



Research article

An inoculum of endophytic fungi for improved growth of a traditional rice variety in Sri Lanka

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Abstract: Use of chemical fertilizers and pesticides in rice cultivation has incurred many environmental and health problems in Sri Lanka. Therefore, there is renewed interest in cultivating traditional rice varieties as they are more amenable to organic farming practices. However, as the yield of these varieties is comparatively low, strategies to enhance their performance should be investigated. As endophytes of plants are reported to promote growth and yield of a number of crop varieties, this study was aimed at studying the endophytic fungal assemblage present in the traditional rice variety Kuruluthuda with a view to evaluate their capacity to enhance plant growth and yield. Twenty seven endophytic fungal species were isolated from different parts of Kuruluthuda rice plants collected from a paddy field cultivated using organic fertilizers in the Gampaha district, Sri Lanka. Two frequently isolated endophytic fungal spp. *i.e.* *Acremonium* and *Arthrobotrys* (frequencies of isolation 60% and 38.6% respectively) were introduced separately and in combination torice seedlings using spore suspension and plate methods to determine their effect on growth and yield under green house and field conditions. All endophyte inoculated plants showed a significant difference ($P \leq 0.05$) in plant growth (height, fresh weight and dry weight), number of tillers and yield when compared with non-inoculated plant under both green house and field conditions. The effect of *Acremonium* and *Arthrobotrys* when introduced in combination showed a significant difference ($P \leq 0.05$) in the fresh weight, dry weight, tiller number and yield (weight of seeds harvested) when compared to their individual effects under field conditions which indicates that the two endophytes in combination can be used as a better inoculum to improve biomass and yield of the plants of rice variety tested. This is the first report of the endophytic mycoflora of the rice variety Kuruluthuda and their potential use for growth promotion and yield enhancement.

Keywords: Fungal endophytes - *Acremonium* - *Arthrobotrys* - Kuruluthuda - Growth enhancement.

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INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food of a large part of the world population (FAO 1994). Improvement of rice grain yield has been dependent on the introduction of new high yielding varieties that are heavily dependent on the use of chemical fertilizers and pesticides. The need for long term, indiscriminate application of chemical fertilizers has led to hazardous influences on the environment as well as on human and animal health. Traditional rice varieties are highly amenable to organic farming practices that cause minimum damage to the environment and sustain soil health (Rathnabharathi 2009). Therefore, there is renewed interest in cultivating traditional rice varieties combined with organic farming practices. However, these varieties are known to be low producers of yield and thus their performance has to be improved to meet the demand of the highly expanding population. Recent studies aimed at improving the productivity of plants using alternative cultivation practices have focused on the possibility of using microorganisms to increase root growth and nutrient uptake of plants, fix nitrogen as well as decrease plant stress and disease incidence (Montesinos 2003).

Endophytes are microorganisms that can colonize internal plant tissues without causing apparent harm to the host (Petrini 1991). They are symbionts in plants and benefit the plant by enhancing growth directly by releasing

phyto-hormones (*i.e.* auxins, gibberallin and cytokinin) which are biosynthesized through their own pathways (Contreras-Cornejo 2009, You *et al.* 2012). These additional concentrations of hormones improve root and shoot biomass and reinforce the root system of plants which facilitates the acquisition of mineral nutrients from the soil (Feng *et al.* 2000) and enable plant roots to increase nitrogen and phosphorus uptake (Ryan *et al.* 2008). Indirectly, they increase the availability of the nutrients in the rhizosphere by mobilization of phosphate from organic phosphate sources (Tarafdar & Claassen 1998) and produce low molecular weight metabolites (*i.e.* mycotoxins, allelochemicals and flavonoids) which are required for the normal functioning of plants under adverse conditions (Strobel 2003).

Diazotrophic bacterial and actinomycete populations of rice are reported to promote plant growth promotion and to possess the potential for nitrogen fixation and disease resistance (Barraquio *et al.* 1997, Tian *et al.* 2004). Fungal endophytes also have been detected in cultivated rice and they have shown to possess antagonistic or plant growth stimulating properties (Yuan *et al.* 2009, Naik *et al.* 2009). Previous studies in Sri Lanka have shown that some endophytes present in the traditional rice varieties Suwandel, Kaluheenati and Herath Banda have the ability to increase the growth of plants significantly (Atugala & Deshappriya 2015, Ponnawila & Deshappriya 2014). However, the endophytic fungal assemblage of the rice variety Kuruluthuda and their effect on plant growth and yield has not been reported in Sri Lanka previously.

Therefore, the present study was carried out with the objectives:

- To isolate the endophytic fungal assemblage present in different plant parts of the rice variety Kuruluthuda.
- To develop method(s) of introducing endophytic fungi into seedlings.
- To evaluate the effect of the most frequently isolated fungal species on the performance of plants under green house and field conditions with a view to develop an inoculum for improved plant growth and productivity.

