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Exploitation of Fungal and Endophytic Bacteria for the Management of Leaf Blight of Ribbon Plant

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Abstract

Leaf blight of ribbon plant caused by *Alternaria alternata* is a serious disease in hanging baskets in households. *In vitro* experiments evaluated the effect of seven isolates of *Trichoderma* species (from ribbon plant rhizosphere) and ten isolates of endophytic bacteria (from ribbon plant phylloplane) were tested against *A. alternata*. All the *Trichoderma* species had varied antagonistic effects against the pathogen. Among them, THA recorded maximum growth inhibition of *A. alternata*. Most of the bacterial isolates tested were short rods and produced bright fluorescence when exposed to UV light. Among the isolates, EBL 5 produced the largest inhibition zone and the least mycelial growth of *A. alternata*. The study identified the *Trichoderma* isolate (THA) and endophytic bacterial isolate (EBL 5) performed well in inhibiting the mycelial growth of test pathogen.

Keywords: *Chlorophytum comosum; Alternaria alternata; Trichoderma* species; Bacterial isolates; Morphological characteristics

Introduction

Ribbon plant (*Chlorophytum comosum*) is a tufted grass-like clump forming, ever green perennial herb belonging to the family Liliaceae. It is native of tropical and southern Africa and is widely used as hanging baskets in households. It is otherwise called as airplane plant or bracket plant or boat lily or spider ivy or spider plant. The economic part is roots used as Chinese traditional medicine for treating bronchitis, fractures and burns.

Ribbon plant is affected by a number of fungal and bacterial diseases. Among the fungal disease, leaf blight caused by *Alternaria alternata* is a serious disease in hanging baskets in households. This is a first report in Tamil Nadu, India [1]. Due to this infection the leaves get ugly appearance (Figure 1). Traditionally, this disease is controlled by the application of synthetic fungicides. But the indiscriminate use of fungicides resulted in the accumulation of residual toxicity, environmental pollution and altered the biological balance in the soil by over killing the non-targeted microorganisms and developed resistance to the pathogen [2].

It is therefore essential to develop an effective cheap and environmentally safe non-chemical method for the management of leaf blight disease. Hence, Biological control has been developed as an alternative to synthetic fungicides and considerable success has been achieved by utilizing antagonistic microorganisms for controlling foliar diseases.

Figure 1: Symptoms of leaf blight of ribbon plant.

The need for alternative control strategies, particularly those involving biological control, has increased greatly in the past two decades. Growth inhibition of *Alternaria* species by the *Trichoderma* and *Pseudomonas* metabolites has been well researched [3-5].

The objectives of the present study were (1) Isolation and identification of pathogen (2) Isolation of fungal antagonists from ribbon plant rhizosphere and tested for its efficacy against *A. alternata* (3) Isolation of endophytic bacteria from ribbon plant and tested for its efficacy against *A. alternata* (4) To study the morphological characteristics of bioagents

Materials and Methods

Isolation, maintenance and identification of pathogen

The infected leaf bits along with some healthy portions were cut into small bits and surface sterilized using 1:1000 mercuric chloride solutions for 30 sec. The bits were washed thoroughly in sterile distilled water for three times to remove traces of mercuric chloride. The molten warm potato dextrose agar (PDA) medium was poured into sterilized Petri plates and allowed to solidify. The surface sterilized leaf bits were placed on PDA medium. These plates were incubated at room temperature for 28 ± 2°C and observed periodically for the fungal growth. Pure cultures were obtained by transferring hyphal tips to

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PDA medium and they were maintained on PDA slants [6]. The slants were incubated at 28 ± 2°C for sporulation for 10-18 days (Figure 2 and 3). The pathogen was identified as *Alternaria alternata* based on the morphological characteristics [7,8]. Further, the identity of the fungus was confirmed at National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India.

