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Research article

Effect of fungal endophytes of rice variety Ld 368 on growth and brown spot disease incidence of rice

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Abstract: Use of chemicals for growth enhancement and disease control in plants has resulted in hazardous influences to the environment and human health. Therefore, less harmful methods should be implemented and the possibility of using microbes for this purpose has been investigated. Endophytic fungal assemblages have been known to enhance plant growth and decrease disease incidence in some crops including rice and thus can be used as an alternative to chemicals. Therefore, this study was aimed to isolate the endophytic fungal communities associated with the rice variety Ld 368 with a view to examine the possibility of using them for plant growth enhancement and management of brown spot disease incidence. Brown spot disease caused by *Bipolaris oryzae* is one of the major rice diseases prevalent in Sri Lanka. Healthy plant parts of variety Ld 368 were used for the isolation of endophytes. 31 endophytic fungal species were isolated, and eight of the most frequently isolated fungal species were tested for their ability to inhibit the growth of *B. oryzae* using dual culture assays. From the fungal species tested, *Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp. inhibited the colony growth of *Bipolaris Oryzae* significantly ($P \leq 0.05$) under *in-vitro* conditions. Based on the results of *in-vitro* tests, spore suspensions of the more effective endophytes were inoculated separately to healthy Ld 368 seedlings to evaluate their efficacy in controlling brown spot disease and to determine their effect on rice plant growth under greenhouse conditions. Two inoculation methods (*i.e.* seedling and soil inoculation) were used to identify the best approach to introduce the endophytic fungi into the plants. Plants inoculated with *Trichoderma* sp.1 and *Chaetomium* sp. using seedling inoculation method showed the lowest disease incidence as well as a significant difference ($P \leq 0.05$) in shoot length and fresh and dry weight of plants. These results indicated that the tested endophytic fungal sp. have the ability to control brown spot disease incidence and enhance plant growth of rice variety Ld 368.

Keywords: Endophytes - *Bipolaris oryzae* - Bio-control - Growth enhancement.

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world and is the staple diet of Sri Lanka occupying 34% of the total cultivated area. Brown spot disease caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker is an important seed-borne disease of rice, causing 6–90% losses in grain yield in both wet and dry seasons (Archana *et al*. 2014). Brown spot disease has become prevalent in some districts in Sri Lanka due to unusual rainfall patterns and climatic changes (RRDI-Bathalagoda 2016).

Bipolariz oryzae attacks the crop from seedling to milk stage (Arshad *et al*. 2013, Sunder *et al*. 2014) and infects leaves, leaf sheath, coleoptile, panicle branches, glumes, and spikelets (Arshad *et al*. 2013) and results in seedling blight, lesions on coleoptile, leaves and glumes and eventually leads to seedling death (Schwanck *et al*. 2015). In severe infections, germination failure or poor germination of seeds, rotting of seeds, roots and coleoptiles (Kamal & Mia 2009), as well as loss in weight, may occur (Kumar *et al*. 2016). When the disease is

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severe or when the cultivar is more susceptible to the disease, spots become larger and may cover the entire leaf, reducing photosynthetic area, nutrient absorption and result in the decrease of tillering nodes (Archana *et al*. 2014, Sunder *et al*. 2014).

The available management strategies for brown spot disease include the use of partially resistant rice cultivars, appropriate plant nutrition management and fungicide application (Dallagnol *et al*. 2014). However, long-term application of fertilizers and fungicides may result in hazardous influences to the environment and human health (Tian *et al*. 2004) and use of partially resistant rice cultivars and plant nutrition management is not feasible due to higher costs (Mithrasena *et al*. 2012). Therefore, use of a bio-control method as an alternative to these methods is a promising approach. Plants host diversity of plant-associated microorganisms that play beneficial roles such as, enhance plant growth, tolerance to abiotic stresses, decrease plant stress and disease incidence (Parsa *et al*. 2016). An example group of such microorganisms is endophytic fungi, which are reported to possess many beneficial effects on plants.

Endophytic fungi are effective in controlling diseases in a number of crops (Kusari *et al*. 2012) and has been reported to control rice blast disease in the traditional rice variety Suwandel in Sri Lanka (Atugala & Deshappriya 2015). Endophytic fungi are also reported to enhance the growth of plants by several mechanisms including the release of auxins, gibberellin and cytokinin (You *et al*. 2012). Although endophytic fungi of rice have been reported to enhance growth and yield of a number of rice varieties (Atugala & Deshappriya 2015, Wijesooriya & Deshappriya 2016), the endophytic fungal assemblage of the rice variety Ld368 and their effect on brown spot disease development and plant growth has not been reported in Sri Lanka previously.

Therefore, the present study carried out to isolate the endophytic fungi present in different healthy plant parts of the Ld 368 rice variety, and to evaluate the effect of the most frequently isolated fungal endophytes on brown spot disease development and rice plant growth under greenhouse conditions.

