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SEEDS ENDOPHYTIC FUNGI OF SOME PLANT SPECIES AND THEIR POTENTIAL FOR PRODUCING INDOLE ACETIC ACID (IAA)

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ABSTRACT: In the present investigation endophytic fungi associated with seeds of plant species was studied and screened for their potential for IIAA production. Altogether 162 endophytic fungi belonging to 14 genera were obtained from the seeds of seven plant species. The dominant endophytic fungal genera isolated from the seeds were *Fusarium, Colletotrichum, Curvularia, Penicillium* and fungi belonging to Mycelia Sterilia. Among the fungal genera colonization frequency of *Fusarium* were found to be highest in all the studied seeds and maximum colonization (%) was observed in the seeds of *Cassia alata* followed by non-sporulating fungi (Mycelia Sterilia). All the endophytic fungal isolates were determined for IAA production. Out of 162 isolates, 47 of them showed positive IAA production. Among the positive isolates four *Fusarium* species showed promising IAA production. Enhanced IAA production was observed when the isolates were cultured in Yeast Extract Broth medium at neutral pH, 7 days incubation period and incubation temperature of 35°C. Further, addition of 0.3% L- tryptophan in the culture medium supplemented with carbon and nitrogen sources increased IAA production *in-vitro*.

KEYWORDS: Seed endophytic fungi, *Fusarium* species, IAA production, L- tryptophan.

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1.INTRODUCTION

Endophytic fungi are microbes colonizing inner plant tissues without causing any immediate negative effects [1]. They can live throughout or a part of a life cycle without causing any damage or disease and are transmitted to the next generation, through tissues of hosts, seeds, vegetative propagules or through spores carried by air, insect or small animals [2]. Endophytes colonized

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virtually in all organs of a given host, some are seed-borne and transmitted through seeds from maternal plant to offspring and such transmission is also called vertical transmission [3, 4]. Clay and Schardl have suggested that the endophytic fungi reside vigorously in the seeds of plant of family Poaceae and Convolvulaceae [5]. Besides, several fungal endophytes have also been reported from seeds of many plant species [6, 7, and 8]. Endophytes may produce a plethora of bioactive metabolites that may be involved in the host-endophyte relationship [9], and may serve as potential sources of novel natural products for exploitation in medicine, agriculture, and industry [10,11]. The endophytic fungi also promote the growth of plants in various ways by secretion of plant growth regulators or phytohormones like cytokines, auxins etc. [12]. The auxins are the group of phytohormones having indole ring compounds and Indole Acetic Acid (IAA) represents one of the most important plant hormones, regulating many aspects of plant growth and development throughout the plant cell cycle, from cell division, cell elongation and differentiation to root initiation, apical dominance, tropistic responses, flowering, fruit ripening [13]. Several endophytes have been reported to produce growth hormone like IAA and Gibberellic Acid and promote the growth of the tissue cultured banana [14, 15]. However, seed borne endophytic fungi and their plant growth promotion ability has not been fully investigated. Therefore the objectives of our present study were to study the diversity of endophytic fungi occurring in seeds of some plant species and their potentials as producer of IAA.

2. MATERIALS AND METHODS

Collection of samples

The study was conducted in Similipal Biosphere Reserve in state of Odisha, India. Healthy seeds were collected from seven plant species namely *Cassia alata, Pongamia pinnata, Semicarpus anacardium, Zingiber roseum***,** *Desmodium pulchellum, Morinda citrifolia* and *Rauwolvia tetraphylla*. The collected samples were brought to the laboratory in sterile polythene bags and were stored at 4°C.

