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Plant Growth Promotion and Biocontrol Potential of a *Streptomyces* sp. Strain N3-3b Isolated from the Rhizosphere of *Chakhao*, a Black Rice Variety of Manipur, India

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Authors' contributions

This work was carried out in close collaboration among all authors. Author DSN proposed, designed and supervised the study. Author SBC wrote the first draft of the manuscript. Authors SBC, RL, KAD and NJ performed the experiments. Author KT performed the statistical analysis. Authors DSN and KT wrote the final version of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To isolate and investigate actinomycete strains from rhizospheric soil of *Chakhao* (black rice) in Manipur, India for biocontrol and plant growth promoting activities.
Study Design: Dual culture, plant growth promoting activities and vigor index.
Place and Duration of Study: Microbial Biotechnology Research Laboratory (MBRL), Department of Biochemistry, Manipur University, India, between August 2010 and July 2013; re-analyzed and reviewed during August 2015-May 2016.
Methodology: Isolates were screened for biocontrol activity and one strain which showed significant antagonistic potential was selected for further studies. The selected strain was subjected to plant growth promoting traits such as IAA, ammonia and siderophore production, and inorganic

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phosphate solubilization. It was further assayed for rice seed germination and growth of rice seedlings under *in vitro* conditions. Characterization of the strain was also done.

Results: Among 122 putative actinomycete isolates, 9 exhibited antagonistic activity against rice fungal pathogens. N3-3b was selected as the most promising biocontrol strain for further studies. The strain exhibited plant growth promoting traits including IAA production, phosphate solubilisation and ammonia production. It promoted seed germination and significantly enhanced growth of seedlings (Vigor index) under *in vitro* conditions. Based on the phenotypic characteristics and 16S rRNA gene sequence analysis, the strain N3-3b was characterized as a member of the genus *Streptomyces*.

Conclusion: Strain N3-3b could be a potential candidate for development as bioinoculant for rice cultivation.

Keywords: Chakhao; actinomycetes; biocontrol; PGP; Streptomyces; Vigor index; Chakhao rice; Manipur.

1. INTRODUCTION

Synthetic agrochemicals pose serious risks to human health and the ecosystem and this warrants development of alternative approaches such as microbial biofertilizers and biocontrol agents including the use of bioinoculants for plant growth promotion and disease control [1]. Microorganisms may facilitate plant growth directly by nitrogen fixation (N fixation), phosphate solubilization (P solubilization), siderophore and phytohormone production or indirectly by antagonism, HCN production, niche competition, ACC deaminase production and induced systemic resistance (ISR) [2]. The importance of native strains and ecological specificity has also been emphasized [3]. Rhizospheres of crop plants are generally richer in microbial population due to release of root exudates. Among the rhizobacteria, the genus *Bacillus* and *Pseudomonas* have been intensively studied but actinobacteria have received comparatively lesser attention [4].

Among the Actinobacteria, *Streptomyces* spp. have shown great potential as prolific producers of bioactive metabolites [5,6,7]. So, native *Streptomyces* strains from rice rhizosphere and other biotopes could potentially be biocontrol agents (BCAs) and plant growth promoting (PGP) agents for rice. Two actinomycete bioinoculants, Mycostop (*Streptomyces griseoviridis* strain K61) and ActinoIron and Actinovate (*Streptomyces lydicus*) are commercially available [8].

Black rice cultivation has a long history especially in Asia [9]. The dark purple color of black rice is due to high anthocyanin content in the pericarp layers [10]. More than 200 black rice cultivars are grown in parts of Asia including Manipur in India [11]. It was known as 'Emperor's Rice' or 'Forbidden Rice' in ancient China as only the royal family was allowed to consume it then.

Besides being a special food item, black rice has several health benefits and is considered a medicinal food, neutraceutical and functional food. It is richer in protein, fiber, vitamin B, niacin, vitamin E and minerals such as Ca, Mg, Fe, and Zn compared to white rice [12]. The high anthocyanin content and associated protective, and antioxidant action makes black rice a 'superfood' [13]. Black rice has been reported to have cardioprotective, anticancer, and antiatherogenic effects [9].

