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Mycoendophytes and its Antifungal Efficacy against *Macrophomina phaseolina* (Tassi) Goid. Incitant of Mulberry (*Morus indica* L.) Charcoal Rot

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A B S T R A C T

Thirty fungal endophytic isolates were recovered from leaf, stem, root and inflorescence of mulberry plant and the morphological characterization was done. The antifungal potential of isolated mycoendophytes against *Macrophomina phaseolina* was evaluated *in vitro* by dual culture technique and revealed that ENF20 isolate reduced the mycelial growth by 82.22 per cent over control followed by ENF12 (80.56 per cent), ENF11 (77.78 per cent), ENF13 (76.11 per cent). The endophyte-pathogen interaction study elucidated the mechanisms of antagonism by antibiosis, competition for substrate, hyperparasitism and neutral reaction. The endophytic fungi interaction with *M. phaseolina* revealed that ENF12 isolate exhibited antibiosis, ENF25 showed competition. Likewise, ENF13, ENF19 and ENF20 expressed mycoparasitism besides ENF3 unveiled neutral reaction.

Introduction

Mulberry (*Morus indica*) is the elite food plant of the silkworm, *Bombyx mori* L. (Zhang *et al*., 2011) and is often affected by a number of diseases and herbivores, which hampered leaf quality and productivity (Kumar and Gupta, 2004). Mulberry diseases caused 5-10 per cent loss in leaf yield by defoliation and additional loss of 20-25 per cent by deterioration in leaf quality (Sukumar and Padma, 1999). The mulberry being a perennial crop the soil borne pathogens seem to rapidly perpetuate and quickly spread to cover extensive areas. In mulberry, charcoal rot is caused by soil borne, necrotrophic fungi *Macrophomina phaseolina*. The infected plants failed to sprout after pruning and dried up completely (Begum *et al.*, 2018). The loss due to this disease was 30-40 per cent (Kumari, 2014).

Since mulberry leaves are used to feed silkworms, the improper use of agrochemicals

to treat this disease could be hazardous to silkworms (Ji *et al*., 2008). Hence, there is a need to explore microbial antagonists like endophytes for management of diseases is unavoidable. Endophytes are microorganisms that colonize on healthy plant tissues intercellularly or intracellularly without causing any apparent symptoms of disease (Wilson, 1995). Mycoendophytes (fungal endophytes) are the most frequently occurring endophytes but relatively less studied and offer tremendous potential of novel secondary metabolites for exploitation in the medicine, pharmaceutical and agriculture industries (Strobel, 2018). However, information and research work on the mulberry endophytes and their use in biological control of mulberry diseases are very scanty. Hence, this study focused on the isolation of mycoendophytes associated with *M. indica* and screening them for antifungal activity against charcoal rot pathogen *M. phaseolina*.

Materials and Methods

Collection and surface sterilization of plant samples

The plant samples were collected from *M. indica*, V1 variety from Forest College and Research Institute, Mettupalayam. The fresh plant materials were used for isolation work to reduce the chances of contamination. The collected plant samples were subjected to surface sterilization within a few hours after sampling.

The surface sterilization of collected plant samples was done according to the method described by Petrini (1986) with modifications. The samples were thoroughly washed in running tap water for 10 minutes to remove the soil particles and adhered debris and finally washed with distilled water. Then the samples were cut into small pieces by a sterilized blade under aseptic conditions followed by washing the samples with Tween 20 (1 drop in 200 ml of Sterile Distilled Water [SDW]) for one minute subsequently samples were rinsed with SDW for 3 times. Each sample was surface sterilized with 70 per cent ethanol for one minute later dipped in 2 per cent sodium hypochlorite solution for two minutes. Finally, samples were immersed in 0.1 per cent mercuric chloride for 30 seconds and the excess mercuric chloride was removed by rinsing with SDW for three times.

Test for effectiveness of surface sterilization

The aliquot of finally rinsed SDW was spread on PDA in Petri plates to confirm the effectiveness of sterilization process (Schulz *et al*., 1993).

Isolation and morphological characterization of mycoendophytes

The surface sterilized samples were macerated with 1 ml of SDW in a sterile mortar and pestle, and each sample was serially diluted in test tubes containing 9 ml of SDW. The dilutions of 10^{-4} and 10^{-5} were plated on Petri plates containing PDA medium. The plates were then incubated at 28 ± 2^{0}C for 10 days and observed for fungal growth. Each colony obtained was subcultured on PDA slants and maintained for further use (Thangavelu and Gopi, 2015).

The isolated fungi were identified on the basis of morphological characteristics according to Domsch *et al.* (1980). Morphological identification was done in consonance with standard taxonomic key included colony growth, color, texture, pigmentation and growth rate.

