© 2023, Centre for Advanced Research in Agricultural Sciences
Research Journal of Agricultural Sciences
Volume 14; Issue 02 (Mar-Apr 2023); pp 368–374

Open Access
ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Full Length Research Article

Endophytic Fungi Associated with some Traditional Rice Varieties of Assam and their Antifungal and Plant Growth Promotion Potential

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Abstract

Endophytic fungi serve as an effective biological method for crop improvement by enhancing growth and reduced disease incidence. In the present study, endophytic fungi associated with some traditional rice varieties was investigated for IAA production and antifungal activity against rice pathogens. The endophytic fungal genera isolated were *Fusarium, Curvularia, Colletotrichum, Monilinia, Mucor Aspergillus, Alternaria, Penicillium, Acremonium* and Mycelia Sterilia. Among them, *Aspergillus, Fusarium* and *Curvularia* were dominant genera with highest colonization frequency. The isolates were screened for IAA production and antifungal activity against rice pathogens. The results indicated that amongst the endophyte *Aspergillus fumigatus* produced 119.09 µg/ml of IAA in medium amended with tryptophan. The endophytic fungi *Curvularia lunata* and *Fusarium oxysporum* showed production of IAA of 24.72 µg/ml and 24.27µg/ml respectively in medium without tryptophan. Five endophytic isolates *Fusarium oxysporum, Curvularia lunata* and *Alternaria* sp. showed good zone of inhibition against *Rhizoctonia solani* and *Helminthosporium oryzae*. Our study indicated that there is great prospect in exploration of seed borne endophytic fungi from indigenous rice as bio-inoculants for sustainable agricultural practices.

***Key words*:** Indigenous rice, Endophytic fungi, IAA production, Antifungal activity

The current population of the World is about 7.9 billion and this number is expected to reach 8.6 billion in 2030. The increasing population demands for increasing food supply and to meet such demand, enormous chemical fertilizers are used in the field of agricultural. The excessive use of this chemical fertilizers may provide large amount of food supply but long term use of these substances may cause permanent damage to the fertility of the soil and it's microbial population [1]. Moreover, plant pathogens and pests plays a negative role in the reduction of 30- 50% annual crop yield which is also a great loss that must be overcome to ensure food securities for an ever-growing human population [2]. In this regard, harmful chemical pesticides and fungicides are also used which causes major threats to the soil. In addition, these chemicals can decrease the soil micro-flora sharply and induce tolerance of the pathogens. This matter is related to environmental pollution and thus, it has become a serious concern for the Government as well as for the environmentalists. Therefore, urgent need came into existence to develop safe and environmental-friendly alternative strategies to protect the agricultural land and improve the productivity of crops. To reduce these negative effects, endophytes could serve to be an ideal candidate as a substitute for chemical fertilizer and pesticides. They are eco-friendly and can be used as most suitable biological means that can help the host plant to attain resistance towards the phytopathogens [3]. It has been observed from the last few decades, endophytes has emerged as the most potent alternative sources which are reservoirs of several bio-active agents that plays important role in promoting healthy plant growth and obtaining good yield. Endophytes are endosymbionts, that can be bacteria or fungi, which live inside the internal tissues of the plant without causing any recognizable diseases. They have the ability of colonizing internal tissues and helps in promoting plant growth and also, helps in controlling plant diseases. Thus, endophytes can be used in agriculture as a tool to improve crops performance [4]. The endophytic microorganism produces secondary metabolites that have enormous potentials in secreting phytohormones which could lead to an improved growth of the host plants [5]. These additional concentrations of hormones enhance root and shoot biomass and support the root system of plants which facilitates the acquisition of mineral nutrients from the soil [6] and enable plant roots to increase

Received: 08 Dec 2022; Revised accepted: 14 Feb 2023; Published online: 10 Mar 2023

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Citation: Paul S, Siddique F, Tayung K. 2023. Endophytic fungi associated with some traditional rice varieties of Assam and their antifungal and plant growth promotion potential. *Res. Jr. Agril Sci*. 14(2): 368-374.

