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Dominant Root Associated Fungi (RAF) from *Drynaria quercifolia* L. either Induce or Retard Growth of PSB Rc10 Rice (*Oryza sativa* L.) in Gibberellic Acid-Inhibited Medium

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Abstract

The testing and use of microorganisms for *in vitro* growth promotion of agriculturally significant crops such as rice has increased but remains underexploited. The current study aims to explore growth-enhancing mechanisms of dominant root-associated fungi (RAF) isolates from *Drynaria quercifolia* and test their effects on rice. The most abundant RAF on five tree-collection sites were cultured *in vitro*. Genomic DNA of the RAF were extracted and the ITS (internal transcribed spacer) region of the 18S ribosomal DNA (rDNA) were sequenced and molecularly identified. Two RAF isolates significantly increased the plant shoot/total length (Meyerozyma guilliermondii: 10.29±4.18/13.46±4.18 cm; *Trichoderma simmonsii*: 10.33±1.38/13.23+1.58 cm), shoot/total fresh weight (*Meyerozyma guilliermondii*: 57.33±15.76/71.00±16.10 mg; *Trichoderma simmonsii*: 63.22±12.23/76.00±10.67 mg) and shoot/total dry weight (*Meyerozyma guilliermondii*: 16.99±6.74/22.78±7.41 mg; *Trichoderma simmonsii*: 16.89±3.33/23.11±5.30 mg) weight compared to the negative control. These results possibly show the ability of the two isolates to produce the hormone gibberellic acid. On the other hand, three of the RAF isolates did not significantly increase seedling growth and biomass. The *Trichoderma yunnanense* (shoot: 0.36±0.16 cm; total: 0.53±0.20 cm), unidentified *Mucoromycotina* isolate $F_5P_1RAF_{16}$ (shoot: 1.87±0.59 cm; total: 2.12±0.58 cm) and the unidentified *Mucoromycotina* isolate $F_9P_2RAF_{21}$ (shoot: 3.26±1.56 cm; total: 5.19±2.00 cm) approximated the growth of rice seedlings inoculated with broth and water negative control (shoot: 4.40+2.27 cm; total: 6.38+2.28 cm). This possibly indicates the inability of these isolates to produce GA or their potential ability to produce growth-retarding metabolites. Preliminary data from this study reveal potential growth-promoting capacity of RAF isolates on rice.

Keywords: *Drynaria quercifolia*, Gibberellic acid (GA3); Growth promotion; Paclobutrazol; Rice; Root associated fungi

Introduction

Rice is one of the Philippines' chief agricultural products and driver of economic growth [1]. It is therefore essential to understand the physiological principles of rice growth and determine the best agricultural alternatives to maximize rice production in the country. Previous studies have revealed the critical role of symbiotic fungi on the eco-physiological status and crop adaptation, as well as in shaping plant community dynamics [2]. Tian *et al*. [3] reported that the continuous interaction between the plant host and fungal symbiont denotes the influence on physiological activity and metabolites. Fungi are able to produce phytohormones such as gibberellic acid (GA_3) as part of their metabolism, which are essential for host growth [4]. GA_3 is reported to promote shoot and root elongation as well as many other physiological processes related to growth and development [5-6]. This research aims to discover the potential of dominant root associated fungi (RAF) to produce GA_3 or other growth-enhancing compounds and determine their effects on rice.

Materials and methods

1) Selection and collection of the plant of interest

Ten basket ferns, *D. quercifolia*, were collected from five tree-collection sites in Don Mariano Marcos Memorial State University's North La Union Campus, Philippines. The university is situated in Bacnotan, La Union (16°43′30.8″N 120°23′37.6″E). *D. quercifolia* was chosen as the plant of interest because its importance is underrated and it is typically considered as a weed. Isolation of RAF from *D. quercifolia* was conducted in the Natural Sciences Research Unit (NSRU) of Saint Louis University, Baguio City, Philippines.

