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In Vitro Growth-promoting Properties of Non-dominant Root Symbiotic Fungi (ND-RSF) from *Drynaria quercifolia* L. and their Effects on PSB Rc10 Rice (*Oryza sativa* L.)

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The use of microorganisms as an environmentally safe method for agricultural crop production and biocontrol has increased in recent years but is still considerably underexploited. This study explored the potential growth-promoting properties of non-dominant root symbiotic fungal (ND-RSF) isolates from *Drynaria quercifolia* L. and their effects on rice. The five molecularly identified ND-RSF isolates induced indole-3-acetic acid (IAA) production spectrophotometrically at 530 nm. The *Trichoderma scalesiae* isolate produced the highest IAA per unit volume of culture broth and the highest IAA per unit of dry weight. All five ND-RSF isolates have phosphate solubilization activity. Three ND-RSF isolates significantly increased the total length of paclobutrazol, a gibberellic acid (GA) inhibitor, treated rice plants. The fresh weight of rice inoculated with *Aspergillus brunneoviolaceus* is significantly heavier than the negative broth and water control. The dry weight of rice inoculated with *Aspergillus aculeatus*, *Aspergillus japonicus*, and *Aspergillus brunneoviolaceus* are significantly higher compared to the positive controls (20 PPM GA, 20 PPM IAA, and non-paclobutrazol treated rice seedlings). The results indicate the ability of these three *Aspergillus* isolates to synthesize the GA hormone. The ability of these ND-RSF isolates to produce growth-promoting hormones auxin and GA, plus their ability to solubilize inorganic phosphate are evidence to their potential growth-promoting abilities toward their host plant. This present study also implies that these ND-RSF can be growth-promoting mutualists to rice.

Keywords: auxin, *Drynaria quercifolia*, growth-promoting properties, rice production, root symbiotic fungi

INTRODUCTION

Rice (*Oryza sativa* L.) is the indispensable food for over half of the globe's population (Muthayya *et al*. 2014). Ninety percent (90%) of total rice production is found in Asia (Evans 1998). This crop has supported more number of consumers than any other crop since it was domesticated over 10,000 years ago (Fairhurst and Dobermann 2002). In the Philippines, rice is a basic food necessity. It provides more than 10% gross value added and about 5% gross domestic product, and it supplies more than 50% household income to millions of Filipino families (Barba *et al*. 2014). Several dynamics threaten the prospect of rice yield in the country – including industrial development, land-use, and contending agricultural uses. These aspects have curbed the space dedicated to rice production. In addition, the diminishing attributes of land systems exacerbate the falling quantity of land resources due to years of mono-cropping traditions (Flinn and De Datta 2004). As a chief part of food

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allocation in the country, a decline in rice production means an increase in rice costs – which could significantly raise people's living expenses – generating more impoverished Filipinos. With the enormous value of rice to the Philippine economy, it is crucial to realize the biological and physiological activities involved in rice metabolic development and uncover the most feasible agricultural substitutes to increase rice production (Onyango 2014).

One of the many possibilities to stimulate growth and enhance rice production is the use of agronomically important microbes (Tiwari *et al*. 1989). This ecologically safe method has augmented in recent years but is still considerably under-utilized (De Souza *et al*. 2015). Soil microbes in the form of bacteria and fungi are the best known in the process of decomposition; therefore, they are highly regarded in agriculture. Previous studies have revealed that symbiotic fungi have significant effects on plant physiology and survival as they shape plant community dynamics (Brundrett 2006). The steady interactions of the fungal symbionts with their hosts create an ensuing influence on one another's physiological dynamics, metabolism, and by-products (Tian *et al*. 2014).

One example of symbiotic influence is the production of phytohormones by fungal species, which is vital in the growth, development, and enhancement of metabolic activities in the host plants that they colonize (Sugiharto *et al*. 2016). Several fungal species induce a number of essential plant hormones like GAs and auxins (*e.g.* IAA) (Hermosa *et al*. 2012). IAA is a phytohormone that promotes cell elongation and apical dominance (Taiz and Zeiger 2006). The GA phytohormones affect the life cycle of plants from the germination of the seed, elongation of the stem, flower development, anther growth, and seed development (Hedden and Kamiya 1997). Furthermore, GA has received critical attention due to its various implications in the agricultural sector – including the induction of hydrolytic enzyme activities to improve crop yield, to overcome dwarfism, and to eliminate dormancy (Rangaswamy 2012). In addition, the fast and strong effects of GA on the above processes lead to increased agricultural harvests, eventually augmenting the food industry (Rios-Iribe *et al*. 2010). Because of these, the search for GA producing fungi has significantly increased in recent years. Various species of fungi including *Gibberella*, *Aspergillus*, and *Neurospora* have been found to synthesize GA (Bilkay *et al*. 2010). Hamayun *et al*. (2010) were able to isolate a GA-producing fungus, *Penicillium* sp. MH7. This fungal isolate was tested for its possible growth promotion in *Chrysanthemum coronarium*. The results of their study show that the culture suspension of the isolate significantly increased the growth of *C. coronarium* due to the presence of a physiologically active GA hormone.

Aside from phytohormone production, some fungi are also considered as P-degrading microorganisms. They are able to transform insoluble phosphorus into its soluble conformation for plant utilization (Khan *et al*. 2014). This feasible alternative can greatly help in plants' assimilation of P because the majority of this element cannot be accessed by plants in their natural setting as they are normally bound tightly with cations (*e.g.* Fe, Al, Ca) (Son *et al*. 2006). Rather than using the conventional chemical processes using sulfuric or phosphoric acid, these P-solubilizing fungi can be a preferred cheaper alternative to access these insoluble forms of phosphorus (Xiao *et al*. 2009). The discovery and use of these P-solubilizing fungi will reduce environmental pollution posed by the traditional chemical processes (Elias *et al*. 2016).

