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Auxin Production, Phosphate Solubilisation and ACC Deaminase Activity of Root Symbiotic Fungi (RSF) from *Drynaria quercifolia* L.

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ABSTRACT
This study aimed to explore the potential growth promoting properties of root symbiotic fungi that can potentially contribute in the agricultural sector. Five root symbiotic fungi (RSF)were isolated from D. quercifolia and were molecularly identified. All RSF isolates were found to produce significantly high IAA compared to the negative control. The unidentified Mucoromycotina isolate ($F_5P_1RSF_{16}$) produced the highest IAA per milliliter of culture broth (9.37±1.48 µg/ml) that is six times higher than thebroth and water control (1.56±0.76 µg/ml). M. guilliermondii produced the greatest IAA per dry weight (0.34+0.02 µg/mg) significantly higher than the other four isolates. M. guilliermondii, T. simmonsii and the unidentified Mucoromycotina ($F_5P_1RSF_{16}$) were found to have phosphate solubilisation activity. The five RSF isolates were also tested positive for ACC deaminase activity. The results of the study possibly indicate the potential use of the isolated RSF for agricultural crop growth and production.
Keywords*: ACC deaminase, auxin, Drynaria quercifolia, growth promotion, phosphate solubilisation, root symbiotic fungi*

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INTRODUCTION

Symbiotic fungi have critical impact in plants' eco-physiological status and adaptation.One excellent example of symbiotic effect is the production of hormones by fungi which is crucial in the maintenance of the normal growth and metabolic activities of the host plant that they colonize [1]. Fungi produce variety of essential phytohormones and natural growth inducers like gibberellic acids (e.g. GA_3), and auxins[2].Aside from auxins and gibberellins, fungi are also known to produce compounds that promote growth even when exposed to environmental stresses. ACC deaminase reduces the "stress ethylene" level that restricts growth in plants [3]. Fungi are also regarded as phosphate solubilizing microorganisms. This means they are capable of converting insoluble phosphorus into its soluble forms for plants. The ability of fungi to synthesize phytohormones plus their capacity to produce compounds that aids on their successful growth even under stressful environments shows their unequivocal importance in the agricultural industry [4]. This research is therefore conceived to identify potential growth promoting properties of root symbiotic fungi found on *D.quercifolia*. These growth enhancing mechanisms may possibly be utilized to improve the growth of important agricultural crops such as rice.

MATERIALS AND METHODS

Collection and identification of RSF isolates

RSF were isolated from *Drynaria quercifolia* found in Don Mariano Marcos Memorial State University – North La Union Campus. This state university is found in Bacnotan, La Union, Philippines. Genomic DNA of the RSF isolates were isolated following the procedure of Liu *et al.*[5] and the ITS region of the 18s

rDNA were sequenced and compared with the available sequences in GenBank of the NCBI for their molecular identification.

Auxin production assay

Sabouraud dextrose broth (SDB) (*TM Media*) was used for the growth of RSF isolates. Zero point one percent (w/v) L-tryptophan (*HiMedia*) was added to SDB and the isolates were grown for seven days. Sterile SDB was used as control. To separate the culture filtrate (CF), 1.5 ml of the mycelia were centrifuged at 10,000 x g for five minutes. Two milliliter of Salkowski reagent (98 ml perchloric acid [35%] and 2 ml ferric chloride solution [0.5M]) was mixed to one ml of the CF and was stored for 30 minutesat room temperature. A UV-VIS Spectrophotometer (APEL PD-303) at wavelength 530 nm was used to quantify the development of an orange-red color in the Salkowski-CF mixture [6]. HPLC water (*Unichrom*) was used as blank. A standard curve in different concentration (0, 10, 20, 30, 40, 50) of commercial 3-indoleacetic acid (*Sigma*) (R^2=0.9922) was used for µg/ml auxin quantification. Cell pellets were oven dried at 50^{o}C for eight hours or more until the weight was constant for IAA production per unit dry weight of the RSF isolates for µg/mg auxin quantification.

