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Biocontrol potential of endophytic *Pseudomonas stutzeri* isolated from watermelon (*Citrullus lanatus* Thunb) against *Colletotrichum orbiculare* causing anthracnose disease in watermelon

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ABSTRACT: The biocontrol potential of bacterial endophytes isolated from watermelon leaf was evaluated against the pathogen causing anthracnose disease in watermelon. Totally ten *Colletotrichum* isolates were isolated using tissue segment method and confirmed as *Colletotrichum* sp. based on the cultural and morphological characteristics. The virulence of the pathogenic isolates was confirmed through pathogenicity test. Among the 10 isolates, WEC 2 was identified as a highly virulent isolate. Eighteen bacterial endophytes were isolated from healthy watermelon plants and identified as *Bacillus* spp. (10 isolates) and *Pseudomonas* spp. (8 isolates) based on the cultural characters. The effect of 18 bacterial endophytes was tested against the highly virulent *Colletotrichum* isolate WEC 2 using dual culture technique. Among the 18 bacterial endophytes, isolate WE 8 was found to be highly potential in inhibiting the pathogenic isolate WEC 2. The next best isolate was WEC 17, which exhibited 57.78 % inhibition of pathogen over control. The least mycelial reduction was depicted by the isolate WE 16. The pathogenic isolate (WEC 2) was further confirmed at molecular level using the universal primers ITS 1 and ITS 4 as *Colletotrichum orbiculare* (WEC 2) and the potential bacterial endophytic isolate WE 8 as *Pseudomonas stutzeri* (WE 8) using ITS 27 F and ITS 1492 R primers.

Keywords: Endophyte, watermelon, anthracnose, *Colletotrichum orbiculare, Pseudomonas stutzeri*, molecular characterization

INTRODUCTION

Watermelon (*Citrullus lanatus* (Thunb.) Matsum and Nakai) is an important cucurbitaceous fruit crop and is widely consumed around the world (Naveen kumar *et al.*, 2017). The crop is grown commercially in areas with long frost-free warm periods in the tropics and subtropics (Prohens and Nuez, 2008). India is the second largest producer of watermelon among the Asian countries (Tiamiyu *et al.*, 2015). Moreover, it is mainly cultivated in Karnataka, Madhya Pradesh, Maharastra, Punjab, Rajasthan, Tamil nadu and Uttar Pradesh. In Tamil nadu, the crop is cultivated in Ariyalur, Erode, Thiruvallur, Pudukkottai, Namakal and Villupuram (Lilly, 2013). Watermelons are susceptible to several diseases that attack the roots, foliage and fruits. The main concern is related to leaf blight (*Alternaria cucumerina*), gummy stem blight (*Didymella bryoniae*), anthracnose (*Colletotrichum orbiculare*) and fusarium wilt (*Fusarium oxysporum* f.sp. *niveum*) (Bulajic *et al.,* 2008). Among the various fungal diseases, anthracnose caused by *C. orbiculare* is considered as one of the important diseases. Economic losses caused by the disease are mainly attributed to lowering of fruit quality and marketability (Zivkovic*et al.*, 2017). Anthracnose caused by the fungi *Colletotrichum* species complex is one of the most significant diseases of cucumber (*Cucumis sativus* L.), melons (*Cucumis melo* L.), pumpkin (*Cucurbita pepo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai). The disease is widespread under both greenhouse and field cultivations and cause severe infection on seedlings, leaves, petioles, stems and fruits of *Cucurbitaceae* and other herbaceous hosts belonging to the families such as *Asteraceae, Fabaceae* and *Malvaceae* (Farr and Rossman, 2013).