MATERIALS AND METHODS

Sample collection

10-week old healthy rice plants and seeds of rice variety Ld 368 and 10-week old plants showing brown spot symptoms were randomly collected from the paddy fields at the Rice Research Station, Bentota, Sri Lanka.

Isolation of endophytic fungi from healthy Ld 368 rice variety

Healthy leaf, stem and root pieces, seeds and seedlings of Ld 368 rice variety were used to isolate endophytic fungi. Each plant part was cleaned by rinsing under running tap water for 10 min and the cleaned plant parts were surface sterilized separately according to the protocols described by Atugala & Deshappriya (2015). After surface sterilization, three consecutive rinses in sterilized distilled water were carried out for each of the plant parts, seeds and seedlings and dried using sterilized filter papers. The edges of the surface sterilized leaf, stem and root segments were trimmed and placed (5 per plate) separately on Potato Dextrose Agar (PDA) plates containing tetracycline (50 mg L^{-1}). 5 replicate plates were prepared for each plant part and incubated at room temperature.

Fungi that grew out from each plant part, seeds and seedlings were sub-cultured onto a fresh PDA medium containing tetracycline (50 mg L^{-1}) and incubated at room temperature. Pure cultures of all isolates were prepared using the Hyphal tip method (Dhingra & Sinclair 1995). The isolated endophytic fungi were identified to the genus level using their macroscopic and microscopic features and identification keys (Domsch *et al*. 1993, Barnett & Barry 1998).

Colonization frequencies (CF), isolation frequencies and dominant fungi percentages were calculated for each isolated endophytic fungal species using the following (Atugala & Deshappriya 2015);

$$Colonization\ frequency\ = \frac{Total\ number\ yielding\ 1\ isolate}{Total\ number\ of\ samples\ in\ that\ trial} \times 100$$

$$Isolation\ frequency\ = \frac{Total\ number\ of\ isolates\ yielded\ in\ a\ given\ trial}{Total\ number\ of\ samples\ in\ that\ trial} \times 100$$

$$Dominant\ fungi\ \%\ = \frac{No.of\ isolates\ \ collected\ from\ the\ samples}{Total\ no.\ of\ leaf/\ stem/\ root/\ seed\ samples} \times 100$$

Isolation of brown spot pathogen from diseased leaves of Ld 368 rice variety

Leaf pieces of 7 cm length, which contained the brown spot necrotic lesions were cut and cleaned under running tap water for 10 minutes. Sections of approximately 1 cm^2 size were cut from the diseased leaf from the edge of the area showing symptoms. Leaf pieces were surface sterilized 70% (v/v) ethanol for 1 min, 0.25% (w/v) NaOCl for 20 min and 70% (v/v) ethanol for 30 s and washed three times with sterilized distilled water and dried on sterilized filter paper. The edges of the sections were trimmed using a sterile scalpel and under aseptic conditions, and the pieces were placed on PDA containing tetracycline (50 mg L^{-1}) distributing 4 pieces on one plate. After 7 days incubation, resultant fungal colonies were separately sub-cultured into PDA plates and incubate at room temperature. The colony morphology and sporulating structures of the brown spot pathogen were observed and identified using identification keys (Domsch *et al*. 1993, Barnett & Barry 1998).

Identification of Morphological features

Colony characteristics such as front and reverse side colony color, colony shape, elevation, texture, type of mycelium, margin, zonation and pigment formation were observed visually after incubation at room temperature.

Identification using microscopic features

Pure cultures of isolated endophytic fungi were observed under light microscope for their microscopic characters, such as sporulating structures, spores, features of hyphae, presence or absence of asci and paraphyses using sticky tape method, in which piece of sticky tape was gently pressed on the colony and placed on a glass slide containing drop of cotton blue.

Identification using molecular methods

i. DNA extraction: For the further identification of endophytic fungal species that showed to have an antagonistic activity aginst the pathogen *i.e. Trichoderma* sp.1, *Trichoderma* sp.2, *Chaetomium* sp. and pathogen *Bipolaris oryzae*, genomic DNA were extracted according to the protocol described by Cenis (1992) and PCR amplified.

ii. PCR amplification: Amplification of the ribosomal DNA, ITS 1 and 2 was done to the extracted fungal DNA with the primers of ITS-5 and ITS-4 primers. PCR was carried out in 25 reaction volumes using 2 mM $MgCl_2$ (Promega Inc, USA), 0.12 mM dNTP (Promega Inc, USA), 1 µM each primer, 0.05 unit μM^{-1} *Taq* polymerases (Go *Taq* flexi, Promega Inc, USA) on a thermal cycler (Techne, flexigene, England). PCR was done under the conditions of initial denaturation at 95°C for 5 min, followed by 30 cycles, of denaturing at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min and the final extension at 72°C for 10 min.

