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Effects of Co-Cultures, Containing N-Fixer and P-Solubilizer, on the Growth and Yield of Pearl Millet (*Pennisetum glaucum* (L.) R. Br.) and Blackgram (*Vigna mungo* L.)

POONGUZHALI, SELVARAJ, MUNUSAMY MADHAIYAN, MUTHU THANGARAJU[1], JEOUNGHYUN RYU, KEUNYOOK CHUNG, AND TONGMIN SA*

Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea
[1]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India

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Abstract Inoculation of the carrier-based mixed bioinoculants of N-fixer (*Azospirillum lipoferum* strain Az204/*Rhizobium* strain BMBS P47) and phosphate-solubilizing bacterium (*Bacillus megaterium* var *phosphaticum* strain Pb1) promoted growth and yield of pearl millet and blackgram under pot-culture conditions. The mixed inoculant of Az204 and Pb1 enhanced germination, seedling vigor, plant height, and seed weight, and resulted in 6% increase in grain yield of pearl millet. Likewise, the mixed inoculant of BMBS P47 and Pb1 increased growth, nodulation, and yield in blackgram. The rhizosphere soil enzyme activities, including nitrogenase, urease, and phosphatase, in both pearl millet and blackgram were significantly increased by the inoculation of the mixed inoculant, compared to that of the individual inoculants. The results clearly indicate the beneficial effect of co-culturing the N-fixer and P-solubilizer in inoculants production.

Key words: Co-inoculation, *Azospirillum lipoferum*, *Bacillus megaterium*, *Rhizobium* sp., mixed inoculant

In developing countries, the cost of production and the hazards associated with the fertilizer industries are two serious limitations. These limitations urge people to constantly look for alternate sources of plant nutrients, both to augment and sustain the crop production. Biofertilizers, an outstanding possibility in this aspect, are products containing living cells of different types of microorganisms, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes [23]. Various groups of beneficial soil organisms, such as N-fixers, P-solubilizers, and vesicular arbuscular mycorrhizal (VAM) fungi, have been well exploited as biofertilizers for improving the fertility status of the soil. Bacteria of the genus *Azospirillum* are free-living nitrogen-fixing rhizobacteria, found in close association with plant roots, exerting beneficial effects on the plant growth and yield of many crops of agronomic importance [15]. *Rhizobium*, the most widely used biofertilizer, associated with the roots of legumes by forming nodules, can fix 100–300 kg N ha^{-1} in a season and leaves substantial amount of nitrogen for the succeeding crop. Efficient and economic use of phosphate fertilizers could be achieved using phosphate-solubilizing microorganisms (PSMs) in legumes, cereals, and other useful crops. Seed or soil inoculation with PSMs improves solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields [12].

As nitrogen and phosphorus are the two major plant nutrients, combined inoculation of N-fixers and PSMs may have greater benefit than either group of organisms alone. Dual inoculation of plants with N-fixer and PSM showed increased nodulation, growth, dry weight yield, and phosphorus and nitrogen uptake [21]. A significant positive effect on grain yield and acetylene reduction activity (ARA) in roots of barley was obtained by combined inoculation of N-fixers and P-solubilizers [6]: Combined inoculation of *Azospurillum* and P-solubilizer (*B. megaterium* var *phosphaticum*) showed higher shoot length, root length, 1,000 grain weight, and grain yield than those of individual inoculation of *Azospirillum* [2]. *Bacillus* spp. forms about 66% of the bacterial community in the rhizosphere soil of hot pepper [14] and they also exhibit multiple antagonistic properties against damping-off fungi, as a biocontrol agent [8].