The structure and ecosystem of plant and soil have been seriously damaged by the overuse of chemical fertilizers and fungicides in agriculture (Abbamondi *et al.*, 2016). Therefore, a great attention has been taken. to reduce the use of these chemicals and to develop microorganisms as biological and environmentally friendly control agents in agricultural disease management. Many microorganisms have been reported to be beneficial to agriculture (Tao *et al.*, 2019). Among them, endophytic bacteria play a significant role in plant growth because of their abilities in solubilizing mineral phosphate and

producing a diverse range of secondary metabolites, such as indole 3- acetic acid (IAA) and siderophore. In addition, endophytic bacteria have the inhibitory effect against plant pathogens and effectively control the plant diseases caused by pathogens, and further promote plant growth (Yang *et al.,* 2021). Hence, the present study was under taken to investigate the biocontrol potential of bacterial endophytes isolated from watermelon against the anthracnose pathogen.

MATERIALS AND METHODS

Isolation and identification of the pathogen

The fungal pathogen was isolated from the infected tissues by employing standard isolation method with slight modification (Zivkovic *et al.*, 2017). The infected tissues were cut into small pieces and surface sterilized with 1% sodium hypochlorite for 30 seconds. Then the leaf bits were serially washed thrice with sterile distilled water (SDW) and dried between two layers of blotting papers. After that the bits were plated onto PDA medium containing 100 ppm streptomycin in sterile Petri dishes and incubated at room temperature (28±2°C). The fungal growth appeared around the bits were sub-cultured on PDA medium and maintained on slants for further studies.

Pathogenicity test

Preparation of spore suspension and inoculation

The pathogenic isolates were tested for their pathogenicity under glass house conditions. One month old watermelon plants were artificially inoculated with conidial suspension ($2x10^6$ cfu/ml) of *Colletotrichum* isolates. On cool evening hours, the plants were squirted with conidial suspension and moistened with damp cotton. Such inoculated plants were covered with polythene bags for seven days. Control plants were retained only by spraying with sterile distilled water. For each isolates three replications were maintained. After seven days of inoculation, these plants were observed for symptom development and the per cent disease index (PDI) was assessed as per the standard grade chart described by Pandey *et al.* (2003). Based on the PDI, virulence of the isolates were assessed and the isolate with maximum PDI was considered as the virulent one. Re-isolation was done from the inoculated plant and compared with the original culture for confirmation of the pathogen. The per cent disease index (PDI) was worked out using the formula Described by Mc Kinney (1923).

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves graded}} \times \frac{100}{\text{Maximum grade}}$$

Table 1. Survey on the incidence of anthracnose disease in different watermelon growing areas of Tamil Nadu

Location	District	Latitude	Longitude	Isolate code	Disease Incidence (PDI)	PDI* under pot culture
Perode	Erode	11.38756	77.629739	WEC 1	29.11	39.25^{d} (38.79)
Chittode	Erode	11.389647	77.63415	WEC 2	28.4	60.15^{a} (50.85)
Kadamathur	Thiruvallur	13.140167	77.505988	WTC 3	34.6	28.49^{f} (32.25)
Chithamur	Pudukkottai	10.37^{0}N	78.89^{0}E	WPC 4	15.32	31.57^{e} (34.18)
Alambadi	Vilupuram	12.006865	79.299048	WVC 5	21.36	57.61^{b} (49.37)
Perumandampalayam	Nammakal	11.114390	78.108255	WNC 6	21.74	25.69^{g} (30.45)
Nochipatti	Nammakal	11.125560	78.117814	WNC 7	19.66	31.57^{e} (34.18)
Tindivanam	Villupuram	12.202319	79.838118	WVC 8	18.34	19.78^{h} (26.40)
Nagamangalam	Ariyalur	11.079468	79.189324	WAC 9	14.83	33.16^{e} (35.15)
K. Kaikatti	Ariyalur	11.123505	79.136905	WAC 10	17.24	42.21^{c} (40.51)

PDI: Per cent Disease Index. *Values are mean of three replications. Values in parentheses are arc sine transformed values. In a column, means followed by a common letter are not significantly different at 5 % levels by LSD.

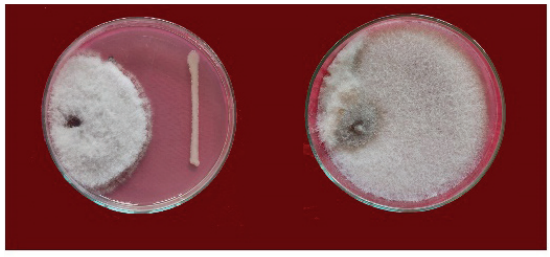
Table 2. Bacterial endophytic antagonists isolated from watermelon and their antifungal activity against *Colletotrichum* sp.

