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Efficient Native Plant Growth Promoting Rhizobacteria and their Role in Plant Growth Promotion and Management of Damping off Disease in Cowpea

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ABSTRACT: Damping-off and collar rot is one of the most important diseases of cowpea causing great menace in cowpea production. Application of chemical fungicides is still being a commonly used approach and rules among all the management approaches. Continuous and non-judicious application of chemical fungicides not only causes environmental hazards and residual toxicity related problems but also may trigger fungicide resistance problem. Disease containment through ecofriendly biocontrol approach, using natural antagonistic plant growth promoting rhizobacteria (PGPR) is now becoming an inevitable component in the integrated management strategy of the disease. Thus, research study was conducted to isolate and evaluate the potent native plant growth promoting rhizobacteria (PGPR) isolates, their potential use for improving growth, yield and management of damping off disease in cow pea. Six potent PGPR strains were isolated and studied for their plant growth promotion, their molecular identification at genus level and management of damping off disease in cowpea. All of the PGPR isolates influenced cowpea growth characteristics. Application of PGPR strains significantly increased root and shoot length, root and shoot biomass by 5.4–53.4%, 9.8–48.6% and 10.8–64.5% and 27.8–103.8% respectively, over the un-inoculated control. Among the six potent PGPR strains Hu3, Hu4, Hu9, Hu14, Hu18 and Hu19; Hu3, Hu18 and Hu19 rhizobacterial isolates were found to be the most effective isolates for rhizosphere competence and plant growth promotion of cowpea seedlings. Also, these isolates Hu3 and Hu18 were more effective in suppressing the pre & post emergence incidence of damping off as compared to other rhizobacteria. The seed and soil treatment with Hu3 and Hu18 rhizobacteria recorded minimum pre-emergence damping-off disease of 5.56 percent in comparison to control. The seed and soil treatment with Hu3 recorded significantly least post-emergence damping off (13.78 percent) comparison to control pot having 50% damping off. The seed and soil treatment with Hu3 recorded significantly maximum (5.33 cm) root length and Hu18 isolate recorded significantly maximum shoot length (21.07 cm) compared to sick pot. Molecular characterization of these isolates showed that these native PGPR rhizobacteria Hu3, Hu9, Hu14, Hu18 and Hu19 were identified as *Bacillus* spp. based on *Bacillus* spp. specific primers. The bacterial bioagents Hu3, Hu18 and Hu19 showed effective results in reducing pre- and post-emergence damping-off disease in *S. rolfsii* pathogen infested soils and also may lead to more seedling vigour and also application of these PGPR rhizobacterial isolates could be a viable supplementary strategy for field level application for maximum benefits through alleviation of biotic stresses and enhancing sustainable crop production.

Keywords: Plant growth promoting rhizobacteria (PGPR), Damping off disease, Plant health management.

INTRODUCTION

Cowpea (*Vigna unguiculata*) is one of the important multipurpose legumes grown in different parts of India as pulse, vegetable, fodder and green manuring crop. Despite the importance of cowpea cultivation throughout the world, abiotic and biotic restrictions are key yield limiting factors, particularly in developing nations where majority of the production occurs. This crop is attacked by numerous pathogens (fungi, bacteria, viruses, nematodes and parasitic plants) constituting major biotic limitations to cowpea productivity in all areas where the crop is grown. These diseases can infect cowpea at many stages, including emergence, vegetative and reproductive stages, producing significant plant damage and yield loss or