In vitro experiments

Isolation and identification of *Trichoderma* species: The Soil samples were collected from ribbon plant rhizosphere at sevent different ribbon plant growing tracts of Tamil Nadu viz., Karur, Chidambaram, Periyakulam, Erode, Coimbatore, Ooty and Nasiyanur. For rhizospheric soil, plant was gently and carefully uprooted, soil tightly adhering the roots was collected, randomly selected, mixed and one forth part was used as composite rhizospheric soil sample of the region. The pH of soil was determined in 1:2 (soil:water) ratio, keeping 30 min as equilibration time.

Collected soil samples were air dried for 4 h and isolation was done by serial dilution technique. *Trichoderma* selective medium (TSM) was used for isolation of the isolates of *Trichoderma* [9]. 1 mL soil suspension was taken with the help of 5 mL sterilized pipette and poured on the Petri plate seeded with TSM. The plates were incubated at 28 ± 2°C for 5 days. Observation on the appearance of colonies was recorded from 3rd to 5th day. Individual colonies were picked up and maintained in pure culture for further study. The *Trichoderma* species were identified and examined under compound microscope on the basis of their cultural and morphological characters [10] and the cultures were maintained on PDA slants at 4°C for further study. Total of eight *Trichoderma* isolates were obtained and identified isolates were designated as THK, THC, TVP, TVE, TVC, THO and THN.

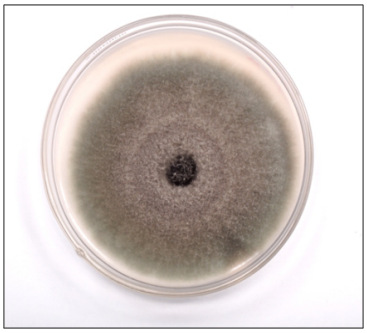


Figure 2: Axenic culture of *Alternaria alternata*.

Figure 3: Conidia of *Alternaria alternata*.

Growth inhibition assay: The antagonistic activity of *Trichoderma* species against *A. alternata* was tested by dual culture technique [11] using PDA medium. Each treatment was replicated four times with five plates per replication. Periodical observations on the growth of *Trichoderma* species and their ability to colonize the pathogen were recorded and also per cent inhibition of mycelial growth of pathogen was calculated by using the formula [12].

Per cent inhibition (I)=C-T/C×100

Where, C-Growth of pathogen in control plates

T Growth of pathogen in dual culture plates

I-Per cent inhibition in mycelial growth

Morphological studies: The colony characteristics such as colony appearance, radial growth, conidial length and width were examined from cultures grown in darkness at 30°C for 96 h on PDA. Four plastic Petri plates, each containing 20 mL of media were used for each *Trichoderma* isolates. For analysis of colony characteristics and growth rate, mycelial discs were taken from the actively growing margin of different *Trichoderma* isolate grown for 3 days on PDA. The 5 mm diameter mycelial disc was placed at approximately 5 mm distance from the edge of 84 mm diameter Petri plate. The Petri plates were incubated in darkness at 30°C, colony radial growth was measured at 24 h intervals until the colony reached the edge of Petri plate. All micro morphological data were examined on cultures grown on PDA and 2% Malt extract agar (MEA) for 7 days at 20 to 22°C. The examination and measurements of conidial length and width were made from slide preparations stained with 3% KOH, which was subsequently replaced by water as the microscope preparation dried. For direct microscopic observations, 30 units of each character were measured with the exception of chlamydospores, of which 30 were not located in some cultures. Phase contrast and interference contrast microscopy were utilized in the study [13].

Isolation and identification of endophytic bacterial isolates: Bacterial endophytes were isolated from leaf portions of ribbon plants. Isolation was done following the method [14]. A total of 10 leaf isolates were obtained from the ribbon plants designated as EBL (Endophytic Bacteria Leaf) (1-10). Based on the dual culture technique, those endophytic bacterial isolates that were effective were used for further study.

Biochemical tests: The endophytic bacterial isolates were identified with various biochemical tests (gram staining, KOH test, motility and fluorescent pigment) given by Bergey's Manual of Systematic Bacteriology [15].

Morphological studies of endophytic isolates of *P. fluorescens*: The morphological characters (shape and colony colour) of endophytic bacterial isolates were performed with the method described [16].

