Seed Borne Endophytic Fungi Associated with Some Indigenous Rice Varieties of North East India and Their Growth Promotion and Antifungal Potential

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ABSTRACT

Background: Seed borne endophytic fungi play an important role in seed germination and plant health. There are ubiquitous and have been found associated in every plant species investigated so far. They colonize plant seeds without causing any disease and are often vertically transmitted. Despite their occurrence, seed borne endophytic fungi are poorly investigated and their role for application in agriculture is still to be elucidated.

Methods: In the present study endophytic fungi associated with seeds of some indigenous rice varieties of North East, India was investigated. The isolates were determined for IAA activity in-vitro and antifungal activity against rice pathogen, Magnaporthe grisea. Crude metabolites obtained from potent isolates were characterized by GCMS analysis to reveal the presence of bioactive compounds.

Result: Our result indicated that commonly isolated fungal genera were Aspergillus, Fusarium, Gliocladium, Mucor, Penicillium, Bipolaris, Basidiobolus and Mycelia sterilia. Amongst them, colonization frequency (CF %) of 

Magnaporthe grisea was highest and was isolated from seeds of almost all the varieties. It was observed that four isolates of Fusarium showed good IAA production in the medium amended with tryptophan. The isolates also displayed antifungal activity against 

Magnaporthe grisea. GCMS analysis of the metabolites indicated presence of several bioactive compounds. The study suggests that seed borne endophytes can be explored as bio-inoculants for crop improvement in future research program.

Key words: Antifungal activity, Indigenous rice, Magnaporthe grisea, Plant growth, Seed borne endophytes.

INTRODUCTION

Rice is a major staple crop in the world and demand for rice is increasing every year (Ray et al., 2013). However, rice production is rapidly declining due to changes in soil nutrition, land-uses, agricultural practices and various diseases. To increase the yield and production, rice growers around the world have resorted to the excessive use of chemical fertilizers and plant protection chemicals. This certainly has enhanced the productivity but also has a detrimental effect on the environment including all plants and animals, leading to development of resistant pathogens and loss of biodiversity (Paul et al., 1995). Therefore, there is a need to develop safe and environment friendly alternative strategies to protect and improve their yield. In the last few decades, endophytic fungi have been emerging as alternative sources for bioactive agents targeted towards plant protection and growth promotion (Wijesooriya and Deshappriya, 2016). They are reported to provide a number of benefits to the host plants in various ways, such as growth promotion, protection against diseases and pests and augmenting absorption of minerals (Hassan et al., 2013).

There are very few studies on endophytic fungi associated with rice cultivars and their applications as plant growth promoter and antagonist against rice pathogens (Atugala and Deshappriya, 2015). Further, very few studies on rice endophytes from India have been undertaken and most of workers have reported bacterial endophytes from rice and their application as plant growth promoter and antagonists against fungal pathogens (Sharma et al., 2015).

Thus, there is paucity of work on fungal endophytes of rice from India. The Eastern Himalayan region of Northeast (NE) India is home to a large number of indigenous rice varieties. There is also evidence that some tradition rice landraces of NE India are resistant to various diseases and pests such as Ball rice of Siang district of Arunachal Pradesh is the most important landrace grown by the Adi tribe, which is highly adapted and possesses multiple resistances to disease and pest (Datta et al., 2006). It has been hypothesized that plants growing in unique environmental settings and ability to resist diseases are expected to harbour novel endophytes that may produce unique metabolites having diversified applications. However, there is meagre information on endophytic fungi associated with these rice varieties. Therefore, the present investigation was undertaken to study the fungal endophytes associated with...
some indigenous rice seeds with an aim to elucidate their growth promoting activities and antagonistic potential against a rice pathogen *Magnaporthe grisea*.

**MATERIALS AND METHODS**

**Sample collection and processing**

Freshly harvested indigenous rice seeds were collected from different places of Northeast India (Table 1). Selection of rice plants was done by considering properties or characteristics like locally adapted, aromatic, early maturity, resistance or tolerance to biotic and abiotic stresses. Collection was done during the month of October, 2018. The samples were collected in sterile polybags separately for each rice variety after the heading stage (when panicle is fully developed), brought to the laboratory and processed within 48 h.

