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Phomopsis azadirachtae a New Destructive Causative Agent of Neem Dieback and its Management through Fungicides and Endophytes

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Abstract

The present study was conducted during *rabi* (October–May, 2023–24) at the Department of Plant Pathology and Central Instrumentation Cell, College of Agriculture, Professor Jayashankar Telangana Agricultural University, Rajendranagar, Hyderabad, Telangana, India to report *Phomopsis azadirachtae* a new destructive causative agent of neem dieback and its management through fungicides and endophytes. Die-back of neem disease is presently a major devastating disease in India, resulting in almost 100% loss of fruit production and drastic reduction in evergreen canopy. Seven isolates of *Phomopsis azadirachtae* infecting neem collected from different districts of Telangana and identified based on morphological and cultural characteristics and molecular studies were carried out. They varied significantly in colony colour, growth pattern on Potato Dextrose Agar medium and isolates identified by molecular method, using ITS (ITS1 and ITS4) sequencing. Fifteen endophytic fungi and eight endophytic bacteria were isolated from neem leaves. Further, Bioefficacy of Endophytes was carried out against die back pathogen (*Phomopsis azadirachtae).* The potential endophytes were identified by ITS sequencing Six systemic fungicides Carbendazim, Hexaconazole, Thiophanate methyl, Tricyclazole, Isoprothiolane, Metalaxyl was evaluated against *Phomopsis azadirachtae* with different concentrations 10 ppm, 100 ppm 250 ppm, 500 ppm, 1000 ppm. Carbendazim and Thiophante methyl both were significantly inhibiting the growth of pathogen. Effect of potential endophytes and their combinations on plant growth promotion followed by roll paper towel method. *In vivo* studies evaluating potential endophytes and their combinations against neem die-back pathogen was carreid out inoculated on 2–3 months seedlings showing the disease incidence. Development of effective, eco-friendly management strategies is most important.

Keywords: Compatibility, *inoculation*, recommended concentration, fall armyworm (FAW)

1. Introduction

Neem (*Azadirachta indica*) is an evergreen deciduous tree. It is commonly called “Indian lilac” or “Margosa” and belongs to Meliaceae family. It is native to Indian sub-continent. It has adaptability to a wide range of climatic, topographic and edaphic factors and compared to other species it is well adapted to stress conditions. Thus, it is referred as “Tree for solving problems”. It has pesticidal properties, affecting over 195 insect species and controlling insect pests resistant to synthetic pesticides. Neem leaves contain chemical compounds beneficial for eye disorders, insect poisons, Vatik disorder and anti-leprotic properties. Its fruits are bitter, purgative, antihemorrhoids and anthelminthic. (Reddy and Neelima, 2022). Pathogens enter trees through wounds or openings, thrive in high humidity and temperature, and compromise the tree’s vascular system, causing water and nutrient flow, leading to dieback (Anjana and Devi, 2022). In spite of its well-known anti-fungal, anti-bacterial and other versatile biological activities, neem is not free from diseases. Many fungal and bacterial pathogens were reported on it. Die-back of neem is caused by *Phomopsis azadirachtae*. In severely affected trees, it has resulted in almost always 100% loss of fruit production. (Sateesh et al., 1997). Neem diseases, including Damping off, Rhizoctonia leaf web blight, Colletotrichium leaf spot and blight, *Alternaria alternata, Pseudacercospora subsessilis, Odium azadirachtae*, root rot, pink disease, and Phomopsis twig blight, significantly damage neem nurseries. Damping off leads up to 20% seedling mortality in India. Other diseases, such as root rot, pink disease, and Phomopsis twig blight, causes serious losses under warm conditions (Ciesla, 1993). Die-back

symptoms were observed in most parts of the southern India by Nagendra (2009). The fungus Phomopsis azadirachtae is causing significant damage to neem trees, impacting the global neem industry, necessitating effective management and prevention to protect India's significant neem exports (Prithvi et al., 2015). Endophytic fungi and bacteria in neem leaf play a crucial role in bio-control against Phomopsis azadirachtae, a pathogen causing twig blight, inflorescence blight, fruit rot, and loss of fruit yield Girish et al. (2008). Sateesh et al. (1997) first to identify neem dieback, caused by seed-borne pathogen Phomopsis azadirachtae, poses a significant threat to neem tree cultivation and biodiversity. Neem dieback is characterized by symptoms such as wilting, leaf drop and dieback of branches, leading to significant economic losses in neem plantations and affecting its utility in agroforestry and traditional medicine (Kumar et al., 2021; Tiwari et al., 2018). Neem, a medicinal plant and natural pesticide, is crucial for sustainable farming practices, ensuring ecological balance and agricultural productivity (Choudhary et al., 2021; Wagh et al., 2022). Fungicides pose environmental concerns, leading to focus on biological agents like endophytic fungi and bacteria for sustainable, eco-friendly disease control, with some showing antagonistic properties against *P. azadirachtae* (Anandaraj et al., 2013; Raja et al., 2018). Endophytes, beneficial microorganisms found in plant tissues, have shown potential in improving plant health and enhancing resistance to fungal pathogens (Rodriguez et al., 2009). Endophytes can enhance plant resistance against pathogens, suggesting a sustainable solution of combining fungicide application with endophytic inoculation (Ravi et al., 2023). Considering the importance of neem dieback, management of disease with effective fungicides and endophytes was carried out against *Phomopsis azadirachtae*. The study highlighted the potential of endophytic fungal and endophytic bacteria as a sustainable and eco-friendly alternative method to chemical control in neem die-back.