2) Isolation, selection and identification of RAF isolates

Root samples of *D. quercifolia* were rinsed with type 1 water, cut to approximately 0.5 to 1.0 cm in length and plated on Potato Dextrose Agar (PDA). Rose Bengal was added to slow down fast-growing filamentous fungi. Chloramphenicol was also mixed into the agar medium to eliminate bacterial contamination. A total of 30 petri plates were prepared. Six plates were prepared per tree-collection site, with ten root segments per plate. The plates were cultured in the laboratory in ambient conditions for seven days. The isolation frequencies of all morpho-species were counted. The most dominant morpho-species per tree-collection site were cultured in PDA slants. In the molecular identification of the morpho-species, the protocol of Liu *et al.* [7] was used for genomic DNA extraction. ITS-1 forward and ITS-4 reverse universal primers were used for the amplification of the ITS region of the 18S rDNA of the RAF isolates. The amplicons were submitted to 1st Base Malaysia for gel extraction and for DNA sequencing. BLASTn algorithm software was used to compare RAF isolate sequences to the available sequences in GenBank.

3) Paclobutrazol-mediated (GA_3 production) assay

One loopful of live mycelial fragments obtained from the RAF isolates' stock culture was initially grown in Sabouraud Dextrose Broth (SDB) for seven days. Simultaneously, rice seeds (PSB Rc10) were obtained from the Philippine Rice Research Institute, Munoz, Nueva Ecija, Philippines. The paclobutrazol-mediated assay followed the procedures of Khan *et al*. [8] with a few modifications. Surface sterilization was done by washing the rice seeds with diluted NaClO (2.5%) for one hour, followed by rinsing with type 1 water (*Unichrom*). The seeds were then pre-germinated in sterilized growth chambers with sterile dH_2O in the dark at

ambient conditions for seven days. To secure even germination, the sterilized seeds were incubated simultaneously. After pre-germination, the seedlings were immersed under aseptic conditions with 20 ppm paclobutrazol for 24 hours. The seedlings were later pre-inoculated to the different RAF isolate mycelial suspensions for another 24 hours. The concentration of the suspension was based on the respective growths of the RAF isolates after seven days of culture in SDB. The positive controls were 20 ppm IAA and GA. Sterile broth solution was the negative control. The rice seedlings were later transferred to test tubes with Murashige and Skoog basal agar medium (*Sigma*). Three replicates of three plants, consisting a total of nine plants were prepared for each treatment. This initial growing phase was regarded as the pre-germination and pre-inoculation phase.

After pre-germination and pre-inoculation, the rice seedlings were grown for two weeks. At the two-leaf stage (1st week) 50 µl of each of the isolate's culture filtrate were spread at the seedlings' apical region and were allowed to grow for another seven days (2nd week) under artificial light (1000 lux) and ambient conditions. The two-week growth period was regarded as 14 days after pre-germination and pre-inoculation (DAPP). The different agronomic traits including shoot-, root-, total- length and shoot-, root-, total-fresh/dry weight were measured and were statistically compared with the positive (IAA and GA_3) and negative control (broth & water).

4) Statistical analysis

SPSS version 20 was used to analyze the effect of RAF isolates on plant traits, and represented using clustered bar graphs. Error bars displayed represent ±standard deviations. The software was also used to conduct one-way Analysis of Variance (ANOVA) with Scheffe post-hoc test for significant differences in growth-promoting capacity of RAF isolates.

Results and discussion

1) The five most abundant RAF isolates

The distribution of the five most abundant root-associated fungi isolated from *D. quercifolia* found in the five tree-collection sites is depicted in Figure 1. RAF isolates $F_2P_3RAF_5$ and $F_3P_3RAF_8$ were found to dominate Sites 1 and 2, respectively, while the most abundant isolates from Sites 3 and 4 were $F_9P_2RAF_{21}$ and $F_5P_1RAF_{16}$, respectively. RAF isolate $F_1P_3RAF_3$ appeared to dominate Site 5. The dominance of the RAF isolates was used as the sole selection criterion since the presence of a dominant species denotes a critical ecological role that may theoretically affect the performance of its plant host [9].