ACC deaminase activity

The RSF isolates were initially cultured in SDB for 16 hours. Three grams of Yeast Carbon Base (*Formedium*) was added to 5g Bacto Agar (*TM Media*). After autoclaving, this N-free agar medium was supplemented with 3.0 mM 1-aminocyclopropanecarboxylic acid (ACC) (*Sigma*) as sole nitrogen source through filter sterilization. Fifty microliters of the culture broth was transferred in the enriched media and was incubated for eight days. The growth of RSF isolates in the enriched culture media indicates the RSF isolates' capacity to use aminocyclopropane-1-carboxylic acid (ACC) deaminase pathway.

Assay for phosphate solubilisation activity

In the determination of phosphate solubilisation activity, the RSF were initially cultured SDB for 16 hours. Fifty microliters of the culture was inoculated to Pikovskaya agar (*HiMedia*) and was incubated for seven days. If halos or clear zones are observed then the isolates have phosphate solubilizing capacities.

Statistical analysis

The levels of IAA were presented using the Chart Builder bar graph with dual Y coordinates and clustered bar graph of SPSS version 20, respectively. Error bars displayed represent $\pm$ standard deviations. Same software was used for the one-way Analysis of Variance (ANOVA) with Scheffe post-hoc test to determine significant differences on the IAA levels.

RESULTS AND DISCUSSION

Table 1 shows the five isolated RSF from *D. quercifolia*. Three of the five RSF isolates were identified up to the species level ($F_1P_3RSF_3$ = *M. guilliermondii*, $F_2P_3RSF_5$ = *T. yunnanense*, $F_3P_3RSF_8$= *T. simmonsii*) using the BLASTn sequence homology database in NCBI GenBank. However, two isolates ($F_5P_1RSF_{16}$ and$F_9P_2RSF_{21}$)were classified as unidentified Mucoromycotina because their sequence similarity to available GenBank sequences is below 95%.

Table 1. Isolated and molecularly identified RSF isolates

RSF Isolate Code	Species name	Accession number[a]
$F_1P_3RSF_3$	*Meyerozyma guilliermondii*	KY474516
$F_2P_3RSF_5$	*Trichoderma yunnanense*	KY474517
$F_3P_3RSF_8$	*Trichoderma simmonsii*	KY474518
$F_9P_2RSF_{21}$	Unidentified Mucoromycotina	KY474527
$F_5P_1RSF_{16}$	Unidentified Mucoromycotina	KY474524

[a] National Center for Biotechnology Information (NCBI)

Auxin production of the RSF isolates

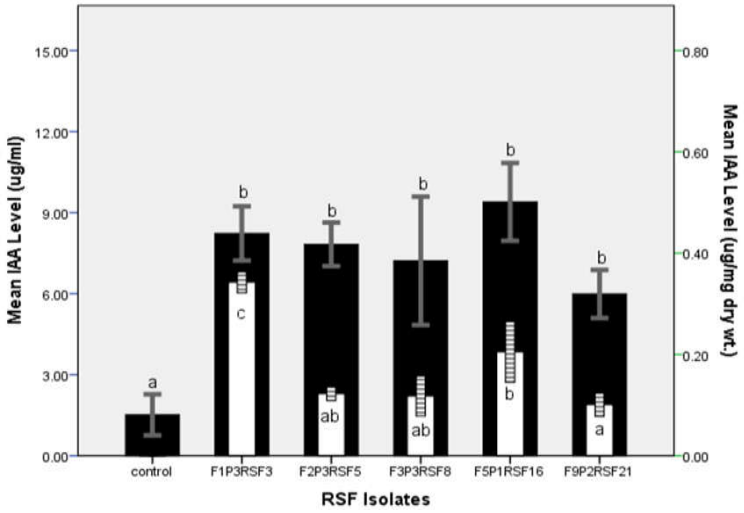
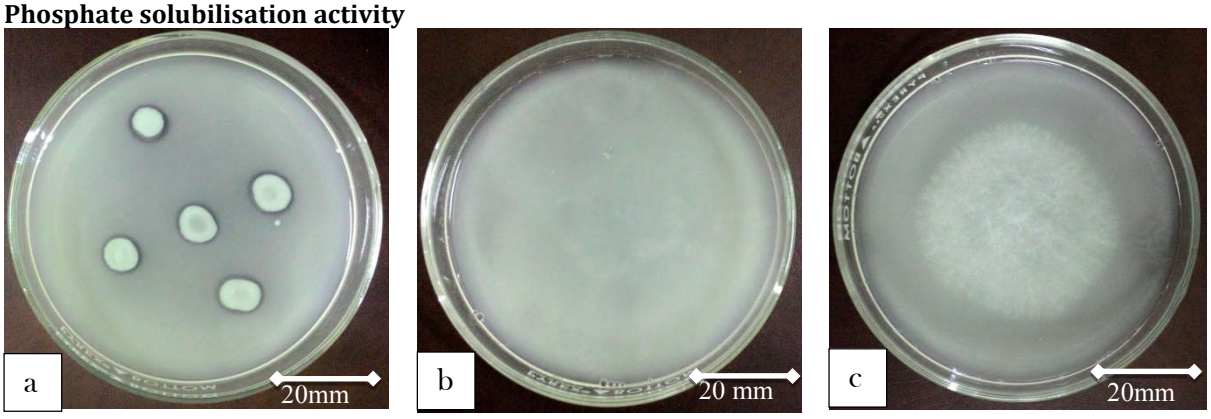