2. Materials and Methods

2.1. Isolation die-back pathogen

Neem infected twigs was collected from different districts of Telangana *i.e.* Hyderabad (AICRP Agroforestry, PJTAU), Siddipet, Karimanagar, Warangal, Jagtial, Jangaon and Sircilla during August to December, 2023. Infected twigs were collected and placed in sterile polythene bags and transferred to laboratory for further analysis. Twigs were made in small pieces (2–3 cm) with a middle transition zone. Diseased twigs were washed with running tap water for an hour. Segments were surface sterilized with 4% sodium hypochlorite for 5 min and rinsed 6–8 times in sterile distilled water. Segments were plated on PDA with 100 ppm chloramphenicol. Plates were incubated in alternating periods of 12 h of darkness and 12 h of light at 25°C–28°C for 4–5 days. The fungus isolates were further subcultured on potato dextrose agar medium and was purified by using single hyphal tip method. Later, the pure cultures of the different isolates of the pathogen were subcultured once in 30 days to maintain the virulent nature of the pathogen. The cultures were maintained on PDA slants and preserved at 4°C for further studies.

2.2. Cultural and Morphological studies of die-back pathogen

Petri-dishes were inoculated with the five mm mycelia disc drawn from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae* was incubated at 26±2°C with 12 h photoperiod for 10 days. Mean colony diameter was recorded by measuring linear growth in three directions at right angles. Five mm mycelia disc was drawn from the margin of mycelial mat of seven-day-old culture of *P. azadirachtae* was inoculated to new petridishes on PDA media. All the isolates were incubated at 26±2°C with 12 h photoperiod for 15 days. After the incubation period total numbers of pycnidia present was counted. The base area of Petri dishes was divided into six equal parts by diagonally marking the lid with a marking pen. Pycnidia present in each part was counted and mean value was taken as total count.

2.3. Molecular identification

Virulent isolates of were selected and named as NDTH, NDTK, NDTW and process for molecular sequencing. Total genomic DNA of these three isolates were checked for quality. ITS (internal transcribed spacer) region of genomic DNA of *P.azadirachtae* (NDTH, NDTK and NDTW) were amplified using ITS1 and ITS4 primers. . The size of PCR amplicon of pathogen was around 600 bp. PCR products of those three fungal isolates were purified and sequenced at Eurofins Genomics, Bengaluru, India and the data were processed using BioEdit and MEGA11 softwares. The sequences generated using BioEdit software was used to perform BLAST against the NCBI GenBank database (https://blat.ncbi.nlm.nih.gov/Blast.cgi#). The top ten NCBI hits for each sequence was observed and the closest homolog of each isolate from the NCBI GenBank database was identified.

2.4. Efficacy of fungicides

Fungicides like Carbendazim, Hexaconale, Tricyclazole, Isoprothiolane, Thiophanate methyl and Metalaxyl were evaluated against *P. azadirachtae* under *in vitro* conditions, using the poison-food technique (Dhingra and Sinclair, 1995). The study used sterile distilled water for stock solution preparations of fungicides added to PDA medium at different concentrations10, 100, 250, 500 and 1000 ppm. Petri dishes was inoculated with mycelial-agar discs from a seven-day-old *P. azadirachtae* and colony diameter was measured. The percentage mycelial growth inhibition (PI) was calculated compared to the control.

2.5. Isolation of endophytes

Endophytes were isolated from neem leaves, surface sterilized by washing firstly with tap water for 15 min, followed by 70% ethanol for 3 min and finally sterilized with distilled water. Sterilized leaf samples placed on PDA for endophytic fungi

whereas for endophytic bacteria isolation on Nutrient agar (NA) was used. Furtherly they were sub-cultured separately for pure cultures of endophytic fungi and endophytic bacteria on PDA, and NA respectively. Total of 23 endophytes were isolated in which 8 are endophytic bacteria and 15 were endophytic fungi.

2.6. Bio-efficacy endophytes against P. azadirachtae under in vitro

P. azadirachtae The Bio-efficacy of fungal and bacterial endophytes were tested by dual culture technique (Dennis and Webster, 1971) against *P.azadirachtae* on PDA medium. A mycelial disc of *P.azadirachtae* from the actively growing 7 days old culture of the fungi was placed at one end and a 6 mm mycelial disc from the actively growing fungal endophyte was placed at the opposite end of the petriplate, whereas in case of bacteria, a mycelial disc of the pathogen (6 mm dia.) was placed at the one end of the petri plate and the bacterial endophyte was streaked 1.0 cm away from the periphery of the plate from the opposite side. The plates were incubated at 28±2°C for 5 days. Efficacy of the endophytic organisms against the of *P.azadirachtae* was rated based on the inhibition zone observed. Percent inhibition over control was calculated by using formula:

PI={C-T)/C}×100

where *I* is the per cent inhibition of mycelial growth over control; *C* is the radial growth of the pathogen in control (mm); *T* is the radial growth of the pathogen in treatment (mm).

2.7. Characterization of endophytes

Characterization of the endophytic fungal and bacterial isolates were carried out by growing each isolate on Potato dextrose agar (PDA) and Nutrient agar (NA) respectively, to study their growth characteristics such as Colony colour, Elevation, Margin, Texture and Gram staining reaction.

2.8. In Vivo evaluation of endophytes against P. azadirachtae

This was carried out by inoculating on two to three month old seedlings of *A.indica* with wounds made by sterile needle. The wounds was inoculated with sterile cotton swabs dipped in conidial suspension of *P. azadirachtae* at spore concentration of 1×10^6 per ml in sterile distilled water. The control plants were inoculated by using sterile distilled water (Virupaksha and Devarana, 2004). The observation was recorded based on the symptom expression at regular intervals 25 days after inoculation. On the inoculated stem, the pathogen produced blight symptoms on twig leaflets and inflorescence and also seedling parameters (seedling length, vigour, root length and shoot length) was recorded.