The potentiality of fungi to synthesize auxins and GA, together with their capacity to produce enzymes and other growth-promoting secondary metabolites that aid their development and their host's successful growth even under stressful environments, show their unequivocal importance in the agricultural industry. Despite these potential growth-promoting activities of fungal symbionts, only about 300 species or 0.02% of these fungal species have been presently described within a phylum (Lee *et al*. 2013). The remaining 99.98% is yet to be studied. This indicates the scarcity of taxonomic literature on symbiotic fungi. Since almost, if not, all plants symbiotically harbor fungi, the task in identifying the agricultural potentials of these microbes is demanding.

To advance this challenging task, the relationship of fungi in the epiphytic fern *Drynaria quercifolia* L. is a potential candidate for a thorough investigation. These epiphytic ferns are often exposed to environmental stresses; hence, their growth and survival on these habitats can be attributed to their interaction with symbiotic fungi. This hypothesis is supported by Selosse and Le Tacon (1998), to where they pointed-out that plant-fungi mutualistic relationships can help plant acclimate to abiotic stresses – including extreme temperature, intense solar radiation, and even desiccation. Aban *et al*. (2017a) published a paper indicating the growth-promoting potentials (IAA production, P-solubilization, and ACC degradation activity) of dominant fungal symbionts found in the epiphytic fern *Drynaria quercifolia* L. They further suggested that the fern's ability of survive even when exposed to multiple abiotic stresses (*e.g.* increased temperature, high light intensity, and low moisture) is due to their symbiotic interaction with the dominant symbiotic fungi found in their roots (Aban *et al*. 2017b). In this present study, it is speculated that even the ND-RSF found in *Drynaria quercifolia* L. have crucial functioning and indispensable roles in the growth and survival of the fern host.

This is the first study that explores the growth-promoting abilities of ND-RSF on *Drynaria quercifolia* L. These growth-enhancing mechanisms may be utilized to improve the growth of important agricultural crops such as rice. Specifically, this present study (1) identified the ND-RSF isolated from *Drynaria quercifolia* L.; (2) determined the plant growth-promoting traits of the ND-RSF, including IAA production and phosphate solubilization; and (3) investigated the effects of the ND-RSF on the initial growth of paclobutrazol- treated rice seedlings.

MATERIALS AND METHODS

Isolation of Root Symbiotic Fungi

A total of 10 ferns (*Drynaria quercifolia* L.) were gathered by taking two ferns from each of the five *Samanea saman* (Jacq.) Merr. (acacia) tree-collection sites located in Bacnotan, La Union, Philippines (GPS coordinates: 16°43′30.8″N 120°23′37.6″E). The roots of these ferns were collected and were washed with water to remove unwanted debris. The roots were transported to the Natural Science Research Unit (NSRU) at Saint Louis University, Baguio City, Philippines for fungal isolation. The samples were placed in zip-locked plastic containers to maintain the roots' integrity during transport.

At the NSRU laboratory, the roots collected were washed again with distilled water. The roots were then cut into 0.5 cm representative segments. A total of 300 representative root segments were prepared from the 10 ferns. The root segments were thoroughly washed with sterile water thrice in sequence and blotted dry on sterile absorbent paper to ensure that only the symbiotic fungi will be isolated from the root segments.

The root segments were mounted at equal distances between and among the segments on potato dextrose agar (PDA) (HiMedia, India). Chloramphenicol was added in the agar medium to suppress bacterial contamination, and rose bengal was mixed to retard the fast-developing filamentous fungal growth. The agar plates were stored and incubated for 7 d in ambient room conditions. Each colony growth within the representative root segments was transferred to PDA slants for molecular identification.

DNA Extraction

The bar-coding genes (genomic DNA) were isolated following the protocol of Liu *et al*. (2000). In a 1.5-mL Eppendorf tube, 1 mL fungal broth was prepared and 500 µL of lysis buffer was added. The lysis buffer consists of: 1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM ethylenediaminetetraacetic acid (pH 8.0), and 400 mM Tris-HCl (pH 8.0). The fungal DNA was freed from the fungal broth immersed in the lysis buffer and, for 10 min, the mixture was allowed to settle at ambient room conditions. A potassium acetate solution (pH 4.8) was prepared by mixing glacial acetic acid and 5 M potassium acetate in distilled water. This solution (150 µL) was added to the existing lysis buffer-fungal broth mixture. This new mixture was spun at 10,000 x g for 60 s. Succeeding centrifugation using the same centrifuge speed was done to the supernatant that was extracted and then was placed in another 1.5 mL Eppendorf tube. The supernatant (500 µL) was transferred to a new 1.5 mL Eppendorf tube, and the crude DNA sample was expected to have been gathered in the tube. A 1/10 volume of the sodium acetate solution (0.3M; pH 5.2) was added to the DNA sample and was thoroughly mixed. For 20 min, the mixture was isolated at –20 °C. The mixture containing the DNA sample was defrosted and was spun for 1 min at 10,000 x g. The supernatant was drawn off and the sediment containing the DNA sample was carefully collected. Subsequent purification of the DNA sample was done by washing it with 70% ethanol solution and was centrifuged again. The supernatant was decanted and the tube holding the DNA samples was air-dried. The DNA sample was immersed again in 50 µL TE buffer and the suspension was kept at 4 °C. To ascertain the purity of the extracts, the DNA samples were run in 1% gel electrophoresis.