Figure 1. **Mean IAA level produced by the RSF Isolates.** Control = broth and water, $F_1P_3RSF_3$ = *M. guilliermondii*, $F_2P_3RSF_5$ = *T. yunnanense*, $F_3P_3RSF_8$ = *T. simmonsii*, $F_5P_1RSF_{16}$ = Unidentified Mucoromycotina, $F_9P_2RSF_{21}$ = Unidentified Mucoromycotina. Means with different letters are significantly different (ρ=0.05) n = 6

All RSF isolates were found to produce IAA spectrophotometrically at 530 nm. Figure 1 shows the level of IAA production of the five RSF isolates. The isolates produced significantly high colorometric indole compared to negative control. The unidentified Mucoromycotina isolate ($F_5P_1RSF_{16}$) produced the highest IAA per milliliter of culture broth (9.37±1.48 µg/ml). This value is six times higher than the broth and water control (1.56+ 0.76 µg/ml). However, this value is comparable to the IAA produced by *M. guilliermondii* (8.23±1.00 µg/ml), *T. yunnanense* (7.82±0.81 µg/ml), *T. simmonsii* (7.21±2.38 µg/ml) and the unidentified Mucoromycotina isolate, $F_9P_2RSF_{21}$ (5.99±0.89 µg/ml). Interestingly, *M. guilliermondii* produced the highest IAA per milligram dry weight (0.34 ± 0.02 µg/mg) which is statistically higher than the four other RSF isolates: *T. yunnanense* (0.12±0.01 µg/mg), *T. simmonsii* (0.12±0.04 µg/mg), unidentified Mucoromycotina isolate $F_5P_1RSF_{16}$ (0.21±0.06 µg/mg)and the unidentified Mucoromycotina isolate $F_9P_2RSF_{21}$ (0.10±0.02 µg/mg). Vast number of literature shows the ability of several species in the genus *Trichoderma* to produce auxin. *Trichoderma* isolates found on *C. brasiliense*[7] and*Trichoderma* isolates from *Zea mays*[8]were auxin producers. Because of these plant growth stimulating mechanisms, various strains of the genus *Trichoderma* have been globally used in agriculture. Despite many studies indicating the ability of some *Trichoderma* species to produce IAA, it is probable that this is the first study to indicate the ability of the two species of *Trichoderma*, the *T. yunnanense* and *T. simmonsii*, to produce the phytohormone, auxin. Aside from species of the genus *Trichoderma*, sparse literature indicates that the genus*Meyerozyma* and some species of the division Mucoromycotina have the potentialfor idole-3-acetic acid production[6]. However, this present study revealed that *M. guilliermondii* and the two unidentified isolates of Mucoromycotina has the ability to produce auxin which indicates that these species are not just merely pathogens but may also have importance in plant growth promotion.

