Original Research Article

Endophytic *Bacillus* and *Xylaria*sp. inhibittwo major rice pathogens

ABSTRACT

Aim: Endophytes living inside the plant tissues are known to play a crucial role in the physiology and developmental functioning of host plants. The aim of the study was the selection of natural suppressors of the brown spot and sheath blight diseases in rice. In this study, three bacterial endophytes *viz*.,*Bacillus subtilis* isolate PAL 11, *B. altitudins* isolate TSL-4 and *B. altitudinis* isolate KAM-11 and one endophytic fungal isolate *Xylaria* sp. isolate KTD-2 were tested for their potential to inhibit *Bipolaris oryzae* and *Rhizoctonia solani* under *in vitro* conditions.

Methodology:*In vitro* evaluation of endophytes against two major rice pathogens viz., *Rhizoctonia solani* and *Bipolaris oryzae* causing sheath blight and brown leaf spot respectively were tested by dual culture technique.

Results: All the tested bacterial and fungal endophytes significantly inhibited both the pathogens under *in vitro* conditions. Among the bacterial endophytes, *B. subtilis* isolate PAL-11 exhibited highest inhibition of both the pathogens. The mycelial inhibition of *B. oryzae* was 56.04 and 74.48 per cent respectively at 5 and 8 days after dual culturingwhereas the inhibition of *R. solani*was80.31 and 80.22 per cent respectively at 3 and 5 days after dual culturing. The fungal endophyte *Xylaria* sp. isolate KTD-2 exhibited 63.2 and 67.95 per cent inhibition of mycelial growth of *B. oryzae*at 5 and 8 days respectively after the pathogen inoculation. The mycelial growth of *R. solani* was also significantly inhibited (54.95 %) by thefungal endophyte at 5 days after pathogen inoculation.

Conclusion: In view of the potential threats caused by the use of fungicides, the bacterial and fungal endophytes tested pave the way for sustainable disease management of rice in an eco-friendly manner.

Key words: Endophytes, Bacillus sp., Xylaria sp., Brown spot, Sheath blight

INTRODUCTION

Rice is the major food crop that feeds half of the world's population [1]. More than 3.5 billion of the world population depends on rice for 20 per cent or more of the daily calorie intake [2]. In view of the alarmingly growing human population, the demand for rice is predicted to rise further. However, rice crop is highly susceptible to biotic and abiotic stresses, leading to significant losses in grain yield and quality [3]. Pest and disease incidences cause severe primary and secondary yield loss ranging from 26 to 38 per cent. Among these, rice diseases alone account for 16 per cent yield loss under various systems of rice cultivation[4]. Rice is infected by more than 70 diseases which are caused by fungi, bacteria, viruses and nematodes [5] during the whole growth period leading to wide spread use of chemical pesticides for disease control. The major diseases causing severe yield loss in rice are blast, sheath blight, brown spot and bacterial leaf blight.

Sheath blight caused by the necrotrophic fungus *Rhizoctonia solani* Kuhn is one of the most serious diseases of rice worldwide, causing considerable yield loss [6]. Among the fungal diseases causing significant yield loss in rice, sheath blight is ranked as the second most important disease after rice blast. Use of high yielding semi dwarf cultivars with dense planting and high dose of nitrogenous fertilizers accelerate the incidence of sheath blight in rice [7]. *R. solani* can infect the plant at any growth stage. The yield loss ranging from 4 to 50 per cent have been reported depending on the crop stage at the time of infection, severity of the disease and environmental conditions [8]. Its diverse host range and ability to remain dormant under unfavourable conditions make the pathogen more difficult to manage

Brown spot of rice caused by *Bipolaris oryzae* is one of the disastrous fungal pathogens causing huge production losses in rice crop [9]. The pathogen attacks the crop from seedling to milk stage and infects leaves, leaf sheath, coleoptile, panicle branches, glumes, and spikelets[10]. *B. oryzae* infects the coleoptiles, leaves forming oval, dark brown lesions, which ultimately destroy the leaves [11]. Apart from reducing plant vigour, number of grains per panicle and kernel weight, it also causes grain discoloration at maturity, thus reducing market value [12]. The infection especially occurs in environments where water supply is scarce and it is often combined with imbalances in plant mineral nutrition, especially the lack of nitrogen and results in yield losses in the range of 4 - 56 per cent [13]. But the epidemics

caused by *B. oryzae* led to Bengal famine during 1942- 43, where 50 to 90 per cent rice grain was lost and about two million people starved to death [14].