Manipur has several endemic varieties of black rice. The most popular cultivar, *Chakhao* (also known as *Chakhao amubi*, literally means delicious black rice) is grown at certain parts of the Imphal valley in Manipur. Due to its poor yield (about 2.5 tons/hectare), *Chakhao* is grown in very limited acreage by farmers in Manipur for ceremonial and cultural purposes [14]. As it is gaining popularity due to its neutraceutical properties, there is urgent need to enhance its productivity. As there are no high yielding varieties yet, research on development of microbial inoculants with biocontrol and PGP potential for *Chakhao* is urgently warranted.

The rice variety *Chakhao* (black rice) endemic to Manipur is rich in nutritional values and contains 18 amino acids, Fe, Zn, Cu and carotene. The dark purple colour is due to its high anthocyanin content and presence of vitamin E renders antioxidant property. It may help prevent cancer, diabetes, heart disease and Alzheimer's disease [15,16]. As the chemical composition of the root exudates of *Chakhao* might be different from that of other rice varieties, *Chakhao* rhizosphere may

harbor different microbial profile. Hence, the present study was aimed at isolation and characterization of bioactive actinomycetes from *Chakhao* rhizosphere and screen their PGP and biocontrol activities.

2. MATERIALS AND METHODS

2.1 Rhizosphere Sample Collection

Rhizospheric soil samples were collected from a rice field in Nambol region of Manipur, India cultivating the local rice variety '*Chakhao*' (black rice). The soil was clayey type with a pH of 5.5. Nambol (24.7° N, 93.84° E) is located at a distance of 18.1 km from Imphal, the capital city of Manipur.

2.2 Isolation of Rhizospheric Actino-mycetes

Soil samples were treated with $CaCO_3$ (10%) and air dried for one week. They were then suspended in 100 ml of sterilized distilled water and were serially diluted. 0.1 ml of each diluted sample was spread plated on Starch Casein Nitrate Agar (SCNA) medium and kept incubated at 30℃ for 4-5 days. Morphologically distinct isolates were selected and subcultured on SCNA plates till pure cultures were obtained.

2.3 Screening for Biocontrol Activity

The isolates were screened for biocontrol activity by dual culture assay [17] against five rice fungal pathogens viz. *Rhizoctonia solani* (MTCC 4633, Sheath Blight Disease) and *Pyricularia oryzae* (MTCC 1477, Blast Disease), *Bipolaris oryzae* (MTCC 3717, Brown Spot Disease), *Rhizoctonia oryzae-sativae* (MTCC 2162, Aggregate Sheath Blight Disease), and *Fusarium oxysporum* (MTCC 287, Root Rot Disease). Mycelial growth inhibition was calculated using the formula: (C-T)/C x 100, where C is the colony growth of pathogen in control (mm), and T is the colony growth of pathogen in dual culture (mm). The isolate showing highest percentage of mycelial growth inhibition was selected for further studies.

2.4 Screening for PGP Traits

2.4.1 Indole-3-acetic acid (IAA) production

The production of IAA was determined according to the method of Bano and Musarrat [18]. The strain was inoculated in SCN broth (SCNB) containing 2 mg/ml of L-tryptophan (trp) (HiMedia) and incubated in a shaker (150 rpm, 30℃, 6 d). The culture broth was centrifuged at 10,000 rpm for 10 min. One ml of the supernatant was mixed with 2 ml of Salkowski reagent. Appearance of pink colour indicated IAA production.

Quantitative assay of IAA production at different trp concentrations (%) was also studied by inoculating the strain in SCNB containing different concentrations (%) of trp (0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4) and kept incubated under shaking conditions (150 rpm, 30℃, 6 d). The culture broth was centrifuged at 10,000 rpm for 10 min. One ml of the supernatant was mixed with 2 ml of Salkowski reagent and incubated for 20 min at room temperature. Optical density (OD) was read at 530 nm and the amount of IAA produced was calculated by comparing with the standard IAA (Rankem) curve.