Location	District	Latitude	Longitude	Isolate code	Mycelial growth (cm)*	Per cent reduction over control
Perode	Erode	11.387549	77.629784	WE 1	5.1^{gh}	43.33 (41.15)
Perode	Erode	11.38756	77.629739	WE 2	4.4^{ef}	51.11 (45.61)
Perode	Erode	11.390023	77.631818	WE 3	4.0^{bc}	55.56 (48.17)
Perode	Erode	11.386433	77.628815	WE 4	5.9^{k}	34.44 (35.91)
Chittode	Erode	11.389655	77.634126	WE 5	5.6^{ij}	37.78 (37.90)
Chittode	Erode	11.388652	77.63447	WE 6	4.1^{cd}	54.44 (47.52)
Chittode	Erode	11.389647	77.63415	WE 7	4.6^{f}	48.89 (44.34)
Kadamathur	Thiruvallur	13.140167	77.505988	WE 8	3.7^{a}	58.89 (50.10)
Pallapattu	Thiruvallur	13.084008	80.017779	WE 9	5.4^{ij}	40.00 (39.21)
Ponneri	Thiruvallur	13.083959	80.017742	WE 10	5.6^{j}	37.78 (37.91)
Ponneri	Thiruvallur	13.0834566	80.01755	WE 11	6.2^{l}	31.11 (33.88)
Nochipatti	Namakkal	11.125560	78.117814	WE 12	4.3^{de}	52.22 (46.25)
Nagamangalam	Ariyalur	11.079468	79.189324	WE 13	5.3^{hi}	41.11 (39.86)
Alangudi	Pudukkottai	10.371200	78.895400	WE 14	4.9^{g}	45.56 (42.43)
Chithamur	Pudukkottai	10.574055	79.009224	WE 15	4.2^{cde}	53.33 (46.88)
Alambadi	Vilupuram	11.993934	79.28052	WE 16	6.9^{m}	23.33 (28.87)
Alambadi	Vilupuram	12.006865	79.299048	WE 17	3.8^{ab}	57.78 (49.45)
Alambadi	Vilupuram	12.01155	79.301629	WE 18	5.1^{g}	43.33 (41.14)
CD (p = 0.05)					0.26	1.22

Values are mean of three replications. Values in parentheses are arc sine transformed values.
In a column, means followed by a common letter are not significantly different at 5 % levels by LSD.

Isolation of antagonistic endophytic bacteria from watermelon

Healthy leaf samples were collected in sterile paper bags from different watermelon growing areas of Tamil Nadu. Leaves were cut into pieces and sterilized with 5% sodium hypochlorite for 3 min, followed by washing in distilled water for 3 times and dried by using sterile filter paper. The leaf bits were then crushed in a sterile mortar and the sap was serially diluted and streaked on to nutrient agar (NA) plates. The sterile water obtained from the last washing step was streaked on NA plates as control. This was followed by the incubation of the plates at 27 °C for 3 days. The selection of bacterial colonies was done based on distinct morphological features (colour, size, shape) and pure cultures were obtained by streak-plate technique (Al-Hussini*et al.*, 2019).



WE 8 Control

Fig 1. Antifungal activity of endophytic bacterial isolates (WE 8) against the *Colletotrichum* sp.

***In vitro* screening of antagonistic bacteria against *Colletotrichum* sp.**

Eighteen endophytic bacterial isolates were investigated *in vitro* for their antagonistic vitality against the virulent isolate (WEC 2) of anthracnose pathogen (*Colletotrichum* sp.) by dual culture technique. A bacterial colony was streaked at one end of a 9-cm Petri dish containing PDA medium. A 5-mm mycelial disc of a 7-day-old culture of the test fungal pathogens was placed on the other end and incubated at 27 °C until mycelial growth of the test fungus covers the entire Petri plate in the control. At the end, the inhibition zone was measured by measuring the distance between the 2 sides of the tested Petri dish (Al-Nadabi *et al*., 2021)**.**

Molecular characterization

Anthracnose pathogen

Total genomic DNA was isolated from mycelium of the virulent isolate (WEC 2) as described by White el at (1990).The isolated DNA was re-suspended in 50 µl of milliQ water or 1X TE buffer and stored at -20°C for further use. To check the quality of isolated DNA, 2.5µl of total DNA was resolved in the 1% agarose gel.

