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MOLECULAR CHARACTERIZATION OF MYCORRHIZA AND ITS POTENTIAL AS BIOCONTROL

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SUMMARY

Orchid mycorrhizal fungi (OMF) are vital biocontrol agents, especially for Odontoglossum ringspot virus (ORSV). The promising study helped identify the mycorrhiza isolate from native tropical orchids and determine its potential as a biocontrol. Sample collection of healthy roots of *Phalaenopsis amabilis* emanated in Yogyakarta, Indonesia, carrying out molecular identification with rDNA-ITS amplification using a set of universal primers ITS1 and ITS4. In vivo, antagonist tests began by inoculating viruses and mycorrhiza to determine the effect of growth and induction of secondary metabolites. The result showed one isolate of *Trichoderma* sp. associated with the molecular analysis has amplified the ITS1-5.8S-ITS4 section by 600–750 bp DNA. The sequenced products revealed insertion and substitution occurrences, which may have caused the variance by strain diversity and potential severity. Indonesian isolates have undergone speciation and separation from other isolates by a substantial distance. The considerable effects were the increase in leaf length, leaf width, root length, leaf count, the number of roots, fresh weight, and a lowering of the virus content. The analysis of the plant growth parameters and virus concentrations provided significant differences among the treatments inoculated with orchid mycorrhiza (Mycorrhiza [M], Mycorrhiza + Virus [MV], and Virus + Mycorrhiza [VM]) and those without orchid mycorrhiza inoculation (Control [C] and Virus [V]). The orchid resistance suggested that the virus infecting plant leaves contain more phenolic chemicals. This study is the first-ever report of the *Trichoderma* sp. isolated from native tropical orchids in Indonesia.

Keywords: Antagonistic test, biocontrol, orchid mycorrhiza fungi, *Phalaenopsis amabilis*, phenolic chemicals, rDNA-ITS

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Key findings: Molecular characterization of mycorrhizal isolates on the roots of *Phalaenopsis amabilis* from Yogyakarta, Indonesia, based on rDNA-ITS has a DNA product size of 600–750 bp located on ITS1-5.8S-ITS4, which is a *Trichoderma* sp. The phylogenetic tree reconstruction showed the isolate has undergone speciation. The results of the mycorrhizal antagonist test against ORSV infection revealed considerable growth of leaf length, width, root length, number of leaves, root volume, fresh weight, and phenolic chemicals.

INTRODUCTION

The induction of endophytic microbes, such as, the Orchid Mycorrhizal Fungi (OMF), could be beneficial in developing and protecting *Phalaenopsis* sp. against diseases (Currah and Zelmer, 1992; Moreno *et al*., 2000) in Indonesia (Mahfut *et al*., 2020a; Mahfut 2023). *Trichoderma* sp. is one form of the OMF that has undergone isolation and identification. The moth orchid (*Phalaenopsis amabilis* L. - Blume) is a native tropical orchid from Indonesia requiring conservation (Sukma *et al.,* 2021). According to their natural habitat, these orchids are decreasing and even threatened to go extinct (Kumalawati *et al*., 2011; Mahfut *et al*., 2021a). Therefore, it is crucial to conserve and protect orchid plants from diseases. The *Odontoglossum ringspot virus* (ORSV) has become a chief causal organism for orchid infections in Indonesia (Mahfut, 2020a; Mahfut *et al*., 2020c).

The OMF presence in plants can protect them from ORSV infection (Otero *et al*., 2013; Minh *et al*., 2016; Mahfut, 2021). Enhanced production of antagonistic enzymes and secondary metabolites can prevent and decrease the severity of disease caused by pathogenic infections (Sukmawati *et al.*, 2021). *Trichoderma* sp. is one of the OMF types incurring isolation and identification. Few studies connect with OMF induction in controlling viral infection in orchids. Several past studies reported the effectiveness of OMF in encouraging the growth traits and disease resistance, such as, enhancing plant height and the number of roots and leaves (Mahfut *et al*., 2019; Tohari *et al*., 2021), increasing leaf thickness (Arifannisa *et al*., 2021), decreasing infection (Izzati *et al*., 2021) and disease intensity caused by ORSV (Minarni *et al*., 2021) on the anatomical structure of orchid roots and leaves (Mahfut *et al*., 2023a; Mahfut *et al*., 2023b). However, information about the effectiveness of OMF secondary metabolites is still unknown.

In this research, the identification of *Trichoderma* sp. through molecular analysis of the rDNA-ITS sequence isolated from *P. amabilis* grown in Indonesia and as a biocontrol considered its potential. Identification of the morphological characters of *Trichoderma* sp. using macroscopic and microscopic observations has prevailed in previous research and published separately. The existing research expects to develop an information guide on cultivating and protecting inhabitant orchids and how to prevent disease occurrence in Indonesia.