Lack of adequate level of resistance, pathogenic variability, diverse host range and ability to survive under unfavourable conditions make these pathogens difficult to manage. Even though many fungicides can manage brown spot and sheath blight to an extent, searching for beneficial microorganisms for biocontrol will improve sustainable integrated pest management. In addition, the use of chemicals is raising several public concerns such as the emergence of fungicide resistant strains, environmental pollution, residual toxicity, soil quality reduction, natural ecosystem damage, and human health issues as well demanding alternate options to chemical management. In the present day scenario, where everyone is shifting towards organic cultivation due to the harmful side effects of the pesticides, use of endophytes plays an important role in the management of different diseases of rice crop

A diverse range of microorganisms dwell in various parts of a plant. In plant-microbe interactions, some microbes exert deleterious effects but most turn out to be highly beneficial to the hosts. Among these, endophytes are diverse plant symbionts that dwell within the host plant tissues without causing any disease symptoms. One of the most important contributions of endophytic mycobiota in agriculture is their antagonistic potential against a broad spectrum of pathogens, insects, and nematodes by different mechanisms including competition, production of antimicrobial compounds and inducing systemic resistance in host plants [15]. Plant benefits extensively by harboring these microorganisms as they promote plant growth as well. These microbes can be exploited for managing pathogens in a sustainable way. The development of biocontrol strategies using endophytes is an emerging area in crop protection to reduce the damage caused by plant pathogens in economically important crops. Therefore, the present investigation was made to study the biocontrol potential of bacterial and fungal endophytes against *R. solani* and *B. oryzae* in *in vitro* conditions.

MATERIALS AND METHODS

Isolation, purification and maintenance of *B. oryzae*

Diseased plants showing typical brown spot symptoms were collected from Integrated Farming System Research Station (IFSRS), Karamana, Thiruvanantha-puramof Kerala Agricultural University. The samples were collected in clean polythene bag and brought to the laboratory for isolation of the pathogen. Samples were thoroughly washed with tap water and then theinfected plant parts were cut in such a way that the leaf bits contain 50 per cent area of healthy part and 50 per cent from the lesion. The leaf bits were then surface sterilized with sodium hypochlorite (1%) for 1 minute followed by rinsing with sterile water with three changes of water. The leaf bits were blot dried on sterile blotting paper and placed on PDA medium aseptically. The inoculated plates were incubated at 25-28ºC for 4-5 days. The fungal mycelia emerging from the leaf bits were sub cultured on PDA medium. The pure culture of *B. oryzae*was obtained by single spore isolation technique[16] and pathogenicity was confirmed by following Koch's postulates. The purified culture was preserved at 4ºC for further use.

Isolation, purification and maintenance of *R. solani*

Diseased plants having typical symptoms of sheath blight were collected from the fields of IFSRS Karamana and the pathogen was isolated following the surface sterilization procedure as mentioned above. The fungal mycelia emerging from the incubated leaf bits were sub cultured on PDA medium. The culture was further purified by hyphal tip method [17].The pathogenicity was confirmed by following Koch's postulates. The purified culture was preserved at 4ºC for further use.

Morphological and cultural characterisation of *B. oryzae* and *R. solani*

Cultural and morphological characterization *B. oryzae* and *R.solani*were carried out in PDA medium. Fungal discs were inoculated on a fresh PDA mediumand incubated at room temperature for 7 days. Mycelial growth, texture, colour and sporulation were observed. For sporulation of *B. oryzae*, alternate light and dark periods and ultraviolet radiations were provided. The sclerotial emergence, size, shape, colour and distribution pattern were also observed in the case of *R. solani*. Mycelia and asexual spores were examined under the microscope for identification of the pathogens [18].