nitrogen and phosphorus uptake [7]. Among food crops, rice serves as the chief staple crop that feeds a large portion of the World's population [8-9]. India is among the topmost rice producer in the World [10]. Among the other parts of India, North East (NE) India seeks special attention for its wide variation of rice cultivars. The NE India is specified by high rainfall, humidity, varied topography, and environmental stresses. Being a biodiversity hotspot area, which is also a part of the eastern Himalayan region, North-east serves to be one of the largest reservoirs of traditional rice varieties. Although, rice is one of the primary gramineous crops that constitutes the main nutrient resource of half of the World's population, but the yield is at serious risk when threatened with such diseases as Rice blast disease, Brown spot disease of rice, Sheath Blight of Rice, Bacterial Leaf Blight and Rice Bakanae disease etc. Although numerous traditional varieties of rice is grown in this region but most of which are prone to diseases and produce low yield. With passing days, demand of rice production is increasing whereas the production rate is decreasing due to many factors like rapid industrialization, urbanization and damage caused by various diseases. In order to meet the high demand, rice growers are bound to use harmful chemicals and pesticides which are deterioting the soil fertility causing soil pollution. However, some of the most spectacular varieties of traditional rice has shown good productivity and multi resistance to severe phyto-pathogen naturally [11]. Many investigations have clear evidence that those traditional rice varieties show multiple resistant to various diseases and pests are mainly associated with endophytic fungi. The Bali rice variety of Siang district of Arunachal Pradesh is highly adapted and possess resistance to various rice pathogens and pest [12]. The Konkowa, Karbisaksaw and Bora dhan varieties of Assam are also possess good yield and resistant to pathogens. But however, such varieties showing resistance are expected to harbour novel endophytes that may produce bio-active compounds having antagonistic activity towards those phyto-pathogens. Traditional rice varieties are highly amenable to organic farming that cause less damage to the environment and sustain soil health [13]. Majority of the study conducted on the traditional rice varieties which are disease resistant are found to be in close association with endopytic fungi which helps in the healthy growth and obtaining satisfactory yields. Even though rice endophytes are studied worldwide but the traditional rice varieties of Assam are yet to be explored. Although, fungal endophytes are the future changing tool in the field of agriculture but there is meagre information collected about them. Therefore, the present investigation was carried to isolate endophytic fungi from some selected important traditional rice varieties of Assam and to screen the fungal isolates for plant growth promotion trait (IAA production) and antifungal activity against rice pathogens *Helminthosporium oryzae* and *Rhizoctonia solani*.

MATERIALS AND METHODS

Sample collection and processing

Four different traditional varieties of rice were collected from four different districts of Assam (Table 1). The selection of the rice varieties were done based on their good yield and disease resistance ability. The collection was done during the month of November. Fresh and healthy seeds were collected from the site of collection and were stored in sterile polybags separately for each rice variety. The rice seeds were processed within 48 hours on reaching the laboratory.

Isolation and identification of endophytic fungi

The samples were washed thoroughly in distilled water and dried over blotting paper. The seeds were then subjected to surface sterilization. For surface sterilization the method suggested by Bills [14] and Strobel [15] were followed. The surface sterilized seeds were dried further and inoculated into freshly prepared Potato Dextrose Agar medium (PDA). To suppress bacterial contamination, Streptomycin (100mg/L) was added to each media. The plates were incubated in 28±1 °C for several days until fungal growth appeared on the plate. After obtaining the pure cultures, the endophytic fungi were identified on the basis of their morphological, cultural and reproductive characters of the fruiting bodies by referring to the manuals of Domsch *et al*. [16], Barnett and Hunter [17] and Hypomycetes [18]. Culture that failed to sporulate were categories as Mycelia Sterilia.

Data analysis

Colonization frequency (CF%) of endophytic fungi was calculated using the formula outlined by Hata and Futai [19]:

$$\text{Colonization frequency (\%)} = \frac{\text{Total number of seeds colonized by endophytes}}{\text{Total number of seeds plated}} \times 100$$

Dominance of endophytic fungi (%) was calculated using the formula outlined by Goveas *et al*. [20]

$$\text{Dominance fungi (\%)} = \frac{\text{Total number of seeds yielding each species during isolation}}{\text{Total number of seeds plated}} \times 100$$

Determination and quantification of IAA

To determine the quantity of IAA produced by the isolates, a colorimetric technique was carried out with Van Urk Salkowski reagent using the Salkowski's method [21]. The optical density (OD) was recorded using a UV/Vis spectrophotometer with a wavelength 530 nm after 30 min of incubation. Observation of the colour change was also measured for qualitative assay. The IAA concentration of each isolate was compared to a standard curve for its quantification.