2.4.2 Phosphate (P) solubilization

P solubilization assay was done using NBRIP-BPB medium [19]. A halo zone surrounding the colony after 4 d of incubation at 30℃ indicated P solubilization.

Quantitative estimation of P solubilization was done according to Kapri and Tewari [20]. The strain was inoculated in 100 ml of NBRIP medium and kept incubated in a shaker (150 rpm, 30℃, 6 d). The culture broth was centrifuged at 10,000 rpm for 10 min. The amount of P in the culture supernatant was estimated using the method of Fiske and Subbarow [21], and expressed as equivalent P (µg/ml). KH_2PO_4 was used as the standard.

2.4.3 Siderophore production

Siderophore production was assayed according to You et al. [22] with few modifications. Agar plug (8 mm) of strain N3-3B was inoculated on SCNA (without iron) amended with CAS-substrate and kept incubated at 30℃ for 6 d. A halo zone with orange colour surrounding the colony was considered as positive for siderophore production.

2.4.4 Ammonia production

Ammonia production was screened in peptone water. The strain was inoculated in 10 ml peptone water and kept in a shaker (150 rpm, 30℃) for 4 d. 0.5 ml of Nessler's reagent was then added in each tube. Development of brown

to yellow colour indicated ammonia production [23].

2.5 *In vitro* Seed Germination Test (Vigor index)

Strain N3-3b was grown on SCNB for 6 d, centrifuged (10,000 rpm, 10 min) and the pellet collected was washed thrice with sterile distilled water (SDW). The pellet was dissolved in SDW and different inoculum sizes were prepared ($3x10^7$, $6x10^7$, $1.2x10^8$, $1.8x10^8$, $2x10^8$ and $2.4x10^8$ cfu/ml). Rice seeds (Variety: *Chakhao*) were surfaced sterilized with 0.2% sodium hypochlorite for 5 min followed by 70% ethanol for 5 min and rinsed four times with SDW. Sterilized seeds were soaked in the cell suspensions prepared earlier and kept overnight. Sterilized seeds soaked in SDW were taken as control. The seeds were dried under laminar flow and then transferred to sterile plates containing wetted filter papers at the rate of 10 seeds per plate. Plates were incubated at 28-30°C and after 4 d, the number of germinated seeds, root lengths and shoot lengths were noted and compared with controls. Four replications were done per treatment and the experiments were repeated twice. Vigor index was calculated using the formula shown as follows [24]:

Vigor index = Percent germination x Seedling length (shoot length + root length).

2.6 Biochemical, Morphological and Molecular Characterization

Biochemical tests viz. catalase production, gelatin liquefaction, citrate reduction, indole production, MR and VP tests, oxidase production, nitrate reduction and sugar fermentation (glucose, sucrose, fructose, lactose, maltose and mannitol) were performed as described by Cappuccino and Sherman [23]. Utilization of sole carbon and nitrogen sources was determined as described by Shirling and Gottlieb [25]. Growth morphologies of the strain were observed in different International *Streptomyces* Project (ISP) media [25]. The colony colour was determined according to the ISCC-NBS colour chart [26]. Growth at different salt concentrations (0-10% NaCl) and pH values (4 to 10) was also evaluated.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene was performed as described by Li et al. [27]. The almost complete 16S rRNA gene sequence of the strain was identified using the EzTaxon-e server database [28] and aligned with the 16S rRNA gene sequences of related species using CLUSTAL X version 2.1 [29]. Phylogenetic analyses were performed using the software package MEGA version 5 [30]. Phylogenetic distances were calculated with the Kimura two-parameter model [31] and tree topologies were inferred using the neighbour-joining method [32]. To determine the support of each clade, bootstrap analysis was performed with 1000 resamplings [33].

2.7 Antibiotic Sensitivity

Antibiotic sensitivity tests were performed using a total of six antibiotics viz. neomycin (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), penicillin (10 µg), streptomycin (10 µg) and rifampicin (5 µg) (HiMedia) for the sensitivity/resistance pattern of the isolate against the antibiotics by paper disc method.

