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THE GROWTH RESPONSES OF ORCHID, *Phalaenopsis* HYBRID TO THE INOCULATION OF *Rhizoctonia solani*

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ABSTRACT

The orchid mycorrhizal association is a symbiotic interaction in which both plants and fungi are mutually beneficial. Mycorrhizal association increases plant access to soil nutrients and is especially important for orchid seed germination. Most orchid mycorrhizal fungi belong to the group *Rhizoctonia,* including *Rhizoctonia solani*, a well-known plant pathogen that affects major crop production worldwide but has also been reported to be an orchid mycorrhiza. However, no systematic study has been done to investigate the type of interaction that develops when pathogenic *R. solani* interact with an orchid; whether a pathogen can alter its association to become mycorrhizal. Thus, this study was conducted to determine the type of interaction between *Phalaenopsis* hybrid and *R. solani* and its effect on the growth and chlorophyll content of *Phalaenopsis* hybrid. Sterile *ex vitro Phalaenopsis* plantlets were inoculated with 4 discs (0.5 cm diameter) of *R. solani*, isolated from diseased rice tissues, and incubated for 9 days. The inoculated root segments were used to observe the fungal hyphae in the root cell. The growth parameters (plant height, leaves length and width, the number of leaves, fresh and dry weights) and chlorophyll content were determined at 0, 3, 6, 7, 8, and 9 days of inoculation. It was observed that no peloton was present in the root cells of all inoculated plants. Severe reduction of growth and chlorophyll content were obtained in inoculated plants compared to control especially after 7 days of inoculation. These results suggested that *R. solani* developed a pathogenic infection to *Phalaenopsis* hybrid as no peloton structure was present in the root cells. *R. solani* infection also reduces the *Phalaenopsis* hybrids growth as well as declined its chlorophyll content.

Key words: Chlorophyll content, growth, mycorrhizae, *Rhizoctonia solani*, *Phalaenopsis* hybrid

INTRODUCTION

The Orchidaceae is the largest family in the flowering plant which includes about 30,000–35,000 species over 700 genera encompassing around 10% of flowering plant species. Nevertheless, there are still hundreds of new species displaying variable floral features, lifestyles, habitat distributions, and trophic patterns that are being discovered and progressed every year (Hossain *et al*., 2013; Herrera *et al*., 2017). For many years, orchid cultivation has been developed from a hobbyist market that is becoming a highly commercial market. Despite the diversity of orchids, only a handful of genera are grown in large quantities as a commercial market such as *Cymbidium, Dendrobium, Oncidium,* and *Phalaenopsis* (Moudi *et al*., 2013). In Malaysia, commercial production of orchids and hybrids is increasing and gives a huge contribution to the economy. It was reported that from the year 2015 to 2017, the export of orchids has augmented from RM11.9 million to RM13.2 million, respectively. The orchids are marketable as ornamental plants especially for cutting flowers in Singapore, Japan, Australia, and Greece (Shabariah *et al*., 2017).

Orchids have several unique characteristics correlated to their reduced size of the embryo and the absence of endosperm which causes difficulty in germination (Moudi *et al*., 2013). Various strategies have been made to induce higher germination rates of orchid seeds. The most successful method is using symbiotic mycorrhizal fungi as well as modulating the factors for germination including nutrients, photoperiod, temperature, and plant hormones (Yamazaki & Miyoshi, 2007; Yeh *et al*., 2019). The orchid-mycorrhizal association is a symbiotic type that is essential for one or both partners, between fungi and

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the roots of a living plant, which is responsible for nutrients transfer (Brundrett *et al*., 2004). Mycorrhizae improve the plant's access to soil resources such as mineral ions and water and help the orchids to survive in an inactive state for years (Parthibhan *et al*., 2017). The interaction begins when the mycorrhizal fungi infect basal cells in the embryo during seed germination. The orchid digested the hyphal to obtain carbon and nutrients necessary for its development. The protocorm (heterotrophic structure) then forms the seedling. The seedling produces its first root and the fungal hyphae grow into host tissues and form specific coiled structures called pelotons within the cortical cells. From this phase, symbiosis with mycorrhizal fungi facilitates the acquisition of nutrients from the substrate (Peterson *et al*., 2004; Rasmussen & Rasmussen, 2009; Dearnaley *et al*., 2012).