The study included variations in disease infection symptoms, in vivo measuring of leaf length and breadth, root length, leaf count, root volume, fresh weight, and testing for total phenolic compounds as a marker of plant resistance to viral infection. Phenolic substances are secondary metabolites from phenylpropanoid metabolism in pentose phosphate and plant shikimic acid (Randhir *et al*., 2004). The relevant research hopes to provide the fundamental knowledge for advancing native orchids and their protection in Indonesia and, wherever practical, preventing disease infestations.

MATERIALS AND METHODS

Plant materials

A sample collection of eight healthy roots from *P. amabilis* commenced at two locations: an orchid garden in Condong Catur and Parakan, Yogyakarta, Indonesia. Preparation of the in vivo antagonist test began by following the methodology of Minarni *et al*. (2021) and Tohari *et al*. (2021). The 12-month-old plant

from Rumah Bunga Rizal, Bandung, Indonesia, sustained soaking in a fungicide solution (2 g/l of water) for 20 min. The plants sown in sterile moss media acquired tending for one month before treatment. The research included five treatments and three replications (without inoculation as Control ©, mycorrhiza inoculation (M), virus inoculation (V), mycorrhiza + virus (MV) inoculation, and virus + mycorrhiza (VM) inoculation).

Molecular analysis

In samples of pure cultures of isolated mycorrhizal endophyte *Trichoderma* sp., genomic DNA isolation employed techniques modified from the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The genomic DNA amplification proceeded by PCR according to manual directions provided with the GoTaq® Green PCR mix from Promega. Mahfut *et al*. (2020b) techniques and a set of universal primers, rDNA-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and rDNA-ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), amplified the DNA. Using electrophoresis on a 2% agarose gel stained with ethidium bromide and a 100 bp Vivantis DNA ladder as a marker helped visualize the PCR results. The visible DNA bands showed the length of the rDNA-ITS base pairs targeted for sequencing.

Phylogenetic analysis

The software suite for sequence analysis DNASTAR Lasergene DM Version 3.0.25's EditSeq and SeqMan application assembled the nucleotide sequences and analyzed the findings of the genomic sequencing. Determining the sequence homology with the information in the DDBJ database attained comparison using the BLAST program. Algorithm Multiple Alignment Parameters DNA using Kimura-2 Parameters, Neighbor-Joining of MEGA 11 Beta, and relationship and phylogenetic analysis also compared the sequences of the isolates. The bootstrap values with 1000 replications became data for the statistical examination of the internal branch.

Inoculation of orchid mycorrhiza and virus

The orchid mycorrhiza inoculation method engaged Mahfut *et al*. (2021c) and Mahfut *et al.* (2023c). Using the PDA media, orchid mycorrhiza grew in a Petri dish. The orchid from the mycorrhiza isolate sustained inoculum on a medium and incubated for seven days. After spending 24 hours in an orchid mycorrhiza-containing Petri disc, the plant's removal from the dish allowed its recovery in the sterile moss-growing medium. The ORSV planting inoculation progressed using an inoculum from the Borobudur Orchid Center inoculum, Magelang. This sample was a positive plantlet leaf for ORSV infection from previous serological and molecular tests (Mahfut *et al*., 2023a). Adding phosphate to crush the orchid leaves in a ratio of 1:10 (m/v) ensued. Phosphate buffers help to kill cells until the virus' release. The plant's leaf surface gained equal pulverizing with carborundum before inoculation. Careful inoculation occurred with fingertips toward the leaf blade. Afterward, re-preserving the plants on sterile moss-growing media continued.

In vivo antagonist test

The inoculated plants proceeded to transfer to the greenhouse. Crops' transfer in glass pots measured 15 cm × 25 cm with sterile fern growing media. After two weeks, the orchid mycorrhiza plants incur inoculation with the virus through mechanical injection (Izzati *et al*., 2021; Minarni *et al*., 2021). This study used a completely randomized design (CRD) with five treatments. Each treatment repeated three times has each replicate with five plantlets, making the number of plantlets used 75. The plantlets' treatments were without inoculation of orchid mycorrhiza and ORSV, herein referred to as Control (C), orchid mycorrhiza inoculated and without ORSV as Mycorrhiza (M), without injecting orchid mycorrhiza but inoculating ORSV as Virus (V), orchid mycorrhiza preoccupied first and then injected with ORSV as Mycorrhiza + Virus (MV), and, finally, ORSV first inoculated then subsequently with orchid mycorrhiza as Virus +

Mycorrhiza (VM). In each treatment, the performing observations on plant growth continued weekly for one month. Probing leaf length included measuring from the base bordering the stem to the tip of the leaf, leaf width at the widest part of the leaf, and root length from the root base to the leaf tip. The number of leaves and number of roots calculation comprised the number of leaves and roots of the plant as a whole, while the fresh weight of the plant proceeded to weigh the plants.