2.8 Statistical Analysis

All data were subjected to one-way ANOVA followed by independent t-test ($P \leq 0.05$) using the SPSS 16 software (SPSS Inc.).

3. RESULTS

3.1 Isolation and Selection

Of 122 actinomycete isolates obtained from *Chakhao* rhizospheric soils, 9 showed antagonistic activity against the tested fungal pathogens. Strain N3-3b was selected for further studies as it exhibited the highest percentage of mycelial growth inhibition.

3.2 Biocontrol Activity

Strain N3-3b could inhibit mycelial growth in the range of 61 to 86%, showing highest inhibition against *Rhizoctonia oryzae-sativae* (86%) and lowest against *Pyricularia oryzae* (61%) (Fig. 1).

3.3 Plant Growth Promotion Activity

Plant growth promotion assays indicated that the strain N3-3b was positive for P solubilization, IAA and ammonia production but negative for siderophore production. Strain N3-3b produced maximum amount of IAA when amended with 1% trp (143.7 µg/ml) (Fig. 2). It could also solubilize maximum amount of P (64.80 µg/ml) after 6^{th} d of incubation (Fig. 3).

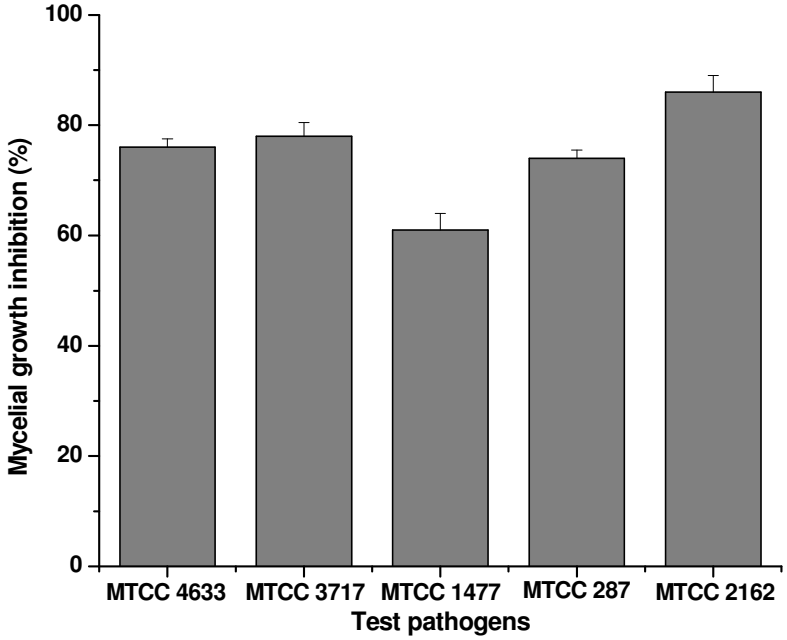

Fig. 1. Percent mycelial growth inhibition of various rice fungal pathogens by *Streptomyces* sp. N3-3b

Note: MTCC 4633, Rhizoctonia solani; MTCC 3717, Bipolaris oryzae; MTCC 1477, Pyricularia oryzae, MTCC 287, Fusarium oxysporium, MTCC 2162, Rhizoctonia oryzae-sativae

