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In vitro Antagonistic Potential of Endophytic Fungi of Soybean (*Glycine Max* (L.) Merril) Against *Macrophomina phaseolina*

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Abstract
Utilization of indigenous endophytes is considered as an environmentally-friendly and ecologically efficient strategy. A total 14 endophytic fungi belonging *Alternaria alternata, Aspergillus niger, Aspergillus* sp., *Curvularia lunata, Cladosporium cladosporioides, Chaetomium* sp., *Phomopsis* sp.1, *Macrophomina phaseolina, Nigrospora sphaerica, Penicillium* sp., *Paecilomyces lilacinus, Fusarium oxysporum, Phomopsis* sp. 2 and *Rhizoctonia* sp. were isolated from soybean (*Glycine max* (L.) Merril) and were screened *in vitro* for the antagonistic activity against charcoal rot of soybean caused by *M. phaseolina*. In this fungal endophytic antagonists tested, *Paecilomyces lilacinus* was found most effective with highest mycelial growth inhibition (61.11%), followed by *Aspergillus niger* and *Penicillium* sp. with of 53.87% and 51.48% mycelial inhibition. These endophytes thus; could be efficient biological control agent in sustainable crop production and offer unique opportunity for crop protection and biological control.

Keywords: Endophytic fungi, antagonistic activity, bio-control agents

Introduction
Plants may be considered complex micro ecosystems where, different niches are exploited by a wide variety of microbes. Such niches include not only the external surfaces of plants, but also, the internal tissues which, endophytic microbe inhabit without apparent harm to the host or external structures (Azevedo *et al*., 2000) [4]. Microbial endophytes are typically defined as plant associated microbes that colonize living internal tissues of plants without causing any visible symptoms or immediate over-negative effects and can be isolated from surface disinfected plant tissue (Wilson, 1995; Zinniel *et al*., 2002; Hung and Annapurna, 2004) [29, 30, 15]. Endophytic microbes include bacteria, actinomycetes and fungi are ubiquitous in most plant species. Endophytes exist in a range of tissue types within a broad range of plants, colonizing the plant systemically, residing latently in intercellular spaces, inside the vascular tissue or within cells (Khan and Doty, 2009) [16]. Relatively steady internal environment inside the plant tissues makes endophytes more bioactive than the rhizospheric or others plant associated microorganisms (He *et al*., 2009) [14]. Endophyte-plant associations have been found to improve plant health and may help host plant to rescue from various biotic and abiotic stresses (Hasegawa *et al*., 2006) [12]. They may also provide fitness benefits to host plants such as tolerance to herbivory, heat, salt, disease and drought and increased below and aboveground biomass etc. (Faeth and Fagan, 2002; Backman and Sikora, 2008) [11, 5]. Thus, endophytic colonization improves the ecological adaptability of the host. Hence, endophytes may be regarded as a true companion of host.
Biological control has been described as a non-hazardous strategy to reduce crop damage caused by plant pathogens when compared to the chemical control of plant diseases (Wang *et al*., 2010) [27]. A major factor influencing plant growth and health is the microbial population living both in the rhizosphere and as endophytes within healthy plant tissue. Endophytes might interact more closely with the host plant and therefore, could be efficient biological control agent in sustainable crop production and offer unique opportunity for crop protection and biological control (Melnick *et al*., 2008) [20].

Hence, with the view of plant health and productivity the proposed studies with special reference to endophytic microbes for soybean crop cultivar JS-335, as model phytosystem, have been initiated.

Materials and methods

Isolation and identification of endophytic fungi

All the sterilized / disinfected segments of each plant parts (stem, roots and leaves) were placed on Potato dextrose agar (PDA) medium supplemented with streptocycline (50µg/ml) to inhibit bacterial growth. Plates were sealed with parafilm to prevent desiccation of the medium and incubated in BOD incubator at 27^0C for 6 to 7 days. The fungal growth was continuously observed. As soon as growth was observed, the hyphal tips were transferred to fresh PDA medium to enhance typical sporulation for better identification. Pure cultures were preserved on PDA slant maintained at 80^0C with proper tags.

Cultures on PDA media were evaluated according to their morphology, mycelium colour, colony appearance and structure, shape of conidiophore and conidia (shape, color, etc.) and characters of conidiogenous cells were observed using a stereo-binocular microscope with 5X, 10X, 40X and 100X objective lenses for magnification. Also, lactophenol or lactophenol blue stains were used to study the characteristics of spores (Barnett and Hunter, 1998; Sutton, 1980) [6, 26]. Also, authoritative monographs and other taxonomic papers analogous to certain genera and species of endophytes were referred for identification of isolated endophytic fungi. Therefore, isolates were identified on the basis of morpho-cultural and microscopic characteristics and for isolates, those would not identified at Department of Plant Pathology, College of Agriculture, Latur, that were identified and confirmed by Division of Mycology, I.T.C.C., IARI, New Delhi. The identified fungal isolates were used for further studies.