2.9. *Plant growth promotion by endophytes*

Plant growth promoting activities of each endophyte was carried out by adopting blotter paper and paper towel method. Ten treated seeds of each treatment (T_1) were placed on a germination paper towel and incubated for 45 days at RT with natural alternate day and night photoperiod (ISTA, 1993). The observations were taken at 45 days after inoculation I. All the treatments from T_1-T_{11} involving individual and combinations applied as seed treatment. The count of the number of seeds germinated was taken on the fourty fifth day. Observations on germination percentage, shoot length, root length, fresh weight, dry weight was recorded subsequently Vigour index was calculated (Abdul and Anderson, 1973). All the treatments were initially treated with pathogen followed by endophytes. Treatments involving combinations are applied as fungal endophyte first followed by bacterial endophyte and applied each treatment with a 30 minutes interval. (Abdul and Anderson 1973). The treatment details are as follows: T_1-Control; T_2- ST of *Phomopsis azadirachtae* NDTWPA; T_3- ST of *Trichoderma harzianum* ELF5; T_4-ST of *Fusarium equiseti* ELF14; T_5-ST of *Bacillus subtilis* ELB4; T_6-ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5; T_7- ST of *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14; T_8-ST of *Phomopsis azadirachtae* NDTWPA+*B. subtilis* ELB4; T_9-ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14; T_{10}-ST of *Phomopsis azadirachtae*NDTWPA+*Trichoderma harzianum* ELF5+*B. subtilis* ELB4; T_{11}- ST of *Phomopsis azadirachtae* NDTWPA+ *Fusarium equiseti* ELF14+*B. subtilis* ELB4.

2.9.1. Germination percentage

Germination of seedlings was recorded 45th day and per cent seedgermination was calculated using the following formula:

GP=(No.of seeds germinated/Total no.of seeds)×100

2.9.2. Seedling vigour index

Seedling vigour index was calculated as suggested by Abdul and Anderson (1973). The formulas used for calculating vigour indices were as follows:

VI=RL+SL×GP

Where, RL is root length (cm), SL is shoot length (cm), GP is germination percentage, VI is vigour index**.**

2.10. In vivo studies for evaluation of potential endophytes against neem die-back pathogen

Azadirachtae indica raised seedlings of 2–3 month old were inoculated with the sterile cotton swabs dipped in spore suspension to the stem region for all the treatments. It was carried to find the Disease incidence of pathogen (*Phomopsis azadirachtae*). Treatments involving combinations are applied as making spore suspension in equal quantity for both pathogen, endophytic fungi and endophytic bacteria (Abdul and Anderson, 1973). The treatment details are as follows: T_1-Control; T_2-Inoculated with *Phomopsis azadirachtae* NDTWPA; T_3- Inoculated with *Trichoderma harzianum* ELF5; T_4-Inoculated with *Fusarium equiseti* ELF14; T_5-Inoculated with *B. subtilis* ELB4; T_6-Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5; T_7-Inoculated with *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14; T_8-Inoculated with *Phomopsis azadirachtae* NDTWPA+*B.*

subtilis ELB4; T9-Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14; T_{10}-Inoculated with *Phomopsis azadirachtae* NDTWPA+ *Trichoderma harzianum* ELF5+ *B. subtilis* ELB4; T_{11}- Inoculated with *Phomopsis azadirachtae* NDTWPA + *Fusarium equiseti* ELF14+ *B. subtilis* ELB4.

3. Results and Discussion

During the survey, it was observed the neem die-back incidence ranged from 75 to 95%. The highest disease incidence of 95% was recorded in Rajendranagar (17.31452° N latitude and 78.41959° E longitude) of Rangareddy district with a height of 32 feets, 34 inches girth and age of plants was 15 years (Table 1). The disease affects trees of all ages and sizes in all agroclimatic zones, with no effect on its severity from climate. Satheesh and Bhat (1999) analyzed seed samples of *Azadirachta indica* from various agroclimatic regions in Karnataka, South India, to identify seed-borne and seed-transmitted pathogens. *Phomopsis azadirachtae* the cause of die-back, was found in all seed components and had a pathogen incidence ranging from 0 to 60%. A total of 7 isolates of *P.azadirachtae* were collected and designated as NDTSi, NDTK, NDTW, NDTSr, NDTJg, NDTJ, NDTH. Identification of the isolated die back fungi was done on the basis of initially, the colour of the colony was white and later on it was turned to greyish black to brown. The fungal mycelium is, branched, septate, and profuse colourless. It produces two types of conidia, alpha and beta in cream to dark-coloured slimy cirri. Alpha conidia are hyaline, fusiform straight, guttulate, smooth and aseptate, while beta conidia are hyaline, filiform, eguttulate, aseptate and resemble hockey sticks. The length of conidia in alpha and beta conidia varied across different regions, with different sizes for each. The colony characteristics varied among different isolates, with NDTK having a wavy margin with sparse mycelium towards the periphery α (3.0×1.6 µm) and ß (24.26×2.05 µm), NDTS having a whitish mycelium with concentric rings at the center α (3.4×2.7 µm) and ß (23.94×2.39 µm), NDTW having a circular margin α (5.4×2.7 µm) and ß (42.32×2.48 µm), NDTH having α (4.8×2.7 µm) and ß (36.30×2.26 µm), NDTS having α (4.2×3.0 µm) and ß (31.39×1.68 µm), NDTJ α (4.6×2.3 µm and ß (33.59×2.38 µm), and NDTJg α (2.5×1.3 µm and ß((30.71×1.52 µm). The pycnidial formation varied among different isolates, with NDTK, NDTSi, NDTW, NDTH, NDTJ, and NDTSr having distinct pcynidial numbers. The mycelial dry weight varied among different isolates, with NDTK having a dry weight of 1.92 g, NDTSi having a dry weight of 1.67 g, NDTW having a dry weight of 2.06 g, NDTH having a dry weight of 2.62 g, NDTJ having a dry weight of 1.54 g, NDTJg having a dry weight of 1.84 g, and NDTSr having a dry weight of 1.72

Table 1: Collection of Neem die-back diseased samples from different districts of Telangana during 2022-2023