***In vitro* bioassay:** The antagonistic activity of the endophytic bacteria against *A. alternata* was tested by the dual culture technique [11] on PDA. The per cent inhibition of mycelial growth was calculated [12].

Inhibition% (I)=C-T/C×100

Where, C-radial growth in control

T-radial growth in treatment

I-per cent inhibition

Statistical analysis

All the experiments were of completely randomized design (CRD) and repeated twice. To determine the effect of each bioagents on the radial growth of pathogen, the per cent reduction compared with the experimental controls was calculated. Data were subjected to analyses of variance and treatment means were compared by an appropriate Duncan's multiple range test (P<0.05). The IRRISTAT package version 92-1, developed by the International Rice Research Institute Biometrics Unit, Philippines, was used for analysis [17].

Results and Discussion

In vitro inhibition of *Alternaria alternata* by fungal antagonists

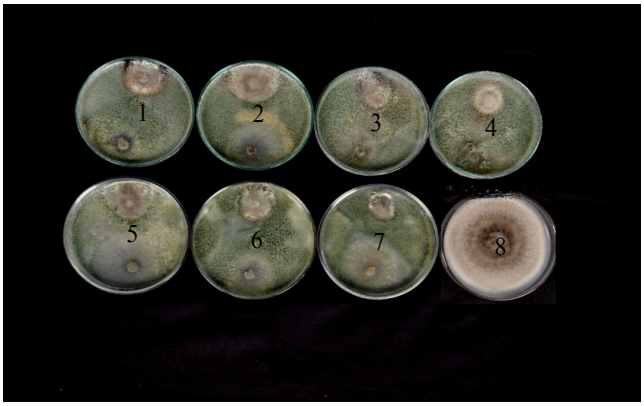
All the native *Trichoderma* species inhibited the mycelial growth of *A. alternata* with reductions ranging from 59.2 to 74.0% (Table 1). Of these, THA exhibited the maximum inhibition of mycelial growth of *A. alternata* (23.00 mm) compared with control (88.3 mm). This was followed by THO and THN. The least mycelial growth was recorded in the isolate TVC (Figure 4). Similarly, *T. harzianum* was highly effective in inhibiting the growth of A. alternata causing brown spot of tobacco [16]. *T. harzianum* was highly inhibited the growth of *A. alternata*, which was followed by *T. viride* [5].

Trichoderma isolate	Mycelial growth (mm)	Reduction over control (%)
THK	27.7 c*	69.0 c
THA	23.0 a	74.0 a
TVP	38.3 e	57.0 d
TVE	35.7 d	59.6 d
TVC	36.0 d	59.2 d
THO	25.0 b	71.7 b
THN	25.7 b	71.0 b
Control	88.3 f	-

THK: *Trichoderma harzianum* Karur; THA: *Trichoderma viride* Annamalai University-Chidambaram; TVP: *Trichoderma viride* Periyakualm; TVE: *Trichoderma viride* Erode; TVC: *Trichoderma viride* Coimbatore; THO: *Trichoderma harzianum* Ooty; THN: *Trichoderma harzianum* Nasiyanur

*Means of four replications. Values in each column followed by the same letter are not significantly different according to the DMRT method (P<0.05).

Table 1: Screening of *Trichoderma* isolates against *Alternaria alternata* in the dual culture test.



1.TVA
2.THAD
3.THD
4.TKD
5.TPKD
6.TVM
7.TVC
8.Control

Figure 4: *In vitro* inhibition of mycelial growth of *A. alternata* by *Trichoderma* species.

Species	Colony character (4th day after inoculation)	Radial growth (mm)	Conidial morphology	
			Shape	L/W (µm)
THK	Dark green sporulation	88.3	Globose to ellipsoidal	2.9×3.2 (3.1)*
THA	Green to bright green sporulation	88.7	Globose to broadly ellipsoidal	3.2×3.3 (3.4)
TVP	Dull green sporulation	86.3	Ellipsoidal	2.8×2.9 (2.9)
TVE	Deep green sporulation	87.0	Subglobose to ellipsoidal	2.3×2.8 (2.6)
TVC	Dark green to dull blackish green sporulation	86.7	Subglobose to ellipsoidal	2.7×2.8 (2.8)
THO	Dark green sporulation	88.0	Globose to ellipsoidal	3.0×3.0 (3.0)
THN	Complete dark green sporulation	88.0	Globose to ellipsoidal	2.9×2.8 (2.9)

*Ratio of length to width.