full production failure (Singh *et al.,* 1979). The greatest losses in cowpea production occur because of seed decay and seedling damping off (Emechebe and Shoyinka 1985). Damping- off occurs in seedlings before and after emergence, and is induced by a number of pathogens including *Sclerotium rolfsii* (Sacc.), *Macrophomina phaseolina* (Tassi.), and *Rhizoctonia solani* (Kuhn.). It is very difficult and cumbersome to manage these notorious soil borne plant pathogens**.** The principal disease symptoms include yellowing of plant leaves and the development of dark brown lesions at the collar region close to the soil line, which ultimately cause the entire plant to wilt (Mahadevakumar *et al.,* 2018). It produces massive sclerotia, which remain in the soil for many years as infected plant debris. Management of this pathogen is complicated because of its broad geographical host range. The pathogen control may be achieved by applying tremendous volume of fungicides but their extreme usage possess harmful impacts on environment as well as on human health (Keinath and DuBose 2017). Therefore, biocontrol agents and plant-based solutions can be used as an inexpensive and environmentally friendly substitute for synthetic disease management methods (Wankhade *et al.,* 2019). Because of their capacity to encourage plant development and soil health, PGPR have recently received a lot of attention in the modern agriculture system (Mohamed *et al.,* 2019). To effectively control these soil borne pathogens, the very promising approach is exploitation of the potent biocontrol agents which have the disease suppression capability and plant growth promoting traits. Plant growth promoting rhizobacteria (PGPR) are a class of biocontrol agents that have been widely used for the bio-suppression of different soilborne diseases (Lugtenberg *et al.,* 2009). These PGPR bacteria are soil-borne bacteria that colonize the rhizosphere/plant roots aggressively and, when administered to seed or crops, improve plant development and yield (Kloepper *et al.,* 1980). These rhizosphere bacteria can directly or indirectly promote plant growth and yield. Direct mechanisms of plant growth promotion may include bacterial compound production or facilitation of nutrient uptake from the environment (Glick *et al*., 1999). Indirect plant growth promotion occurs when PGPR reduces or prevents the harmful effects of plant diseases on plants by producing inhibitory chemicals or strengthening the host's inherent resistance (Persello Cartieaux *et al*., 2003). The following are the direct growth-promoting mechanisms: (i) nitrogen fixation; (ii) phosphorus solubilization; (iii) iron sequestration via siderophores; and (iv) phytohormone synthesis such as auxins (indole acetic acid (IAA)), cytokinins, gibberellins, and (v) ethylene concentration reduction (Kloepper *et al*., 1989). The indirect mechanisms of plant growth promotion by PGPR include (i) antibiotic production; (ii) rhizosphere iron depletion; (iii) synthesis of antifungal compounds; (iv) development of fungal cell wall lysing enzymes; (v) competition for root site locations; and (vi) induced systemic resistance (Glick *et al.,* 1999). Functionally, PGPR incorporates various direct and indirect mechanisms such as plant-microbe symbiosis, increased plant nutrient absorption, develops colonization space competition and decreased plant pathogen activity (Lugtenberg *et al.,* 2002). Pathogen suppression through PGPR's is accomplished through the production of enzymes (chitinase, protease, cellulase), antibiotics, volatile organic compounds, hydrogen cyanide and ammonia (Saharan and Nehra, 2011). PGPR's exert biocontrol effects by antagonistic action, signal interference, quorum sensing inhibition, biofilm formation inhibition, induced systemic resistance and systemic acquired resistance etc (Patten and Glick, 1996). In addition to their ability to suppress the plant pathogens, these bacteria have the capacity to decompose organic matter in soil which plays an important role in plant production (Mohamed *et al*., 2019), also provide nutrients to the plant and enhance their growth (Xiang *et al.,* 2017). PGPR's isolated and screened from rhizospheric soils have been used as agricultural inputs to increase plant development and yields by lowering plant diseases (biological control) and have been commercialized as pesticide alternatives, microbial bio-inoculants and bio-fertilizers (Adesemoye and Kloepper 2008). Keeping in view the beneficial effects of PGPR, an experimental study was conducted to isolate, identify the bacterial strains and to evaluate their efficiency as PGPR on growth performance and damping off disease suppression caused by *Sclerotium rolfsii* in cowpea under laboratory and greenhouse conditions.

MATERIAL AND METHODS

Isolation of rhizospheric bacteria:

Soil samples were collected from different field crops like pointed gourd, paddy and other forest, pasture lands and also grass. Isolation of rhizospheric bacteria was done by soil-dilution plate technique using nutrient agar medium. Colony count was taken after 72 hours of incubation at 28–30°C. Different types of colonies appeared in Nutrient agar medium and individual characteristic colony was picked followed by streaking in the same agar plates to attain single isolated colony. In this way, 75 pure cultures were obtained. Out of which six pure cultures Hu3, Hu4, Hu9, Hu14, Hu18, and Hu19 were selected and evaluated for plant growth promotion ability and their potentiality to act as biocontrol agent for damping off disease suppressiveness. These cultures were maintained in nutrient agar slants for further experiments.

Pot experiments for evaluating plant growth promoting potentiality of native rhizobacteria:

Layout, Design and Treatments. In this experiment, six native PGPR bacteria were applied along with one control (water treated) and the total seven treatment combinations were laid out with three replications in a Randomized Block Design.