Fungal cultivation and metabolites extraction

The fungal isolates showing good result in IAA production were selected for extraction of their secondary metabolites. The extraction process was carried out by the method outlined by Bhardwaj *et al*. [22]

Determination of antifungal activity

Both *Helminthosporium oryzae* causing Brown spot disease of rice and *Rhizoctonia solani* causing Sheath blight of rice were used as test organisms for the antifungal bioassay. The test organisms were obtained from Assam Agricultural University, Jorhat (Assam), India. Dual culture technique was followed for antagonistic test against rice pathogens on Potato Dextrose Agar (PDA) plates. Five-day-old mycelia disks (5mm diameter) of test pathogens were placed at the centre of the petri-plates containing PDA medium and the selected endophytes were inoculated on four different corners of the Petri plates. Plates were incubated at 28 ± 1 °C for 5days and the activities of inhibition were observed. The inhibition of the growth of fungal pathogen was also determined by Agar well Diffusion method. The crude extracts that were obtained from fungal isolates were dissolved in 100µl of Di-methy Sulphoxide (DMSO) and loaded into the wells (7mm diameter). The plates were incubated at 37˚C for 5-7 days and the zone of inhibition was measured.

Table 1 Rice varieties and their sites of collection from different parts of Assam

Sampling site	District	Name of rice variety	GPS Location
Rangia	Kamrup	Keteki Joha	26°26'N 91°36'E
Jonai	Dhemaji	Gendali Towa	27°48'N 94°58'E
Lakhimpur	Lakhimpur	Ranjit	27°23'N 94°10'E
Goalpara	Goalpara	Upendra black rice	26°25'N 90°21'E

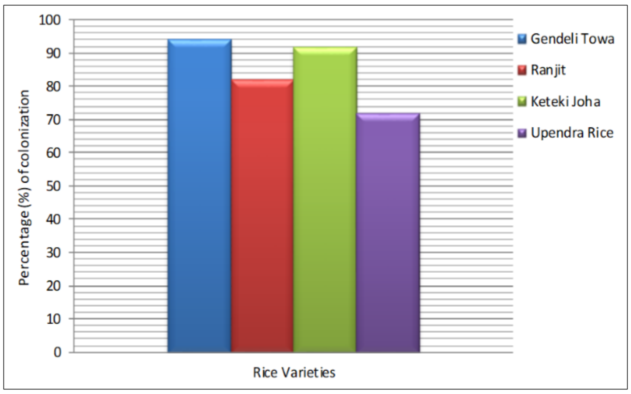

Fig 1 Percentage colonization of fungal endophytes in four different rice varieties

RESULTS AND DISCUSSION

Isolation and Identification of endophytic fungi

In the present investigation, fungal endophytes associated with traditional rice varieties collected from different location of Assam were studied. The name of the rice varieties and their site of collection along with their GPS location are presented in (Table 1). A total of 40 endophytic fungal isolates were obtained from the seed samples of the rice varieties i.e., Keteki Joha, Gendeli Towa, Ranjit and Upendra Black rice. Out of the 40 isolates, 10 isolates were obtained from Keteki Joha, 12 isolates from Gendeli Towa Rice variety, 9 isolates each from Ranjit and Upendra black rice variety. The endophytes isolated from the studied samples belong to fungi of the class Zygomycetes, Ascomycetes and Deuteromycetes. However, the class Ascomyectes was dominant which was followed by Deuteromycetes and Zygomycetes. The isolates consisted of 38 numbers of filamentous and 2 numbers of non-filamentous yeast. The result showed that all the rice varieties were found colonized with endophytic fungi and colonization of each rice variety was found to be diverse. Among the rice varieties, the colonization of endophytic fungi in Gendeli Towa (94%) was found to be highest followed by Keteki Joha and Ranjit (Fig 1). Percentage colonization of Upendra rice variety was found to lowest (70%) among all the studied rice varieties (Fig 1, Table 3). Both the rice varieties i.e., Gendali towa and Upendra black rice were collected from different geographical sites. Gendali Towa was collected from Dhemaji district of Assam whereas Upendra black rice was collected from Goalpara district of Assam. The fungal endophytic genera isolated were *Fusarium*, *Curvularia*, *Colletotrichum*, *Monilinia*, *Mucor*, *Aspergillus Alternaria*, *Penicillium*, *Acremonium* and Mycelia Sterilia. *Fusarium* was the dominant fungal genera present in Keteki Joha while *Aspergillus* was the dominant genera present in both Gendeli towa and Upendra Black Rice (Table 2). The fungal genera *Curvularia* was the dominant in Ranjit rice variety (Table 2).