DNA Amplification and Sequencing

The internal transcribed region (ITS) of the 18S rDNA found the extracted DNA samples were amplified using universal primers ITS-1 (5'TCC GTA GGT GAA CCT GCG G-3') for the forward primer and ITS-4 (TCC TCC GCT TAT TGA TAT GC') for the reverse primer. To begin the amplification of the samples, a polymerase chain reaction (PCR) mixture was prepared by adding the following: 1 µL of isolated DNA samples, 22 µL of nuclease-free water, 1 µL reverse primer, 1 µL forward primer, and 25 µL of the Vivantis PCR Master Mix. The PCR mixture was pre-denatured in the PCR machine at 95 °C for 5 min. The succeeding step was done by subjecting the PCR mixture to 35-repeated PCR cycles of 95 °C for 30 s to 60 °C for 60 s and 72 °C for 60 s amplification stage. The final extension phase optimized the amplification procedure by exposing the PCR mixture to 72 °C for 6 min. The PCR-generated amplicons (approximately 550 base-pair length) were tested in 1% gel electrophoresis. The amplicons were sent to the 1st Base sequencing facility (Malaysia) for cleaning and sequencing.

Molecular Identification

The Basic Local Alignment Search Tool nucleotide search program was used to look for nucleotide sequence

similarity on the sequenced ITS of the 18S rDNA of the RSF isolates. These sequences were compared with the available sequences in GenBank of the National Center for Biotechnology Information (NCBI) for proper molecular identification.

Determination of ND-RSF

The determination of ND-RSF is based on the calculation of the colonization and occurrence rates computed by Aban *et al*. (2017b). The colonization percentage was obtained using the formula: % colonization = NSCz / NSCt x 100, where NSCz is the root representative segments colonized and the NSCt is the total number of segments collected. With the given formula, the five most dominant RSF from each of the five *Samanea saman* (Jacq.) Merr. (acacia) tree collection sites in their study were calculated. *Trichoderma yunnanense* is the most dominant in Site 1 (n = 17). *Trichoderma simmonsii* (*n* = 18), undescribed Mucoromycotina isolate F9P2RSF21 (n = 21), and another undescribed Mucoromycotina isolate F5P1RSF16 (n = 34) are the dominant RSF isolates in Sites 2, 3, and 4, respectively. The most dominant RSF isolate in Site 5 is *Meyerozyma guilliermondii* (n = 14) (Aban *et al*. 2017b). Any RSF isolates with lower calculated n in each of the tree-collection sites were considered ND-RSF. This became the basis of dominance and non-dominance of root symbiotic fungi in the five *Drynaria quercifolia* L. tree-collection sites.

Auxin (IAA) Production Assay

The protocol of Xin *et al*. (2009) was followed with few modifications for the auxin production assay. L-tryptophan (0.1% (w/v) (HiMedia, India) was supplemented to Sabouraud dextrose broth (SDB) and the ND-RSF isolates were incubated for 7 d. Sterile SDB was used as the control. The ND-RSF cells (1.5 mL) were pelleted by centrifugation at 10,000 x g for 5 min. Salkowski reagent [2 mL 0.5M $FeCl_3$ (Eisen-Golden Laboratories; USA), 98 ml 35% $HClO_4$ (Apha Chemika, India)] was mixed with 1 mL of the culture suspension. Salkowski reagent was used as a rapid colorimetric assay that recognized indolic compounds, with IAA having an optimal peak of 530 nm (Glickmann and Dessaux 1995). The CF-Salkowski's mixture was covered with foil to prevent unwanted contamination and was kept in the dark for oxidation consistencies. An ultraviolet-visible spectrophotometer (APEL PD-303) at 530 nm was utilized to measure the intensity of the pink color that developed in the mixture after 30 min. High-performance liquid chromatography water (Unichrom, Belarus) was used as blank. To quantify the IAA produced by the fungal isolates, cell pellets were oven-dried at 50 °C for 8 h or more until the dry weight was consistent for the calculation of auxin production of the ND-RSF per unit dry weight. The cell pellets' dry weight was measured using a Discovery Semi-Micro and Analytical Balance (Ohaus DV114C; USA). Likewise, the intensity of pink color was used to develop a standard calibration curve (10, 20, 30, 40, and 50 µg/mL) using commercial IAA (Sigma; USA). The procedure for IAA production was done in three replicates.

Assay for Phosphate Solubilizing Activity

The procedure in the study of Oliveira *et al.* (2009) was used for the assay on phosphate solubilization. The ND-RSF were grown in SDB for 16 h; then, 50 µL of the culture broths were grown to Pikovskaya agar (HiMedia, India) and were incubated for 7 d. Halos or clear zones indicate phosphate solubilizing capacities. The phosphate solubilization activity was done in five replications per Petri plate.

Paclobutrazol-mediated (GA Production) Assay

Rice seeds were initially surface-sterilized with 2.5% sodium hypochlorite (Zonrox) for 1 h and were rinsed with type 1 water (Unichrom; Belarus). The sterilized rice seeds were pre-germinated using Simple Nutrient Addition Program (SNAP) hydroponic solution for 7 d (Santos and Ocampo 2005). To secure equal germination, the sterilized seeds were grown simultaneously at room temperature. After 7 d and under aseptic conditions, the seedlings were immersed with 20 ppm paclobutrazol (Quali-Pro; USA) for 24 h. Simultaneous to the pre-germination of rice seeds, ND-RSF isolate suspensions were prepared. These were done by incubating the ND-RSF isolates on SDB for 7 d. Any extracellular compounds naturally produced by the ND-RSF become the integral component of the prepared suspension.

