Phenetic Characterization of Nitrogen Fixing
Azotobacter from Rhizospheric Soil of Southern Rajasthan

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Abstract

The present research was conducted to characterize the indigenous plant growth promoting (PGP) Azotobacter strains isolated from plant root interface of semi-arid regions of Rajasthan (India) and to study their potential to be used as bio-fertilizers. A total of 172 Azotobacter strains were isolated, purified and based on the morphological test i.e. gram staining, pigmentation, cyst formation, fluorescence etc, broadly classified as Azotobacter. Further the secluded strains were examined for biochemical analysis and plant growth promoting characters. All the isolates showed different biochemical characteristics and significant PGP traits. IAA activity of the Azotobacter strains ranges from 54.5-6000 µg/mL. Ammonia, HCN and siderophore was produced by 92.4%, 78.4% and 80.23% of the total isolates respectively. Solubilization of phosphate was observed in 97.6% of the total isolates. These strains were also characterized for qualitative and quantitative N₂ fixation abilities and the result indicated that 114 strains showed positive results on nitrogen free malate agar medium (NFMM) containing bromothymol blue (BTB) and able to produce 18.93-475.6 N-moles C₂H₂ mg protein⁻¹ h⁻¹ of acetylene reduced by Azotobacter strains. In vitro pot studies revealed that the selected native Azotobacter strains having high ARA results significantly increase the plant growth characters. Shoot length, root length, root number and chlorophyll content and leaf number increases by 45.62%, 17.60%, 97.49%, 49.69% and 27.83% respectively in pot inoculated with AZO23-3 as compared to control. These effective strains can further be utilized for development of effective microbial formulations.

Keywords: - Biochemical Test, Cyst, Pigmentation, Antibiotic sensitivity, Acetylene Reductase Assay, Pot study

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(Received: September 29, 2020; accepted: December 16, 2020)

Citation: Jain D, Kaur G, Bhojiya AA, et al. Phenetic Characterization of Nitrogen Fixing Azotobacter from Rhizospheric Soil of Southern Rajasthan. J Pure Appl Microbiol. 2021;15(1): 428-436. doi:10.22207/JPAM.15.1.40

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Journal of Pure and Applied Microbiology 428 www.microbiologyjournal.org
INTRODUCTION

Excessive use of inorganic chemical-based fertilizers is not only cost intensive but also creates the problem of soil, agriculture and environmental management strategies. Organic farming is the most important component for improving plant, soil health and to overcome the uses of chemical fertilizers. To search an alternative approach to usage of chemical fertilizers, the use of biofertilizer is gaining momentum in agriculture. Biofertilizers are organic fertilizers, which are composed of beneficial living microbial inoculants. These microorganisms promotes growth of plants and the healthy structure of soils by converting nutritionally nitrogen, phosphorus, potassium and many essential elements from unavailable to available form for plants. Bio-inoculates also entail organic fertilizers and low input agricultural tool which manifest in an available form due to the symbiosis of micro-organisms and their association with plants.

One of important bio-inoculants is Azotobacter which is gram negative, aerobic and free-living N₂-fixing bacteria non-symbiotically fixes the atmospheric nitrogen, degrades cellulose, phosphates and most importantly it degrades lignin in trace amounts. It is oval or spherical in shape and capable for forming thick-walled cysts under favorable condition. Azotobacter strains binds with the beyond reach atmospheric nitrogen and liberate it in the form of ammonium ions which is utilized by plants to fix in the soil with an average of 20 kg N/ha per year. Moreover, Azotobacter is also able to multiply at 4.8-8.5 pH range and fixes atmospheric N₂ and the optimum pH range is 7.0-7.5. The other beneficial effects of Azotobacter is that it helps in increasing the overall nitrogenase activity of soil and also posses keratinolytic activity. The genus Azotobacter is an adjunct to Pseudomonadaceae family which is a subclass of γ-Proteobacteria. This subclass is comprehend of seven species: Azotobacter vinelandii, A. chroococcum, A. salinestris, A. nigricans, A. beijerinckii, A. paspali, and A.armeniacus. Azotobacter survives in soil for longer duration due to the formation of cyst. Azotobacter also stimulates plant growth by facilitating the plants to uptake essential nutrients from the surrounding and they also produces phytohormones like auxins, gibberellins, cytokinins. Therefore considering the above properties, Azotobacter sp. is used as a beneficial biological agent, extensively seek for a various crops like wheat, rice, sorghum, sugarcane, maize, sorghum, and maize. To develop commercial biofertilizer the initial steps are isolation and characterization of native Azotobacter strains. Consequently, this study was designed to isolate and characterize Azotobacter for their various plant growth promoting characteristics and N₂ fixing attributes.

