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Management of bacterial blight in rice (*Oryza sativa*) through combined application of endophytes and rhizosphere antagonist

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ABSTRACT

Biocontrol agents and plant growth promoting microbes have emerged as an effective alternative for chemical management of plant diseases in addition to realising an increase the crop yield. Present study was made to explore endophytic microbes and rhizospheric *Streptomyces* of rice plant to develop biocontrol strategy for the management of bacterial blight (BB) of rice (*Oryza sativa* L.) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). *In-vitro* studies revealed that few promising endophytic microbes (E_1 and E_2) and rhizospheric *Streptomyces* (S_1 and S_2) could suppress *Xoo* effectively in dual culture assay *in- vitro*. The combination of antagonistic microbes (E_1+S_1+S_2) showed highest (58.71%) inhibition of BB pathogen. Pot experiments were conducted to study the effect of the promising endophytic microbes on disease reduction, yield and yield attributing characteristics of rice. Results revealed the lowest disease incidence in plants treated with combination of E_1+S_1+S_2(10.29 %) compared to other treatments. Similarly, the highest yield (50.06g per hill) and other yield attributing characters of rice plants were recorded with microbial treatment E_1+S_1+S_2. These observations suggested far better superiority of rhizosphere antagonists plus endophytes than either of the two alone.

Key words: Bacterial blight, Endophytes, *Streptomyces*, Yield

The use of biocontrol agents having suppressing effect on disease-causing agents and to increase the production of major crops, provide a strategically viable option for the host plant resistance *vis-a-vis* disease management (Bora *et al.* 2019). Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases of rice causing severe losses during conducive conditions (Ou 1985). Traditional methods employed to minimize the pathogen-borne losses have limitations. Therefore, viable, economic and eco-friendly disease management tools need to be developed. Biocontrol agents and Plant Growth Promoting Microbes (PGPM) are known to enhance crop productivity in a sustainable manner (Sharma *et al.* 2013, Beneduzi *et al.* 2012, Bora *et al.* 2020).

The bacterial community inhabiting rhizosphere is usually considered as a source of formation of the community of endophytic bacteria. At the same time, it is known that the mechanisms used by endophytic bacteria to improve plant growth are similar to those of rhizospheric bacteria. However, endophytes have been used to enhance plant growth and yield, besides protection against plant diseases *via* several mechanisms (Kumar *et al.* 2015, Bora *et al.* 2019). Ability of *Streptomyces* to suppress some plant pathogens is also well documented (Rizk *et al.* 2007, Park *et al.* 2011). Our study aimed to manage BB of rice as well as increase yield of rice using endophytes and rhizospheric *Streptomyces* of rice ecosystem. The major objectives of our study included isolation and characterization of various endophytic microbes and rhizosphere-based *Streptomyces* of rice ecosystem, and later combining them for management of BB of rice with emphasis on enhancement in yield and yield attributing characters.

MATERIALS AND METHODS

The above study was carried out under laboratory conditions at Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam (Replicated seasons of 2017-18). Diseased leaves showing typical bacterial blight symptoms were collected from rice fields of Regional Agricultural Research Station (RARS), Titabar. The pathogen, *Xoo* was isolated using Sucrose Peptone Agar (SPA) and Modified Wakimoto's media. Pure culture of *Xoo* was preserved on Nutrient Agar (NA) slants at 4°C for future use. Pathogenicity test was conducted by clip inoculating

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10^7-10^8 cfu/ml of bacterial inoculums on 1 month old rice seedlings (Kauffman *et al.* 1973).

Isolation of endophytic and rhizospheric antagonists

Endophytes were isolated from the healthy leaf, stem and root samples of rice plants using the protocol suggested by McInroy and Kloepper (1995). Rhizospheric *Streptomyces* were isolated from the rice rhizosphere using the protocol suggested by Maleki *et al.* (2013).

