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Antagonistic Potential of Endophytic *Trichoderma asperellum* against *Alternaria alternata* causing Leaf Blight in Watermelon

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ABSTRACT: The present study was conducted to assess the potential of fungal endophytes against *Alternaria alternata* causing leaf blight disease in watermelon. Totally of sixteen *Alternaria* isolates were isolated using tissue segment method and confirmed as *Alternaria* sp. based on the cultural and morphological characteristics. The virulence of the pathogen isolates was confirmed through pathogenicity test. Among the 16 isolates WTA 7 was identified as highly virulent isolate. Fifteen fungal endophytes were isolated from healthy watermelon plants and identified as *Trichoderma* spp. and *Fusarium* spp. based on the cultural and conidial characters. The effect of 15 endophytes were tested against the highly virulent *Alternaria* isolate WTA 7 using dual culture technique. Among the 15 endophytes, isolate WTD 5 was found to be highly potential in inhibiting the pathogenic isolate WTA 7. The next best isolate was WTD 6, which exhibited 70 % inhibition of pathogen over control. The least mycelial reduction was depicted by the isolate WF13. The pathogenic isolate (WTA 7) and the potential endophytic isolate WTD5 were further confirmed at molecular level using the universal primers ITS 1 and ITS 4 as *Alternaria alternata* (WTA 7) and *Trichoderma asperellum* (WTD 5) respectively. Thus, it is concluded that *Trichoderma asperellum* inhibit the mycelial growth of *Alternaria. alternata* under *in vitro* condition.

Keywords: Watermelon, Leaf blight, *Alternaria alternata*, *Trichoderma asperellum*, Molecular characterization, Antagonism, Biological control.

INTRODUCTION

Watermelon (*Citrullus lanatus* Thunb) is an economically important fruit crop that belongs to family Cucurbitaceae. The annual production was recorded as 77.5 million tonnes worldwide (Food and Agriculture organisation, 2021). India ranks 2nd after China with respect to production of watermelon in Asia, producing around 2, 55,000 tonnes annually. In India mainly cultivated in the following states *viz.*, Maharashtra, Karnataka, Tamil Nadu, Punjab, Rajasthan, Madhya Pradesh and Uttar Pradesh. In Tamil Nadu, watermelon production is about 381.55 tonnes, which is 7.53% of India's total share (National Horticulture Board, 2019-20). Watermelon production is drastically reduced by various diseases, such as, *Fusarium oxysporum, Podosphaera xanthii, Didymella bryoniae, Colletotrichum orbiculare, Nigrospora sphaerica*, and bacterial diseases (Burdman and Walcott, 2012; Ismail *et al.*, 2021; Noh *et al*., 2014). Among various diseases, leaf blight caused by the genus *Alternaria* is devastating disease that results in reduced quality, fruit size and yield (Chopra *et al*., 1974; Kim *et al*., 1994; Zhao *et al*., 2016). Jackson and Weber, (1959) reported that *Alternaria cucumerina* was associated with causal agent leaf blight in watermelons. Besides *Alternaria cucumerina* various other *Alternaria* species are also associated with leaf blight diseases *viz.*, *A.cucumerina, A. alternata, A. tenuissima, A. gaisen* and *A. infectoria*. Various management strategies are being utilized by the farming community for the management of foliar diseases. Utilization of chemicals for the management of the diseases cause environment pollution, health hazardous and also cost expensive. To eliminate the indiscriminate use of chemicals for the management of crop diseases and their influence on the

environment, an alternative approach using bio control agents is the need of the day.

Endophytic fungi are living inside the plant species through asymptomatic manner, which exhibits symbiotic relationship with its host plants. Which in turn provide increased competitiveness and resistance towards herbivores, pathogens, and various abiotic stresses (Pavithra *et al*., 2020). Hence, the present study was aimed to isolate and characterize the fungal endophytes that naturally colonize the watermelon and their effect on leaf blight (*A. alternata*) pathogen.