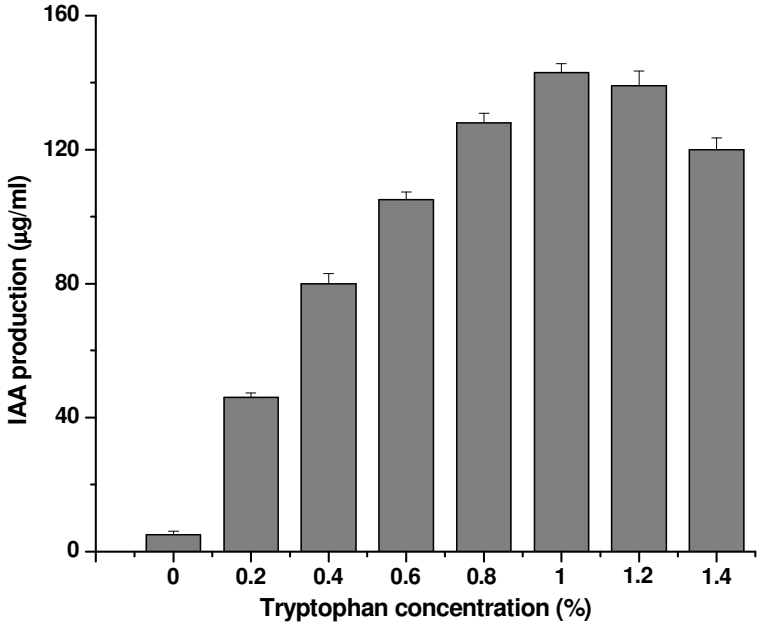

Fig. 2. IAA production by N3-3b at different tryptophan concentrations

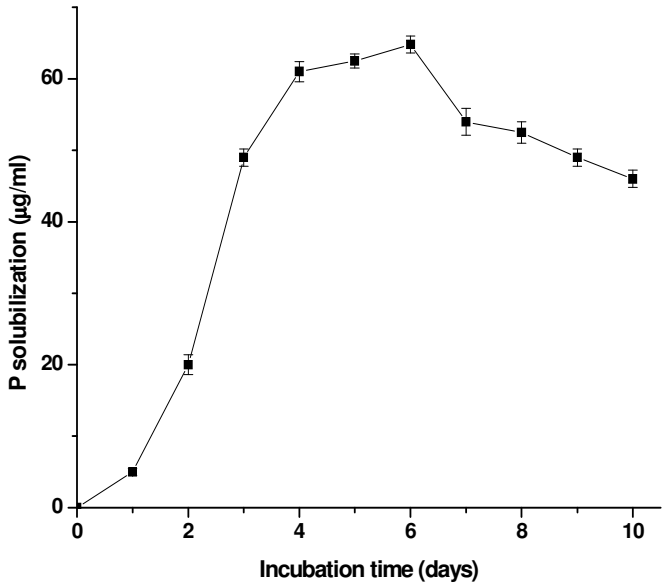

Fig. 3. P solubilization by N3-3b at different time intervals

3.4 *In vitro* seed germination test (Vigor Index)

Among the different inoculum densities, inoculum size corresponding to $6x10^7$ cfu/ml showed the highest vigor index. N3-3b treated seeds showed higher germination percentage, vigor index and significant increases in shoot and root lengths ($P \leq 0.05$) over the control. Treatment of seeds with inoculum size corresponding to $6x10^7$ cfu/ml showed significant increase in root and shoot lengths ($P \leq 0.05$) of rice seedlings over other inoculum sizes (Table 1).

3.5 Biochemical, Morphological and Molecular Characterization

The strain N3-3b was positive for nitrate reduction, indole production, citrate utilization, catalase activity, lipase production and gelatin liquefaction tests. It utilized galactose, lactose, ribose, mannitol, mannose, maltose, raffinose and fructose as sole carbon source. It could utilize asparagine, tryptophan, adenine, arginine, leucine, aspartic acid, glutamic acid and tyrosine as sole nitrogen source. The strain N3-3b was able to grow at a wide range of pH (5-10) and could tolerate up to 5% NaCl (Table 2) The cultural characteristics of N3-3b on different ISP media were observed using the ISCC-NBS colour chart (Table 3).

Strain N3-3b showed highest 16S rRNA gene sequence similarity (99.68%) with *Streptomyces castelarensis*. Based on the phylogenetic and genotypic data, N3-3b was found to represent a strain of the genus *Streptomyces* which has now been referred to as *Streptomyces* sp. strain N3-3B (Fig. 4).