MATERIAL AND METHODS

Isolation of Azotobacter sp

The Azotobacter sp. was isolated on Nitrogen free Jensen’s medium by serial dilution and spread plate technique. The serial soil dilution of soil samples were done upto 10⁻⁶ and afterwards spread on Jenson medium plates. The inoculated petriplates were kept in invert position for incubation at 28±2°C for about 48 h. The Azotobacter colonies were large, ovoid, pleomorphic in shape and were purified on Azotobacter medium and preserved in glycerol stocks.

Biochemical analysis of Azotobacter sp

Pure strains of Azotobacter sp. were isolated from soil samples and were characterized according to procedure mentioned in Bergey’s Manual of Systematic Bacteriology. The cyst formation was induced according to the procedure of Socolofsky and Wyss and the staining of cysts were done by the method of Vela and Wyss. Under biochemical characteristics, starch hydrolysis, H₂S production, TSI test, Nitrate reduction, Urease, Catalase and Oxidase reaction were studied. Carbon source i.e., glucose, mannitol, lactose and sucrose utilization were also assayed. Antibiotic sensitivity of Azotobacter isolates were determined by disk agar diffusion method using standard concentration of antibiotic discs of Ampicillin, Rifampicin, Kanamycin, Chloramphenicol and Penicillin.

PGP activities

IAA Production

Quantification measurement of IAA production was followed by the method as described by Gordon and Weber. The produced IAA concentration in the culture was measured by plotting the standard graph of IAA obtained in the range of 10-100μg/ ml.
NH₃ Production

Ammonia production was tested for Azotobacter strains as described by Cappuccino and Sherman²⁰. Overnight grown bacterial culture was inoculated in 10 ml peptone broth and incubated at 35±0.1°C for 48 h in shaking position. 0.5 mL of Nessler’s reagent was added after incubation.

HCN Production

Azotobacter culture was streaked on nutrient agar medium, which contained glycine (4.4g per liter) and in the same petriplate a 0.5% picric acid solution (in 2% sodium carbonate) soaked Whatman filter paper was placed inside the lid. These plates were sealed with parafilm and incubated at 25 ± 2°C for 4 days and the color change of filter paper was observed (Castric, 1977)²¹.

Siderophore Production

Siderophore production was assayed on the Chrome azurol S agar medium as described by Schwyn and Neilands²².

Phosphate solubilizing activity

The potential to solubilize insoluble phosphates on the modified Sperber’s medium was determined by measuring clear zone around the colonies²³.

Estimation of Nitrogen production and fixation

Qualitative estimation

For qualitative estimation of nitrogen production Nitrogen free Malate media, containing with bromothymol blue (BTB) as an indicator, was used for detection of nitrogen fixing activity. Plates were inoculated with purified isolates and incubated at 37°C up to 24 h. Those isolates producing blue colored zone on Nitrogen free Malate media were considered nitrogen fixers and they were further tested by quantification of nitrogen fixation by Kjeldahl and acetylene reduction activity (ARA) method.

Nitrogen Production

Total nitrogen fixation by the Azotobacter isolates in the growing medium was quantified by the method²⁴.

Total Nitrogen was calculated and expressed as % (percentage) using the formula:

Quantitative estimation by ARA

Quantification of nitrogen fixation was carried out by ARA assay following the method of Stewart et al.²⁵. Briefly, five ml of modified Ashby’s medium²⁶ were inoculated with Azotobacter and incubated at 30°C for 24 h. Further, the cotton plugs of the tubes were replaced with rubber stopper and 5 ml head space was injected with 10% (v/v) acetylene and incubated at 30°C for 2 h. The C₂H₄ production was measured using gas chromatography and the ARA values were recorded as N moles C₂H₄ mg protein⁻¹ h⁻¹.

In vitro studies on the effect of selected Azotobacter on growth of maize seedling

The pot experiment with selected Azotobacter strains having high nitrogenase activities were conducted in plastic pots filled with sterile perlite/vermiculite/coco-peat mixture in triplicate as per CRD design on maize (FEM-2 variety)²⁷. Various plant growth parameters like leaf number, chlorophyll content of leaf, average shoot length, root length and root numbers, were analyzed.

