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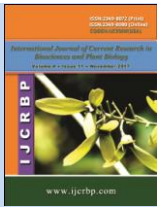

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Isolation and Characterization of Endophytic Fungi from *Bauhinia* sp., *Delonix regia* and *Crotalaria* sp.

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Abstract

Endophytic fungi are a group of unexplored microorganisms with an abundant source of natural bioactive compounds of industrial and pharmaceutical importance. The current study reports a total of five different endophytic fungi *viz.*, *Phoma sp.*, *Phomopsis sp.* (brown colonies), *Phomopsis sp.* (white colonies), *Drechslera biseptata*, *Nigrospora sphaerica* isolates obtained from the leaf of *Bauhinia sp.*, *Delonix regia* and *Crotalaria sp.* The highest colonization frequency was observed in *Phoma sp.* (75%) followed by *Drechslera biseptata* (61%). Extra hyphal clearing was observed for all the isolates grown on media containing glucose as the sole carbon source indicating the presence of amylase whereas *Phoma sp.* and *Nigrospora sphaerica* were able to produce laccase. *Phoma sp.* and *Phomopsis sp.* (white) were able to produce protease. The isolates also produced ammonia. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was inhibited by *Phoma sp.*, *Phomopsis sp.* (white), *Phomopsis sp.* (brown), *Drechslera biseptata* and *Nigrospora sphaerica* at a concentration of 5mg/ml. *Proteus mirabilis* was inhibited by all the isolates except *Drechslera biseptata*. Interspecific activities of the isolated endophytic fungi indicated *Nigrospora sphaerica* was the dominant organism.

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Introduction

Endophytes are microorganisms (bacteria, fungi, yeast) that colonize the intercellular tissues of healthy plant tissues without causing any symptoms of disease (Tan and Zhou, 2001). They have been associated with plants for over 400 million years and their association can be obligate or facultative without causing harm to their host plants and also mutualism and antagonism with their hosts (Krings et al., 2007; Kumaresan and Suryanarayanan, 2002). Several endophytic fungi have mutualistic relationship with the host plant thereby limit the damage caused by pathogens by inducing resistance intrinsic to the host plant (Eaton et al., 2011). In the case of symbiotic association there is an increase in biomass as well as better development of aerial parts (Das et al., 2012). They are important components of the plant micro-ecosystem (Rodriguez et al., 2009; Zhang et al., 2006; Tan and Zhou, 2001) and are ubiquitous, colonize all plants and have been isolated from mosses (Davey and Currah, 2006), lichens (Li et al., 2007 a,b; Suryanarayanan et al., 2005) shrubs (Petrini et al., 1982), ferns (Swatzell et al., 1996), grasses (Muller and Krauss, 2005; Su et al., 2010), coniferous trees(Sun et al., 2011; Albrectsen et al., 2010; Mohamed et al., 2010). In the long co-evolution of the endophytes and

their host plant, the endophytes have adapted themselves to the microenvironment by genetic variation including uptake of some plant DNA into their own genome. This could have led the endophytes to biosynthesize various bioactive compounds such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols and peptides (Poorani et al., 2015). The secondary metabolites produced by fungal endophyte is large than that of any other endophytic microorganisms class (Zhang et al., 2006) and proves to be an alternative for producing valuable bioactive compounds efficiently (Lee et al., 2014). These bioactive compounds function as antibacterial (Praptiwi et al., 2013), anticancer (Mohana et al., 2012), antimalarial (Elfita et al., 2011), anti-inflammatory (Zhang et al., 2014) agents that also promote host growth and resistance to environmental stress (Saikkonen et al., 2010) and decompose litter (Purahong and Hyde, 2011; Sun et al., 2011). Enzymes such as pectinases, cellulases, lipases, amylases, laccases, and proteinases are produced as a part of their mechanism to overcome the defense of the host plant against microbial invasion and to obtain nutrients for their development (Correa et al., 2014). These play major role as industrial enzymes and also in biodegradation of components of lignocellulosic material (Lee et al., 2014; Sunitha et al., 2013). The fungal communities live inside the healthy tissue of medicinal plants and increase the absorption of soil nutrients and also change their nutrient cycle (Krishnamurthy et al., 2008). Fungal endophytes promote plant growth through the production of ammonia and phytohormones, particularly indole acetic acid (IAA) (Hassan et al., 2013; Bal et al., 2013; Spaepen and Vanderleyden, 2011) that acts as plant growth promoter.