In recent years, biofertilizers emerging as an important component of the integrated nutrient supply system have held

*Corresponding author
Phone: 82-43-261-2561; Fax: 82-43-271-5921;
E-mail: tomsa@chungbuk.ac.kr

a great promise to improve crop yields through environmentally better nutrient supplies. However, their application somehow has not achieved constant effects [25]. Also, establishment of a threshold population of viable inoculant is an important prerequisite for plant-microbe interactions like growth enhancement and biocontrol of bacteria [13]. Even though the N-fixers *Azospirillum*, *Rhizobium*, and PSM *Bacillus megaterium* are recommended to farmers, they are presently being produced and supplied as individual biofertilizer packets. The technology to combine the two organisms and produce a common inoculant, serving as N-fixer and P-solubilizer, will reduce production cost, facilitate application, and help promote the biofertilizer technology. The present experiment was undertaken to evaluate the performance of such bioformulations prepared with an N-fixer (*Azospirillum*/*Rhizobium*) and P-solubilizer (*Bacillus megaterium*) on the promotion of plant growth and improvement of soil properties under greenhouse conditions.

Azospirillum lipoferum strain Az204, *Rhizobium* strain BMBS P47, and *Bacillus megaterium* var *phosphaticum* strain Pb1 were obtained from the culture collection center of the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Individual cultures were grown on their respective broth with constant shaking at 120 rpm at 28±2°C. Glucose peptone agar [24], slightly modified by the addition of 5 g l^{-1} yeast extract, was used to prepare the mixed inoculant of *Azospirillum* and *Bacillus* strains (MI-1). Yeast extract mannitol agar with slight modification (mannitol reduced to 50% concentration and glycerol added at 5 ml l^{-1} of broth) was used for producing mixed inoculant containing *Rhizobium* and *Bacillus* strains (MI-2). At the time of application, the population of individual bacteria in lignite-based formulation was found to be 1.5×10^8 CFU g^{-1}. Blackgram (*Vigna mungo* L., cultivar MCU8) and pearl millet [*Pennisetum glaucum* (L.) R.Br., cultivar CoH Cu 8]

Table 1. Efficacy of individual and mixed bacterial inoculants on seed germination and seedling vigor of pearl millet.

Treatments	Germination per cent	Vigor index*	% increase over control
Control	90.0 (71.6)^b	2,722.8±105.0^c	-
Az204	93.2 (74.9)^b	3,022.5±152.0^b	11.0
Pb1	93.1 (75.0)^b	3,214.2±195.2^b	18.1
Az204+Pb1**	98.0 (82.1)^a	3,638.5±230.3^a	33.6
MI-1	98.3 (83.0)^a	3,722.0±293.2^a	36.7
LSD ($P\leq0.05$)	4.74	288.24	

Values are mean of three replications of 50 seeds each. Values in parentheses are arc sine transformed values. In the same column, significant differences according to LSD at $P\leq0.05$ level are indicated by different letters, and data followed by the same letter in a column are not significantly different from each other.

*Average of 10 seedlings; **Co-inoculation of *Azospirillum* and *Bacillus megaterium*; MI-1: Mixed inoculant prepared with *Azospirillum* and *Bacillus megaterium*.

Table 2. Efficacy of individual and mixed bacterial inoculants on seed germination and seedling vigor of blackgram.

Treatments	Germination per cent	Vigor index*	% increase over control
Control	92.0 (73.6)^c	2,036.4±17.2^b	-
BMBS P47	95.2 (77.5)^{bc}	2,,107.8±27.2^b	3.5
Pb1	95.2 (77.4)^{bc}	2127.4±107.2^b	4.5
BMBS P47+Pb1**	97.3 (81.3)^{ba}	2,541.5±161.0^a	24.8
MI-2	98.1 (82.2)^a	2,580.9±85.3^a	26.7
LSD ($P\leq0.05$)	4.04	246.28	

Values are mean of three replications of 50 seeds each. Values in parentheses are arc sine transformed values. In the same column, significant differences according to LSD at $P\leq0.05$ level are indicated by different letters, and data followed by the same letter in a column are not significantly different from each other.

*Average of 10 seedlings; **Seed treatment with *Rhizobium* and *Bacillus megaterium* mixed at the time of inoculation (co-inoculation); MI-2: mixed inoculant prepared with *Rhizobium* and *Bacillus megaterium*.

