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Gibberellin and indole acetic acid production capacity of endophytic fungi isolated from *ZEA MAYS* L.

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Abstract

Endophytic fungi are endosymbionts of majority of plants and having role in plant growth promotion by producing important secondary metabolites such as gibberellin and auxin. A total of 29 different endophytic fungal strains were isolated from the roots (21) and leaves (8) of *Zea mays* L. and screened for GA and IAA producing capacity. Out of 29 fungal strains, 18 were found to be growth promoters and 6 growth inhibitors of rice seedlings. MR 1-3-1 and MR 2-4-4 were the best shoot growth promoter strains. The presence of plant growth promoting hormone was later on confirmed using double beam spectrophotometer. For the GA_3 detection and quantification, a very simple bioassay was done using wheat seeds with embryo removed. Maximum GA_3 (52.26 ng/mL) was found in the culture filtrate of MR 2-4-4. Similarly maximum IAA (2.02 µg/mL) was detected in the culture filtrate of ML 1-5. The present work suggests that these endophytic fungi can be used as bio-fertilizer in the agricultural fields because extensive use of artificial fertilizers, cause deterioration of environment and have hazardous effects for plants. Moreover, artificial fertilizers are very costly and must be supplied continuously to the fields while endophytic fungi stay in the field for years and safe to the plant and environment.

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Introduction

A sustainable agricultural industry is required to feed the ever increasing population of the earth. Huge quantities of fertilizers are utilized by the agriculture industry for crop growth and higher yields. However, such fertilizer application cause huge damages to the environment and are hazardous to human health. Biological fertilizers may be a very good alternative as they are environment friendly and also need less financial inputs. Apart from this, biological fertilizers need no repetition like artificial fertilizers which must be supplied to the field continuously.

A diverse flora of endophytic fungi is found in the internal tissues of majority of higher plants without causing any damage to the host plant (Addy *et al.,* 2005). Among the 770 species of endophytic fungi isolated so for from various plants, most common and identified strains are, *Alternaria infectoria*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *Aspergillus* sp., *Nigrospora oryzae*, *Nigrospora sphaerica*, *Phomopsis* sp., *Penicillium* sp., *Cordana musae*, *Guignardia* sp., *Rhizoctonia* sp., species of *Xylaria* and *Phialocephala sphaeroides* (Wilson *et al.*, 2004). It has been estimated that nearly 13, 00,000 endophytic fungal species are still undiscovered (Dreyfuss and Chapela, 1994).

Endophytic fungi are used by the plants as an indirect defense against herbivorous organisms (insects, birds and mammals), heat, drought and salt stresses, diseases, and increased below and above ground biomass stresses (Carroll, 1986; Lekberg *et al.*, 2005). Ecological adaptability and tolerance of the host plant to biotic and abiotic stresses may also be enhanced by the presence of endophytic fungi (Schulz and Boyle, 2005). In return the plant provides carbohydrates energy resources to the endophytic fungi (Lekberg and Koide, 2005). More than 50,000 secondary metabolites are discovered so far in the microbial world (Berdy, 2005). These secondary metabolites constitute different classes of biologically active compounds such as steroids, alkaloids, terpenoids, quinones, phenols, xanthones, tetralones, isocoumarins, benzopyranones, flavonoids, cytochalasins, enniatins phenylpropanoids, lignans, aliphatics, peptides, phenolics, and volatile organic compounds. (Tan and Zou, 2001; Schulz *et al.*, 2002; Gunatilaka, 2006; Zhang *et al.*, 2006).

Phytohormones are chemical messengers that work as signal molecules and control plant growth and development. They are involved in controlling responses of the plants to environmental changes and also the internal genetic potentials that are controlled by some principal agents. Some rhizospheric fungi also secrete growth promoting phytohormones (Hamayun *et al.*, 2009; Khan *et al.*, 2009). GAs are said to be responsible for all sort of plant growth and development but their most important function is the improvement in the stem growth and biomass (Nishijima *et al.*, 1995). GAs is produced by *Gibberella fujikuroi*, *Aspergillus fumigatus*, *Neurospora crassa*, *Sphaceloma manihoticola*, *Sphaceloma* sp., *Azospirillum brasilense*, *Chrysosporium pseudomonarium*, *Penicillium funiculosum*, *Sesamum indicum*, *Phaeosphaeria* sp. and *Penicillium citrinum* (Rademacher, 1994; Khan *et al.*, 2008).