In vitro* evaluation of the endophytes against *B. oryzae* and *R. solani

Endophytic bacterial cultures of *Bacillus subtilis* isolate PAL 11 (Acc. No. OR81210), *B. altitudins* isolate TSL-4 (Acc. No. OR81212) and *B. altitudinis* isolate KAM-11 (Acc. No. OR81213) and endophytic fungal isolate *Xylaria* sp. isolate KTD-2 (OR787561) maintained at Plant Pathology laboratory atIFSRS Karamana, Kerala Agricultural University were used for the study. The bacterial cultures preserved in glycerol stock at -20ºC were sub-cultured on Tryptic soy agar (TSA) and the fungal isolate stored in PDA slants wassub-cultured on fresh PDA medium for the study.

Evaluation of the endophytic bacteria against *B. oryzae* and *R. solani*

*In vitro*evaluation of the bacterial endophytes against *B. oryzae* and *R. solani*was checked by dual culture technique[19]. TSA and PDA mediamixed in 1:1 proportion was used for the study. Culture disc (5 mm) from an actively growing culture of the pathogen was placed at the centre of the mixed medium in a Petri plate. Each bacterial antagonist was streaked at two sides of the pathogen disc 2.5 cm away from the centre.Culture disc without antagonistic bacteria served as the control. Five replications were maintained for each pathogen and the plates were incubated at 28±2ºC. The growth of the pathogen was recorded both in the treatment plate and control plate. In the case of dual culture with *R. solani,* observations were recorded at 3 and 5days after incubation; whereas in case of *B. oryzae*, observation were recorded 5 and 8 days after incubation.

The results were expressed as percent inhibition of the mycelial growth of the pathogen over control [20].

$$\text{Inhibition percentage (\%)} = \frac{c - t}{c} \times 100$$

Where,

c = Growth of the pathogen in the control plate

r = Growth of the pathogen in the treatment plate

Evaluation of the endophytic fungus against *B. oryzae*and *R. solani*

The antagonism of the endophytic fungus *Xylaria*sp. isolate KTD-2 against the pathogens *B. oryzae* and *R. solani*was also checked by dual culture technique[19].5 mm diameter culture disc of the endophytewas placed at one end of the Petri plate, 1

cm away from the edge. After 3 days of incubation, culture disc (5 mm)of the pathogen was placed at the opposite side, 1 cm away from the edge. Pathogen without antagonistic fungi served as the control. Five replications were maintained for each pathogen and the plates were incubated at 28±2 ºC. Growth of the pathogen was recorded both in the treatment plate and control plate. In the case of dual culture with *R. solani*observations were recorded 3 and 5days after incubation where as in case of *B. oryzae*, observations were recorded 5 and 8 days after incubation. The inhibition of pathogen growth by the fungal endophyte was calculated as above.

RESULTS AND DISCUSSION

Morphological and cultural characterisation of *B. oryzae* and *R. solani*

*B. oryzae*initially produced greyish white mycelium turning to dark grey when fully grown (Fig. 1). The mycelia arefluffy on the PDA medium and took8-9 days for attaining full plate growth. Conidiophores and conidia were septate. Conidia are brown in colour with 5-10 septations and its shapevaried from oval to cylindrical, slightly curved with a bulge in the middle and tapering towards the ends(Fig 2).Many previous research findings explained that *B. oryzae* isolates generally show different morphological characters in culture. In a study conducted to isolate and characterize *B. oryzae* from nine states across India, 17 isolates were formed into four groups such as black with fluffy growth, grey with fluffy growth and white spots, grey with fluffy growth and grey with suppressed growth based on colony morphology and growth pattern [21]. Similarly, *B. oryzae* isolated from Fogera area of Ethiopa was grouped into nine categories based on its colony morphology [22]. The conidia of *B. oryzae*are generally 5-10 septate with oldest conidia at the base [23]. The conidia are brownish in colour, slightly curved towards the end and broadest in the middle [24].