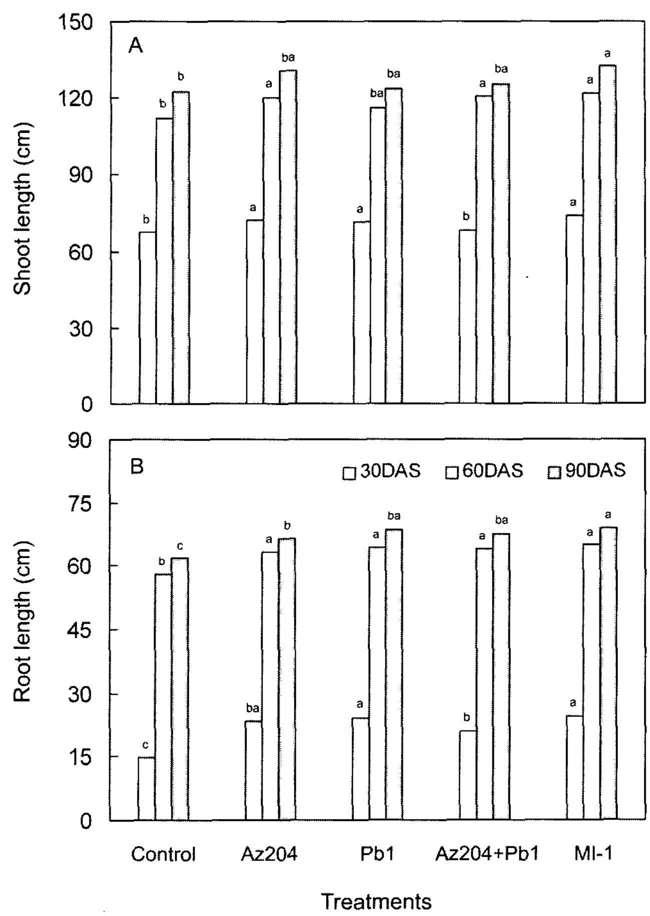

Fig. 1. Effect of individual and mixed inoculants of *Azospirillum* and *Bacillus megaterium* on the growth of shoot (A) and root length (B) of pearl millet.

Each value represents the mean of three replicates. Significant differences according to LSD at $P\leq0.05$ mentioned on the columns, and the data with the same letter are not significantly different from each other. The LSD values are 8.12, 7.66, and 8.83 (A) and 2.71, 2.46, and 2.15 (B) for 30, 60, and 90 DAS, respectively.

were used as test plants of MI-1 and MI-2, respectively, for both germination and pot-culture studies.

Surface sterilized seeds were treated with the bioformulations and sown in sterile aluminum trays (20×20×10 cm, 50 seeds/tray) containing fine moist sand, and then placed in a germination room and maintained at 20°C. Germination of the seeds was recorded daily from day 2 to day 7, and the vigor index was calculated as the product of percentage germination and seedling length in cm, and the results expressed in whole numbers [4].

In pearl millet, the highest effect on seedling vigor (3722.02) and germination percentage (98.31) was observed with the seeds treated with MI-1, followed by the co-inoculation treatment of Az204 and Pb1 (Table 1). The seeds that were not treated with any bioinoculant strain showed poor performance. The different combination of inoculants of BMBS P47 and Pb1 treatments had higher effect on seedling vigor and germination percentage of blackgram than individual inoculations and their control. Among the different individual and mixed bioinoculant treatments, MI-2 showed 82.22% germination and the highest seedling vigor of 2,580.94; it was a 26.74% increase over the control (Table 2). The treatments, mixed inoculant and individual inoculants mixed at the time of inoculation, showed no significant differences, supporting the notion that the use of mixed inoculants will reduce the time and risk involved in individual inoculant preparations.

