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## RESEARCH ARTICLE

# ISOLATION AND PRELIMINARY SCREENING OF ENDOPHYTIC FUNGI FROM *Ficus carica* FOR BIOCONTROL AND PHOSPHATE SOLUBILIZATION

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## ABSTRACT

Fungal endophytes are a special group of symbionts that invade the internal environment of the host organism without causing any pathogenic symptoms. They increase the vigor of the plant while protecting them from several biotic and abiotic stress conditions such as herbivory, disease, and drought. In this study, endophytic fungi were isolated from fig (*Ficus carica*), a beneficial plant belongs to the family of *Moraceae*. This study was conducted to determine the potential of fungal endophytes of *F.carica* as biocontrol agents against selected phytopathogens *Ganoderma boninense*, *Magnaporthe oryzae*, and *Fusarium verticillioides*. Besides, endophytic fungi isolates were also screened to assess their ability as a phosphate-solubilizing agent. Results obtained revealed that a total of 11 fungal endophytes was successfully isolated from leaf, root, and stem samples of *F.carica*. The cultural-morphological identifications were carried out on the unknown fungal isolates. For the antagonistic test, S2-1 and R3-4 show a promising potential against those phytopathogens with more than 20% Percentage Inhibition Radial Growth (PIRG). Endophyte S2-1 exhibits the competition mechanism, while R3-4 expresses the antibiosis mechanism in suppressing the mycelium growth of phytopathogens. As for phosphate solubilization, 8 of 10 isolates show positive results as phosphate solubilizer with the highest PSI value  $3.02 \pm 0.05$  (S2-4).

## KEYWORDS

Fungal endophytes, *Ficus carica*, *Moraceae*, biocontrol, *Ganoderma boninense*, *Magnaporthe oryzae*, *Fusarium verticillioides*, phosphate, phytopathogens, antibiosis, mycelium.

## 1. INTRODUCTION

*F. carica* L. belongs to the family of *Moraceae*, with over 1400 species classified into about 40 genera (Mawa et al. 2013). It is one of the most abundant genera of angiosperms with over 800 species of trees, epiphytes, and shrubs in tropical and subtropical regions around the world (Vinson, 1999). The fruit of *F. carica* itself contains high amounts of dietary fibers and polyphenols that necessary for the promotion of good health. In addition, the entire fig tree, including the fruits, leaves, roots, latex, and leafy branches have been utilized ailments for many types of illness, which include eye vision problems, indigestion, and diarrhea (Barolo et al. 2014).

Endophytes are defined as microorganisms associated with living plant tissues that produce no indication of its presence in the plant and cause no harm to the host (Nisa et al. 2018). Endophytic fungi spend the whole or part of its life cycle colonizing intercellular inside the healthy tissues of the host plants. Many pieces of research show the contributions of endophytic fungi as a biocontrol agent in plants.

For example, in an investigative study performed on sunflower plants supplemented with endophytic fungi *Penicillium citrinum* and *Aspergillus terreus*, the plants showed disease resistance against *Sclerotium rolfsii* and improved the biomass yield of sunflower plants (Waqas et al. 2015). Endophytic fungi, isolated from oil palm, collectively known as *GanoEF1*, has shown the potential to suppress *Ganoderma boninense* infection in oil

palm seedlings (Idris et al. 2010).

Other than that, endophytic microorganisms also contribute to several processes related to plant growth and development, such as nitrogen fixation and phosphate solubilization (Santoyo et al. 2016). Another contribution is shown by the growth-promoting activity of the endophytic fungi *Piriformospora indica* resulted in enhanced barley grain yield by increasing shoot fresh weight by 1.65 times higher compared to the control plant (Waller et al. 2005).

A recent *in vitro* study reported that endophytic fungi *Penicillium* and *Aspergillus* from the root of *Taxus wallichiana* solubilized phosphate by utilizing the substrates calcium, aluminium, and iron phosphate along with the production of phosphatase and phytase enzymes (Adhikari and Pandey, 2019). This proves the potential of endophytic fungi as a plant growth promoter.

The potential of endophytes fungi as the biological control and phosphate solubilizing agent has been stated in many studies before. However, the research of endophytic fungi, specifically in *F. carica*, is still green compared to the other plants. Thus, in our study, we aim to isolate the endophytic fungi from different parts of *F. carica* and evaluate their potential as a biocontrol agent and phosphate solubilizer.

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## 2. MATERIALS AND METHODS

### 2.1 Plant materials

Endophytic fungi were isolated from 5 months old *F. carica* that appear to be healthy without any disease symptoms. The plant samples were obtained from Banting, Selangor. The samples of the stem, leaves, and roots were carefully cut and stored in sterilized plastic. For the root, it was cut according to the region of maturation, region of elongation, and apical meristem. A sterilized plastic bag was brought to the laboratory where they will be further processed.