In the present study*R. solani*presented fast growth on the PDA medium and reached 9 cm growth within 3-4 days. The mycelia were white to cream in colour. The growth pattern of the mycelia on the medium were flat and also produced moderate quantity of aerial mycelium on the colony surface as well as on the lid and produced some sclerotia on the lid.The sclerotial emergence started after 96 h and majority of sclerotia were irregular in shape and some were spherical.The

sclerotialcolour was white at first and became light to dark brown after maturation and exuded droplet on the sclerotial surface. The sclerotia were firmly attached to the hyphae and producedsuperficially at the periphery of the Petri plate (Fig. 3). Many of the previous research findings stated that *R. solani*exhibited a range of morphological variations in culture. The mycelia colour varied from white to cream to brown and dark brown in many isolates of *R. solani*[25]. Mycelial growth nature of *R. solani* isolates varied between areal and flat growth patterns [26]. The mycelial growth is comparatively faster in *R. solani*. The growth was rapid if mycelia have grown to Petri dish diameter (90 mm) on the third day, slow if mycelial growth is less than 90 mm on the third day[27]. *R. solani*, isolated from infected rice plants from Bangladesh, exhibited significant variation in sclerotial size, shape and distribution. The brown to black sclerotia having exuded droplet were attached superficially on the mycelium. The distribution of sclerotia within the colonies was highly variable. Sclerotia were produced either near the inoculum or peripheral region or near the margin or scattered and so on. Some isolates produced a moderate quantity of aerial mycelium on the colony surface as well as on the lid and produced at least some sclerotia on the lid [28]. In a study on the diversity of field isolates of *R. solani* from sheath blight-infected rice fields of south India, most of the isolates (75.4%) were fast growers and reached 90mm radial growth on PDA within 48h. The sclerotial emergence was started after 72h in majority of the isolates and the sclerotia matured from fourth day onwards. Two types of sclerotia i.e.micro andmacro sclerotia with light and dark brown colour, were observed. Two types of sclerotial attachments, i.e. firm and loose, were observed in the study. Sclerotia shape also varied from irregular to spherical or globose [29].

Fig. 1. Pure culture of *B. oryzae*

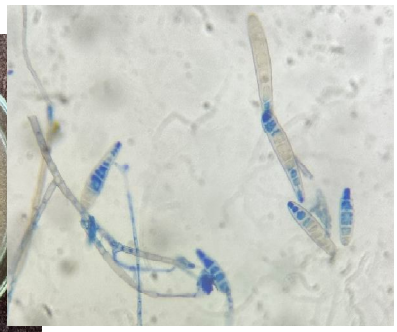

Fig. 2. Conidia and Conidiophores of *B. oryzae*

Fig. 3. Pure culture of *R. solani*

Evaluation of endophytic bacteria against *B. oryzae* and *R. solani*

All the tested bacterial endophytes significantly inhibited the mycelial growth of both the pathogens in dual culture. The highest inhibition of mycelial growth of *B. oryzae* was exhibited by the bacterial endophyte *B. subtilis* isolate PAL-11 and it was 56.04 per cent and 74.48 per cent respectively at 5 and 8 days after incubation followed by *B. altitudinis* isolate TSL-4 with 47.80 and 71.15 per cent inhibition at 5 and 8 days after incubation respectively (Table 1) (Fig. 4). In the case of dual culture of endophytes with *R. solani* also, the maximum inhibition of 80.31 and 80.22 per cent respectively at 3 and 5 days after inoculation was obtained when the bacterial endophyte *B. subtilis* isolate PAL-11 was used. It was followed by *B. altitudinis* isolate TSL-4 with 57.31 and 67.69 per cent inhibition respectively at 3 and 5 days after inoculation (Table 2) (Fig. 5).