Two pot-culture experiments were conducted to study the response of pearl millet to MI-1 (I) and blackgram to MI-2 (II), with the experimental design being completely randomized with three replications. Seed treatments with mixed inoculants improved the shoot and root growth of pearl millet. Although no significant variations existed between the treatments, they showed significant increases when compared to control. Treatment of seeds with MI-1 improved the shoot length (8.1% over control) and root length (10.93% over control), followed by Az204 individual inoculation treatment under pot-culture conditions in pearl millet (Figs. 1A and 1B). Increased growth of plants might have been due to increased efficiency of roots in extracting the nutrients from the soil that are made available by the activity of inoculated bacteria.

The efficacy of MI-1 was also superior in recording the maximum earhead length (15.2% over control), earhead weight (46.3% over control), and 100 grains weight (6%

Table 3. Effect of mixed bioinoculant on yield parameters of pearl millet.

Treatments	Earhead length (cm)	Earhead weight (g)	Grain yield (g/100 seed)
Control	12.5±0.71[b]	4.32±0.20[d]	1.67±0.09[b]
Az204	13.7±1.27[a]	5.32±0.15[b]	1.70±0.14[b]
Pb1	13.5±1.18[ab]	4.82±0.39[c]	1.70±0.15[b]
Az204+Pb1*	13.9±1.37[a]	6.03±0.20[a]	1.70±0.05[b]
MI-1	14.4±0.94[a]	6.32±0.39[a]	1.77±0.10[a]
LSD ($P\leq0.05$)	1.07	0.45	0.31

Each value represents mean±S.E of three replicates per treatment. In the same column, significant differences according to LSD at $P\leq0.05$ level are indicated by different letters, and data followed by the same letter in a column are not significantly different from each other.

*Seed treatment with *Azospirillum* and *Bacillus megaterium* mixed at the time of inoculation.

MI-1: Mixed inoculant prepared with *Azospirillum* and *Bacillus megaterium*.