Sl. No.	Village	Mandal	District	Lattitude	Longitude	Height (ft)	Girth (inch)	Age (Y)	DI	Temperature (°C)		Relative humidity (%)	
										Maxi	Min	Maxi	Min
1.	Pandilla	Husnabad	Siddipet	18.10190	78.852070	22	32	20	75%	32.8	18.6	90.8	35.9
	Husnabad	Husnabad		18.10190	78.852070	26	31	25	85%				
2.	Nawabpet	Chigurumamidi	Karimnagar	18.438560	79.128840	30	24	10	90%	30.4	20.2	90	25
	Thimmapur	Thimmapur		18.438560	79.128840	29	24	12	75%				
3.	Repaka	Illantakunta	Sircilla	18.386620	78.802250	16	12	7	85%	27.2	12.9	91.2	37.3
4.	Elkathurthi	Elakathurthy	Warangal	18.000060	79.588170	39	28	15	90%	30.6	14.4	95	28
5.	Polasa	Jagtial	Jagtial	18.794910	78.912870	30	42	15	87%	33.2	17.1	84.7	28.5
6.	Narsapur	Tharigopula	Jangaon	17.721870	79.172290	36	31	18	84%	31.2	15.6	94.2	34.6
7.	Rajendranagr	Rajendranagr	Rangareddy	17.314520	78.419590	32	34	15	95%	31	15	95	28
CD						1.441	1.467	0.84	4.156				
SEm±						0.481	0.49	0.28	1.388				
SEd±						0.681	0.693	0.397	1.963				
C.V						2.886	2.96	3.191	2.825				

g. *P. azadirachtae* isolates showed cultural, morphological, pathogenic and biochemical variation (Table 2).

Significant differences in the mycelial type, colour of the colony, texture, radial growth and number of pycnidia were observed among the isolates. (Girish and Bhat, 2008), Similarly Conidia of two types, in a cream to dark yellow coloured slimy cirrhi: alpha-conidia hyaline, fusiform, straight, 2-4 guttulate, smooth, aseptate, 4.8-11×1.6-3.2 µm, germinate readily, beta-conidia hyaline, filiform, hamate, eguttulate, aseptate, 16-25.6×1.6-2.0 µm germination unknown. (Girish and Bhat, 2008). Then the mycelial dry weight was recorded of each isolates *i.e*. NDTK with 1.92 g, NDTSi with 1.67 g, NDTW with 2.06 g, NDTH with 2.62 g, NDTJ with 1.54 g, NDTJg with 1.84 g, NDTSr with 1.72 g (Table 2). Pycnidia present in each part was counted and mean value was taken as total count, then pycnidial number of each isolates was recorded as for NDTK with 15, NDTSi with 15, NDTW with 22, NDTH with 18, NDTJ with 13, NDTJg with 14, NDTSr with 16, whereas formation of pycnidia is also differs for isolates such as NDTK, NDTH, NDTSi are submerged and scattered, NDTW is distinct and scattered, NDTJ is submerged in concentric rings, likewise NDTJg, NDTSr are distinct with concentric rings. The virulent pathogens of three different districts were selected named as NDTH, NDTK, NDTW

3.1. Genomic DNA isolation and PCR amplification

PCR products of those three isolates were purified and sequenced at Eurofins Genomics, Bengaluru, India and the data were processed using BioEdit and MEGA11 softwares. The consensus sequences generated using BioEdit software were used to perform NCBI-BLAST against the NCBI GenBank database (https://blat.ncbi.nlm.nih.gov/Blast.cgi#) The top ten NCBI hits for each sequence were observed and the closest homolog of each isolate from the NCBI GenBank database was identified. Briefly, the fungal pathogen of *P.azadirachtae* (NDTHPA) showed 99.25% identity and (NDTKPA) showed 99.63% identity, whereas (NDTWPA) showed 98.70% identity. Nagendra prasad *et al*. (2009) found *Phomopsis azadirachtae* is a seed-borne and was isolated from twigs, seeds, and

Table 2: Cultural characteristics of neem die-back pathogen (*P. azadirachtae*)

Isolate designation	Colony colour	Colony characteristics	Radial growth (mm)	Pycnidial formation	Pycnidial Number	Conidia length		mycelial dry weight (g)
						α (µm)	β (µm)	
NDTK	Greyish white	greyish white mycelium, margin was wavy with sparse mycelium towards periphery.	89	submerged and scattered	15	3.0×1.6	19.50×1.06	1.92
NDTW	white	greyish mycelium with concentric rings at centre, circular margin	90	distinct and scattered	22	5.4×2.7	19.52×1.20	2.06
NDTH	Creamish white	whitish raised wooly mycelium, concentric rings in centre, sparse mycelium toward periphery.	90	submerged and scattered	18	4.8×2.7	17.35×1.12	2.62
NDTJ	white	white raised wooly mycelium, concentric rings of pcynidia, margin was circular.	88	submerged in concentric rings	13	4.2×3.0	15.57×1.03	1.54
NDTJg	white	greyish white mycelium, margin was wavy with sparse mycelium towards periphery.	87	distinct with concentric rings	14	4.6×2.3	16.72×0.78	1.84
NDTSr	greyish white	whitish raised wooly mycelium, concentric rings, margin is circular.	87	distinct with concentric rings	16	2.5×1.3	16.19×1.08	1.72

embryos. The genus-specific primers (5.8S r-DNA) were used for PCR detection. The amplification of 141 bp DNA in the diseased trees indicated the causal organism. The current technique can detect very low propogules within 4–5 days, compared to the 15–21 days required for conventional isolation and identification. This method offers a faster and more accurate for detecting dieback disease. Likewise, Satheesh and Bhat (1998) developed PCR assay for the rapid detection and identification of *P. azadirachtae* from neem plant tissues. The primers amplify DNA from diseased neem explants and mycelia of different *P. azadirachtae* isolates, making them potentially useful for specific identification in neem tissues.