Table 2: Morphological characteristics of *Trichoderma* species isolated from ribbon plant rhizosphere.

Antibiosis, mycoparasitism, food competition, secretion of chitinolytic enzymes, production of inhibitory compounds and induced host-plant resistance are the main mechanisms in biological control [18-20]. The inhibitory effect of *T. viride* against *A. alternata* might be due to the production of antibiotic substances includes viridin, gliotoxin, glioviridin, dermin and trichodermin [19].

Morphological characterization of *Trichoderma* species

Differences in micromorphological characteristics of seven *Trichoderma* species were described in Table 2. Conidia of *Trichoderma* species THA (3.2×3.3 µm; L/W ratio of 3.4 µm) were larger than those of the other species and had a shape of globose to broadly ellipsoidal. The most obvious difference in conidia was their shape, which is to some extent reflected by the L/W ratio of the conidia. Conidia of *Trichoderma* species THK (2.9×3.2 µm), THO (3.0×3.0 µm) and THN (2.9×2.8 µm) were conspicuously globose to ellipsoidal and had a length/width (L/W) ratio of 3.1, 3.0 and 2.9 µm, respectively. Conidia of *Trichoderma* species TVE (2.3×2.8 µm) and TVC (2.7×2.8 µm) were subglobose to ellipsoidal and had a L/W ratio of 2.6 and 2.8 µm, respectively and those of TVP (2.8×2.9 µm) were ellipsoidal and had a L/W ration of 2.9 µm.

In this study growth characters, conidial morphology and size could separate *Trichoderma* species THA from other species. Recently, *Trichoderma* sp.1 can be distinguished from other *Trichoderma* species by the characters of colony morphology, phialide, conidial morphology and size [13]. Similar observation was made by several workers [20,21].

Identification of effective endophytic bacteria

Biochemical tests: The results of the gram reaction and biochemical tests performed for the identification of endophytic bacterial isolates showed that all the isolates produced similar result with regard to gram staining and KOH test (EBL 1-EBL 8) showed negative, whereas fluorescent pigment test showed positive results. Hence they are identified as *P. fluorescens*. The isolates EBL 9 and EBL 10 showed positive in gram staining and KOH test, whereas fluorescent pigment

showed negative results. Hence, they are identified as *B. subtilis*. Generally all the isolates showed positive results in motility test.

In vitro inhibition of *A. alternata* by the bacterial isolates

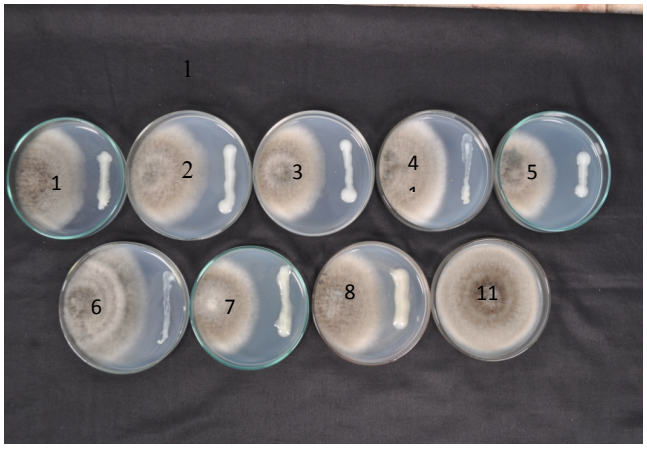
Table 3 lists the varying degrees of antagonism to *A. alternata* by the endophytic bacterial isolates. Among the isolates tested, *P. fluorescens* EBL 5 produced the widest inhibition zone, 13.6 mm, with a minimum of 25 mm of mycelia growth of *A. alternata*, reducing mycelial growth by 71.7% compared with the control (Figure 5). This was followed in order by *P. fluorescens* EBL 2, EBL 8. The other isolates (EBL 1, 3, 4, 6, 7, 8 and 9) were less effective in inhibiting mycelial growth of *A. alternata in vitro.*

