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# Isolation of fungal endophytes of rice and their antagonistic effect against some important rice fungal pathogens *in vitro*

**Lalngaihawmi, Susanta Banik and Pezangulie Chakruno**

**Abstract**
Fourteen endophytic fungi *viz. Cladosporium cladosporioides, Penicillium citrinum, Fusarium moniliforme, Trichoderma asperellum, Penicillium pinophilum, Aspergillus niger, Aspergillus flavus, Drechslera specifera, Penicillium oxalicum, Geotrichum candidum, Curvularia lunata, Aspergillus amstelodami, Talaromyces* sp., and *Chaetomium ochraceum* isolated from rice plant were screened under *in vitro* to see their antagonistic effect on three important rice fungal pathogens *viz. Pyricularia oryzae, Bipolaris oryzae* and *Ustilaginoidea virens.* In dual culture, *A. niger* was found most promising as antagonist against *P. oryzae* with 79.63 per cent inhibition of radial growth followed by *A. amstelodami* (70.37%), *T. asperellum* (69.81%), and *P. citrinum* (69.63). The highest per cent inhibiton of mycelial growth of *B. oryzae* was observed for *T. asperellum* with 77.77 that is closely followed by other endophytes like *A. niger* (76.47%), *A. flavus* (74.81%), *G. candidum* (72.22%) and *C. lunata* (72.22%). The highest per cent inhibition of *U. virens* was again observed for *T. asperellum* (53.54%) followed by *A. niger* (44.52%), *G. candidum* (44.52%) and *A. amstelodami* (43.86%).

**Keywords:** Antagonist, *Endophytic fungi*, fungal pathogens, rice, rice blast

## Introduction
The term endophyte originating from the Greek words *endon* meaning within and *phyton* meaning plant refers to a microorganism, usually a bacterium or a fungus that lives within a plant. In other words, microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence are defined as endophytes (Wilson, 1993; Saikkonen *et al*., 2004) [30, 20]. Plants benefit extensively by harbouring these endophytic microbes; they promote plant growth (Compant, *et al.*, 2005) [5] and confer enhanced resistance to various pathogens (Clay and Schardl, 2002; Höflich, 2000; Arnold *et al*., 2003) [4, 9, 1] by producing antibiotics (Ezra, *et al*. 2004) [7]. Endophytes also produce unusual secondary metabolites of plant importance (Taechowisan *et al*., 2005) [26]. It has been suggested that the presence of a mutualistic endophyte acts as a "biological trigger" to activate the stress response system more rapidly and strongly than non mutualistic plants (Redman, 2002) [19].

Endophytes may also benefit host plants by preventing pathogenic organisms from colonizing them. Extensive colonization of the plant tissue by endophytes creates a "barrier effect", where the local endophytes outcompete and prevent pathogenic organisms from taking hold. Endophytes may also produce chemicals which inhibit the growth of competitors, including pathogenic organisms. Endophytic microorganisms provide advantages to the host plant by enhancing the physiological activity of the plant or through other modes of action and thus may serve as a source of agroactive compounds, biocontrol agents, or plant growth promoters (Shimizu *et al*., 2009; Dombou *et al*., 2002) [24, 6]. Thus, envisaging the potential of endophytic microorganisms in plant disease management, the present work has been undertaken to isolate endophytic fungi from rice and to explore their biocontrol potential against important rice pathogens *in vitro*.

## Materials and Methods
The present investigation was carried out in the laboratory of Department of Plant Pathology, School of Agricultural Sciences and Rural Development, Nagaland University, Medziphema Campus, Nagaland. A local rice variety (Kemenya) cultivated in and around Medziphema town was collected for the isolation and identification of fungal endophytes and pathogens. Identified cultures of rice fungal endophytes were also obtained from the Department of Plant Pathology Laboratory.