Fungal test pathogen

Endophytic fungi were screened for their *in vitro* antagonistic activity against the isolated fungal pathogen of soybean (*M. phaseolina*). The isolated plant pathogen *M. phaseolina* was used as test pathogen. Stock cultures of the test pathogen were maintained on potato dextrose agar (PDA) at 4°C. Working cultures were established by transferring a stock agar plug containing the mycelium of each isolate onto PDA and incubated at 28 ± 2 °C for 7 days.

In vitro evaluation of endophytic fungi against *M. phaseolina*

Isolated endophytic fungi were screened for their antagonistic activity against test pathogen. Stock cultures of each isolate were maintained on PDA at 4°C. The assay for antagonism was performed on PDA by dual culture method (Dennis and Webster, 1971) [10]. A 5 mm of mycelial agar disc from test pathogen culture and endophytic test fungal disc were placed on sterilized PDA plate opposite to each other away from 5 cm and incubated for 7 days at 28 ± 2 °C. PDA plates inoculated only with test pathogens were maintained as control. The experiment was performed in three replications.

Experimental details

Design : C.R.D (Completely Randomised Design)
Replications : Three
Treatments : Fourteen

Table 1: Treatments details

Tr. No.	Treatments	Tr. No.	Treatments
T_1	*Alternaria alternata*	T_9	*Nigrospora sphaerica*
T_2	*Aspergillus niger*	T_{10}	*Penicillium* sp.
T_3	*Aspergillus* sp.	T_{11}	*Paecilomyces lilacinus*
T_4	*Curvularia lunata*	T_{12}	*Fusarium oxysporum*
T_5	*Cladosporium cladosporioides*	T_{13}	*Phomopsis* sp. 2
T_6	*Chaetomium* sp.	T_{14}	*Rhizoctonia* sp.
T_7	*Phomopsis* sp. 1	T_{15}	Control (untreated)
T_8	*Macrophomina phaseolina*		--

Analysis of antagonistic activity

Determination of per cent growth inhibition

Observations on linear mycelial growth of the test pathogen and test fungal endophytes were recorded at an interval of one day, continued till untreated control plates were fully covered with mycelial growth of the test pathogen and then, averaged finally % inhibition of the test pathogen with the test fungal endophytes, over untreated control was calculated by applying following formula, (Arora and Upaddhyay, 1978) [3].

$$PGI(I) = \frac{\text{Colony growth in control plate} - \text{Colony growth in intersecting plate}}{\text{Colony growth in control plate}} \times 100$$

Results and discussion

Isolation and identification

A total of 14 endophytic fungi isolated from soybean plant samples (leaves, stems and roots), five isolates from leaves, five isolates from stems and four isolates from roots were obtained. Amongst them isolated from the leaves were *Curvularia lunata*, *Cladosporium cladosporioides*, *Nigrospora sphaerica*, *Penicillium* sp. and *Paecilomyces lilacinus* and from stems were *Alternaria alternata*, *Phomopsis* sp. 1, *Rhizoctonia* sp., *Phomopsis* sp. 2 and *Macrophomina phaseolina*. From root were *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus* sp. and *Chaetomium* sp., respectively.

Isolated fungal strains such as *Aspergillus niger*, *Aspergillus* sp., *Fusarium oxysporum*, *Chaetomium* sp., *Curvularia lunata*, *Cladosporium cladosporioides*, *Penicillium* sp. and *Alternaria alternata*, were identified at Department of Plant Pathology, College of Agriculture, Latur by observing morpho-cultural and microscopic characteristics such as colony appearance, mycelium color and structure, shape of conidia and conidiophore (color, shape, etc.) and characters of conidiogenous cells using stereo-binocular microscope. Also its authoritative monographs and other taxonomic papers relating to particular genera as well as species of endophytes

were referred for identification of fungal endophytes. Fungal isolates such as *Nigrospora sphaerica*, *Paecilomyces lilacinus*, *Phomopsis* sp. 1, *Rhizoctonia* sp., *Phomopsis* sp. 2 and *Macrophomina phaseolina* were identified and confirmed by Division of Mycology, I.T.C.C., IARI, New Delhi. The identified fungal isolates were used for further studies.