Auxin is an important plant hormone, discovered in 1928 by Frits Went, which controls many developmental processes and growth of plants such as cell division, cell elongation and differentiation, phototropic and geotropic responses, formation of flowers, fruit ripening, process of senescence and apical dominance. It is believed that to regulate these phenomena, auxin induce some variations in genes expression (Guilfoyle *et al.*, 1998). IAA is produced by *Aspergillus niger*, *Herbaspirillum seropedicae, Acetobacter diazotrophicus,* species of *Erwinia, Rhizobium, Pseudomonas*, *Rhizopus, Azospirillum* and *Bacillus* (Ahmad *et al.*, 2008). The current study on endophytic fungi aims, to investigate their biodiversity, to isolate strains which produce more plant growth promoting phytohormones (IAA and GAs) and to reduce the excessive use of artificial fertilizers in the near future, as they are very costly, deteriorate environment and has negative impacts on plants.

Materials and methods

Isolation of endophytic fungi from Zea mays roots and leaves

The water-cleaned plant samples were suspended in Tween 80 solution (2-3 drops in 50 ml autoclaved distilled water) and kept in shaking incubator set at 120 revolutions per minute (rpm) for 5 minutes at room temperature (Seena and Sridhar, 2004). Samples were surface-sterilized by suspending in 70% ethanol for 30 seconds, 3 % Sodium Hypo Chloride (NaOCl) for 5 minutes and again in 70% ethanol for 30 seconds. The root samples were dried between sterilized filter papers and cut into 0.25 cm pieces. Cork borer was used to cut leaves into 3–4mm diameter discs with and without midrib (Hallmann *et al.*, (2007). Surface sterilized roots and leaves pieces were placed on Hagem medium plates (10 pieces/plate) containing antibiotic streptomycin (80 ppm) and incubated at 25°C till the emergence of fungi (Khan *et al.*, 2009a). To obtain pure cultures and for longer storage, PDA medium plates and slants were inoculated by the fungal isolates and fully-grown pure cultures were stored at 4°C in refrigerator. For GA and IAA collection, Czapek broth medium (pH 7.3 ± 0.2) was used, incubated in a shaking incubator set at 30°C with 120 rpm for 7 days (Hamayun *et al.*, 2009; Khan *et al.*, 2009).

Screening bioassay of fungal culture filtrates on rice seedlings

Different strains were selected based on growth pattern and colonial morphology to avoid unnecessary repetition. 29 strains were selected out of 107 for preliminary screening bioassay experiment. 8 strains were isolated from the *Zea mays* L. (Maize) leaves and 21 from the *Zea mays* L. roots. The fungal culture filtrates were harvested by first using sterilized filter paper and then centrifuged at 5000xg at 4°C for 15 min, for the separation of pellet and supernatant. The separated pellets and supernatants were kept at -70°C in refrigerator for lyophilization. All the supernatants were first diluted in 1ml autoclaved distilled water and then applied on rice seedlings (Hamayun *et al.*, 2009; Khan *et al.*, 2009). Rice Seeds (Fakhr-e-malakand variety) were provided by rice programmed, Agricultural Biotechnology Institute (ABI) National Agricultural Research Center Islamabad (NARCI). Mature, healthy and uniform sized seeds were chosen by physical appearance. Rice seeds were first washed with Tween 80 detergent and then rinsed 3 times with sterilized distilled water to remove the detergent. Clorox (5.25% sodium hypochlorite) and 70% ethanol were used for surface sterilization of seeds. Finally the seeds were shifted to sterilized container, containing water and kept in the culture room in the dark at room temperature for 5 days until the emergence of radicle and plumule. The germinated seeds, having uniform sized radicle and plumule were shifted to the flasks containing 30ml of 0.8% water-agar medium. These flasks were then kept in growth chamber (day/night cycle: 14 h—28 °C ± 0.3; 10 h—25 °C ± 0.3; relative humidity 70%; 6 plants per treatment) for 10 days. At two leaf stage of seedlings, 100µl of fungal supernatants were applied at the seedling tips with the help of micropipette. Along with the supernatants, two other control treatments were also used, one with autoclaved distilled water and the other with Czapek broth medium. Root and shoot lengths were recorded after 5 days of application of fungal supernatants (khan *et al.*, 2009a).