Observations of leaf phenol compounds

Evaluating phenolic compounds used the method of Evdokimenko *et al*. (2021). The first step began with preparing the phenol standard calibration curve using gallic acid as a gauge solution. The concentrated series of pure gallic acid used was 10, 20, 30, 40, 50, 60, 70, 80, and 100 ppm added to distilled water. Acquiring 0.5 ml of each concentration continued to be placed in a 100 ml volumetric flask, then added with 2.5 ml of Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The mixed solution, put into a cuvette, led to observing the absorption value at a wavelength of 765 nm. Knowing the absorption value helped develop a standard curve to assess the regression equation between gallic acid concentration and absorption value. Sample preparation occurred in all treatments on days 0, 2, 4, 6, and 8. Leaf extract creation emerged by weighing and crushing 0.5 grams of orchids. The extract, dissolved in 10 ml 80% methanol, homogenized by vortex, sustained centrifuging at 1500 rpm for 10 min, taking the supernatant after.

Statistical analysis

Quantitative data based on the plantlet resistance induction test against ORSV using Orchid Mycorrhiza reached analysis by ANOVA to check for significant differences among the treatments. If notable differences occur, the Duncan Multiple Range Test (DMRT), used at a 5% probability level to further compare and separate the means, aided in determining the difference between treatments.

RESULTS AND DISCUSSION

Sample collection

Eight collected healthy *P. amabilis* root samples came from two separate sites, the Condong Catur and Parakan, Yogyakarta, Indonesia. Referring to Currah and Zelmer (1992) and Carvalho *et al*. (2018), positive results were evident for *Trichoderma* sp. in an orchid garden in Parakan, which had a colony characteristic of white to gray color, phialide-shaped ampulliform at a size of 4.0–6.6 × 1.7–3.1 µm, conidia shape subglobose to ovoidal and size 2.7–3.1 × 2.5–3.0 µm.

Molecular analysis

The ITS rDNA amplification showed a band size of 600–750 bp, similar to results in past studies (Bayer *et al.*, 1996; Mahfut *et al*., 2020b). The nuclear ribosomal DNA (nrDNA) region known as the internal transcribed spacer (ITS) plays a vital role in revealing the information required for reconstructing the phylogenetic trees at various taxonomic levels and determining intrageneric relationships (Bayer *et al.*, 1996). The DNASTAR Lasergene DM Version 3.0.25 helped combine and analyze the sequencing findings. As a result, it was apparent that the sequences were typical of *Trichoderma* sp. isolates, a non-pathogenic (endophyte) associated with orchid roots (OMF). *Trichoderma* sp. isolates from Yogyakarta, Indonesia, incurred sequencing analysis, and the results showed 618 total bases with 56.18% GC content. Thus, according to BLAST, *Trichoderma* sp. isolate indicated kinship with the highest identity percentage of 99.19%. It also continued with selecting 11 sequences from Indonesia (West Sumatera), China, Australia, and Mexico, as well as, one outgroup from Indonesia (Lembang, West Java) to establish the affiliation of the OMF.

The results of similarity analysis using BLAST software showed that the *Trichoderma* sp. isolates originating from Yogyakarta are similar to those from West Sumatra, China, Australia, and Mexico. Based on the past research conducted by Shan *et al*. (2002), if

the similarity level of the genomic sequences among the *Hypocreaceae* isolates reached more than 90%, then it would be the same species isolated in the genus *Hypocreaceae*. Meanwhile, if the isolates are in the same genus, the similarity level is <97%. The homologous sequences analysis results using the BLAST-NCBI program obtained homologous sequences with the highest kinship (99.19%). All the successions in the *Trichoderma* sp. sample indicated the base content of GC. The alignment results revealed nucleotide differences among the *Trichoderma* sp. isolates and 11 others due to mutation. The results of the alignment analysis of the 12 nucleotide sequences displayed that there was a mutation in the *Trichoderma* sp. isolate.

The analysis of 12 other *Trichoderma* sp. isolates, selected according to each country's distribution area, showed that Indonesian isolates were highly different from other countries, with a similarity index (SI) ranging from 99.02% to 99.19%. Given that the isolates from Indonesia have undergone speciation and complete separation from those from other countries. Nucleotide sequence alignment of 11 different *Trichoderma* sp. isolates revealed that point mutations, mainly insertions and substitutions, were more common in Indonesian isolates. Total mutations were 73 bases, 23 deletions, 24 insertions, 11 transitions, and 15 transversions.

These variations revealed the synthesis of amino acids. Some Indonesian isolates had considerably different percentages of the overall 194 amino acids from those of other isolates. Alanine, glutamic acid, valine, glutamine, lysine, and tyrosine experienced considerable enhancement in number compared with other isolates, with increases of 7.14%, 4.17%, 6.55%, 2.38%, 4.17%, and 1.79%, respectively, of the total average. In addition, the average amount of other amino acids reduced, including aspartic acid, arginine, tryptophan, threonine, and leucine, by 5.95%, 14.88%, 2.38%, 6.55%, and 5.36%, respectively.

