Effect of fungal endophytes of soybean (Cv. JS-335) on growth parameters and charcoal rot disease incidence of soybean

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Abstract
Use of chemicals for growth enhancement and disease control in plants has resulted in hazardous influences to the environment and human health. Therefore, less harmful methods should be implemented and the possibility of using microbes for this purpose has been investigated. Endophytic fungal assemblages have been known to enhance plant growth and decrease disease incidence in some crops including soybean and thus can be used as an alternative to chemicals. A total seven effective fungal endophytes in vitro, which were evaluated to assess their efficacy against M. phaseolina, by sick soil method, in polybags, under screen house condition, they also influenced on growth parameter of soybean (Cv. JS-335), thereby improving seed germination, root length, shoot length and seedling vigour index (SVI) in soybean. In the present study, the results were obtained on per cent mortality (pre-, post emergence and average) and reductions, over untreated control. Fungal endophytes viz., Paecilomyces lilacinus, Aspergillus niger, Fusarium oxysporum and Penicillium sp. were found most effective in reducing the mortality (PRES, POESM and average) over untreated control, against M. phaseolina in soybean.

Keywords: Seed germination, Root length, Shoot length, Seedling vigour index (SVI)

Introduction
Soybean (Glycine max (L.) Merril) is one of the most important leguminous and extensively used as oilseed crop, grown in India. Among many soybean growing countries, India ranks fifth in respect of production and the major states growing soybean are viz., Madhya Pradesh, Maharashtra, Rajasthan, Andhra Pradesh and Karnataka. However, the production and productivity of soybean has been reducing day by day, for which diseases caused by fungi, bacteria, viruses and phytoplasma’s, etc. are responsible.

Among varied fungal diseases infecting soybean, root rot / charcoal rot / stem rot caused by Macrophomina phaseolina, is one of the most devastating fungal disease, affecting the crop at all stages of crop growth but generally, it infects at post flowering stage. This disease of soybean has been reported to cause 70 per cent yield losses in India (Kumar et al., 2019) [11]. The overall average root rot incidence of soybean was 26.16 per cent during kharif, 2017-18 in the Marathwada region of Maharashtra state (Agale et al., 2018) [2]. When temperature is between 28o C - 32o C, the disease cycle for M. phaseolina begins with germination of microsclerotia. Usually, symptoms in adult plants are brown and red lesions on roots and stem and produces dark mycelia and black microsclerotia. The stem shows longitudinal dark lesions and plant becomes defoliated and wilted.

Nearly all plants that existing in the World were found to be related with endophytes. The term “endophyte” is derived from the Greek word “Endon” means within and “Phyte” means plant. This term was first used by Anton De Bary in 1866. Endophytes are the microbe that is residing in plant tissues without causing harmful effects to the host plants. They are everywhere in most plant species, especially in field grown plants and found in mainly all vascular plant species examined and includes fungi, bacteria and actinomycetes. Under natural conditions, endophytes promote plant growth by using various mechanisms. These include phosphate solubilization activity, indole acetic acid (IAA) production and therefore the production of a siderophore. A kind of other beneficial effects on growth of the plants are attributed to endophytes and that includes, the stomatal regulation, osmotic adjustment,
modification of root morphology, enhanced intake of minerals and changes of nitrogen accumulation and also metabolism (Compant et al., 2005) [3]. Endophytes have the capacity to produce antibiotics, that act as antifungal, antibacterial, antiviral and insecticidal properties, which strongly inhibit growth of other microorganisms, including plant pathogens. So, endophytic fungal assemblages have been known to increase plant growth and reduce disease incidence in some crops including soybean and thus, can be used as an alternative to chemicals. Therefore, this study was subjected to the endophytic fungal communities associated with soybean crop with a view to examine the possibility of using them for plant growth enhancement and management of charcoal rot disease incidence.

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 streptomycin (50µg/ml) to inhibit bacterial growth. Plates were sealed with paraffin to prevent desiccation of the medium and incubated in BOD incubator at 27°C 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°C 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) [4,20]. 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 morphocultural 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.