Rhizoctonia solani is the most extensively studied among *Rhizoctonia* species. They are widely distributed in both farmland and forests. Most of the species are pathogenic to various crops, including agronomical, ornamental, and forestry species, thus causes great losses to the yield (Agarwai *et al*., 2010). Symptoms of the disease caused by *R. solani* in different host plants include leaf blights, cankers lesions on sprouts, rots on roots, leaf spots, damping off, shoots and fruits, stolons, and sclerotial diseases. Remarkably, despite being pathogenic to the valuable crops, especially rice, some strains of *R. solani* were also reported to form symbiotic mycorrhizal relationships with orchid plants (Mosquera-Espinosa *et al*., 2013; Abdeljalil *et al*., 2016). Hence, it is necessary to determine whether the same fungal species but isolated from pathogenic sources can form this mycorrhizal association or remain pathogenic in the *Phalaenopsis* hybrid.

Losing chlorophyll content leading to necrotic lesions are frequently observed on infected leaves as an early plant response during the plant-pathogen infection. The necrotic lesions develop gradually from small green watersoaked lesions during an early infection phase and then rapidly enlarged, causing destruction and cell death (Borges *et al*., 2013; Aung *et al*., 2018). The ability of plants to withstand the pathogenic interaction is very important for plant survival. Pathogen activity resulting in damage to photosynthetic machinery, loss of photosynthetic tissue, and vasculature disorders that affect water and sugar transport, have adverse effects on photosynthesis, thus reducing its growth (Mitra & Baldwin *et al*., 2014). Therefore, the purpose of this study is to determine the type of interaction between *Phalaenopsis* hybrid and *R. solani* and its effect on the physical appearance of *Phalaenopsis* hybrid. Moreover, the growth and chlorophyll content of the *Phalaenopsis* hybrid were also assessed.

MATERIALS AND METHODS

Plant materials

Cultures of *Phalaenopsis* hybrid (five-month-old) were chosen as the model plants (Figure 1). The plantlets were purchased from *Phalaenopsis* Waltex Biotech Sdn. Bhd. Ulu Yam, Selangor. The plantlets were transferred into 12 cm diameter pots filled with 3 g mosses and were grown in a moisture chamber for 2 weeks. Plantlets were then relocated to the greenhouse where they were acclimatized under normal daylight for 2 weeks under a plastic shaft and wetted once daily. Three replications of control and infected plants for each treatment were put on separate covered shelves. The experiments were carried out for 9 days. The growth and chlorophyll content were determined at 0, 3, 6, 7, 8, and 9 days of the experimental period. The experiments were conducted using a Randomized Complete Block Design (RCBD).

Rhizoctonia solani isolation

Rhizoctonia solani were obtained from diseased rice tissues from paddy fields and was isolated at Faculty of Science and Marine Environment (FSME), Universiti Malaysia Terengganu (UMT). Segments of diseased tissues were collected and washed thoroughly under running tap water. The tissues were cut (1 cm) then surface sterilized with 70% ethanol for 1 min followed by soaking in 25% Clorox for 2 min. Then, the tissues were rinsed with sterile distilled water 2 to 3 times. The sterilized tissues were then cultured onto potato dextrose agar (PDA) media and were incubated at 27 ± 2°C. After 3 days of incubation, the fungi were examined under a microscope and the identification was confirmed based on cultural characteristics such as an absence of conidia, right angle hyphae, and septation in hyphal branches, mycelia contain brown shades and presence of small dark brown round shaped sclerotia (Ogoshi *et al*., 1975). The identified isolate was sub-cultured onto fresh PDA media to obtain a pure culture. The pure cultures of

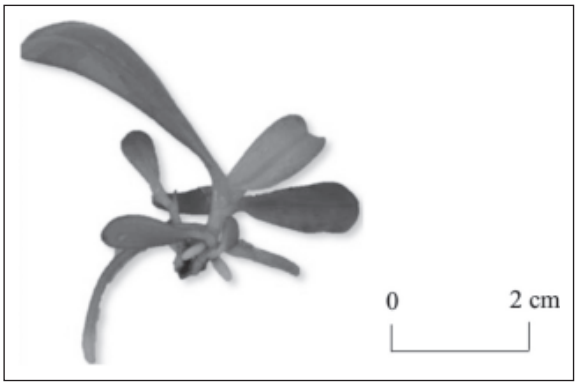

Fig. 1. *Phalaenopsis* hybrid.