Based on research, the variations in nucleotides can change the production of amino acids (Mahfut *et al*., 2020b). The occurrence of the mutation can cause changes in amino acids in *Trichoderma* sp. isolates and other comparative isolates. Amino acid variations in *Trichoderma* sp. isolates were due to the alterations in amino acids and their frequencies. *Trichoderma* sp. isolates had a higher amino acid content with a total frequency of 168% of the amino acid content. Mahfut *et al*. (2020b) also stated that the degree of similarity in the nucleotide sequences of fungi groups ranged from 83%–99% for strains within the same fungus and 39%–53% for different fungi. An analysis of the genetic distance in *Trichoderma* sp. isolates also transpired that infect plants (Table 6). The probe revealed that the *Trichoderma* sp. isolate has a very different genetic expanse than other isolates. The genetic distance based on nucleotide sequence is consistently higher than those based on amino acids.

A suggestion also implied the resulting amino acid variations significantly impacted how the Indonesian isolates adapted to their environment. The construction of a phylogenetic tree used in the relationship analysis of the isolates indicated that the isolates from Indonesia were on separate branches and distant from 11 other segregates (Figure 1). The Yogyakarta, Indonesia isolates showed a close relationship with those from Mexico (KU990874.1), China6 (MH284216.1), and Australia3 (MK870380.1) at a genetic distance of 99. Several other isolates showed a far relationship, namely, China 4 (MW369599.1), China3 (MN486570.1), China2 (MK791709.1), China1 (MH284485.1), and Australia1 (MK870719.1) at genetic distances of 21, six, and five, respectively. The phylogenetic tree detailed that *Trichoderma* sp. from Yogyakarta formed a separate branch with other *Trichoderma* sp. The group of isolates molded due to human activity can also be due to geographical areas that were relatively very close. Based on the phylogenetic tree, the grouping of isolates resulted from several mutations that occurred in *Trichoderma* sp. isolates and have led far toward the species and a fungal evolution. The factors that led to the emergence of new species and the process of spreading fungi were the progression of fungal diversity, the

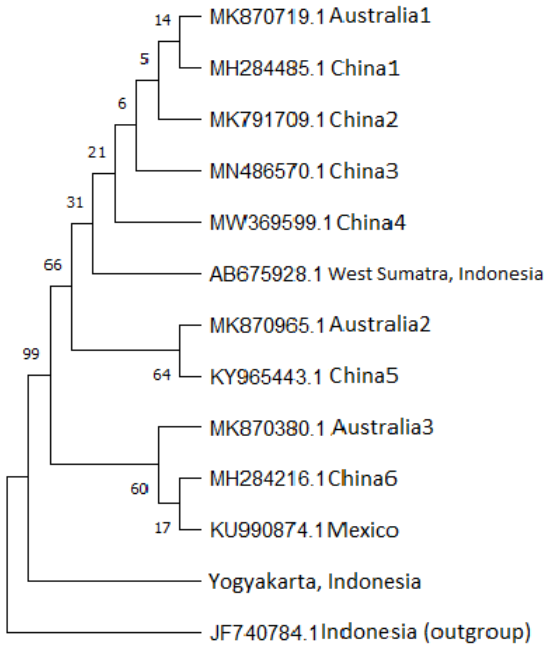


Figure 1. Reconstruction of phylogenetic trees of *Trichoderma* sp. isolates based on nucleotide sequences with Maximum Parsimony method with Bootstrap-1000 approach.