Seed treatment with native rhizobacteria. The native rhizobacteria were mass multiplied by inoculating on sterilized nutrient broth media and incubated at 30°C in an incubator for 48 hours. After 48 hours of incubation, the broth was centrifuged for 20 minutes at 10,000 rpm/min to sediment the bacterial cells. The pellet was then centrifuged in distilled water for 10 minutes at 10,000 rpm. The bacterial cell pellet was then adjusted to 1×10^8 cfu/ml using a UV spectrophotometer set to 620 nm. Seeds were treated by soaking them in bacterial suspension for one hour at a temperature of 25 ± 2°C and air dried before being sown in the pot. The effect of seed treatment on growth of the plant was studied. Seeds soaked in distilled water were served as control. Pot trials were conducted in the net house of the Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, West Bengal. Surface sterilized cowpea seeds (cv. Kashi Kanchan) were treated with six different bacterial suspension and seeds treated with water served as control. Twelve cowpea seeds were sown in each pot containing 1.5 kg of soil per pot. Pots were placed in the net house under normal lighting and temperature conditions. The treatment details are given below: T1: Seeds treated with rhizobacteria Hu3; T2: Seeds treated with rhizobacteria Hu4; T3: Seeds treated with rhizobacteria Hu9; T4: Seeds treated with rhizobacteria Hu14; T5: Seeds treated with rhizobacteria Hu18; T6: Seeds treated with rhizobacteria Hu19; T7: Seeds treated with water served as control. Every day, both treated and untreated pots were irrigated with sterilized water. The plants were uprooted at 14 days after sowing and root length, shoot length and root and shoot biomass data were recorded.

Pot experiments for evaluating rhizobacteria mediated damping off disease suppression. The inoculum of *Sclerotium rolfsii* – the pathogenic fungus that cause the damping off and collar rot disease on cowpea seedlings was prepared on sand maize meal media (1 part partially broken maize grain + 3 part sand + distilled water to moisten the media). The flasks containing the sterilized media were inoculated with mycelial disc of *S. rolfsii* (6 mm diameter) and incubated at 26°C for 14 days. This inoculum was used for soil inoculation at 25 g kg^{-1} soil in all the pot experiments. The pathogen inoculated pots were kept under moist condition for 3 days and after that the rhizobacterial suspension of 10 ml were added in different treatments, and treatment details are mentioned below: T1: *S. rolfsii* pre-inoculated soil + seed and soil treatment with rhizobacteria Hu3; T2: *S. rolfsii* pre-inoculated soil + seed and soil treatment with rhizobacteria Hu4; T3: *S. rolfsii* pre-inoculated soil + seed and soil treatment with rhizobacteria Hu9; T4: *S. rolfsii* preinoculated soil + seed and soil treatment with rhizobacteria Hu14; T5: *S. rolfsii* preinoculated soil + seed and soil treatment with rhizobacteria Hu18; T6: *S. rolfsii* preinoculated soil + seed and soil treatment with rhizobacteria Hu19; T7: *S. rolfsii* preinoculated soil + seed and soil treatment with water. Surface sterilized cowpea seeds (cv. Kashi Kanchan) were treated with six different bacterial suspension and water treated seeds served as control. Twelve cowpea seeds were sown in each pot containing 1.5 kg of soil per pot. In the net house, pots were placed under ambient light and temperature. Every day, both treated and untreated pots were irrigated with sterilized water. The pre and post emergence damping off of seedlings data were taken upto 14 days and the plants were uprooted at 14 days after sowing and root length, shoot length and root and shoot biomass data were also recorded.

Molecular characterization of the rhizobacteria:

DNA isolation. Genomic DNA of the selected rhizobacterial isolates was extracted using Proteinase K lysis protocol (Shahriar *et al.,* 2011). Overnight growth bacterial culture of 2ml was centrifuged at 10,000 rpm for 5 min. Supernatant was removed. The step is repeated for one to two times. 50µl Proteinase K was added. The centrifuge tubes were placed in water bath at 54ºC for 15 min followed by 80ºC for 15 min. The tubes were transferred immediately to cold ice 0ºC for 5 min. After that, it was centrifuged at 13,000 rpm for 5 min. The supernatant was collected to the new centrifuge tube for further use.