Pathogenicity tests of the fungal sp isolated from diseased plants

Pathogenicity of the isolated fungal sp was confirmed on healthy rice plants grown under greenhouse conditions using a spore suspension. A suspension of, 1×10^5 spores ml^{-1} of the isolated organism was prepared using a 14 days old culture maintained in PDA. 0.05% of tween 80 was added to the suspension.

Leaves of 45 days old rice plants maintained under greenhouse conditions, were wiped with 70% ethanol and were slightly wounded using a sterilized needle and sprayed with 2 ml of the prepared spore suspension. Plants sprayed with sterilized distilled water containing 0.05% Tween 80 served as the controls. All treated plants were covered with transparent polythene bags with wetted cotton wool to provide adequate humidity and observed after 7 days for disease symptom development.

Antagonistic activity of isolated endophytic fungi

i. Dual culture assay: A 5 mm diameter disc from growing edge of a 5-day old *Bipolariz oryzae* and each endophytic fungal culture maintained on PDA were placed at the two opposite ends of a PDA plate. A sterile PDA disc replaced the endophytic fungal disc in the control plates. There were 5 replicates for each endophyte tested. All plates were incubated at room temperature and the effect of endophytic fungi on mycelial growth of *B. oryzae* was evaluated 2 days after incubation by measuring the diameter of the *B. oryzae* colony (R2) on the test and the diameter of the *B. oryzae* colony in the control plate (R1).

ii. Percentage growth inhibition (I%) was calculated using the equation, $I\% = (R1 - R2) \div R1$ (Rahman *et al*. 2009, Khalili *et al*. 2012).

Effect of volatile metabolites of endophytes on the growth of Bipolaris oryzae

To determine the effects of volatile metabolites of endophytic fungi on mycelial growth of *Bipolaris oryzae*, discs (5 mm diameters) cut from the margin of the 7 days old endophytic fungi and *Bipolaris oryzae* cultures

were placed in the center of two separate bottom portions of petri dishes containing PDA, plates with the pathogen were placed in an inverted position over the plate containing each endophytic fungal sp. The two bottoms held together with the pathogen at top, was sealed with parafilm and incubated at room temperature for 7 days. A control plate was maintained without endophytic fungi in the bottom plate. There were 5 replicates for each fungus and the percentage of growth inhibition was calculated.

Tests for the effect of endophytes on brown spot disease incidence under greenhouse conditions

Three endophytic fungal isolates *i.e. Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp. that showed a significant antagonistic activity against the brown spot pathogen under *in vitro* conditions were used for the greenhouse experiments. Healthy Ld 368 rice seeds were surface sterilized (75% ethanol for 30 s, 1% NaOCl for 10 min and 70% ethanol for 30 s) and soaked overnight in sterile distilled water. The seeds were germinated by wrapping them with a wet sterilized cloth and incubating under room temperature for five days. Inoculation of endophytes to plants was carried out using two methods:

i. Method I- Seedling Inoculation- plate method: Seedlings were inoculated with endophytes *Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp. by using the plate method (Wijesooriya & Deshappriya 2016) and incubated at room temperature. Seedlings were placed on fresh PDA plates as a control.

The success of inoculation was confirmed by placing 15 randomly selected seedlings subjected to each treatment including controls on PDA plates supplemented with tetracycline (50 mg L^{-1}) after surface sterilization (75% ethanol for 30 s, 1% NaOCl for 10 min and 70% ethanol for 30 s). *Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp. inoculated seedlings and control seedlings were planted in pots (8 cm in height and 9.5 cm in width) filled with sterilized soil which had been autoclaved for 20 min (121°C and 15 lb in^{-2}) separately.

ii. Method II- Soil inoculation method: Soil samples collected from a field were autocalved for 20 min at 121°C and 15 lb in^{-2}. 15 ml (Tarafdar & Gharu 2006) of spore suspensions (1×10^5 spores ml^{-1}) of *Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp. were added to pots (8 cm in height and 9.5 cm in width) filled with sterilized soil (250 g) for planting and control was prepared adding sterilized distilled water. 6 days old healthy non-inoculated Ld 368 seedlings germinated as mentioned above were planted in pots (5 seedlings per pot). There were 5 replicates for each inoculation and plants were maintained under the average temperature of 30 ± 5°C during day and 20 ± 5°C during night in the greenhouse.

To prepare the pathogen inoculum a spore suspension of 1×10^7 spores/ ml of *Bipolaris oryzae* was prepared using a 14-day old culture maintained in PDA. 0.05% tween 80 was added to the spore suspension. Leaves of the 21 days old endophyte treated and control plants were wiped with 70% ethanol and leaves were slightly wounded using a sterilized needle and sprayed with *B. oryzae* spore suspensions. Control plants were sprayed with sterilized distilled water added to fresh uncontaminated PDA plate. Each plant was covered separately with clean, transparent polythene bags and wet cotton wool was incorporated to provide moisture. Disease development on the rice leaves was observed daily, and the disease incidence was calculated using the following formula,

$$Disease\ incidence\ (I) = \frac{Number\ of\ diseased\ plants}{Total\ number\ of\ plants\ (healthy\ and\ infected)} \times 100$$

Effect of fungal endophytes on rice plant growth

The effect of the most frequently isolated fungal endophytes *i.e. Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp., on the growth of the rice variety Ld 368 was tested. Ld 368 healthy seeds were surface sterilized and germinated as mentioned earlier and the fungal endophyte isolates were inoculated using Seedling method (plate method) and soil method.