RESULT AND DISCUSSION

Isolation and characterization of the Azotobacter strains

Total of 172 Azotobacter strains were isolated from different rhizospheric soil samples on Azotobacter selected media. The phenetic characteristics of Azotobacter strains are summarized (supplementary sheet Table 1). Most of the bacterial colonies isolated were circular (even) in shape, whitish in colour and the size ranges between 1.0-4.0 mm in size Jimenez et al.²⁸, reported that most Azotobacter isolates showed circular form, entire margin with whitish (cream color), smooth, irregular, shining, 3-8mm diameter colonies. All the bacterial isolates were gram negative. Out of 172 isolates, 22 produced water soluble yellow pigment, 10 produced brown pigment, 2 isolates produced red pigment. Out of 172 isolates, 27 showed a green fluorescence, 3 presented a yellow fluorescence and 5 displayed a red fluorescence. Only two isolates did not form cyst, remaining 170 isolates formed cyst. Shaikh and Mohd. Shakir²⁹ performed cyst formation test on 39 Azotobacter vinelandii, and found that 38 out of 39 Azotobacter produced cyst.

All the bacterial isolates were characterized by detecting their biochemical characteristics. Results of the investigation results indicated that out of 172 isolates, 129 isolates were found to hydrolyze starch; 146 isolates were
### Table 1. Qualitative and quantitative estimation of nitrogen fixation by *Azotobacter* strains