**Isolation and identification of rice fungal endophytes**

All the isolation techniques carried out in this experiment were performed in an aseptic condition inside the laminar air flow chamber. The rice samples for the isolation and identification of fungal endophytes were carefully collected from the field and brought to the laboratory to carry out the required isolation procedures. The rice plant collected was carefully washed in running water and surface sterilization was done by sequential immersion in 70% ethanol for 5 minutes and sodium hypochloride (0.9% available chlorine) for 20 minutes. The samples were again washed in sterile water three times to remove the surface sterilizing agents (Tian, 2004) [28]. The surface sterilized samples were then cut into 1cm long fragments approximately. Ten fragments obtained from different parts of the leaves, shoots and roots were then placed in PDA (Potato Dextrose Agar- distilled water 1000ml, potato 200g, dextrose 20g, agar agar 20 g) surface in separate plates and incubated at 25ºC for 2-3 days. The fungal colonies thus obtained were further purified and maintained in PDA slants. Identification of all the fungal endophytes isolated in this investigation was made by ITCC (Indian Type Cultural Collection Centre), Division of Plant Pathology, IARI (Indian Agricultural Research Institute), New Delhi – 110012.

**Isolation of *Pyricularia oryzae* and *Bipolaris oryzae***

Rice plant infected with the pathogen *Pyricularia oryzae* (blast of rice) and *Bipolaris oryzae* (brown spot of rice) were carefully collected in polythene bags for the isolation of the pathogen. The infected plant parts were then cut in such a way that the leaf bits contain 50% area of healthy part and 50% from the lesion. The leaf bits were then surface sterilized with 0.1% sodium hypochloride for 60 seconds followed by rinsing with sterile water 2-3 times. The leaf bits were then blot dried with the help of sterile blotting paper and were placed on PDA plates aseptically. The inoculated plates were incubated at 25ºC for 2-3 days. The fungal colonies thus obtained were further purified and maintained in PDA slants.

**Isolation of *Ustilaginoidea virens***

Rice plant infected with the pathogen *Ustilaginoidea virens* (false smut of rice) was also collected carefully in polythene bags in such a way that the spore or smut balls of the pathogen are not damaged. Few spores was scrapped off from the spore ball and transferred in Petri plates containing PDA under aseptic condition. The inoculated plates were incubated at 25ºC for 2-3 days. The fungal colonies thus obtained were further purified and maintained in PDA slants. Identification of the fungal rice pathogens was made based on the cultural and morphological characters and with the help of literatures available.

**Antagonistic effect of fungal endophytes of rice on the radial growth of important rice pathogens *in vitro***

Fourteen fungal endophytes *viz. Cladosporium cladosporioides, Penicillium citrinum, Fusarium moniliforme, Trichoderma asperellum, Penicillium pinophilum, Aspergillus niger, Aspergillus flavus, Drechslera specifera, Penicillium oxalicum, Geotrichum candidum, Curvularia lunata, Aspergillus amstelodami, Talaromyces* sp., and *Chaetomium ochraceum* were screened under *in vitro* condition by using dual culture method against *Pyricularia oryzae, Bipolaris oryzae* and *Ustilaginoidea virens* the causal organisms of blast disease, brown spot disease and false smut disease of rice respectively. Culture discs of 5mm diameter each of the fungal endophytes and the pathogens were corked out with the help of sterilized cork borer from the margin of three days old active culture and transferred into PDA medium contained in Petri plates (90mm diameter) on opposite sides approximately at 10mm from the periphery of the plate. A control plate having only the test pathogen was also kept for comparison. The Petri plates were then incubated at 25ºC. The experiment combinations were placed in Completely Randomized Design (CRD) and each treatment was replicated three (3) times. Colony diameter of the test pathogens was measured and per cent inhibition was calculated. Recording of the antagonistic effect was done 7 days after incubation. The respective inhibition was calculated as follows $PI = \frac{(C-T)}{C} X$ 100, where, PI - % inhibition, C - Radial growth in control, T - Radial growth in treatment.

The experimental data were analyzed statistically by applying the technique of analysis of variance, using software provided by Microsoft Excel and the significance of different sources of variance was tested by Error mean square using Snedor's 'F' test of probability at 5 % level of significance.