3.2. Efficacy of fungicides against Phomopsis azadirachtae

In vitro studies were carried out with effective fungicides *i.e* Carbendazim, Hexaconazole, Tricyclazole, Isoprothiolane, Thiophanate methyl, Metalaxyl. Among them Carbendazim and Thiophanate methyl was more effective fungicides against *P.azadirachtae* pathogen (Table 3). Carbendazim was inhibiting with 92.9 per cent at 10 ppm, 94% at 100 ppm and 250 ppm, whereas 100% at 500 and 1000 ppm.Thiophanate methyl was also inhibiting at maximum level compare to other fungicides 89.3% at 10 ppm, 93% at 100 ppm and 250 ppm, 100% at 500 ppm and 1000 ppm Hexaconazole inhibits with 83% at 10 ppm, 88.2% at 100 ppm, 88.5% at 250 ppm, 92.2% at 500 ppm, 94% at 1000 ppm.

Table 3: Effect of fungicides against die back pathogen of neem (*P. azadirachtae*)

Concentrations (ppm)	Fungicides											
	Carbendazim		Hexaconazole		Tricyclazole		Isoprothiolane		Thiophanate methyl		Metalaxyl	
	MG	IP	MG	IP	MG	IP	MG	IP	MG	IP	MG	IP
10	6.3	92.9	15.3	83	59.6	20.53	41.5	53.8	8	89.3	51.3	43
100	5	94	10.6	88.2	5	93	28.3	68.5	5	93	50.60	43.7
250	5	94	10.3	88.5	5	94	12	86.6	5	93	35.6	60.4
500	0	100	7	92.2	5	94	8.3	90.7	0	100	24	74
1000	0	100	5	94	5	94	5	94	0	100	23.1	73
Control	90	-	90	-	90	-	90	-	90	-	90	-
CD	0.231	5.062	0.545	4.703	1.322	4.521	1.279	4.148	0.254	5.005	2.155	3.072
SEm±	0.072	1.586	0.171	1.473	0.414	1.416	0.401	1.3	0.079	1.568	0.675	0.962
SEd±	0.102	2.243	0.241	2.084	0.586	2.003	0.567	1.838	0.112	2.218	0.955	1.361
C.V.	3.839	2.856	3.068	2.862	4.508	3.102	3.648	2.859	3.825	2.857	3.167	2.834

MG: Mycelial growth (mm); IP: Inhibition Percent

Isoprothiolane inhibits with 53.8% at 10 ppm, 68.5% at 100 ppm, 86.6% at 250 ppm, 90.7% at 500 ppm, 94% at 1000 ppm. Tricyclazole inhibits with 20.53% at 10 ppm, 94% at 100 ppm, 94% at 250 ppm, 500 ppm, 1000 ppm. The least inhibiting fungicide was Metalaxyl inhibits with 43% at 10 ppm, 43,7% at 100 ppm, 60.4% at 250 ppm, 74% at 500 ppm, 73% at 1000 ppm. Girish et al. (2009) studied using six systemic fungicides. Carbendazim was most effective in inhibiting the growth of *the P. azadirachtae* pathogen, followed by thiophanate methyl. The concentrations tested were 0.25 ppm and 0.75 ppm respectively. Both carbendazim and thiophanate methyl were found to be good for controlling die-back of neem disease

3.3. Endophytes from neem leaves

Fifteen fungal endophytes designated as ELF1–ELF15 and eight bacterial endophytes ELB1–ELB8 were isolated from neem leaves. Gangwar et al. (2016) isolated 35 endophytic actinomycetes from roots, leaves, and stems of *Azadirachta indica* revealed that healthy living tissues harbor a variety of endophytic bacteria. among, 87% of isolates being *Streptomyces* sp.

3.4. Bio-efficacy of bacterial endophytes against P.azadirachtae

Among the 8 bacterial endophytes tested, ELB 4 isolate showed the highest per cent growth inhibition of 66.6% over control and minimum per cent growth inhibition of 50% over control was recorded by ELB 3 isolate. All 8 bacterial endophytes were screened, eight isolates includes leaves endophytic bacteria *viz.,* ELB 4 (66.6%), ELB 1 (63.3%), ELB 2 (61.4%), ELB 8 (60%), ELB 7 (59.2%), ELB 5 (57%), ELB 6 (54.4%), ELB 3 (50%) inhibition against *P.azadirachtae* (Table 4). Similarly, Girish (2009) evaluated bacterial and fungal antagonists were tested against *P. azadirachtae* using a dual culture method. *Bacillus subtilis* showed significant inhibitory effects against *P. azadirachtae* growth.

3.5. Bio-efficacy of fungal endophytes against P. azadirachtae

Among the 15 fungal endophytes tested, ELF5 isolate showed highest percent growth inhibition of 74.7% over control and minimum per cent growth inhibition of 56.2% was recorded by ELF 12 (Table 5). The inhibition per cent of endophytic fungi ranged from 56.2% to 74.7%. Out of 15 fungal endophytes

Table 4: Bio-efficacy of endophytic bacterial isolates against *P. azadirachtae* under *in vitro*

Sl. No	Isolate ID	Radial growth of pathogen (mm)*	Percent growth inhibition over control*
1.	ELB1	33	63.3 (52.6)
2.	ELB2	34.6	61.4 (51.5)
3.	ELB3	45	49.9 (44.9)
4.	ELB4	30	66.6 (54.6)
5.	ELB5	38.6	57.0 (49.0)
6.	ELB6	41	54.4 (47.5)
7.	ELB7	36.6	59.2 (50.3)
8.	ELB8	36	59.9 (50.7)
9.	Control	90	-
CD		1.821	2.971
SEm±		0.602	0.983
SEd±		0.852	1.39
C.V.		2.831	2.885

Table 5: Bio efficacy of fungal endophytes against *P. azadirachtae* under *in vitro*

Sl. No.	Isolate ID	Radial growth of pathogen (mm)*	Percent growth inhibition over control*
1.	ELF1	38	57.3 (49.2)
2.	ELF2	30.3	66.2 (54.4)
3.	ELF3	31	65.5 (54.0)
4.	ELF4	30	66.6 (54.6)
5.	ELF5	22.6	74.7 (59.8)
6.	ELF6	32	64.4 (53.3)
7.	ELF7	29.6	67.9 (55.5)
8.	ELF8	34.3	61.8 (51.8)
9.	ELF9	29	67.7 (55.3)
10.	ELF10	30	66.2 (54.4)
11.	ELF11	31.3	65.1 (53.7)
12.	ELF12	39.3	56.2 (48.5)
13.	ELF13	34	62.0 (52.0)
14.	ELF14	26	71.0 (57.4)
15.	ELF15	29	67.7 (55.3)
16.	Control	90	-
CD		1.557	3.208
SEm±		0.537	1.105
SEd±		0.759	1.563
C.V.		2.983	2.927

screened, except two endophytic fungi *viz.,* ELF 1 (57.3%) and, ELF12 (56.2%) showed more than 60% inhibition against *P. azadirachtae* (Table 5). Similarly, studies were done by, Girish et al. (2009) with antagonistic endophytic bacteria and fungi, *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa*, *P. oleovorans*, *Trichoderma harzianum* and *T. viride* against *P. azadirachtae* under *in vitro*.