### 2.2 Isolation of endophytic fungi and surface sterilization.

The collected plant samples were washed with running tap water to remove the dirt and air-dried. Next, the isolation was carried out in a laminar air flow. Then, the samples were soaked in sterilized distilled water for 10 minutes. Small fragments of leaves, roots, and stems were cut into 0.5 cm x 0.5 cm using sterilized blades. Next, all the plant's part was subjected to surface sterilization by soaking in 80% ethanol (leaves for 3 minutes, stem and root for 5 minutes). Next, the samples were rinsed again in sterilized distilled water and treated with sodium hypochlorite for five minutes. Then, the samples were rinsed about 8 to 10 times with sterile distilled water. After that, the samples were blotted on sterile filter paper to remove excess moisture. Other than that, the imprint method was used to verify the effectiveness of surface sterilization (Schulz and Boyle, 2005). Each sterilized tissue was imprinted on Potato Dextrose Agar (PDA) before placed on Petri dishes containing PDA supplemented with 25 µg/mL chloramphenicol. Surface sterilization is considered successful if no bacteria growth is present in the imprint media.

Endophytic fungi are slow to emerge; thus, plates are incubated for 7-10 days at 35°C. The plates were appropriately sealed with parafilm to avoid desiccation of the medium and any contamination during the period. The incubation period for each isolate was recorded, and the day of the first visual growth observed from the plating date was considered as an incubation period of growth.

### 2.3 Calculation of colonization frequency

Next, the Colonization Frequency (CF %) of endophytic fungi was measured after seven days of incubation (Suryanarayanan et al. 2003; Photita et al. 2001).

$$\text{Percentage of Colonization Frequency (\%)} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segments observed}} \times 100$$

### 2.4 Preservation of endophytic fungi

In order to obtain a pure culture of endophytic fungi, the colonies were grown on water agar for seven days at 30°C. The hyphal tips of each colony were transferred to fresh PDA without any antibiotic with great precaution. The purified fungal isolates were transferred separately to PDA slants with proper labeling and kept at 4°C.

### 2.5 In vitro antagonistic properties of endophytic fungi in *F. carica* against plant pathogenic fungi

The antagonistic potential of the endophytic fungi was determined using the *in vitro* culture method against selected phytopathogens. Previous to the dual culture test, all endophytic fungi isolate, and selected phytopathogens (*G.boninense*, *M.oryzae*, and *F. verticillioideis*) were incubated on PDA to ensure actively growing mycelium and same age colonies for the antagonistic assays. Briefly, a 5 mm mycelial disc from the margin of actively growing colony of 7 days old *G. boninense*, *M. oryzae* and *F. verticillioideis* was placed at about 1 cm from the wall of a 9 cm PDA plate and at the opposite side, a similar-sized disc of the fungal isolate was placed. Then, the plates were incubated at 25 °C for seven days. Each antagonistic assay was performed in 3 replicates for each endophytic fungus tested.

Percentage inhibition radial growth (PIRG) between endophytic fungi isolates and selected phytopathogens were observed and calculated using the following formula (Whipps, 1997).

$$\text{PIRG (\%)} = \frac{R_2 - R_1}{R_2} \times 100$$

R2: Pathogen mycelia radius in the control plate (cm)

R1: Pathogen mycelia radius in pathogen endophytes dual culture (cm)

The fungal isolates were screened *in vitro* for their phosphate solubilization activity on Pikovskaya's agar medium to evaluate the potential of endophytic fungi on phosphate solubilization (Iman, 2008). A spot inoculation of fungal isolates was made onto the Pikovskaya's agar in triplicate under aseptic condition and incubated at 28°C for seven days. Uninoculated Pikovskaya's agar plate served as control. Comparative solubilization index measurement was carried out seven days after incubation by measuring clear zone and colony diameters in centimeter. Phosphate solubilization index was determined by using the following formula (Premono et al. 1996):

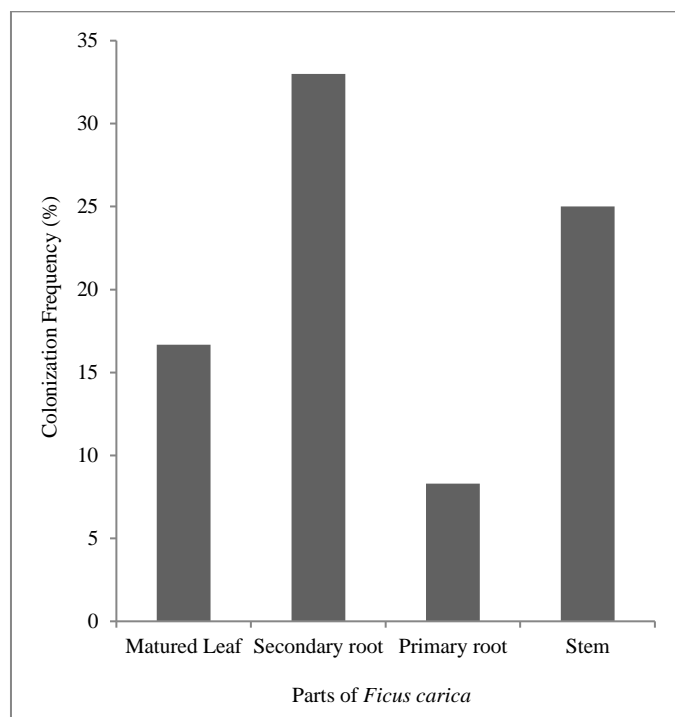
$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