MATERIALS AND METHODS

Isolation and identification of leaf blight pathogen. Infected watermelon leaves and stems exhibiting leaf blight lesions were collected and stored under refrigerator at 4°C for isolation of pathogen. By using tissue separation method, leaf showing leaf blight area were recovered from disease tissue (Zheng *et al*., 2015). Numerous fungal colonies were obtained, only with the morphologies of *Alternaria* was selected as isolates. Pure cultures of these isolates were obtained by single spore purification and periodically sub cultured onto new PDA plates for culture maintenance. In addition, these isolates were identified based on morphological and molecular confirmation.

Pathogenicity test

Preparation of spore suspension and inoculation. The pathogenic isolates were tested for their pathogenicity under glass house condition. One month old watermelon plants were artificially inoculated with conidial suspension (2×10^6 cfu/ml). 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).

$$\text{PDI} = \frac{\text{Sum of all numerical ratings}}{\text{Total no. of leaves graded}} \times \frac{100}{\text{Maximum grade}}$$

Isolation of Endophytic fungi from watermelon crop. Endophytic fungi was isolated from asymptomatic plant samples (leaf and stems) according to the protocol followed by joncy *et al*. (2019) with slight modification. After surface sterilization the sample were macerated with 1 ml of sterile water by using pestle and mortar. The homogenized samples were serially diluted in sterile test tube containing 9 ml of sterile water. The dilution with 10^{-3} and 10^{-4} were plated on Petri plate containing PDA medium supplemented with streptomycin. The observation were made on plates incubated after 7 days at 28±2° for fungal growth and were sub cultured for further use.

In vitro* screening of antagonistic fungi against *Alternaria sp.

Dual culture experiments were performed to determine the inhibitory activity of 15 isolates of endophytes against *Alternaria* sp. Arnold *et al*., (2001). An agar plug (5 mm in diameter) of each endophytic fungus was excised from a 5-day-old colony on the PDA and placed on one side of the PDA plate. Similarly, pathogen mycelial plugs were excised from 5-day-old colony on the PDA, placed on the opposite side of the PDA plate, and incubated at 25°C for 5 days. The pathogen mycelial plug, also placed alone, served as a control. After 7 days, radial growth of pathogen's mycelial growth was measured. The per cent inhibition was calculated as described by Nuangmek *et al*., (2008) by using the following formula: $[(A-B)/A] \times 100$, where, A represents the radial growth of pathogen in the control and B represents the radial growth of pathogen after treatment. Three replicas were maintained for each treatment and the experiment was repeated twice. Effective fungal antagonists for mycelial growth of pathogens were selected and used in subsequent experiments.

Molecular characterization

(i) Leaf blight pathogen. Total genomic DNA was isolated from mycelium of the virulent isolate (WTA 7) as described by Lee and Taylor (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 electrophoresis.

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 from *Alternaria* 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 for *Alternaria* sp. 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.

(ii) Effective fungal antagonist. Total genomic DNA was isolated from *Trichoderma* sp. as described by Lee and Taylor (1990).

Polymerase chain reactions were performed with a total volume of 50 µl using the Emerald Amp® GT PCR master mix using *Trichoderma* 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 for *Trichoderma* sp. were 5 mins at 95°C for initial denaturation, followed by 35 cycles of 30s at 95°C for denaturation, annealing at 52°C for 30s, 54°C for 45s,

52°C for 1 min, extension at 72°C for 1 min, and a final cycle at 72°C for 10 min. The PCR products were resolved by electrophoresis in 1% agarose gel and it was sequenced at Bioserve Biotechnologies India Pvt Ltd.

Statistical Analysis. 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 pathogen. Leaf blight pathogen (*Alternaria* sp.) was isolated from the disease infected leaves of watermelon collected during the survey (Table 1 & Fig. 1). Numerous fungal colonies were obtained from infected leaves, totally of 16 *Alternaria* isolates were confirmed through microscopic examination based on their conidial structure. All isolates resembled as loose cottony and grey to black colour on PDA medium after incubation at 26° for seven days in the dark (Fig. 2).