Plants are constantly involved in interactions with a wide range of endophytic microorganism living inside the plant system. These endophytes are sheltered from environmental stresses and act as plant growth promoters and biocontrol agents [30]. *Bacillus* species are among the most common species of bacteria that found to colonize plants endophytically [31]. Several investigators successfully applied *Bacillus* spp. to control diseases in many crops including rice. The antagonistic potential of endophytic *Bacillus* spp. against important rice diseases have already been investigated including *B. subtillus against Pyriculariaoryza*[32], *B. amyloliquefaciens, B. methylotrophicus* and *B. subtilis* against *Xanthomonas oryzae* pv*. oryzae*, *B. subtilis* against *R. solani*[33], *B. velezensis*against *B. oryzae*[34] and

so on. *B. velezensis* LS123N isolated from rice exhibited control against multiple rice pathogens in planta assays including bacterial blight, rice blast, bakanae disease, *Pythium* disease, and brown spot disease. Field trials further demonstrated that LS123N significantly reduced naturally occurring brown spot disease in both seedlings and mature rice plants, and increased rice yields [34]. Similarly, endophytic *B. subtilis and B. amyloliquefaciens*isolated from rice plants significantly inhibited*B. oryzae* in *invitro* studies and controlled the brown spot incidence in the field conditions. Further the consortia developed from the two endophytes were also found to be effective in managing the disease [35]. In a similar experiment conducted to evaluate the efficacy of endophytes to control seed borne pathogens of rice, *Bacillus* spp. were more effective in managing the diseases including brown spot and bacterial leaf blight [36]. Adding to these findings, in the present investigation also, *B. oryzae*was significantly inhibited by *Bacillus* spp.

R. solani inciting sheath blight of rice has emerged as an economically important rice pathogen causing considerable yield loss. Unfortunately, at present, there is no known rice variety which is either immune or havinghigh degree of resistance to sheath blight disease [37]. Therefore, biological control can be a potent option. In the present study all the bacterial endophytes tested were found to inhibit the *R. solani* in dual culture. Many previous investigations support these findings. For example,in a research study, out of all the isolated endophytesfrom rice, *B. subtilis* var. *amyloliquefaciens* (FZB24) resulted in maximum inhibition (36 %) of *R. solani*in *in vitro*study and rice plants treated with this endophyte exhibited 55 per cent reduction of disease over control under glasshouse conditions along with increase in secretion of defense related enzymes [38]. Similarly, plant growth promoting *B. subtilis* isolated from soil showed 80.73 per cent inhibition of mycelial growth of *R. solani* in dual culture and lowest disease severity when paddy seeds were treated in pot culture experiment [39]. *B. altitudinis*isolated from *Ocimumtenuiflorum*when challenged inoculated with *R. solani* in rice had significantly reduced sheath blight disease incidence, infected tillers, recorded maximum induction of defense-related enzymes (phenyl ammonia lyase, peroxidase, and polyphenol oxidase), and enhanced dry matter accumulation [40]. The antagonistic potentialof *B. altitudinis*against rice blast disease was also already reported[41].

Some other endophytic *Bacillus* spp. such as *B. methylotrophicus*[42]and *Lysinibacillussphaericus*[43]) were also found to inhibit*R. solani*.

Table 1. Inhibition of mycelial growth of*B. oryzae* by endophytic bacteria,*Bacillus* spp.

Sl. No.	Treatments	Mycelial growth of *B. oryzae* (cm)			
		5 days after inoculation		8 days after inoculation	
		B. oryzae diameter (cm)	Inhibition percentage (%)	*B. oryzae* diameter (cm)	Inhibition percentage (%)
1	*B. subtilis* isolate PAL-11	2.00	56.04	2.28	74.48
2	*B. altitudinis* isolate TSL-4	2.38	47.80	2.58	71.15
3	*B. altitudinis*KAM-11	2.45	46.15	2.70	69.75
4	Control	4.55	0.00	8.93	0.00
	S.Ed CD (0.05%)	0.08 0.17		0.08 0.18	

Table 2. Inhibition of mycelial growth of *R. solani*by endophytic bacteria, *Bacillus* spp.