MATERIALS AND METHODS

Sample collection and microscopic observation of roots

Nine week old healthy, intact plants of the traditional rice variety Kuruluthuda were randomly collected from a field treated with organic fertilizers at Eldeniya, Sri Lankaduring the Maha season (September to February) and transported to the laboratory in clean polythene bags. Roots were cut into 7 cm long pieces and washed under running tap water for 20 minutes. Squashed preparations of root segments were observed under the light microscope and the presence of root inhabiting endophytic fungi was confirmed by trypan blue staining method (Yuan *et al.* 2009).

Isolation and identification of endophytic fungi of the rice variety Kuruluthuda

Leaf pieces stem pieces, root segments and seeds of Kuruluthuda were used to isolate endophytic fungi. Each plant part was cleaned separately by rinsing under running tap water for 10-15min and the cleaned plant parts were surface sterilized using the sterilization protocols reported previously (Atugala & Deshappriya 2015).

After treatments with surface sterilizing agents, each plant part and seeds were washed with three consecutive changes of sterilized distilled water (SDW). The edges of the surface sterilized leaf, stem and root segments were trimmed and twenty five pieces of each of the plant parts and seeds were transferred aseptically into petri plates containing Malt Extract Agar (MEA) supplemented with tetracycline (50 mg.l⁻¹). After 10 days incubation at room temperature (RT) (30°C), endophytic fungal species that grew out were subcultured onto fresh Potato Dextrose Agar (PDA) plates supplemented with tetracycline (50 mg.l⁻¹). Pure cultures of each isolated colony was obtained by hyphal tip method (Tutte 1969) and stored at 4°C. The fungal cultures were identified based on morphological characters (*i.e.* features of hyphae and sporulating structures, colony morphology, texture and color) to genus level using standard keys of identification (Domschet *et al.* 1993).

Isolation frequencies and percentages of dominant fungi were calculated as following (Fisher & Petrini 1991, Goveas *et al.* 2011):

$$\text{Isolation frequency} = (\text{Total number of isolates yielded in a given trial} / \text{Total number of sample segments in the same trial}) \times 100$$

$$\text{Percentage dominance} = (\text{Number of isolates collected from the samples} / \text{Total number of leaf/stem/root/seed samples}) \times 100$$

Inoculation of endophytic fungal species into seedlings

The effect of most frequently isolated fungal endophytes *i.e.* *Acremonium* and *Arthrobotrys* on rice plant growth and yield was determined by inoculating each fungus separately and together to three day old seedlings of the rice variety Kuruluthuda.

Seed germination

Seeds were surface sterilized as described in 2.2 above. The surface sterilized seeds were soaked overnight in SDW and allowed to germinate by wrapping with a wet cloth for three days at RT (30°C).

Inoculation of seedlings

Inoculation of seedlings was carried out using two methods, (a) spore suspension method and (b) plate method.

(a) Spore suspension method:

10 day old cultures of *Acremonium* and *Arthrobotrys* grown on PDA plates were used to prepare the spore suspensions. 5 ml of SDW was added to each culture and the spores were dislodged using a sterilized glass spreader. The resulting spore suspensions were transferred to sterilized petri dishes and spore concentrations adjusted to 1×10^6 spores/ml.

Inoculation of the seedlings was done in the following manner:

- (i) Immersion of seedlings in 40 ml of each quantified fungal spore suspension separately in a petri plate.
- (ii) Immersion of seedlings in a mixture of 20 ml each of spore suspensions (40 ml in total) in a petri plate.
- (iii) Immersion of seedlings in 40 ml of SDW in a petri plate (control).

Ten seedlings were used for each treatment and they were immersed in the spore suspensions/SDW overnight at RT (30°C). There were ten replicates for each treatment.

(b) Plate method:

10 day old *Acremonium* and *Arthrobotrys* cultures grown on PDA were used for the plate method and the inoculation was carried out as follows;

- (i) Seedlings were placed on each fungal culture plate separately and incubated for 2–3 days at RT (30°C).
- (ii) Seedlings were placed on *Acremonium* culture plates for 2 consecutive days and subsequently on *Arthrobotrys* culture plates for 2 consecutive days at RT (30°C).
- (iii) Seedlings transferred onto PDA+tetracycline plates without the fungus served as the controls.

Ten seedlings were used for each treatment and there were ten replicates for each treatment.

Confirmation of entry and establishment of inocula

To test whether the inoculations were successful, randomly selected 15 seedlings subjected to each treatment of both methods including controls were surface sterilized (75% ethanol for 30 s, 1% NaOCl for 10 min and 70% ethanol for 30 s) and transferred onto PDA plates supplemented with tetracycline (50 mg.l⁻¹).

Determination of the effect on plant growth and yield

The effect of the endophytes was evaluated under greenhouse conditions as pot experiment and in the field using seedlings inoculated with the fungal species separately and together by both methods of inoculation.

Pot experiments in greenhouse

Fifty seedlings selected randomly from the five replicates of each treatment were washed with SDW and planted in polythene bags (31 cm of diameter and 24.4 cm of height) filled with soil treated with organic fertilizers transported from a paddy field, which had been autoclaved for 20 min (121°C and 15 lb.in⁻²). Overall eighty bags were prepared at a rate of five seedlings per bag. Plants were watered regularly and maintained under a temperature range 30°C (day) and 20°C (night) in the greenhouse for 3 months.

