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Harnessing the Antifungal Potential of Seed Bacteriome against Major Seed and Soil-borne Pathogens in Blackgram

Chandana K. C. [a++*], S. Lakshmi [b#], T. Anand [c†], S. Kavitha [c†] and C. Sharmila Rahale [d‡]

[a] Department of Seed Science and Technology, Tamil Nadu Agriculture University, Coimbatore, India.
[b] Open Distance and Learning, TNAU, Coimbatore, India.
[c] Seed Centre, TNAU, Coimbatore, India.
[d] Centre for Agricultural Nanotechnology, TNAU, Coimbatore, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Blackgram is an important pulse crop in India. One of the major constraints on blackgram production is the attack of pathogens, which leads to yield loss. Nowadays, with the understanding of harmful effects of chemical fungicides, biocontrol methods are gaining more importance. One such method is biopriming with endophytes to control pathogens and enhance yield. In the present study, the endophytes of blackgram seeds was explored and tested for its biocontrol potential

[++]M.Sc Scholar;
[#]Professor, Directorate;
[†]Associate Professor;
[‡]Assistant Professor;
**Corresponding author: E-mail: kcchandana1999@gmail.com;*

against major seed- and soil-borne pathogens and growth promotion in blackgram. A total of 14 bacterial endophytic isolates were obtained and screened against *Macropomina phaseolina* and *Fusarium oxysporum.* Among them, BSE5 isolate was proven to be more efficient in inhibiting the mycelial growth of *M. phaseolina and F. oxysporum,* with per cent inhibition of 22.5 and 50.6, respectively, following BSE9. Both the isolates showed improved plant growth parameters like germination percentage, root length, shoot length, dry matter production and vigour index. Molecular characterisation of BSE5 and BSE9 confirmed that the isolates are *Pseudomonas aeruginosa and Bacillus xiamenensis.*

Keywords: Blackgram; endophytes; Fusarium oxysporum; Macropomina phaseolina; seed priming.

1. INTRODUCTION

Blackgram (*Vigna mungo* L.) belongs to the Leguminosae family and is an important pulse crop grown throughout India. It contains high levels of protein (around 25%), which is almost three times higher than that of cereals. India is the world's largest producer and consumer of blackgram. It is grown on 4.12 million ha, with an average yield of 2.23 million tonnes and a productivity of 538 kg/ha. The pulse crop is grown as an irrigated, rain-fed, or rice fallow crop [1,2].

The crop is susceptible to attack by a number of pathogens, including various seed, soil and foliar pathogens. Among them, *Macrophomina phaseolina* and *Fusarium oxysporum* are more destructive and known to cause more than 60% yield loss [3]. The occurrence of the pathogen is influenced by two factors: high soil temperature and low soil moisture level [4]. The pathogen may prevent seed germination at the pre-emergence stage, causing the plants to die prematurely [5].

Although the current disease management strategies to combat *M. phaseolina* and *F. Oxysporum* rely on breeding for resistance genotypes, optimizing cultural practices or use of fungicides, these strategies have achieved low to moderate success. A promising alternative strategy to manage these outbreaks in field may be through the use of biological antagonists. Among the biological antagonists, endophytes have been used for the management of various plant pathogens. Endophytic microbes, particularly bacteriome of seeds, have recently gained significant importance owing to the diversity of roles that they play, eventually resulting in improved plant growth as well as plant fitness. Bacterial endophytes isolated from plants have been known to have antifungal activity against several disease causing fungi [6,7].

Seed treatment might be carried out in order to stop the infection of these fungi. Now-a-days seed treatment with biocontrol agents is gaining attention as there is growing awareness against the hazardous effect of fungicides on environment and also human health [8]. Seed treatment can be done by coating the seeds with biocontrol formulations [9], or by biopriming the seeds with biocontrol agents [10]. With this background the present investigation is proposed to identify and characterize the seed-borne endophytes and to test their efficacy against *M. phaseolina* and *F. oxysporum* and growth promotion in blackgram under *in vitro* conditions.