Sl. No	Treatments	Mycelial growth of *R. solani* (cm)			
		3 days		5 days	
		R. solani diameter	Inhibition percenta	*R. solani* diameter	Inhibition percenta

		(cm)	ge (%)	(cm)	ge (%)
1	*B. subtilis* isolate PAL-11	0.95	80.31	1.78	80.22
2	*B. altitudinis* isolate TSL-4	2.05	57.51	2.90	67.69
3	*B. altitudinis* KAM-11	2.15	55.44	3.05	66.02
4	Control	4.83		8.98	
	S.Ed CD (0.05%)	0.08 0.18		0.05 0.11	

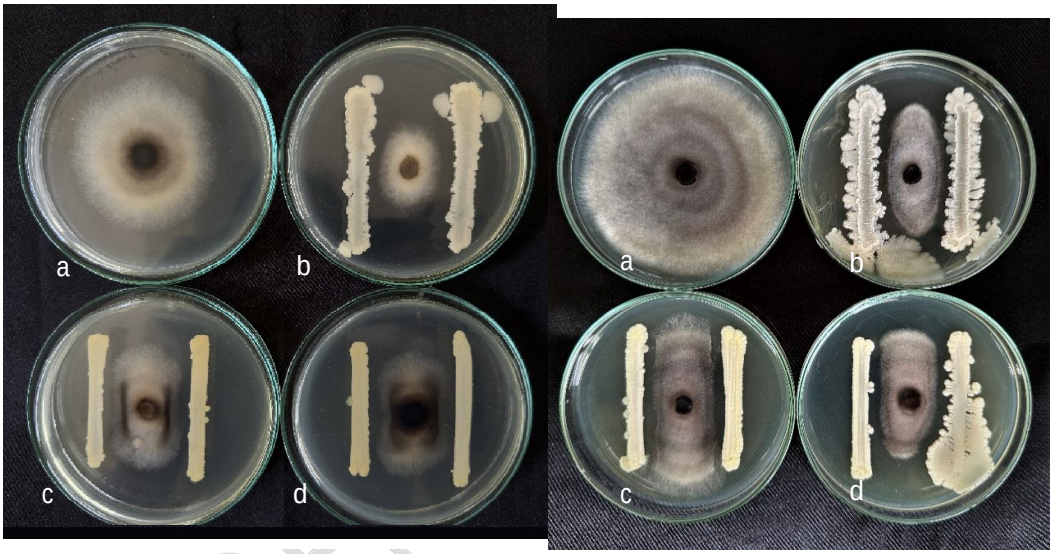

Plate 1 **Plate 2**

Fig 4: Inhibition of mycelial growth of *B. oryzae* by endophytic bacteria at 5 days (plate 1) and 8 days (plate 2) after incubation; a. control, b. *B. subtilis* isolate PAL-11, c. *B. altitudinis* isolate TSL-4, d. *B. altitudinis* KAM-11