R. solani were maintained on PDA and were incubated at 27 ± 2°C (2 to 3 days) in the Microbiology Laboratory, FSME, UMT.

***Rhizoctonia solani* inoculation**

After 2 weeks of transplantation, the moss at the base of orchid plantlets was pushed aside to uncover the portion of the root. The plantlets were inoculated with 4 discs of *R. solani* mycelia (0.5 cm diameter each) which were placed near to the root at 1 cm from the stem. Roots were covered by moss after inoculum (Yao *et al*., 2002). The plant morphological changes, peloton structure observation as well as growth, and chlorophyll content were assessed at 0, 3, 6, 7, 8, and 9 days.

Screening for peloton structure

Sections of the infected and control root segments (1 cm) were randomly sampled for evaluation of *R. solani* infection. The roots transverse sections were made manually using a sterile razor blade (Gillete, India) and the sections were stained with lactofuchsin (Larone, 1995). The stained roots were examined under a microscope (100×) for the presence of peloton structure. The fungal hyphae form coiled around the plant root cells indicated the formation of peloton structure.

Growth parameters

The plant height leaves length and width of all leaves were measured using a 15 cm ruler. The fresh weights were obtained using an analytical balance. Plants were dried to a constant weight in an oven at 80°C for dry weight measurements. All the growth parameters were measured in inoculated plants as well as their respective control.

Determination of chlorophyll content

Chlorophyll content was determined based on the method by Harbone (1984). Approximately 0.15 g fresh leaf tissues were ground up with 6.0 mL of 80% acetone at 0-4°C in pre-chilled mortar and pestle. The mixture was centrifuged at 10 000 rpm (Eppendorf 5840R) for 10 minutes at 0-4°C. The supernatant obtained was read at 663 nm and 645 nm using a spectrophotometer (Shimadzu UV-1601). The total chlorophyll content was calculated using the formula:

$$\text{Total chlorophyll} = \frac{20.4\ A645 + 8.67\ A633 \times \text{acetone volume (mL)}}{\alpha \times 1000\ \text{mL} \times \text{leaves weight (g)}}$$

Where α=1.0.

Statistical analysis

Data obtained were analyzed using GraphPad Prism 5 software. The comparison of growth and chlorophyll content of *Phalaenopsis* hybrid were tested using two-way ANOVA at α=0.05 as a significant level. The data were expressed as means ± standard error.

RESULTS AND DISCUSSION

The changes in the morphological characteristic of the *Phalaenopsis* hybrid

The first symptoms of the infection begin with a small spot on leaves (Figure 2A) which can expand to large circular spots with concentric rings accompanied by an irregular emergence of plants. This was followed by wilting and yellowing of leaves especially after 3 days of inoculation (Figure 2B). The roots became rot and change to brown at the end of the inoculation period (Figure 2D). After 6 days of inoculation, the leaves of the *Phalaenopsis* hybrid showing shriveled, twisted, and thin (Figure 2C) and slowly die at 9 days of inoculation period. These results were consistent with the results obtained by Sweet and Uchida (2015) on the interaction of *Fusarium* spp. and *Dendrobium* orchid cultivar UH 800. They found that *Fusarium* infection usually causes leaf spots, leaf blights, or leaf rots of *Dendrobium* orchid. Leaves are infected when young, and the extremity of the disease is based on the age and humidity of the shoots. Foliar symptoms started with chlorotic 2-5 mm circular leaf spots that lead into necrotic small and sunken brown to blackish brown leaf spots. In severe infections, these spots can be fused to form blights that sometimes kill the shoot. It was also observed that the young shoots of *Phalaenopsis* hybrid were easily affected and may decay if infection occurs at shoot emergence. These symptoms can be caused by the production of toxic metabolites and associated with toxins that cause pathogenicity in the plants. The results of the study showed that toxins lead to the cell membrane and cytoplasm rupture (Geoffrey & Kerstin, 2017). Thus, new growth is destroyed by *R. solani* and multiple plant deaths can cause severe losses.