Amplification and sequencing of ITS region of rDNA

Polymerase chain reaction (PCR) was performed in a total volume of 50 µl using the Emerald Amp® GT PCR master mix using genomic DNA isolated from *Colletotrichum* sp. as a template. The intermediate regions of the 5.8S ribosomal gene were amplified using primer pairs ITS1 (5'TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3'). PCR cycle includes 4 minutes at 94 °C for Initial denaturation, followed by 35 cycles of 1 minute at 94 °C, 1 minute at 55 °C for annealing and 1 minute at 72° C with a final 7 minute extension at 72° C. The PCR products were resolved by electrophoresis in 1% agarose gel and it was sequenced at Bioserve Biotechnologies India Pvt Ltd, Bangalore.

Molecular characterization of potential bacterial antagonist

The genomic DNA from potential antagonistic bacterial isolates was isolated using the standard protocol of Cetyl trimethyl ammonium bromide (CTAB) method (Wilson, 2001).

Two milli liter of actively grown broth culture was taken in 2 ml centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4°C. The supernatant was removed and the pellet was suspended in 1 ml TE buffer. To that, 0.5 ml of 1-butanol was added and vortexed well to mix the cells thoroughly (to remove extra cellular materials) and centrifuged at 6000 rpm for 5 min at 4°C. The supernatant (both supernatant layer and aqueous layer) was discarded and the pellet was re-suspended in 2 ml of TE buffer and centrifuged at 6,000 rpm at 4°C for 5 min to remove all traces of butanol. Again the pellet was re-suspended in 1ml TE buffer. To that, 100 µl of lysozyme (10 mg ml^{-1} freshly prepared) was added and incubated at room temperature for 5 min. After incubation, 100 µl of 10 % SDS and 25 µl of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37°C for one hour. To the above mixture, 200 µl of 5M NaCl was added and mixed well. To this mixture, 150 µl of CTAB solution was added, mixed well and incubated at 65°C for 10 min. The mixture was extracted with 1ml of phenol: chloroform mixture in the ratio of 25:24, mixed well and centrifuged at 14000 rpm for 15 min at 4°C. The aqueous layer was transferred carefully to a 2 ml micro-centrifuge tube and DNA was precipitated by adding equal volume of ice cold iso-propanol by incubating overnight at - 20°C. The DNA was pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. The pellet was washed with 70 per cent ethanol for 2 times and dried under vacuum or kept on water bath at 50- 55°C for 10 mins and re-suspended in 50 µl of TE buffer. The DNA was stored at -20°C for further use. The bacterial genome DNA was verified on agarose gel electrophoresis method as described earlier.

16S rDNA sequencing of endophytic bacteria

Polymerase Chain reaction was performed in a total volume of 50 µl using Emerald Amp® GT PCR master mix using genomic DNA of endophytic bacteria as a template. The small sub unit 16S region was amplified with universal primers. The primer pair used were 27F + 1492r. PCR conditions followed were, initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing temperature at 50°C for 30 sec and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The reaction was carried out in Eppendorf MasterCycler gradient PCR machine. The PCR products were then resolved by electrophoresis in 1% agarose gel. Later the PCR products were purified using FavorPrep

GEL/ PCR purification kit and sequenced at Bioserve Biotechnologies India Pvt. Ltd. Bangalore. (Isik*et al.*, 2014).