Considering the aforesaid properties, the present study was taken to isolate endophytic fungus from the leaves of *Bauhinia sp., Delonix regia* and *Crotalaria sp.* from the Stella Maris College campus and evaluate their enzymatic and antibacterial activities.

Materials and methods

Sample collection

Fresh and healthy leaves of *Bauhinia sp, Delonix regia* and *Crotalaria sp* were collected from Stella Maris College campus, Chennai. The plants were authenticated by Dr. Bhuvaneshwari, Head, Department of Plant Biology and Biotechnology, Lokanathan Narayana Swami Government College, Ponneri, Chennai and deposited in the herbarium (MLHA1132, MLHA1133, MLHA1134) at Marina labs.

Surface sterilization of leaves

Twenty five segments of leaves from each sample was cut and were thoroughly washed in running tap water and surface sterilized by in 70% ethanol for five seconds, followed by 4% sodium hypochloride for 90 seconds and finally rinsed in sterile distilled water for 10 seconds (Sowparthani and Kathiravan, 2011).

Isolation of endophytic fungi

Five surface sterilized leaf segments were placed in Petri dishes containing Potato Dextrose Agar (PDA) amended with streptomycin (50mg/L) to eliminate bacterial growth and incubated at 28°C for 7 days. The hyphal tips, which grow out from leaf segments were isolated and sub cultured on a new PDA plate and incubated at 28°C to get pure culture (Shubhpriya and Preeti, 2015).

Fungal identification

The endophytic fungal isolates were stained with lactophenol cotton blue and were morphologically identified based on spore morphology with the help of standard manual (Ellis, 1971; Sutton, 1980; Onions et al., 1981; Udhayaprakash, 2004).

Data analysis of colonization frequency

The colonization frequency of each endophytic fungus was calculated by the formula (Kannan et al., 2014).

$$CF\% = \frac{\text{No. of colonized segments}}{\text{Total n. of segments observed}} \times 100$$

Extracellular enzyme assay

The endophytic fungal species from the leaves of *Bauhinia, Delonix regia* and *Crotalaria* were tested for the production of extracellular enzymes such as amylase, laccase, lipase, protease and cellulase (Amirita et al., 2012).

Amylase

The isolates were inoculated in Glucose yeast peptone

(GYP) agar medium supplemented with 2% soluble starch. After 3-5 days of incubation at 37°C, the fully formed cultures were flooded with 1% iodine. The plates were observed for the formation of halos around the colony.

Laccase

The isolates were inoculated in GYP agar medium supplemented with 0.005% 1-naphthol and incubated for 3-5 days at 37°C. The change in the colour of the medium was observed.

Lipase

The lipase activity was tested by growing the isolates on the peptone agar media amended with sterile 10ml of 1% (v/v) Tween 20 and incubated for 5-7 days at 37°C. The plates were observed for the formation of halos around the colony.

Protease

The protease activity was determined by growing the isolates on 1000ml of GYP agar media supplemented with 0.4% gelatin. Eight grams of gelatin was dissolved separately in 100ml distilled water and sterilized. This was added to the media prepared earlier. The plates were inoculated with the fungal isolates and incubated for 3-5 days at 37°C. The culture was flooded with saturated aqueous ammonium sulphate solution. The plates were observed for the formation of clear zone around the culture.

Cellulase

The isolates were grown on yeast extract peptone agar medium amended with 0.5% Na-carboxymethyl cellulose and incubated for 5-7days at 37°C. After incubation the plate were flooded with 0.1% Congo red and destained with 1M sodium chloride for 15 min. The plates were observed for the formation of clear zone around the culture.

Ammonia production

Ammonium production was determined for the fungal strains by growing them in peptone water for 72 h at 28°C. 1 ml Nessler's reagent was added to the culture media. Change of colour to faint yellow indicates minimum ammonia production and change of colour deep yellow to brownish colour indicates maximum ammonia production (Amr et al., 2015).

Extraction of bioactive compounds

The isolated endophytic fungus were inoculated in Potato Dextrose Broth (PDB) and incubated for 7days at 120 rpm. The broth was then filtered through two-folds of cheese cloth. The filtrates were extracted with equal volumes of ethyl acetate in a separating funnel. The organic solvent extracts were evaporated in a rotary evaporator and redissolved in ethyl acetate (Sowparthani and Kathiravan, 2011).