Treated and control seedlings were planted in pots (5 seedlings per pot) filled with autoclaved soil. There were 5 replicates for each treatment and plants were maintained under the average temperature of 30±5°C during the day and 20±5°C during the night in the greenhouse. Fresh weight, dry weight and shoot lengths of randomly selected 5 plants from each treatment were measured at 1 week interval. First initial fresh weights of the plants were measured and dry weights were measured by drying plants in an oven at 60°C for 24 hours until a constant weight was obtained (Vibhuti *et al*. 2015). The length between the starting point of the shoot and end point of the flag leaf was measured as shoot length. Results were statistically analyzed using two-way ANOVA and Tukey's pairwise comparisons.

RESULTS

A total of 31 fungal species was isolated from 150 samples of the root, stem, leaf, seeds and seedlings of healthy Ld 368. Higher colonization (92%) and isolation frequency (40 %) were shown by seeds while lowest colonization (20%) and isolation (4%) frequency shown by leaves (Table 1). *Chaetomium* sp. was the dominant fungus present in Ld 368 rice variety (Table 2).

Table 1. Frequency of isolation and colonization of endophytic fungi from different parts of healthy Ld 368 rice plants.

Plant part		Total number of samples	Isolation frequency (%)	Colonization frequency (%)
Seed		25	40	92
Seedling	shoot	25	48	20
	root	25	36	8
Root		25	20	96
Stem		25	32	60
Leaves		25	4	20
Total		**150**	**20.7**	**58.7**

Table 2. List of isolated endophytic fungi, % dominance and colonized plant part of healthy Ld 368.

Endophytic fungi	% Dominance
Chaetomium sp.	28.8
Sterile mycelia (SM 1)	23.3
Trichoderma sp. 1	16.7
SM 2	16.7
Penicillium	13.7
Trichoderma sp. 2	13.3
SM 6	13.3
Aspergillus	12.5
Fusarium sp.1	9.6
SM 3	6.7
Fusarium sp.2	4.0
SM 4	3.3
SM 5	3.3
SM 7	3.2
Colletotrichum	2.5
SM 8	1.6
Curvularia	1.3

In vitro tests for the effect of endophytes on the brown spot pathogen

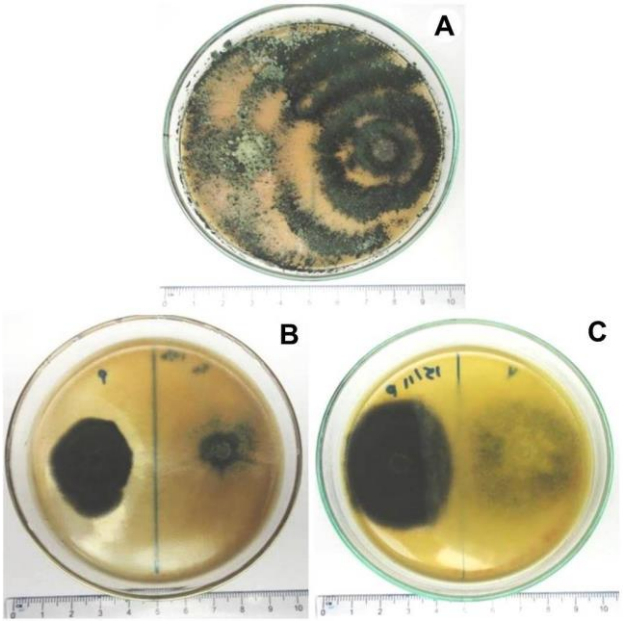

Figure 1. Endophytes and *Bipolaris oryzae* in dual culture showing antagonism (10 days old cultures) colonies on the right hand side represent endophytes: **A**, *Trichoderma* sp. 1; **B**, *Trichoderma* sp. 2; **C**, *Chaetomium* and colony on the left hand side represent *Bipolaris oryzae*.