Gel Electrophoresis and PCR amplification. To prepare 20 ml of 1% agarose gel, 0.2 gm of pure agarose was weighed and mixed with 20 ml of TBE buffer in a conical flask. The flask was then heated for 1 minute to mix the agarose properly. It was cooled down and 2 µl of ethidium bromide was mixed and shaken well for mixing. Then slightly warm agarose solution was poured into the mold. While agarose solution was cooling, an appropriate comb was selected for forming the sample slot in the gel. Gel should be allowed to set completely (30-45 mins at room temperature). Then the comb was removed and the gel was immersed on the electrophoretic apparatus. Then the sample mixture (3 µl loading dye and 5 µl sample) were loaded slowly into the slots of submerged gel using a micropipette. A marker (4 µL) was also loaded on the gel. The lid of the gel tank was closed and attached the electrical leads so that the sample will migrate towards the positive anode. The required voltage was applied then. When the samples were migrated to a sufficient distance through the gel then the electric current should be turned off. After that, the bands were captured. The rhizobacterial isolates were identified by amplifying the genomic DNA at an annealing temperature 65°C with Bacillus specific primer (Bac F GGGAAACCGGGGCTAATACCGGAT and R 1378r CGGTGTGTACAAGGCCCGGGAACG) (Garbeva *et al.,* 2003).

RESULTS AND DISCUSSION

Plant Growth Parameters induced by native rhizobacteria. The six potent rhizobacterial isolates (Hu3, Hu4, Hu9, Hu14, Hu18, and Hu19) were selected

through in-vitro studies based on plant growth promotion potentialities and secondary metabolites production abilities. All of the rhizobacterial isolates influenced cowpea growth characteristics (Table 1). The increase in germination percentage due to seed bacterization with native rhizobacterial isolates ranged between 3.35 – 13.78% over un-inoculated control. The relative increase in root and shoot length due to bacterial isolates ranged between 5.4–53.4% and 9.8–48.6%, respectively, over the un-inoculated control while the subsequent increase in the root and shoot biomass ranged between 10.8–64.5% and 27.8–103.8%, respectively. Hu3, Hu18 and Hu19 rhizobacterial isolates were found to be the most effective isolates for rhizosphere competence and plant growth promotion of cowpea seedlings. When seeds were treated with the six potent rhizobacterial isolates, highest germination percentage was recorded in HU4 treated seeds, whereas, highest root and shoot length were recorded in HU3 and HU18 treated plants, respectively while highest fresh root weight and shoot weight were observed in HU18 and HU19 treated cowpea seedlings, respectively.

Table 1: Germination percentage and plant growth parameters of cowpea seeds treated with native PGPR rhizobacterial isolates.

Treatments	Isolate	Germination (%)	Root Length (in cm)	Shoot Length (in cm)	Root Fresh Wt (in g)	Shoot Fresh Wt (in g)
T1	HU3	88.9(70.5)	4.80	19.47	0.147	1.713
T2	HU4	91.7(73.2)	3.30	16.10	0.120	1.383
T3	HU9	83.3(65.9)	3.50	15.63	0.107	1.343
T4	HU14	86.1(68.1)	3.30	14.60	0.103	1.213
T5	HU18	86.1(68.1)	4.53	19.78	0.153	1.843
T6	HU19	86.1(68.1)	3.50	19.23	0.127	1.933
T7	Water treated control	80.6(63.8)	3.13	13.30	0.093	0.947
SeM ±		**4.27**	**0.21**	**0.60**	**0.01**	**0.12**
CD (P=0.05)		**NS**	**0.64**	**1.86**	**0.03**	**0.37**

All the native rhizobacterial isolates significantly increased root and shoot length, root and shoot dry weight, and also improved the germination percentage and vigour index of inoculated cowpea seedlings. Plant growth promotion could be the outcome of the beneficial functions of the applied PGPR isolates, such as nitrogen fixation, plant growth hormone synthesis and P solubilization. As the inoculated plants received no extra N or soluble P, the growth promotion can be attributed to the bacterial-assisted growth enhancement phenomena. It is preferable to inoculate PGPR with multi-functional traits rather than single trait (Imran *et al.,* 2014). IAA is involved in cell division, cell enlargement, and root initiation; it increases root surface area and, as a result, access to soil nutrients through improved root development (Dey *et al.*, 2004). Along with P-solubilization (Rajput *et al.,* 2013), auxin production has been recommended as a primary strategy of promoting early growth in wheat (Khalid *et al.*, 2004). Plant's responses to different isolates varied, which could be linked to individual characteristics and rhizospheric abilities. The large increase in growth, both in shoot and root, following isolate application is obvious evidence that the bacterial isolates were able to offer greater nutrient flow to the plant host, resulting in an increase in plant biomass. Thus, from the present investigation it may be concluded that Hu3, Hu18 and Hu19 rhizobacterial isolates may be exploited for plant growth promotion of cowpea seedlings.