Field trials

Autoclaved (at 121°C and 15 lb.in⁻² for 20 min) coconut coir was layered on plastic trays (31 cm × 20 cm) and seedlings subjected to each treatment were planted in the trays (150 seedlings per tray). Plants were allowed to grow under greenhouse conditions for 2 weeks with regular watering of the coconut coir. 14 day old plants were introduced into a pre-leveled field prepared for planting and they were hand-planted separating the groups of plants subjected to different treatments according to randomized block design (Fig. 1).

Plants were allowed to grow in the field for 4 months until maturation under controlled conditions: the water depth was maintained evenly at 5 inches with 2 weekly additions of a liquid organic fertilizer mixture supplemented with dolomite (green manure, cow dung and dolomite, 7:2:1).

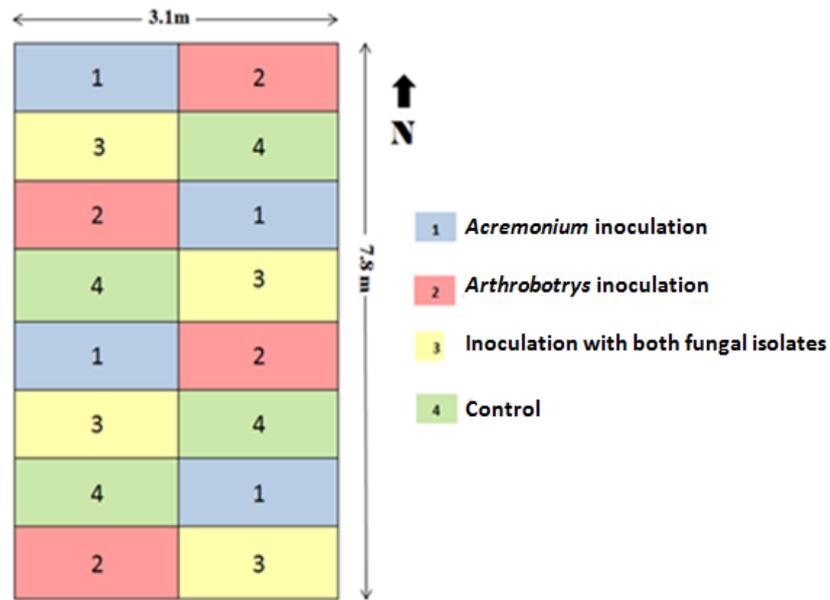


Figure 1. Lay out of the field trials.

Parameters Evaluated

Height, fresh and dry weights of the plants under greenhouse and field conditions were measured for each treatment separately at 2 weeks, 4 weeks and 10 weeks after planting. The length of the crown to the end point of the flag leaf was measured as plant height. Dry weight of the plant was measured by oven drying at 60°C until a constant weight was reached. Number of tillers per plant of the plants grown under field conditions was measured at the panicle initiation stage (10 weeks after planting). Weight of the seeds per plant was measured at harvesting stage. The results were analyzed statistically using ANOVA and pair wise comparisons of treatments were done using T-test.