Table 2 Percentage dominance of endophytic fungal genera in different rice varieties

Endophytic fungi	Dominance (%)			
	KJ	GT	RT	UBR
Acremonium sp.	-	2.1	-	-
Alternaria sp.	-	6.3	-	13.8
Aspergillus sp.	-	44.6	29.2	41.6
Colletotrichum sp.	8.8	-	12.2	-
Curvularia sp.	35.5	12.7	36.6	-
Fusarium sp.	51.1	8.5	14.6	-
Monilinia sp.	6.6	-	-	-
Mucor sp.	2.2	-	-	-
Penicillium sp.	-	6.3	-	-
Mycelia Sterilia KJ1	-	8.5	-	-
Mycelia Sterilia KJ2	-	8.5	-	-
Mycelia Sterilia KJ3	2.2	-	-	-
Mycelia Sterilia KJ4	-	-	-	8.3
Mycelia Sterilia KJ5	-	-	-	2.7
Mycelia Sterilia KJ6	2.2	-	-	-
Mycelia Sterilia KJ7	-	2.1	-	-
Mycelia Sterilia KJ8	-	-	-	-
Mycelia Sterilia KJ9	-	-	2.4	-
Mycelia Sterilia KJ10	-	-	2.4	-

KJ= Keteki Joha, GW= Gendeli Towa, RAN= Ranjit, UBR= Upendra Black Rice

IAA production by endophytic isolates

IAA production by the endophytic fungal isolates was determined for both tryptophan dependent and independent broth cultures. Out of 25 endophytic fungal isolates, 18 isolates shown the pink colouration with different concentration which clearly indicates the Indole-3-acetic acid (IAA) production (Table 4). The concentration of the pink ranges from light to deep pink or light to dark brown. The optical density of each isolates was recorded at 530 nm after 30 mins with the help of a UV/Vis spectrophotometer. The quantity of Indole-3-acetic acid (IAA) production was easily determined from the standard curve prepared with known concentrations of Indole-3-acetic acid (IAA). By plotting the optical density (OD) of each isolates in the standard curve equation, the quantity of IAA produced can be detected. The quantity of IAA produced is measured in µg per ml. Among the 18 isolates showing IAA production, 3 isolates that produced the highest amount of IAA were *Aspergillus fumigates* (119.09 µg/ml), Unidentified sp2 (88.63

µg/ml) and Unidentified sp1 (65.27 µg/ml) when supplied with L-tryptophan. It is quite interesting to mention that some of the isolates were even able to produce IAA without any precursor i.e., L-tryptophan. *Curvularia lunata* (24.72µg/ml), *Fusarium oxysporum* (24.27 µg/ml) and *Curvularia* sp.1 (18.18 µg/ml) had shown good amount of IAA without amendment of L-tryptophan in the growth medium (Table 4). Similarly, some other isolates were also able to produce IAA without tryptophan and the range of concentration of IAA production was different variable.

Table 3 Occurrence of the Endophytic fungi in different rice varieties of Assam

Endophytic fungal genera	Number of Fungal Isolates in different rice varieties				
	KJ	GW	RAN	UBR	CF (%)
Fusarium	23	04	06	00	16.5
Curvularia	11	06	15	00	16.0
Aspergillus	00	21	12	15	24.0
Colletotrichum	04	00	05	00	4.5
Monilinia	03	00	00	00	1.5
Mucor	01	00	00	00	0.5
Alternaria	00	03	00	05	4.0
Penicillium	00	00	00	00	1.5
Acremonium	00	00	00	00	0.5
Mycelia Sterilia KJ1	00	00	04	00	2.0
Mycelia Sterilia KJ2	00	00	04	00	2.0
Mycelia Sterilia KJ3	01	00	00	00	0.5
Mycelia Sterilia KJ4	00	00	00	02	1.0
Mycelia Sterilia KJ5	00	00	00	01	0.5
Mycelia Sterilia KJ6	01	00	00	00	1.5
Mycelia Sterilia KJ7	01	00	00	00	1.5
Mycelia Sterilia KJ8	00	01	00	00	1.5
Mycelia Sterilia KJ9	00	00	01	00	1.5
Mycelia Sterilia KJ10	00	00	01	00	1.5