In vitro antifungal assay

The isolated endophytes were screened for *in vitro* antagonistic activity against

M. phaseolina by using dual culture technique (Dennis and Webster, 1971). In this method, 5 mm size discs of culture of endophytic isolates (5 days old culture) and simultaneously the same size of another disc having *M. phaseolina* (5 days old culture) was placed opposite to each other and close to the periphery of 90 mm Petri plates containing PDA. The plates inoculated only with the pathogen served as control. Three replications were maintained for each treatment. The plates were incubated at 28 ± 2^0C until 100 per cent growth coverage in control. After the incubation period, the area between the two colonies at the interaction point was measured. The inhibition percentage was calculated using the formula given by Fokkema (1976).

$$\text{Inhibition percentage} = \frac{C\text{-}T}{C} \times 100$$

Here, 'C' represents the growth diameter of the pathogen in the control plate and 'T' represents the pathogen diameter growth on the dual plate, where both the test endophyte and pathogen were inoculated.

Endophyte-pathogen interaction

From the visual interpretation of *in vitro* dual culture technique, the type of interaction between an endophyte and pathogen was evaluated according to the method followed by Mejia *et al.* (2008).

Statistical analysis

The Completely Randomized block Design method was adopted. All multiple comparisons were first subjected to Analysis of Variance (ANOVA). Duncan's multiple range test (DMRT) (Duncan, 1955) was applied to the transformed values and then transferred to the original means (Gomez and Gomez, 1984). The data were analyzed statistically by using software package SPSS 16.0 Command Syntax Reference.

Results and Discussion

Isolation and identification of endophytic fungi

The endophytic fungi from mulberry (*M. indica*, variety: V1) were explored and their morphological characterization was done. A total of 30 endophytic fungal isolates were obtained from healthy tissues of *M. indica*. Among them, 13 isolates from leaf, 3 from stem, 13 from root and 1 from inflorescence were isolated. No microbial growth was observed on plates inoculated with finally rinsed SDW which proved the effectiveness of sterilization. The isolation and characterization of fungal endophytes from various plants were performed by several workers (Kannan *et al*., 2017; Yu *et al*., 2018; Lu *et al*., 2012). The efficiency of surface sterilization was proved by Ramalashmi *et al*. (2018).

The morphological characteristics of isolated fungal endophytes were observed. Majority of the young fungal colonies were appeared in whitish shade, whereas older colonies exhibited in white with different shades of colors. Similarly, most of the colony reverse colors of the isolates were seemed to be in creamy to white shades except ENF7, ENF8, ENF9, ENF11, ENF12, ENF14, ENF20, ENF22 and ENF25. The texture of the isolates was generally noticed as velvety, cottony and rarely powdery in nature. Among all isolates, ENF11, ENF12 and ENF16 were produced yellow pigmentation over the media. The growth rate of the endophytic fungal isolates was ranges from very slow to medium (Table 1). The morphological characterization of endophytic fungal isolates was carried out by Tolulope *et al.*, (2015) suggested that the endophytic fungal colonies were initially white in color that became dark

with later stages of growth. The colony morphological character such as texture, colony reverse and pigmentation has also been studied according to Nisa *et al.,* (2018).

In vitro screening of endophytic fungal isolates against *M. phaseolina*

The thirty isolated fungal endophytes were individually screened against mulberry charcoal rot pathogen, *M. phaseolina*. In dual plate assay, the minimum mycelial growth of pathogen was noticed in ENF20 isolate by 16 mm diameter. ENF19 and ENF11 recorded the mycelial growth of 17.50 mm and 18.00 mm respectively. ENF7 (22.50 mm), ENF12 (20.00 mm) and ENF13 (21.50 mm) isolates also showed inhibition of pathogen growth (Plate 1).

Among all, the ENF20 isolate showed maximum growth inhibition of 82.22 per cent over control followed by ENF12 (80.56 per cent), ENF11 (77.78 per cent), ENF13 (76.11 per cent) inhibited the growth of pathogen. The ENF7 isolate exhibited 75 per cent of growth inhibition next to ENF9 (75.56 per cent).

The minimum growth inhibition was noticed in ENF26 (27.23 per cent) (Table 2). Molecular characterization of the best performing endophytes *viz.,* ENF7, ENF9, ENF11, ENF12, ENF13, ENF19, ENF20 and ENF25 are to be carried out to reveal their identity.