In-vitro efficacy of microbial anatagonists against Xoo

The inhibitory effect of the isolated endophytes and Rhizospheric *Streptomyces* isolates against *Xoo* were tested *in vitro* by using a modified dual culture assay method (Aspiraz and Cruz 1985). The per cent inhibition was calculated following the formula described by Myee and Datar (1986) as: Per cent inhibition =

$$\frac{\text{Mean of inhibition zone (mm)}}{90} \times 100$$

On the basis of its percentage inhibition, 2 endophytes and 2 rhizospheric *Streptomyces* were further selected for compatibility test *in-vitro* using NA and PDA as basal media. Various morphological, cultural, biochemical and molecular characterization schemes were adopted to identify the isolates.

In-vivo efficacy for the management of BB of rice

Selected combination of endophytic microbes and Rhizospheric *Streptomyces* showing highest inhibition (%) against *Xoo in-vitro* were evaluated for suppression of BB in pot grown rice plants under green shade net condition (cv. TN1). Five replications were maintained for each treatment following completely randomised design. Selected endophytic microbes and Rhizospheric *Streptomyces* were applied as seed treatment (100 seeds/20ml of solution), root treatment for 1 hour prior to transplanting (100 seedlings/100 ml of solution), soil treatment was done 30 days after transplanting (100ml /plant) and spray application (2% solution), 30 days after transplanting with subsequent sprays undertaken at 45, 60 and 75 DAT.

Per cent disease incidence (PDI) and per cent disease reduction were assayed in treated rice plants.Disease severity percentage was calculated based on 0-9 scale of Standard Evaluation System for rice (IRRI 2002). Moreover, yield and plant attributing characters were recorded.

Statistical analysis

Data from *in-vitro* and *in-planta* experiments were analyzed and subjected to Analysis of Variance (ANOVA). Critical differences were estimated to compare different treatments and the per cent values were transformed by angular transformation.

RESULTS AND DISCUSSION

The bacterial blight (BB) pathogen (*Xoo*) was isolated from symptomatic rice leaves and ooze test was done to confirm the bacterial presence in the diseased tissues. Light yellow, circular, convex, opaque and smooth colonies typical to *Xoo* were observed on Modified Wakimoto's and SPA plates. Morphologically the bacterium was rod shaped and retained red colour when counter-stained with safranin revealing its gram negative reaction. Biochemically it showed positive reaction to KOH test, Citrate utilization, Gelatin liquefication, Catalase test, Levan production test. Similar results of biochemical tests were also obtained by Arshad *et al.* (2015) during identification of *Xoo.* The bacterium appeared to be rod shaped under a Field Emission Scanning Electron Microscope (FESEM).16S rRNA sequencing results of the bacterium showed maximum homology (>97%) with *Xanthomonas oryzae* pv. *oryzae*. Pathogenicity test of *Xoo* was carried out on healthy rice plants (var. TN1) which revealed highly virulent reaction, producing typical disease symptoms in the inoculated plants within 10 days. Similar results were obtained by Kauffman *et al.* (1973) , wherein successful inoculation of the *Xoo* was obtained by leaf clipping method.

Isolation and of endophytes and rhizosphere antagonists characterisation

As many 20 bacterial and 6 fungal endophytes were isolated and identified based on various cultural and micro-morphological studies. Moreover, 16 rhizospheric *Streptomyces* were isolated from different locations. *In -vitro* tests were conducted with the endophytic microbes and rhizospheric *Streptomyces* against *Xoo*. Among all the isolates, 2 endophytes (E_1 and E_2) and 2 rhizospheric *Streptomyces* (S_1 and S_2) were found to be most effective in inhibiting *Xoo* which were further characterised.