2) Molecular identification of the five most abundant RAF per site

Five RAF isolates found in *D. quercifolia* were identified using the molecular method. Table 1 shows the closest type match using BLASTn algorithm in NCBI and their proposed species name, based on available sequences on GenBank. Accession numbers were also provided for RAF sequence retrieval in NCBI. Three of the five isolates can be identified up to the species level *(Meyerozyma guilliermondii, Trichoderma yunnanense* and *Trichoderma simmonsii*) since the sequences of these isolates obtained the highest total score (highest sections of similarity between the query and the hit) to the entire list of type match hits in the BLASTn database and an identity threshold >97% [10]. However, two of the RAF isolates (*Mucoromycotina* isolate $F_5P_1RAF_{16}$ and *Mucoromycotina* isolate $F_9P_2RAF_{21}$) showed sequence similarity below 95% and were accordingly treated as unidentified species [11].

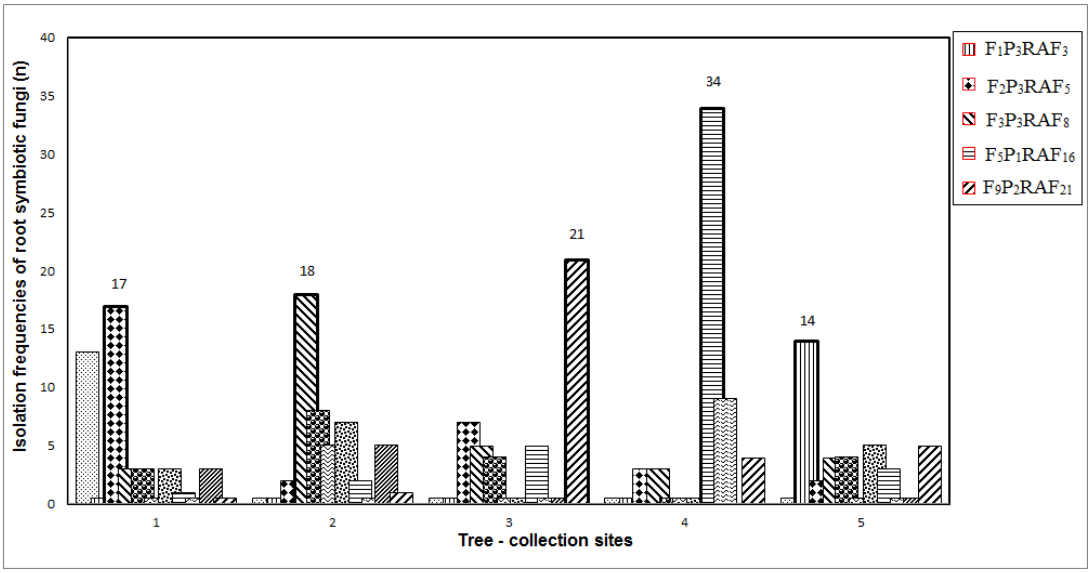

Figure 1 Abundance of RAF in the five tree-collection sites.
The most abundant RAF isolates per site were presented with bold outline. Site 1: $F_2P_3RAF_5$, Site 2: $F_3P_3RAF_8$, Site 3: $F_9P_2RAF_{21}$, Site 4: $F_5P_1RAF_{16}$, Site 5: $F_1P_3RAF_3$.

Table 1 Five molecularly identified RAF isolates found in *D. quercifolia*

Isolate code	Proposed species name	Closest TYPE match	Total score	Identity	Accession[a]
$F_1P_3RAF_3$	*Meyerozyma guilliermondii*	*Meyerozyma guilliermondii*	946	98%	KY474516
$F_2P_3RAF_5$	*Trichoderma yunnanense*	*Trichoderma yunnanense*	940	99%	KY474517
$F_3P_3RAF_8$	*Trichoderma simmonsii*	*Trichoderma simmonsii*	1003	99%	KY474518
$F_5P_1RAF_{16}$	Unidentified *Mucoromycotina*	*Mucor fusiformis*	686	89%	KY474524
$F_9P_2RAF_{21}$	Unidentified *Mucoromycotina*	*Mucor fusiformis*	686	89%	KY474527

[a] GenBank - National Center for Biotechnology Information (NCBI) databank

3) Paclobutrazol-mediated assay (GA production)