Based on the *in vitro* assay, nearly eight isolates had excellent antifungal activity (>70 per cent) against *M. phaseolina* followed by ten isolates had high activity (50-70 per cent). The remaining twelve isolates were grouped under the category of moderate antifungal activity (20-50 per cent). The antifungal nature of endophytic fungal isolates was reported by several workers. Yu *et al.,* (2018) evaluated the antifungal activity of endophytic fungal isolate *Oidium* sp. (ty-64) isolated from *Camellia oleifera* had strong inhibitory action against anthracnose pathogen. Aramsirirujiwet *et al*., (2016) studied the antagonistic behaviour of endophytic fungal isolates of rainbow plant against five plant pathogenic fungi (*Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia* sp., *Alternaria brassicicola* and *Phytophthora palmivora*) by dual culture technique.

Endophyte-pathogen interaction

Dual culture tests revealed that isolated endophytes reduced the growth of pathogen *in vitro*. The visual assessment of the interactions between endophytes and pathogen suggested that endophytes could antagonize the pathogen through several mechanisms such as antibiosis, competition for substrate, mycoparasitism and neutral growth.

Based on the growth responses in dual plate, the interaction between endophyte and pathogen was classified into four groups such as antibiosis (chemical reaction) with presence of reactive zone, competition for substrate thus forms the faster growth of one fungus, mycoparasitism when one fungi that have the ability to parasite other fungi and neutral or intermingling growth which had no effect on inhibition (Mejia *et al.,* 2008).

In dual plate, growth of the pathogen was inhibited by endophytic fungi, ENF12 isolate through antibiosis mechanism. It was revealed that the endophytic fungi produced some biologically active compounds which inhibited the growth of pathogen. The antibiosis mechanism between endophytic fungi, *Monographella nivalis* and Dutch elm disease pathogen, *Ophiostoma novo-ulmi* was explained by Blumenstein (2015).

Table.1 Morphological characteristics of endophytic fungal isolates of *Morus indica* L

S. No.	Fungal isolates	Plant part used	Colony observation		Colony reverse	Texture	Pigmentation	Growth rate
			Young colony	Old colony				
1	ENF1	Leaf	White	White	Cream	Velvety	No pigmentation	Medium
2	ENF2	Stem	White	White with small knots	White	Cottony	No pigmentation	Medium
3	ENF3	Stem	White	White	Cream	Cottony	No pigmentation	Medium
4	ENF4	Root	White	Greyish white	Grey	Cottony	No pigmentation	Medium
5	ENF5	Leaf	Olive green	Green	Cream	Powdery	No pigmentation	Slow
6	ENF6	Root	White	White with gummy exudates	White	Velvety	No pigmentation	Medium
7	ENF7	Root	Yellow centre with white margins	Yellow	Yellow	Velvety	No pigmentation	Medium
8	ENF8	Leaf	Fern green shade centre with white margins	Dark green	Pink	Velvety	No pigmentation	Medium
9	ENF9	Root	Grey with crateriform elevation	Greyish black	Black	Powdery	No pigmentation	Slow
10	ENF10	Root	Emerald green centre with white margins	Dark green	White	Velvety	No pigmentation	Slow
11	ENF11	Root	Sandal yellow centre with white margins and crateriform elevation	Dark sandal with golden exudates	Yellow	Cottony	Yellow	Slow
12	ENF12	Leaf	Sandal with spiral topography	Brown	Yellow	Powdery	Yellow	Slow
13	ENF13	Stem	Olive green with white margins	Dark green	Cream	Powdery	No pigmentation	Slow
14	ENF14	Root	Grey with white	Greyish black	Black	Cottony	No pigmentation	Slow
15	ENF15	Leaf	White	White with orange resting spores	White	Cottony	No pigmentation	Medium

16	ENF16	Leaf	Light green	Olive green	Cream	Powdery	Yellow	Medium
17	ENF17	Root	White	Snow white	White	Velvety	No pigmentation	Medium
18	ENF18	Leaf	Grey	Greyish black	Grey	Velvety	No pigmentation	Medium
19	ENF19	Root	White	White	White	Velvety	No pigmentation	Medium
20	ENF20	Flower	White	Greyish white	Greyish black	Velvety	No pigmentation	Medium
21	ENF21	Root	White	White	White	Cottony	No pigmentation	Medium
22	ENF22	Leaf	White with brown exudates and filamentous/flower like mycelium	Greyish white	Black	Velvety	No pigmentation	Slow
23	ENF23	Root	White	White	Cream	Cottony	No pigmentation	Slow
24	ENF24	Leaf	White	White with blackish grey	Grey	Velvety	No pigmentation	Medium
25	ENF25	Root	Green with white margins	Dark green	Green	Powdery	No pigmentation	Slow
26	ENF26	Leaf	White	Whitish yellow with brown gummy exudates	Cream	Velvety	No pigmentation	Medium
27	ENF27	Root	Grey	Greyish green	Grey	Cottony	No pigmentation	Slow
28	ENF28	Leaf	White	Snow white with spiral morphology and with White exudates	White	Velvety	No pigmentation	Medium
29	ENF29	Leaf	White	Whitish green	Cream colour	Powdery	No pigmentation	Very slow
30	ENF30	Leaf	White	Whitish green	Whitish yellow	Cottony	No pigmentation	Slow