The gram staining revealed that the endophytic bacterium E_1 was gram negative and rod shaped. In King's B medium it showed fast growing greenish yellow colony which were opaque, smooth edged and convex. Biochemical characterization of E_1 revealed positive reactions to Gelatin liquefication test, Catalase test, Oxidase test, Citrate utilization test, Nitrate reduction test, Arginine dihydrolase test, KOH test and Levan production test. Molecular characterization using 16S rRNA sequencing of the endophyte exhibited maximum homology (95%) with *Pseudomonas putida*. These results of biochemical tests are in conformity with the results of tests conducted by Kumar *et al.* (2016) for identification of *P. putida*.

Cultural charactistics of E2 on PDA media was found to have Conidia aseptate, cylindrical to ovoid, formed in chains, pale to bright green to yellow-green or olivaceous in colour. The length of the conidia varied from 5.5 to 7.15 µm and the width varied from 1.8 to 2.87 µm.18S rRNA sequencing results showed maximum homology (>97%) with *Metarhizium anisopliae*. Sasan and Bidochka (2013) reported that *M. robertsii* can endophytically colonize roots of switch grass and haricot beans.

Similarly, Gram staining of S_1 and S_2 showed both the bacteria as gram positive filamentous strands. Colony size varied from small to medium, rough edges and

colony of S_1 appeared grey, while colony of S_2 appeared dusty pink. Biochemically, S_1 showed positive reaction in Starch hydrolysis test, Oxidase test, Catalase test, Gelatin liquefication and negative reaction for Citrate utilization test, Nitrate reduction test, Arginine dihydrolase test, Indole production, Levan production and KOH test, whereas S_2 showed positive response for 5 biochemical tests namely, Starch hydrolysis test, Catalase test, Gelatin liquefication test, Oxidase test and Indole production. Moreover, FESEM of S_1 and S_2 also revealed that the bacterial cells were filamentous. S_1 and S_2 were identified using 16S rRNA sequence analysis which showed maximum homology with *Streptomyces fimicarius* (>97%) and *Streptomyces laurentii* (>97%) respectively. Trejo *et al.* (1977) isolated *S. laurentii* which produced pinkish blush on white aerial mycelium and light rose pigmentation.

In-vitro evaluation of antagonistic ability of endophytes and rhizospheric Streptomyces

Both the endophytes and rhizospheric microbes either alone or in combination were found to have inhibited *Xoo* under *in vitro* condition and also exhibited positive compatibility for E_1+E_2, S_1+S_2, E_1+S_1, E_1+S_2, E_1+S_1+S_2, E_2+S_1, E_2+S_2 and E_2+S_1 +S_2. Highest inhibition was shown by combination of E1+S1+S2 (58.71%) followed by E2 +SI+S2 (56.62%) after 48 hr of inoculation (Table 1). *P. putida* (E1) has been recognized as an efficient BCA against many phytopathogens and antagonism is attributed to siderophore production, active colonization and antibiotic production (Bora *et al.* 2018.). Fungal endophyte *M. anisopleae* (E_2) produces secondary metabolites which have antimicrobial activity (Ravindra *et al.* 2014). Endophytic *Streptomyces* spp. and their metabolites are promising option in controlling various fungal and bacterial phytopathogens (Vurukunda *et al.* 2018).

Table 1 Inhibition (%) of Xoo by effective endophytic microbes (E1 and E2) and rhizospheric Streptomyces (S1 and S2)

Isolates	Zone of inhibition (mm dia.)		Per cent inhibition	
	24 hr	48 hr	24 hr	48 hr
E_1	23.53	38.97	26.14 (30.72)*	43.3 (41.15)
E_2	17.69	24.90	19.65 (26.28)	27.67 (31.69)
E_1+E_2	34.53	42.62	38.37 (38.23)	47.36 (43.45)
S_1	17.40	24.54	19.33 (26.06)	27.27 (31.44)
S_2	21.00	28.00	23.33 (28.86)	31.10 (33.90)
S_1+S_2	32.07	39.35	35.63 (36.63)	43.72 (41.38)
E_1+S_1	40.60	49.42	45.11 (42.19)	54.91 (47.81)
E_1+S_2	43.46	50.60	48.29 (43.97)	56.22 (48.56)
E_1+S_1+S_2	47.32	52.84	52.58 (46.43)	58.71 (50.01)
E_2+S_1	31.92	40.80	35.46 (36.51)	45.33 (42.3)
E_2+S_2	32.65	41.93	36.28 (36.99)	46.58 (42.99)
E_2+S_1+S_2	42.84	50.96	47.60 (43.62)	56.62 (48.79)
Control	No inhibition		0 (0.57)	0 (0.57)
SEd ±	-	-	1.82	1.74
CD (P=0.05)	-	-	3.78	3.61