The production of GA_3 by the RAF isolates were measured based on several agronomic traits (shoot- root- total plant height and weight) of rice seeds grown in GA_3-inhibited culture medium. The visual appearance of the rice seedlings inoculated with RAF isolates at 14 DAPP is displayed in Figure 2. The length of rice seedlings in the MS medium without paclobutrazol (GA_3 inhibitor), and those inoculated with 20ppm GA_3, 20ppm IAA, *M. guilliermondii* isolate, and *T. simmonsii* isolate are noticeably higher than the rice seedlings inoculated with broth and water. As expected, paclobutrazol-treated rice seedlings exposed to 20ppm GA_3 had the longest shoot length, but also exhibited a distinctly thin and spindly shoot growth, typical of increased exposure to GA_3. Shorter rice seedlings were observed in *T. yunnanense* and the two unidentified *Mucoromycotina* isolate ($F_9P_2RAF_{21}$ and $F_5P_1RAF_{16}$) treatments. The differences in the mean length of rice seedlings at 14 DAPP were computed and statistically analyzed (Figure 3).

Figure 2 Visual appearance of the rice seedlings inoculated with RAF isolates at 14 DAPP. Abbreviations: (?) = unidentified, DAPP = days after pre-germination and pre-inoculation

Figure 3 presents the mean shoot, root and whole plant length of rice seedlings inoculated with RAF isolates and grown in GA-inhibited medium 14 DAPP. Inoculation of the rice seedlings with RAF isolates has contrasting effects. Two RAF isolates significantly increased shoot length compared with the negative control. Rice seedlings with *M. guilliermondii* isolate-inoculation attained mean shoot length of 10.29±4.18 cm and mean total length of 13.46±4.18 cm, while the *T. simmonsii* isolate reached an mean shoot length of 10.33±1.38 cm and total pant length of 13.23±1.58 cm. These values are more than twice the shoot (4.40±2.27 cm) and total length (6.38±2.28 cm) of seedlings inoculated with broth and water. The above-mentioned results suggest the ability of *M. guilliermondii* and *T. simmonsii* to produce GA_3. On the contrary, three RAF isolates possibly retard the shoot length growth of rice seedlings although the numerical mean values are comparable with the negative control. At 14 DAPP, the *T. yunnanense* isolate-inoculated rice seedling only had 0.36±0.16 cm shoot length growth while the two unidentified Mucoromycotina-isolated rice seedlings, $F_5P_1RAF_{16}$ and $F_9P_2RAF_{21}$ only allowed shoot length growth to 1.87±0.59 cm and 3.26±1.26 cm, respectively. Likewise, 2 weeks after germination, a significantly lower total plant height was observed in *T. yunnanense*-inoculated rice seedling (0.53±0.20 cm) compared to the negative control (6.38±2.28 cm) which signifies the possible growth inhibitory effects of the three RAF isolates.

Several isolates of *Trichoderma* has been found to enhance seedling development as it is considered an essential beneficial and biocontrol fungus [12]. A similar work was conducted by Doni *et al.* [13] where the *Trichoderma* species isolated from corn tested for their potential rice germination and growth. Based on the rice seedling height as a growth parameter, there was a significant increase compared to control indicating that corn likely carries *Trichoderma* isolates with potential agricultural applications.

The growth promoting potentials of *Trichoderma* were also observed in other crops aside from rice. Yedidia *et al.* [14] reported increased cucumber growth after exogenous application of *T. harzianum* suspension and Yadav *et al.* [15] documented the effects of *T. harzianum* on shoot growth of *C. arietinum*. In this study, the species *T. simmonsii* was reported to increase rice seedling shoot and total length possibly because of its GA_3 production ability.

Although it has been highly proposed that the secretion of phytohormones by some species of the genus *Trichoderma* promotes plant growth, the study of Shi *et al.,* [16] found out that certain linear proteins are formed by this genus responsible in the restriction of the development of roots in *Arabidopsis*. In their study, the fungus *T. longibrachiatum* SMF2 produced a growth-inhibitory compound, Trichokonin, therefore decreasing root cell division and elongation in *Arabidopsis*. In this present study, *T. yunnanense* and the two unidentified isolates of Mucoromycotina ($F_5P_1RAF_{16}$ and $F_9P_2RAF_{21}$) were found to possibly inhibit the growth of rice seedlings perhaps due to their secretion of growth-inhibiting compounds.