3.6. Identification of bacterial endophytes

A total of 8 bacterial endophytes were observed for morphological and colony characteristics *viz.*, size, shape, colour, margin, texture, elevation. gram staining reaction. Out of these, 5 isolates (ELB2, ELB3, ELB4, ELB5, ELB6) showed Gram positive reaction and 3 isolates (ELB1, ELB7, ELB8) showed Gram negative reaction. The colony colour of the bacterial isolates ranged from white to orange with varying shades. Maximum six isolates showed creamy whithish coloured colonies and two isolates showed whitish coloured colonies. The endophytes also showed variation in their colony morphology. It was observed that the colonies of 4 were circular, 4 were irregular in shape. With respect to colony size, among all the isolates, 3 isolates were small, one was large and 3 were medium in size (Table 6). These isolates were characterized using 16S r-DNA restriction analysis and clustered according to their geographical distribution. The similarity index confirmed the endophytes.

3.7. Identification of fungal endophytes

A total of 15 fungal endophytes were observed for morphological, cultural and microscopic characters. Out of these isolates, maximum of four fungal endophytes showed cottony type of mycelium, three were powdery, three isolates showed velvety mycelium, two isolates showed fine cottony type of mycelium, isolate ELF 12 was dense cottony and isolate ELF 15 was hairy. The colony colour of endophytes also showed variation in shades of white and green. Six isolates were white, two isolates were olive green, two isolates showed greenish white, whereas one isolate ELF10 was moss green, and one isolate ELF4 was light brown in colour, one isolate ERF 8 showed light red, one isolate ELF12 was grey, whereas one isolate ELF 14 was creamy white in colour (Table 7). Similarly, Kusari et al. (2012) identified and characterized endophytic fungus from *Azadirachta indica A. Juss*, producing natural insecticides with antifeedant and growth-regulating properties.

3.8. Molecular identification of potential endophytes

The best three potential endophytes with high antagonistic activity were selected and sequenced with ITS for fungal and 16S rDNA for fungal and bacterial gene sequencing and identified as *Trichoderma* spp. (ELF5)*, Fusarium equisetti* (ELF14), *Bacillus.*

PCR products of best three potential endophytic isolates were purified and sequenced at Eurofins Genomics, Bengaluru, India and the data were processed using BioEdit and MEGA11

Table 6: Morphological and colony characters of endophytic bacterial isolates isolated from neem leaves

Isolate no	Colony colour	Colony size	Shape	Elevation	Margin	Texture	Cell shape	Gram staining	Appearance	Optical property
ELB1	creamy white	small	irregular	raised	wavy	mucoid	Spherical	Gram negative	rough	opaque
ELB2	creamy white	medium	irregular	Flat	wavy	Dry	Rod	Gram positive	shiny	transparent
ELB3	creamy white	medium	circular	raised	wavy	mucoid	Rod	Gram positive	rough	opaque
ELB4	Cream	medium	circular	raised	entire	Moist	Spherical	Gram positive	shiny	opaque
ELB5	Whitish	small	circular	Flat	entire	Dry	Spherical	Gram positive	smooth	opaque
ELB6	creamy white	large	circular	raised	entire	Moist	Spherical	Gram positive	shiny	opaque
ELB7	Whitish	medium	irregular	Flat	wavy	mucoid	Rod	Gram negative	rough	opaque
ELB8	creamy white	small	irregular	Flat	wavy	mucoid	Spherical	Gram negative	smooth	opaque

Table 7. Morphological and colony characters of endophytic fungal isolates isolated from neem leaves

Isolate No	Colony characteristics	Surface texture	Upper colour	Reverse colour	Radial growth (mm)	No. of days to fill plate
ELF1	White dense mycelium, circular margin	Cottony	white	yellow	87.5	5
ELF2	Cottony mycelium, dense ring at centre	Cottony	white	white	90	5
ELF3	Whitish mycelium, dense at centre, circular margin	Cottony	white	Pale white	88	5
ELF4	Light brownish mycelium, whitish margin	Powdery	Light brown	Light yellow	82.5	6
ELF5	Dark green dense mycelium at centre	Very fine cottony	Greenish white	Light brown	89.5	4
ELF6	Olive green mycelium, scattered	Velvety	Olive green	cream	88.7	5
ELF7	Light green mycelium, dense at margin	Cottony	Green	white	88.5	5
ELF8	Light reddish mycelium at centre, whitish margin	Powdery	Light red	white	87.5	6
ELF9	White cottony mycelium	cottony	white	cream	90	4
ELF10	Moss green mycelium, circular colonies scattered entirely	Powdery	moss green	yellow	87	6
ELF11	White mycelium	Velvety	white	Dull white	88.5	5
ELF12	Greyish white mycelium, dark at centre	Dense cottony	Grey	brown	86.3	7
ELF13	Olive green mycelium and scattered, tough circular colonies	Velvety	Olive green	cream	87.8	6
ELF14	whitish yellow mycelium, distinct concentric rings, dense at centre	Fine cottony	Creamy white	yellow	87.5	7
ELF15	Wooly mycelium, dense at centre, sparse towards periphery	Hairy	white	Cream	87	5