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

Sr. No.	Location	District	Latitude	Longitude	Isolate code	Per cent Disease Index (%)
1.	Perode	Erode	11.387549	77.629784	WEA 1	25.34
2.	Perode	Erode	11.390023	77.631818	WEA 2	22.60
3.	Chittode	Erode	11.389655	77.634126	WEA 3	24.10
4.	Chittode	Erode	11.388652	77.63447	WEA 4	19.81
5.	Kavinad	Pudukkottai	10.381120	78.775401	WPA 5	25.14
6.	Ponneri	Thiruvallur	13.083959	80.017742	WTA 6	27.52
7.	Pallapattu	Thiruvallur	13.084008	80.017779	WTA 7	32.52
8.	Alangudi	Pudukkottai	10.371200	78.895400	WPA 8	19.36
9.	Alambadi	Vilupuram	11.993934	79.28052	WVA 9	14.98
10.	Alambadi	Vilupuram	12.01155	79.301629	WVA 10	20.74
11.	Muthur	Namakkal	11.102112	78.101626	WNA11	23.14
12.	Perumandampalayam	Namakkal	11.114390	78.108255	WNA12	21.46
13.	Nochipatti	Namakkal	11.125560	78.117814	WNA13	20.24
14.	Tindivanam	Vilupuram	12.202319	79.838113	WVA14	16.80
15.	Nagamangalam	Ariyalur	11.079468	79.189324	WAA15	19.54
16.	K.Kaikatti	Ariyalur	11.123505	79.136905	WAA16	20.18

Fig. 1. Symptoms of leaf blight (*Alternaria* sp.) in watermelon.

Mycelium in Petri plate

Morphology of conidia under the light microscope

Fig. 2. Isolate of leaf blight pathogen (*Alternaria* sp.).

Pathogenicity test

Pot culture experiment was conducted to study the virulence of different *Alternaria* isolates. The results showed that all the isolates induced the leaf blight symptoms on the inoculated watermelon leaves as like the natural symptoms. Among the 16 isolates, WTA 7 isolate collected from Thiruvallur district was found to be more virulent one with the PDI of 63.42 per cent. The isolate WEA 1 from Erode stood next with the PDI of 44.25 per cent whereas infection in other isolates ranged from 41.31 to 24.91 per cent. The virulent isolate (WTA 7) exhibited initial development of dark brown necrotic spots on leaves. The adjacent spots later coalesced with one another to form large irregular patches, which finally dried up (Table 2). The isolate (WAA15) was identified as least virulent isolate, which exhibited a PDI of 24.91 per cent.

Table 2: Pathogenicity of different isolates of *Alternaria* spp on watermelon plants under pot culture conditions.

Sr. No.	Isolate code	Per cent Disease Index (%)
1.	WEA 1	44.25^{c} (41.69)
2.	WEA 2	41.31^{d} (39.99)
3.	WEA 3	29.79^{g} (33.07)
4.	WEA 4	35.71^{e} (36.69)
5.	WTA 5	32.34^{ef} (34.65)
6.	WTA 6	58.61^{b} (49.95)
7.	WTA 7	63.42^{a} (52.78)
8.	WPA 8	28.79^{gh} (32.44)
9.	WVA 9	25.92^{h} (30.60)
10.	WVA 10	29.79^{g} (33.07)
11.	WNA11	31.56^{f} (34.17)
12.	WNA12	29.62^{g} (32.97)
13.	WNA13	42.12^{cd} (40.46)
14.	WVA14	46.79^{c} (43.15)
15.	WAA15	24.91^{h} (29.94)
16.	WAA16	33.52^{e} (35.37)
	CD (P = 0.05)	2.134

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 fungal endophytes from plant sample. Endophytic fungi were isolated from healthy leaves and stem of watermelon crop. Totally 15 isolates were isolated by using serial dilution method on potato dextrose agar medium. Out of 15 endophytic fungi, two different fungal colonies were identified based on their morphological characters. The fungal colonies were cultured on separate Petri plates containing PDA media for getting pure culture and maintained at 4^{o}C for further studies (Table 3).