**Isolation and identification of endophytic fungi**

Healthy rice seeds were selected and washed thoroughly under tap water to remove the debris and left for drying over blotting paper. After drying, the seeds were surface-sterilized by following the method suggested by Ebeltaghy et al. (2000). The surface sterilized dried seeds were then inoculated into freshly prepared Potato Dextrose Agar (PDA) medium. The plates were incubated at 26±2°C for the growth of endophytic fungi. After obtaining the pure cultures, endophytic fungi were identified on the basis of cultural characteristics, morphology of fruiting bodies and spores by referring to fungal identification manuals, Compendium of soil fungi (Domsch et al., 1980) and Hyphomycetes (Seifert and Gams, 2011). Cultures that failed to sporulate repetitively on different media were categorized as sterile mycelia. The colonization frequency of endophytic fungi was calculated as the total no. of representative rice seeds colonized by endophyte divided by the total no. of seeds inoculated. Colonization rate was expressed as percentage.

**Determination for IAA production**

The quantitative analysis of Indole-3-Acetic Acid (IAA) was performed by the method suggested by Bric et al., (1991). A standard curve was drawn for comparison to determine auxin production by the isolates. HPLC analysis was done to confirm the presence of IAA. The crude extracts were dissolved in methanol (HPLC grade) and chromatographic separations were performed at ambient temperature on a C8 column (Symmetry 4.6 9 150 mm, 5 lm, Waters) fitted with a C8 guard column (Symmetry3.9 9 20 mm, 5 lm, Waters) using gradient elution. Eluent ‘A’ consisted of 2.5: 97.5 % (v/v) acetic acid: H2O, pH 3.8 (the pH was adjusted by addition of 1 mol L⁻¹ KOH) and eluent ‘B’ consisted of 80: 20 % (v/v) acetonitrile: H2O. The mobile phase started with eluent ‘A’; eluent ‘B’ at 80: 20 %, changing to 50: 50 %, 0: 100 % and 80: 20 % in 25, 31 and 33 min, respectively. The total run time was 36 min. The flow rate of the mobile phase was 1 mL min⁻¹; the injection volumes were 20 µL and the fluorimetric detector was set to excitation and emission wavelengths of 280 and 350 nm, respectively.

**Extraction of secondary metabolites**

The fungal isolates that showed positive and good IAA production *in vitro* were selected for extraction of secondary metabolites. The extraction of secondary metabolites was carried out by using the method described by Bhardwaj et al. (2015).

**Determination of antifungal activity**

The rice pathogen *Magnaporthe grisea* was isolated from infected rice plants by using modified medium (100 ml Rice leaf extract, 3g sucrose and 4g agar). A mycelia disc of 0.6 cm diameter of the fungal pathogen was transferred onto the center of PDA plates. The inhibition of fungal growth was observed by Agar Well Diffusion method. The crude extracts obtained from fungal isolates were dissolved in 100 µL of Dimethly Sulphoxide (DMSO) and loaded into the wells, 3 cm away from the fungal discs. The plates were incubated at 28°C for 4 days and the zone of inhibition was recorded and compared with a control plates.

**Effect of fungal endophytes on growth of rice plants**

A pot experiment was conducted using an indigenous rice variety “Eri” which showed no endophytic colonization to assess the effect of fungal endophytes on the growth of rice plants. Healthy seeds were selected, surface sterilized and germinated by wrapping with a wet sterilized cloth and incubating them under room temperature for five days. Thereafter, fungal endophytes were inoculated following two methods: In the first method, one-week old pure cultures of selected fungal isolates were grown in PDB at 28°C and seeds were inoculated with individual fungal broths by coating with 20% sugar solution as an adhesive before sowing. In the second method, 15 ml of spore suspension (3x10⁵ cfu mL⁻¹) of the selected fungal isolates were added to the pots filled with sterilized soil before sowing. Treated and control seedlings were planted in pots (5 seedlings per pot) filled with autoclaved soil. For this experiment, 5 replicates for each treatment were maintained in the greenhouse at average day and night temperature ranging between 30±5°C and 20±5°C respectively. Root and shoot

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**Table 1:** Sampling sites and rice variety collected from different parts of North East India.

| Sampling Site  | State            | Name of rice variety                      | GPS Location       |
|---------------|------------------|------------------------------------------|--------------------|
| West Siang    | Arunachal Pradesh| Bali, Ankar, Jyoti and Dhaya Dara.       | 28°24‘N 94°33‘E    |
| Dhemaji       | Assam            | Konkwa and Boga Dhan.                    | 27°48‘N 94°58‘E    |
| Karbi Anglong | Assam            | Karbi Saksaw                             | 26°11‘N 93°34‘E    |
| Imphal        | Manipur          | Chakhaos (Black Rice)                    | 24°807‘N 93.9384‘E |
| West Garo Hills| Meghalaya        | Boro Dhan and Gorioya Dhan               | 25.5167‘N 90.2167‘E|
Seed Borne Endophytic Fungi Associated with Some Indigenous Rice Varieties of North East India and Their Growth Promotion...

lengths of 5 randomly selected plants from each treatment were measured at one-week interval.