Surface sterilization protocol for wheat seeds and bioassay for Gibberellic acid

The seeds of all cereal grains contain embryo and endosperm surrounded by special cellular layer called aleurone layer. Gibberellic acid is (a seed germinating hormone) presents in the embryo in bound form and released when seed imbibes water. After releasing GA_3 travels to the aleurone layer, where it starts activation of those cells whose are responsible for the synthesis of alpha- amylase (starch digesting enzyme). From here the alpha-amylase enzyme travels to the endosperm tissue where the complex carbohydrate molecules (starch) are broken down to simpler molecules such as glucose (Coombe *et al.*, 1967). In this bioassay we used wheat seeds without embryo i. e wheat germ removed.

Healthy wheat seeds of local variety were chosen for

the bioassay of GA_3. Before surface sterilization 10 seeds were taken for each petri plate. All seeds were cut into two halves. The half pieces containing embryo, were discorded while the remaining halves were further cut into two pieces, making quarters of the seeds. Then these wheat seed quarters were surface sterilized by soaking in 70% ethyl alcohol for 60 seconds, 0.1% mercuric chloride ($HgCl_2$) solution for 2 minutes and again in 70% ethyl alcohol for 60 seconds. Seed quarters were rinsed three times with autoclaved distilled water and shifted to petri plates containing 10 ml of acetate buffer (pH 4.5) and GA_3 in different concentrations. The plates were kept at room temperature and allowed to incubate. After 48 hours, the solution was filtered to test tubes, using sterilized filter paper. 200 µl of Benedict´s solution was added to each test tube. All the test tubes were heated for 30 minutes, using water bath, for the presence and absence of glucose. The amount of glucose is calculated manually by comparing the color to the known concentrations of glucose, as well as using PerkinElmer Lambda 25 double beam spectrophotometer. This bioassay is very simple, inexpensive and easy because it gives clear cut results (Sandra, 1975).

DATA analysis

The data were subjected to Duncan Multiple Range Test by using IBM SPSS software version 21.0 (SPSS Inc, Chicago, USA)

Results

Endophytic fungi isolated from the Zea mays L.

A total of 29 different endophytic fungal strains were isolated from the roots (21) and leaves (8) of *Zea mays* L. These fungal strains were different from each other on the basis of external morphology, color, texture, reproductive structures (sporangium, spores) mycelium depth in the medium etc. The culture filtrates of all these endophytic fungal strains were screened for plant growth-promoting hormones by applying them on rice seedlings Fakhr-e-malakand variety.

Table 1. Screening bioassay of fungal cultural filtrates obtained from the roots of *Zea mays* L. on rice seedlings.