| S.No. | Isolates   | N\textsubscript{2} Fixed (PPM) | Nmole\textsubscript{C\textsubscript{2}H\textsubscript{4}} mg protein\textsuperscript{-1} hr\textsuperscript{-1} |
|-------|------------|-------------------------------|--------------------------------------------------|
| 1.    | AZO1-1     | 48.95                         | 59.30159                                         |
| 2.    | AZO1-2     | 48.95                         | 87.79894                                         |
| 3.    | AZO1-4     | 80.68                         | 62.86772                                         |
| 4.    | AZO2-1     | 35.36                         | 56.95238                                         |
| 5.    | AZO2-3     | 123.73                        | 57.48148                                         |
| 6.    | AZO2-11    | 42.16                         | 49.24868                                         |
| 7.    | AZO2-12    | 395.65                        | 433.0931                                         |
| 8.    | AZO2-13    | 42.16                         | 34.08466                                         |
| 9.    | AZO2-15    | 42.16                         | 201.3968                                         |
| 10.   | AZO3-6     | 60.28                         | 27.30159                                         |
| 11.   | AZO3-7     | 80.68                         | 59.66138                                         |
| 12.   | AZO4-5     | 55.75                         | 50.74074                                         |
| 13.   | AZO4-7     | 28.56                         | 84.87831                                         |
| 14.   | AZO4-11    | 17.23                         | 125.1534                                         |
| 15.   | AZO5-2     | 67.08                         | 22.46651                                         |
| 16.   | AZO6-1     | 37.62                         | 30.7619                                          |
| 17.   | AZO6-2     | 89.74                         | 43.82011                                         |
| 18.   | AZO6-3     | 64.82                         | 76.8254                                          |
| 19.   | AZO7-1     | 53.49                         | 41.60847                                         |
| 20.   | AZO8-2     | 64.82                         | 399.7778                                         |
| 21.   | AZO10-1    | 85.21                         | 36.10582                                         |
| 22.   | AZO11-1    | 48.95                         | 22.67725                                         |
| 23.   | AZO11-2    | 37.62                         | 55.49206                                         |
| 24.   | AZO12-2    | 58.02                         | 22.35979                                         |
| 25.   | AZO13-1    | 53.49                         | 62.16931                                         |
| 26.   | AZO13-2    | 60.28                         | 29.8836                                         |
| 27.   | AZO14-1    | 151.22                        | 53.33333                                         |
| 28.   | AZO17-1    | 112.4                         | 51.78836                                         |
| 29.   | AZO17-4    | 53.49                         | 53.67196                                         |
| 30.   | AZO17-7    | 64.82                         | 55.24868                                         |
| 31.   | AZO17-8    | 80.68                         | 78.13757                                         |
| 32.   | AZO17-10   | 67.08                         | 56.78307                                         |
| 33.   | AZO17-11   | 78.41                         | 133.1217                                         |
| 34.   | AZO17-12   | 139.59                        | 41.01587                                         |
| 35.   | AZO19-1    | 80.68                         | 124.6243                                         |
| 36.   | AZO19-2    | 44.42                         | 43.60847                                         |
| 37.   | AZO20-1    | 123.73                        | 290.0106                                         |
| 38.   | AZO20-2    | 46.69                         | 372.836                                          |
| 39.   | AZO20-3    | 46.69                         | 107.9577                                         |
| 40.   | AZO21-1    | 105.6                         | 121.8201                                         |
| 41.   | AZO22-1    | 39.89                         | 304.6772                                         |
| 42.   | AZO23-2    | 193.98                        | 86.61376                                         |
| 43.   | AZO23-3    | 35.36                         | 475.6402                                         |
| 44.   | AZO23-6    | 114.67                        | 48.2963                                          |
| 45.   | AZO23-7    | 51.22                         | 39.84127                                         |
| 46.   | AZO25-1    | 68                            | 54.93122                                         |
| 47.   | AZO25-1    | 56.4                          | 54.93122                                         |
| 48.   | AZO25-1    | 79.32                         | 80.06349                                         |
found to produce H₂S; 163 isolates hydrolyze urea; 151 isolates gave positive reaction for oxidase test; 153 isolates reduces nitrate and 117 isolates gave positive reaction for TSI test. All the isolates gave positive reaction for catalase activity. The results of biochemical properties of Azotobacter species observed by Tejera et al.30 were in close agreement with the results observed in the present study. These findings clearly indicated the distribution of Azotobacter species in different soil ecosystem. Ahmad et al.31 studied the biochemical tests in Azotobacter species and reported that Azotobacter are positive for Catalase, and Starch hydrolysis. Similarly, Shrivastava32 also reported all the isolates were positive for catalase. Most of the Azotobacter isolates utilize sucrose, lactose, mannitol and glucose as carbon source used in the present study. Out of 172 isolates, 18 isolates utilize 4 sugars; 13 isolates utilize 3 sugars; 42 isolates utilize 2 sugars and 78 isolates utilize 1 sugar as carbon source. For antibiotic resistance, 172 isolates of Azotobacter strains were tested by the antibiotic disk method on Jenson medium plates. Table 2 in supplementary table sheet shows the resistance pattern of 172 Azotobacter isolates against Ampicillin, Rifampicin, Kanamycin, Chloramphenicol and Penicillin antibiotics discs. Multiple antibiotic resistances were seen in a majority of the isolates. Out of 172 strain 105 isolates showed sensitivity and 87 shows resistance against Ampicillin. Only 3 strains showed resistance and 169 were sensitive for Rifampicin. 122 isolates were sensitive and 50 are resistant for Kanamycin. Only one strain out of 172 shows resistance for Chloramphenicol. 85 Azotobacter strains were found resistant to penicillin while 87 were sensitive to penicillin. Bhaduri et al.32 studied among 18 isolates of Asymbiotic N₂-fixers (Azotobacter) and symbiotic N₂-fixers (Rhizobium) spp for the antibiotic resistance and susceptibility of against different antibiotics including Chloramphenicol and Rifampicin. The antibiotics ampicillin, chloramphenicol, kanamycin, penicillin and rifampicin resistance tested on 117 isolates of A. chroococcum were tested by the antibiotic disc method by Sindhu et al.33.

Plant growth promoting (PGP) activities

Pink color appearance in bacterial culture after addition of orthophosphoric acid and Salkowski reagent to supernatant in LB broth confirmed that bacterial strains are positive for IAA production. In the present study IAA activity ranges from 54.5-6000µg/mL. Maximum IAA production 6000µg/ml was observed for isolate AZO 17-6 followed by 1500µg/mL for isolates AZO1-1, AZO1-3, AZO2-8, AZO4-11, AZO17-2, AZO17-3, AZO17-4, AZO22-2 and AZO22-4. Minimum IAA production 54.5µg/mL was observed for isolate AZO 53-1. Ahmad et al.35 reported that Azotobacter spp produced 38.82 µg/mL IAA in culture medium supplemented with Tryptophan at the rate of 5mg/