Table 1. *In vitro* seed germination test (Vigor index)

Treatment	Inoculum size (x 10^8cfu/ml)	Germination percent	Root length* (cm)	Shoot length* (cm)	Vigor index
Control		95	4.07±0.34a	1.2±0.59a	500.65
	0.3	95	4.41±0.11b	1.24±0.04a	536.75
	0.6	100	4.8±0.06c	1.75±0.08c	655
	1.2	100	4.58±0.05b	1.48±0.08b	606
	1.8	95	4.50±0.07b	1.6±0.04b	610
	2	100	4.57±0.04b	1.47±0.07b	604
N3-3b	2.4	95	4.29±0.13a	1.18±0.04a	519.65

**Values with the same letter within a column are not significant at P≤ 0.05*

Table 2. Biochemical and physiological characteristics of the strain N3-3b

Test	Result	Test	Result	Test	Result
Citrate utilization	+	Sole C- source utilization		Sole N- source utilization	
Indole production	+	Galactose	+	Asparagine	+
Gelatin liquefaction	+	Lactose	+	Tryptophan	+
		Ribose	+	Adenine	+
Methyl Red (MR) Test	-	Mannitol	+	Arginine	+
Voges Proskauer (VP) test	-	Mannose	+	Leucine	+
Catalase production	+	Maltose	+	Aspartic acid	+
Nitrate reduction	+	Raffinose	+	Glutamine	+
Lipid hydrolysis	+	Fructose	+	Tyrosine	+
Oxidase production	-	pH tolerance	5-10	NaCl tolerance (%)	0-5

Table 3. Cultural characteristics of N3-3b on different ISP media as observed using ISCC-NBS colour chart (Kelly, 1964)

Medium	Growth	Colour of the mycelium		Pigmentation
		Aerial	Substrate	
ISP2	++	Yellowish grey	Pale yellow	-
ISP3	+++	White grey	Yellowish white	-
ISP4	+++	Light yellow	Mild orange yellow	-
ISP5	-	-	-	-
ISP6	+	Yellow white	Yellow white	-
ISP7	+	Bluish white	-	-

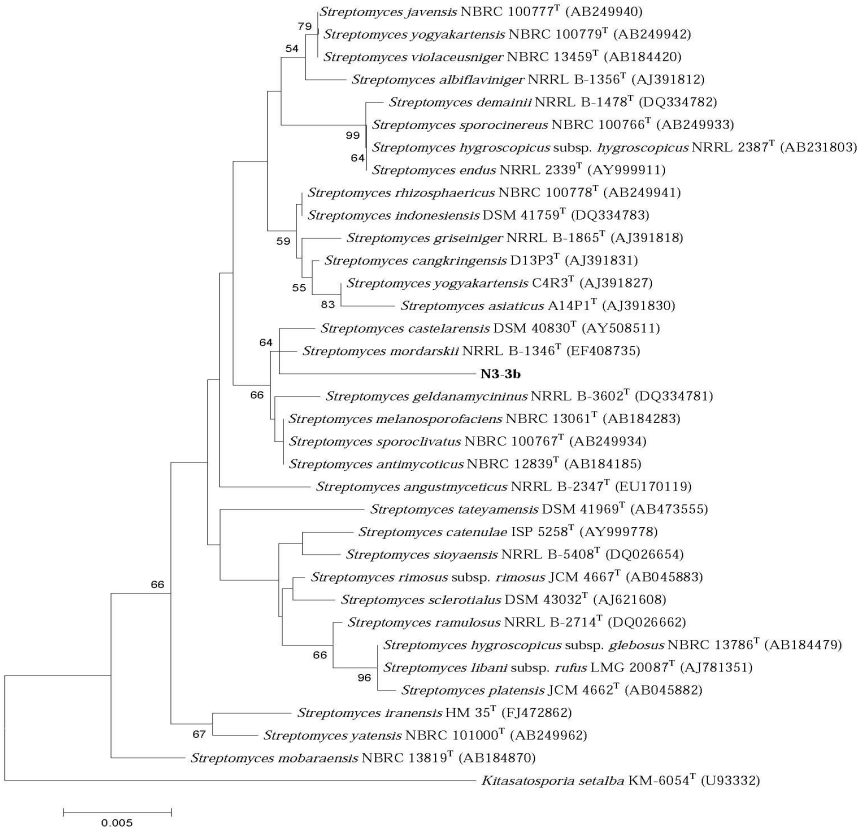

Fig. 4. Neighbour-joining tree showing phylogenetic relationship of strain N3-3b with its closely related strains

3.6 Antibiotic Sensitivity

Streptomyces sp. N3-3b was sensitive to neomycin and streptomycin, but resistant to chloramphenicol, ampicillin, penicillin and rifampicin.