Plant health management & damping off disease suppression mediated by native rhizobacteria. The pre and post emergence incidence of damping off was notably reduced in response to seed and soil treatment with native rhizobacteria as compared to control (Table 2). The study also showed that the rhizobacteria Hu3 and Hu18 were more effective in suppressing the pre & post emergence incidence of damping off as compared to other rhizobacteria evaluated in the present investigation. The seed and soil treatment with Hu3 and Hu18 rhizobacteria recorded minimum pre-emergence damping-off disease of 5.56 percent in comparison to control pot having 19.44 percent of pre-emergence damping-off disease. The seed and soil treatment with Hu3 recorded significantly least post-emergence damping off (13.78 percent) comparison to control pot having 50% damping off. The seed and soil treatment with Hu3 recorded significantly highest percentage decrease of pre-emergence damping-off (71.3 percent) and post-emergence damping off disease (72.4 percent) followed by Hu18 with pre- and post-emergence damping off disease 71.3 percent and 65.1 percent respectively (Fig. 1). The seed and soil treatment with Hu3 recorded significantly maximum (5.33 cm) root length compared to sick pot having (2.07 cm) root length which was followed by seed and soil treatment with Hu9 rhizobacteria (2.23 cm). The seed and soil treatment with Hu18 recorded significantly maximum shoot length (21.07 cm) compared to sick pot having (8.57 cm) shoot length (Table 3 and Fig. 2). According to Ahmadzadeh *et al.* (2004), several Bacillus species have the ability to prevent bacterial and fungal root rot diseases in a variety of crops. Such findings were found during our research on damping off disease in cowpea seedlings. It is also in agreement with the findings that

Bacillus spp. can effectively control the soil borne pathogens (Thakur *et al.,* 2022) which is due to the fact that Bacillus spp. can successfully inhibit the growth of pathogens by producing several metabolites (terpenes and polypeptide) and cell wall degrading enzymes such as chitinases (Shoda, 2000). Huge studies on the use of rhizobacteria as biocontrol agents point to improved phosphorus uptake, which makes plants more strong and resistant to disease invasion (Lioussanne, 2010). The enhanced P-solubilization ability of the rhizobacteria Hu18, Hu3 and Hu19 may also be attributed with their enhanced antagonistic potentials. This finding supports previous research showing specific biocontrol agents are promising elements for managing soil-borne diseases on a variety of plants (Roberts *et al*., 2005; Harman, 2006; Sahar *et al.,* 2009). However, the bacterial bioagents Hu3, Hu18 and Hu 19 showed positive effects in reducing pre- and post-emergence damping-off disease in *S. rolfsii* pathogen infested soils, that may lead to enhanced seed germination vigour and ultimately more crop yield and may be in future, can be used as microbial consortia for sustainable plant health management.

Table 2: Influence of native PGPR rhizobacterial isolates on percent decrease of pre & post emergence damping off.

Treatments	Isolate	Pre-emergence damping off (%)	Post-emergence damping off (%)
T1	HU3 + *S. rolfsii*	5.56 (13.64)	13.78 (21.79)
T2	HU4 + *S. rolfsii*	11.11 (19.47)	23.91(29.27)
T3	HU9 + *S. rolfsii*	11.11 (19.47)	26.67 (31.01)
T4	HU14 + *S. rolfsii*	11.11 (19.47)	22.22 (28.12)
T5	HU18 + *S. rolfsii*	5.56 (13.64)	17.41 (24.67)
T6	HU19 + *S. rolfsii*	8.33 (16.78)	21.48 (27.61)
T7	only *S. rolfsii*	19.44 (26.16)	50 (45.00)
SeM ±		**2.71**	**2.63**
CD (P=0.05)		**8.34**	**8.10**

Table 3: Plant growth parameters of cow pea seeds treated with potential PGPR rhizobacterial isolates when challenged with fungal pathogen, *S. rolfsii*

Treatments	Isolate	Root Length (in cm)	Root Fresh Wt (in g)	Shoot Length (in cm)	Shoot Fresh Wt (in g)
T1	HU3 + *S. rolfsii*	5.33	0.12	20.17	1.17
T2	HU4 + *S. rolfsii*	2.47	0.06	14.33	0.79
T3	HU9 + *S. rolfsii*	2.23	0.05	13.67	0.77
T4	HU14 + *S. rolfsii*	2.27	0.05	10.27	0.54
T5	HU18 + *S. rolfsii*	3.93	0.11	21.07	1.05
T6	HU19 + *S. rolfsii*	2.67	0.08	18.60	1.42
T7	Only *S. rolfsii* (Ck)	2.07	0.04	8.57	0.42
SeM ±		**0.46**	**0.01**	**0.94**	**0.08**
CD (P=0.05)		**1.43**	**0.03**	**2.89**	**0.26**