Anti-bacterial activity

Anti-Bacterial activity of the endophytic fungi was tested against *Klebsiella pneumonia* (ATCC 700603), *Protease mirabilis* (ATCC 25933) and *Pseudomonas aeruginosa* (Clinical isolate) by agar well diffusion method (Bauer et al., 1959). 100µl of the inoculum of the test pathogen was spread on Muller Hinton Agar plates. A 5mm well was made in each corner of the plate with equal distance using a sterile cork borer. Crude extract of the five different fungal strains were added at a concentration of 5µg/ml. The plates were incubated at 37°C for 24 hrs. Streptomycin (10µg/ml) was used as positive control. After the incubation, the zone of inhibition around the well was recorded and expressed as millimeter (mm).

Interspecific activity by Dual culture method

The interspecific activity of isolated endophytic fungi was carried out within themselves by dual culture method (Senthilmurugan et al., 2013) in the following combination:

1. *Phoma sp.* vs *Phomopsis sp.* (white)
2. *Phoma sp.* vs *Phomopsis sp.* (brown)
3. *Phoma sp.* vs *Drechselra biseptata*
4. *Phoma sp.* vs *Nigrospora sphaerica*
5. *Phomopsis sp.* (white) vs *Phomopsis sp.* (brown)
6. *Phomopsis sp.* (white) vs *Drechslera biseptata*
7. *Phomopsis sp.* (white) vs *Nigrospora sphaerica*
8. *Phomopsis sp.* (brown) vs *Drechslera biseptata*
9. *Phomopsis sp.* (brown) vs *Nigrospora sphaerica*
10. *Drechslera biseptata* vs *Nigrospora sphaerica*

Seven day old grown cultures of the endophytic fungus

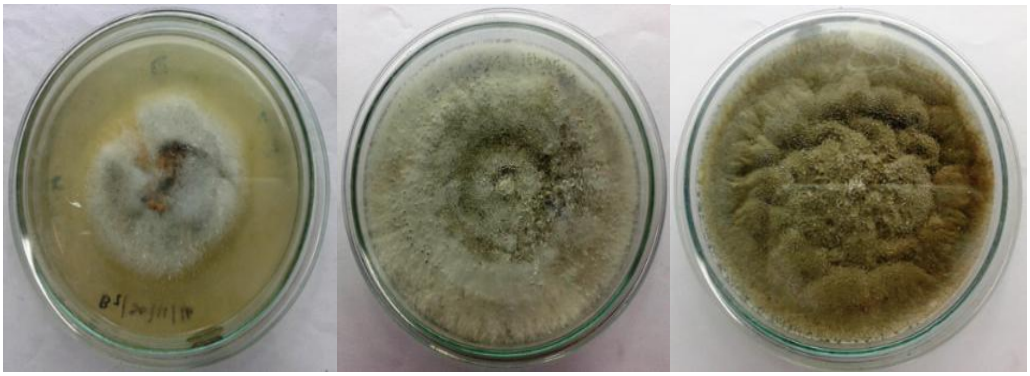
were removed from the edges of the old colony aseptically by using 5mm cork borer. These blocks were placed separately at one end of the Petri plates containing potato dextrose agar medium. At the other end of the plate another seven day old culture of other species of fungi was placed aseptically. Individual plates were maintained for each culture which served as a control. The plates were incubated at room temperature for 4-7 days and the radial growth of fungi was measured.

Results and discussion

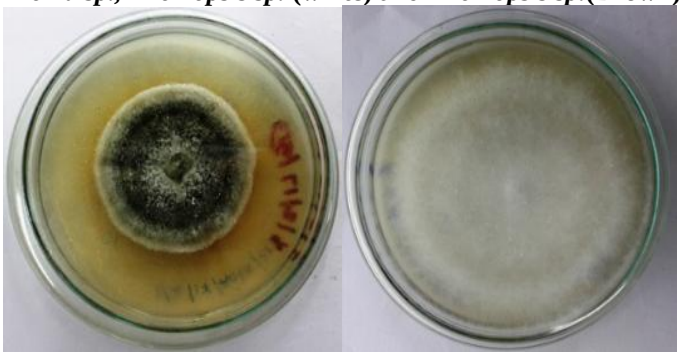
Isolation of endophytic fungi and identification based on spore morphology

A total of 8 different endophytic fungi were isolated from the leaf of *Bauhinia sp.*, *Delonix regia* and *Crotalaria sp* (Figs. 1 and 2). *Phoma sp.* (AS1) and *Phomopsis sp.* (brown colonies) (AS2) were isolated from leaf of *Bauhinia sp*. Mariana et al. (2011) has reported *Phomopsis sp.* from *Bauhinia* which is similar to the reports of the present study. Poorani et al. (2015) has reported *Phoma sp.* which is in concurrence with the present study.