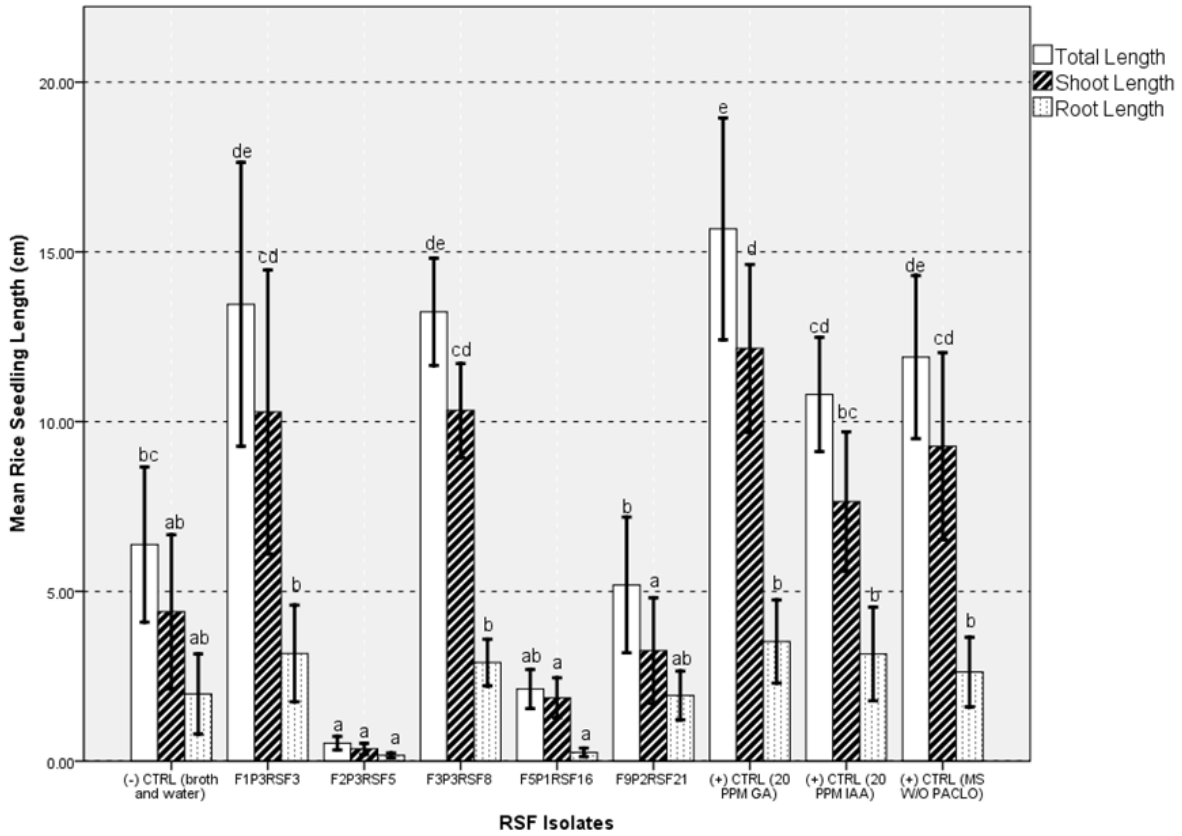

Figure 3 Mean rice seedling length (cm) at 14 DAPP.
Control = broth and water, $F_1P_3RAF_3$ = *M. guilliermondii*, $F_2P_3RAF_5$ = *T. yunnanense*, $F_3P_3RAF_8$ = *T. simmonsii*, $F_5P_1RAF_{16}$ = Unidentified Mucoromycotina, $F_9P_2RAF_{21}$ = Unidentified Mucoromycotina. Means with different letters are significantly different (ρ=0.05) n = 9. Abbreviation: DAPP = days after pre-germination and pre-inoculation.