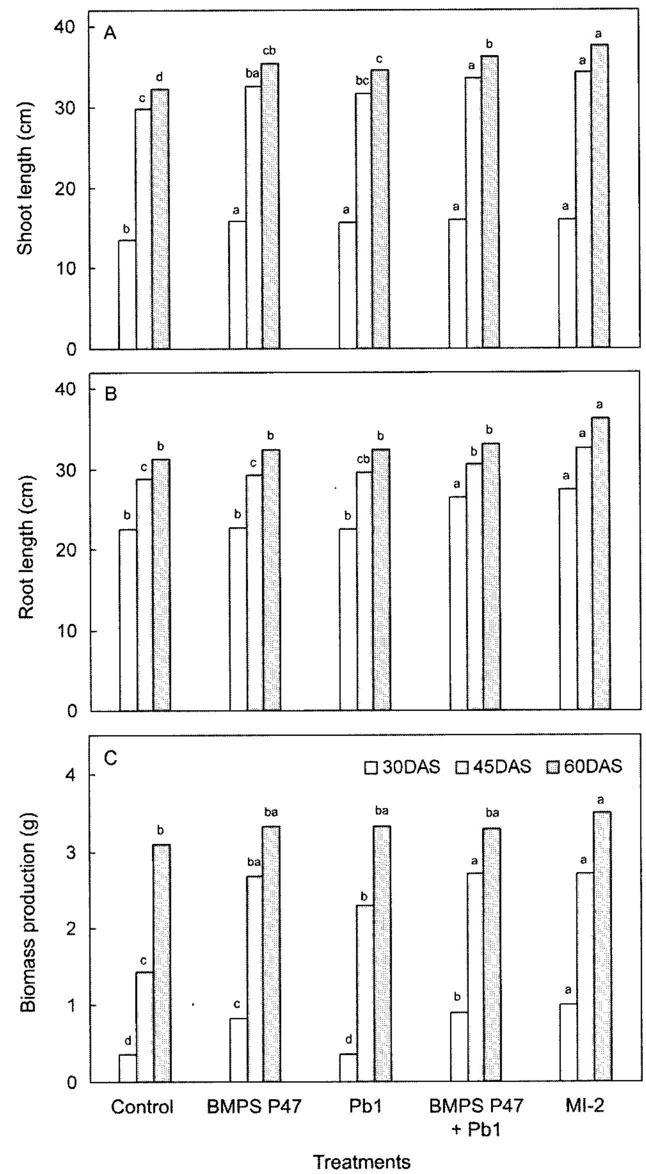

Fig. 2. Effect of individual and mixed inoculants of *Rhizobium* and *Bacillus megaterium* on the growth of shoot (A), root length (B), and biomass production (C) of blackgram.

Each value represents the mean of three replicates. Significant differences according to LSD at $P\leq0.05$ mentioned on the columns, and the data with the same letter are not significantly different from each other. The LSD values are 2.18, 1.81, and 1.07 (A), 2.18, 1.04, and 1.97 (B), and 0.06, 0.38, and 0.23 (C) for 30, 45, and 60 DAS, respectively.

over control), followed by other treatments in pearl millet (Table 3). The inoculation of the mixed inoculant stimulated the population of *A. lipoferum* and *B. megaterium* in the rhizosphere. The increase of the microbial population in the rhizosphere might have influenced the nutrient uptake; biomass and grain yield than single inoculation. Similar results have also been reported in cotton [18]. Belimov *et al.* [6] confirmed the above observation through isotope studies; combined inoculation of nitrogen fixing and phosphate-solubilizing organisms enhanced the absorption of P and N in barley plants. The release of soluble 'P' from inorganic and organic rock phosphate [5] and the plant growth-promoting substances produced by phosphobacteria [3] result in improved plant growth.

In the pot-culture experiment with blackgram, biometric observations such as shoot and root length and biomass increased significantly compared to control, although variations among them were not significant. Compared to root length, treated seeds showed significant differences in shoot length at 45 and 60 DAS within them, whereas the variations in biomass were minimum. An increase in shoot length (16.1% over control), root length (15.4% over control), and biomass production (9% over control) was observed with the inoculation of MI-2 (Figs. 2A, 2B and 2C). Also, the plants showed higher nodule number (130% over control), nodule dry weight (31.14% over control), number of pods/plant (23.1% over control), number of seed/pod (30% over control), and 100 seed weight (16.7% over control), followed by BMBS P47+Pb1 treatments (Table 4). The increase of yield may be attributed to a greater supply of available phosphorus to plants and rhizobia by phosphate-solubilizing bacteria and enhanced dinitrogen fixation by *Rhizobium*, influencing the synthesis and secretion of organic compounds by the plants, which also benefit these microorganisms [7].

Experiments on the enzyme activities of the rhizosphere soils revealed greater efficiency of mixed inoculants. Nitrogenase activity was estimated from the rhizosphere soils of pearl millet as described by Hardy *et al.* [11] and approximately six weeks after planting, and the nodule ARA was estimated from each treatment in blackgram [22]. The amount of ethylene formed was determined by injecting 1 ml of gas mixture into the preconditioned gas chromatography (GC) using Systronics 4010GC with a Porapak-Q column and FID detector. The phosphatase activity of rhizosphere soils was measured by determining the intensity of the p-nitrophenol formed [19]: The intensity of the p-nitrophenol formed by enzymatic oxidation was measured by reading absorbance at 420 nm using a UV spectrophotometer (Beckman DU-64). The urease activity was measured by reading absorbance at 440 nm, which indicates the amount of ammoniacal nitrogen (NH_4-N) catalyzed [20].