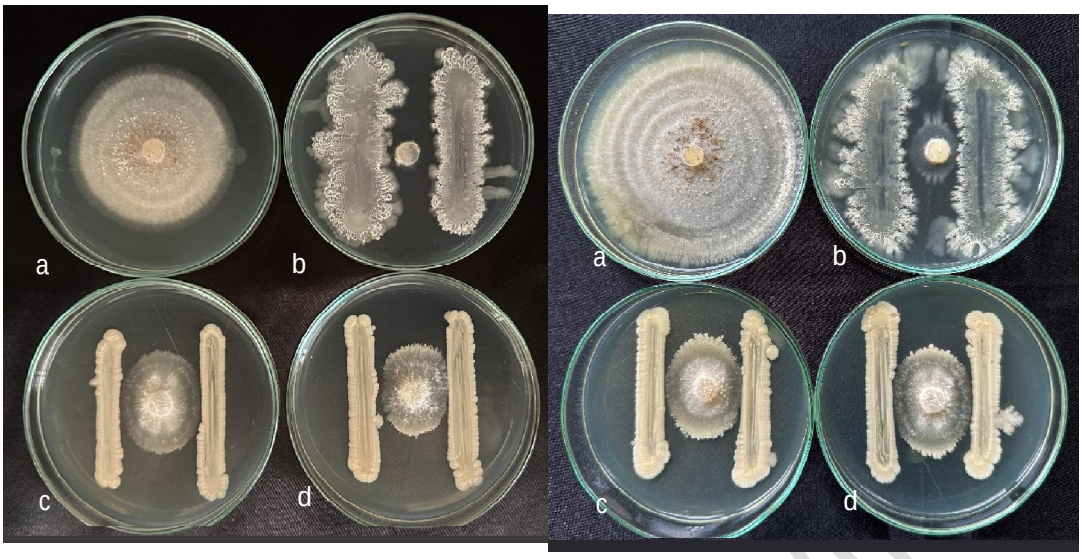

Plate 1 **Plate 2**

Fig. 5 Inhibition of mycelial growth of *R. solani* by endophytic bacteria at 3 days (plate 1) and 5 days (plate 2) after incubation; a. control, b. *B. subtilis* isolate PAL-11, c. *B. altitudinis* isolate TSL-4, d. *B. altitudinis* KAM-11

Evaluation of endophytic fungi against *B. oryzae* and *R. solani*

Mycelial growth of *B. oryzae* was significantly inhibited by the fungal endophyte *Xylaria* sp. isolate KTD-2 and the inhibition was 63.2 and 67.95 per cent respectively at 5 and 8days after pathogen inoculation (Table 3) (Fig. 6). The mycelial growth of *R. solani* was also significantly reduced by the endophyte and the inhibition was 54.95 per cent at 5 days after pathogen inoculation (Table 4) (Fig. 7).Different genera of fungal endophytes have been isolated from rice plants and many were identified to inhibit pathogens [44].Xylariaceous endophytes have been reported in all major groups of plants including conifers, monocots, dicots, ferns, and liverworts [45]. In the present investigation the endophytic *Xylaria* sp. isolate KTD-2 was found to inhibit rice pathogens *B. oryzae* and *R. solani.* The antagonistic potential of endophytic *Xylaria* against many plant pathogens have been already reported. For example, *X. regalis* isolated from *Tuja plicata* exhibited antagonistic activity against *F. oxysporum* and *Aspergillus niger*[46].The antagonistic potential of*Xylaria*sp. F0010 against many diseases such as rice blast (*Magnaporthe grisea*), rice sheath blight (*Cortisiumsaski*), wheat leaf rust (*Puccinia recondita*) and barley powdery mildew (*Blumeriagramminis* f. sp. *hordei*) was also reported [47]. Similarly,

reduction in severity of early blight in tomato caused by *Alternariasolani* by the application of endophytic *X. feejeensis* SRNE2BP[48] and inhibition of *Collectotrichumgloeosporioides*by endophytic *Xylaria* spp. [49] was also studied previously. These research works confirm the potential of *Xylaria*sp. to be used as a potent biocontrol agent in managing brown spot and sheath blight diseases of rice.

Table 3. Inhibition of mycelial growth of *B. oryzae* by fungal endophyte *Xylaria* sp. isolate KTD-2

Sl. No	Treatments	Mycelial growth of *B. oryzae* (cm)			
		5 days		8 days	
		B. oryzae diameter (cm)	Inhibition percentage (%)	*B. oryzae* diameter (cm)	Inhibition percentage (%)
1	Dual culture	2.58	63.2	2.88	67.95
2	Control	7.02		9	
	S.Ed	0.06		0.03	
	CD (0.05%)	0.13		0.06	

Table 4. Inhibition of mycelial growth of *R. solani* by fungal endophyte *Xylaria* sp. isolate KTD-2