RESULTS

Microscopic observations of endophytic fungi in Kuruluthuda

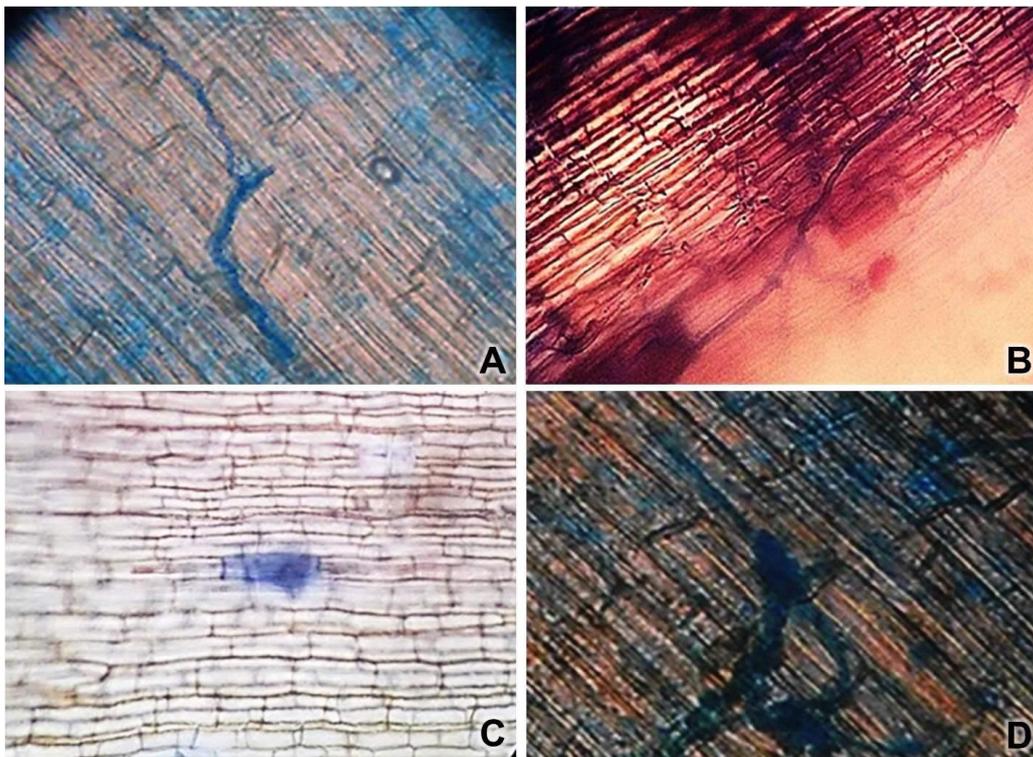


Figure 2. Endophytic fungal structures in Kuruluthuda roots: **A–B**, Trypan blue stained hyphae of endophytic fungal species (10×40×2); **C–D**, Sclerotium-like structures of colonized endophytic fungi (10×40×3).

Root segments stained with trypan blue were examined for the presence of endophytic fungal structures. Intra and intercellular hyphae were seen in the root cortex parallel to the longitudinal axis of the roots (Fig. 2A & B), and intra-cellular, sclerotium-like structures were observed in roots (Fig. 2C & D).

Table 1. Occurrence of endophytic fungi in different parts of Kuruluthuda plants.

Plant part	Total number of samples	Number of fungal species isolated	Percentage frequency of isolation
Root	25	13	52
Stem	25	6	24
Leaf	25	5	24
seed	25	3	12
Total	125	27	21.6

Note: Fungi isolated from surface sterilized plant parts placed on MEA plates. There were 25 replicates for each plant part.

Isolation and identification of endophytic fungi from Kuruluthuda rice variety

A total of 27 endophytic fungi were isolated from 125 samples of root, stem, leaf and seeds of plants of Kuruluthuda assessed. Endophytic fungi were isolated at a higher frequency from root segments (Table 1). *Acremonium*, *Arthrotrichum*, *Colletotrichum* and *Humicola* showed ubiquitous colonization as they were isolated at least from 3 different plant parts and *Acremonium* was the most dominant endophytic fungus present in Kuruluthuda (Table 2).

Table 2. Dominant endophytic fungal percentage and their colonized plant part of Kuruluthuda.

Endophytic fungi	Colonized plant part	Percentage dominance
<i>Acremonium</i>	seed, stem, leaf	60.0
<i>Arthrotrichum</i>	root, stem, leaf	38.6
<i>Aspergillus</i> sp1	root	10.4
<i>Aspergillus</i> sp2	root	20.8
<i>Aureobasidium</i>	root	0.8
<i>Chaetomium</i>	root	10.4
<i>Colletotrichum</i>	stem, leaf, seed	26.4
<i>Curvularia</i>	root	13.6
<i>Fusarium</i>	seed	3.2
<i>Humicola</i>	root, stem, leaf	33.6
<i>Penicillium</i> sp1	root	6.4
<i>Penicillium</i> sp2	stem	8.0
<i>Phoma</i> sp1	root	8.0
<i>Phoma</i> sp2	stem	4.8
<i>Rhizoctonia</i>	leaf	0.8
<i>Rhizopus</i> sp1	stem	4.0
<i>Trichoderma</i> sp1	root	5.6
<i>Trichoderma</i> sp2	root	1.6
Unidentified genus 1	root	13.6
Unidentified genus 2	root	8.8
Sterile Mycelia (SM) 1	stem	4.0
SM 2	root	0.8
SM 3	root	2.4
SM 4	leaf	4.8
SM 5	root, leaf	9.6
SM 6	seed	0.8

Effectiveness of the methods used for endophyte inoculation to rice seedlings

Two of the most frequently isolated fungal endophytes *i.e.* *Acremonium* and *Arthrotrichum* were inoculated to seedlings separately and in combination to test their effect on the growth and productivity of the rice variety

Kuruluthuda using spore suspension and plate methods. To test for successful entry and colonization of seedlings, re-isolations were carried out. Fungal species identical to those that were inoculated were re-isolated from the seedlings inoculated using both methods. The inoculated fungal species were not isolated from the non-inoculated seedlings treated with SDW (controls). These results confirmed that both methods of inoculation of the endophytes into seedlings were successful.