Figure 4 shows the fresh weight of the RAF isolate-treated seedlings at 14 DAPP. The shoot/total fresh weight of seedlings applied with the *M. guilliermondii* (57.33±15.76/71.00±16.10 mg) and *T. simmonsii* (63.22±12.23/76.00±10.67 mg) isolates was significantly greater than the broth and water control (40.22±11.46/50.89± 9.18 mg) and was comparable to the fresh weight

of rice seedlings treated with 20 ppm GA_3 (52.44±8.06/66.56±8.43 mg), 20 ppm AA (51.00± 12.55/67.33±12.27 mg) and MS medium without paclobutrazol amendment (51.00±9.90/ 64.44±9.15 mg). This indicates the potential ability of *M. guilliermondii* and *T. simmonsii* to produce the phytohormone, GA_3 or other biomass inducing phytohormones and compounds which subsequently increasedd shoot and total biomass of the rice seedlings. Consequently, rice seedlings inoculated with the *T. yunnanense* isolate have significantly lower shoot/total fresh (6.94+2.78/9.89+3.62 mg) weight, while the unidentified *Mucoromycotina* isolate $F_5P_1RAF_{16}$ – inoculated seedlings had significantly lower total fresh weight (28.78+8.90 mg) compared to the negative control. This implies the possible inability of the aforementioned RAF isolates to produce GA_3 or their potential ability to produce growth-inhibiting compounds.