S.NO.	Strain	Plant hight (cm)	Shoot length (cm)	Root length (cm)	Growth status
1	Control (Czk)	12.87 ± 2.75 ab	08.62 ± 2.1 ab	4.25 ± 0.75 a	NA
2	Control (DW)	12.75 ± 0.59 a	07.80 ± 0.53 a	4.95 ± 0.89 abcd	NA
3	MR 1-1	19.50 ± 2.52 cdefg	12.42 ± 1.1 bcde	6.82 ± 1.44 abcde	Promoted
4	MR 1-1-10	13.95 ± 1.20 abc	09.42 ± 0.53 abcd	4.52 ± 0.78 ab	Promoted
5	MR 1-2-2	16.15 ± 1.9 abcdef	07.85 ± 1.06 abcd	5.80 ± 1.02 abcde	Promoted
6	MR 1-2-3	15.90 ± 1.32 abcd	08.77 ± 0.95 ab	5.87 ± 0.69 abcde	Promoted
7	MR 1-3-1	23.87 ± 2.85 g	14.77 ± 1.3 e	9.10 ± 1.78 e	Promoted
8	MR 1-3-2	18.57 ± 1.56 abcdefg	10.80 ± 0.97 abcde	7.77 ± 0.94 abcde	Promoted
9	MR 1-3-4	19.57 ± 2.31 cdefg	10.90 ± 1.24 abcde	8.67 ± 1.23 de	Promoted
10	MR 1-3-5	20.75 ± 0.25 defg	13.25 ± 0.75 cde	7.50 ± 1 abcde	Promoted
11	MR 1-3-6	19.27 ± 1.05 cdefg	10.90 ± 1.02 abcde	8.37 ± 0.97 cde	Promoted
12	MR 1-4-2	21.62 ± 1.89 efg	12.97 ± 0.8 bcde	8.65 ± 1.09 de	Promoted
13	MR 1-5-1	17.50 ± 0.43 abcdef	08.85 ± 0.2 ab	8.65 ± 0.57 de	Promoted
14	MR 2-1-1	19.00 ± 1 bcdefg	11.52 ± 0.71 bcde	7.47 ± 0.62 abcde	Promoted
15	MR 2-3-4	21.47 ± 1.78 efg	13.50 ± 1.3 de	7.97 ± 0.65 abcde	Promoted
16	MR 2-4	20.37 ± 1.98 defg	12.42 ± 1.33 bcde	7.95 ± 1.05 abcde	Promoted
17	MR 2-4-1	18.37 ± 2.3 abcdefg	12.22 ± 3.2 e	6.15 ± 1.5 abcde	Promoted
18	MR 2-4-4	22.32 ± 1.65 fg	13.57 ± 0.59 de	8.75 ± 1.71 e	Promoted
19	MR 2-4-5	21.35 ± 1.27 efg	13.25 ± 0.58 cde	8.10 ± 1.56 bcde	Promoted
20	MR 2-4-6	19.12 ± 1.69 bcdefg	11.30 ± 1.04 bcde	7.82 ± 0.73 abcde	Promoted
21	MR 2-4-7	13.92 ± 1.69 abc	09.17 ± 1.06 abc	4.75 ± 0.72 abc	Promoted
22	MR 3-2-1	15.95 ± 2.85 abcde	09.67 ± 1.52 abcd	6.30 ± 1.54 abcde	Promoted
23	MR 4-2-1	18.70 ± 1.90 abcdefg	11.22 ± 1.76 bcde	7.47 ± 0.37 abcde	Promoted

(cm) = Centimeters, Czk= Czapek Medium, DW = Distilled Water. Values with different letters in the same column in that group are significantly different at the 5% level by DMRT (Duncan's Multiple Range Test). Values within the table refers to the mean ± SE (n = 4).

Fungal filtrates were applied on rice seedlings to know the growth promoting and inhibiting activities of endophytic fungi. Shoot and root lengths were noted after 5 days application of fungal cultural filtrates. Out of 21 endophytic fungal strains isolated from roots, 18 were found to be growth promoters, 3 neutral and no one growth inhibitor. Only 5 were noted to be best growth promoter strains (enhance more than 80% shoot length) (Table 1). Among 8 fungal strains isolated from leaves, 5 were found as slightly growth promoters, 1 (ML 1-2) as growth inhibitor while 2 as growth neutrals (Table 2). Two negative controls (one Czapek medium and one Distilled Water) were also taken and compared with fungal filtrates.

Table 2. Screening bioassay of fungal cultural filtrates obtained from the leaves of *Zea mays* L. on rice seedlings.

S.NO.	Strain	Plant hight (cm)	Shoot Length (cm)	Root length (cm)	Growth status
1	Control (Czk)	12.87 ± 2.76 ab	08.62 ± 2.09 abc	4.25 ± 0.75 a	NA
2	Control (D.W)	12.75 ± 0.59 a	07.80 ± 0.53 ab	4.95 ± 0.89 a	NA
3	ML 1-1	19.92 ± 1.64 bc	12.25 ± 0.88 c	7.67 ± 0.92 ab	Promoted
4	ML 1-2	13.17 ± 0.63 ab	07.00 ± 0.71 a	6.17 ± 1.08 ab	Promoted
5	ML 1-5	18.92 ± 3.44 abc	09.95 ± 1.53 abc	8.97 ± 1.97 b	Promoted
6	ML 1-5-2	16.30 ± 1.92 abc	09.75 ± 1.25 abc	6.55 ± 0.88 ab	Promoted
7	ML 2-1-2	18.02 ± 1.52 abc	11.60 ± 1 bc	6.42 ± 0.9 ab	Promoted
8	ML 2-2-1	19.97 ± 2.42 bc	12.20 ± 1.3 c	7.77 ± 1.18 ab	Promoted
9	ML 2-2-2	21.92 ± 2.74 c	12.17 ± 1.24 c	9.75 ± 1.65 b	Promoted
10	ML 2-3	16.25 ± 2.06 abc	10.35 ± 1.2 abc	5.90 ± 1.15 ab	Promoted