The seedlings were transferred to the different ND-RSF isolate suspensions for another 24 h. Broth (SDB) and water plus 20 ppm IAA (Sigma; USA) and GA (Spectrum; USA) were used as negative and positive controls, respectively. The rice seedlings previously inoculated in ND-RSF suspensions were transferred to test tubes with 2-mL Murashige and Skoog (MS) basal agar medium (Sigma; USA). Three replicates of three plants, consisting a total of nine plants, were prepared for each treatment. After a week or at a two-leafed stage, 50 µL of each of the isolate's culture suspension was spread at the apex of the rice seedlings. After the treatment, the rice seedlings were again allowed to grow for 7 d under artificial light (~1000 lux measured using Lutron LX-105 light meter) and at ambient conditions. Different agronomic traits including total plant-, shoot-, root length, and weight (fresh and dry) were recorded and were compared with the positive (IAA and GA) and negative control (broth and water). In the measurement of shoot, root, and total

rice seedling length, a metric ruler (Prince) was used to measure the length in cm. The seedlings' fresh and dry weight were measured using a Discovery Semi-Micro and Analytical Balance (Ohaus DV114C; USA). To measure the dry weight, the rice seedlings were oven-dried at 50 °C for 8 h or more until the dry weight was consistent. For the measurements of all agronomic traits, all replicates per treatment were uprooted and measured for calculation of the average mean.

Data Presentation and Analysis

The levels of IAA and the different growth indicators were presented using the Chart Builder bar graph with dual Y coordinates and clustered bar graphs of SPSS version 20, respectively. The same software was used for the one-way analysis of variance with Scheffe *post hoc* test to determine significant differences in the growth-promoting capacities of the RSF isolates. Error bars displayed represent ± standard deviations.

RESULTS

The five ND-RSF isolates were identified and submitted to the NCBI (Table 1). All the five isolates have an NBCI identification percentage of 99% and, hence, can be identified to their closest type match in the available sequences in GenBank of NCBI. Below are the five ND-RSF isolates with their specific extraction code and accession number: (1) *Aspergillus aculeatus* (Extraction Code: F1P3ND-RSF4; Accession Number: MK035984); (2) *Trichoderma asperellum* (Extraction Code: F2P2ND-RSF6; Accession Number: MK035985); (3) *Trichoderma scalesiae* (Extraction Code: F3P1ND-RSF11; Accession Number: MK035986); (4) *Aspergillus japonicus* (Extraction Code: F4P3ND-RSF12; Accession Number: MK035987); and (5) *Aspergillus brunneoviolaceus* (Extraction Code: F9P2ND-RSF19; Accession Number: MK035983). Meanwhile, *Aspergillus aculeatus* and *Trichoderma asperellum* have an isolation frequency of n = 5, *Trichoderma scalesiae* and *Aspergillus japonicus* have an isolation frequency of n = 7, and *Aspergillus brunneoviolaceus* has an isolation frequency of n = 1. Since their measured isolation frequencies (n) for the five isolates were lower than the calculated n of the dominant RSF, they were considered as ND-RSF.

Auxin (IAA) Production

All ND-RSF isolates produced auxin spectrophotometrically at 530 nm. The levels of auxin produced by the five ND-RSF isolates are shown in Figure 1. All the isolates produced significantly high indole acetic acid based on Salkowski's colorimetric test ($p < 0.05$). The *T. scalesiae* isolate (F3P1ND-RSF11) produced the highest IAA per mL of culture broth (11.07b ± 3.18 µg/mL). This value is more than seven times higher than the broth and water control (1.56a ± 0.76 µg/mL). Though, this value is statistically similar to the auxin produced by *A. aculeatus* (6.89b + 0.20 µg/mL), *T. asperellum* (8.52b ± 0.49 µg/mL), *A. japonicus* (9.16b ± 1.21 µg/mL), and the *A. brunneoviolaceus* isolate (10.00b ± 5.08 µg/mL). The *T. scalesiae* isolate (F3P1ND-RSF11) also produced the highest IAA per mg dry weight (0.21c ± 0.06 µg/mg), which is statistically higher than the four other ND-RSF isolates: *A. aculeatus* (0.09b + 0.00 µg/mg), *T. asperellum* (0.06b ± 0.01 µg/mg), *A. japonicus* (0.10b ± 0.01 µg/mg), and the *A. brunneoviolaceus* isolate (0.09b ± 0.07 µg/mg).

Phosphate Solubilization Activity

All the five ND-RSF isolates in *D. quercifolia* were found to have phosphate solubilization activity (Figure 2). The isolates developed clearing zones after growing in Pikovskaya agar for 7 d.

Table 1. Molecular identification and computed isolation frequencies of ND-RSF.