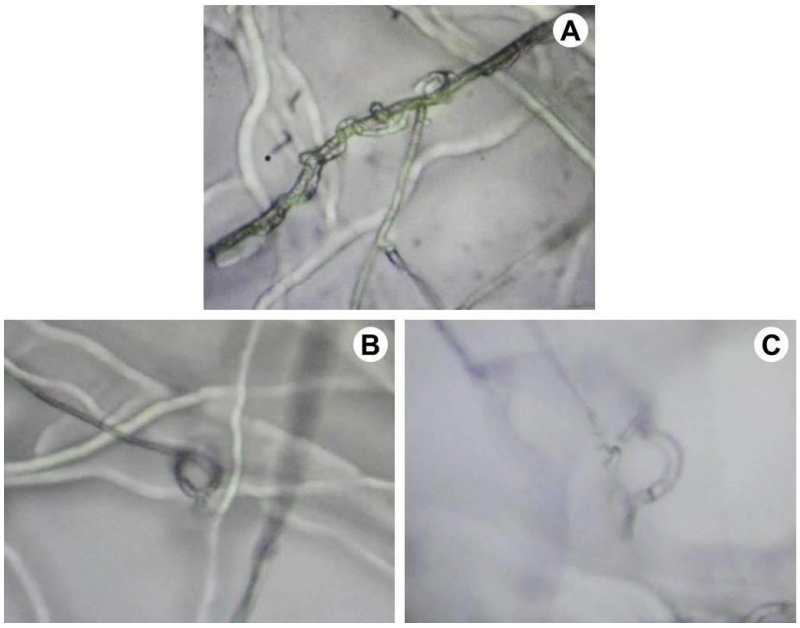

Figure 2. Structures formed by endophytes in mycoparasitism of *Bipolaris oryzae*: **A**, Fungal endophytes hyphae coiling around the thicker hyphae of *Bipolaris oryzae* (10×40×2); **B**, Loops formed by endophytes to trap *Bipolaris oryzae* (10×40×3); **C**, Endophytes forming clamps on pathogen mycelia (10×40×4).

i. Dual culture assays: All fungal isolates tested showed a significant inhibition (P≤ 0.05) of the growth of *Bipolaris oryzae* on PDA. However, *Trichoderma* sp.1, *Trichoderma* sp.2and *Chaetomium* sp. showed the highest inhibitory effects (Fig. 1A, B & C) with inhibitory percentages of 64.4%, 47.0% and 29.5% on the colony growth of *Bipolaris oryzae* respectively (Table 3). When the dual cultures were observed under the microscope, hyphae of *Trichoderma* sp.1 showed coiling around the hyphae of *Bipolaris oryzae* (Fig. 2A) and *Trichoderma* sp.2, *Chaetomium* sp. showed formation of loops (Fig. 2B) and clamps (Fig. 2C), which would trap the pathogen hyphae.

Table 3. Results of dual plate method.

Endophytic fungal isolate	Diameter of *Bipolaris oryzae* colony (cm)	% inhibition
Control	5.53 ± 0.77	0
Trichoderma sp. 1	1.97± 0.21	64.4[a]
Trichoderma sp. 2	2.93 ± 0.15	47.0[b]
Chaetomium	3.90 ± 0.26	29.5[c]
Fusarium sp. 2	4.00 ± 0.20	27.7[cd]
SM 7	4.13 ± 0.30	25.3[cd]
Fusarium sp. 1	4.20 ± 0.20	24.0[cd]
SM 8	4.20 ± 0.72	24.0[cd]
SM 2	4.33 ± 0.30	21.7[cd]

Note: n=5; mean ± SD; Mean values sharing common letters in each row are not significantly different p ≤ 0.005.

ii. Effect of volatile compounds produced by endophytic fungi on pathogen growth: All fungal endophytes tested inhibited the growth of *B. oryzae* colony. *Trichoderma* sp.1 showed the maximum % inhibition, while *Chaetomium* sp. showed the minimum inhibition of pathogen growth (Table 4). One way ANOVA showed a significant difference (P ≤ 0.05) in colony growth inhibition between control and all fungal endophytes.

Table 4. Effect of metabolites on the growth of *Bipolaris oryzae*.

Fungal endophyte	Diameter of pathogen growth (cm)	% Inhibition of the pathogen
Control	7.97 ± 0.51	0
Trichoderma sp. 1	5.70 ± 0.80	28.5[a]
Trichoderma sp. 2	6.30 ± 0.47	20.9[a]
Chaetomium	7.90 ± 0.24	0.8[b]

Note: n=5; mean ± SD; Mean values sharing common letters in each row are not significantly different P ≤ 0.005.

DNA extraction and PCR amplification of fungal DNA

PCR amplification was performed to DNA extracted from *Trichoderma* sp.1, *Trichoderma* sp.2, *Chaetomium* sp. and pathogen *Bipolaris oryzae* using the rDNA primers ITS 5 and ITS 4. PCR amplified products and a 100 bp ladder were visualized as shown in figure 3. All the endophytes and pathogen were given approximately 600–700 bp size bands in the gel except *Trichoderma* sp.1.