The Primers sequence used for amplification of 16S rRNA region, 27f - 5′ AGAGTTTGATCTGGCTCAG 3′ (forward primer) 1492r - 5′ TACGGYTACCTTGTTACGACT 3′ (reverse primer)

Statistical analysis

All the experiments were done in triplicates. The treatment mean differences were analysed using ANOVA and Duncan`s Multiple Range Test with a 5% significance level (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Isolation, cultural and morphological characterization of anthracnose pathogen

Anthracnose pathogen (*Colletotrichum* sp.) was isolated from the infected leaves of watermelon collected during the survey (Table 1). Numerous fungal colonies were obtained from infected leaves, totally of 10 *Colletotrichum* isolates were confirmed through microscopic examination based on their conidial structure. All isolates resembled as dark gray to white mycelium on PDA medium after incubation at 26^{o}C for seven days in the dark.

Pathogenicity test

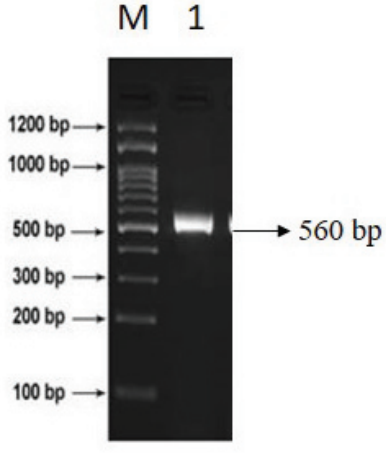
Pot culture experiment was conducted to study the virulence of different *Colletotrichum* isolates. The results showed that all the isolates induced symptoms on the inoculated watermelon leaves as like the natural symptoms. Among the 10 isolates, WEC 2 isolate collected from Erode district was found to be highly virulent with the PDI of 60.15 per cent. The isolate WVC 5 from Vilupuram stood next with the PDI of 57.61 per cent, whereas infection in other isolates ranged from 19.78 to 42.21 per cent (Table 1). The virulent isolate (WEC 2) exhibited initial development of dried up infected area and became dark brown, while necrotic areas broke and shattered at the centre of the lesion. The isolate (WVC 8) was identified as least virulent isolate, which exhibited a PDI of 19.78 per cent.

Isolation of bacterial endophytes from watermelon

Endophytic bacteria were isolated from healthy leaves and stems of watermelon crop. Totally 18 isolates were isolated by using serial dilution method on nutrient agar medium. Out of 18 endophytic bacteria, two different bacterial colonies were identified based on their morphology and biochemical characterization. The bacterial colonies were cultured on separate Petri plates containing NA media for getting pure culture and maintained at 4^{o}C for further studies (Table 2).

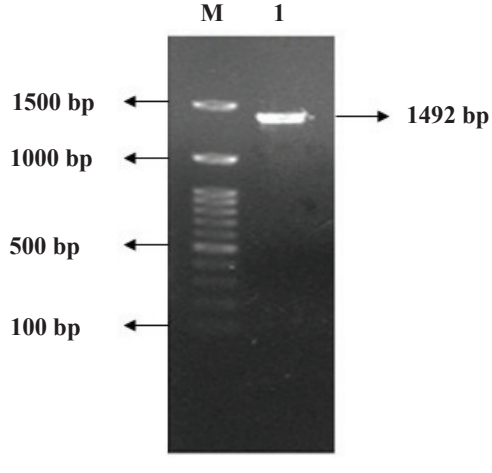
***In vitro* antifungal activity of endophytic bacterial isolates against *Colletotrichum* sp.**

In vitro antagonistic effects of bacterial endophytes were tested against *C. orbiculare* using the dual culture assay. Among the eighteen endophtyic bacteria, WE 8 isolate showed the maximum growth reduction of *C. orbiculare* by 58.89 per cent over the control and it was followed by WE 17 isolate which recorded PDI of 57.78 per cent. The minimum mycelial growth reduction



M- Marker (1000bp); 1- *Colletotrichum orbiculare* (WEC 2)

Fig 2. Molecular identification of virulent isolate of pathogen (*Colletotrichum* sp.)