Evaluation of fungal endophytes (pot culture)
Seven effective fungal endophytes were thus selected based on results of in vitro experiment and used for further studies. The effective fungal endophytes were tested against charcoal rot disease causing, M. phaseolina, in pot culture experiment, under screenhouse conditions. Sand: maize medium was used for mass multiplication of M. phaseolina. Sand: maize medium (3 part partially broken grain + one-part sand + distilled water to moisten the medium) was prepared, filled into polypropylene bags (9 x 12 cm) and autoclaved at 20 lbs pressure for 30 min, for two consecutive days. After autoclaving cooled at room temperature, then sand: maize medium in the bags were inoculated with 8 - 10 mycelial discs (5 mm dia.) obtained from 6 to 7 days old pure culture of M. phaseolina and inoculated at room temperature, for 14 to 15 days. Black colored nursery polybags (size 20x30 cm) filled with the autoclaved potting mixture of soil : sand (3:1) was inoculated (each @ 50g / Kg mixture) with the test pathogen (M. phaseolina) mass multiplied (sand : maize medium) culture, watered lightly and incubated in screenhouse for two weeks, so as to proliferate the pathogen and made the potting mixture sick. After 14 to 15 days of incubation, these polybags were sown with soybean seeds, which were sterilized using (2 - 3% sodium hypochlorite solution, for 5 min.), soaked in the potato dextrose broth of mass multiplied with each effective endophytic fungi for 10 to 12 hours. Then, such seeds were sown (10 seeds / bag), watered regularly and maintained in the screenhouse. In the control treatment, seeds submersion was performed using sterilized distilled water was maintained as untreated control.

Experimental details
Design : C.R.D (Comletely Randomised Design)
Replications : Four
Treatments : Eight

| Tr. No. | Treatments | Tr. No. | Treatments |
|---------|------------|---------|------------|
| T1 | Aspergillus niger | T4 | Fusarium oxysporum |
| T2 | Curvularia lunata | T5 | Penicillum sp. |
| T3 | Nigrospora sphaerica | T6 | Phomopsis sp. 2 |
| T7 | Fusarium oxysporum | T8 | Control (untreated) |

Observations on seed germination, pre-emergence seed rot (PRESR) and post-emergence seedling mortality (POESM) were recorded, respectively at seven days and thirty days after sowing and average mortality was calculated. Per cent seed germination, PRESR, POESM and average mortality were enumerated by following formulae.

Germination (%) = \( \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}} \times 100 \)

% Pre-emergence mortality = \( \frac{\text{No. of seeds un-germinated}}{\text{Total no. of seeds sown}} \times 100 \)

% Post-emergence mortality = \( \frac{\text{No. of seedlings died}}{\text{Total no. of seedlings emerged}} \times 100 \)

% Av. mortality = \( \frac{\text{PRESR} + \text{POESM}}{2} \)

Further, per cent disease control / reduction over untreated control was calculated by applying following formula

% Disease control = \( \frac{\text{C} - \text{T}}{\text{C}} \times 100 \)

Where, C = per cent average mortality (pre- and post emergence) in treatment polybags.

T = per cent average mortality (pre- and post emergence) in untreated control polybags.

Observations on root length and shoot length (cm) were noted at 30 days after sowing. For the purpose, five soybean seedlings / treatment / replication were uprooted gently, washed under gentle flow of tap water and air dried. Root length was measured from collar region to tip of the tap / main root and shoot length from collar region to tip of the seedling / plant. Also, seedling vigour index (SVI) was calculated by following formula.

SVI = per cent seed germination x (Root length + shoot length in cm)
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 Carvalaria 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 Macrophoma phaseolina. From roots were Fusarium oxysporum, Aspergillus niger, Aspergillus sp. and Chaetomium sp., respectively. Isolated fungal strains such as Aspergillus niger, Aspergillus sp., Fusarium oxysporum, Chaetomium sp., Carvalaria 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 Macrophoma 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 (Sutton, 1980; Miller and Roy, 1982; Piemental et al., 2006; Seifert et al., 2011; Anitha et al., 2013; Dalal et al., 2014) [20, 13, 14, 17, 3, 6].

Efficacy of endophytic fungi (pot culture)
A total seven effective fungal endophytes in vitro were evaluated as described earlier in materials and methodology to assess their efficacy against M. phaseolina, by sick soil method, in polybags, under screen house condition. The results were obtained for per cent mortality (pre-, post emergence and average) and reductions, over untreated control are presented in Table 1 and represented in PLATE 1 and fig. 1.