2. MATERIALS AND METHODS

2.1 Seeds and Pathogens

Freshly harvested seeds were collected from farmer's fields in different blackgram growing areas of Tamil Nadu, India. The pure cultures of *Macrophomina phaseolina* and *Fusarium oxysporum* were obtained from the Department of Plant Pathology, Agricultural College and Research Institute (AC&RI), Madurai, Tamil Nadu, India.

2.2 Isolation of Bacterial Seed Endophytes

Freshly harvested blackgram seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution for one min. Then, the seeds were washed 3 times with sterile distilled water for one min. The last washed water was plated onto nutrient agar medium to assess the success of surface sterilization. The sterilized seeds were then macerated with 3ml Na_2HPO_4 buffer using an autoclaved pestle and mortar. The macerated sample was taken and serially diluted. 1ml aliquot was taken 10^{-8} dilution and plated in nutrient agar medium. The plates were incubated at 28±2°C for 5 days, during

which they were observed for the growth of endophytes [11]. Colonies that developed were then isolated and cultured to obtain pure cultures for further screening. The bacterial isolates were identified using various biochemical and molecular methods.

2.3 Screening of Bacterial Seed Endophytes against Pathogens

Dual plate technique was followed for assessing the efficiency of the bacterial endophytes isolated from blackgram seeds against *M. phaseolina* and *F. oxysporum* [12]. A fully grown mycelial disc (8 mm) was cut using cork borer from the 5-days old culture plate. Disc was placed 1cm away from the periphery of the Petri plate containing Potato Dextrose Agar (PDA) media. On the opposite side of the plate, bacterial endophytic isolates was streaked using an inoculation needle. Control plates were maintained only with mycelial disc of pathogen without bacterial streak. Three replications were maintained. The plates were incubated at 28±2°C for five days and the observation of the mycelial growth (cm) and inhibition zone (cm) was recorded when full growth of the pathogen was obtained in the control plates. The per cent growth inhibition of test fungus by bacterial endophytes was calculated by using formula given by Vincent [13].

$$I = \frac{(C-T)}{C} \times 100$$

Where, I = % inhibition in mycelial growth; C = growth of pathogen in control plates; T = growth of pathogen in dual culture plates.

2.4 Effect of Bacterial Seed Endophytes on Germination and Growth Promotion in Blackgram

The bacterial seed endophytes were cultured in 100ml of NA broth for 24 hours at 34^{o}C. After 24 hours, the broth was centrifuged at 10,000 rpm for 20 min. The bacterial cells were then suspended in sterilized PBS buffer and 0.5% of carboxymethyl cellulose (CMC) was added. The seeds were surface sterilized with 5% sodium hypochlorite solution and sterile water wash 3 times. These surface sterilized seeds were then soaked in the bacterial suspension at a ratio of 1:0.3 (v/v) and kept in a shaker in for 3 hours. The seeds were then drained and dried under laminar condition to bring back to original moisture condition. These seeds were then placed in a germination sheet at a rate of 25 seeds per sheet (13+12) and then rolled and kept in sterile distilled water. On the seventh day, germination %, root and shoot length of the seedlings were recorded in each treatment and the seedling vigour index was also calculated.

Seedling vigour index I: Seedling vigour index was computed using the following formula given by Abdul Baki and Anderson [14]. Seedling vigour index I = Germination (%) × Mean seedling length (cm)

Seedling vigour index II: Seedling vigour index II was computed as per the formula suggested by Reddy and Khan [15] as given below: Seedling vigour index II = Germination (%) × Seedling dry weight (g).

2.5 Cultural and Biochemical Characterisation of Elite Bacterial Endophytes

Elite bacterial endophytes were observed for its colour and colony characteristics. The selected endophytic bacterial strains were biochemically characterized by testing for HCN production and IAA production.