Table 3: Isolation of fungal endophytic antagonists from watermelon.

Sr. No.	Location	District	Latitude	Longitude	Isolate code
1.	Perode	Erode	11.387549	77.629784	WTD1
2.	Alambadi	Villupuram	11.993934	79.28052	WTD 2
3.	Chittode	Erode	11.389655	77.634126	WTD 3
4.	Chittode	Erode	11.388652	77.63447	WTD4
5.	Kadamathur	Thiruvallur	13.140167	77.505988	WTD5
6.	Pallapattu	Thiruvallur	13.084008	80.017779	WTD6
7.	Ponneri	Thiruvallur	13.083959	80.017742	WTD7
8.	Alangudi	Pudukkottai	10.371200	78.895400	WTD 9
9.	Alangudi	Pudukkottai	10.371200	78.895400	WTD 9
10.	Chithamur	Pudukkottai	10.574055	79.009224	WTD10
11.	Kavinad	Pudukkottai	10.3632	78.8120	WTD11
12.	Nochipatti	Namakkal	11.125560	78.117814	WTD12
13.	Muthur	Namakkal	11.102112	78.101626	WF13
14.	K. Kaikatti	Ariyalur	11.123505	79.136905	WF14
15.	Nagamangalam	Ariyalur	11.079468	79.189324	WF15

Antifungal activity of endophtyic fungal isolates against *A. alternata* under *in vitro.* *In vitro* antagonistic effects of fungal endophytes were tested against *A.alternata* using the dual culture assay. Among the fifteen endophtyic fungi, WTD 5 isolate showed the maximum growth reduction of *A. alternata* by 73.33 per cent over the control and it was followed by WTD 6 isolate which recorded PDI of 70 per cent. The minimum mycelial growth reduction of 21.11 per cent was observed in the WF 13 isolate (Table 4 & Fig. 3). Matrood *et al*. (2020) studied the antagonistic activity of *Trichoderma* sp and reported that *Trichoderma* sp. inhibited the mycelial growth of *A. alternata* under *in vitro.* Camacho-Luna *et al*., (2021) reported that *T.asperellum* resulted in the growth inhibition of *Alternaria* sp by 56%, while *T. atroviridae* showed only 20% inhibition of the mycelial growth of the pathogen.

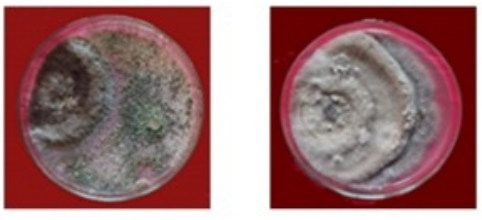


Fig. 3. Antifungal activity of endophytic fungal isolate against the *Alternaria* sp.

Table 4: *In vitro* antifungal activity of endophtyic fungal isolates against *A. alternate.*

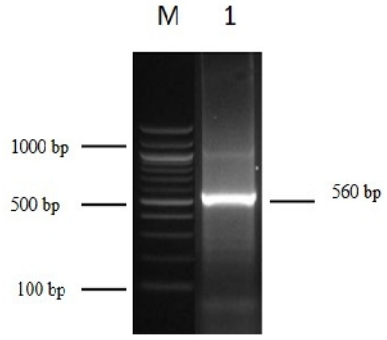
Sr. No.	Treatments	Mycelial growth of *Alternaria alternata* (cm)*	Per cent growth inhibition over control (%)
1.	WTD1	5.90^{e}	34.44 (35.93)
2.	WTD 2	5.20^{d}	42.22 (40.52)
3.	WTD 3	5.10^{d}	43.33 (40.59)
4.	WTD4	3.80^{cd}	57.77 (49.47)
5.	WTD5	2.40^{a}	73.33 (58.89)
6.	WTD6	2.70^{ab}	70.00 (56.79)
7.	WTD7	3.20^{bc}	64.44 (53.39)
8.	WTD 9	3.60^{c}	60.00 (50.77)
9.	WTD 9	2.90^{b}	67.77 (55.41)
10.	WTD10	3.10^{b}	65.55 (54.06)
11.	WTD11	5.20^{d}	42.22 (40.52)
12.	WTD12	5.40^{de}	40.00 (39.23)
13.	WF13	7.10^{h}	21.11 (27.35)
14.	WF14	6.90^{g}	23.33 (28.88)
15.	WF15	6.30^{f}	30.00 (33.21)
16.	Control	9.00	-
	CD (P=0.05)	0.19	2.469

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.