Phosphate solubilisation activity



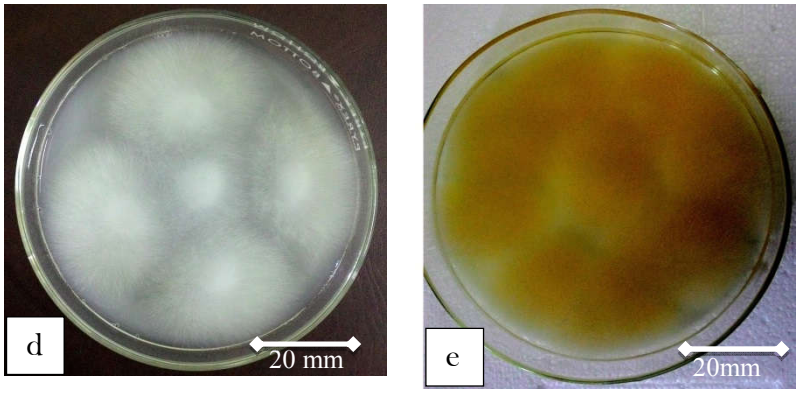
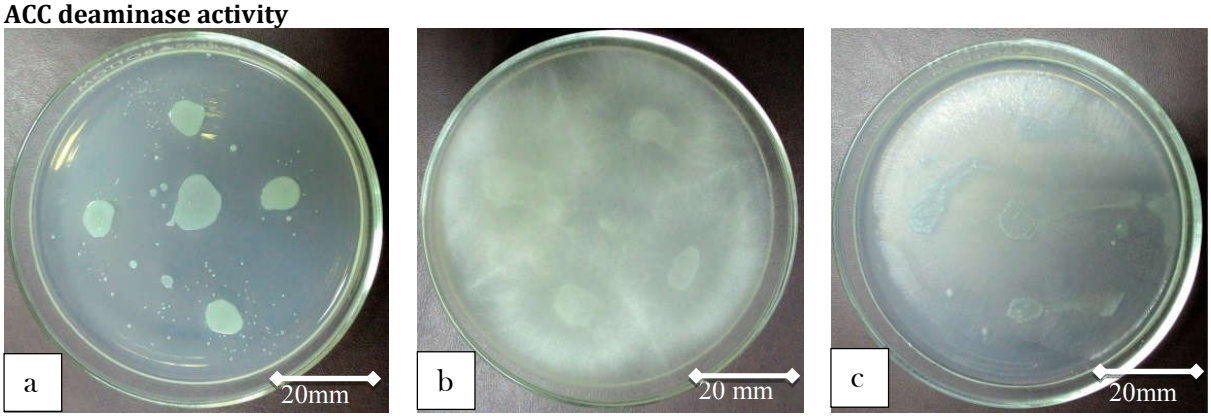


Figure 2. Halos surrounding some RSF isolates on Pikovskaya Agar. (a) *M. guilliermondii* (positive clearing zones), (b) *T. yunnanense* (negative clearing zone), (c) *T. simmonsii* (positive clearing zone), (d) Unidentified Mucoromycotina ($F_5P_1RSF_{16}$) (positive clearing zones), (e) Unidentified Mucoromycotina, ($F_9P_2RSF_{21}$) (negative clearing zone).

Aspresented in Figure 2, three of the five RSF isolates in *D. quercifolia* were found to have phosphate solubilisation activity. *M. guilliermondii*, *T. simmonsii* and the unidentified Mucoromycotina ($F_5P_1RSF_{16}$) isolate developed clearing zones after growing in Pikovskaya agar for seven days. A number of published data showed the ability of some species in the genus *Trichoderma* to solubilize inorganic phosphate. *Trichoderma* isolates found in Egyptian soils were also found to solubilize inorganic phosphates which were determine by the formation of clearing zones around RSF colonies on Pikovskaya's agar after 4D incubation [9]. The study of Yadav *et al.*[10]exhibitedthe potential phosphate solubilization activities of the *T. harzianum* inoculum and its efficacy as bio-fertilizer since it significantly increased the shoot, root and total height and weight of chickpea. Relative to this, the phosphate solubilisation ability of *T. simmonsii* isolated from *D. quercifolia* in this study could possibly indicate potential growth stimulating capacity which is useful in agriculture.

On the other hand, the ability of soil yeasts and other filamentous fungi to solubilize inorganic phosphate is underexploited. On this detail, the ability of phosphate-solubilizing yeast, *M. guilliermondii* was explored by Nakayan *et al.*[11] and their results show that the CC1 strain of *M. guilliermondii* displayed superior seed vigor and increased the biomass and nutrient uptake of both Chinese cabbage and maize. Sharma *et al.*[12] also showed phosphate-solubilizing filamentous fungi (e.g. *Mucor* sp.) in the group Mucoromycotina has shown to increase growth of test plants by up to 20% after culture filtrate inoculation. In this present study, *M. guilliermondii* and the unidentified Mucoromycotina isolate ($F_5P_1RSF_{16}$) also showed their potential phosphate solubilisation capacity which stipulates their prospective ability as agricultural crop growth enhancers.