2.5.1 HCN production

HCN production test was carried out using Lorck's [16] method. The selected isolates were grown on nutrient Agar that also contained 4.4 g of glycine per liter of the medium. A piece of Whatman filter paper No. 1 was soaked in a 0.5% (w/v) solution of picric acid and placed under the lid. This procedure was used to collect any HCN gas that the experiment may have produced. The Petri dishes were carefully parafilm wrapped to stop HCN gas from escaping. The capped plates were then incubated for 5 to 7 days at room temperature. The color of the filter paper would noticeably change from yellow to red-brown if HCN were to be manufactured. This alteration in color was a sign that the endophytic bacterial isolates were producing hydrogen cyanide gas.

2.5.2 IAA production

Indole-3-acetic acid (IAA) production was tested following the protocol described by Bric [17]. To determine IAA production, endophytic bacteria were cultured in LB broth supplemented with L-tryptophan at a concentration of one microgram per ml. After incubating for 72-hours, bacterial

cultures were subjected to centrifugation at 10,000 g for 10 min. This 1ml supernatant was mixed with 2ml of Salkowsky reagent and allowed to react for 20 min at room temperature. The presence of IAA was indicated by a distinct change in colour, turning the solution brownish-pink. This change in colour served as an observable marker for the presence of Indole-3-acetic acid in the bacterial culture.

2.6 Molecular Charaterisation of Effective Endophytes

Molecular characterization of bacterial seed endophytes involved extracting bacterial DNA using a modified CTAB method. Bacterial growth was initiated in nutritional broth at 34°C for 24 h. For DNA extraction 1ml of culture was centrifuged at 7000 rpm for 10 min. The resulting pellet was mixed with sterile water and centrifuged at 7000 rpm for 10 min. The supernatant was discarded. The pellet was suspended in 675 µL of genomic DNA buffer and incubated for 30 min at 37°C, with intermittent vortexing at 10-min intervals. Subsequently, 75 µL of 10% SDS was added, and the mixture was incubated for two hours at 65°C with intermittent vortexing every ten minutes. The final step involved centrifuging the solution at 11,000 rpm for 10 min at 4°C, and the resulting supernatant was collected into fresh eppendorf tubes. To further purify the DNA, an equal volume of a mixture containing phenol, chloroform, and isoamyl alcohol (25:24:1) was added to the tubes. After gently vortexing the tubes 20-30 times, they were centrifuged again at 11,000 rpm for 10 min at 4°C. To precipitate the DNA, 0.6 volumes of isopropanol were added, and the DNA was left to incubate at -20°C for 60 min. The tubes were centrifuged at 12,000 rpm for 15 min at 4°C and the resulting pellet was retrieved. This pellet underwent two washes with 500 µL of 70% ethanol and was subsequently air-dried at 37°C. The DNA was stored at -20°C, and the pellet was reconstituted in 50 ml of TE buffer. Verification of the presence of total genomic DNA was achieved through agarose gel electrophoresis.

The polymerase chain reaction was executed utilizing universal bacterial primers 27F and 1492R which amplifies at 16s rRNA region. The thermocycler conditions encompassed an initial denaturation at 94°C for 5 min, followed by denaturation at 96°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 5 min, and a final extension at 72°C for 10 min. The amplified products were subsequently resolved using 1.2% agarose gel electrophoresis and visualized under a gel documentation unit. The amplified PCR products were subjected to sequencing, employing the Sanger dideoxy sequencing method.