Pre-emergence seed rot (PRESR)
The results (PLATE 1, Table 1 and Fig. 1a) revealed that all of the test fungal endophytes significantly influenced pre-emergence seed rot (PRESR) which were ranged from 10.00 to 21.65 per cent, as against 40.00 per cent in untreated control. However, it was significantly least with Paecilomyces lilacinus (10.00 %), followed by Aspergillus niger (12.00 %), Fusarium oxysporum (15.00 %) and Penicillium sp. (16.00 %), respectively. Rest of the fungal endophytes resulted with comparatively maximum PRESR in the range of 20.00 to 21.65 per cent, but it was significantly highest in untreated control (40.00 %).

Post-emergence seedling mortality (POESM)
Similar trend as that of pre-emergence seed rot (PRESR) was observed in respect of post-emergence seedling mortality (POESM), which ranged from 12.40 to 25.00 per cent, as against 44.55 per cent in untreated control. However, it was significantly least with Paecilomyces lilacinus (12.40 %), followed by Aspergillus niger (15.65 %), Fusarium oxysporum (18.32 %) and Penicillium sp. (20.64 %), respectively. Rest of the fungal endophytes resulted with comparatively maximum POESM in the range of 24.18 to 25.00 per cent, but it was significantly highest in untreated control (44.55 %).

Table 1: Effect of fungal endophytes on mortality caused by M. phaseolina in soybean

| Tr. No. | Treatments            | Seed Germination* (%) | Rot/ mortality (%)* | Av. Mort. (%) | (% Reduction* | Av. Redn. (%) |
|---------|-----------------------|-----------------------|---------------------|---------------|---------------|---------------|
|         |                       |                       | PRESR               | POESM         | PRESR         | POESM         |
| T<sub>1</sub> | Aspergillus niger     | 88.00 (69.73)         | 12.00 (20.26)       | 15.65 (23.30) | 13.82 (21.82) | 70.00 (56.78) | 64.87 (53.65) | 67.43 (55.20) |
| T<sub>2</sub> | Curvularia lunata     | 79.66 (63.19)         | 20.34 (26.80)       | 24.18 (29.45) | 22.26 (28.15) | 49.15 (44.51) | 45.72 (42.54) | 47.43 (43.52) |
| T<sub>3</sub> | Nigrospora sphaerica  | 78.35 (62.27)         | 21.65 (27.72)       | 24.89 (29.92) | 23.27 (28.84) | 45.87 (42.63) | 44.13 (41.62) | 45.00 (42.13) |
| T<sub>4</sub> | Fusarium oxysporum    | 85.00 (67.21)         | 15.00 (22.78)       | 18.32 (25.34) | 16.66 (24.08) | 62.50 (52.23) | 58.87 (50.10) | 60.68 (51.16) |
| T<sub>5</sub> | Paecilomyces lilacinus| 90.00 (71.56)         | 10.00 (18.43)       | 12.40 (20.61) | 11.20 (19.55) | 75.00 (60.00) | 72.16 (58.15) | 73.58 (59.06) |
| T<sub>6</sub> | Penicillium sp.       | 84.00 (66.42)         | 16.00 (23.57)       | 20.64 (27.02) | 18.32 (25.34) | 60.00 (50.76) | 53.67 (47.10) | 56.83 (48.92) |
| T<sub>7</sub> | Phomopsis sp. 2       | 80.00 (63.43)         | 20.00 (26.56)       | 25.00 (30.00) | 22.30 (28.31) | 50.00 (45.00) | 43.88 (41.48) | 46.94 (43.24) |
| T<sub>8</sub> | Control               | 60.00 (50.76)         | 40.00 (39.23)       | 44.55 (41.87) | 42.27 (40.55) | 0.00 (0.00)  | 0.00 (0.00)   | 0.00 (0.00)   |
| SE ±     | 0.84                  | 0.79                  | 0.81                | -             | 0.74 (0.64)  | -             | -             |
| CD (P=0.01) | 2.46                  | 2.34                  | 2.39                | -             | 2.18 (1.89)  | -             | -             |

Average mortality influenced by the test fungal endophytes was ranged from 11.20 to 23.27 per cent. However, it was significantly least with Paecilomyces lilacinus (11.20 %), followed by Aspergillus niger (13.82 %), Fusarium oxysporum (16.66 %) and Penicillium sp. (18.32 %). Rest of the test fungal endophytes recorded average mortality in the range of 22.26 to 23.27 per cent, but it was significantly highest in untreated control (42.27 %).