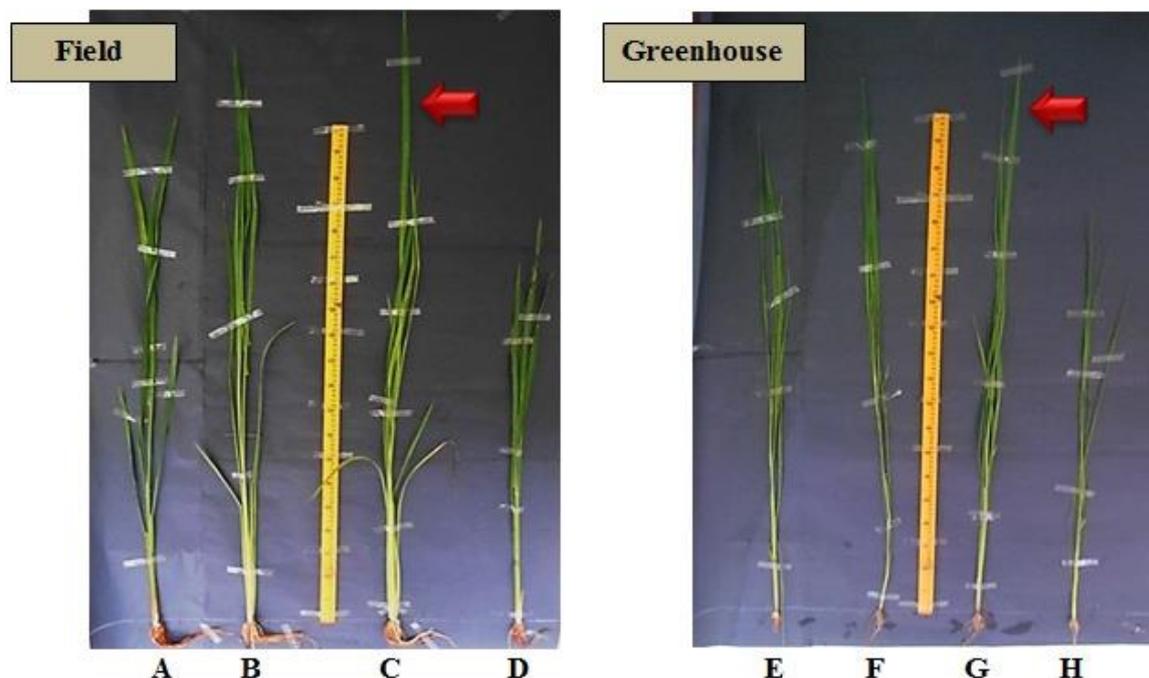


Figure 3. Variation in shoot length after 10 weeks: A–D, Kuruluthuda plants grown under field conditions; E–H, Kuruluthuda plants grown under greenhouse conditions; A,E, *Acremonium* inoculated plants; B,F, *Arthrobotrys* inoculated plants; C,G, *Acremonium*&*Arthrobotrys* inoculated plants; D,H, non-inoculated plants. Plants inoculated with both endophytic fungi show a significant increase of plant height (arrowed).

Effect of *Acremonium* and *Arthrobotrys* on growth and yield of Kuruluthuda

A significant difference ($P \leq 0.05$) in plant height as well as fresh and dry weights was observed for the plants subjected to all 3 treatments (i. *Acremonium* inoculation, ii. *Arthrobotrys* inoculation, iii. *Acremonium* and *Arthrobotrys* combined inoculation) when compared with non-treated plants under both field and greenhouse conditions after two, six and ten weeks (Table 3,4 & 5) (Fig 3). Plant height, fresh weight and dry weight of all treated and non-treated plants increased with time up to 10 weeks and finally became constant at panicle initiation stage.

Table 3. Effect of endophyte inoculation on plant height 2, 6 and 10 weeks after planting.

Treatment	Mean plant height (cm) \pm SE					
	After 2 weeks		After 6 weeks		After 10 weeks	
	Field	Greenhouse	Field	Greenhouse	Field	Greenhouse
<i>Acremonium</i> inoculation	18.7 \pm 0.28 ^a	17.9 \pm 0.37 ^a	71.8 \pm 0.98 ^a	64.9 \pm 1.44 ^a	106.0 \pm 1.21 ^a	93.6 \pm 2.03 ^a
<i>Arthrobotrys</i> inoculation	18.5 \pm 0.38 ^a	19.1 \pm 0.50 ^{a,b}	79.5 \pm 0.91 ^b	78.5 \pm 1.64 ^{a,b}	113.7 \pm 0.74 ^b	108.5 \pm 1.93 ^b
<i>Acremonium</i> & <i>Arthrobotrys</i> inoculation	19.2 \pm 0.37 ^a	20.0 \pm 0.45 ^b	81.6 \pm 1.01 ^b	84.0 \pm 0.90 ^b	117.1 \pm 0.84 ^b	110.2 \pm 1.88 ^b
Non inoculation	14.5 \pm 0.32 ^b	14.3 \pm 0.41 ^c	58.3 \pm 0.93 ^c	51.0 \pm 1.16 ^c	98.3 \pm 0.84 ^c	73.6 \pm 1.24 ^c

Note: n=20; mean \pm SE; Mean values sharing common letters in each row are not significantly different $p \leq 0.05$.

Plants inoculated with *Arthrobotrys* showed a significant difference ($P \leq 0.05$) in plant height, fresh weight, dry weight, tiller number and yield compared to the plants inoculated with *Acremonium* (Table 3,4,5,6 & 7) (Fig 3 A,B & E,F). There was a significant difference ($P \leq 0.05$) in fresh and dry weights in plants inoculated with both fungal species separately and in combination in both greenhouse and field trials as compared with non-inoculated plants. This was shown as early as 6 weeks (Table 4 & 5). There was no significant difference ($P \leq 0.05$) in plant height and in tiller number between *Arthrobotrys* single inoculation and combined inoculation of both fungal species. However it was significantly different ($P \leq 0.05$) when compared to *Acremonium*

inoculated plants after 6 and 10 weeks of planting (Table 3 & 6). Combined effect of two endophytic fungi showed an increase of the fresh weight and dry weight than their individual effects after 6 and 10 weeks of planting (Table 4 & 5).