3. RESULTS

3.1 Isolates of Bacterial Seed Endophytes

A total of fourteen endophytic bacterial isolates were obtained from blackgram seeds collected from different locations. The isolated bacterial endophytes were subjected to dual culture against *Macrophomina phaseolina* and *Fusarium oxysporum.*

3.2 Effect of Bacterial Seed Endophytes on *Fusarium oxysporum*

The results revealed that out of fourteen isolates, seven inhibited the mycelial growth of *F. oxysporum* at different levels. Among the seven isolates, BSE5 isolate recorded the maximum inhibition of *F. oxysporum* with an inhibition zone of 2.9 cm and the mycelial growth of 4 cm dia as against 50.6 per cent reduction over control. This was followed by BSE9 and BSE8 isolates with an inhibition zone of 1.5 cm and 1.0 cm and the mycelial growth of 5.5 and 6.0 cm, respectively (Table 1; Fig. 1). The lowest inhibition zone (0.2 cm) was observed in isolate BSE4 and there was no inhibition zone observed in isolates BSE6, BSE7, BSE10, BSE11, BSE12, and BSE13. Moreover, the pathogenic fungi have grown over the streaked bacterial endophyte, and are thus considered ineffective against *F. oxysporum.*

3.3 Effect of Bacterial Seed Endophytes on *Macrophomina phaseolina*

Among the isolates tested, the BSE5 isolate showed maximum inhibition against *Fusarium oxysporum*, which has proven to show maximum inhibition against *Macrophomina* as well. The isolate BSE5 recorded the highest inhibition zone of 0.56 cm with the mycelial growth of 6.3 cm (22.5 per cent inhibition over control) followed by isolates BSE9, BSE12 and BSE4, which recorded the inhibition zone of 0.3, 0.3 and 0.1cm and the mycelia growth of 6.4, 6.9 and 6.5 cm (Table 1; Fig. 2). Very less inhibition zone was observed in the isolate BSE7 (0.06 cm). The remaining nine isolates were unsuccessful in inhibiting the mycelial growth of *Macrophomina* and were considered ineffective. The isolates

BSE5 and BSE 9 were effective against both *Fusarium* and *Macrophomina*. Thus, these were considered the most effective endophytes. These endophytes were further characterised.

3.4 Effect of Bacterial Seed Endophytes on Germination and Growth Promotion in Blackgram

Among the isolates primed with seeds, the BSE5, BSE9 and BSE12 isolates gave the highest germination percentage, recording 94 per cent seed germination. The germination percentage of BSE6, BSE8, BSE11, and BSE14 was on par with BSE5, BSE9 and BSE12. Lowest seed germination was recorded in BSE4, which is on par with control and BSE2. Further, BSE5 recorded highest root (22.4 cm) and shoot length (24.3 cm), followed by BSE9 with root (20.9 cm) and shoot length (23.7 cm) (Table 2). Lowest root (14.8 cm) and shoot length (18.5 cm) was observed in control, followed by BSE4 (14.8; 17.9 cm). Highest vigour index I was recorded in BSE5 (4390), followed by BSE9 (4192) and least seedling vigour was recorded in control (2796) followed by BSE4 (2722). Moreover, BSE5 recorded highest vigour index II (20.9), followed by BSE9 (21.3) and lowest was recorded in control (18). The isolate BSE5 showed maximum growth promotion compared to all other endophytes, followed by BSE9. Thus, BSE5 and BSE9 were selected for further study and characterization.

3.5 Cultural and Biochemical Characterisation of Elite Bacterial Endophytes

The isolate BSE5 colonies appeared as smooth with a bluish-green fluorescent colour under UV light. Whereas, isolate BSE9 produced smooth white colonies. BSE5 isolate tested positive for the production of both HCN and IAA. However, BSE9 tested negative for the production of HCN but positive for the production of IAA.

3.6 Molecular Characterisation of the Elite Bacterial Endophytes

Molecular characterization of the effective isolates was done to identify the genus and species of the obtained isolates. The primer amplified the region of 1500 base pair. The amplified DNA samples were purified and sequenced. The obtained sequence was blasted in NCBI data base. Isolate BSE5 was identified as *Pseudomonas aeruginosa* and isolate BSE9 was identified as *Bacillus xiamenensis*.