Type of interaction between *R. solani* with *Phalaenopsis* hybrid

In this study, the first step in understanding the interactions, either mutualistic or antagonistic is the identification of the peloton structure in the root cell after inoculation with *R. solani*. Observation on the infected orchid roots showed that there was no

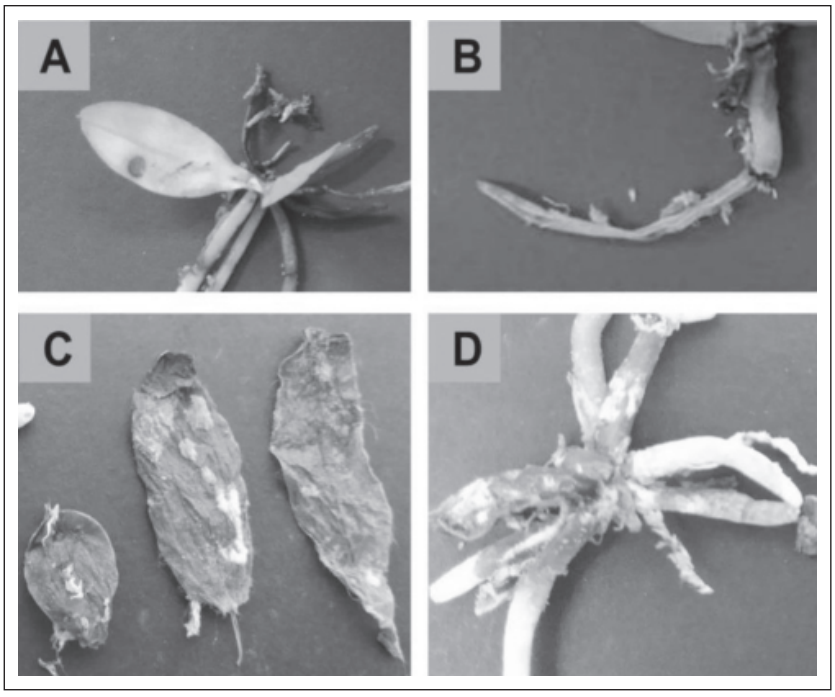

Fig. 2. Symptoms of Interaction between *R. solani* and *Phalaenopsis* hybrid: A) Leaves showing yellowish, leaf spots, and leaf blight. B) Root rot. C) Leaves showing shriveled, twisted, and thin. D) Brown roots.

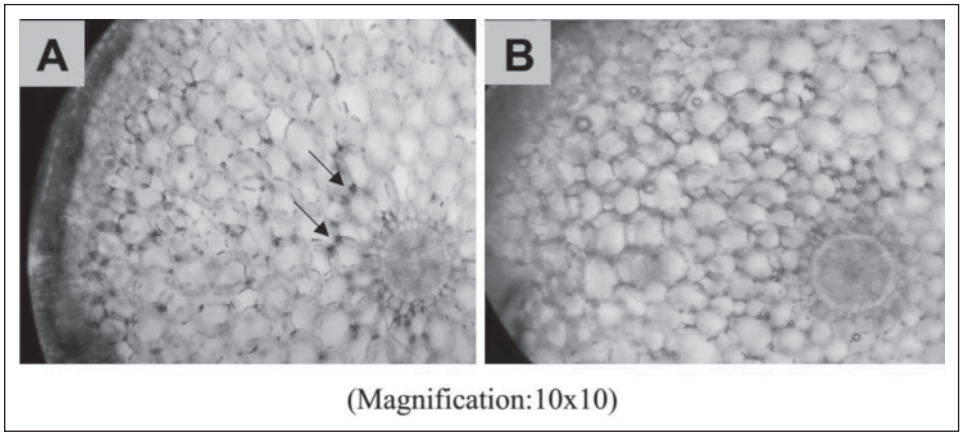

Fig. 3. Root cells of *Phalaenopsis* hybrid inoculated with *R. solani* (arrows point to *R. solani* compact infection cushions) (A) and control (B).

peloton formed in the root cells of *Phalaenopsis*, thus indicating that the interaction is pathogenic or there is no mycorrhizal symbiosis between *Phalaenopsis* hybrid roots with *R. solani* (Figure 3). The orchid-mycorrhizal association is a mutualistic symbiosis where both plants and fungi get benefit from each other (Abdeljalil *et al*., 2016). The typical indicator of an orchid mycorrhizal interaction is the presence of the structure called the peloton, which is hyphae masses of tightly interwoven coils, of which the formation is influenced by the type of orchid and orchid's habitat.