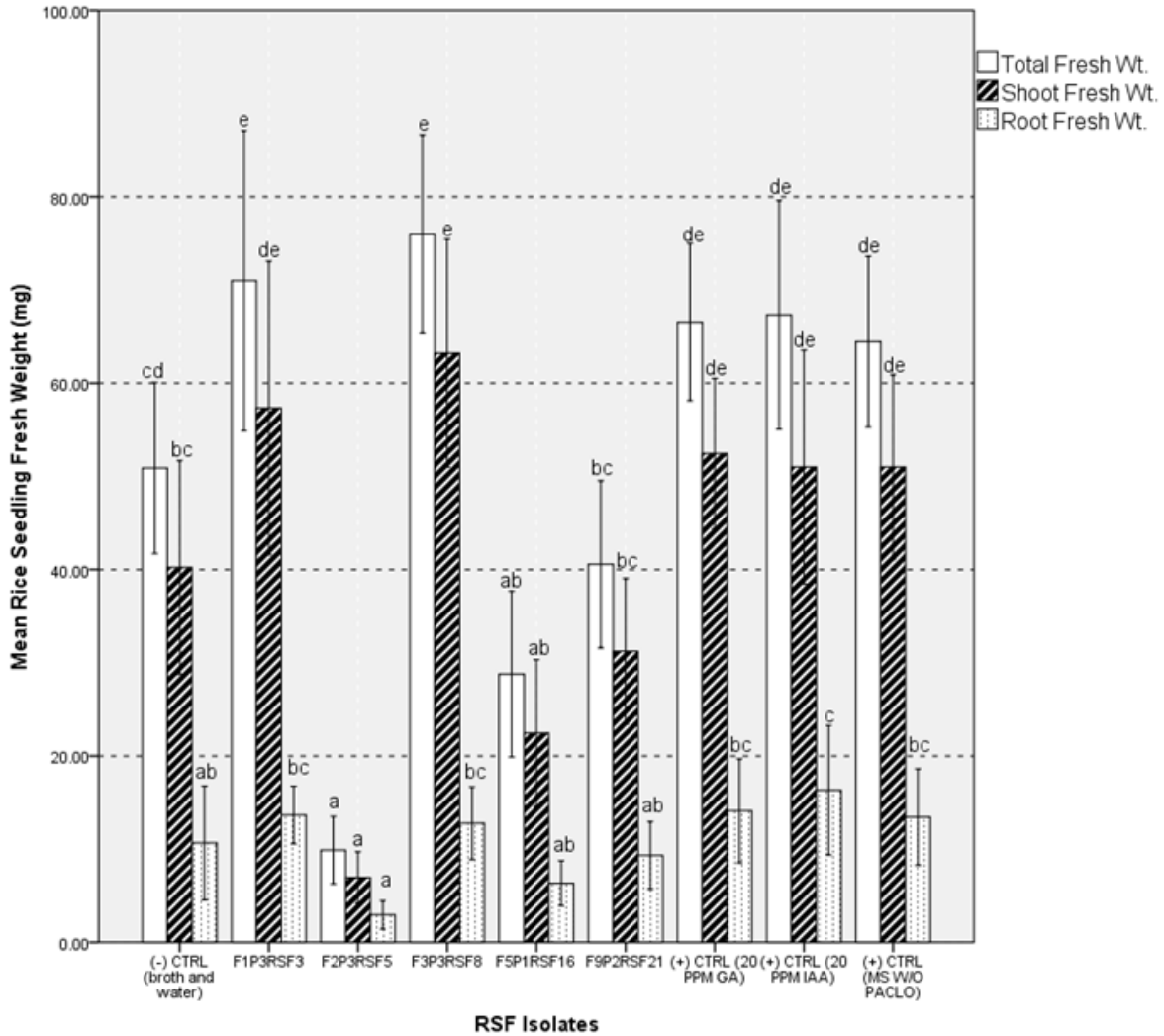

Figure 4 Mean rice seedling fresh weight (mg) at 14 DAPP.
Control = broth and water, $F_1P_3RAF_3$ = *M. guilliermondii*, $F_2P_3RAF_5$ = *T. yunnanense*, $F_3P_3RAF_8$ = *T. simmonsii*, $F_5P_1RAF_{16}$ = Unidentified Mucoromycotina, $F_9P_2RAF_{21}$ = Unidentified Mucoromycotina. Means with different letters are significantly different (ρ=0.05) n = 9. Abbreviation: DAPP = days after pre-germination and pre-inoculation.

Figure 5 displays the dry weight of rice seedlings treated with RAF isolate culture filtrate (CF). The rice seedlings applied with *M. guilliermondii* (16.99±6.74/22.78±7.41 mg) and *T. simmonsii* (16.89±3.33/23.11±5.30 mg) had significantly higher shoot/total dry weight compared to the broth and water control (11.67±3.46/15.11±3.66 mg). It is therefore probable that these two isolates are GA_3-producers or have the ability to produce other phytohormones or compounds that induce plant biomass production. The rice seedlings inoculated with the *T. yunnanense* isolate had significantly lower shoot (1.91±0.81 mg) and total (3.01±1.28 mg) dry weight compared to the negative control. It is probable that this RAF isolate lack GA_3 production ability or it consequently produces compounds capable of inhibiting plant biomass production.