## 3. RESULTS

### 3.1 Characterization of endophytic fungi collection

A total of 11 isolates were observed from 48 plant samples (Table 1). The endophytic fungi frequency was varied according to the respective organ (leaf, root, and stem). The highest colonization frequency of endophytic fungi was observed in the secondary roots of *F. carica* with 33%, whereas the primary root of *F. carica* showed the lowest colonization frequency with approximately 8.3% (Figure 1). Next, to gain an insight into the cultural characteristics of the endophytic fungi collection, we observed the colony surface color, colony reverse color, colony form, colony margin, colony elevation, and colony growth (Table 1). From the result obtained, both endophytic fungi isolated from leaf shows circular colony form and filiform colony margin. However, endophyte L1-2 expresses white with yellow ring surface color compared to L1-1, which is pure white (Figure 2). Next, for the endophytic fungi isolated from secondary roots (Figure 3), all of them have a circular colony form.

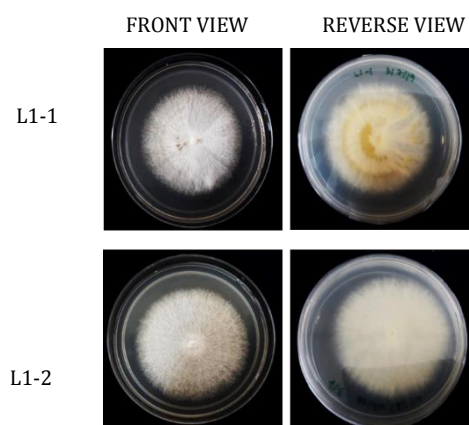
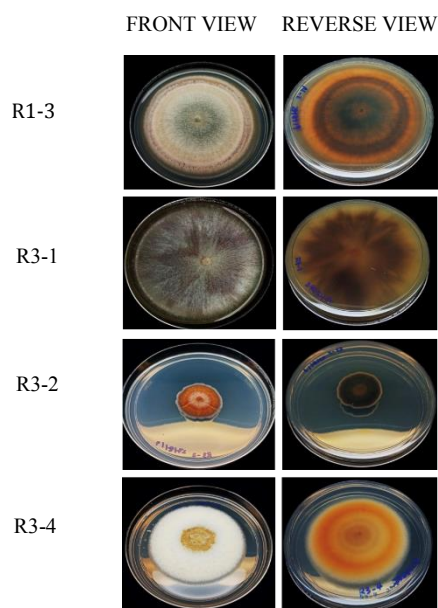
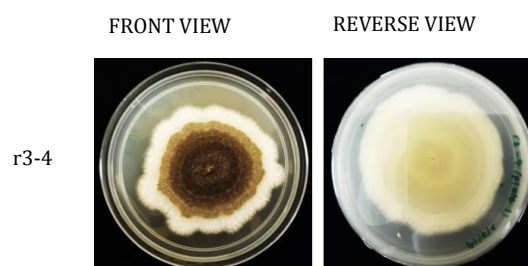
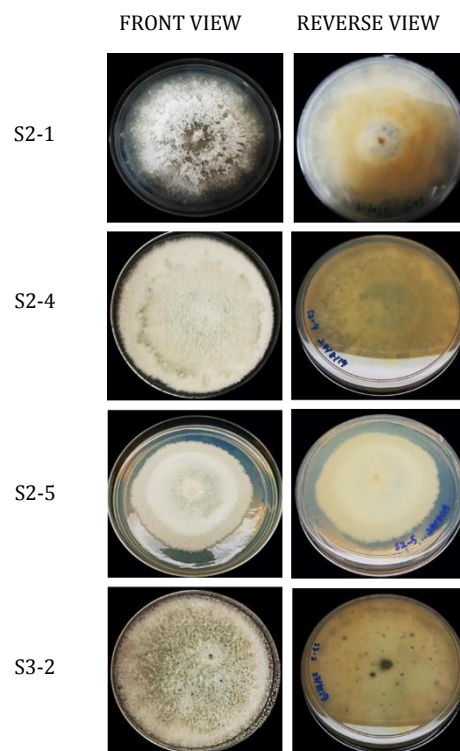
They also have an entire colony margin except for endophyte R3-1 with a filiform colony margin. Besides, only endophytic fungi R1-3 shows flat colony elevation compared to others with umbonate colony elevation. Among all the isolated endophytes, R3-2 has the smallest growth diameter size, which is 2.33 cm. Next, the only endophytic fungi isolated from primary roots show black surface color indicates many spores with white hyphae (Figure 4). Then, among endophytic fungi isolated from stems, endophyte S2-1 is different morphologically when it is the only one with irregular colony form, undulate colony margin, and umbonate colony elevation (Figure 5). Apart from those endophytic fungi isolated from leaf has a more substantial growth diameter, which is around 8.4 cm.