RSF isolate code	Closest type match	Ident.[a]	NCBI[b] accession	IF[c] (n)
F1P3ND-RSF4	*Aspergillus aculeatus*	99%	MK035984	5
F2P2ND-RSF6	*Trichoderma asperellum*	99%	MK035985	5
F3P1ND-RSF11	*Trichoderma scalesiae*	99%	MK035986	7
F4P3ND-RSF12	*Aspergillus japonicus*	99%	MK035987	7
F9P2ND-RSF19	*Aspergillus brunneoviolaceus*	99%	MK035983	1

[a]Ident. – identification percentage (NCBI GenBank)
[b]NCBI – National Center for Biotechnology Information
[c]IF – isolation frequencies showing RSF non-dominance (Aban 2019)

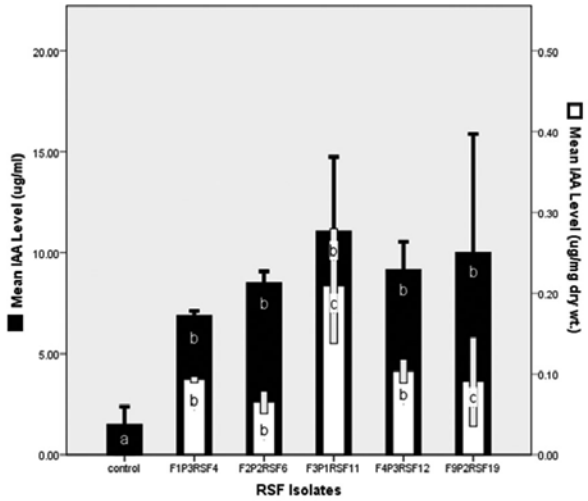

Figure 1. Mean IAA level produced by the ND-RSF Isolates found in *D. quercifolia*. Control – broth and water, F1P3ND-RSF4 – *Aspergillus aculeatus*, F2P2ND-RSF6 – *Trichoderma asperellum*, F3P1ND-RSF11 – *Trichoderma scalesiae*, F4P3ND-RSF12 – *Aspergillus japonicus*, F9P2ND-RSF19 – *Aspergillus brunneoviolaceus*. Means with different letters are significantly different ($p = 0.05$; n = 6).

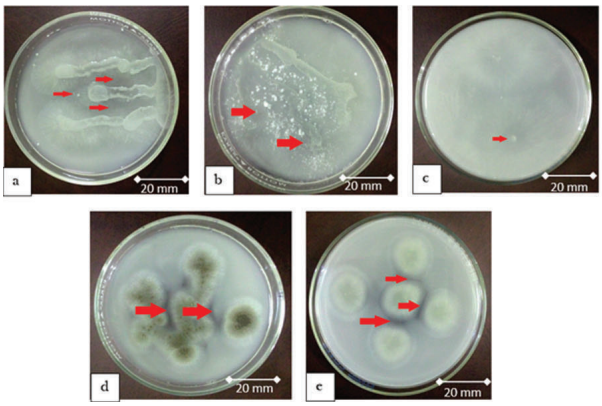

Figure 2. Halos (pointed by red arrows) surrounding some ND-RSF isolates on Pikovskaya agar indicating a positive phosphate solubilizing activity. (a) F1P3ND-RSF4 – Aspergillus aculeatus (partial clearing zones), (b) F2P2ND-RSF6 – Trichoderma asperellum (partial clearing zone), (c) F3P1ND-RSF11 – Trichoderma scalesiae (partial clearing zone), (d) F4P3ND-RSF12 – Aspergillus japonicus (positive clearing zones), (e) F9P2ND-RSF19 – Aspergillus brunneoviolaceus (positive clearing zone).

Paclobutrazol-mediated (GA Production) Assay

Figure 3 presents the mean shoot, root, and whole plant length of paclobutrazol-treated rice seedlings inoculated with ND-RSF isolates at 14 d after planting (DAP). As gleaned from the figure, the inoculation of the rice seedlings with ND-RSF isolates has contrasting effects. Three ND-RSF isolates – namely, *Aspergillus aculeatus*, *Aspergillus japonicus*, and *Aspergillus brunneoviolaceus* – significantly increased the total length of rice seedlings compared to the negative control (total length: F value 90.10 > F crit 2.07; $p < 0.05$). Rice seedlings inoculated with the *Aspergillus aculeatus* isolate attained mean total length of 17.73 ± 2.41 cm, rice seedlings inoculated with *Aspergillus japonicus* isolate reached 15.80 ± 1.41 cm total plant length, and the rice seedlings inoculated with *Aspergillus brunneoviolaceus* attained a mean total length of 18.15 ± 1.86. These values are more than twice the total length (6.38 ± 2.28 cm) of rice seedlings inoculated with the control setup broth and water. It can further be noted that the three aforementioned isolates are significantly taller than the two positive controls (20 PPM IAA and non-paclobutrazol treated rice seedlings in MS medium). The above-mentioned results suggest the ability of *Aspergillus aculeatus*, *Aspergillus japonicus*, and *Aspergillus brunneoviolaceus* to produce the hormone GA. Consequently, after 14 DAP, no growth was observed in the rice seedlings inoculated with *Trichoderma asperellum* and *Trichoderma scalesiae* – suggesting the two isolates' inability to synthesize GA or their potential synthesis of growth suppressing metabolites to the rice plant host.

Figure 4 reveals the fresh weight of paclobutrazol-treated rice seedlings inoculated with ND-RSF isolate suspensions at 14 DAP. The total fresh weight of rice seedlings inoculated with the *Aspergillus brunneoviolaceus* (62.75 ± 9.00 mg) isolate are significantly heavier than the negative broth and water control (and 50.89 ± 9.18 mg) and is comparable to the fresh weight of rice seedlings treated with 20 ppm GA (52.44 ± 8.06 mg and 66.56 ± 8.43 mg), 20 ppm IAA (51.00 ± 12.55 mg and 67.33 ± 12.27 mg) and non-paclobutrazol treated rice seedlings in MS medium (51.00 ± 9.90 mg and 64.44 ± 9.15 mg) (total fresh weight: F value 87.83 > F crit 2.07; $p < 0.05$). This indicates the potential ability of *Aspergillus brunneoviolaceus* to produce the phytohormone GA, which subsequently increased the shoot and total fresh weight of the rice seedlings. Since no growth was observed in the rice seedlings inoculated with *Trichoderma asperellum* and *Trichoderma scalesiae* after 14 DAP, the measured value for the rice seedlings' fresh weight was zero.