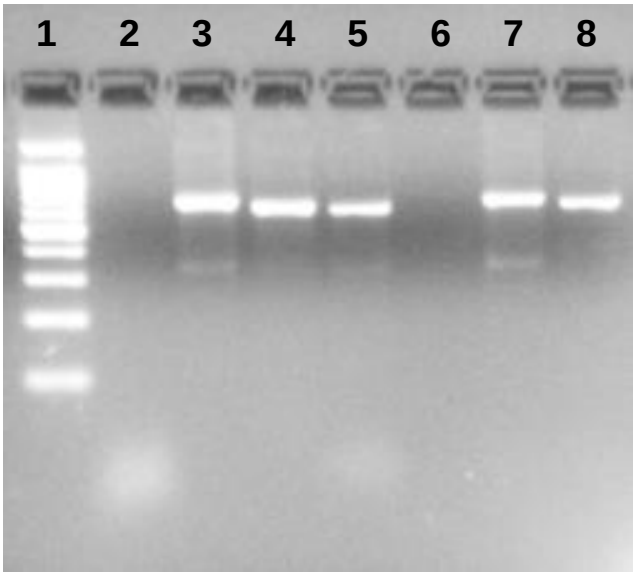

Figure 3. Gel image of PCR products of fungal endophytes and pathogen amplified using ITS 5 and ITS 4. [Well 1: 100 bp ladder, Well 2: *Trichoderma* sp. 1, Well 3: *Trichoderma* sp. 2, Well 4: Unidentified sp. 4, Well 5: *Chaetomium*sp., Well 6: PCR (-) control, Well 7: PCR (+) control, Well 8: *Bipolarizoryzae* (pathogen)]

In planta tests for the effect of endophytes on the development of brownspot disease

Symptoms developed on the plants onto which only *Bipolaris oryzae* was inoculated. Disease symptoms were also observed in all plants treated with each endophyte *Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp. using soil and seedling inoculation methods when inoculated with *Bipolaris oryzae*. However, the disease incidence was lower in plants inoculated with *Trichoderma* sp. 1 and *Chaetomium* using both soil and seedling inoculation methods (Table 5). There was no disease development in control plants sprayed with sterilized distilled water. Plants grown from seedlings treated with *Trichoderma* sp. 1 and inoculated with *Bipolaris oryzae* showed the lowest disease incidence compared to other treatments.

Table 5. Disease incidence in the Ld 368 plants of each endophyte treated plants.

Method of inoculation of endophytes	Treatment	Disease incidence %	
		Plants sprayed with pathogen spore suspension (1×10^7 spores ml^{-1})	Plants sprayed with sterilized distilled water (Controls)
Soil inoculation	*Trichoderma* sp. 1	10	0
	Trichoderma sp. 2	75	0
	Chaetomium	50	0
	Sterilized distilled water	83	0
Seedling inoculation	*Trichoderma* sp. 1	5	0
	Trichoderma sp. 2	25	0
	Chaetomium	15	0
	Sterilized distilled water	80	0

Effect of fungal endophytes on the growth of Ld 368 rice variety

A significant increase ($P \leq 0.05$) in plant height, fresh weight and dry weight were observed in plants inoculated with the endophytes compared with non-treated plants. The highest increase in plant height, fresh weight and dry weight was shown in plants inoculated with *Trichoderma* sp.1.

Table 6. Mean shoot length (cm) of plants after 2 and 4 weeks.

Week	Treatment	Inoculation method	
		Soil inoculation	Seedling inoculation
After 2 weeks	*Trichoderma* sp.1	13.42 ± 1.24^{a}	14.40 ± 0.62^{a}
	Trichoderma sp.2	13.26 ± 1.39^{a}	$13.72 \pm 0.81^{a\,b}$
	Chaetomium	13.68 ± 0.64^{a}	$12.58 \pm 0.43^{a\,b}$
	non-inoculated	12.60 ± 0.66^{a}	12.02 ± 0.66^{b}
After 4 weeks	*Trichoderma* sp.1	19.86 ± 0.61^{a}	21.90 ± 0.58^{a}
	Trichoderma sp.2	14.90 ± 0.55^{c}	16.72 ± 0.45^{b}
	Chaetomium	$18.68 \pm 0.83^{a\,b}$	16.94 ± 0.55^{b}
	non-inoculated	13.60 ± 0.29^{c}	13.10 ± 0.29^{c}

Note: n=5; mean ± SE; Mean values sharing common letters in each row are not significantly different $P \leq 0.05$.

Plants inoculated with endophytes did not show a significant increase in their shoot length after the second week depending on the inoculation method but after four weeks endophytes inoculated via seedlings plants showed a significant increase in their shoot length compared to plants inoculated using soil inoculation method (Table 6).

DISCUSSION

In this study, the fungal endophytes present in different parts of the plant and in seeds of the rice variety Ld 368 was isolated and identified and subsequently their effect on rice plant growth and their ability to control brown spot disease was studied.