Table 4. Effect of endophyte inoculation on fresh weight 2, 6 and 10 weeks after planting.

Treatment	Mean fresh weight (g) ± SE					
	After 2 weeks		After 6 weeks		After 10 weeks	
	Field	Greenhouse	Field	Greenhouse	Field	Greenhouse
<i>Acremonium</i> inoculated	0.28 ± 0.01 ^a	0.18 ± 0.02 ^a	3.41 ± 0.98 ^a	1.71 ± 0.38 ^a	7.25 ± 0.22 ^a	5.20 ± 0.19 ^a
<i>Arthrobotrys</i> inoculated	0.39 ± 0.02 ^b	0.20 ± 0.02 ^a	3.85 ± 0.78 ^b	1.82 ± 0.51 ^b	7.86 ± 0.20 ^b	5.25 ± 0.22 ^a
<i>Acremonium</i> & <i>Arthrobotrys</i> inoculated	0.40 ± 0.03 ^b	0.21 ± 0.02 ^a	4.21 ± 0.53 ^c	2.15 ± 0.48 ^c	9.31 ± 0.19 ^c	5.74 ± 0.13 ^b
Non inoculated	0.13 ± 0.01 ^c	0.08 ± 0.01 ^b	2.24 ± 0.37 ^d	1.14 ± 0.36 ^d	6.24 ± 0.12 ^d	3.87 ± 0.17 ^c

Note: n=20; mean ± SE; Mean values sharing common letters in each row are not significantly different $p \leq 0.05$.

Table 5. Effect of endophyte inoculation on dry weight 2, 6 and 10 weeks after planting.

Treatment	Mean dry weight (g) ± SE					
	After 2 weeks		After 6 weeks		After 10 weeks	
	Field	Greenhouse	Field	Greenhouse	Field	Greenhouse
<i>Acremonium</i> inoculated	0.049 ± 0.002 ^a	0.034 ± 0.002 ^a	1.095 ± 0.23 ^a	0.478 ± 0.02 ^a	2.478 ± 0.05 ^a	1.115 ± 0.05 ^a
<i>Arthrobotrys</i> inoculated	0.050 ± 0.003 ^a	0.050 ± 0.02 ^b	1.214 ± 0.16 ^b	0.584 ± 0.035 ^{a,b}	2.707 ± 0.021 ^b	1.244 ± 0.036 ^b
<i>Acremonium</i> & <i>Arthrobotrys</i> inoculated	0.052 ± 0.003 ^a	0.055 ± 0.02 ^b	1.586 ± 0.12 ^c	0.748 ± 0.11 ^c	3.421 ± 0.002 ^c	1.586 ± 0.01 ^c
Non inoculated	0.030 ± 0.001 ^b	0.014 ± 0.001 ^c	0.894 ± 0.08 ^d	0.361 ± 0.013 ^d	1.987 ± 0.04 ^d	0.758 ± 0.02 ^d

Note: n=20; mean ± SE; Mean values sharing common letters in each row are not significantly different $p \leq 0.05$.

A significant difference ($P \leq 0.05$) in tiller number and yield per plant was observed in the plants subjected to all 3 treatments compared with non-treated plants under field conditions (Table 6 & 7). Combined inoculation of *Acremonium* and *Arthrobotrys* showed an increase of yield than the plants subjected to other two treatments (Table 7). However data indicated that the overall effect of *Arthrobotrys* was higher than that of *Acremonium*. There was no significant difference in plant height between plants grown under greenhouse and field condition but the fresh weight and dry weight increased in all treated and non-treated plants under field condition when compared to greenhouse condition (Table 3).

Table 6. Mean number of tillers at panicle initiation stage. (After 10 weeks of planting)

Treatment	Mean number of tillers ± SE
<i>Acremonium</i> inoculated	5.5 ± 0.14 ^a
<i>Arthrobotrys</i> inoculated	6.0 ± 0.14 ^b
<i>Acremonium</i> & <i>Arthrobotrys</i> inoculated	7.3 ± 0.16 ^b
Non inoculated	4.6 ± 0.11 ^c

Note: n=20; mean of tillers ± SE; mean values that do not share a letter are significantly different $p \leq 0.05$.

Table 7. Mean yield/plant (g) of Kuruluthuda variety. (After 12 weeks)

Treatment	Mean yield per plant (g) ± SE
<i>Acremonium</i> inoculated	5.99 ± 0.12 ^a
<i>Arthrobotrys</i> inoculated	7.57 ± 0.12 ^b
<i>Acremonium</i> & <i>Arthrobotrys</i> inoculated	9.65 ± 0.23 ^c
Non inoculated	4.54 ± 0.17 ^d

Note: n=20; mean of yield/plant ± SE; mean values that do not share a letter are significantly different $p \leq 0.05$. The mean weight of seeds per plant is considered as yield.