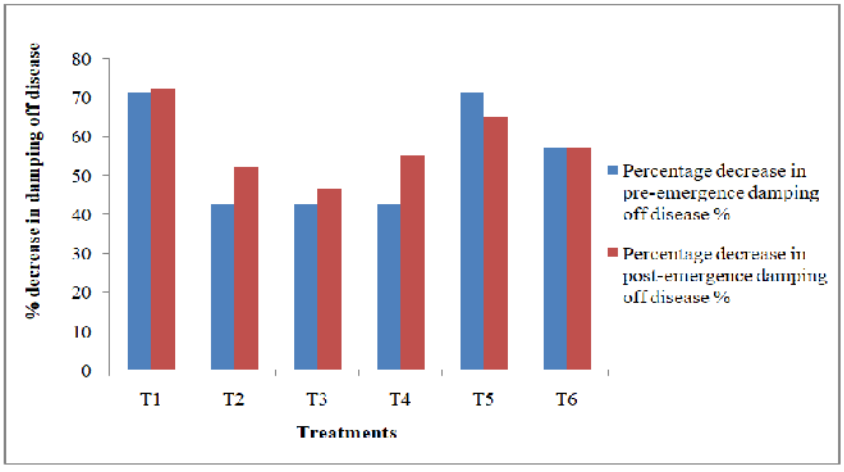

Fig. 1. Influence of native PGPR rhizobacterial isolates on percent decrease of pre and post emergence damping off disease.

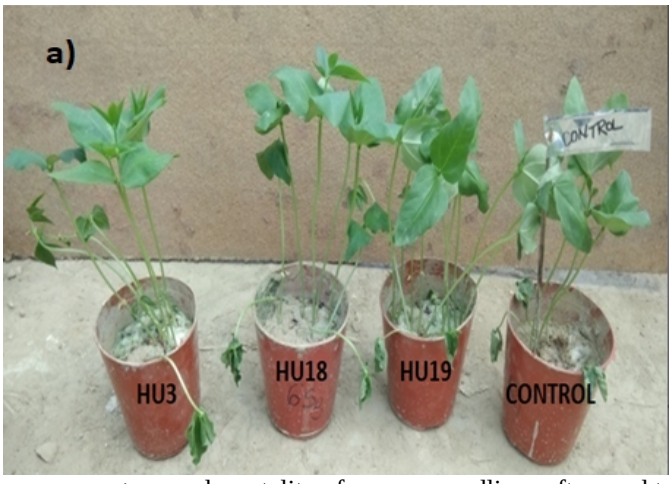

Fig. 2. (a) Damping off disease percentage and mortality of cowpea seedlings after seed treatment with potent PGPR rhizobacterial isolates (Hu3, Hu18, Hu19 and control) with *S. rolfsii*.

Molecular characterization of the potent rhizobacterial isolates. DNA of the rhizobacterial isolates were extracted and were amplified with Bac F and R1378 primers specific for Bacillus spp. Among twelve number of native rhizobacterial isolates, only seven rhizobacteria produced specific amplification with the *Bacillus* spp. specific primers (expected size of about 1300 bp). Thus, native rhizobacteria Hu3, Hu2, Hu7, Hu9, Hu14, Hu18 and Hu19 were identified as Bacillus spp. based on Bacillus spp. specific primers.

CONCLUSION

The results of this study clearly indicate that Hu3, Hu18 and Hu19 PGPR rhizobacterial isolates were found to be the most effective isolates for rhizosphere competence and plant growth promotion of cowpea seedlings and incase of damping off pathogen disease suppression, Hu3 and Hu18 rhizobacteria found to be excellent bacterial bioagents for successful management of this soil borne phytopathogen. This study has extended the range of the PGPR strains that have promising results and it can be used as biocontrol agents to alleviate plant disease stress further increasing crop productivity.

FUTURE SCOPE

In the near future, these native rhizobacterial isolates may be efficiently used as bio-inoculants for integrated disease management for other soil borne plant pathogens and also it can act as plant growth promoters in field applications. Future research should concentrate more on the functional characterization of PGPR for field applications.

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Conflicts of Interest. None.

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