4. DISCUSSION

Microorganisms isolated from rhizosphere soil may be better adapted to crop plants and provide better disease control and growth promotion than organisms isolated from other sources such as composts, ordinary soils and harsh environments etc. as rhizosphere isolates are already closely associated with the plant system as well as adapted to the local environment [34].

In the present study, we explored the potential of rhizospheric actinomycetes from rhizosphere of an endemic black rice variety *Chakhao*, for biocontrol and plant growth promotion potential. *Streptomyce*s sp. N3-3b exhibited significant antagonistic activity against major rice fungal pathogens. Antagonistic property could be due to production of hydrolytic enzymes or antibiotics. Antifungal antibiotics and fungal cell wall degrading enzymes produced by *Streptomyces* species have been reported to inhibit the growth of pathogens and protect plants from infection [35,36].

Streptomyces sp. N3-3b showed plant growth promotion traits including IAA production, P solubilization and ammonia production. It produced maximum IAA (143.7 µg/ml) when supplemented with 1% trp which was similar to the report of Khamna et al. [37]. The strain produced much higher levels of IAA than those reported for some bacterial strains by various authors, Khamna et al. [37], Shrivastava et al. [38] and Harikrishna et al. [39]. This seems to be a positive feature for N3-3b to be developed as a bioinoculant for rice cultivation. Growth promotion of rice plants by *Streptomyces* sp. N3-3b may be due to the production of IAA. When *Streptomyces* sp. En-1 which produce IAA inoculum was applied onto *Arabidopsis*, it significantly increased the biomass indicating the distinct phytostimulating effects; however, when administrated with another *Streptomyces* sp. IFB-A02 or IFB-A03 which does not produce IAA there was no significant improvement of growth as compared to control [40].

N3-3b could solubilize a significant amount of inorganic phosphate (64.8 µg/ml). This is comparable to the report of Sadeghi et al. (41) who observed that *Streptomyces* sp. C solubilized inorganic phosphate up to 92 µg/ml. The present strain from *Chakhao* rice solubilized much higher levels of P than those reported by Passari et al. [42] (3.2-32.6 µg/ml of P). Hamdali et al. [43] reported a P solubilizing *Streptomyces griseus* as a PGP bacterium.

N3-3b also promoted seed germination under *in vitro* conditions. The highest vigor index was found at inoculum size of $6x10^7$ cfu/ml of the cell suspension. The culture filtrate of *Streptomyces* sp. S-580 has been reported to promote the germination of rice seeds [44]. Several *Streptomyces* spp. have been reported to enhance seed vigor indices and seedling growth promotion. The results clearly indicated that *Streptomyces* sp. N3-3b has great potential to be a plant growth promoting and biocontrol agent. The significant improvement in seedling length and plant growth may be due to the high production of IAA by *Streptomyces* sp. N3-3b. IAA stimulates cell division and enhances cell enlargement and extension and plays a major role in normal shoot and root growth [45], and seed bacterization with IAA producing bacteria significantly enhanced early seedling germination and seedling vigor [46]. Other factors that might have contributed to rice growth promotion by strain N3-3b could be P solubilization, ammonia production and fungal antagonism.

5. CONCLUSIONS

The results of the present study indicated that *Streptomyces* sp. strain N3-3b from *Chakhao* (Manipuri black rice) rhizospheric soil holds promise for development as a biocontrol and PGP agent for rice cultivation. The utilization of such beneficial rhizobacterial actinomycetes may lead to increased crop yields while reducing the use of agrochemicals. Such an approach is indeed an attractive trend towards introducing sustainable, green, and eco-friendly agriculture. Further work on PGP potential of N3-3b on *Chakhao* and other rice cultivars under pot trial and field conditions are underway and shall be published separately.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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