also showed improved total bacterial population (16S rRNA gene) in the rhizosphere of other mungbean lines but the improvement was statistically non-significant when compared with respective un-inoculated control plants. The varieties showed non-significant effect on the rhizosphere bacteria either with inoculation or without inoculation.

## Discussion

Incorporation of mungbean into existing cropping systems of Pakistan can be helpful to increase the profitability of small landholders in the country. Under agro-ecological conditions of Pakistan, the nodulation in mungbean is poor due to poor performance of indigenous rhizobial strains in our soils. In mungbean, using productive varieties that can establish effective symbiosis with inoculated rhizobium and rhizobacterial strains is possibly the most cost-effective way to achieve improvement in its productivity. The use of biofertilizers containing efficient bacterial strains can be possible solution to increase the nodulation and ultimately the yield of mungbean crop (Ahmad *et al.*, 2013a). Present study was conducted to evaluate the efficiency of multistrain biofertilizer for increasing the nodulation, growth and yield of different mungbean genotypes under field conditions in Bahawalpur. The effect of this fertilizer on rhizosphere bacterial population was also studied through 16S rRNA analysis. The results showed that inoculation with multistrain biofertilizer containing efficient strains of *Rhizobium* and *P. fluorescens* and *B. subtilis* significantly improved the nodulation in mung bean genotypes. The increase in nodulation was however varied with different mungbean genotypes. The better performance of these strains in some mungbean genotypes might be due to better symbiotic association of these strains with specific genotypes. The bacterial strains respond differentially to different crop varieties and species under low nutrient soil conditions (Imran *et al.*, 2015). They suggested paying equal attention for selecting plant genotype and bacterial strains to get better nodulation and yield of legume crop. Moreover, the improvement in nodulation due to inoculation might be due to stimulation of root growth of mungbean by PGPR which ultimately increased the surface area for rhizobial infection during the process of nodulation, so more nodules formed in inoculated plants (Ahmad *et al.*, 2011, 2014). In another study, Sindhu and Dadarwal (2001) reported improvement

in nodulation due to co-inoculation and they linked this increase in nodulation with release of hydrolytic enzymes i.e. cellulases by PGPR. These enzymes are responsible for the production of more infection sites on root hairs by rupturing the cell wall and making it more feasible for rhizobial infection (Hubbell *et al.*, 1978).

More nodulation means the increased availability of nitrogen that results improvement in growth and yield of leguminous crops. In present study, the inoculation increased the root and shoots growth that resulted in enhanced yield of mung bean genotypes. On average, the inoculation in current study has a positive effect on grain yield as compared to earlier estimates (Ronner *et al.*, 2016) however the benefit of inoculation was minimal in some specific cases as observed in some earlier studies (Heerwaarden *et al.*, 2017). The increased growth and yield due to combined used of *Rhizobium* and *Pseudomonas* strains can be the result of better nutrient availability, presence of microbial metabolites such as indole acetic acid, siderophores, microbial enzymes and exopolysaccharides which were produced by inoculated strains (Ahmad *et al.*, 2011, 2013b). The results of our study are consistent with the report by Kyei-Boahen *et al.* (2017) which showed that inoculation of cowpea increased nodulation, grain yield and grain N contents under field conditions. Similarly, the increased growth and yield due to better reproductive growth and fruit setting has also been reported in previous studies (Samavat *et al.*, 2012; Ahmad *et al.*, 2013a). It has been demonstrated in previous studies that low phosphorus availability in soils constrains nitrogen fixation and crop productivity. This might be due to important role P both nodulation, nitrogen fixation and plant growth processes through enhancement in root hair formation and root development (Nziguheba *et al.*, 2016), along with nodule initiation as well as energy source for nitrogen fixation process that may directly affect the nitrogenase activity and photosynthesis (Hogh-Jensen *et al.*, 2002; Kyei-Boahen *et al.*, 2017). The increased growth and yield due to inoculation with P solubilizing bacterial strains might also be due to increased P uptake leading to higher reproductive growth and flower development. The more P uptake as a result of indigenous phosphate solubilization by colonized *P. fluorescens* might be one of the reasons for improved growth and yield of inoculated crops (Egamberdieva *et al.*, 2010; Sanchez *et al.*, 2014).

Inoculation with multi-strain biofertilizer has improved the indigenous bacterial population in the rhizosphere of different mungbean lines as observed by the abundance of 16S rRNA gene (total bacteria) in present studies. The increased bacterial population due to inoculation was due to the better root colonization of inoculated strains (Ahmad *et al.*, 2011). More colonization of bacterial strains due to



inoculation resulted in better root proliferation that improved the quality of root exudates which ultimately provided more nutrition for bacteria growing in the rhizosphere.

Different mungbean genotypes responded differently to inoculation with multistrain biofertilizer. The better performance of biofertilizer in some mungbean genotypes might be due to better symbiotic association of bacterial strains with specific genotypes that is an evidence for support of the idea that crop, soil, and climate-specific biofertilizer should be developed for better performance of biofertilizers under specific agro-ecological conditions.

## Conclusions

The use of multistrain biofertilizer prepared through combined use of *Rhizobium* and PGPR strains containing ACC-deaminase could be an effective approach to improve growth, nodulation and yield of mungbean genotypes. The response of different genotypes to the inoculation varied significantly. So, research for the development of inoculum for different advanced genotypes should be continued and more emphasis should be deployed to develop biofertilizers with efficient strains to use them under different climate and soil conditions.

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