*Values in parentheses are arcsine-transformed values

Evaluation of endophytic microbes and rhizospheric Streptomyces against BB

On the basis of per cent inhibition *in-vitro*, 10 treatment combinations of selected endophytic microbes and Rhizospheric *Streptomyces* were tested under pot condition to evaluate their efficacy in managing BB of rice (var. TN1). One treatment was maintained as un-inoculated control and another one as inoculated control. Lowest disease incidence (10.29%) was observed in plants inoculated with T_7 (E_1+S_1+S_2) and highest disease incidence (74.99%) was recorded in T_1 (Inoculated control). Disease severity was significantly lowest in the plants inoculated with T_7 (9.73%) and highest disease intensity was recorded in T_1 (70.73%) (Table 2). Efficacy of *P. putida, M. anisopleae* and *Streptomyces* spp against plant pathogens were earlier reported by Zablotowicz *et al.* (1991). These biocontrol agents (BCA) also reduce disease severity through enhancing plant defense (Bora *et al.* 2019). Combination of bioagents displayed far better efficacy in the management

Table 2 Effects of different treatments on BB incidence (%) and severity (%) in pot grown rice

Treatment	PDI	Disease reduction (%)	Disease severity (%)	Scale
T_1	74.99 (59.93)	0.00 (0.00)	70.73 (57.23)*	9
T_2	13.14 (21.22)	80.06 (63.43)	11.63 (19.91)	3
T_3	17.57 (24.73)	76.57 (61.00)	24.30 (29.53)	5
T_4	25.47 (30.26)	66.03 (54.33)	29.71 (33.02)	7
T_5	16.59 (23.97)	77.87 (61.89)	23.23 (28.79)	5
T_6	16.08 (23.58)	78.55 (62.38)	22.73 (28.45)	5
T_7	10.29 (18.63)	86.27 (68.19)	9.73 (18.15)	3
T_8	23.99 (29.27)	68.00 (55.55)	27.33 (31.5)	7
T_9	19.62 (26.28)	73.83 (59.21)	24.83 (29.87)	5
T_{10}	14.95 (22.71)	82.47 (65.20)	18.16 (25.18)	5
SEd ±	1.32	-	1.87	-
CD (P=0.05)	2.73	-	3.89	-

*Values in parentheses are arcsine-transformed values. Data are mean of three replications. T_1= Inoculated control; T_2= Uninoculated control; T_3= E_1+E_2; T_4= S_1+S_2; T_5= E_1+S_1; T_6= E_1+S_2; T_7= E_1+S_1+S_2; T_8= E_2+S_1; T_9= E_2+S_2; T_{10}= E_2+S_1+S_2

Table 3 Effects of different treatments of endophytic microbes (E1&E2) and rhizospheric *Streptomyces* (S1&S2) on yield and yield attributing characters of rice