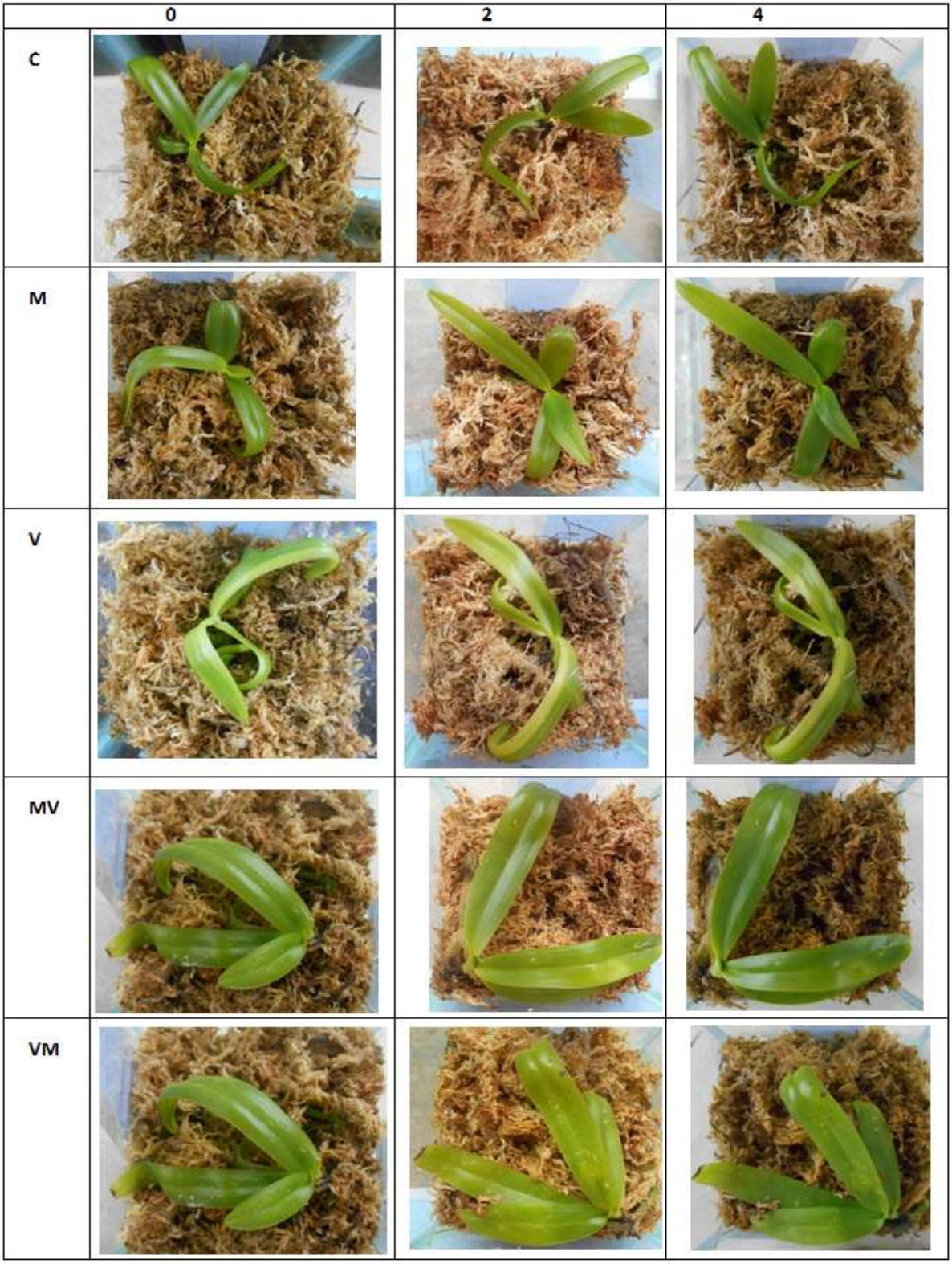
introduction of susceptible host genes in the crossing process, and climate change that occurs in each region (Mahfut *et al*., 2016; Mahfut *et al*., 2020b).

Nucleotide sequences can be applicable in the Maximum Parsimony method with the Bootstrap-1000 methodology to categorize the data based on specific parameters to learn about evolution from the variations in the data that have occurred over time. The analysis's findings incur categories based on how they attained distribution worldwide. A long branch that separated the two groups showed that the isolates from Indonesia had undergone significant evolution, resulting in speciation occurrences. The ability of the mycorrhizal to adapt to environmental variations and its host range facilitates such changes in sequences through mutation. The existence of amino acid variations caused by mutations that modify the activity of genes that mycorrhiza arranges significantly impacts adaptable organisms (Bayer *et al.*, 1996; Shan *et al*., 2002). The phylogenetic tree showed the kinship based on the origin and distribution history of the organisms. The nucleotide and amino acid sequences of *Trichoderma* sp. isolates from Indonesia were in separate branches and were entirely different from isolates from Asia, Australia, and America.

Orchid mycorrhiza and virus inoculation

The findings of orchid mycorrhiza inoculation and virus, performed in vivo, resulted from observing plant growth for one month (Figure 2). The orchid mycorrhiza antagonist test against ORSV showed significant effects by enhancing leaf length and width, root length, leaf count, root volume, and fresh weight and reducing virus concentration. The observed results and analysis of plant growth parameters and viral concentrations revealed significant differences in the treatments inoculated with orchid mycorrhiza (Mycorrhiza [M], Mycorrhiza + Virus [MV], Virus + Mycorrhiza [VM]) and those not inoculated with mycorrhiza orchids (Control [V], Virus [V]).

Increased plant growth in each treatment may refer to orchid mycorrhiza forming a peloton in plant root tissue, thereby increasing the root mass. According to Mahfut (2020b), mycorrhiza hyphae orchids that enter the orchid root network will form clumps as



Fig*ure* 2. Plantlet development as a result of inoculation of orchid mycorrhiza and virus; 0: Week 0 (inoculation start); (2) 2nd week; (4) 4th week; (C) control; (M) Mycorrhiza; (V) Virus; (MV) Mycorrhiza + Virus; (VM) Virus + Mycorrhiza.

pelotons. Inoculation treatment of orchid mycorrhiza on orchids has enhanced the efficiency of nitrogen (N) absorption, boosting plant growth. Elemental N is the building block of amino acids, proteins, enzymes, nucleic acids, nucleoproteins, and alkaloids. The lack of N will also decline cell division and proliferation (Currah and Zelmer, 1992; Tohari *et al*., 2021).