ACC deaminase activity



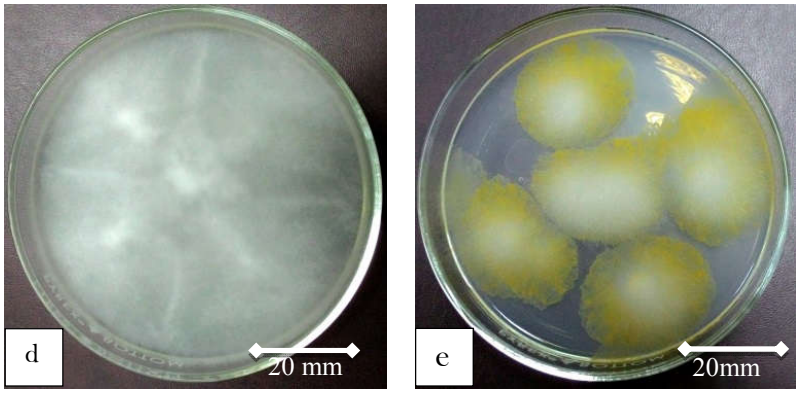


Figure 3. Growth of RSF isolates on ACC-enriched Yeast Carbon Base. (a) *M. guilliermondii,* (b) *T. yunnanense,* (c) *T. simmonsii* (d) Unidentified Mucoromycotina ($F_5P_1RSF_{16}$) (e) Unidentified Mucoromycotina, ($F_9P_2RSF_{21}$).

Figure 3 shows the growth of the five RSF isolates in YCB amended with 3.0 mM ACC. All the isolates were able to use ACC indicating their capacity to deter the stress-inducing hormone, ethylenethat may potentially inhibit development of their host plant [3].Published literature showed the ACC deaminase action of some species in the genus *Trichoderma*. Viterbo *et al.* [13] evaluated the ethylene and ACC biosynthesisactivity of *T. asperellum* isolate T203. Their study revealed that the isolate was able to produce ACC deaminase leading to root elongation promotion in *B.napus*. Brotman *et al.*[14] further showed that many *Trichoderma* species are able to promote plant development to up to 300% through the ACC deaminase mediated activity of the fungus coupled with IAA production. On the other hand, there are a few published data showing the ACC deaminase activity of yeasts including the genus *Meyerozyma*. In the study conducted by Nutaratat *et al.*[15], ACC deaminase activity were not detected in nine yeast species including the*M.guilliermondii* isolate. Their results appear to oppose the result of this present study where the *M. guilliermondii* isolate was found to have ACC deaminase activity. This present study additionally shows the ACC deaminase activity of two Mucoromycotina isolates ($F_5P_1RSF_{16}$ and $F_9P_2RSF_{21}$). ACC deaminase activity does not only involve growth promotion in plants but could also play a multi-level role in various plant processes[16]. Therefore, understanding the physiochemical functions of this enzyme is key to critical agri-biotechnological implications.

CONCLUSION

All the RSF isolates produced significantly high IAA using Salkowski's test. The unidentified Mucoromycotina isolate ($F_5P_1RSF_{16}$) produced the highest IAA that is 6x higher than the broth and water control. On the other hand, the root symbiotic yeast, *M. guilliermondii* produced the highest IAA per milligram dry weight which is statistically higher than the four other RSF isolates. The ability of the RSF isolates to produce IAA denotes their possible growth promoting mechanisms important in the field of agriculture. Furthermore, three of the five RSF isolates were found to have phosphate solubilisation activity. *M. guilliermondii*, *T. simmonsii* and the unidentified Mucoromycotina ($F_5P_1RSF_{16}$) have inorganic phosphate solubilizing capacity on Pikovskaya's agar indicating their prospective ability as agricultural crop growth enhancers. All the isolates have ACC deaminase activity which can potentially help their host grow under environmental stresses by restricting the stress ethylene level that inhibits the development of their host.

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