DISCUSSION

There is renewed interest in the cultivation of traditional rice varieties using organic farming practices in Sri Lanka due to numerous health problems such as the Chronic Kidney Disease of Unknown Etiology (CKDU) which is spreading fast amongst farmers in the country as well as various environmental problems. The traditional rice varieties, whilst responding well to organic cultivation practices, produce low yields and thus, finding means of yield improvement is important. Research based on the development of effective organic fertilizers consisting of applicable microorganism preparations for increased yield is a viable solution to the problem. Endophytic fungi that grow as symbionts in plants have been reported to enhance growth in a number

of crops as well as in organic rice cultivation (Angelard *et al.* 2010). The ability of endophytic fungi to improve plant growth and reduce rice blast incidence in traditional rice varieties of Sri Lanka has also been reported. (Atugala & Deshappriya 2015, Ponnawila & Deshappriya 2014).

Therefore, the present study was carried out to test whether fungal endophytes of the Sri Lankan traditional rice variety Kuruluthuda would show a similar capacity for growth and yield enhancement of rice plants under green house and field conditions. Determination of the endophytic association was initialized by isolation of the endophytic fungal assemblage present in different parts of plants of the variety Kuruluthuda. As the use of fresh plant material is a critical requirement for successful isolation, fresh plant samples collected from the field were used for fungal isolations within 48 hours. Direct observation of trypan blue stained squashed root preparations indicated the co-existence of endophytic fungi inside roots without causing apparent damage to plant tissue.

Since the endophytes are a group of microorganisms that can spend a part of their life cycle in different parts of the same plant (Petrini *et al.* 1992), most parts of the plant *i.e.* roots, stem, leaves and seeds were used for the isolations. A suitable surface sterilization regime is essential to eliminate epiphytic microbes present on the plant surface whilst maintaining the viability of the endophytes. Therefore, the most effective surface sterilizing regimes developed in a previous study (Atugala & Deshappriya 2015) were used for the effective surface sterilization of the plant parts used for isolations. Once surface sterilized, the plant parts were plated on MEA medium supplemented with tetracycline (50 mg/L) as soil and endophytic fungi are reported to grow more effectively on MEA resulting in a higher fungal yield and species richness (Bills & Polishook 1993). Tetracycline added to the medium restrained bacterial growth until emergence of fungal colonies from the plant segment.

Isolation of endophytic fungi from healthy *Oryza sativa* plants has been reported previously to yield a few fungal genera such as *Fusarium*, *Aspergillus*, *Curvularia*, *Penicillium*, *Gilmaniella* and *Arthrobotrys foliicola* at a high frequency (Zakaria *et al.* 2010). Most fungal genera isolated from Sri Lankan traditional rice varieties Suwandel and Kaluheenati belonged to the class ascomycetes and they have been identified as being members of commonly observed genera of soil fungi, *e.g.* *Fusarium*, *Penicillium*, *Rhizopus*, *Rhizoctonia*, *Absidia*, *Aspergillus* and *Paecilomyces* (Atugala & Deshappriya 2015). In a comparison of endophytic assemblages between traditional and newly improved rice varieties in Sri Lanka, higher fungal colonization and species richness have been shown by the traditional rice varieties Suwandel and Herath Banda compared to the newly improved variety Bg 352. *Fusarium* and *Arthrobotrys* have been identified as the most frequently colonized endophytic fungi in Herath Banda traditional variety (Ponnawila & Deshappriya 2014). In the present study, 27 different fungal genera were isolated from the variety Kuruluthuda. However, unlike in the previous studies, the most frequently isolated genera were *Acremonium*, *Arthrobotrys*, *Colletotrichum* and *Humicola*. *Acremonium* was mostly isolated from leaf and stem, *Arthrobotrys* from roots, *Colletotrichum* from stem and *Humicola* from leaves. Colonization and isolation frequencies of isolates in each plant part revealed that Kuruluthuda has a rich endophytic fungal diversity that is ubiquitous within the whole plant. Nevertheless, most isolates were restricted to specified areas of the plants. A surprisingly rich endophytic community was associated with roots but *Fusarium* and sterile mycelia species were restricted only to seeds.

The combined and individual effects of the two most frequently isolated fungal endophytes *Acremonium* and *Arthrobotryson* growth and production of variety Kuruluthuda was tested under field and greenhouse conditions. Previous studies have shown that fungal inoculation into immature roots of seedlings was successful when they were in direct contact with fungal cultures (Fischer 1962, Herre *et al.* 2007) or immersed in a spore suspension (Champion *et al.* 1973, Abdel-Motaal *et al.* 2010). In this study, both methods were used and the ability to reisolate the same fungal species from the seedlings as that of the inoculated species confirmed the success of both spore suspension and plate methods in introducing the endophytes to plant seedlings. This is significant as successful and easy introduction of inoculum into plants is an essential aspect of development of a biofertilizer.