**Figure 1:** The colonization frequency of different parts of *F. carica*. The bar indicates the percentage value of colonization

**Table 1:** Cultural-morphological characteristics of endophytic fungi isolated from *F. carica*

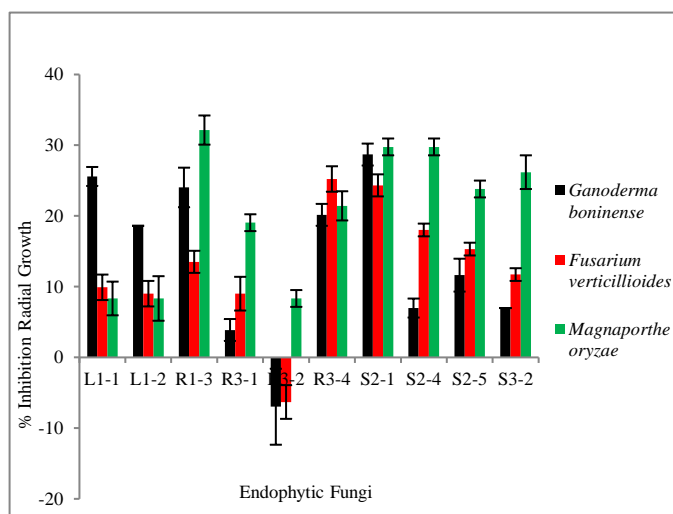
Fungi Species (isolates code)	Colony colour	Reverse colour	Colony form	Colony margin	Colony elevation	Average diameter (cm)
L1-1	White with yellow ring	Yellowish	Circular	Filiform	Flat	6.33 cm
L1-2	White	White	Circular	Filiform	Raised	6.43 cm
R1-3	White brown	Brown with orange ring	Circular	Entire	Flat	7.86 cm
R3-1	Brown yellowish	Brown yellowish	Circular	Filiform	Umbonate	7.85 cm
R3-2	Orange-brown	Black	Circular	Entire	Umbonate	2.33 cm
R3-4	White with yellow spores	Creamy orange	Circular	Entire	Umbonate	5.26 cm
r3-4	Black with white hyphae	White	Irregular	undulate	Flat	6.56 cm
S2-1	White with slightly yellowish	Creamy slightly yellowish	Irregular	Undulate	Umbonate	8.46 cm
S2-4	White	White with black dots	Circular	Filiform	Raised	8.43 cm
S2-5	White	White	Circular	Filiform	Raised	8.40 cm
S3-2	White With black dots	White with black dots	Circular	Filiform	Raised	8.43 cm


**Figure 2:** Cultural-morphological characteristics of endophytic fungi isolated from the matured leaf of *F. carica*. The left side shows the front view of the isolates, while the right side shows the reverse view of respective isolate.

**Figure 3:** Cultural-morphological characteristics of endophytic fungi isolated from the secondary root of *F. carica*. The left side shows the front view of the isolates, while the right side shows the reverse view of the respective isolates.

**Figure 4:** Cultural-morphological characteristics of endophytic fungi isolated from the primary root of *F. carica*. The left side shows the front view of the isolates, while the right side shows the reverse view of the respective isolates.

**Figure 5:** Cultural-morphological characteristics of endophytic fungi isolated from the stem of *F. carica*. The left side shows the front view of the isolates, while the right side shows the reverse view of respective isolates.