Dual inoculation of soybeans with mixed cultures of microorganisms stimulated nodulation and the nitrogenase activity of nodules and enhanced nitrogen fixation, as studied by ^{15}N dilution methods, in comparison to nodule bacteria alone [16]. Mixed inoculant of BMBS P47+Pb1 showed higher activities of nitrogenase (64.35 nmol of ethylene released/h/g of nodule), urease (808.72 μg NH_4-N/g of soil/day), and phosphatase (38.16 μg PNP/g of dry soil/sec) in rhizosphere soil (Figs. 3A, 3B and 3C). Increased nodulation and significant increase in nodule dry weight and nodule activity of blackgram were noted in the present study. The nodule-promoting efficiency in bean plants was mainly attributed to increased phosphate availability by phosphobacteria *P. putida* [10]. In green gram (*Vigna radiata*), the production of flavanoid-like compounds by plant roots, because of co-inoculation with rhizobacteria, probably augments nodule formation by bradyrhizobia [17]. Enhanced phosphate solubilization in the rhizosphere promoted the population of *Rhizobium tropici* as well as nodule formation, which increased nitrogen fixation and subsequently the growth of common bean [9]. Dual inoculation of PSB with *A. brasilense* increased the root-associated acetylene reduction activity and crop yield in sorghum plants over single inoculation of *Azospirillum* [1]. However, in the present study, the inoculation of MI-1 increased the rhizosphere soil enzyme activities such as nitrogenase

Table 4. Effect of mixed bioinoculant on yield parameters of blackgram.

Treatments	Nodule number (# per plant)	Nodule dry weight (mg)		No. of pods (# per plant)	No. of seed (# per pod)	100 seed weight (g)
		Per plant	Per nodule			
Control	7.7±0.32^{d}	12.1±1.46^{b}	1.58±0.11^{ba}	26.0±1.18^{c}	10.0±0.94^{b}	3.60±0.28^{c}
BMBS P47	11.0±0.94^{c}	15.6±1.70^{a}	1.42±0.07^{b}	30.0±1.74^{ba}	12.0±0.71^{ba}	3.80±0.33^{bc}
Pb1	7.3±0.63^{d}	11.8±0.86^{b}	1.61±0.12^{a}	28.0±0.94^{bc}	11.0±0.66^{ba}	3.70±0.24^{c}
BMBS P47+Pb1*	13.7±1.26^{b}	15.8±1.78^{a}	1.15±0.03^{c}	30.0±1.37^{ba}	12.5±0.24^{a}	4.00±0.19^{ba}
MI-2	17.7±1.26^{a}	15.9±1.35^{a}	0.90±0.03^{d}	32.0±2.69^{a}	13.0±1.60^{a}	4.20±0.28^{a}
LSD (P≤0.05)	1.62	1.44	0.18	2.73	2.00	0.22

Each value represents mean±S.E. of three replicates per treatment. In the same column, significant differences according to LSD at P≤0.05 levels are indicated by different letters, and data followed by the same letter in a column are not significantly different from each other.

*Seed treatment with *Rhizobium* and *Bacillus megaterium* mixed at the time of inoculation; MI-2: mixed inoculant prepared with *Rhizobium* and *Bacillus megaterium*.