Molecular characterization of *Alternaria* sp. The virulent isolate of *Alternaria* sp. (WTA 7) was observed macroscopically and microscopically, *Alternaria* sp. was confirmed by morphological and cultural characters at genus level. In the present study, its identity was done by molecular technique using ITS sequence analysis.

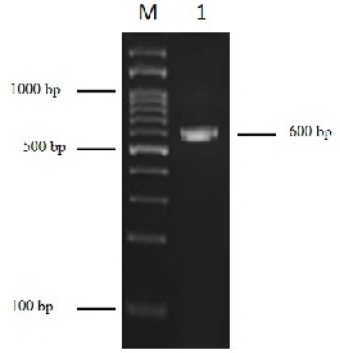
DNA from *Alternaria* sp. was isolated using CTAB method. Single band of intact genomic DNA was visualised in the agarose gel. The Internal transcribed spacer (ITS) region of *Alternaria* sp. isolate WTA 7 was amplified with primers ITS 1 and ITS 4 and the products obtained was visualised as a single band in agarose gel stained with ethidium bromide. The size of the PCR fragments was approximately 560 bp (Fig. 4).The PCR products 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). Zhao *et al*., (2016) reported that 31 *Alternaria* isolates produced a 546-bp fragment, which had more than 99 % similarity with the sequences of isolates retrieved from the GeneBank database. Tozlu *et al*. (2018) identified and confirmed the *Alternaria alternata* isolates by using universal primer ITS1 (5′TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′TCCTCCGCTTATTGATATGC-3′), which had 99 % and 95 % similarity with the sequences of isolates retrieved from the GeneBank database.

Molecular characterization of effective endophytic antagonistic fungi. Effective endophytic fungal isolate was characterized melocularly by PCR using the universal primer pair of ITS 1 and ITS 4 which produced amplicon size of 600 bp (Fig. 5). The PCR product was sequenced at Eurofin Genomics Bangalore, India and the sequenced obtained was submitted in the NCBI database. The accession number allotted for the effective isolate WTD 5 (*Trichoderma asperellum)* was Acc. No. OL468809. Matas- baca *et al*. (2021) reported that universal primers ITS 1 & ITS4 amplified 600 bp fragment for *Trichoderma asperellum*. The phylogenetic analysis revealed that the isolate matched with *Trichoderma* sp, which clustered by 98.11% identity with the species *Trichoderma asperellum*. García-Núñez *et al*., (2017) used universal primers ITS 1 & ITS4 for theidentification of *Trichoderma asperellum and* obtained amplicon at 600 bp. The results from GenBank through a BLAST analysis of the amplified sequences of the two primer pairs showed that the isolate, was 99 % identical to *T. asperellum*.



M- Marker (1000bp); 1- *Alternaria alternata* (WTA 7).

Fig. 4. Molecular characterization of virulent isolate of pathogen (*Alternaria* sp.).



M- Marker (1000bp); 1- *Trichoderma asperellum* (WTD.

Fig. 5. Molecular characterization of effective endophytic fungi (WTD 5). 5)

CONCLUSION

The present study illustrated the effectiveness of 15 endophytic fungi against leaf blight pathogen *A.alternata*. Among the 15 endophytic fungal isolates, one isolate WTD 5 showed the maximum antagonistic activity against the pathogen and was confirmed as *Trichoderma asperellum* through molecular approach. The biocontrol agents are alternative method for management of diseases and it is free from hazardous for environment and human health.

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Conflict of Interest. None.

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