The dry weight of paclobutrazol-treated rice seedlings inoculated with ND-RSF isolate culture suspension was shown in Figure 5. The rice seedlings applied with *Aspergillus aculeatus* (27.26 ± 2.82 mg), *Aspergillus japonicus* (29.00 ± 4.24 mg), and *Aspergillus brunneoviolaceus* (28.00 ± 1.63 mg) have significantly higher total dry weight compared to the negative broth and water control (15.11 ± 3.66 mg) (total dry weight: F value 90.235 > F crit 2.07; $p < 0.05$). Interestingly, these three isolates also appear to have higher total dry weight compared to all the positive controls, namely: 20 PPM GA (20.22 ± 4.18 mg), 20 PPM IAA (20.44 ± 3.91 mg),

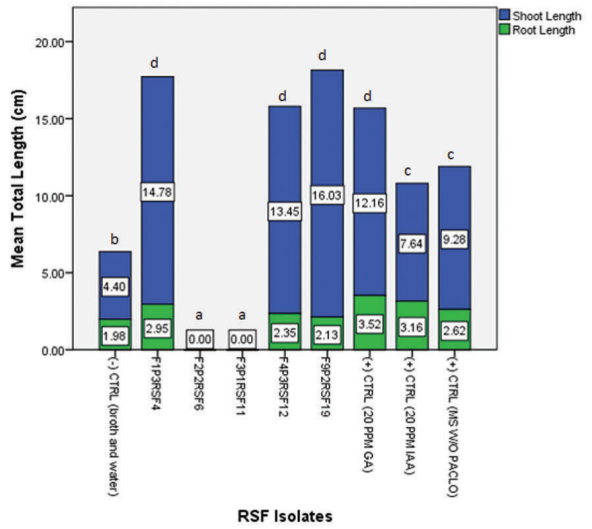

Figure 3. Mean rice seedling lengths (cm) at 14 DAP. Negative control – broth and water, F1P3ND-RSF4 – *Aspergillus aculeatus*, F2P2ND-RSF6 – *Trichoderma asperellum*, F3P1ND-RSF11 – *Trichoderma scalesiae*, F4P3ND-RSF12 – *Aspergillus japonicus*, F9P2ND-RSF19 – *Aspergillus brunneoviolaceus*. Means with different letters are significantly different (p = 0.05; n = 9).

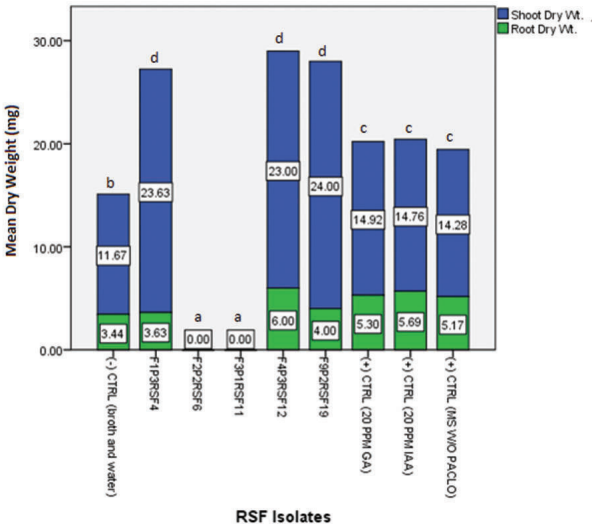

Figure 5. Mean rice seedling dry weights (mg) at 14 DAP. Control – broth and water, F1P3ND-RSF4 – *Aspergillus aculeatus*, F2P2ND-RSF6 – *Trichoderma asperellum*, F3P1ND-RSF11 – *Trichoderma scalesiae*, F4P3ND-RSF12 – *Aspergillus japonicus*, F9P2ND-RSF19 – *Aspergillus brunneoviolaceus*. Means with different letters are significantly different (p = 0.05; n = 9).

and non-paclobutrazol treated rice seedlings (19.44 ± 3.88 mg). It is, therefore, probable that these three isolates are GA-producers. However, the rice seedlings inoculated with the two *Trichoderma* species did not grow, hence their computed dry weight was zero.

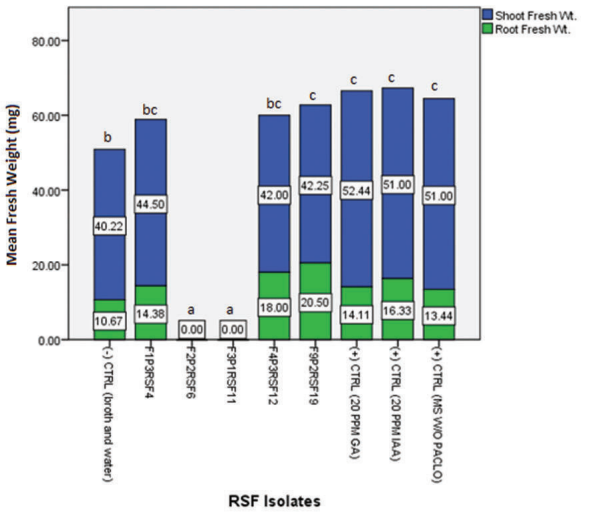

Figure 4. Mean rice seedling fresh weights (mg) at 14 DAP. Control – broth and water, F1P3ND-RSF4 – *Aspergillus aculeatus*, F2P2ND-RSF6 – *Trichoderma asperellum*, F3P1ND-RSF11 – *Trichoderma scalesiae*, F4P3ND-RSF12 – *Aspergillus japonicus*, F9P2ND-RSF19 – *Aspergillus brunneoviolaceus*. Means with different letters are significantly different (p = 0.05; n = 9).