Treatment	Yield per hill (g)	No. of tillers per hill	Shoot length (cm)	No. of panicles per hill	Panicle length (cm)	Panicle weight (g)	Root dry weight (g)	Shoot dry weight (g)	Root.: shoot ratio	Root length (cm)	Test weight (g)
T_1	23.10	8.33	82.50	9.33	20.66	16.56	11.80	32.23	0.36	30.50	21.16
T_2	48.40	20.66	110.50	19.66	27.96	28.10	45.13	63.56	0.71	42.73	24.83
T_3	45.50	16.66	104.16	14.66	25.16	24.33	37.83	62.02	0.61	38.50	24.10
T_4	41.83	14.33	101.60	14.00	24.4	21.00	30.82	58.16	0.53	36.30	23.83
T_5	47.00	16.66	104.50	16.33	25.53	24.46	39.33	62.43	0.63	39.16	24.33
T_6	47.63	19.00	106.93	18.00	25.83	27.60	42.72	62.83	0.68	41.06	24.26
T_7	50.06	21.66	112.80	20.66	28.76	29.16	46.54	63.76	0.73	43.60	25.00
T_8	42.76	15.00	102.26	14.33	24.66	21.23	33.17	59.23	0.56	37.73	23.86
T_9	44.80	16.00	104.00	14.66	25	23.10	35.16	59.6	0.59	38.06	24.09
T_{10}	48.00	19.66	108.16	18.33	27.06	28.00	43.54	63.10	0.69	41.83	24.63
SEd ±	0.34	0.94	1.32	0.91	1.97	1.27	1.50	1.71	-	1.48	0.93
CD (P=0.05)	0.71	1.96	2.74	1.88	4.09	2.63	3.10	3.55	-	3.07	1.93

of diseases on account of multiple functions and synergistic effect of more number of other micro-organisms, collectively resulted in reduction of BB of rice. Bora *et al.* (2013) reported that combined application of more than one compatible BCA produced a greater control of bacterial wilt disease of different crops under varied field conditions.

Effect on the yield and yield attributing characteristics

The application of effective endophytic microbes and rhizospheric *Streptomyces* in combinations has significant effect on rice yield and yield attributing characteristics (Table 3). Significant difference in all the yield attributing characters was observed between inoculated control and treated plants. The highest number of panicles in rice plant was observed in T_7 (20.66) with highest panicle length (28.7 cm) and weight (29.16 g). Lowest was recorded in T_1. Data showed that T_7 also recorded highest root dry weight (46.54g) and lowest root weight was recorded in T_1 (11.8g). The field study revealed that T_7 exhibited highest shoot weight, root-shoot ratio, root length, test weight leading to the highest yield (g per hill) in plants inoculated with T_7 (50.06 g).

Kaur *et al.* (2017) exploited the endophytic *Pseudomonas* sp. for improving growth and productivity in rice under sustainable management system. Anwar *et al.* (2016) demonstrated the ability of rhizospheric *Streptomyces* to enhance the plant growth which results in yield enhancement of wheat crop. Actinobacteria, such as *Streptomyces* spp., produce siderophores and plays significant role in solubilizing phosphate, and also produces array of enzymes, viz. amylase, chitinase, cellulase, invertase, lipase, pectinase, protease, phytase, and xylanase which make the complex nutrients into simple mineral forms (Vurukonda *et al.* 2018). Various microbes involved in this study, showed promising efficacy against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) both *in-vitro* and *in-vivo*.

In our study, endophytic *P. putida, M. anisopleae* and rhizospheric *Streptomyces* showed satisfactory control over BB pathogen *Xoo* both *in vitro* and *in vivo* condition. they were also found to be promising in enhancing plant growth parameters and increasing rice yield. The synergistic biochemistry amongst bioactive compounds released *in- vivo* by different microorganisms, possibly render them more effective towards growth and development as observed in our rice plants treated with combination of divergent microbes. New research advances in plant–bacteria interactions would decipher the plants ability to shape their rhizosphere and endorhiza microbiome in a synergised sequence to safeguard soil health as well as plant health.

Data are mean of three replications. T_1= Inoculated control; T_2= Uninoculated control; T_3= E_1+E_2; T_4= S_1+S_2; T_5= E_1+S_1; T_6= E_1+S_2; T_7= E_1+S_1+S_2; T8= E_2+S_1; T_9= E_2+S_2; T_{10}= E_2+S_1+S_2

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