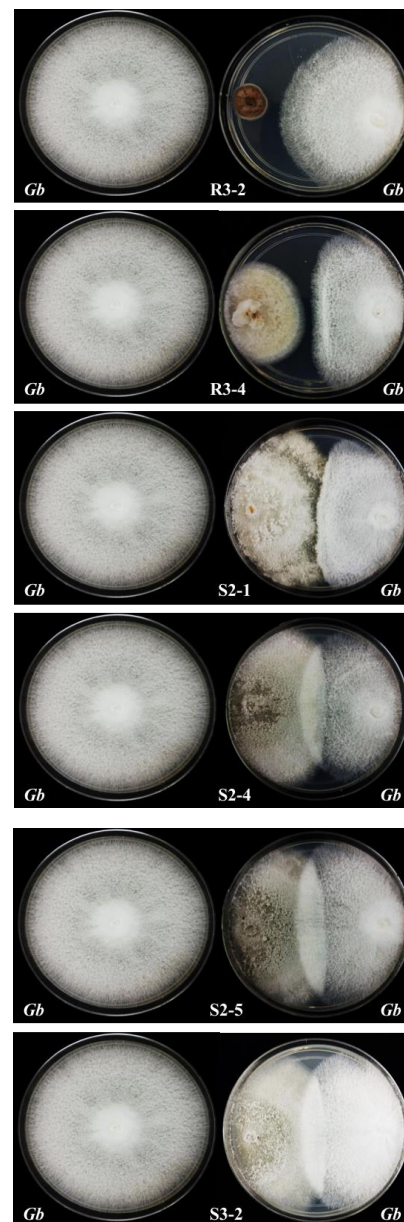
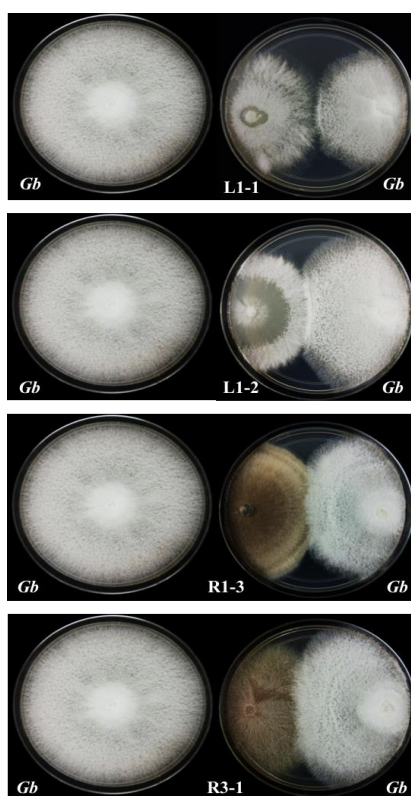


### 3.2 *In vitro* antagonistic properties of endophytic fungi in *F. carica* against plant pathogenic fungi

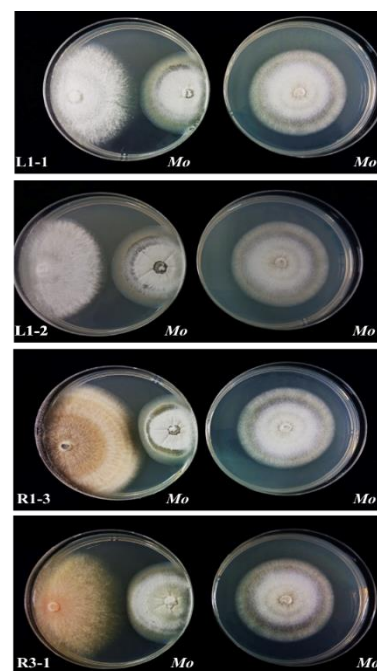
Next, to evaluate whether the endophytic fungi have antagonistic properties, *in vitro* dual culture was performed against three selected phytopathogens, which are *G. boninense*, *M. oryzae*, and *F. verticillioides*. Dual culture tests revealed that S2-1 has the highest percentage inhibition radial growth (PIRG) against *G. boninense* with 28%, whereas the lowest is R3-1 with 3.87% (Figure 6, 7). Next, R1-3 exhibits good antagonistic capability against *M. oryzae* with PIRG value more than 30%, while the lowest PIRG value is L1-1, L1-2, and R3-2 with 8.33% inhibition (Figure 6). There is a significant difference in the size of *M. oryzae* in dual culture with R1-3, which is smaller compared to control (Figure 8). In addition, S2-1 and S2-4 also show high PIRG value against *M. oryzae*, which PIRG value is around 29.76%. Moreover, endophytes R3-4 and S2-1 were identified as the most potent antagonists against *F. verticillioides* with PIRG value of 25.22% and 24.32%, respectively (Figure 6, 9). This result can be seen by a clear inhibition zone where the growth of *F. verticillioides* is restricted in dual culture plates between R3-1 and *F. verticillioides*. Overall, S2-1 and R3-4 showed promising potential as antagonistic agents against these three selected phytopathogens (Figure 6).