A significant decrease in the average absorption of viral titers in each treatment disclosed that the symbiosis between orchid mycorrhiza and plantlets can reduce viral titers. The presence of a viral infection does not affect the absorption of nutrients in the plantlet because the plantlet has the symbiotic orchid mycorrhiza first. Otero *et al*. (2013) reported that orchid mycorrhiza critically support orchid growth. Mahfut (2021) also explains that the formation of pelotons in root tissue is vital in supplying nutrients for plant growth, and later, plants deliver photosynthetic products in the form of carbon essential for the survival of orchid mycorrhiza.

In vivo antagonist test

The in vivo antagonist test showed that the lowest increase in leaf length was visible in the control device, and those inoculated with viruses emerged with an increase in leaf length with an average of 0.2 ± 0.6 cm (control) and 0.1 ± 0.6 cm (V) (Table 1). It might be due to viral inoculation, which can reduce the ability of plants to grow and develop plants. Plantlets inoculated with orchid mycorrhiza had an average value of leaf length increases, i.e., 0.2 ± 1.0 cm (M), 0.1 ± 1.0 cm (MV), and 0.1 ± 1**.**

The test results further specified that orchid mycorrhiza inoculation in the three treatments had the highest average leaf length increase compared with the others. It indicates that orchid mycorrhiza was able to boost plantlet growth and development. Plants associated with orchid mycorrhiza will have a faster growth rate, as the presence of orchid mycorrhiza helps plants acquire the nutrients necessary for their progress. Tohari *et al*. (2021) mentioned that the symbiosis of mutualism between orchid mycorrhiza and plants occurs through the exchange of nutrients required for their growth. Orchid mycorrhiza supplies nutrients, water, and minerals to plants, while plants provide carbon from photosynthesis from these plants to orchid mycorrhiza.

The observations revealed that an increase in leaf length effectively occurs in the third and fourth weeks (Table 1). The results showed a significant increase in extent among the treatments inoculated by orchid mycorrhiza and those not injected in the third week while in the fourth week. The overall treatment did not differ significantly. The outcomes further indicated that orchid mycorrhiza has a fruitful effect in increasing the length of plant leaves. It may be due to the faster growth of orchid mycorrhiza, making them mix with plants more quickly. The faster orchid mycorrhiza interacts with host plants, the nutrient absorption capacity increases, leading to increased growth. Arifannisa *et al*. (2021) reported that the foremost mechanism of orchid mycorrhiza is to infect the host plant root system and produce hyphae intensively interacting, thus increasing the ability of the plant to absorb more nutrients.

The results of plant leaf width also showed a significant difference in the final mean value of observations on inoculated orchid mycorrhiza with an average value of leaf width increase of 0.1 ± 1.5 cm (M), 0.08 ± 1.2 cm (MV), and 0.1 ± 1.8 cm (VM) (Table 2). The effectiveness of increasing leaf width also occurred in the third and fourth weeks. Minarni *et al*. (2021) explained that in addition to the compatibility between host plants and fungi, the ability of orchid mycorrhiza to increase leaf width also acquires influence from the degree of sporulation and colonization of fungi and environmental factors. The observations also showed that viral inoculation reduces the effectiveness of orchid mycorrhiza in increasing leaf width. Infections and diseases can interfere with plants' physiological activities, resulting in stunted growth (Minh *et al*., 2016). The virus-infected orchids also significantly decreased leaf width (Table 2). It refers to pathogenic viral infections producing toxins and fusaric acid that can interfere with the cell membranes' permeability, thus preventing water movement.

Table 1. Increase in leaf length per week. C: Control, M: Mycorrhiza, V: Virus, MV: Mycorrhiza + Virus, VM: Virus + Mycorrhiza.

Treatments	Average of increased length of leaves per week (cm)				
	0	1	2	3	4
C	0	0.2 ± 0.2a	0.3 ± 0.6a	0.1 ± 0.6a	0.2 ± 0.5a
V	0	0.1 ± 0.3a	0.2 ± 0.5a	0.1 ± 0.6a	0.4 ± 0.4b
M	0	0.2 ± 0.5a	0.3 ± 1.0a	0.2 ± 0.7a	0.5 ± 0.8b
MV	0	0.1 ± 0.3a	0.2 ± 0.6a	0.3 ± 0.5b	0.7 ± 1.0b
VM	0	0.1 ± 0.6a	0.3 ± 0.6b	0.1 ± 0.7a	0.2 ± 1.2a

Note: Numbers followed by the same letter in the same column indicate not significantly different.

Table 2. Increase in leaf width per week. C: Control, M: Mycorrhiza, V: Virus, MV: Mycorrhiza + Virus, VM: Virus + Mycorrhiza.