Table.2 *In vitro* antagonistic activity of endophytic fungal isolates against *Macrophomina phaseolina*

Isolate No.	Mycelial growth of pathogen (mm)	Per cent growth inhibition over control
ENF1	37.00 (6.08)^{efg}	58.89 (50.12)
ENF2	37.50 (6.12)^{efg}	58.34 (49.80)
ENF3	55.00 (7.42)^{ijk}	38.89 (38.58)
ENF4	33.50 (5.79)^{def}	62.78 (52.40)
ENF5	38.00 (6.16)^{efg}	57.78 (49.48)
ENF6	45.50 (6.75)^{hi}	49.45 (44.68)
ENF7	22.50 (4.74)^{abc}	75.00 (60.00)
ENF8	54.00 (7.35)^{ij}	40.00 (39.23)
ENF9	24.00 (4.90)^{abcd}	73.33 (58.91)
ENF10	35.00 (5.92)^{efg}	61.11 (51.42)
ENF11	17.50 (4.18)^{ab}	80.56 (63.83)
ENF12	20.00 (4.47)^{abc}	77.78 (61.88)
ENF13	21.50 (4.64)^{abc}	76.11 (60.74)
ENF14	36.50 (6.04)^{efg}	59.45 (50.44)
ENF15	59.50 (7.71)^{jk}	33.89 (35.60)
ENF16	23.50 (4.85)^{abcd}	73.89 (59.27)
ENF17	40.50 (6.36)^{fg}	55.00 (47.87)
ENF18	31.00 (5.57)^{cdef}	65.56 (54.06)
ENF19	18.00 (4.24)^{ab}	55.00 (47.87)
ENF20	16.00 (4.00)^{a}	82.22 (65.06)

ENF21	52.00 (7.21)^{ij}	42.22 (40.52)
ENF22	58.50 (7.65)^{jk}	35.00 (36.27)
ENF23	54.00 (7.35)^{ij}	40.00 (39.23)
ENF24	60.50 (7.78)^{jk}	32.78 (34.92)
ENF25	23.50 (4.85)^{abcd}	73.89 (59.27)
ENF26	65.50 (8.09)^{k}	27.23 (31.45)
ENF27	59.00 (7.68 ^{jk}	34.45 (35.94)
ENF28	60.00 (7.75 ^{jk}	33.34 (35.27)
ENF29	57.00 (7.55 ^{jk}	36.67 (37.27)
ENF30	28.00 (5.29)^{bcde}	68.89 (50.12)
Control	90.00 (9.49)^{l}	0.00 (49.80)

Values are mean of three replications;
Means followed by a common letter are not significantly different at 5% level by DMRT;
Values in the parentheses are square root and arcsine transformed values respectively

Plate.1 *In vitro* screening of endophytic fungal isolates against *M. phaseolina* by dual culture assay