Unfortunately, the inoculated *Phalaenopsis* roots in this study were accompanied by the occurrence of the pathogen infection cushions in the root tissues (Figure 3A). Such an incident was also observed in root pathogens such as *F. oxysporum* as an initial event for the invasion of plant tissues (Mendgen *et al*., 1996). Infection cushions produced in this study enable the *R. solani* vegetative hyphae to penetrate different types of plant cell walls. The morphogenetic events leading to the formation of the infection structure often depend on specific signals provided by the plant surface and are

prerequisites for a mode of penetration. Physiological changes such as targeted secretion of enzymes or an increase of pressure within the infection structure support the penetration process. Following this, the cell membrane disappeared, mitochondria and cell wall were no longer smooth, the shapes of chloroplast changed, and the endoplasmic reticulum swelled. Also, complete disruption was observed in the epidermal cell resulting in decay and degradation of the primary cell wall (Abdeljalil *et al*., 2016).

Rhizoctonia-like fungi have been previously reported as orchid mycorrhiza of the epiphytic orchids, *Appendiculla* sp., *Calanthe vestita,* and *Bulbophyllum beccarii* (Suryantini *et al*., 2015). In contrast, some of the epiphytic orchids were also found to be pathogenic in *Cattleya skinneri, C. aurantiaca,* and *Brassavola nodosa* with *Rhizoctonia* fungus (Ovando *et al*., 2005) as observed in this study. No peloton structures spotted in this study might be related to the fungi toxic phytoalexins, cell wall-degrading enzymes, and defense mechanism activation during plant-pathogen interaction, therefore control or limit colonization of fungal (Quoc & Chau *et al*., 2017).

Effect of *Rhizoctonia solani* inoculation on the growth of *Phalaenopsis* hybrid

Rhizoctonia solani infection did not significantly affect (p>0.05) plant growth parameters at earlier stages of experimental periods except for plant height. Fresh and dry weights of *Phalaenopsis* were unaffected at 0 to 7 days of experiments; however, the fresh and dry weights of control plants were significantly higher (p<0.05) compared with inoculated plants after 7 days of experiments (Figures 4 & 5). Daami-Remadi *et al.* (2008) also reported similar observations when a significant reduction in fresh and dry weights of potato, *Solanum tuberosum* infection with *R. solani*. This may be related due to starch translocation and nutrient movement from leaves to roots leading to these reduce yields. The decrease in the dry matter content of the plant in this study indicates that less biomass was transported into the generative organs during the growth period of the plants (Lemoine *et al*., 2013). All these features were promptly related to the photosynthesis rates of the plants. A previous report by Debona *et al*. (2014) revealed that reductions in photosynthesis may be proportional to decreases in yield of wheat plants infected with *Pyricularia oryzae*. Fungal infection can also affect the net carbon assimilation rate by increasing leaf respiration, which is necessary for supplying the demand caused by the accelerated metabolic activity of the host cells (Lucas *et al*., 1998).

Figures 6 & 7 illustrate the changes in leaf width and leaf length of *Phalaenopsis* hybrid inoculated with *R. solani* and its respective controls. The leaf width and leaf length of *Phalaenopsis* hybrid were not significantly affected (p>0.05) by *R. solani* inoculation throughout 6 and 7 days of inoculation, respectively. The leaf width of control plants increased significantly (p<0.05) after 6 days of inoculation periods whereas the inoculated leaves slowly decreased. Alternatively, leaf length was remarkably decreased in inoculated plants compared with control. The results obtained in this study were parallel with the research by Sandras *et al*. (2010). They found that the fungus *Verticillium dahliae* significantly reduces the total photosynthetic leaf area on the sunflower plant at 50 days after inoculation. It was postulated that during the infection process, the plant tries to cope with the situation by decreasing its leaf area because of the high energy costs and nutrient requirements associated with their production and maintenance (Geoffrey & Kerstin, 2017).