In order to determine the entire endophytic fungal assemblage of Ld 368 rice variety, isolations were carried out using roots, stems, leaves, seeds and seedlings of Ld 368. Use of fresh samples is required for successful isolation, therefore, samples collected from the field were used for isolations within 48 hours. Leaf pieces of wheat (Larran *et al*. 2002), stems and leaf pieces of maize (Fisher *et al*. 1992) have used to isolate endophytic fungi with different surface sterilizing protocols. In order to isolate a maximum number of colonized endophytes while eliminating microbes present on the plant surface, the surface sterilization regimes developed in a previous study (Atugala & Deshappriya 2015) were used for effective surface sterilization of the plant parts used for the isolations. Endophytic fungi of Ld 368 were isolated placing the surface sterilized tissues on PDA supplemented with tetracycline. Tetracycline was added as an antibiotic agent to prevent the bacterial growth until the emergence of fungal colonies from the plant segments.

Thirtyone different fungal genera were able to isolate from roots, stems, leaves, seeds and seedlings of healthy disease free Ld 368 rice variety. *Trichoderma* sp.1, *Trichoderma* sp.2, *Chaetomium* sp., *Fusarium* sp.1, *Fusarium* sp.2, sterile mycelia (SM 2), SM 7 and SM 8 were the most commonly isolated fungal genera while sterile mycelia were the most common inhabitors of seeds of Ld 368. Isolation of endophytic fungi from healthy rice plants has been reported to yield 35 and 31 different genera of endophytic fungi associated with 80 samples of leaves, stems, roots and seeds of traditional rice varieties Suwandel and Kaluheenati respectively (Atugala & Deshapriya 2015).

Fungal endophytes thus isolated from various crops have been reported to enhance plant growth as well as to reduce disease incidence. Endophytic *Fusarium moniliforme* isolated from roots, nodes and stems of *Zea mays* enhanced root growth and histological modifications in leaves and shoots of corn seedlings (Yates *et al*. 1997) and *Gliocladium catenulatum* the endophytic fungus isolated from the *Theobroma cacao* L. reduced the incidence of Witches' Broom Disease in cacao seedlings (Rubini *et al*. 2005).

Fungal endophytes could be used to enhance the growth of rice plants (Wijesooriya & Deshappriya 2016) due to their plant growth promoting ability (Yates *et al*. 1997, Maciá-Vicente *et al*. 2009). Therefore, in this study, the effect of more frequently isolated fungal endophytes on Ld 368 rice plant growth was tested under greenhouse conditions. The endophytes were inoculated into soil and seedlings to determine their effect on plant growth. Previous studies have shown that successful inoculation of endophytes into seedlings can be done through placing them on the respective cultures grown on artificial media (Atugala & Deshapriya 2015) or by inoculating endophytes into the sterile soil (Tarafdar & Gharu 2006). Endophytes inoculated plants using both soil and seedling inoculation methods showed a significant increase ($P \leq 0.05$) in plant height, fresh weight and dry weight when compared with non-inoculated plants after 4 weeks.

Even though the during second week plant did not show a significant increase in plant height, fresh weight and dry weight when compared with non-inoculated plants depending on the inoculation method, plants showed a significant increase after 4 weeks, that could be due to the colonization rate of the endophytic fungi (Maciá-Vicente *et al*. 2009). These results are in accordance with the previous studies carried out in Sri Lanka (Ponnawila & Deshappriya 2015, Wijesooriya & Deshappriya 2016) and it indicates that the plant growth can be enhanced significantly by inoculating endophytic fungi.

The sterilized soil was used to grow the rice plants in the greenhouse experiments and thus, the direct or indirect impact of endophytes on rice plant growth may be correlated with this significant increase in plant height, fresh weight and dry weight. *Trichoderma* sp.1 and *Chaetomium* sp. inoculated plants showed a significant increase in plant height, fresh weight and dry weight when compared to other treatments.

Therefore, these endophytes may have some mechanisms to enhance the plant growth by inducing the plant nutrient uptake and producing chemicals which can enhance the plant growth. It has found that *Trichoderma* sp. have the ability to enhance plant growth by solubilizing many plant nutrients from their solid phase compounds and increasing the plant's nutrients uptake efficiency (Altomare *et al*. 1999). Wheat seedlings grown in sterilized

soil mixed with 15 ml of *Chaetomium globosum* culture result a significant improvement in plant biomass, root length, plant phosphorous (P) concentration, seed and straw yield and seed P content, therefore Tarafdar & Gharu (2006) suggested that *C. globosum* produces phosphatases and phytases, which mobilize phosphorous (P) and enhance the plant growth.

In the present study, inoculating the seedlings with the endophytes using seedling method was more successful than introducing the inoculum through soil, the concentrations of inoculants in the inoculated soils might have an impact on the results or the biotic factors of a sterile soil may have an influence on the fungal growth in the soil compared to non-sterile soil (Tefera & Vidal 2009).

Endophytic fungi can be potentially used as effective bio-control agents and they can play an important role in ecological agriculture (Mejía *et al*. 2008). Therefore, In this study, fungal endophytes isolated from healthy plant organs of Ld 368 were tested for their ability to control the growth of the brown spot pathogen, *Bipolaris oryzae* both *in vitro* and *in planta*.