P. fluorescens Pf1 was found to inhibit the growth of the pathogen *A. palandui* causing leaf blight of onion [22]. *P. fluorescens* was effective bioagent in reducing the mycelial growth of *A. helianthi* causing leaf blight of sunflower [23]. Antimicrobial metabolite production was tested by determining the antagonistic activity of Bacillus subtilis

Isolates	Mycelial growth (mm)	Per cent reduction over control	Inhibition zone
EBL1	38.0 f*	57.0	5.0 d*
EBL2	26.6 b	70.0	12.3 a
EBL3	40.6 g	54.0	2.3 f
EBL4	31.3 d	65.0	10.3 b
EBL5	25.0 a	71.7	13.6 a
EBL6	29.0 c	67.2	11.0 a
EBL7	34.6 e	60.1	7.6 c
EBL8	27.6 b	68.4	11.3 a
EBL9	42.0 g	52.4	2.0 e
EBL10	40.6 g	54.0	1.9 e
Control	88.3 h	-	-

*Means of four replications. Values in each column followed by the same letter are not significantly different according to the DMRT method (P<0.05)

Table 3: Screening of endophytic bacterial isolates against *Alternaria alternata*.



1.EBL1
2. EBL2
3. EBL3
4. EBL4
5. EBL5
6. EBL6
7. EBL7
8. EBLB
11. Control

Figure 5: *In vitro* inhibition of mycelial growth of *A. alternata* by endophytic bacterial isolates.

Isolates	Colony type	Colony colour	Cell shape	Growth type	Reaction to UV light
EBL1	Round	Yellowish	Short rod	Fast	Bright
EBL2	Round	Yellowish green	Short rod	Fast	Bright
EBL3	Round	Dull Yellowish	Short rod	Slow	Bright
EBL4	Round	Yellowish green	Short rod	Fast	Bright
EBL5	Round	Yellowish green	Short rod	Fast	Bright
EBL6	Round	Greenish yellow	Short rod	Fast	Bright
EBL7	Round	Greenish yellow	Short rod	Fast	Bright
EBL8	Round	Yellowish green	Short rod	Fast	Bright
EBL9	Circular undulated margin	Cream colour	Rod	Fast	No
EBL10	Circular undulated margin	Cream colour	Rod	Slow	No

(EBL-Endophytic Bacteria Leaf)

Table 4: Cultural characteristics of endophytic bacterial isolates.

isolated from soil samples against *A. porri* and *A. solani*. The antagonist was found to be effective in inhibiting both the pathogens under study [24]. Several authors reported that the effectiveness of endophytic bacteria against wide range of plant pathogens [25-27]. *P. fluorescens* isolate EBS 20 produced higher levels of extracellular metabolites like siderophore, salicylic acid and HCN when compared with other isolates which was highly effective in inhibiting the growth of Pythium aphanidermatum inciting chilli damping-off [28]. Similarly, antifungal compounds such as pseudobactin, HCN, salicylic acid and 2-hydroxy phenazine produced by fluorescent pseudomonas suppressed plant pathogenic fungi [29,30].

The ten endophytic bacterial isolates tested showed variations in colony type, colony colour, growth type and reaction to UV light. The isolates (EBL 1- EBL 8) were short rod in shape and produced bright fluorescence when exposed to UV light. The isolates EBL 9 and EBL 10 were rod shaped and it did not produce bright fluorescence when exposed to UV light. Colony type varied from round to circular undulated margin. Colony colour varied from yellowish to greenish yellow, yellowish green and dull yellow. The growth type varied from fast to slow (Table 4).

In the present study native isolate of *Trichoderma harzianum* and endophytic isolate of *Pseudomonas fluorescens* were isolated from ribben plant rhizosphere and phylloplane was found to be highly effective in inhibiting the mycelial growth of *Alternaria alternata* causing leaf blight of ribbon plant.

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