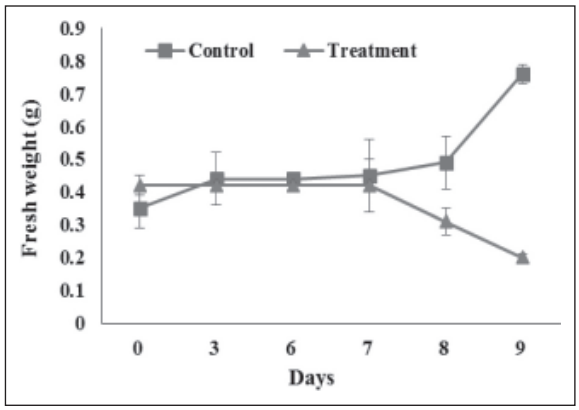

Fig. 4. Changes in fresh weight (g) of *Phalaenopsis* hybrids inoculated with *R. solani* and control. Data are means ± standard errors (n=3).

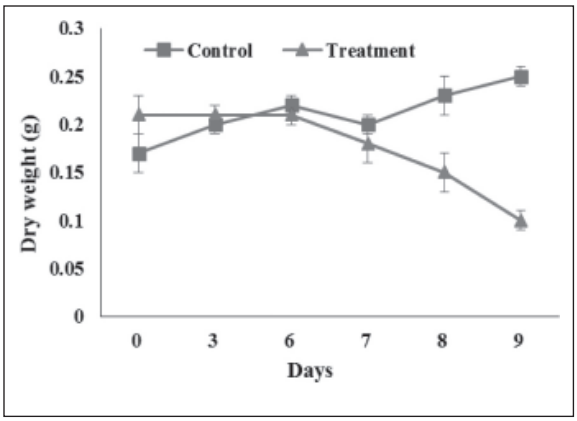

Fig. 5. Changes in dry weight (g) of *Phalaenopsis* hybrids inoculated with *R. solani* and control. Data are means ± standard errors (n=3).

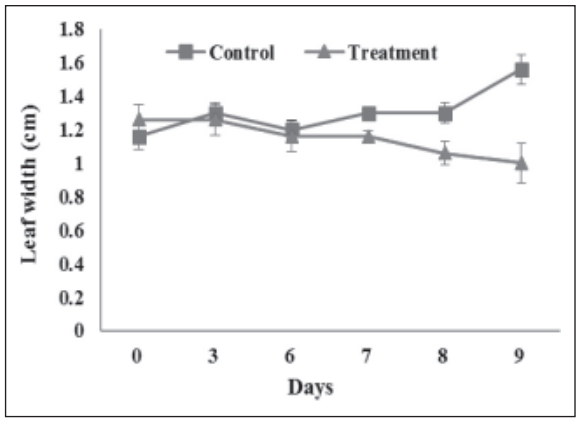

Fig. 6. Changes in leaf width (cm) of *Phalaenopsis* hybrids inoculated with *R. solani* and control. Data are means ± standard errors (*n*=3).

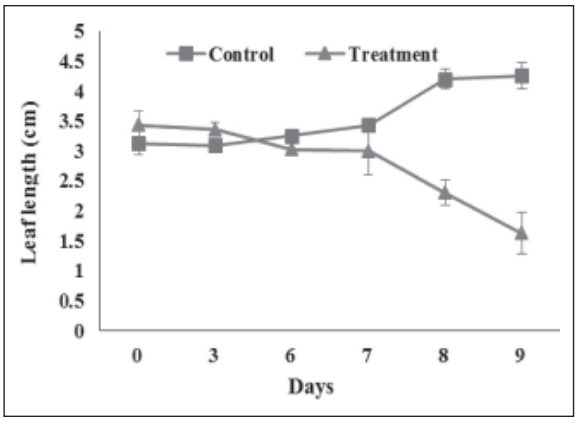

Fig. 7. Changes in leaf length (cm) of *Phalaenopsis* hybrids inoculated with *R. solani* and control. Data are means ± standard errors (*n*=3).