Phoma sp. (AS3) and *Phomopsis sp.* (white colonies) (AS4) were isolated from *Delonix regia*. Zuoping et al. (2014) has isolated 24 colonies of *Phomopsis sp.* and *Phoma sp.* from the plant *Delonix regia* which is concordance with the present findings. *Drechslera biseptata* (AS5), *Nigrospora sphaerica* (AS6), *Phoma sp.* (AS7) and *Phomopsis sp.* (white colonies) (AS8) were isolated from *Crotalaria* in the present investigation. In contrast, Umashankar et al. (2014) has reported *Alternaria sp*., *Penicillium sp*., *Aspergillus flavus* from *Crotalaria sp*. and Govindappa et al. (2011) has reported *A. niger* and *F. oxysporum* from *Crotalaria pallida.*



***Phoma sp., Phomopsis sp.* (white) and *Phomopsis sp.*(Brown)**

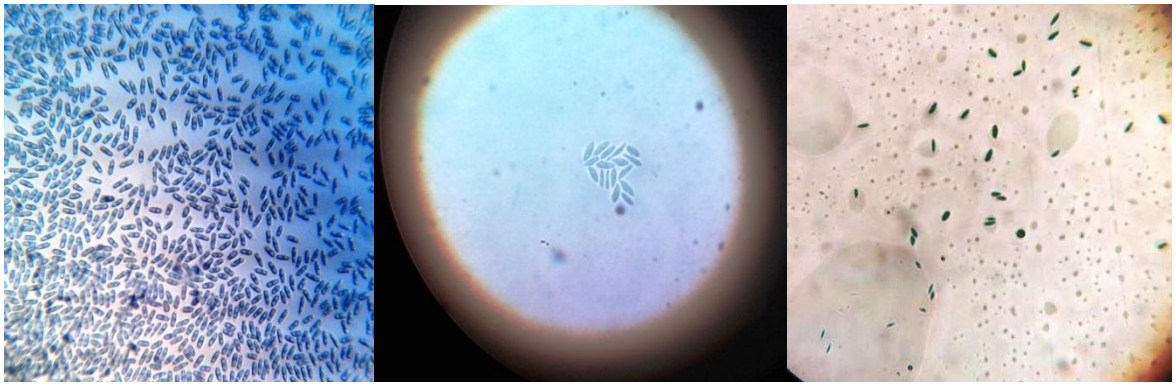


Drechslera biseptata* and *Nigrospora sphaerica

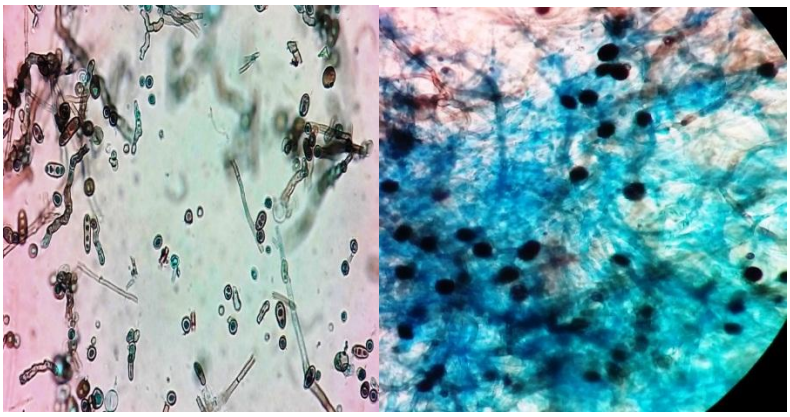
Fig. 1: Colony morphology of the endophytic fungi.