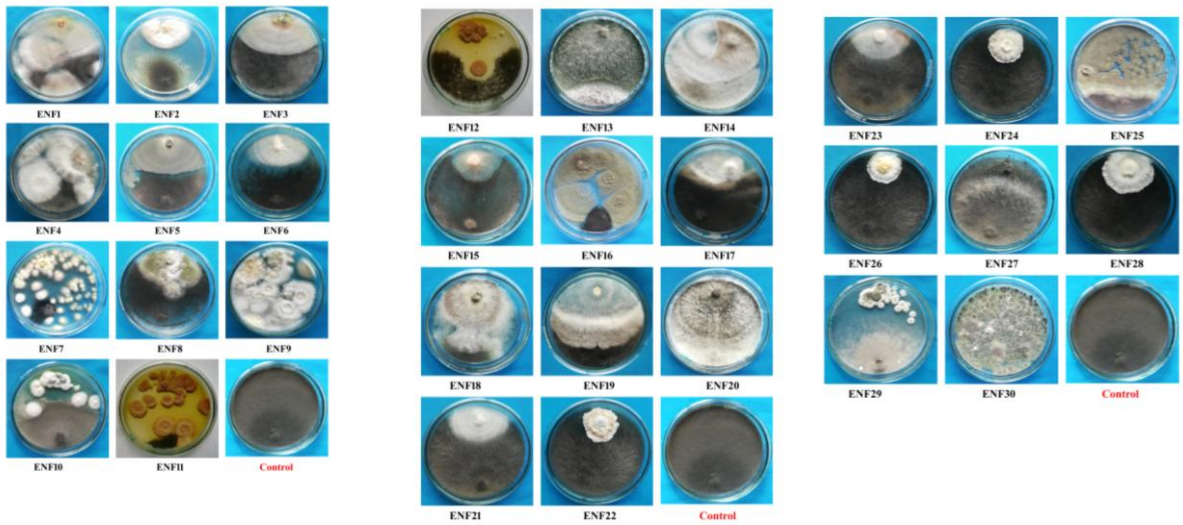
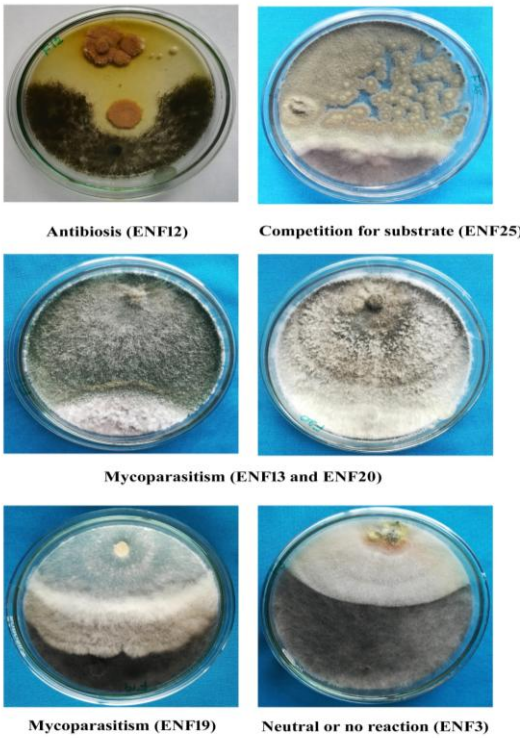


Plate.2 Mechanisms of antifungal activity



The antibiosis was due to the production of bioactive compounds which revealed that if such compounds were present in the host plant; this could contribute a defense mechanism against fungal pathogens. Similarly, the bioactive elements produced by endophytic fungi against plant pathogenic fungi was reported in many crops (Liu *et al*., 2001; Park *et al*., 2005; Inácio *et al*., 2006; Kim *et al*., 2007).

In this study, ENF25 isolate suppressed the growth of pathogen by faster growth thus creates the competition for substrate. Endophytes improved plant health as producing competition for the nutrients with pathogens in colonized plant (Compant *et al.*, 2010). The endophytic fungi from elm such as *Neofusiccocum luteum* and *Sordaria* sp. smothered the extension of the pathogen's colony through a faster growth by competition for the substrate (Blumenstein, 2015). The overall competitive ability of a species perhaps referable to a combination of factors involving growth rates, metabolite production, niche overlap and interactions with environmental conditions (Lee and Megan, 1999). The antagonism by competition was studied by Mendoza *et al*. (2015) who documented that the antagonistic *Trichoderma* sp. stopped the growth of *M. phaseolina* at the site of contact by forming a barrier that prevents pathogen development.

In the present study, ENF13, ENF19 and ENF20 isolates showed mycoparasitism against *M. phaseolina.* These isolates were over grown on the pathogen's colony. ENF27 endophytic isolate was failed to antagonize the pathogen in this case pathogen over grow on endophyte. Fungal endophytes parasitized around the hyphae of pathogens by various means such as coiling, twisting, penetrating the hyphae of pathogens and secreting lyase to decompose the cell wall of pathogens. Microbial predations are the one of the mode

of action of endophytes to suppress the plant pathogens (Gao *et al.,* 2010). On the other hand, hyperparasitism doesn't confirm the parasitism behaviour based on the visual observations. In this case, mycoparasitism determined which ''won" or ''lost" the interaction and by which type of activity. Endophytes were considered to win if they inhibited the growth of the pathogen, showed more mycelial growth than the pathogen, or parasitized the pathogen. Endophytes were considered to ''lose" if the pathogen ''won" (showed the reverse outcome). In this work, many of the tested endophytic fungal isolates did not showed inhibition against pathogen. ENF3 and ENF5 isolates displayed neutral growth. In dual plate assay, if there was no visible interaction or "neutral" interaction, the endophyte was not examined further and thus could be excluded at an early stage of the investigations (Mejia *et al.,* 2008) (Plate 2).

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