Isolation and identification of endophytic fungi

Seed coat/pods were removed, healthy seeds were selected and initially washed thoroughly with distilled water and shade dried. The dried seeds were taken for isolation of endophytic fungi following surface sterilization protocol. At first, the seeds were washed with sterile distilled water followed by dipping in 70% ethanol for 3 minutes. It was then transferred to solution containing 1% sodium hypochloride (NaOCl) for 1 minute. The seeds were then rinsed twice in sterile distilled water and soaked under sterile filter paper. The dried surface seeds were transferred to the Potato Dextrose Agar (PDA) plates. 10 seeds per plate from each plant species were inoculated under aseptic condition in laminar airflow chamber. Inoculated plates were then kept in BOD incubator at 30±1°C for a week. Fungi growing out of plated seeds were immediately transferred into freshly prepared PDA slant. The fungal isolates were identified by both morphological traits and

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microscopic observation referring standard identification manuals [16]

Determination for IAA production

The fungal isolates were cultured in freshly prepared Potato Dextrose broth (PDB) supplemented with L-tryptophan at concentrations of 5 g L^{-1}. The isolates were inoculated into the sterilized PDB medium and incubated in dark at 29° C for 7 days. After the incubation, the cultures were centrifuged at 3000 rpm to remove the fungal cells and the supernatants were collected for determination of IAA. IAA was estimated according to the procedure of Gordon and Paleg [17] with slight modification. Briefly, 25 ml of supernatant was collected in a 100 ml conical flask and the p^H was adjusted to 2.8 using 1N HCL. 25 ml of diethyl ether was added to it and incubated in dark for 4 hours. IAA extraction was done at 4° C in a separating funnel using Ethyl acetate. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, 2 ml of methanol was added. Again, 0.5 ml of methanol extract, to this 1.5 ml of double distilled water and 4 ml of Sapler's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % Perchloric acid) were added and incubated in dark for 1 hour. The intensity of pink colour developed was read at 535 nm in a UV-VIS spectrophotometer.

Condition optimization for IAA production

Selected endophytic fungi that showed IAA activity were further investigated to assess the effect of various cultural conditions on the production of IAA. The effect of L-tryptophan concentrations on IAA production was studied by inoculating the selected fungal isolates in PDB medium supplemented with L-tryptophan at concentrations of 0.1, 0.3, 0.5 and 0.7 % at pH 7. Effect of incubation time was studied by inoculation the isolates in PDB medium supplemented with 0.3% L-tryptophan at pH 7 and incubation for 9 days. Production of IAA was measured at every 48 hour intervals (3, 5, 7 and 9 days). Effect of pH was studied by inoculating the isolates at different pH range (3, 7 and 11). Effects of different media were studied by inoculating the isolates in PDB, Czapek- dox Broth (CDB), Malt Extract Broth (MEB) and Yeast Extract Broth (YEB) media. Effect of different carbon and nitrogen sources on IAA production were studied by growing the isolates in PDB medium containing L-tryptophan and supplemented with different concentration (0.1, 0.3 and 0.5 gm) of carbon sources (Sucrose, Starch and Fructose). Similarly, the medium was supplemented with different nitrogen sources (Sodium Nitrate, Calcium Nitrate and Potassium Nitrate) with different concentrations (0.1, 0.3 and 0.5 gm). In all cases the cultures were incubated in dark at 29 °C for 7 days (except for incubation time and heat shock study) and IAA was determined by the method as described above.