| S.No. | Treatment | Average Shoot Length (cm) | Average Root Length(cm) | Average Root Number | Average Leaf number | Total Chlorophyll (µg/ml) |
|-------|-----------|---------------------------|-------------------------|---------------------|---------------------|--------------------------|
| 1     | Control   | 13.7±0.12                 | 34.6±0.66               | 9.96±0.58           | 6±1.0               | 35.74±0.95               |
| 2     | AZO2-12   | 18.75±0.20                | 39.7±0.95              | 18.93±1.15          | 6.67±0.58           | 51.57±1.90               |
| 3     | AZO8.2    | 17.12±0.50                | 37.37±0.96             | 13.67±0.58          | 6.67±1.53           | 46.76±1.49               |
| 4     | AZO22-1   | 15.59±0.38                | 36.75±1.05             | 14.67±0.58          | 6.0±0.0             | 39.91±1.20               |
| 5     | AZO23-3   | 19.95±0.51                | 40.69±1.03             | 19.67±0.58          | 7.67±0.58           | 53.50±1.15               |
| 6     | AZO6A1    | 16.56±0.52                | 37.12±0.98             | 15.33±1.53          | 6.0±0.58            | 45.72±1.27               |

Value ± SD; Data are recorded after 30 days of germination.
mL. Kannapiran and Ramkumar\textsuperscript{36} have reported that the amount of IAA produced by \textit{Azotobacter chroococcum} and \textit{Azotobacter beijerinckii} were 23.6 and 17.6 μg/mL respectively. Deshmukh and Vidhale\textsuperscript{37} isolated four \textit{Azotobacter} species from the rhizospheric soil in the alluvial valley of Purna river and its tributaries occupy parts of Amravati and found to produce high level (9.2 to 40.0 mg/mL) of IAA. According to Mali and Bodhankar\textsuperscript{38}, 25 isolates of \textit{Azotobacter chroococcum} isolated from the rhizosphere soil of groundnut were tested for production of IAA in 55 μg/mL.

The production of ammonia is an essential PGPR trait that can influence the plants growth indirectly. Estimation of ammonia production examined by the development of faint yellow to dark brown color after addition of Nessler’s reagent to overnight grown bacterial culture in peptone broth indicated 92.4% ammonia production by the bacterial isolates. These results are similar to those of Ahmad et al.\textsuperscript{39}, who revealed the production of ammonia commonly detected in the \textit{Azotobacter} isolates. HCN production was indicated by the change in yellow to brown color of the filter paper strips. Out of 172 isolates, 78.4% isolates gave positive reaction for HCN production. For siderophore production, formation of yellow orange halo around the colony on CAS agar medium showed production, formation of yellow orange halo reaction for HCN production. For siderophore production 114 isolates were studied on FEM-2 medium indicating fixation of nitrogen by them. Further, these 114 \textit{Azotobacter} isolates were quantified for nitrogen production by Kjeldahl method. Nitrogen production ranges from 17.23-395.6 ppm. Maximum nitrogen production 395.6 ppm was observed for isolate AZO2-12 followed by 198 ppm for isolate AZO34-2. Minimum nitrogen production 17.23 ppm was observed for isolate AZO4-11.

All the 114 \textit{Azotobacter} isolates were screened for acetylene reduction ability (ARA). The comparison of nitrogenase activity of all efficient nitrogen-fixing isolates is shown in Table 1. All the isolates showed nitrogenase activity ranging from 18.93-475.6 Nmoles C\textsubscript{2}H\textsubscript{4} mg protein\textsuperscript{-1} hr\textsuperscript{-1}. Maximum nitrogenase activity was observed for isolate AZO23-3 \textit{i.e} 475.6 Nmoles C\textsubscript{2}H\textsubscript{4} mg protein\textsuperscript{-1} hr\textsuperscript{-1} and of AZO DP-5 was observed to be the least \textit{i.e} 18.93 Nmoles C\textsubscript{2}H\textsubscript{4} mg protein\textsuperscript{-1} hr\textsuperscript{-1}. In present study the obtained ARA ranges were significantly higher than other observations and corroborated with Rodelas et al.\textsuperscript{42} who reported the ARA results of pure cultures of strains were between 9.70 to 257.73 nmol C\textsubscript{2}H\textsubscript{4} h\textsuperscript{-1} vial\textsuperscript{-1}. According to Tejera et al.\textsuperscript{28} \textit{A. chroococcum} isolates from soil showed ARA production within ranged between 79.6 to 329.5 nmol C\textsubscript{2}H\textsubscript{4} h\textsuperscript{-1} culture.