KJ= Keteki Joha, GW= Gendeli Towa, RAN= Ranjit, UBR= Upendra Black Rice
CF (%) = Colonization frequency. No of seeds plated per variety = 50

Table 4 Indole Acetic Acid (IAA) production by endophytic F = fungi with and without tryptophan

Endophytic fungal isolates	IAA Concentration (µg/ml)	
	With Tryptophan (100 µg/ml)	Without Tryptophan
Alternaria sp.	6.81	--
Aspergillus flavus	9.90	6.45
Aspergillus fumigates	119.09	--
Colletotrichum sp	8.54	--
Curvularia ahvazensis	16.45	9.18
Curvularia asianensis	13.63	7.72
Curvularia lunata	34.18	24.72
Curvularia sp1	20.00	18.18
Curvularia sp2	21.81	17.63
Fusarium metavorans	6.45	--
Fusarium oxysporum	8.90	24.27
Fusarium solani	7.18	--
Monilinia sp	6.54	--
Mycelia sterilia KJ1	7.90	--
Mycelia sterilia KJ2	7.27	--
Penicilium chrysogenum	15.45	8.72

Table 5 Antifungal activities of potent fungal isolates against *Rhizoctonia solani*

Endophytic fungi	Zone of inhibition (mm)
Fusarium oxysporum	22.33 ± 1.16
Mycelia sterilia KJ1	23.00 ± 2.00
Curvularia lunata	18.33 ± 0.57
Mycelia sterilia KJ2	19.33 ± 1.53
Alternaria sp.	17.33 ± 2.08

n = 3; ± =Standard deviation

Antifungal activity of the fungal endophytes

The antifungal activity showed that out of 18 endophytic fungal isolates, 14 isolates shown good antifungal against *Helminthosporium oryzae* and 12 of the isolates showed resistance against *Rhizoctonia solani*. The isolates that showed good antifungal activities against *Rhizoctonia solani* in dual-culture assay was further determined by agar-cup diffusion assay with crude extract metabolites. The result indicated out of the 12 isolates, 5 of them showed promising antifungal activity against the test pathogen. The five isolates were *Fusarium oxysporum*, Mycelia Sterilia KJ1, *Curvularia lunata*, Mycelia Sterilia KJ2 and *Alternaria* sp. All the 5 isolates showed clear zone of inhibition against *Rhizoctonia solani* (Fig 3). The zone of inhibition shown by each isolate was measured and recorded Amongst the isolates, Mycelia Sterilia KJ1 showed the highest zone of inhibition of (23.00± 2.00) mm followed by *Fusarium oxysporum* (22.33 ± 1.16 mm) and Mycelia Sterilia KJ2 (19.33±1.53 mm) (Table 5). *Curvularia lunata* and *Alternaria* sp have also showed considerable zone of inhibition against *Rhizoctonia solani*.