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3. RESULTS AND DISCUSSION

Isolation and identification of seed endophytes

In the present investigation endophytic fungi associated with seeds of seven plant species namely *C. alata, P. pinnata, S. anacardium, D. pulchellum*, *Z. roseum, M. Citrifolia* and *R. tetraphylla* were studied. Altogether 162 endophytic fungal isolates belonging to 14 genera were obtained from the seeds of seven plant species (Table 1). The result indicated that all the seeds were found colonized with endophytic fungi. However, colonization was found to be variable within the seeds of different plant species. The occurrence of endophytic fungi in seed and their role in plant growth and health has also been reported by many workers (Ernst et al., 2013; Schardl et al., 2004). This indicates that seed borne endophytic fungi are ubiquitous in nature. The seeds of *C. alata* were found colonized with fungi belonging to genus *Fusarium.* Altogether three species of *Fusarium* were obtained and those were *F. oxysporum, F. solani* and *Fusarium* sp. Among them, the colonization frequency of *F. oxysporum* was found to be highest (30%) followed by *F. solani* (11%) and *Fusarium* sp. with colonizing frequency of 7% (Table 1). The occurrence of *Fusarium* as endophytes has been reported from several plant species both wild and cultivated and in different plant parts [18, 19]. Such results suggest that the genus *Fusarium* is widely distribution in different lifestyle. Similarly, from the seeds of *P. pinnata*, *Colletotrichum* sp. and fungi belonging to sterile mycelia were obtained as endophytes with colonization frequencies of 2% and 8% respectively. It was very interesting to observe that seeds of *S. Anacadium* were found colonized only with sterile mycelia with colonization frequency of 17% while seeds of *Z. roseum* were found colonized with fungi belonging to various genera namely *Fusarium, Penicillium, Curvularia, Torula, Aspergillus, Mucor, Trichoderma* and *Pestiloptiosis* and sterile mycelia. Among them the colonization frequencies of *Curvularia* sp. and *Penicillium* sp. was found to be highest (6% each). Again, the seeds of *D. pulchellum* were found colonized with four different endophytic fungi, of which *Fusarium* sp. and *Colletotrichum* sp. showed maximum colonization frequency (Table 1). Similarly, seeds of *M. citrifrifolia* were found colonized with four different endophytic fungi namely *Bipolaris* sp., *Gliocladium* sp., *Colletotrichum* sp. and sterile mycelia and maximum colonization were showed by fungi belonging to sterile mycelia (10%). The seeds of *R. tetraphylla* also showed maximum colonization frequency by fungi belonging to sterile mycelia (14%) while the other fungi obtained as endophytes were *Fusarium* sp., *Penicillium* sp. and *Sporotrichum* sp. Among the plants, the seeds of *Z. roseum* harboured different fungi as endophytes while fungi of the genus *Fusarium* were host specific to seeds of *C. alata*. Further, seeds of *S. anacardium* were found colonized only with fungi belonging to sterile mycelia. Among the endophytes, colonization of sterile mycelia was found in seeds of all the plant species except *C. alata*. The occurrence of different fungal genera in studied plant seed samples suggests rich distribution and colonization of endophytes.

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Table 1: Occurrence of endophytic fungi isolated from the seeds of the selected plant species

Colonization Frequency (%)								
Endophytic fungi	Ca	Pa	Sa	Zr	Dp	Mc	Rt	Total
F. solani	11	--	--	--	--	--	--	1
F. oxysporum	30	--	--	--	--	--	--	30
Fusarium sp.	07	--	--	02	06	--	04	19
Nigrospora sp.	--	--	--	--	02	--	--	02
Colletotrichum sp.	--	02	--	--	06	04	--	12
Curvularia sp.	--	--	--	06	--	--	--	06
Aspergillus sp.	--	--	--	04	--	--	--	04
Penicillum sp.	--	--	--	06	--	--	02	08
Trichoderma sp.	--	--	--	02	--	--	--	02
Torula sp.	--	--	--	02	--	--	--	02
Mucor sp.	--	--	--	02	--	--	--	02
Gliocladium sp.	--	--	--	--	--	02	--	02
Sporotrichum sp.	--	--	--	--	--	--	02	02
Pestiloptiosis sp.	--	--	--	02	--	--	--	02
Bipolaris sp.	--	--	--	--	--	02	--	02
Sterile mycelia	--	08	17	02	05	10	14	56

*** Colonization frequency (CF %) was calculated as the number of seeds colonized by a specific fungus divided by total number of segments plated X 100. Total number of seeds plate was 100 for each plant species.** Ca = *C. alata*, Pa = *P. pinnata*, Sa = *S. annacardium* , Zr = *Z. roseum,* Dp = *D. pulchellum*, Mc = *M. citrifolia*, Rt = *R. tetraphylla*

Most of the fungal genera isolated as endophytes represent fungi belonging to plant pathogenic group. Therefore, existence of these fungi as endophytes is highly speculative in nature. Petrini proposed to extend the term 'endophyte' to account for those latent pathogens that can live asymptomatically in their hosts for some time in their life [20]. Therefore, when a fungus is isolated as an endophyte it does not exclude the possibility that it can be a weak pathogen or a virulent strain detected during quiescence.