Data analysis of colonization frequency

The colonization frequency of the endophytic fungus is represented in Table 1. The percentage of *Phoma sp*isolated from the leaves of *Bauhinia* was 75% followed by *Phomopsis sp.* (25%). The percentage of *Phoma sp.* isolated from the leaves of *Delonix regia* was 63% followed by *Phomopsis sp.* (37%). The percentage of *Drechslera biseptata* isolated from the leaves of *Crotalaria sp.* was 61 % followed by *Nigrospora sphaerica* (24%), *Phomopsis sp.* (12%) and *Phoma sp.* (3%).



***Phoma sp.*, *Phomopsis sp.* (white) and *Phomopsis sp.* (Brown)**



Drechslera biseptata* and *Nigrospora sphaerica

Fig. 2: Spore morphology of the endophytic fungi.

Table 1. Colonization frequency of endophytic fungi.

Plant source (Leaves)	Fungal isolate	Colonization frequency
Bauhinia sp.	*Phoma sp.* (AS1)	75%
	Phomopsis sp. (Brown) (AS2)	25%
Delonix regia	*Phoma sp.* (AS3)	63%
	Phomopsis sp. (White) (AS4)	37%
Crotalaria sp.	*Drechslera biseptata* (AS5)	61%
	Nigrospora sphaerica (AS6)	24%
	Phoma sp. (AS7)	3%
	Phomopsis sp. (White) (AS8)	12%

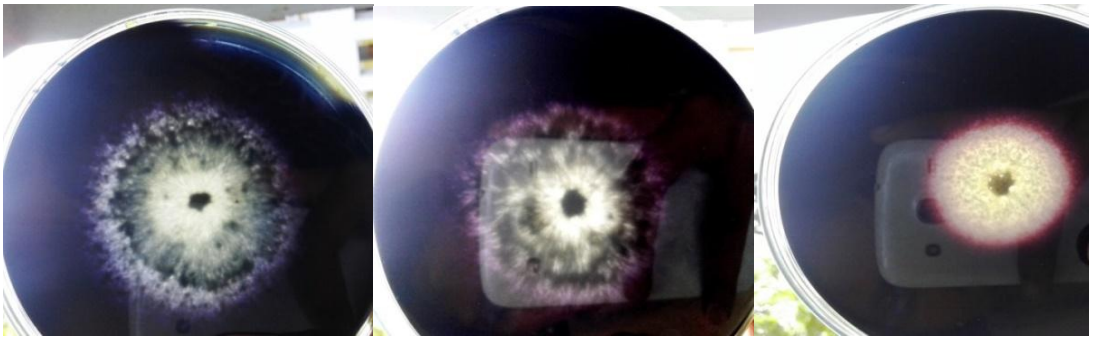
Extracellular enzyme assay

Amylase activities were shown by all the five endophytic fungi as seen from the zone of clearance (Fig. 3; Table 2). The results of the present study are in concordance with that of Shubhpriya and Preeti (2015), Amirita et al. (2012) who have reported amylase production by *Nigrospora sp.* and *Nigrospora sphaerica* respectively. Sunitha et al. (2013) has reported that *Drechslera sp.* showed the positive activity of amylase which is similar to the present study. In the present investigation, Laccase activities were shown by *Phoma sp.* and *Nigrospora sphaerica* (Fig. 4). Sunitha et al. (2013) has reported that *Phoma sp.* showed the positive activity for the enzyme laccase whereas Shubhpriya and Preeti (2015) has reported positive activity for laccase by *Nigrospora sp.* in concordance with the present findings. Protease activity was seen in *Phoma sp.* and *Phomopsis sp.* (white) (Fig. 5). Orlandelli et al. (2015) have reported protease activity in *Phoma herbarum* JF766995 is similar to the current study. None of the fungal isolates produced cellulase and lipase.

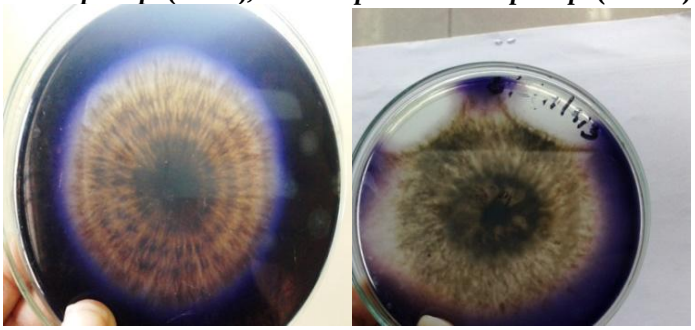
Ammonia production

All the five endophytic fungi had the ability to produce

ammonia in the growth media as seen from the colour change to yellow when compared with the control plates that were colourless which indicates that these organisms influence the growth of the plants. Amr et al. (2015) has reported ammonia production by *Penicillium chrysogenum* Pc_25, *Alternaria alternata* Aa_27 and isolate Sh_26 similar to the current study which has influenced the growth of maize roots along with IAA.