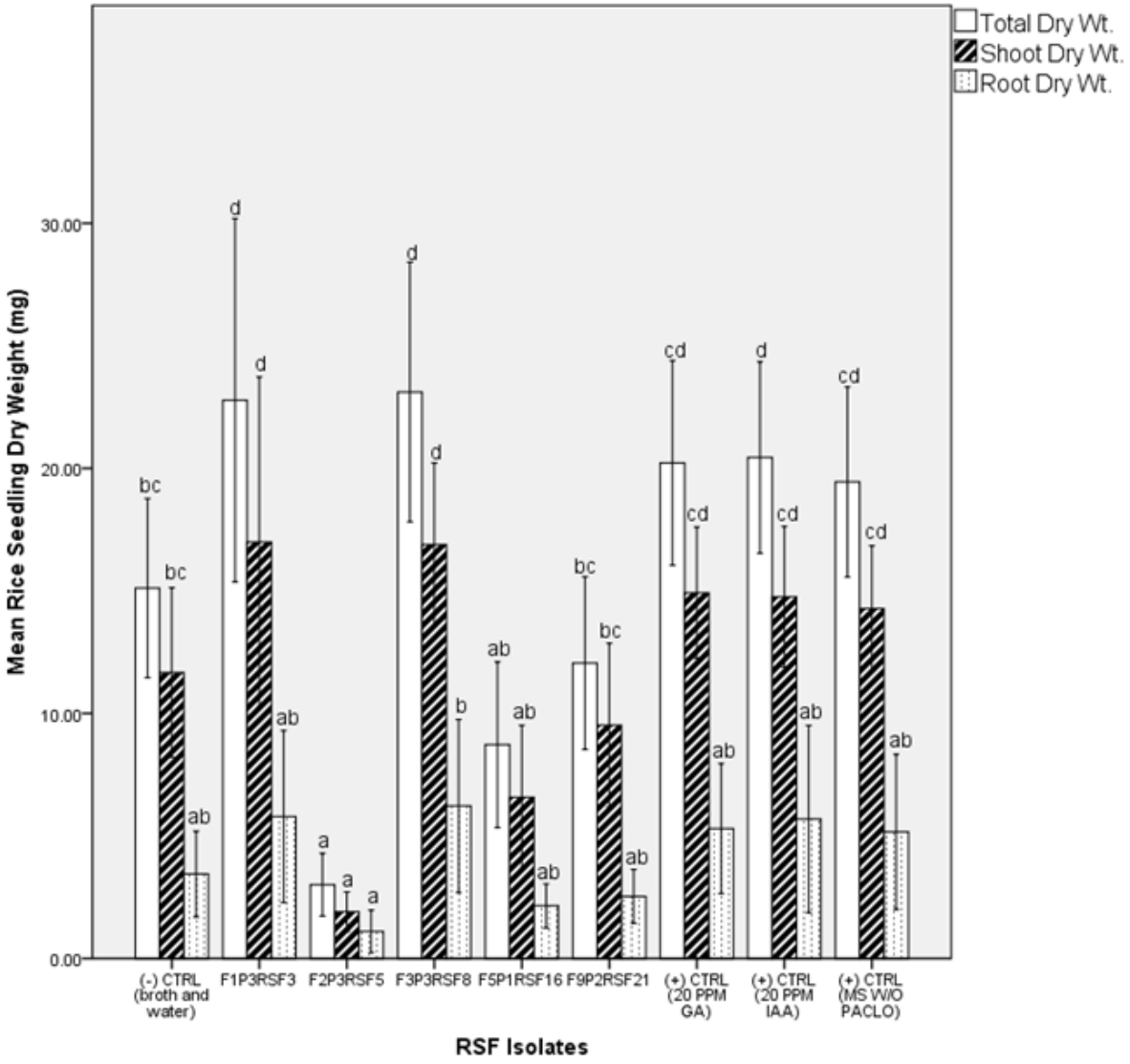

Figure 5 Mean rice seedling dry weight (mg) at 14 DAPP.
Control = broth and water, $F_1P_3RAF_3$ = *M. guilliermondii*, $F_2P_3RAF_5$ = *T. yunnanense*, $F_3P_3RAF_8$ = *T. simmonsii*, $F_5P_1RAF_{16}$ = Unidentified Mucoromycotina, $F_9P_2RAF_{21}$ = Unidentified Mucoromycotina. Means with different letters are significantly different (ρ=0.05) n = 9. Abbreviation: DAPP = days after pre-germination and pre-inoculation.

Species of the genus *Trichoderma* are also known to essentially affect plant biomass and yield [17]. Contreras-Cornejo *et al.* [18] discovered the ability of *T. virens* to enhance biomass in *A. thaliana*. Yadav *et al.* [15] significantly increase the weight of chickpea. However, Hoyos-Carvajal *et al.* [19] confirmed that some strains of *Trichoderma* are unable to produce plant-growth promoting metabolites. Nevertheless, the study of Nakayan *et al.* [20] revealed that strains of *M. guilliermondii* induced growth promotion in corn and lettuce under greenhouse conditions. The fungal isolate significantly increased the dry-weight, nutrient uptake and seed vigor index of the plants under investigation which validates the potential of *M. guilliermondii* for increased productivity of some agricultural crops. These previous works are consistent with the results of this study where *T. simmonsii* and *M. guilliermondii* were also found to increase the fresh and dry weight of rice seeds inoculated with isolates' culture suspensions.

Conclusions

Two RAF isolates (*M. guilliermondii* and *T. simmonsii*) significantly increased plant shoot and total length, fresh and dry biomass in comparison to the negative control in a GA-inhibited medium. These results possibly indicate the ability of *M. guilliermondii* and *T. simmonsii* to produce the GA_3 hormone. The results of this study reveal the potential growth-promoting capacity of RAF isolates on rice. As a recommendation, further studies can be conducted to test these RAF isolates on other agricultural crops to confirm their potential agricultural significance.

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