Sl. No	Treatments	Mycelial growth of *R. solani* (cm)			
		3 days		5 days	
		R. solani diameter (cm)	Inhibition percentage (%)	*R. solani* diameter (cm)	Inhibition percentage (%)
1	Dual culture	4.02	10.77	4.05	54.95
2	Control	4.50		9	
	S.Ed	0.03		0.04	
	CD (0.05%)	0.07		0.08	

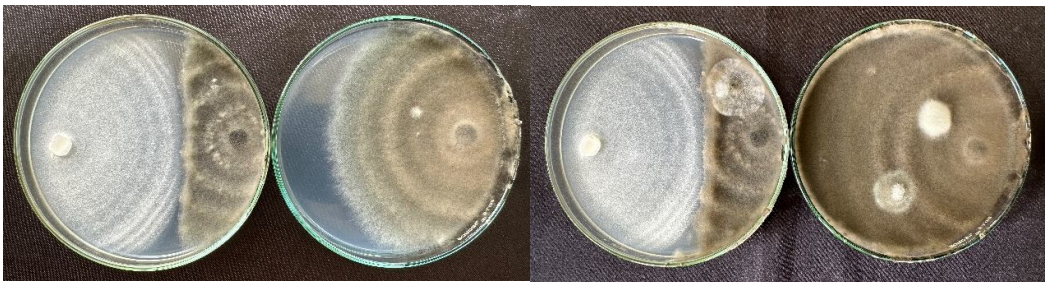

Plate 1 **Plate 2**

Fig 6: Inhibition of mycelial growth of *B. oryzae* by endophytic fungi *Xylaria*sp isolate KTD-2 at 5 days (plate 1) and 8 days (plate 2) after incubation

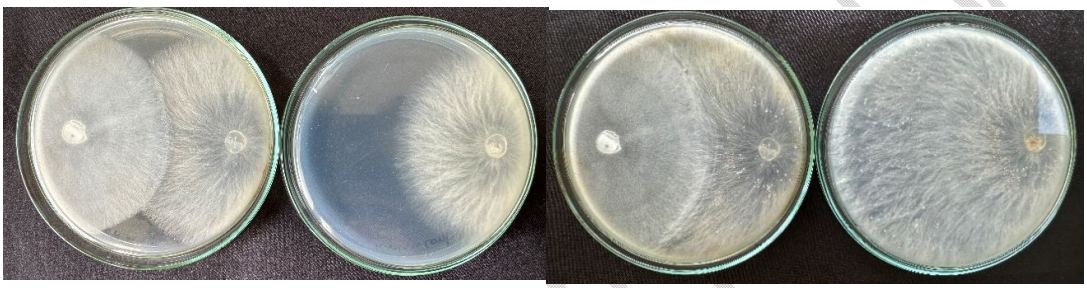

Plate 1 **Plate 2**

Fig 7: Inhibition of mycelial growth of *R. solani* by endophytic fungi *Xylaria*sp isolate KTD-2 at 3 days (plate 1) and 5 days (plate 2) after incubation

CONCLUSION

Brown spot caused by *B. oryzae* and sheath blight caused by *R. solani*are the two major diseases of rice causing severe threat to global rice production. Lack of adequate resistance, variability of the pathogen and ability of these pathogens to survive under extreme conditions make these pathogens difficult to manage. Despite the fact that pesticides are recommended for disease control, they are not considered to be long-term remedies because of concerns about fungicide residues, exposure risks, toxicity to non-target organisms, and other health and environmental issues. Therefore, endophytic microorganisms having biocontrol potential as well as plant growth promotion represent an alternative viable option to chemical management. In the present investigation, endophytic *Bacillus*sp. as well as *Xylaria* sp. were found to significantly inhibit *B. oryzae* and *R. solani*under *in vitro* conditions.These findings strongly suggest that these endophytes can be developed

as potent biocontrol agents against the major pathogens of rice after field evaluation studies.

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