Czk= Czapek Medium, D.W = Distilled Water. Values with different letters in the same column in that group are significantly different at the 5% level by DMRT (Duncan's Multiple Range Test). Values within the table refers to the mean ± SE (n = 4).

Discussion

Most of the endophytic fungi form a mutualistic relationship with their host which is very advantageous for both partners (Tejesvi, 2007). During mutualistic relationship, host plants get benefit in the form of enhanced growth and development, immunity against abiotic and biotic stresses such as draught, heat, pathogens, enhancement of phosphorus absorption, nitrogen fixation, phosphate solubilization and production of Phytohormones (auxin, gibberellins, abscisic acid and cytokinin) which help in the plant growth and development (Firakova *et al.*, 2007).

Table 3. Optical density of fungal culture filtrates for the presence and quantification of GA_3.

S. NO.	Sample NO.	Sample ID	Optical Density	GA_3 (ng/mL)
1	10	MR 1-3-5	0.4517	49.63
2	11	MR 1-3-1	0.2387	25.94
3	12	MR 2-3-4	0.1356	14.48
4	13	MR 2-4-5	0.4515	49.60
5	19	MR 2-4-4	0.4754	52.26
Control	-	-	0.0041	-0.15

Enhanced growth of rice seedlings, using fungal culture filtrates, clearly indicates the presence of plant growth promoting hormones (GA and IAA) and which were later on confirmed by using PerkinElmer Lambda 25 spectrophotometer (Hamayun, 2008; Choi *et al.*, 2005). Fungal culture filtrates had been known and will continue, to be a rich source of biologically active secondary metabolites (Tejesvi,

2007). Screening procedure for the determination and identification of novel and biologically active microbial secondary metabolites is a simple and well known method (Higgs *et al.*, 2001; Kumar *et al.*, 2005). A similar procedure was used by Rim *et al.* for the detection, identification and quantification of plant growth promoting secondary metabolites in the *Fusarium proliferatum* (Rim *et al.*, 2006). It was observed that out of 29 fungal strains, 18 were found to be growth promoters of rice seedlings, only 6 were found to be growth inhibitor and the rest of strains were neutral or slightly growth promoters. The best shoot growth promoter strains were MR 1-3-1and MR 2-4-4 while no strain was found to be shoot growth inhibiter.

Table 4. Optical density of fungal culture filtrates for the presence and quantification of IAA.

S. NO.	Sample	Sample ID	Optical density	IAA (mg/mL)
1	5	ML 1-5	0.3532	2.02

Similarly out of 29 strains, fungal culture filtrates of ML 2-2-2A, MR 1-3-5, MR 1-3-1, MR 2-3-4, MR 2-4-5, MR 2-4-4, MR 1-4-2 and MR 2-4 were found to be best growth promoter of overall plant length (root + shoot length) while no strains was growth inhibiter. While strains ML 2-2-2A, ML 1-5, MR 1-3-1, MR 2-4-5, MR 1-3-6, MR 1-5-1, MR 1-3-4, MR 2-4-4 and MR were observed to be enhanced root length promoters and strains MR 1-1-10 was root length inhibiter.

Presence of plant growth promoting hormone was later on confirmed using double beam spectrophotometer. For the GA_3 detection and quantification, a very simple bioassay was done using wheat seeds with embryo removed. A similar protocol, with some modifications, for the GA_3 detection in the wheat seeds was used by Sandra Lyn Biroc (Sandra, 1975).