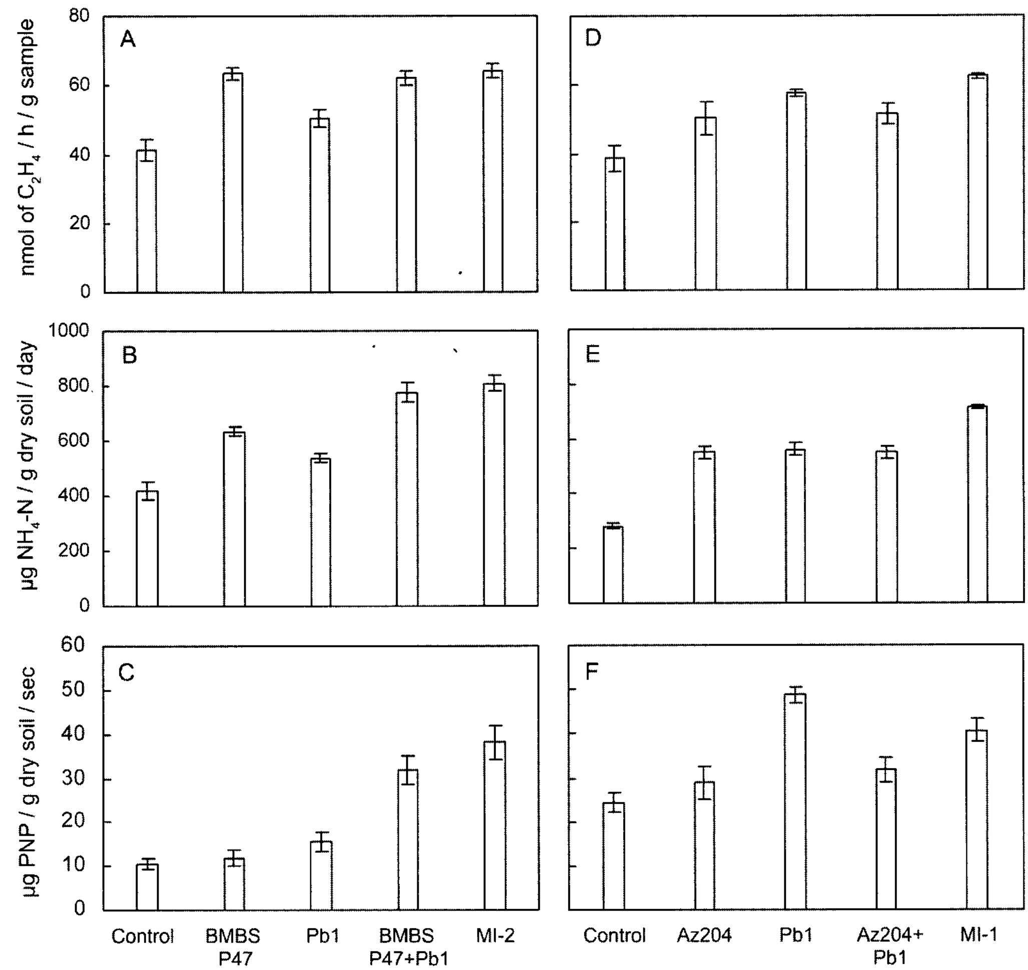

Fig. 3. Effect of individual and mixed inoculants on nitrogenase (A and D), urease (B and E), and phosphatase (C and F) activities in rhizosphere soils of blackgram and pearl millet.

The enzyme activities were compared between individual and mixed bacterial inoculants of *Azospirillum* and *Bacillus megaterium* in pearl millet and *Rhizobium* and *Bacillus megaterium* in blackgram. Each value represents the mean of three replicates. Error bar indicates ±SE.

(62.48 nmol of ethylene released/h/g of soil), urease (716.2 µg NH_4-N/g of soil/day), and phosphatase (40.59 µg PNP/g of dry soil/sec), when compared to control and *Azospirillum* individual inoculations. However, the activity of phosphatase was higher with Pb1 inoculation (48.83 µg PNP/g of dry soil/sec) than the mixed inoculant (Figs. 3D, 3E, and 3F). Therefore, the interactions between the various groups remain unexplained, and the variations may be attributed to different ecological factors and rhizosphere factors.

Although the several reports discussed above pointed out the beneficial effects of co-inoculating N-fixers and PSMs, we attempted in the present study to prepare mixed inoculants of both organisms. The studies clearly indicated that the combined inoculation of the N-fixers and P-solubilizers as a mixed inoculant increases the growth and yield attributes than individual inoculations. These results are expected to increase the possibility of using the mixed inoculant in achieving the dual goal of yield increase coupled with saving the cost involved in production. However, the experiments were conducted in pot cultures. Although pot experiments are associated with a large number of artifacts and have many limitations, they must be conducted prior to field inoculation trials. Furthermore, to evaluate the effects of bacterial inoculation, it is essential to conduct field experiments.

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