Table 1. Effect of bacterial seed endophytes against the mycelial growth of *Fusarium oxysporum* and *Macrophomina phaseolina*

Isolate	*Fusarium oxysporum*			*Macrophomina phaseolina*		
	Inhibition zone (cm)	**Diameter of mycelial growth (cm)**	**Per cent inhibition over control**	**Inhibition zone (cm)**	**Diameter of mycelial growth (cm)**	**Per cent inhibition over control (%)**
Control	0	8.1	--	0	8.13	--
BSE 1	0.5	6.3	22.2	0	7.9	2.8
BSE 2	0.4	6.1	24.7	0	8.13	0.0
BSE 3	0	7.93	2.1	0	7.93	2.5
BSE 4	0.2	6.7	17.3	0.1	6.5	20.0
BSE 5	2.9	4	50.6	0.56	6.3	22.5
BSE 6	0	6.92	14.6	0.09	6.43	20.9
BSE 7	0	6.4	21.0	0.06	6.69	17.7
BSE 8	1	6	25.9	0	7.3	10.2
BSE 9	1.5	5.5	32.1	0.3	6.4	21.3
BSE 10	0	7.32	9.6	0	8	1.6
BSE 11	0	7.02	13.3	0	7.12	12.4
BSE 12	0	7.5	7.4	0.3	6.9	15.1
BSE 13	0	6.7	17.3	0	7.9	2.8
BSE14	0.6	6.5	19.8	0	7.12	12.4
CD (0.05)	**0.047**	**0.352**	**1.342**	**0.010**	**0.381**	**0.751**
SEd	**0.023**	**0.172**	**0.655**	**0.005**	**0.186**	**0.367**

Fig. 1. Effect of bacterial seed endophytes on the growth of *Fusarium oxysporum*

Table 2. Effect of bacterial seed endophytes on germination and growth promotion in blackgram

Isolate	Germination percentage (%)	Root length (cm)	Shoot length (cm)	Dry matter production (g)	Vigour index I	Vigour index II
Control	84	14.8	18.5	0.21	2796	18
BSE1	86	17.2	21.3	0.25	3311	22
BSE 2	84	15.3	18.7	0.23	2856	19
BSE 3	87	17.7	21.7	0.26	3428	23
BSE 4	83	14.8	17.9	0.23	2722	19

Isolate	Germination percentage (%)	Root length (cm)	Shoot length (cm)	Dry matter production (g)	Vigour index I	Vigour index II
BSE 5	94	22.4	24.3	0.27	4390	25
BSE 6	92	18.7	22.5	0.26	3790	24
BSE 7	89	16.3	19.3	0.24	3168	21
BSE 8	92	19.9	22.9	0.26	3938	24
BSE 9	94	20.9	23.7	0.28	4192	26
BSE 10	88	18.1	21.6	0.25	3494	22
BSE 11	92	19.8	21.8	0.25	3827	23
BSE 12	94	20.4	22.3	0.26	4014	24
BSE 13	88	17.3	20.4	0.24	3318	21
BSE 14	92	18.7	21.8	0.25	3726	23
SEd	**2.253**	**0.462**	**0.541**	**0.008**	**89.87**	**0.568**
CD (0.05)	**4.602**	**0.945**	**1.105**	**0.016**	**183.55**	**1.159**

Fig. 2. Effect of bacterial seed endophytes on the growth of *Macrophomina phaseolina*