Dual culture method was used to assess the effect of endophytic fungi on the growth of pathogen mycelium by measuring the radial growth of the pathogen colonies. Percentage of inhibition was the important parameter to evaluate the antagonistic activity of the endophyte against the pathogen. Among the screened isolated strains, some endophytes showed inhibitory activity against the test fungal pathogen through mechanisms such as competition, compared to the control. *Trichoderma* sp. 1 and *Trichoderma* sp. 2 showed the most effective inhibition 64% and 47% respectively. Competition between *Trichoderma* spp and *Bipolaris oryzae* plays an important role. These fungi showed competition for nutrients and space with *Bipolaris oryzae* pathogen in the dual culture test as the evident antagonism. *Trichoderma* isolates have the ability to grow faster while efficiently competing for space and nutrients than the pathogenic fungi, inhibiting the growth of the target organisms (Khalili *et al*. 2012). Similarly, Abdel-Fattah *et al*. (2007) used a dual culture method to study the antagonistic behavior of *Trichoderma harzianum* against *Bipolaris oryzae* and *Trichoderma harzianum* showed 48% inhibition. *Chaetomium* sp. showed 29.5% inhibition of the pathogen. Shanthiyaa *et al*. (2013) found that *Chaetomium* sp. produces several fungistatic metabolites such as chaetomin, chaetocin, chaetoglobosin A and B, chaetoviridin A and B that are active against many soilborne plant pathogens and exhibit higher exo- and endo-glucanase activity *in vitro*. Other antifungal mechanisms may also be involved during the inhibition. The mycoparasitic activity of bio controlling fungal endophytes was observed under the high power of the light microscope and various structures that used by the antagonist to parasitize the pathogen were observed in this study.

Loops formation to trap pathogen mycelia, coiling around thick pathogen mycelia to obtain nutrients were observed and coiling may result in the formation of haustoria which develop into pathogen hyphae and facilitate the nutrient uptake.

Volatile compounds produced by *Trichoderma* sp.1 and *Trichoderma* sp.2 showed 28.5% and 20.9% inhibition percentages respectively against the pathogen *B. oryzae*. Khalili *et al*. (2012) tested the effect of volatile compounds produced by 45 *Tichoderma* isolates obtained from paddy fields against the *B. oryzae*. *T. harzianum* strains, *T. virens* and *T. atroviride* showed 72%, 66% and 75% inhibitory action against *B. oryzae* respectively.

Gel electrophoresis of the PCR-amplified rDNA gene of the fungal genus exhibited a single band for each genus which varied in size corresponding to the primer pairs used. Different sizes of PCR products were obtained for *Trichoderma* sp. 2, *Chaetomium* sp. and *Bipolaris oryzae* ITS region amplified using ITS 5 and 4 primers but primers may have not worked for the *Trichoderma* sp.1, since there was no amplified band in the gel. Amplification of ITS region was successful for the fungal isolates and brown spot pathogen. The size of the PCR product of ITS region of *Bipolaris oryzae* with the use of ITS 5/ITS 4 primers was visualized as 600–680 bp in size by Goh *et al*. (1998). Green-house experiments were carried out to test the ability of fungal endophytes to reduce disease incidence.

According to Abdel-Fattah *et al*. (2007), diseased rice seedlings treated with *Trichoderma harzianum* showed 53.5% and 46.9% brown spot disease incidence after 7 and 21 days of *T. harzianum* application respectively. In the present study lowest disease incidences 5% and 15% were observed with rice seedlings treated with *Trichoderma* sp. 1 *Chaetomium* sp. using the seedling inoculation method after 10 days, therefore, these endophytes may be having a potential to prevent pathogen growth in rice plants. So it is possible to consider that these isolates are endophytic strains as nonpathogenic mutualistic endophytes on rice, but further experiments are needed to determine their genetic relationships and mutualistic associations with the host plant and effect against the brown spot.

The ability of isolated endophytes to inhibit brown spot pathogen growth, promote host plant growth and reduce disease severity was attempted in this study. Further *in-vitro* and field trials have to be carried out to obtain effective results and gain knowledge about their bio-control strategies and crop performance. Based on the results, optimized an inoculum consisting of fungal endophytes *i.e. Trichoderma* sp. 1, *Trichoderma* sp. 2 and *Chaetomium* sp. could be developed to control the brown spot disease as well as to enhance the growth of Ld 368 rice variety.

CONCLUSION

- Higher diversity of fungal endophytes is present in the most parts (*i.e.* root, stem, leaf and seed) of the Ld 368 rice variety.
- Seedling application of endophytic fungi is more effective than soil application.
- Endophytic fungi, *Trichoderma* sp.1, *Trichoderma* sp.2 and *Chaetomium* sp.can increase rice plant growth and reduce the disease incidence of the brown spot disease of rice caused by *Bipolaris oryzae*.

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