Determination of IAA production and optimization

All the endophytic fungal isolates were determined for IAA production. Out of 162 isolates, 47 of them showed positive IAA production. In many instances endophytic fungi have been reported to produce plant growth promoting activities like ammonia and IAA production [21, 22]. The efficacy of the production was determined by observing the reaction mixture which turns into deep pink

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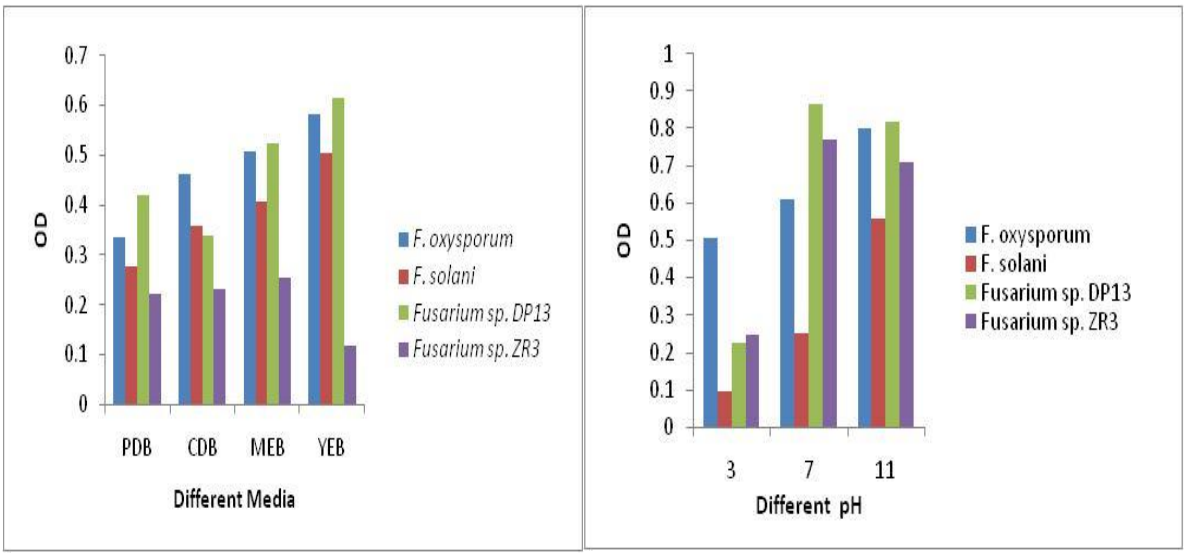
colour. Based on the colour intensity four isolates were selected for further study and optimization. The isolates used for further study were *F. oxysporum* and *F. solani* isolated from *C. alata* and *Fusarium* sp. DP13 and *Fusarium* sp. ZR3 isolated from *D. pulchellum* and *Z. roseum* respectively. The isolates were grown under different cultural conditions and their effects on IAA production were observed.

Effect of media, pH, incubation time and temperature

The selected isolates were grown in different liquid media namely, PDB, CDB, MEB and YEB and incubated in dark at 29° C for 7 days. After the incubation period the crude metabolites obtained in each case were determined for IAA. The result indicated that *Fusarium* sp. DP13showed maximum IAA production in YEB medium followed by *F. oxysporum* and *F.solani* while *Fusarium* sp. ZR3 showed the least production (Fig 1, A). Previous workers have also reported IAA production by endophytic *Fusarium* isolates [15, 22]. Effect of pH on IAA production was observed by growing the isolates in PDB medium at different pH (3, 7 and 11). The result indicated that *Fusarium* sp. DP13showed maximum production at pH 7 followed by *Fusarium* sp. ZR3. However, in comparison all the isolates showed increased IAA activity at pH 11 (Fig. 1, B). Again, maximum IAA activity was observed when the isolates grown in PDB medium were incubated for 7 days. Longer incubation beyond this period showed decreased IAA activity in some of the isolates (Fig. 1, C). Similarly, decreased IAA activity was observed when the isolates were incubated at higher temperature and maximum activity was observed when incubated at the temperature of 35° C. Among the isolates, *F. oxysporum* showed maximum IAA activity at 35° C followed by *F. solani*. All the isolates did not showed any IAA activity at 65° C (Fig. 1, D).