These results were in conformity with several earlier workers (Barnett and Hunter, 1998; Sutton, 1980; Miller and Roy, 1982; Halnin, 1998; Piemental *et al*., 2006; Seifert *et al*., 2011; Kulakarni and Dalal, 2012; Anitha *et al*., 2013; Dalal *et al*., 2014; Pieterase *et al*., 2018) [6, 26, 21, 13, 22, 24, 8, 2, 18, 23].

In vitro evaluation of endophytic fungi against *M. phaseolina*

A total of 14 endophytic fungi were screened for their antagonistic potentials against charcoal rot pathogen of soybean. The antagonistic effectiveness of the fungal isolates varied from poor to moderate to strong. Results from the dual culture assay showed that the fungal pathogen showed promising ability to inhibit the mycelial growth of *M. phaseolina.*

Results (Table 2) revealed that, all the fungal endophytes evaluated, exhibited antifungal activity against *M. phaseolina* and significantly inhibited its growth over untreated control. *Paecilomyces lilacinus* was found most effective with highest mycelial growth inhibition (61.11%) of the test pathogen. The second and third best antagonists were *Aspergillus niger* (53.87%) and *Penicillium* sp. (51.48%). These were followed by *Phomopsis* sp. 2 (49.75%), *Curvularia lunata* (47.41%), *Nigrospora sphaerica* (46.05%), *Fusarium oxysporum* (44.94%), *Aspergillus* sp. (44.82), *Chaetomium* sp. (44.57), *Macrophomina phaseolina* (43.58%), *Phomopsis* sp.1 (42.96%), *Alternaria alternata* (41.61%), then least growth inhibition were seen in *Cladosporium cladosporioides* (41.00%) and *Rhizoctonia* sp. (40.86%). Thus, the fungal endophytes *viz*., *P. lilacinus*, A. *niger*, *P*. sp., *Phomopsis* sp. 2, *Curvularia lunata*, *Nigrospora sphaerica* and *Fusarium oxysporum* were found most potential fungal endophyte antagonists against *M. phaseolina*. Thus, in the order of merit, the most potential fungal endophytic (bio-control agents) as antagonist against *M. phaseolina* were *Paecilomyces lilacinus* > *Aspergillus niger* > *Penicillium* sp. > *Phomopsis* sp. 2 > *Curvularia lunata* > *Nigrospora sphaerica* > *Fusarium oxysporum* > *Aspergillus* sp. > *Chaetomium* sp. > *Macrophomina phaseolina* > *Phomopsis* sp.1 > *Alternaria alternata* > *Cladosporium cladosporioides* > *Rhizoctonia* sp.

Plate 1: *In vitro* efficacy of endophytic fungi of soybean against *M. phaseolina*

Table 2: *In vitro* anatagonistic activity of endophytic fungi against *M. phaseolina*

Tr No.	Treatments	Colony diameter* (mm) of pathogen	% inhibition*
T_1	*Alternaria alternata*	52.55	41.61 (40.17)
T_2	*Aspergillus niger*	41.51	53.87 (47.21)
T_3	*Aspergillus* sp.	49.66	44.82 (42.02)
T_4	*Curvularia lunata*	47.33	47.41 (43.51)
T_5	*Cladosporium cladosporioides*	53.10	41.00 (39.81)
T_6	*Chaetomium* sp.	49.88	44.57 (41.88)
T_7	*Phomopsis* sp.1	51.33	42.96 (40.95)
T_8	*Macrophomina phaseolina*	50.77	43.58 (41.31)
T_9	*Nigrospora sphaerica*	48.55	46.05 (42.73)
T_{10}	*Penicillium* sp.	43.66	51.48 (45.84)
T_{11}	*Paecilomyces lilacinus*	35.00	61.11 (51.41)
T_{12}	*Fusarium oxysporum*	49.55	44.94 (42.09)
T_{13}	*Phomopsis* sp. 2	45.22	49.75 (44.85)
T_{14}	*Rhizoctonia* sp.	53.22	40.86 (39.73)
T_{15}	Control (Untreated)	90.00	00.00 (00.00)
	SE±	0.62	0.50
	CD (P=0.01)	1.82	1.50

Our findings are in congruence with several reports in various crop plants (Lahlali and Hijri, 2010; Kumar and Kaushik, 2013; Dalal *et al*., 2014; Deepa and Sally, 2015; Sreeja, 2016; Ankita *et al*., 2018; Zuhria *et al*., 2016; Brunda *et al*., 2018; Wati *et al*., 2019) [19, 17, 18, 9, 25, 1, 31, 7, 28].