***Phomopsis sp.* (white), *Phoma sp.* and *Phomopsis sp.* (Brown)**

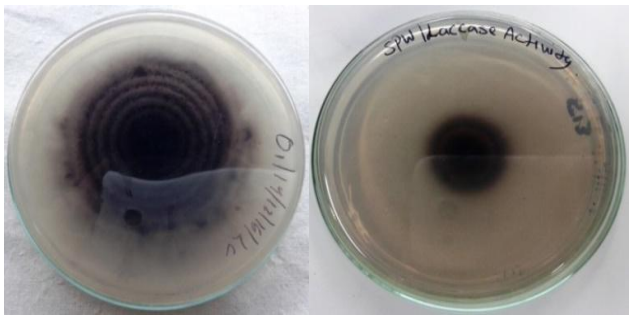


Drechslera biseptata* and *Nigrospora sphaerica

Fig. 3: Amylase activity of endophytic fungi.

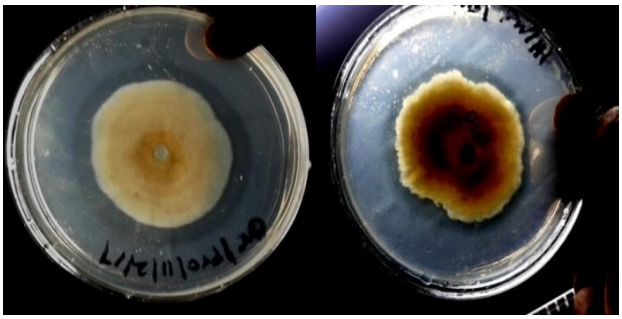
Table 2. Extracellular enzyme activities of the endophytic fungi.

Enzymes	*Phoma sp.*	*Phomopsis sp.* (white)	*Phomopsis sp.* (brown)	*Drechslera biseptata*	*Nigrospora sphaerica*
Amylase	+	+	+	+	+
Laccase	+	-	-	-	+
Lipase	-	-	-	-	-
Cellulase	-	-	-	-	-
Protease	+	+	-	-	-



Phoma sp.* and *Nigrospora sphaerica

Fig. 4: Laccase activity of endophytic fungi.



***Phoma sp.* and *Phomopsis sp.* (White)**

Fig. 5: Protease activity of endophytic fungi.

Antibacterial activity of endophytic fungi

The antibacterial activities of endophytic fungi are represented in Table 3. In the present study it was found that *Klebsiella pneumonia* was inhibited by *Phoma sp.*, *Phomopsis sp.* (white), *Phomopsis sp.* (brown),

Drechslera biseptata and *Nigrospora sphaerica* at a concentration of 5mg/ml. Results similar to the present study has been reported by Sandhu et al. (2014), where *Drechslera sp.* and *Phoma sp.* showed inhibition against *Klebsiella pneumoniae*. In the present study it was found that *Proteus mirabilis* was inhibited by *Phoma sp.*, *Phomopsis sp.* (white), *Phomopsis sp.* (brown) and *Nigrospora sphaerica* at a concentration of 5mg/ml. Liang et al. (2014) has reported that *Phomopsis sp.* showed the best inhibitory responses against bacterial pathogens. In the present study it was found that *Pseudomonas aeruginosa* was inhibited by *Phoma sp.*, *Phomopsis sp.* (white), *Phomopsis sp.* (brown), *Drechslera biseptata* and *Nigrospora sphaerica* at a concentration of 5mg/ml. *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Proteus mirabilis* are Gram negative organisms that are resistant to antibiotics. Antibacterial activity exhibited by the endophytic fungus isolated in the current study suggests that potent antibacterial compounds can be isolated from them.

Table 3. Antibacterial activity of endophytic fungi.