softwares. The consensus sequences generated using BioEdit software were used to perform NCBI-BLAST against the NCBI GenBank database (https://blat.ncbi.nlm.nih.gov/Blast.cgi#) The top ten NCBI hits for each sequence were observed and the closest homolog of each isolate from the NCBI GenBank database was identified (Table 7). Briefly, the potential fungal endophyte (ELF5) showed 97.97% identity to *Trichoderma harzianum,* and (ELF14) showed 100% identity to *Fusarium equisetti*, whereas for potential bacterial endophytic isolate ELB4 showed 98.57% identity to *Bacillus subtilis*. Similarly, in addition, Souza et al. (2017) reported the genera *Trichoderma, Aspergillus, Fusarium and Colletotrichum* as potential producers of antibiotic activity. Differences were observed among strains from the same species with respect to their ability to produce active metabolites, as observed for *F. oxysporum* (CBMAI 45, CBMAI 47 and CBMAI 53), Glomerella spp. (CBMAI 63, CBMAI 67 and CBMAI 163) and *Trichoderma spp.* (CBMAI 43 and CBMAI 52) based on rDNA ITS sequences BLAST analysis. Likewise, Amaresan *et al*. (2014) identified *Bacill*us spp., *Achromobacter* spp., *Providencia* spp., *Microbacterium* spp. And *Arthrobacter* spp. From 16sr DNA partial sequence isolates, including BECR2, BECR12, BECL10, BECL11, BECR14, BECS7, BECL5 and BECR13. In the present investigation also on the basis of 16Sr DNA partial sequence the isolates ELB 4 were identified as *Bacillus subtilis*

3.9. Effect of potential endophytes and their combinations on plant growth promotion by following rolled paper towel method

3.9.1. Germination percentage

In rolled paper towel method, among all the treatments the highest per cent germination of 90% was recorded by treatment T_1 (Control) and T_3 (ST of *Trichoderma harzianum*) with 80% was on par with T_4 (ST of *Fusarium equiseti*) with 70%, T_6 (ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum*) with 70% and T_9 (ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14) with 70%. The per cent germination of 60% was observed with treatment T5 (ST of *B. subtilis* ELB4), T_7 (ST of *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14), T_8 (ST of *Phomopsis azadirachtae* NDTWPA+ *B. subtilis* ELB4) it was followed by the treatments T10 (50%), T_{11} (40%) and T_2 (30%).

3.9.2. Shoot and root length

Among the treatments, highest shoot length of 5.36 cm was recorded with treatment T_3 (ST of Trichoderma harzianum ELF5), T_8 (ST of *Phomopsis azadirachtae* NDTWPA+ *B. subtilis* ELB4) with 5.13 cm, T_9 (ST of *Phomopsis azadirachtae* NDTWPA +*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14) with 4.6 cm was on par with T_4 (ST of *T. harzianum* ERF2+*B. subtilis* ESB9) with 4.56 cm and T_{11} (ST of *Phomopsis azadirachtae* NDTWPA + *Fusarium equiseti* ELF14+ *B. subtilis* ELB4) with 4.36 cm. The shoot length of 4.16 cm was observed with treatment T_{10} (ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*B. subtilis* ELB4), followed by treatments T_1 (3.50 cm), T_6 (3.43 cm), T_5 (2.83 cm), T_7 (2.19 cm). The lowest shoot length of 6.5 cm was recorded by treatment T_2 (ST of *Phomopsis azadirachtae* NDTWPA). Whereas, among all treatments, the highest root length was observed in treatment T_1 (Control) with 6.16 cm was on par with T3(ST of *Trichoderma harzianum* ELF5), followed by treatment T_9 (ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14) with 5.43 cm, T_8 (ST of *Phomopsis azadirachtae* NDTWPA+*B. subtilis* ELB4) with 5.26 cm. The treatment T_6 was recorded with root length of 5.06 cm followed by T_4 (4.96 cm), T_7 and T_5 (4.66 cm), T10 (4.6 cm) and T_{11} (4.39 cm). The lowest root length of 2.83 cm was recorded in treatment T_2 (ST of *Phomopsis azadirachtae* NDTWPA).

3.9.3. Vigour indices

Among all the treatments evaluated, the highest vigour index (908.8) was observed with treatment T_3 (ST of Trichoderma harzianum ELF5) followed by treatment T_1 (Control) with 869.67, T_9 (ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5 + *Fusarium equiseti* ELF14) with 702.1, followed by T_4 (666.4), T_8 (623.4), T_6 (594.3), T_5 (449.58), T_5 (438), T_7 (411.42) and T_{11} (350.28). The lowest vigour index of 151.8 was recorded in treatment T_2 (ST of *Phomopsis azadirachtae* NDTWPA). To isolate and identify the disease-causing pathogen, conventional approaches need at least 15 to 21 days**.** *Phomopsis azadirachtae*, the pathogenic fungus that causes neem dieback disease (Bhat et al., 1998), is a both seed borne and seed transmitted (Sateesh et al., 1997). Neem seeds are only viable for a short time, so it is important to know the mycoflora and pathogens that are present in neem seeds. It is significant that *Phomopsis azadirachtae* was found in neem seed because of its negative effects on neem trees. Seeds are used to spread *P. azadirachtae*. Thus, it spreads from seed to seedling and may cause a large-scale disease outbreak. The embryo, cotyledons, and seed coat all contained the infection. Embryos only contained *P. azadirachtae* on the transfer of pathogens from seeds to seedlings, the pathogen can cause seed rot, seedling rot, weak seedling growth, as well as seedlings with fibrous root systems and those without root systems (Girish and Bhat, 2008) (Table 8).

3.10. In vivo studies for evaluation of potential endophytes against neem die-back pathogen

Azadirachtae indica raised seedlings of 2–3 month old were inoculated with the sterile cotton swabs dipped in spore suspension to the stem region for all the treatments. It was carried to find the Disease incidence of pathogen (*Phomopsis azadirachtae*). Treatments involving combinations are applied as making spore suspension in equal quantity for both pathogen, endophytic fungi and endophytic bacteria. (Abdul and Anderson, 1973). The following treatments were same as in roll paper towel method.