Treatments	Average of increased leaf width per week (cm)				
	0	1	2	3	4
C	0	0.1 ± 0.2a	0.1 ± 0.5a	0.2 ± 0.5a	0.2 ± 0.6a
V	0	0.07 ± 0.1a	0.03 ± 0.1a	0.1 ± 0.7a	0.09 ± 1.3a
M	0	0.1 ± 0.3a	0.2 ± 0.4a	0.3 ± 1.3b	0.6 ± 1.5b
MV	0	0.08 ± 0.2a	0.1 ± 0.2a	0.2 ± 1.0a	0.3 ± 1.2a
VM	0	0.1 ± 0.5a	0.3 ± 0.6a	0.4 ± 1.8b	0.7 ± 1.5b

Note: Numbers followed by the same letter in the same column indicate not significantly different.

Table 3. Increase in root length per week. C: Control, M: Mycorrhiza, V: Virus, MV: Mycorrhiza + Virus, VM: Virus + Mycorrhiza.

Treatments	Average of increased root length per week (cm)				
	0	1	2	3	4
C	0	0.05 ± 0.1a	0.1 ± 0.6a	0.1 ± 0.3a	0.09 ± 0.1a
V	0	0.03 ± 0.07a	0.06 ± 0.1a	0.06 ± 0.1a	0.05 ± 0.1a
M	0	0.1 ± 0.1a	0.08 ± 0.1a	0.2 ± 0.8a	0.2 ± 1.0a
MV	0	0.04 ± 0.1a	0.06 ± 0.1a	0.5 ± 0.7b	0.4 ± 1.0b
VM	0	0.03 ± 0.06a	0.1 ± 0.2a	0.6 ± 1.3b	0.5 ± 1.1b

Note: Numbers followed by the same letter in the same column indicate not significantly different.

Observations of increased root length showed significant differences in the mean length increase among the treatments inoculated by orchid mycorrhiza and those not injected. The highest increase in root length was evident in orchid mycorrhiza inoculation treatment, with an average length increase of 0.03 ± 1.3 cm (VM), 0.04 ± 1.0 cm (MV), and 0.08 ± 1.0 cm (M). The effectiveness of root length extension also occurred in the third and fourth weeks (Table 3). The investigations on leaf number also significantly differed in the average increase in leaf volume among the treatments inoculated with orchid mycorrhiza and the inoculated. In general, in all the treatments, the number of leaves decreased in the second week of observation. The leaves become rotten as a plant adaptation to orchid mycorrhiza inoculation therapy, either a single orchid mycorrhiza inoculation or equivalent to viral infection.

In the single viral inoculation treatment, the leaves do not decompose. This adaptation process halted at the beginning of the third week, and then the plant experienced a significant increase in leaf number. From the third week, there was a noteworthy increase in the number of leaves in orchid mycorrhiza inoculation treatment, with an increase of six (M), six (MV), and three leaves (VM). The

lowest increase in leaf volume was apparent at one virus inoculation (V) and control, with an increase in leaf count of two (Table 4). Based on data on the leaf volume increase, it is acceptable that orchid mycorrhiza inoculation proved effective in plant leaf growth. Tohari *et al*. (2021) reported that the orchid mycorrhiza inoculation on *Phalaenopsis* can increase the length, width, and number of leaves. This increase in orchid growth was foreseeable because orchid mycorrhiza can enhance nutrient absorption, produce plant growth hormones, and also boost the chlorophyll content.

The observations on root volume showed a significant difference in the average increase in root volume between inoculation and non-inoculation treatments of orchid mycorrhiza. Generally, the number of roots in the entire treatment decreased in the first and second weeks of observation (Table 5). Based on visual observations, the roots and leaves decay as a process of adapting to the injection of the virus and orchid mycorrhiza. This adjustment process stops at the beginning of the third week. Starting from the third week, the highest increase in root volume appeared in orchid mycorrhiza inoculation treatment with a total addition of four (M), four (MV), and two (VM) roots. The lowest increase in root numbers emerged in one viral inoculation (V) and the control treatment. Orchids need mycorrhiza infection to complete their life cycle. The orchid mycorrhiza is crucial in plant growth by enhancing its ability to absorb macro- and micro-nutrients (Izzati *et al*., 2021).

The assessments on the fresh weight of the plants came from the measurements at the beginning and end of the observation; it was due to the condition of the plants needing to be in an aseptic state. The results showed an increase in fresh plant weight between treatments (Table 6). At the start of the observation, it was visible that there were many rotten roots and leaves, probably due to the adaptation of the plant to the inoculation of the virus and orchid mycorrhiza. On average, in each treatment, the highest fresh weight was apparent in small plants treated with orchid mycorrhiza inoculation. It may be because orchid mycorrhiza forms a peloton in the plant root tissue, thus increasing the root mass. According to Mahfut (2021), the hyphae of the orchid mycorrhiza that enters the orchid root network forms a clump called peloton. This peloton mass causes the weight of the roots inoculated with orchid mycorrhiza to be higher than other treatments.