Fungal isolates	*Klebsiella pneumonia*		*Pseudomonas aeruginosa*		*Protease mirabilis*	
	Zone of inhibition (mm)					
	5mg	Control (10 mg)	5mg	Control (10 mg)	5mg	Control (10mg)
Phoma sp	**15**	35	12	32	5	28
Phomopsis sp (white)	10	30	15	34	4	25
Phomopsis sp (brown)	13	30	14	30	5	28
Drechslera biseptata	12	30	**18**	34	-	20
Nigrospora sphaerica	14	30	11	34	3	25

Control – Streptomycin.

Interspecific activity

The interspecific activity of isolated endophytic fungi is represented in Fig. 6. In the study of interspecific activity of *Phoma sp.* vs *Phomopsis sp.* (white), *Phoma sp.* had a growth of 2.5cm whereas *Phomopsis sp.* (white) had 3cm. In the case of *Phoma sp.* vs *Phomopsis sp.* (brown), *Phoma sp.* had a growth of 2.8cm whereas *Phomopsis sp.* (brown) had 3.8cm. The control plates of *Phoma sp.*, *Phomopsis sp.* (white) and *Phomopsis sp.* (brown) had a growth of 5cm each. In the case of *Phoma sp.* vs *Drechslera biseptata*, *Phoma sp.* had a growth of 3.5cm whereas, *Drechslera biseptata* had 2cm. The control plates of *Phoma sp.* had a growth of 5cm and *Drechslera biseptata* had a growth of 4cm. In the study of interspecific activity among *Phoma sp.* vs *Nigrospora sphaerica*, *Phoma sp.* had a growth of 2.5cm whereas *Nigrospora sphaerica* had 4cm. The control plates of *Phoma sp.* and *Nigrospora sphaerica* had a growth of 5cm each.

In the study of interspecific activity of *Phomopsis sp.* (white) vs *Phomopsis sp.* (brown), *Phomopsis sp.* (white) had a growth of 2.9cm whereas *Phomopsis sp.* (brown) had 3.1cm. The control plates of *Phomopsis sp.* (white) and *Phomopsis sp.* (brown) had a growth of 5cm each. In the case of *Phomopsis sp.* (white) vs *Drechslera biseptata* it was found that *Phomopsis sp.* (white) had a growth of 3.5cm whereas *Drechslera biseptata* had 2cm. The control plates of *Phomopsis sp.* (white) had a growth of 5cm while *Drechslera biseptata* had 4cm. In the case of *Phomopsis sp.* (white) vs *Nigrospora sphaerica*, *Phomopsis sp.* (white) had a growth of 2.3cm whereas *Nigrospora sphaerica* had 4.2cm. The control plates of *Phomopsis sp.* (white) and *Nigrospora sphaerica* had a growth of 5cm each.

In the interspecific activity of *Phomopsis sp.* (brown) vs *Drechslera biseptata*, *Phomopsis sp.* (brown) had a growth of 3cm whereas *Drechslera biseptata* had 2cm. The control plates of *Phomopsis sp.* (brown) had a growth of 5cm and *Drechslera biseptata* had a growth of 4cm. In the case of *Phomopsis sp.* (brown) *vs Nigrospora sphaerica*, *Phomopsis sp* (brown) had a growth of 2.9cm whereas *Nigrospora sphaerica* had 4cm. The control plates of *Phomopsis sp.* (brown) and *Nigrospora sphaerica* had a growth of 5cm each.

In the study of interspecific activity among the isolated endophytic fungi *Drechslera biseptata* vs *Nigrospora sphaerica*, *Drechslera biseptata* had a growth of 2.1cm whereas *Nigrospora sphaerica* had 4.2cm. The control plates of *Drechslera biseptata* had a growth of 4cm whereas *Nigrospora sphaerica* had a growth of 5cm.The results of the current study indicates that *Nigrospora sphaerica* was the dominant organism in the interspecific studies.