Characterization of secondary metabolites by GCMS analysis

GCMS analysis of the potent fungal isolates showing antifungal activity was carried out using a Perkin Elmer Turbo Mass Spectrophotometer (USA), model Claurus 680 Gas chromatography/Claurus 600 Mass spectrometer (GC with Liquid Auto sampler). Library search for the peaks were carried out using library of National Institute of Standard and Technology (NIST).

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate for the root and shoot length between treated and control rice seedling by using SPSS software and considered significant when p < 0.05.

RESULTS AND DISCUSSION

Seed borne endophytic fungi from indigenous rice varieties

In the present study, seed endophytic fungi associated with indigenous rice varieties collected from 10 different places of Northeast India were investigated. The sampling sites and name of the rice varieties with their GPS location is presented in (Table 1). Although there are reports on bacterial seed endophytes from rice and their function roles but there is paucity of works on seed borne endophytic fungi from rice. A total of 225 endophytic fungal isolates belonging to 8 different fungal genera and non-sporulation fungi categorized as Mycelia sterilia were isolated from the collected seeds (Table 2). This indicates occurrence of rich diversity of seed borne endophytic fungi in rice. Among the rice varieties, seeds of Bali rice were found to have maximum (88%) where seeds of Boro Dhan rice had the minimum (10%) colonization frequency of endophytic fungi (Fig 1). Both the rice varieties were collected from different geographical sites. Bali rice was collected from West-Siang district of Arunachal Pradesh while Boro Dhan was collected from West Garo hills of Meghalaya. This shows that colonization of endophytic fungi differs among the rice seeds varieties and within the geographical sites. The finding collaborates with the result obtained by Göre and Bucak (2007) who reported that colonization frequency of endophytes in seed fluctuates with geographical factors. The endophyte fungal genera isolated were Aspergillus, Fusarium, Gliocladium, Mucor, Penicillium, Bipolaris, Basidiobolus and Mycelia sterilia. Among the fungal

Table 2: Occurrence of endophytic fungi in the seeds of different indigenous rice varieties of North-East India.

| Endophytic fungal genera | AM | BA | AN | JY | DD | KON | CH | BO | KS | BD | Total | *CF (%) |
|--------------------------|----|----|----|----|----|-----|----|----|----|----|-------|---------|
| Curvularia               | 03 | 08 | 02 | 02 | 00 | 04  | 00 | 00 | 01 | 00 | 20    | 04      |
| Aspergillus              | 00 | 02 | 22 | 00 | 00 | 00  | 00 | 00 | 02 | 03 | 29    | 5.8     |
| Fusarium                 | 22 | 01 | 03 | 02 | 00 | 14  | 01 | 00 | 01 | 44 | 5.8   | 8.8     |
| Gliocladium              | 00 | 00 | 00 | 00 | 00 | 00  | 01 | 00 | 02 | 01 | 16    | 2.4     |
| Mucor                    | 00 | 00 | 02 | 00 | 00 | 00  | 00 | 00 | 00 | 05 | 01    | 0.2     |
| Penicillium              | 00 | 00 | 01 | 00 | 00 | 00  | 00 | 00 | 00 | 00 | 00    | 0.8     |
| Bipolaris                | 00 | 00 | 00 | 00 | 00 | 00  | 00 | 00 | 00 | 00 | 00    | 0.6     |
| Basidiobolus             | 00 | 00 | 00 | 00 | 00 | 03  | 00 | 00 | 00 | 00 | 00    | 0.6     |
| Mycelia Sterilia         | 11 | 30 | 09 | 16 | 14 | 02  | 12 | 03 | 04 | 06 | 107   | 21      |

Note: AM= Ampack, BA= Bali, AN= Ankar, JY= Jyoti, DD= Dhaya Dara, KON= Konkuwa, CH= Chakhao, BO= Boro Dhan, KS= Karbi Saksaw, BD= Boga Dhan. * CF (%) = Colonization frequency. No of seeds plated per variety= 50.
genera the colonization frequency (% CF) of *Fusarium* was found to be highest (8.8%) and was isolated almost from all the rice varieties with maximum isolates recovered from Ankar, an indigenous rice variety of West-Siang district of Arunachal Pradesh (Table 2). In many instances, the genus *Fusarium* has been obtained as endophytes from different parts of rice (Leewijit et al., 2016).