In the present study, endophyte inoculated plants showed a significant increase of plant height, fresh weight and dry weight ($P \leq 0.05$) when compared with non-inoculated plants during a 10 week period under both field and greenhouse conditions. These results are in agreement with previous studies carried out in Sri Lanka (Ponnawila & Deshappriya 2014, Atugala & Deshappriya 2015) and indicate that rice plant growth can be enhanced by introducing their endophytic myco flora.

Mechanisms of growth enhancement were not studied in the present study. However evidence from previous studies have indicated that fungal endophytes promote plant growth and performance in a number of ways

including the production of siderophores, supplying biologically fixed nitrogen and by secretion of plant growth regulators (Herre *et al.* 2007, Kedar *et al.* 2014). The endophytic fungi of *Potentilla fulgensis* is reported to have the metabolic machinery to secrete plant growth hormones such as cytokinins, auxins and gibberellins which promote seed germination and growth (Bhagobaty & Joshi 2009).

Khan *et al.* (2015) reported that two fungal endophytes, *Fusarium* and *Alternaria* isolated from the leaves of *Solanum nigrum*, increased the chlorophyll content, root-shoot length, and biomass production of Dongjin rice plants when inoculated at the seedling stage. Gas chromatography/ mass spectrometry analysis for the culture filtrates of each fungal endophytic isolate revealed that both *Fusarium* and *Alternaria* could produce 54 and 30 $\mu\text{g}\cdot\text{mL}^{-1}$ of indole acetic acid, respectively.

In addition, Kedar *et al.* (2014) revealed a significant effect on shoot height, root length, fresh and dry weights of shoots and roots of experimental maize plants inoculated with the endophytic fungus *Phoma* compared to non-inoculated plants. It was also found that the roots of germinated seeds treated with the *Phoma* extract showed development of additional root hairs allowing the absorption of more nutrients which subsequently resulted in increased growth and biomass of plants.

Based on these reports, it can be concluded that endophytes have a number of mechanisms to enhance plant growth and the similar trend of growth enhancement observed in the present study may also be attributed to one or more of such mechanisms.

In most previous studies, individual effects of two endophytic fungi on host plant performances have been tested. Waqas *et al.* (2012) reported that the endophytic fungi *Phomaglomerata* and *Penicillium* sp. significantly promoted the shoot growth of rice as well as increased plant biomass and related growth parameters of cucumber plants compared to non-inoculated plants. However, the combined effect of two endophytes has not been evaluated in these studies. In the present study, the combined effect of two endophytic fungal species showed an increase of the fresh and dry weights when compared with their individual effects on Kuruluthuda plants under both field and greenhouse conditions. It can be concluded that *Acremonium* and *Arthrobotrys* may have a synergistic effect that results in improved plant weight. A significant difference of plant height could not be observed between *Arthrobotrys* single inoculation and combined inoculation with both fungal species. However, there was a significant increase in the height of plants inoculated with *Arthrobotrys* when compared with those inoculated with *Acremonium* indicating that *Arthrobotrys* might have the ability to improve plant height than *Acremonium*.

Although tillers of the Kuruluthuda variety were formed 4 weeks after planting, the number of tillers was counted at the panicle initiation stage to obtain an accurate count of tillers per one bush. An increased number of tillers could be observed for the plants inoculated with both fungi. It is reported that endophytic fungi associated with many agricultural crops improve crop production significantly (Yuan *et al.* 2009). In this study also endophytes inoculated Kuruluthuda plants yielded a significantly higher amount of seeds when compared with non-inoculated plants. Plants inoculated with both *Acremonium* and *Arthrobotrys* gave a higher yield as well as improved fresh and dry weights. Therefore it can be concluded that the combination of two endophytic fungi can result in increased biomass and rice yield. These results were evident as early as six weeks after planting and remained to be so up to the harvesting stage (12 weeks). There was no difference in plant height between plants grown under greenhouse and field conditions but increased fresh weight and dry weight could be observed under field conditions when compared to greenhouse condition. The enhanced effect of endophyte inoculation shown by plants grown under field conditions when compared with those grown under greenhouse conditions was both an interesting and encouraging result.

Based on these very promising results, an inoculum consisting of the two fungal endophytes *i.e.* *Acremonium* and *Arthrobotrys* could be utilized to enhance the biomass and yield of the rice variety Kuruluthuda. This is the first report on the endophytic fungal assemblage present in the variety Kuruluthuda and their potential to be used for improved productivity of the rice variety under field conditions. The possibility of developing such an inoculum as a biofertiliser for other rice varieties after more stringent tests could be the solution to the many problems associated with the use of chemical fertilizers in rice cultivation in Sri Lanka and worldwide.

CONCLUSIONS

- Kuruluthuda traditional rice variety consists of a high diversity of fungal endophytes in most parts of the plant *i.e.* root, stem, leaf, and seeds.

- Spore suspension method and plate method can be used for successful inoculation of fungal endophytes into seedling roots.
- *Acromonium* and *Arthrobotrys* can improve the growth of rice plants of the variety Kuruluthuda and yield under field conditions.
- Combined effect of the two endophytes enhances the fresh weight, dry weight, number of tillers and yield of rice plants significantly as compared to their effect when inoculated separately.

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