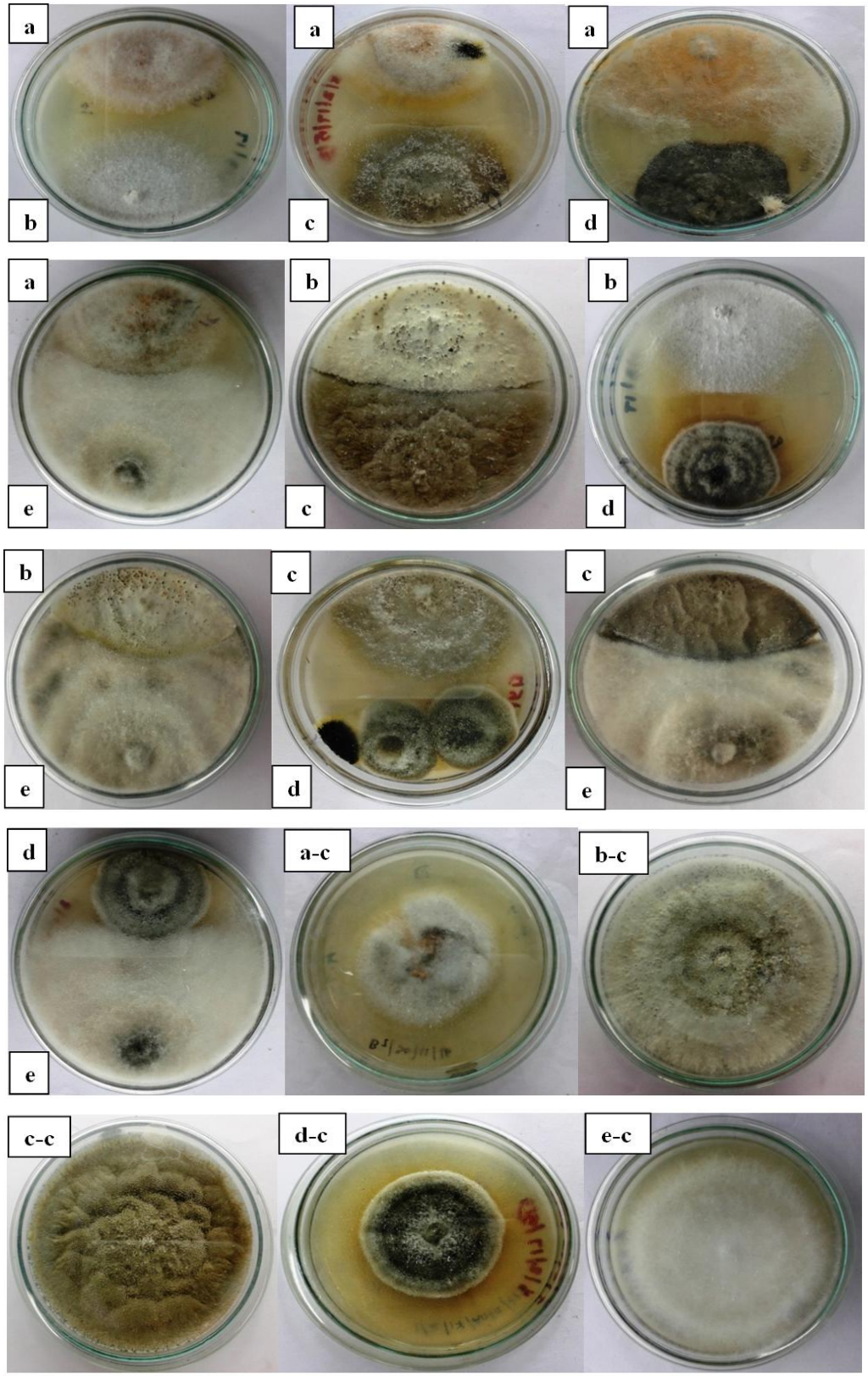


Fig. 6: Interspecific activity of endophytic fungi. *Phoma sp.*; b- *Phomopsis sp.* (white); c- *Phomopsis sp.* (brown); d- *Drechslera biseptata*; e- *Nigrospora sphaerica*; a-c- *Phoma sp.* control; b-c- *Phomopsis sp.* (white) control; c-c- *Phomopsis sp.* (brown) control; d-c - *Drechslera biseptata* control; e-c - *Nigrospora sphaerica* control.

Conclusion

In the present study *Phoma sp* was present in all the leaves and were predominant in *Bauhinia* and *Delonix regia*. *Drechslera biseptata* was predominant in *Crotalaria sp*. Amylase was produced by all the endophytic fungi. Emergence of resistance to antibiotics require development of new antibiotics and in this view the potent bioactive secondary metabolites from *Phoma sp.* and *Drechslera biseptata* obtained in the current study may be used for drug development paving way for natural products.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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