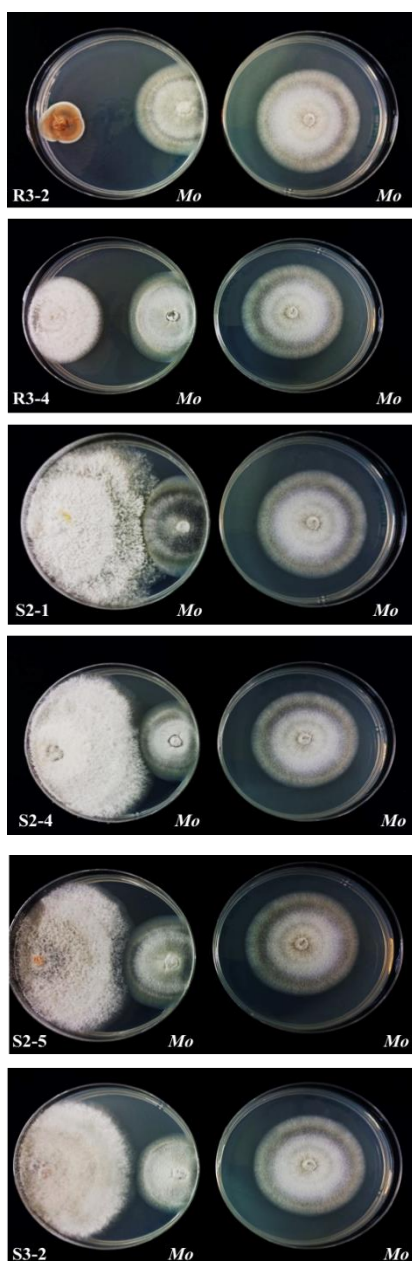


**Figure 6:** The percentage inhibition radial growth (PIRG) of endophytic fungi against selected phytopathogens *Ganoderma boninense*, *Fusarium verticillioides*, and *Magnaporthe oryzae*. Values are the means average of three replicates. Bars represent standard deviation.

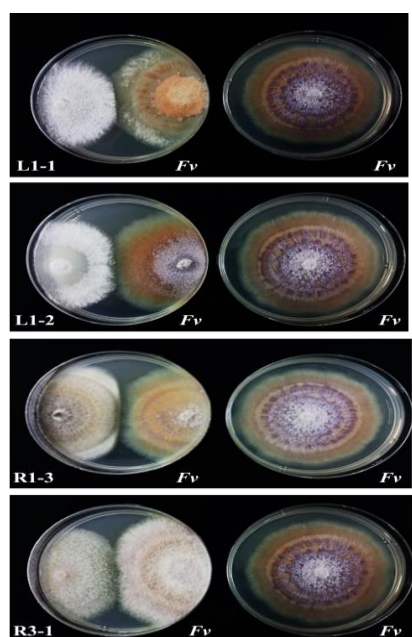


**Figure 7:** Dual culture plates of endophytic fungi isolates against *Ganoderma boninense* (Gb). All the dual culture plates are compared with the negative control plate of *G. boninense* (right).





**Figure 8:** Dual culture plates of endophytic fungi isolates against *Magnaporthe oryzae* (*Mo*). All the dual culture plates are compared with the negative control plate of *M. oryzae* (left).

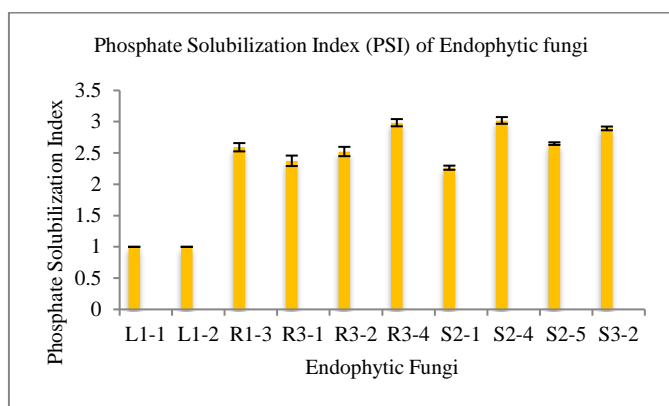


**Figure 9:** Dual culture plates of endophytic fungi isolates against *Fusarium verticillioides* (*Fv*). All the dual culture plates are compared with the negative control plate of *F. verticillioides* (left).