**Result and Discussion**

**Isolation and identification of fungal endophytes**

On the basis of characters studied, the fungal endophytes of rice were identified in the laboratory. All the identified fungal species and taxonomic position are given in Table 1. The identification results were confirmed by the ITCC, New Delhi. The identified fungal endophytes of rice are *Cladosporium cladosporioides, Penicillium citrinum, Fusarium moniliforme, Trichoderma asperellum, Penicillium pinophilum, Aspergillus niger, Aspergillus flavus, Drechslera specifera, Penicillium oxalicum, Geotrichum candidum, Curvularia lunata, Aspergillus amstelodami, Talaromyces* sp., and *Chaetomium ochraceum.*

Endophytic fungi are widespread in all phyla of the kingdom Fungi. Most of the endophytic species belong to the phylum *Ascomycota*, and they are often closely related to fungi known to cause diseases, either in healthy tissue or as secondary invaders of damaged tissues (Schardl *et al*., 1997) [23]. It is evident from earlier work that endophytes mostly belong to the phylum *Ascomycota.* Naik *et al.* (2009) [16] reported that *Chaetomium* sp., *Penicillium* sp., *Fusarium* sp. and *Cladosporium cladosporioides* were dominant endophytes of rice in their studies and all of which belong to phylum *Ascomycota*. *Cladosporium* sp. and *Fusarium* sp. were also isolated by Fisher and Petrini (1992) [8] from rice grown under dry and wet conditions. *Cladosporium* sp., *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. were also isolated by Laskar *et al.* (2012) [12] from rice in Assam. Tian *et al.* (2004) [28] also isolated *Fusarium* sp., *Penicillium* sp., and *Aspergillus* sp. from rice.

**Isolation and identification of rice fungal pathogens**

Three locally important fungal pathogens of rice were isolated and identified as *Pyricularia oryzae, Bipolaris oryzae* and *Ustilaginoidea virens* the causal organisms of blast disease, brown spot disease and false smut disease of rice respectively. The pathogens were isolated from the leaves and stems and were purified several times to ensure consistency. The pathogens were identified by observing and studying the nature of disease symptom and its fungal structures observed under microscope

**Antagonistic effect of fungal endophytes of rice on the radial growth of important rice pathogens *in vitro***

Fourteen fungal endophytes *viz. C. cladosporioides, P. citrinum, F. moniliforme, T. asperellum, P. pinophilum, A. niger, A. flavus, D. specifera, P. oxalicum, G. candidum, C. lunata, A. amstelodami, Talaromyces* sp., *C. ochraceum* isolated from rice were screened under *in vitro* condition by using dual culture technique against *Pyricularia oryzae, Bipolaris oryzae* and *Ustilaginoidea virens* the causal organisms of blast of rice, brown spot of rice and false smut of rice respectively.

**Table 1:** Fungal endophytes of rice and their taxonomic position

| Sl. No. | ITCC Ref. No. | Endophyte species | Kingdom | Phylum | Class |
|---|---|---|---|---|---|
| 1. | 9575.14 | *Cladosporium cladosporioides* | Fungi | Ascomycota | Dothideomycetes |
| 2. | 9576.14 | *Penicillium citrinum* | Fungi | Ascomycota | Eurotiomycetes |
| 3. | 9578.14 | *Fusarium moniliforme* | Fungi | Ascomycota | Sordariomycetes |
| 4. | 9580.14 | *Trichoderma asperellum* | Fungi | Ascomycota | Sordariomycetes |
| 5. | 9582.14 | *Penicillium pinophilum* | Fungi | Ascomycota | Eurotiomycetes |
| 6. | 9376.14 | *Aspergillus niger* | Fungi | Ascomycota | Eurotiomycetes |
| 7. | 9377.17 | *Aspergillus flavus* | Fungi | Ascomycota | Eurotiomycetes |
| 8. | 9378.14 | *Drechslera specifera* | Fungi | Ascomycota | Dothidiomycetes |
| 9. | 9380.14 | *Penicillium oxalicum* | Fungi | Ascomycota | Eurotiomycetes |
| 10. | 9381.14 | *Geotrichum candidum* | Fungi | Ascomycota | Saccharomycetes |
| 11. | 9382.14 | *Curvularia lunata* | Fungi | Ascomycota | Euascomycetes |
| 12. | 9383.14 | *Aspergillus amstelodami* | Fungi | Ascomycota | Eurotiomycetes |
| 13. | 9407.14 | *Talaromyces* sp. | Fungi | Ascomycota | Eurotiomycetes |
| 14. | 9408.14 | *Chaetomium ochraceum* | Fungi | Ascomycota | Sordariomycetes |