(A) (B)

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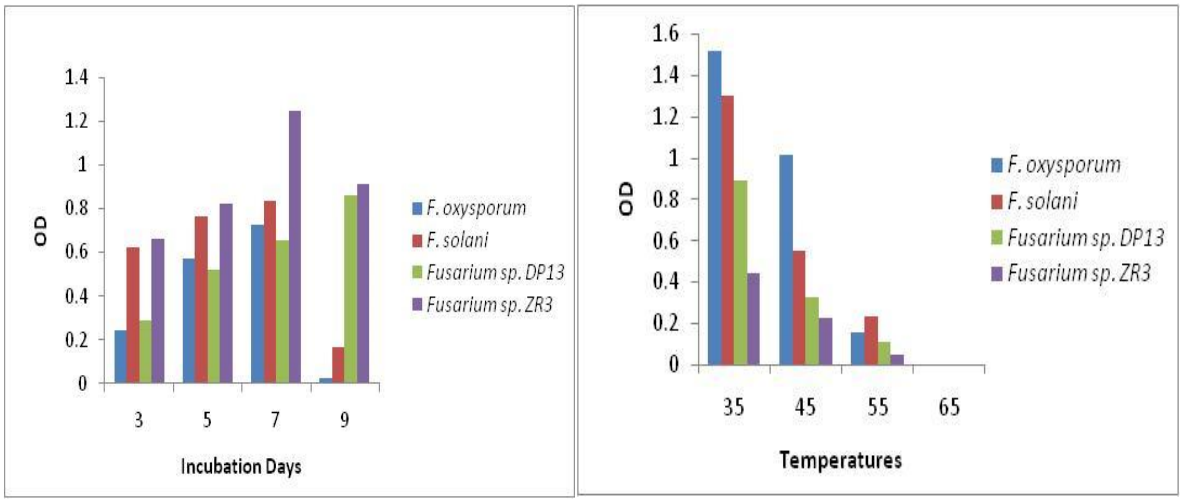

(C) (D)

Fig. 1: Effect of different cultural condition (A) Media (B) pH (C) Incubation time and (D) Temperature on IAA production by *F. oxysporum*, *F. solani*, *Fusarium* sp. (DP13) and *Fusarium* sp. (ZR3)

Effect of L- tryptophan, carbon and nitrogen sources on IAA production

Tryptophan is considered as a precursor for IAA biosynthesis and its addition of in culture medium has been reported to enhance IAA production. The isolates were grown in PDB medium supplemented with different concentrations of L- tryptophan (0.1, 0.3, 0.5 and 0.7 %) and the crude metabolites were evaluated for IAA production. The results indicated maximum IAA activity was observed at 0.3% tryptophan concentration thereafter it gradually decreases (Fig. 2, A). The finding collaborates with that of Khalid et al. (2004) who studied the effect of L-tryptophan concentration for the production of IAA and observed that Ltryptophan-derived auxin biosynthesis was enhanced several folds. Nutrients like carbon and nitrogen sources have been found to influence microbial growth and metabolites production. Therefore, effect of different carbon and nitrogen sources were studied on the selected isolates for IAA production. The results indicated that *F. oxysporum* showed highest IAA activity in the medium supplemented fructose and sucrose at low concentration (Fig. 2, B). Similarly, high IAA activity was observed in *F. oxysporum* when the medium was supplemented with low concentration of calcium nitrate, potassium nitrate and sodium nitrate as nitrogen sources (Fig. 2, C).