**IAA production by endophytic fungi**

There are several reports that endophytic fungi produce growth hormones like Gibberellins and Indole acetic Acid (IAA) and promote host-plant growth (Mehmood et al., 2018). Therefore, in the present study endophytic fungal isolates were screened for IAA production *in vitro* by amending the medium with and without tryptophan. The result indicated that four isolates of *Fusarium* showed good IAA production in-vitro in the medium amended with tryptophan. The IAA production in the culture medium was determined by Salkowski reagent and the isolates showed variable concentration of IAA. Production of IAA was much higher in the medium amended with tryptophan for all the endophytic isolates as compared to cultures without tryptophan (Table 3). This clearly indicates role of tryptophan in IAA production. Tryptophan is considered as a precursor for IAA biosynthesis and its addition in the culture medium enhances IAA production (Hoffman et al., 2013). In our present study, all the isolates that produced considerable IAA *in vitro* were identified to be *Fusarium* species. Production of IAA by endophytic *Fusarium* has also been reported recently by several workers (Mehmood et al., 2018; Shah et al., 2019).

**Growth promotion of rice plants by endophytic fungi**

*Fusarium* strains are reported to improve seed germination and also produce secondary metabolite that exerts beneficial effect to the host plant (Vujanovic et al., 2000 and Zhang et al., 2013). Therefore in the present study, the endophytic *Fusarium* isolates that showed promising IAA production were evaluated for rice plant growth potential *in vitro* through pot experiment. It was observed that in both the treatment process (T1 and T2) the isolates showed higher growth promotion in term of increased shoot and root length in the experimental rice as compared to the control (Table 4). The result indicated that T2 (soil amended with fungal extracts)
showed more root and shoot lengths as compared to T1 (seed coated with fungal extract). In many instances, application of endophytic *Fusarium* species has been reported to promote plant growth and enhance root and shoot lengths (Mehmood et al., 2018). Hence, the growth promotion activity of *Fusarium* may be due to the production of phytohormones.

**Antifungal activity of endophytic fungi**

Endophyte fungi are reported to produce secondary metabolites that inhibit several phytopathogenic microbes (Mejía et al., 2008). Therefore, in the present study endophytic *Fusarium* isolates were evaluated for their antifungal activity against a rice pathogen, *Magnaporthe grisea*. The result obtained from Agar Well Diffusion assay showed that ethyl acetate extracts of all the isolates possess good antifungal activity against the tested rice pathogen (Fig 3). Previous studies have also shown that extracts from endophytic fungi exhibited antifungal activity against wide range of pathogens (Johanna et al., 2012). Several mechanisms have been underlined for antagonistic activities of endophytic fungi against phytopathogens. One of the important mechanisms is production of secondary metabolites. Endophytic fungi of the genus *Fusarium* are reported as sources of bioactive metabolites (Marie and Toghue, 2019). Thus, the antifungal activity of the endophytic *Fusarium* isolates may be due to production of diverse bioactive metabolites and this has been revealed by GCMS analysis. The major identified compounds present in the ethyl acetate extract of the endophytic *Fusarium* isolates are presented in the (Table 5). Some of these compounds have been reported to have antimicrobial and antifungal activities against wide range of human and plant pathogenic microorganisms (Padmapriya and Maneemegalai, 2016; Chinaka et al., 2018).

**CONCLUSION**

The results presented in this study revealed endophytic fungi associated with seeds of ten different indigenous rice varieties collected from North East India. All the seeds were found colonized with endophytic fungi showing a dominance of genus *Fusarium*. Amongst the endophytes, four *Fusarium* isolates showed good IAA production together with rice plant growth promoting capability. The isolates also showed good *in vitro* antifungal activity against rice pathogen, *Magnaporthe grisea*. Thus, the study indicated that seed borne endophytic fungi inhabiting indigenous rice varieties could be further studied for their potential use in agricultural practices.
Seed Borne Endophytic Fungi Associated with Some Indigenous Rice Varieties of North East India and Their Growth Promotion... explored for being used as bio-inoculants for growth and yield of commercial rice cultivars.

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