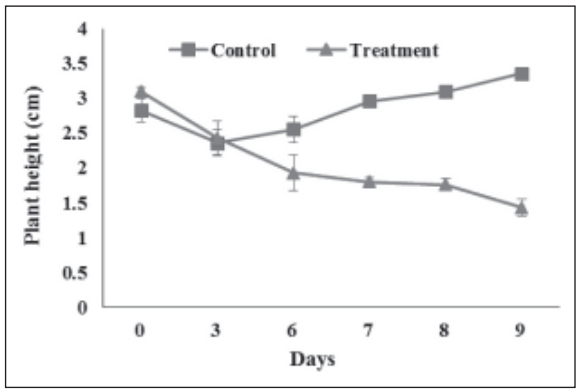

Fig. 8. Changes in plant height (cm) of *Phalaenopsis* hybrids inoculated with *R. solani* and control. Data are means ± standard errors (*n*=3).

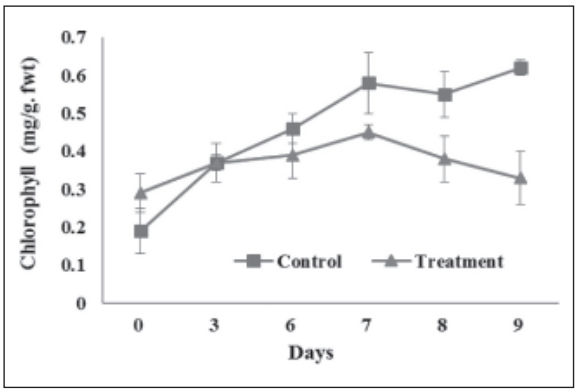

Fig. 9. Changes in chlorophyll content (mg/g.fwt) of *Phalaenopsis* hybrids inoculated with *R. solani* and control. Data are means ± standard errors (*n*=3).

The results of plant height in this study (Figure 8) were in agreement with the research by Taheri and Tarighi (2011), who reported that *R. solani* infection significantly reduces the height of tomato plants. This may owe to a pathogenic fungus infection that leads to a reduction of shoot growth would inhibit cell division and expansion. Besides, the reduction of plant height might as well related to some phenolic and glucosidic phytotoxic substances produced by the pathogen and involved in the inhibition of plant shoot growth. Consequently, the above symptoms in the infected plants including low plant growth, leaf area, and dry matter accumulation, followed by tissue wilting, and finally plant death might be due to the energy demands of the host-parasite interaction (Chávez-Arias *et al*., 2019).

Effect of *Rhizoctonia solani* inoculation on chlorophyll content of *Phalaenopsis* hybrid

The chlorophyll content in control plants was constant at the earlier stage of inoculation and slowly increase at the later stage of the experiment. A different pattern of responses was observed in inoculated plants, where the chlorophyll content was significantly depleted ($p<0.05$) after 7 days (Figure 9). These results suggest that plants switch off photosynthesis and another assimilatory metabolism to initiate respiration coupled with other processes required for defense (Berger *et al*., 2007). A decline in 9% leaf photosynthesis was also spotted in sunflower hybrid after 66 days of inoculation with *V. dahliae* (Sandras *et al*., 2010). Also, Berger *et al*. (2007) described similar results in *Phaseolus vulgaris* plant infected by *Colletotrichum lindemuthianum* race 23. A low leaf chlorophyll content in this study may be because *R. solani* can produce a pathogenicity factor, which alters the plant's metabolism and favors leaf photosynthetic pigments degradation as observed in *Physalis Peruviana* L. against *F. oxysporum* (Wu *et al*., 2008).

CONCLUSION

The results of this study indicated that the type of interaction between the *Phalaenopsis* hybrid with *R. solani* is pathogenic. *Rhizoctonia solani* manages to inhibit *Phalaenopsis* hybrids growth by reducing its fresh and dry weights as well as decrease the leaf area and plant height. The decrease in chlorophyll content was subsequently reduced the dry biomass. It is interesting to explore whether *R. solani* can play roles as mycorrhiza at different growth stages of *Phalaenopsis* hybrid especially during seed germination, protocorm emergence as well as a young seedling.

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