M- Marker (1000bp); 1- *Pseudomonas stutzeri* (WE 8)

Fig 3. Molecular identificationof effective endophytic bacteria (WE 8)

of 23.33 per cent was observed in the WE 16 isolate (Table 2 & Fig.1). Rakotoniriana *et al.* (2012) isolated thirty-one endophytic bacteria from healthy leaves of *Centella asiatica* and screened against *Colletotrichum higginsianum*. The result showed that endophytic bacteria *Pseudomonas fluorescens* BCA08 inhibited the mycelial growth of *Colletotrichum higginsianum* by 82 per cent. According to Silva *et al.* (2016),the endophytic bacteria isolated from the guarana seed had growth inhibition percentage of 52.41% against *C. gloeosporioides.*

Molecular characterization of *Colletotrichum* sp.

The virulent isolate of *Colletotrichum* sp. (WEC 2) was observed macroscopically and microscopically and confirmed as *Colletotrichum* sp. by morphological and cultural characters at genus level. Further, its identity was confirmed through molecular technique using ITS sequence analysis.

The Internal transcribed spacer (ITS) region of *Colletotrichum* sp. isolate WEC 2 was amplified with primers ITS 1 and ITS 4 and the products obtained was detected as a single band in agarose gel stained with ethidium bromide. The size of the PCR fragments was approximately 600 bp (Fig 4). The DNA fragment obtained was sequenced at Bioserve Biotechnologies India Pvt, Ltd. The full length 16S rRNA sequences obtained was BLAST searched in the database of National Centre for Biotechnology Information (NCBI) and identified as *C. orbiculare*. Zivkovic *et al.* (2017) reported that *Colletotrichum* Serbian isolates LC1, LC2 and LC3 produced approximately 600-800 bp fragment, which had 100 % similarity with *C. orbiculare* isolate retrieved from the GeneBank database. Kumar *et al.*, (2010) identified and confirmed 21 isolates of *Colletotrichum* sp. using universal primers (ITS 1 and ITS 4) which produced a fragment of 600 bp and it had 95 % to 100 % similarity with the sequences of isolates retrieved from the GeneBank database.

Molecular characterization of effective endophytic antagonistic bacteria

The 16S rDNA sequence analysis is one of the commonly used molecular methods for the identification of bacteria at species level. The 16S rRNA from four virulent isolates WE 8 isolated using CTAB method. Single band of intact genomic DNA was visualized on the agarose gel. The 16S rRNA region of these bacterial isolates was amplified with primer pairs 27f and 1492r using a thermo cycler and the products produced were visualized as a single band in agarose gel strained with ethidium bromide. The size of the PCR fragments was approximately 1492 bp length for primer pair 27f and 1492r. The PCR products were sequenced at Bioserve Biotechnologies India Pvt, Ltd. The full length 16S rRNA sequence obtained for potential bacterial species was BLAST searched in the database of National Centre for Biotechnology Information (NCBI). The potential isolates were molecularly identified at the species level as *Pseudomonas stutzeri* (Fig. 3). Similarly, Islam *et al*. (2016) isolated ten bacterial isolates from the endophere region of cucumber and assigned isolate code *viz.,* PP B1,PPB2,PPB3,PPB4,PPB5,PPB8,PPB9,PPB9,PPB1 0,PPB11 and PPB12 and they were identified through morphological and biochemical methods. Further they were molecularly characterized with partial sequencing of 16s rRNA and identified as *Pseudomonas stutzeri, Bacillus subtilis, Stentotrophomonas maltphilia* and *Bacillus amyloliquefaciens*. Isolate PPB1 had 99% homology with *Pseudomonas stutzeri* and was submitted to Gen Bank under accession number KJ959616. Yuwantiningsih *et al.* (2015) isolated the endophytic bacteria from the forest crop and molecularly identified through universal primers (27F and 1492R) which produced fragments of 1500 bp and it had 99 % similarity.

CONCLUSION

The present study illustrated the effectiveness of 18 endophytic bacteria against anthracnose pathogen,C*olletotrichum orbiculare*. Among the 18 endophytic bacterial isolates, one isolate WE 8 showed the maximum antagonistic activity against the pathogen and was confirmed as *Pseudomonas stutzeri* through molecular approach.

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