Reduction in mortality (pre-, post- and average mortality) All of the fungal endophytes tested (Table 1 and Fig. 1b) were found effective in reducing pre-emergence, post-emergence and average mortality, over untreated control. However, the most effective fungal antagonistic endophyte found was Paecilomyces lilacinus, with significantly highest reduction in PRESR (75.00 %), POESM (72.16 %) and average mortality reduction (73.58 %). This was followed by Aspergillus niger (70.00 %, 64.87 % and 67.43 %, respectively), Fusarium

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oxysporum (62.50 %, 58.87 % and 60.68 %, respectively) and Penicillium sp. (60.00 %, 53.67 % and 56.83 %, respectively). Rest of the test fungal endophytes recorded reductions of PRESR in the range of 45.87 to 50.00 %, POESM in the R. solanacearum, 065.92, respectively), Aspergillus niger, eters of soybean. Whereas, A. niger, o, (2019) - (2018) reported the e there found most effective in reducing the e) under pot culture. were reported earlier by several workers. Lahlali and Hijri dase, including charcoal / root / stem rots in many crops were reported earlier by several workers. Lahlali and Hijri (2010) \([12]\) reported effectiveness of endophytic fungi Trichoderma atroviride against Rhizoctonia solani, in potato. Deepa and Sally (2015) \([8]\) evaluated and reported in vivo efficacy of seventeen endophytic fungi isolated from tomato against bacterial wilt (R. solanacearum) under pot culture. Urooj et al. (2018) \([6]\) reported the effect of endophytic Penicillium species on root rotting fungi on sunflower in earthen pots in screenhouse and reported that, P. lividum alone showed no infection of M. phaseolina on sunflower roots. Zuhria et al. (2018) \([23]\) evaluated in vivo efficacy of 15 species of endophytic fungi from soybean against S. rolfsii and reported that, Trichoderma sp. showed lowest disease incidence (21.25%). Priyadarshini et al. (2018) reported the effectiveness of endophytic fungi such as Trichoderma sp.1 and Chaetomium sp. of rice against Bipolaris oryzae. Sudarma et al. (2019) \([19]\) also evaluated in vivo efficacy six exophytic and endophytic fungi isolated from sugar apple against Lasiodiplodia theobromae and reported highest inhibition ability in endophytic fungi of leaves 4 (Aspergillus sp.) and fruit exophytic 5 (A. niger) with 0% infection. Wati et al. (2019) \([22]\) evaluated in vivo efficacy of five endophytic fungi isolated from Artemisia annua L. against R. solani and reported the highest inhibition ability in Phoma sp.

Effect of fungal endophytes on growth parameters
Results (Table 2) revealed that, all of the fungal endophytes tested for the management of M. phaseolina (polybag culture) also influenced on growth parameters of soybean (Cv. JS-335), thereby improving seed germination, root length, shoot length and seedling vigour index (SVI) in soybean. However, the Paecilomyces lilacinus resulted with significantly highest seed germination (90.00 %), root length (12.59 cm), shoot length (23.12 cm) and SVI (3213.9). This was followed by Aspergillus niger (88 %, 12.35 cm, 22.49 cm and 3065.92, respectively), Fusarium oxysporum (85 %, 11.54 cm, 21.56 cm and 2813.5, respectively) and Penicillium sp. (84 %, 11.90 cm, 19.28 cm and 2619.12, respectively). Rest of the fungal endophytes, were also found effective in improving plant growth parameters of soybean. Whereas, untreated control showed significantly minimum seed germination (60.00%), root length (08.45 cm), shoot length (14.49 cm) and (SVI) (1736.4).

Similar results regarding effect of fungal endophytes on growth parameters in many crops were reported earlier by several workers. (Senthilkumar et al., 2009; Urooj et al., 2018; Priyadarshani et al., 2018; Abro et al., 2019) \([18,8,16,1]\).
Conclusion
Fungal endophytes were found effective to improve seed germination and growth parameters in polybag culture and significantly reduced pre-, post-emergence and average mortality, caused by *M. phaseolina* in soybean. However, *Paecilomyces lilacinus, Aspergillus niger, Fusarium oxysporum* and *Penicillium* sp. were found most promising.

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