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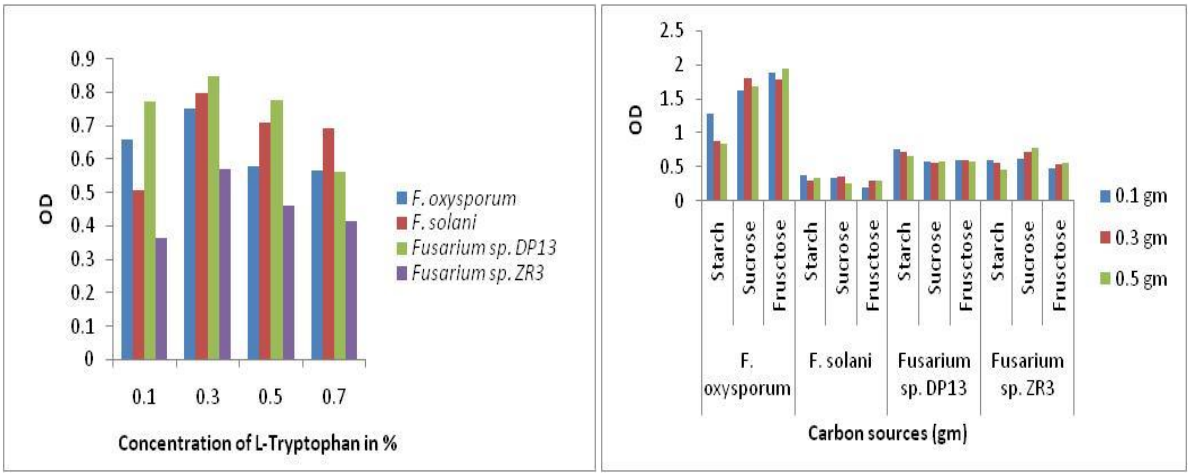
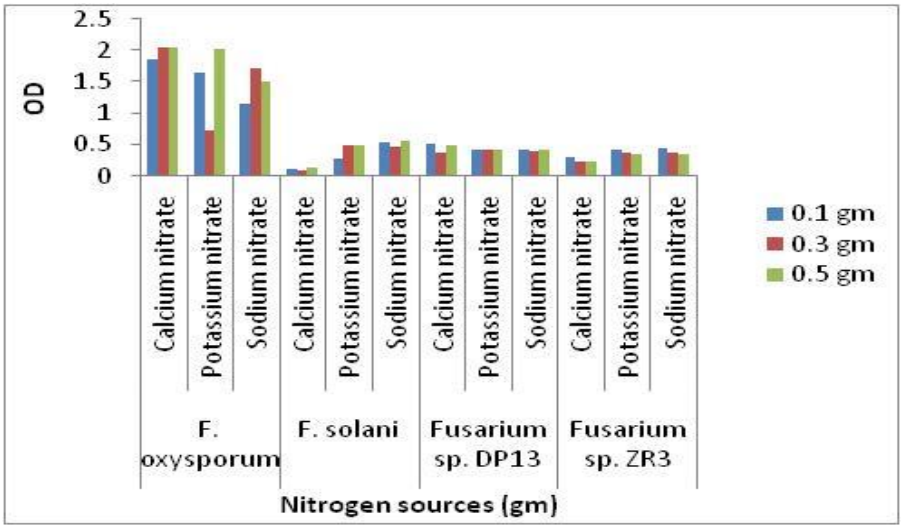


(A) (B)



(C)

Fig. 2: Effect of Tryptophan (A) Carbon sources (B) and Nitrogen sources (C) on IAA production by *F. oxysporum*, *F. solani*, *Fusarium* sp. (DP13) and *Fusarium* sp. (ZR3)

4. CONCLUSION

The present study revealed endophytic fungi associated with seeds of seven plant species. All the seed were found colonized with endophytic fungi. Among the isolated fungi, the colonization frequency of genus *Fusarium* was found to be highest. Maximum production of IAA production was observed in three different species of *Fusarium*. The study indicates the seed borne endophytes fungi might benefit the host in growth and development. The isolates with good IAA production showed the potential to be used as plant growth promoter.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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