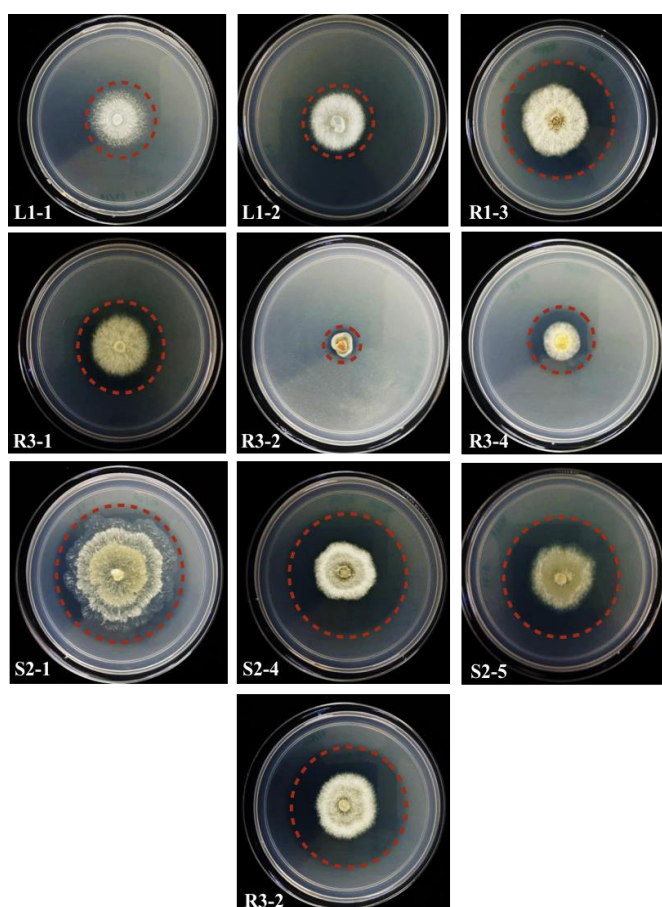
### 3.3 Endophytic fungi isolates are capable of solubilizing phosphate *in vitro*.

Next, to examine whether the endophytic fungi can solubilize phosphate, we performed *in vitro* phosphate solubilizing assay. Our result indicates that S2-4 has the highest phosphate solubilization index (PSI), which is 3.02, whereas the lowest is L1-1 and L1-2 with PSI value 1.00 (Figure 10). Endophytic fungi with PSI value 1.00 may have the solubilization zone as wide as the colony diameter. S2-4 has the most significant formation and the dimension of clear halo around the colonies. As for L1-1 and L1-2, we can barely see the formation of the halo zone around the colonies (Figure 11).





**Figure 10:** The phosphate solubilization index (PSI) of endophytic fungi isolates. Values are the mean average of three replicates. Bars represent standard deviation.



**Figure 11:** The phosphate solubilization activity of endophytic fungi on Pikovskaya's media. Red dashed lines indicate the halo zone diameter of endophytic fungi.

#### 4. DISCUSSION

A total of 11 endophytes were successfully isolated from 48 segments of *F. carica* with the secondary root showing the highest colonization frequency. This finding is in line with the previous studies that found a decrease of the endophytic population from underground parts to above-ground parts (Company et al. 2011). The study of *Cirsium arvense* by Wearn et al. also supported the same hypothesis whereby the highest number of endophyte species were found to be harbored in roots compared to the leaves (Wearn et al. 2012). However, in some studies, the endophytic population showed high colonization in stems instead of the roots, as found by Bezerra et al. in the study of *Bauhinia forficata* (Bezerra et al. 2015).

Next, we determined in vitro antagonistic properties of endophytic fungi in *F. carica* against selected plant pathogenic fungi, namely, *G. boninense*, *M. oryzae*, and *F. verticillioides*. *G. boninense* is the major cause of basal stem rot (BSR) disease, mainly in an oil palm plantation in Malaysia. BSR

infects not only mature oil palms but also seedlings and younger plants and contributes to the serious manifestation of the disease (Susanto et al. 2005). As for *M. oryzae*, it is the rice blast fungus that produced the world's most damaging plant diseases, especially in cereal (Fernandez and Orth, 2018). Next, *F. verticillioides* is phytopathogen that is commonly associated with maize. The fungus caused seedling decay, stalk rot, ear rot, and mycotoxin contamination in maize (Murillo-Williams and Munkvold, 2008).

In general, antagonism can be explained as any activity of one organism, which is in some way adversely affects another growth in association with it (Khara and Hadwan, 1990). According to Siameto et al., there are numerous types of antagonism that fungal antagonists could exhibit, such as mycoparasitism, competition, and the production of extracellular metabolites (antibiosis) (Siameto et al. 2010). Our result indicated that some of our endophytic fungi express competition antagonism and antibiosis antagonism against these phytopathogens.

In a competition mechanism, endophytic fungi will compete with pathogens for food and space in the medium plate, often causing a difference in the size of the culture. This mechanism was exhibited by S2-1 by overgrowing against *G. boninense*. Next, R1-3, R3-1, S2-1, S2-4, S2-5, and S3-2 also clearly constrain the growth of *M. oryzae* mycelium. On the other hand, S2-1, S2-5, and S3-2 also display the competition mechanism against *F. verticillioides* by reducing the growth of *F. verticillioides* and changing the colony morphology of *F. verticillioides*. Hence, the endophytic fungi isolates are speculated to have competed with pathogens for nutrient and space, altering the growth and morphology of the pathogens. This finding is supported by the research from Berlian et al. which specified that the nutritional needs of pathogen *Fusarium oxysporum* were disturbed by the mechanism of competition in dual culture plate with endophytic fungi and causing a decrease in the germination percentage of its spores as much as 20-30% (Berlian et al. 2013).