The effect of all the fungal endophytes significantly differed in terms of inhibition of radial growth of the pathogens (Table 2.) The growth of the pathogens in dual culture plates were observed to progress until they came in contact with leading edges of the fungal endophytes, after which it ceased to grow and only the fungal endophytes continued to grow. The growth of the pathogen was seen to be overrun by the fungal endophytes in many cases. The per cent inhibition over control was calculated 7 days after incubation (DAI) based on the fungal growth in control plate. The results thus obtained have been presented in Table 2. Among the fungal endophytes, *A. niger* was found most promising as antagonist against *P. oryzae* with 79.63 per cent inhibition of radial growth followed by *A. amstelodami* (70.37%), *T. asperellum* (69.81%), and *P. citrinum* (69.63). The per cent inhibition recorded by the rest of the fungal endophytes against *P. oryzae* ranged from 34.44 in case of *C. cladosporioides* to 68.14 in case of *G. candidum.* Per cent inhibition of radial growth of *P. oryzae* by *A. niger in vitro* is significantly different from all other endophytes. However, inhibition of radial growth of *P. oryzae* by endophytes *A. amstelodami* (70.37). *T. asperellum* (69.81), *P. citrinum* (69.63), *G. candidum* (68.14) and *Talaromyces* sp. (64.44) are found to be statistically *at par*. The per cent inhibition of radial growth of *B. oryzae in vitro* was observed highest by the endophyte *T. asperellum* (77.77%) that is closely followed by other endophytes like *A. niger* (76.47%), *A. flavus* (74.81%), *G. candidum* (72.22%) and *C. lunata* (72.22%). The per cent inhibition recorded by the rest of the fungal endophytes ranged between 28.70 in case of *C. ochraceum* and 72.03 in case of A. *amstelodami*. However, inhibition of radial growth of *B. oryzae* by the endophyes *T. asperellum, A. niger, A. flavus, G. candidum, C. lunata* and *A. amstelodami* are found to be statistically *at par*. The per cent inhibiton of mycelial growth of *U. virens* was again observed highest by *T. asperellum* (53.54%) followed by *A. niger* (44.52%), *G. candidum* (44.52%) and *A. amstelodami* (43.86%). The per cent inhibition recorded by the rest of the fungal endophytes against *U. virens* ranged from 19.98 in case of *C. cladosporioides* to 39.33 in case of *A. flavus.* However, inhibition of radial growth of *U. virens* by the endophytes *A. niger, A. flavus, D. specifera, P. oxalicum, G. candidum, C. lunata* and *A. amstelodami* are found to be statistically *at par*.

**Table 2:** Antagonistic effect of fungal endophytes of rice on the radial growth of important rice pathogens *in vitro*

| Fungal endophytes | Radial growth (mm) and per cent inhibition (PI) 7 DAI at 25ºC | | | | | |
|---|---|---|---|---|---|---|
| | *Pyricularia oryzae* | | *Bipolaris oryzae* | | *Ustilaginoidea virens* | |
| | Growth | PI | Growth | PI | Growth | PI |
| *Cladosporium cladosporioides* | 59.00 | 34.44 (35. 93) * | 46.67 | 48.14 (43.93) | 20.67 | 19.98 (26.55) |
| *Penicillium citrinum* | 27.33 | 69.63 (56.66) | 27.17 | 69.81 (56.67) | 18.50 | 28.37 (32.18) |
| *Fusarium moniliforme* | 52.83 | 41.30 (39.99) | 53.17 | 40.92 (39.77) | 19.33 | 25.16 (30.11) |
| *Trichoderma asperellum* | 27.17 | 69.81 (56.67) | 20.00 | 77.77 (61.84) | 12.00 | 53.54 (47.03) |
| *Penicillium pinophilum* | 44.67 | 50.37 (45.21) | 58.83 | 34.63 (36.05) | 16.67 | 35.46 (36.55) |
| *Aspergillus niger* | 18.33 | 79.63 (63.17) | 21.17 | 76.47 (60.98) | 14.33 | 44.52 (41.85) |
| *Aspergillus flavus* | 38.17 | 57.59 (49.37) | 22.67 | 74.81 (59.87) | 15.67 | 39.33 (38.84) |
| *Drechslera specifera* | 35.50 | 60.55 (51.09) | 59.33 | 34.07 (35.71) | 16.50 | 36.12 (36.94) |
| *Penicillium oxalicum* | 39.50 | 56.11 (48.51) | 28.67 | 68.22 (55.69) | 16.00 | 38.05 (38.09) |
| *Geotrichum candidum* | 28.67 | 68.14 (55.64) | 25.00 | 72.22 (58.19) | 14.33 | 44.52 (41.85) |
| *Curvularia lunata* | 39.17 | 56.48 (48.72) | 25.00 | 72.22 (58.19) | 16.33 | 36.77 (37.33) |
| *Aspergillus amstelodami* | 26.67 | 70.37 (57.02) | 25.17 | 72.03 (58.07) | 14.50 | 43.86 (41.47) |
| *Talaromyces* sp. | 32.00 | 64.44 (53.39) | 30.50 | 66.11 (54.40) | 18.17 | 29.65 (32.99) |
| *Chaetomium ochraceum* | 44.50 | 50.55 (45.32) | 64.17 | 28.70 (32.39) | 20.17 | 21.91 (27.91) |
| Control | 90.00 | 0.00 (0.00) | 90.00 | 0.00 (0.00) | 25.83 | 0.00 (0.00) |