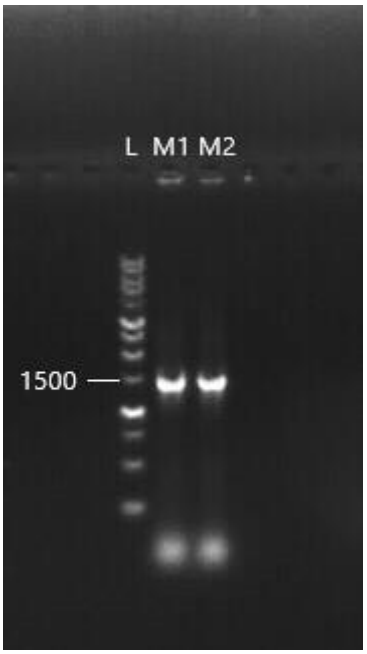

Fig. 3. Agarose gel electrophoresis showing the single band of 1500 bp of the polymerase chain reaction (PCR) amplification of 16S rDNA. LANE L- 1000 bp DNA marker; Lane M1- isolate BSE5; M2- isolate BSE9

4. DISCUSSION

Endophytic bacteria showing beneficial influence on the growth, development, and protection of plants against diseases facilitates sustainable approaches in agriculture [18-20]. Twenty five bacterial strains were screened in various dual culture assays, from which *P. aeruginosa* PPB3 and *A. aneurinilyticus* PPB9 were selected for superior antagonistic activity against *F. oxysporum* [21]. The formation of inhibition zone between the endophyte and the pathogen on agar medium is usually considered as a result of production of antibiotics, pH change and competition for nutrients [22]. *Pseudomonas fluorescens* produces antibiotic 2,4-diacetyl phloroglucinol which inhibited the growth of *Septoria tritici* [23]. In the present study also, *Pseudomonas aeruginosa* (BSE5) showed maximum inhibition of *F. oxysporum* and *M. phaseolina. P. aeruginosa* produces HCN, which play an important role in biological control and thus can be the mechanism behind inhibition of *M. phaseolina* and *Fusarium oxysporum.* This result is in accordance with study conducted by [24], were *P.aeruginosa* P4 tested positive for HCN production which correlated with the *in vitro* antibiosis of *F. oxysporum*. *P. aeruginosa is* also known to produce siderophores, compounds that can limit iron availability to soil-borne fungi [25], inhibit fungal spore germination [26] further aiding in biocontrol.

The endophyte *Bacillus xiamenensis* (BSE9) has been observed to inhibit both *M. phaseolina* and *F. oxysporum*. Similar results were recorded by Xia [27], where the pathogen and disease control were observed using *Bacillus xiamenensis.* The mechanism behind this inhibition was due to generation of extracellular enzymes that can hydrolyze the fungal cell wall possessing broad spectrum of antibiotic resistance, competition for nutrients in rhizosphere, production of antibiotics and induction of systemic resistance (ISR) against pathogen infections in plants. *Bacillus spp.* has known to exert fungicidal effects, such as inhibition of germination or the lysis of fungal mycelia by production of various extracellular antimicrobial compounds as diffusible and/or volatile molecules that act on phytopathogenic fungi [28].

In the current study, both *P. aeruginosa* (BSE5) and *Bacillus xiamenensis* (BSE9) have been observed to produce IAA, which can be attributed to the enhancement in plant growth. *Delftia lacustris* and *Rahnella aquatili*, two rhizosphere endophytes have been observed to produce β-Indole acetic acid, which enhanced plant growth and also ameliorated damage caused by phytoplasma-associated diseases at the sugarcane seedling stage [29]. Similar results were obtained by Hwang [30], where the endophytes producing IAA have

shown to improve plant growth even under abiotic stress.

5. CONCLUSION

Collectively, explorative study of blackgram seed micro biome has proven the presence of several beneficial endophytes having potential pathogen suppression ability and growth promotion activity in blackgram. The efficient endophytes, *P. aeruginosa* (BSE5) and *Bacillus xiamenensis* (BSE9) have proven to have inhibitory effects against phytopathogenic fungi, *M. phaseolina* and *F. oxysporum* and have shown significant growth promotion activity in blackgram seedlings. Further studies can be taken up by conducting field trials and developing a formulation for commercial application. The use of these endophytes in seed biopriming will reduce the harmful effects of commercial seed treatment fungicides, leading to sustainable agricultural production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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