DISCUSSION

Five ND-RSF were molecularly identified: (1) *Aspergillus aculeatus*, (2) *Trichoderma asperellum*, (3) *Trichoderma scalesiae*, (4) *Aspergillus japonicus*, and (5) *Aspergillus brunneoviolaceus*. According to Park *et al*. (2017), the *Aspergillus* species are recognized to liberate varied kinds of primary and secondary metabolites. These primary metabolites, generally speaking, are directly connected to growth and subsequent development of a host organism. These metabolites also commonly act in normal physiological functions. Consequently, the *Trichoderma* species – according to Harman *et al*. (2004) – are fungi common in root ecosystems. Recent evidence shows that the genera can induce both localized and systemic resistance in plants to different pathogens. Interestingly, they can also have a considerable effect on the host plant's physiology and metabolism.

Understanding that these two genera have physiological capabilities to support their host's metabolic growth, three modes of growth promotion are investigated in the study. The first is auxin production. IAA is a naturally occurring plant hormone recognized to induce wall extensibility, enhance effective turgor, and yield elongation growth when exogenously applied in plants (Liu *et al*. 1992). In the assay for the production of indoles using Salkowski's test, L-tryptophan was added to the culture broth. The amendment of this amino acid is needed for the synthesis of IAA through the tryptophan dependent pathway. In the natural epiphytic environment where these ND-RSF isolates

thrive, their host (*Drynaria quercifolia* L.) presumably provides the tryptophan to the ND-RSF isolates so that these symbionts no longer have to spend high energy to synthesize the compound. In return, the ND-RSF isolates convert tryptophan to IAA to benefit their *Drynaria quercifolia* L. host, which may be an observable mutualistic fungal-host relationship (Xin *et al*. 2009).

Several species of *Trichoderma* are able to produce auxin. *Trichoderma* symbionts of *C. brasiliense* (Resende *et al*. 2014), *Trichoderma* isolates from *Zea mays* (Doni *et al*. 2014), *Trichoderma* isolates from Columbia (Hoyos-Carvajal *et al*. 2009), and the *T. harzianum* isolated by Yadav *et al*. (2011) were able to synthesize IAA. However, literature works regarding the presence of auxin-producing *Trichoderma* on ferns are scarce (Brotman *et al*. 2013). The production of auxin by *Trichoderma* involves various plant physiochemical processes – including jasmonate mediated regulation, lateral root growth, cell proliferation and organ formation, and enhanced plant biomass (Brotman *et al*. 2013). It may also involve multilevel of root and shoot systems of communication that leads to root branching promotion and increased ability to uptake nutrients (Lopez-Bucio *et al*. 2015). Additional proposed growth promotion mechanisms include *Trichoderma* inhibiting auxin inhibitors during root-cell development (Bjorkman 2004) and the production of other IAA analogs through the activation of auxin-dependent pathways (Vinale *et al*. 2014). Due to the production of these phytohormones, various species in the *Trichoderma* genera are used in agriculture worldwide. This is a pioneering study to indicate the ability of the two specific species of *Trichoderma – T. asperellum* and *T. scalesiae* – to produce the phytohormone, auxin.

The second mode of growth promotion inspected is phosphate solubilization. The element phosphorus is one of the vital macronutrients in agricultural production. The ability of microorganisms, particularly fungi, to solubilize phosphate increases the availability of inorganic phosphates to plants (Elias *et al*. 2016). However, P-solubilizing fungi constitute only 0.1–0.5% of the total described fungal community (Sharma *et al*. 2013). Amusingly, all ND-RSF in the current paper are capable of solubilizing inorganic phosphate *in vitro*. In the natural epiphytic environment where these ND-RSF isolates symbiotically thrive with their host *Drynaria quercifolia* L., phosphate solubilization activity is important primarily because of the lack of nutrients available on epiphyte substrates. The observed P-solubilization activity of the isolates could potentially help their *Drynaria quercifolia* L. host to assimilate more phosphorus in their P-deficient substrate common in epiphytic habitats. This, in turn, helps maintain the normal growth and development of their host despite being exposed to hostile epiphytic conditions.

Previous studies indicate the ability of diverse fungal genera to solubilize inorganic phosphate (Singh *et al*. 2015). In the study of Elias *et al*. (2016), the genera *Aspergillus*, *Penicillium*, and *Fusarium* are phosphate solubilizers of tricalcium phosphate after growing for 2 wk. The ability of certain *Trichoderma* species to dissolve inorganic phosphate has been published in some literature. *Trichoderma* species of soils in Egypt were found to convert inorganic P to soluble forms determined by the formation of halos around fungal clusters on Pikovskaya's agar after a 4d growth (Yasser *et al*. 2014). In the study of Hoyos-Carvajal *et al*. (2009), 20% of the *Trichoderma* isolates have phosphate solubilization capacity that possibly stimulated the growth of *P. vulgaris*. Moreover, the study of Yadav *et al*. (2011) bared that the *T. harzianum* inoculum has potential phosphate solubilization activities and can be used as an effective biofertilizer since it increased the chickpea's weight and height.