Analysis of leaf phenol compounds

The resistance mechanism in plantlets from induction is increased phenolic compounds in the leaves inoculated by the virus (Table 7). Based on the results, it was well-defined that the plants inoculated with the virus have shown increased levels of total phenolic compounds. Increased phenolic levels authenticated the plant resistance to withstand the rate of ORSV pathogen infection. Minh *et al*. (2016) reported that enhanced phenolic compounds are one of the indications that can increase plant resistance to various pathogens.

The highest phenol content occurred in the treatments of mycorrhiza-inoculated co-viruses, i.e., MV and VM, with values of 0.097059 and 0.087059 ppm, respectively. The highest phenol concentrations were at a single jab of virus (V) and mycorrhiza (M), 0.054706 and 0.037647 ppm, respectively. The lowest phenol content (0.020588 ppm) in plantlets manifested with the control treatment. The results further revealed that in the absence of the first mycorrhiza inoculation, the increase in total phenol content was not too high compared with viral infection before mycorrhiza inoculation. It also proves that raising the total concentration of phenolic compounds in plantlets can increase if the plant has pathogen infections. Evdokimenko *et al*. (2021) also reported an increase in phenol content in *Spathoglottis plicata* caused by *Rhizoctonia* to 36.0 ppm, while those not caused by mycorrhiza were 33.2 ppm, and control plants had only a total phenol content of 9.67 ppm.

Table 4. Increase in the number of leaves per week. C: Control, M: Mycorrhiza, V: Virus, MV: Mycorrhiza + Virus, VM: Virus + Mycorrhiza.

Treatments	Average of increased number of leaves per week (cm)				
	0	1	2	3	4
C	16a	16a	16a	17a	18a
V	9a	9a	10a	11a	12a
M	14a	16a	11a	17a	17a
MV	21b	21b	17a	22b	23b
VM	13a	15a	12a	12a	15a

Note: Numbers followed by the same letter in the same column indicate not significantly different.

Table 5. Increase in the number of roots per week. C: Control, M: Mycorrhiza, V: Virus, MV: Mycorrhiza + Virus, VM: Virus + Mycorrhiza.

Treatments	Average of increased number of roots per week (cm)				
	0	1	2	3	4
C	16a	14a	14a	14a	15a
V	10a	10a	9a	10a	11b
M	14a	14a	12a	10a	14b
MV	26a	27a	28a	22b	26b
VM	11a	12a	13a	13a	15a

Note: Numbers followed by the same letter in the same column indicate not significantly different.

Table 6. Fresh weight gains every week. C: Control, M: Mycorrhiza, V: Virus, MV: Mycorrhiza + Virus, VM: Virus + Mycorrhiza.

Treatments	Average of fresh weight gain per week (cm)				
	0	1	2	3	4
C	0	0.98 ± 1.03b	*	*	1.38 ± 1.4b
V	0	0.45 ± 0.62b	*	*	0.8 ± 2.33b
M	0	0.91 ± 0.96b	*	*	1.63 ± 1.69b
MV	0	3.47 ± 3.67b	*	*	3.23 ± 3.92b
VM	0	0.21 ± 0.27a	*	*	0.52 ± 0.54b

Note: Numbers followed by the same letter in the same column indicate not significantly different. (*): not observed in the second and third weeks due to technical reasons to ensure that the plants do not die/ contaminate.

Table 7. Levels of phenol compounds in leaves treated with inoculation of orchid mycorrhiza and viruses.

Treatments	Concentration	Absorbance	Phenol Mass
Control	0.020588	0.035	0.000411765
Mycorrhiza	0.037647	0.064	0.000752941
Virus	0.054706	0.093	0.001094118
Virus + Mycorrhiza	0.087059	0.148	0.001741176
Mycorrhiza + Virus	0.097059	0.165	0.001941176

CONCLUSIONS

Based on rDNA-ITS, the mycorrhizal isolates on the roots of *P. amabilis* from Yogyakarta, Indonesia, have classification as *Trichoderma* sp. The isolate has a DNA fragment of 600–750 bp at the ITS1–5.8S–ITS4 locus. The Indonesia isolate has undergone speciation, and according to the phylogenetic tree, it has a location far from the others in the cluster. The

mycorrhizal antagonist test against ORSV significantly affected growth traits, such as, leaf length and breadth, root length, number of leaves, root volume, and fresh weight. The observed plant growth parameters and virus concentrations were significantly different among the treatments that contained orchid mycorrhiza (Mycorrhiza [M], Mycorrhiza + Virus [MV], and Virus + Mycorrhiza [VM]) and treatments without (Control [C] and Viruses [V]). Increased total phenolic compounds also imply the plant's resistance to pathogens.

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