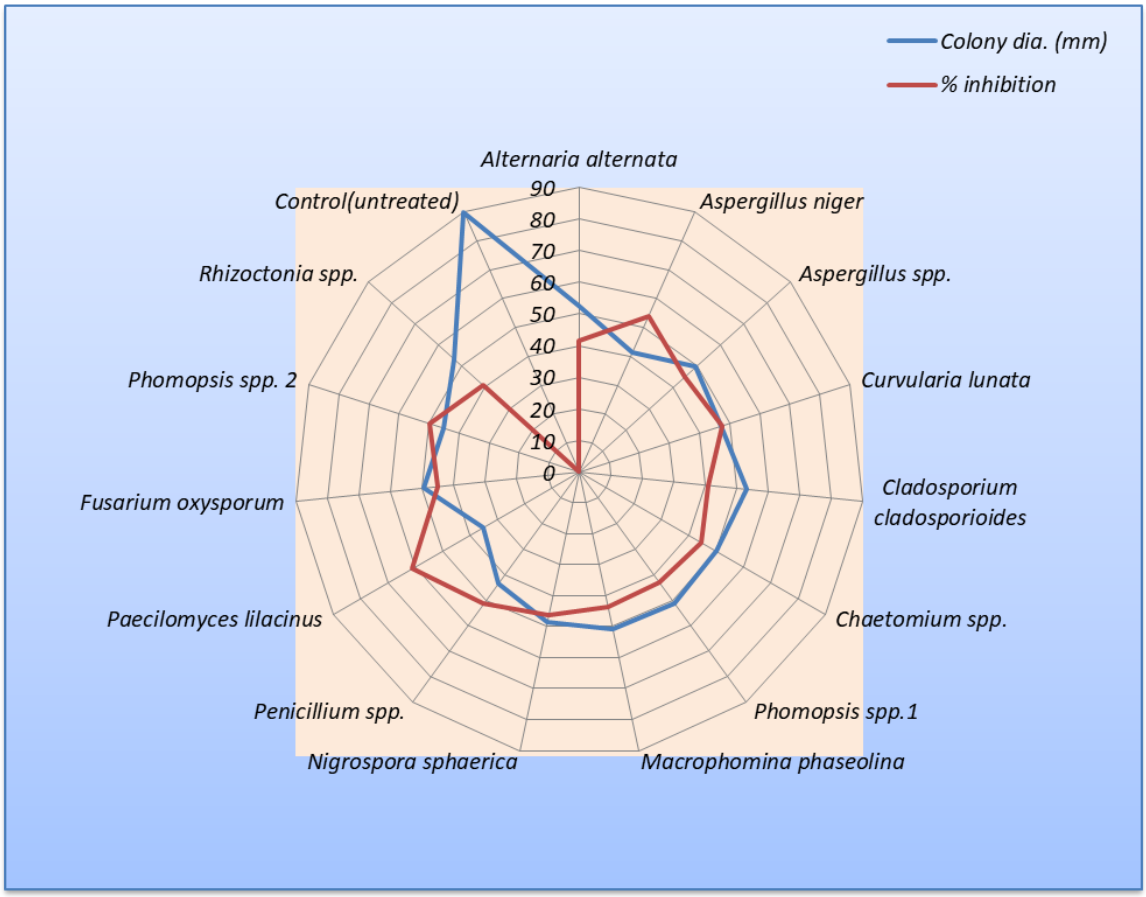

Fig 1: *In vitro* efficacy of endophytic fungi against *M. phaseolina*, causing charcoal rot of soybean

Conclusion

Endophytes are potential bio-control agents as all of the fourteen fungal endophytes evaluated *in vitro* were proved potential antagonists against *M. phaseolina*. However, *Paecilomyces lilacinus* followed by *Aspergillus niger*, *Penicillium* sp., *Phomopsis* sp. 2, *Curvularia lunata*, *Nigrospora sphaerica* and *Fusarium oxysporum* were most effectual.

Endophytic strains as they possible dual ability of antagonizing fungal pathogen and plant growth promotion; with the view of plant health and productivity. Thus, the antifungal or fungistatic action exhibited by the fungal endophyte antagonists against *M. phaseolina*, causing charcoal rot of soybean, may be attributed to the various mechanisms such as competition, hyper parasitism, antibiosis, production of various secondary metabolites and production of pectolytic enzymes, by the antagonistic fungal endophytic micro-organisms. Therefore, these promising endophytes may be commercially formulated effective bio-control agents for the management of soil-borne fungal pathogens of soybean.

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