Table 8: Effect of potential endophytes and their combinations on plant growth promotion by rolled paper towel method at 45 DAI

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Vigour index
T_1	90	6.16	3.50	869.67
T_2	30	2.83	2.23	151.8
T_3	80	6	5.36	908.8
T_4	70	4.96	4.56	666.4
T_5	60	4.66	2.83	449.58
T_6	70	5.06	3.43	594.3
T_7	60	4.66	2.19	411.42
T_8	60	5.26	5.13	623.4
T_9	70	5.43	4.6	702.1
T_{10}	50	4.6	4.16	438
T_{11}	40	4.397	4.36	350.28
CD	2.949	0.234	0.194	27.843
SEm±	0.999	0.079	0.066	9.433
SEd±	1.413	0.112	0.093	13.34
C.V	2.799	2.797	2.956	2.915

Values expressed are mean of three replications. *DAI- days after incubation; T_1: Control; T_2: ST of *Phomopsis azadirachtae* NDTWPA; T_3: ST of *Trichoderma harzianum* ELF5; T_4: ST of *Fusarium equiseti* ELF14; T_5: ST of *B. subtilis* ELB4; T_6: ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5; T_7: ST of *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14; T_8: ST of *Phomopsis azadirachtae* NDTWPA+*B. subtilis* ELB4; T_9: ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14; T_{10}: ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*B. subtilis* ELB4; T_{11}: ST of *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14+*B. subtilis* ELB4

3.10.1. Disease incidence

In *In vivo* studies, among all the treatments the highest disease incidence of 90% was recorded by treatment T_2 (Inoculated with *Phomopsis azadirachtae* NDTWPA), followed by T_{11} (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14+ *B. subtilis* ELB4) with 60%, T_7 (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14) with 40%, T_8 (Inoculated with *Phomopsis azadirachtae* NDTWPA+ *B. subtilis* ELB4) and T_{10} (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*B. subtilis* ELB4) with 30% and T_6 (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5) with 20%. There was no disease incidence in following treatments such as T_1 (Control), T_3 (Inoculated with *Trichoderma harzianum* ELF5), T_4 (Inoculated with *Fusarium equiseti* ELF14), T_5 (Inoculated with *B. subtilis* ELB4) (Table 9).

Table 9: *In vivo* studies of potential endophytes against neem die-back pathogen

Treatments	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Root dry weight (g)	shoot dry weight (g)	DI
T_1	47.9	36.7	8	4.16	1.16	1.33	0
T_2	28	25.33	3.427	2	1	0.66	90
T_3	46.763	30.7	11.53	5.227	4.66	2.16	0
T_4	48.3	36.53	7.503	4.833	2.50	1.833	0
T_5	40.13	27.96	5.33	3.503	2.16	1.33	0
T_6	37.36	34.3	9.93	5.5	3.66	1.5	20
T_7	35.4	32.6	9.33	4.66	3.16	1.503	40
T_8	43.73	30.863	4.66	2.157	1.33	1	30
T_9	44.1	49.763	11	5.503	3.503	1.833	10

Table 9: Continue...

Treatments	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Root dry weight (g)	shoot dry weight (g)	DI
T_{10}	25.3	36.83	7.66	3	2.5	1.66	30
T_{11}	29.73	26.96	7.503	3.83	2.66	1.66	60
CD	1.896	1.593	0.373	0.189	0.122	0.071	2.051
SEm±	0.642	0.54	0.126	0.064	0.041	0.024	0.695
SEd±	0.908	0.763	0.179	0.091	0.059	0.034	0.983
C.V	2.867	2.789	2.801	2.749	2.84	2.776	4.728

Values expressed are mean of three replications. *DAI- days after incubation; T_1: Control; T_2: ST of *Phomopsis azadirachtae* NDTWPA; T_3: ST of *Trichoderma harzianum* ELF5; T_4: ST of *Fusarium equiseti* ELF14; T_5: ST of *B. subtilis* ELB4; T_6: ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5; T_7: ST of *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14; T_8: ST of *Phomopsis azadirachtae* NDTWPA+*B. subtilis* ELB4; T_9: ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14; T_{10}: ST of *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*B. subtilis* ELB4; T_{11}: ST of *Phomopsis azadirachtae* NDTWPA+*Fusarium equiseti* ELF14+*B. subtilis* ELB4

Similarly in the current study we observed that Disease Incidence against neem die-back was less in the treatment T_9 (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*Fusarium equiseti* ELF14) with 10%, T_6 (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5) with 20%, T_8 (Inoculated with *Phomopsis azadirachtae* NDTWPA+ *B. subtilis* ELB4) and T_{10} (Inoculated with *Phomopsis azadirachtae* NDTWPA+*Trichoderma harzianum* ELF5+*B. subtilis* ELB4) with 30%.Overall above results includes T_9, T_6, T_8, T_{10} were the best treatments, controlling the neem die-back disease Table 8. Likewise, Kumar et al. (2015) found that when a microbial consortium (*T. harzianum*+*B. subtilis*) was used as a carrier with vermicompost, tomato seedling growth parameters significantly increased as compared to when B. subtilis OTPB1 or *T. harzianum* (OTPB3) were treated separately and the control group. In comparison to the control, the consortium enhanced fresh weight, shoot length, and root length by 35.5%, 40.8%, and 54.5% respectively.

4. Conclusion

The study identified potential endophytic fungal and endophytic bacterial isolates against pathogen under *in vitro* conditions. Evaluation of *in vivo* studies of potential endophytes and their combinations of plant growth promotion on seedlings against pathogen showing disease incidence and also seed treatment with potential endophytes and their combinations under *in vitro* conditions against pathogen using roll paper towel. Further *in vitro* screening of effective fungicides using poison food technique against virulent pathogen can fully revealed their potentiality for sustainable management.

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