Next, for the antibiosis mechanism, it can be defined as the main mechanism for biological control in which the antagonist generates a substance that could be an antibiotic, lytic enzyme that degrades plant cell wall, volatile substance, or toxin that destroys the pathogen (Mousa and Raizada, 2016). Ghorbanpour et al. stated that fungi exhibit antibiosis by synthesizing antibiotics, volatile and non-volatile compounds, and cell wall degrading enzymes, which was able to destroy mycelia or reproductive organs such as sclerotia, conidia or sporangia of pathogens (Ghorbanpour et al. 2018). This may also contribute to the presence of a clear inhibition zone, as found in the dual culture of R3-4 against *G. boninense*, *M. oryzae*, and *F. verticillioides*.

Thus, we speculated that endophytic fungi R3-4 produce certain antibiotic to reduce the mycelium growth of the pathogen. This speculation is in agreement with the finding of the interaction between endophytic fungi *Acremonium zeae* isolated from maize against *Aspergillus flavus* and *Fusarium verticillioides*, whereby a clear zone can be observed in this microbe-microbe interaction (Wicklow et al. 2005). Other research by Silva et al. shows fungal endophyte *Phomopsis cassiae* isolated from *Cassia spectabilis* has antifungal potential by the presence of a clear inhibition zone against *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* (Silva and Vidor, 2000). This type of fungus has antibiotic Cadinane sesquiterpenes against pathogens.

On the other hand, endophytic fungi are capable of acting as plant growth-promoting (PGP). PGP traits include the production of vital enzymes like 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD), urease, catalase, siderophore, indole acetic acid (IAA) formation, and phosphate solubilization. From these, we only test the phosphate solubilization of endophytic fungi. According to the scale, values under 1.0 were categorized as very low solubilizers, 1.0 to 2.0 as low solubilizers, 2.0 to 3.0 as medium solubilizers, and values above 3.0 were classified as high solubilizers. Result obtained indicates endophyte S2-4 is a high solubilizer fungus with PSI value  $3.02 \pm 0.05$ . A similar result can be seen in the phosphate solubilization assay of endophytic fungi *Aspergillus niger*, isolated from leaf and root of orchid *Pomatocalpa decipiens*. *A. niger* produced a clear halo zone with high phosphate solubilization index of  $22.7 \pm 0.46$  and  $36.2 \pm 5.6$ , respectively, when tested on Pikovskaya's media (Sahoo, 2018).

Phosphate solubilizers showed a clear zone on solid media containing tricalcium phosphate ( $\text{Ca}_3\text{PO}_4$ ). The development of halo zones around the fungal colonies may be due to the production of organic acids, which will acidify the cell into the surrounding environment and causes the release of mineral ions P by substitution of  $\text{H}^+$  cations bound to phosphate (Park et al. 2009). This scenario will make phosphate to be available to plants.

Overall, endophytes R3-4 and S2-1 have a promising potential as a biocontrol agent against phytopathogens. R3-1 shows the behavior of inhibition through the antibiosis mechanism, while S2-1 expresses the obvious behavior of inhibition through competition mechanism. Plus, R3-1 and S2-1 also have phosphate solubilization capability even though they are not as high as S2-4. It is also hypothesized that S2-4 excretes the highest organic acid with the largest formation of the halo zone compared to others and has the promising potential as plant growth-promoting fungi (PGPF). However, due to our limitation, we only performed the screening of isolated endophytic fungi in order to evaluate their potential as biocontrol agents and phosphate solubilizers in agriculture. In the future, molecular characterization of the endophytic fungi and deciphering the mechanism of antagonism and other plant growth-promoting traits are important to be carried out in order to explore their full potential in agriculture.

## 5. CONCLUSION

In conclusion, 11 endophytic fungi were successfully isolated from *F. carica* and morphologically characterized based on colour, form, margin, elevation, and diameter of the fungal colony. The result gives the impression that fungal isolates were different species due to different characteristics. Besides, it was also found out that every endophytic fungal isolate was able to exhibit antagonistic properties against three selected phytopathogens., granting a variation of efficacy and mechanisms. Furthermore, the endophytic fungi also showed a definite potential as a plant growth-promoting factor, specifically in phosphate solubilization.

However, as this study was conducted in a preliminary stage, extensive research such as in molecular aspect should be done in assessing the ability and potential of endophytic fungi as a biocontrol agent and plant growth-promoting agents. This further research will significantly contribute to enhancing the health and productivity of plants in agricultural practices.

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NMR contributed to the acquisition of data, analysis, and interpretation of data, drafting, and revising the article. KIAHA contributed to the drafting and revising of the article. NSAA contributed to the study conception and design, drafting the article and final approval of the version to be submitted and any revised version.

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