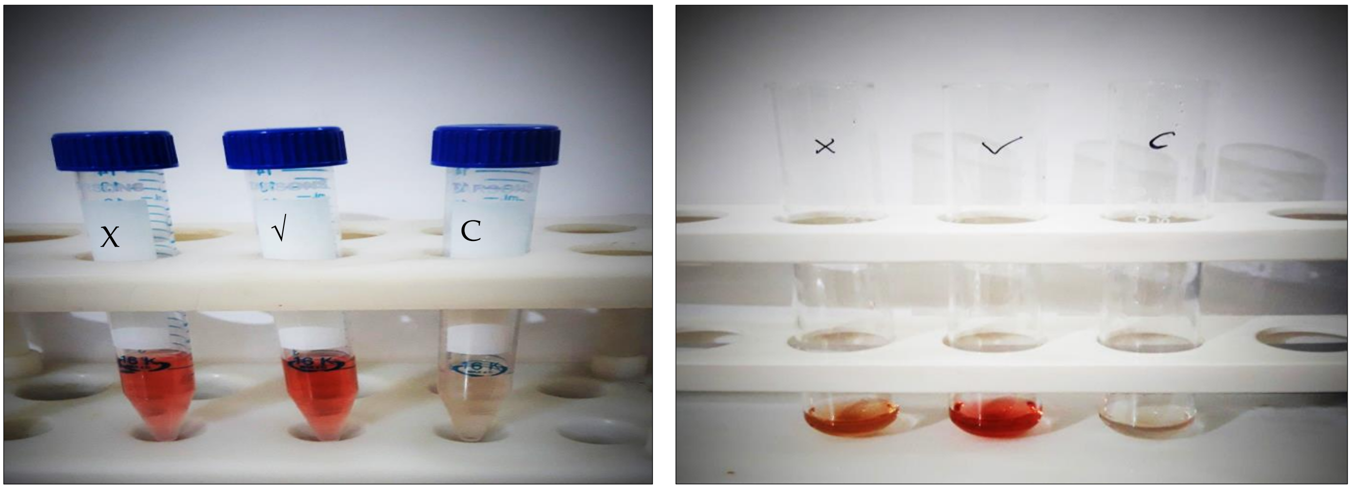

Fig 2 Indole Acetic Acid production (× indicates no tryptophan and √ indicates tryptophan added)

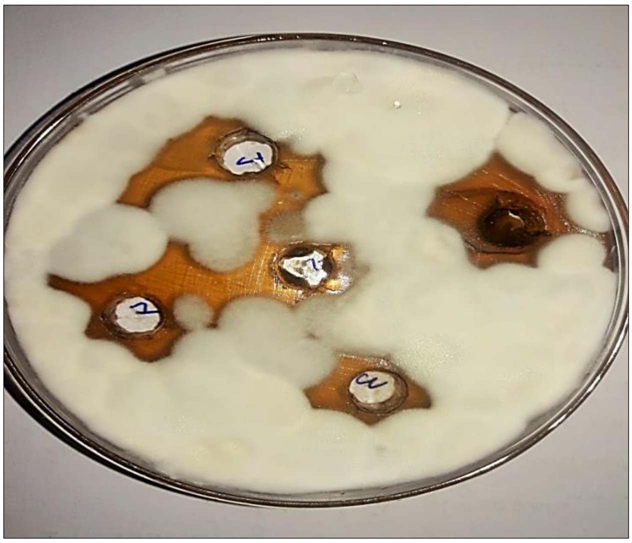

Fig 3 Antifungal activities of potent fungal isolates against *Rhizoctonia solani*

In the present investigation, fungal endophytes isolated from the seeds of some traditional rice varieties were studied and similar work was reported by Atugala and Deshappriya [5]. The colonization of endophytic fungi of different rice varieties differs within geographical sites [23]. Despite great diversity and abundance of endophytes in traditional rice varieties of North East India, only a few of them were studied so far. In the present study, endophytic fungi isolated from traditional rice varieties were fungi of the genera *Fusarium, Curvularia, Aspergillus, Penicillium, Acremonium, Alternaria, Monilinia, Colletotrichum,Mucor* and Mycelia Sterilia. Our present finding collaborates with the results reported by Atugala and Deshappriya [5]. The present study revealed Keteki Joha rice variety was dominated by endophytic fungus, *Fusarium oxysporum* with high rate of colonization while in case of Gendeli Towa rice seed variety was found colonized which diverse fungal endophytes. *Aspergillus fumigatus* was the most dominating species with high colonization frequency in Gendeli Towa rice variety. Similar reports were also obtained by Cao and Zhou [24]. Amongst the four rice varieties, two rice seeds were hybrid varieties namely Ranjit and Upendra black rice. Similarly, endophytic fungi associated with hybrid rice were also studied by previous worker [5]. In Ranjit rice variety, the most dominating fungal gemera was *Curvularia* which was previously reported to be dominatant endophyte in the investigation of Lalngaihawmi *et al*. [25]. Two unidentified species were isolated from Upendra black rice varieties and similar results were also investigated by Atugala and Deshappriya [5]. Out of the 40 fungal endophytic isolates, 25 of the morphologically distinct isolates were screened for Indole Acetic Acid Production. The screening for IAA production was done using broth culture of the isolates with and without the supply of tryptophan (Fig 2) and the similar kind of work was reported by Mohite [26]. Out of 25 isolates studied, 18 isolates had shown positive response towards Indole Acetic Acid Production and among them 3 of the isolates produced the highest amount of IAA i.e., *Aspergillus fumigatus* 119.09 µg/ml of IAA followed by unidentified sp2 with 88.63µg/ml of IAA and Unidentified sp1 with 65.27µg/ml of IAA. The results were in accordance to many previous workers [27]. Out of 25 isolates, 9 of the isolates had shown the production of IAA without any supply of tryptophan in the culture medium. Among the 9 isolates, *Curvularia lunata* had shown highest production of IAA without tryptophan i.e., 24.72 µg/ml, followed by *Fusarium oxysporum* (24.27µg/ml) and Unidentified sp1 (20.36 µg/ml) (Table 4). This result was similar to report made by Mohite [26]. Some of the isolates that showed positive for IAA also possess good anti-fungal activities against the test phytopathogens.