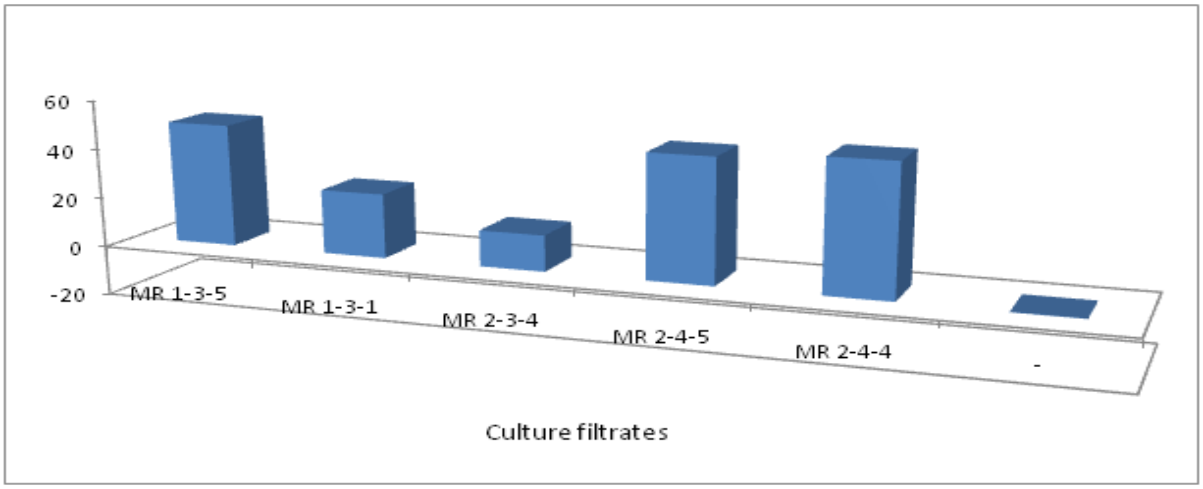

Fig. 1. Gibberellic acid (GA_3) concentration of different fungal strains isolated from three maiz plant after 7 days of incubation in Czapek medium in shaking incubator set at 120 rpm at 30°C. Highest concentration of GA_3 (52.26 ng/mL) was found in strain MR 2-4-4.

This bioassay for the detection and quantification of gibberellin in the culture filtrates of endophytic fungi, using wheat seeds, was never tried before. Wheat seeds were selected for GA_3 bioassay because of its easy availability and handling. Gibberellic acid is a seed germinating hormone present in the embryo in bound form and released by the embryo during seed germination, after imbibing water. GA_3 after releasing from the embryo travels to the aleurone layer where it activates those cells which are responsible for the synthesis of α-amylase. This enzyme is responsible for the digestion of starch. The α-amylase enzyme then

travels to the endosperm tissue and convert starch into glucose. Glucose molecules are then used in respiration to obtain energy for the germination process. In this bioassay wheat seeds were used with embryo removed to overcome the source of GA_3 production by the seeds. Presence of GA_3 in fungal culture filtrates was responsible for the conversion of endospermic starch into simple sugar (glucose) which was easily detected with Benedict´s test. Maximum GA_3 (52.26 ng/mL) was found in the culture filtrate of MR 2-4-4. In the similar study on endophytic fungi, Hamayun *et al.* showed the presence and quantification of GA_3 only up to 3.21 ng/mL (Hamayun *et al*, 2009). All the 29 strains were also checked for the synthesis of IAA using Salkowski reagent. Out of 29 fungal culture filtrates checked, only 1 strain (ML 1-5) was found to have IAA (2.02 µg/mL).

Conclusion

From this study we can conclude that endophytic fungi are cosmopolitan and diverse group of living organisms. They are found in most parts of the plant body (root, stem and leaves) and play very important role for supporting their host plants.

They are responsible for the synthesis of secondary metabolites such as GA_3 and IAA so, these can be used as bio-fertilizer because extensive use of artificial fertilizers in agricultural fields cause deterioration of environment and have hazardous effects for plants. They are very costly and must be supplied continuously to the fields. So use of endophytic fungi is suggested as bio-fertilizers in the future because they are safe to the plant and environment, and stay in the field for years.

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