| | | | | | | |
|---|---|---|---|---|---|---|
| SEm± | | 2.17 | | 2.27 | | 3.20 |
| CD(p=0.05) | | 6.04 | | 6.30 | | 8.88 |

*values in the parentheses are Arc Sine transformed values. DAI= Days after inoculation

In the present investigation concerning inhibitory effect of fungal endophytes of rice on important rice pathogens *viz. P. oryzae, B. oryzae* and *U. virens*, all the endophytes were reported to have antagonistic effect on the pathogens *in vitro*, though at different levels. It is evident from earlier works that endophytes have antagonistic effect on the plant pathogenic fungi (Liu *et al*., 2001; Tian *et al*., 2004; Park *et al*., 2005; Inacio *et al*., 2006; Kim *et al*., 2007) [13, 28, 17, 10, 11]. The rice pathogen *P. oryzae* was reported earlier to be inhibited by fungal endophytes like *Fusarium* sp., *Penicillium* sp. and *Aspergillus* sp. (Tian *et al*., 2004) [28]. Several endophytes from rice are found to have antagonistic properties against rice fungal pathogens (Li *et al*. 2005, Naik *et al*., 2009) [14, 16]. Rice fungal endophytes *C. cladosporioides*, *Chaetomium* sp., *Fusarium* sp. (Naik *et al*., 2009) [16], *Trichoderma* sp. (Ping *et al*., 2009) [18], *Aspergillus* sp. (Tian *et al*., 2004) [28] have already been shown to have inhibitory activity against various rice pathogens.

The growth inhibition of *P. oryzae, B. oryzae* and *U. virens* in the presence of fungal endophytic species could be attributed to microbial acid production which is important for suppressing plant pathogens (Takijima, 1964; Browning *et al*., 2004; Browning *et al*., 2006) [27, 2-3]. Fungal endophytes are known to produce indole derivatives like 6-isoprenylindole-3-carboxylic acid (Lu *et al*., 2000) [15], siderophore (Li *et al*., 2005) [14], antibiotics (Strobel, 2002; Schulz and Boyle, 2005; Wang *et al*., 2007) [25, 21, 29] which might show growth inhibition properties against different plant pathogenic fungi and constitute a defence mechanism against fungal pathogens of crop plants.

Naik *et al.* (2009) [16] reported that endophytic fungi like *Chaetomium* sp. and *Penicillium* sp. are suitable candidates for the extraction of biologically active compounds. Schulz *et al.* (2002) [22] also reported that fungal endophytes possess exoenzymes necessary to colonize their hosts and that they grow well in apoplastic washing fluid of their hosts. It has been suggested that fungal endophyte-plant host interactions are characterized by a finely tuned equilibrium between fungal virulence and plant defence. There has been no report of inhibition of *B. oryzae* and *U. virens* by rice fungal endophytes earlier. Moreover we also report for the first time the inhibitory activity of rice endophytes *Talaromyces* sp., *C. lunata, G. candidum* and *D. specifera* against the rice pathogens *P. oryzae, B. oryzae* and *U. virens*.

Thus, from the overall results of the present investigation based on the evidence obtained, it can be concluded that fungal endophytes of rice are potential candidates for the management of different rice pathogens and this study paves the way for further exploration towards developing an effective and eco-friendly solution for management of different rice fungal pathogens through plants own endophytes.

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