Among the 40 morphological distinct isolates, 18 isolates were selected for the preliminary screening for antifungal activity using Dual-culture method against *Rhizoctonia solani* (Sheath blight of rice) and *Helminthosporium oryzae* (Brown spot of rice). Similar investigations were also reported by several workers where endophytic fungal isolates from rice have demonstrated antifungal activities against rice pathogens [28], [24]. Out of the 18 isolates, 14 of them have shown good result against *Helminthosporium oryzae* and 12 of them have shown resistance against *Rhizoctonia solani*. Similar results were also observed by many previous workers [28-29]. Among the isolates that showed good antifungal activities against *Rhizoctonia solani* during the preliminary screening, 5 of the isoloates were selected for Agar-cup Diffusion Assay. These five isolates are *Fusarium oxysporum*, Mycelia Sterilia KJ1, *Curvularia lunata*, Mycelia Sterilia KJ2 and *Alternaria* sp, and all of them has shown clear zone of inhibition against *Rhizoctonia solani* (Fig 3) (Table 5). Our findings were similar to reports made by Ratnaweera *et al*. [30]. The evaluation depends on the capability of these fungal endophytes to produce compounds having anti-fungal activities confirmed the potentiality of these groups for screening of bioactive natural products. Therefore, endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential. It is a known fact that endophytic fungi existing in the plant are potential sources of antimicrobial substances [31]. The indication of peak (λ-max) in the crude metabolites of the endophytic isolates of class Mycelia Sterilia KJ1 suggested the presence of active substances, which could be purified for the anti-fungal agents. From the present investigation, it can be concluded that endophytic fungi associated with the rice seed help in the growth and development of host plant and also helps in disease resistance against plant pathogens. Similar antifungal activity against phyto-pathogens by fungal endophytes had been reported mostly from the rice plants. Thus, it can be attributed that the anti-fungal properties of most of the rice plants is not only the contribution of the plant but the metabolites secreted by the endophytic fungi colonizing the host plant. In our present investigation, we found that the metabolites produced from the endophytic fungi possess anti-fungal activities against various rice pathogens which may otherwise lead to poor yield and low productivity. This shows its potentiality in contribution towards biological control of phyto-pathogens that can help in increasing the yield of the rice plant naturally without using harmful chemicals and fertilizers. Even though rice endophytes are studied worldwide but the traditional rice varieties particularly from Assam are yet to be explored. Moreover, the rice varieties of Assam are severely attacked by Blast, Brown spot and Sheath blight diseases resulting in poor development of the plant with decrease in its yield. Therefore, the present investigation was to screen the endophytes for their ability to promote growth and development of the rice plant varieties of Assam along with disease resistance capabilities against rice pathogens.

CONCLUSION

The present study on fungal endophytes associated with rice seeds that showed positive response for Indole-3-acetic acid (IAA) production and anti-fungal activities against phyto-pathogens indicate that they can be used and exploit for biofertilizers and or biocontrol agents in sustainable agricultural practices.

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