The study of Anil and Lakshmi (2010) found out that *Trichoderma* cultures in the forest tree rhizospheres have the capacity to dissolve tricalcium phosphate and, under glasshouse conditions, the isolates were able to increase the shoot and root length together with the biomass of their test plant in P-deficient soil. Rice inoculated with *Trichoderma* isolates (*T. harzianum* and *T. asperlloides*) in greenhouse conditions also show increased biomass, indicating a high efficiency in phosphate solubilization by the isolates (Borges-Chagas *et al*. 2015). Based on their empirical data, Altomare *et al*. (1999) found out that the ability of *Trichoderma* to increase plant growth through phosphate solubilization involves both chelation and reduction. These operations also play a vital role in plant pathogen biocontrol. The P-solubilization capacity of *T. asperellum* and *T. scalesiae* isolated from *Drynaria quercifolia* L. in this study potentially show growth-promoting ability making it useful in the agricultural industry. The potential phosphate solubilization capacities of the ND-RSF isolates stipulate their prospective abilities as agricultural crop growth enhancers. Normally, the mechanisms employed by these phosphate solubilizing ND-RSFs include (1) discharge of dissolving acids, (2) extracellular enzymatic activities, and (3) discharge of phosphorous during substrate catabolism (Sharma *et al.* 2013).

The third mode of growth promotion investigated is GA hormone production. The phytohormone GA is linked to various physiological processes, from seed germination to elongation of stems, and previous reports have shown that fungal isolates with the capacity to synthesize GA can induce growth promotion in their host plants (Bilkay *et al*. 2010; Hamayun *et al*. 2010). GA production ability of the different ND-RSF isolates was assessed on paclobutrazol-treated rice seedlings. Since paclobutrazol is a GA-inhibiting compound, GA

production is qualitatively assumed when growth occurred in a paclobutrazol-mediated environment. According to Hamayun *et al*. (2009), the use of rice plants for *in vitro* growth promotion screening is recommended as they can effortlessly grow under laboratory conditions such as growing in MS basal agar media. An increase in both plant height and biomass can, therefore, be linked to the presence of growth-promoting metabolic compounds in the ND-RSF isolate culture suspension applied. Sparse literature can prove the potential ability of some closely related taxa of these *Aspergillus* isolates to produce GA. Remarkably, all the *Aspergillus* isolates in the present study are suggested to produce GA as they were able to encourage rice seedling growth in GA-inhibited (paclobutrazol-applied) medium.

On the contrary, the *Trichoderma* isolates in the present study suppressed the growth of rice seedlings exposed in a GA-inhibited environment. These results suggest two things. First, it could indicate the inability of these *Trichoderma* isolates to produce GA. Similar to the present results, none of the rhizosphere or endophytic isolates of *Trichoderma* in the study of Resende *et al.* (2014) produced GA. However, these outcomes were contrary to the study of Al-Askar *et al*. (2016) where they showed the ability of their *Trichoderma* isolates to synthesize the phytohormone, GA. They even evaluated the effect of such production of GA on potato tuber and, surprisingly, their *Trichoderma* treatments induced 100% germination. Moreover, their *Trichoderma* treatments also have a significant impact on shoot weight, root weight, and the plant hosts' vigor.

Second, the suppression of growth of rice seedlings by the *Trichoderma* isolates in the present study could also indicate their capacity to synthesize growth suppressing metabolites in rice plants. Though these *Trichoderma* isolates appear to inhibit their hosts' growth, some literature works show antithetical results. Some species of *Trichoderma* can enhance plant growth as they are regarded to be important fungal symbionts and potential biocontrol agents (Brotman *et al*. 2013). In the paper published by Doni *et al*. (2014), *Trichoderma* isolated from corn were found to affect seedling germination in rice. Based on the seedlings' growth parameters, there was a significant upsurge in the length and biomass of rice compared to the growth of the control plants. These indicate that *Trichoderma* symbionts in corn have mechanisms of growth specifically beneficial to rice plants. Literature also shows that *Trichoderma* symbionts can affect other important agricultural crops. Significant improvement in cucumber growth was reported after being inoculated with culture suspensions of *Trichoderma harzianum* (Yedidia *et al*. 2001). The same fungal species also positively affected the growth of *C. arietinum* (Yadav *et al*. 2011).

Due to the diversity of the genus *Trichoderma* and due to varied types of plant hosts, the effect of the symbiosis of one fungal species to two different hosts may differ. Likewise, the effect of two dissimilar species to a singular host may also be different. Hoyos-Carvajal *et al*. (2009) further pointed out that not all *Trichoderma* isolates have the capacity to synthesize natural plant growth regulators.

CONCLUSION

This present study provided various evidence of growth promotion exemplified by the different ND-RSF isolated in *Drynaria quercifolia* L. The production of the phytohormone auxin was apparent across all ND-RSF isolates but on varying amounts. These ND-RSF isolates were also found to perform other growth-promoting activities such as solubilization of unassimilable forms of phosphate. Interestingly, two contrasting effects were observed when these isolates were inoculated to rice grown in a GA-inhibited environment. The *Aspergillus* species encouraged the growth of rice, suggesting their ability to produce GA hormones. The *Trichoderma* species – on the contrary – stopped the growth of the rice plants suggesting their inability to produce GA or their capacity to synthesize growth suppressing metabolites to rice. These biochemical tests prove the existence of fungal symbionts with growth-promoting activities. These fungal symbionts do not only prove their importance in growth enhancement but also their significance on their host's metabolism and survival especially when exposed to environmental stresses. This project is promising in the agricultural industry by potentially increasing rice production.

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