Nitrogen fixation potentiality of the selected isolates of \textit{Azotobacter} sp was estimated by Akhter et al.\textsuperscript{43}, ranging from 04.95 to 10.55 mg N/g substrate. Kizilkya\textsuperscript{44} reported that the \textit{Azotobacter} fix nitrogen in the range of 3.50 to 29.35 μg N mL\textsuperscript{-1} and yield of maize seedling

\textbf{Estimation of Nitrogen production and fixation}

\textit{Azotobacter} strains were preliminary screened for nitrogen fixation on nitrogen free Malate agar medium containing Bromothymol Blue (BTB) as an indicator. Out of 172 bacterial isolates, 114 isolates produced blue color zone on medium indicating fixation of nitrogen by them. Further, these 114 \textit{Azotobacter} isolates were quantified for nitrogen production by Kjeldahl method. Nitrogen production ranges from 17.23-395.6 ppm. Maximum nitrogen production 395.6 ppm was observed for isolate AZO2-12 followed by 198 ppm for isolate AZO34-2. Minimum nitrogen production 17.23 ppm was observed for isolate AZO4-11.

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\textbf{Studies of In vitro effect of Azotobacter on growth and yield of maize seedling}

The experiment of pot culture was conducted in plastic pots filled with sterile planting mixture. Plant growth promoting activity of \textit{Azotobacter} isolates were studied on FEM-2 variety of Maize. Cultivable seeds were treated with \textit{Azotobacter} inoculant thorough seed bacterization method and sown. Five \textit{Azotobacter} strains with higher ARA values were selected for pot studies. Pot experiment data were recorded after 30 days of germination were summarized in Table 2.

O Maize seedlings raised from bacterized seeds with selected \textit{Azotobacter} isolates, observed higher growth in treated ones as compared with absolute control. This indicates the positive effect
of *Azotobacter* strains on maize plantlet. Leaf number, total chlorophyll content, Shoot length, root number, root length, have been significantly increased in the maize plantlet inoculated with the selected *Azotobacter* strains as compared to uninoculated control.

Shoot length, root length, root number and chlorophyll content increases by 45.62%, 17.60%, 97.49% and 49.69% respectively in pot inoculated with AZO23-3 followed by AZO2-12 (36.86%, 14.74%, 90.06% and 44.29%, respectively). Leaf number increases by 27.83% in pot inoculated with AZO23-3 followed by AZO2-12 and AZO8.2 (11.17%). All the *Azotobacter* strains significantly influenced the observed parameters as compared to uninoculated control and contributed to plant growth. Similar finding was observed by Mahato and Neupane who reported *Azotobacter* seed bacterization in maize promote the growth of treated plants and find increased growth in root and shoot lengths. Perdomo et al. reported *Azotobacter* bacterial inoculation in cotton positively influenced plant growth parameters and reduced 50% nitrogen fertilization doses. These findings advocated the application of *Azotobacter* for improvement of plant growth due to their intrinsic abilities of fixing atmospheric nitrogen and expressing plant growth-promoting substances.

The plant growth promoting traits and the nitrogen fixation by the local *Azotobacter* strains is a very critical for the selection of such strains for biofertilizer formulations in order to replace the ineffective strains. The results indicated that inoculation by multi PGP *Azotobacter* strains significantly improved the plant growth under *in vitro* condition and may be used for commercial production. Hence, the dedicated field studies are required to confirm the efficacy of these *Azotobacter* strains.

**ACKNOWLEDGMENTS**

The financial assistance from All India Network Project on soil biodiversity and biofertilizers and Rastriya Krishi Vikas Yojana (RKVY) research project are highly acknowledged. The support of Dean, RCA and Director of Research, MPUAT is also acknowledged.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORS’ CONTRIBUTION**

DJ conceived and designed the experiments; GK, AAB, SC performed laboratory experiments; DJ, SKK, RHM and DR wrote the manuscript. All authors read and approved the final manuscript.

**FUNDING**

This study was supported by the AINP-SBB grant (10-t-37) from ICAR, New Delhi and RKVY grants (10-CSA-65A-V) from Government of Rajasthan.

**DATA AVAILABILITY**

All datasets generated or analyzed during this study are included in the manuscript and also in the Supplementary Files.

**ETHICS STATEMENT**

This article does not contain any studies with human participants or animals performed by any of the authors.

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**SUPPLEMENTARY INFORMATION**

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.15.1.40

Additional file: Additional Table S1- S2.
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