

Growth responses, enzyme activities, and component changes as influenced by *Rhizoctonia* Orchid mycorrhiza on *Anoectochilus formosanus* Hayata

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ABSTRACT. *Rhizoctonia* spp. of binucleate R02 and multi-nucleate R04 (*Rhizoctonia solani*, AG-6) of orchid mycorrhizal fungi (OMF)—collected and isolated from terrestrial orchid roots in Taiwan, including *Anoectochilus formosanus* Hayata, an orchid native to Taiwan—enhanced the growth of *A. formosanus* plants to a high degree, both *in vitro* and *ex vitro*. OMF (R02 or R04) inoculation used oatmeal agar (OMA) *in vitro*, in which mycorrhizal seedlings were larger than non-mycorrhizal controls on Hyponex # 3 agar medium. Both *Rhizoctonia* isolates (R02, R04) inoculated alone or mixed could significantly enhance plant grown *ex vitro* in terms of plant height, leaf number, root length, and fresh weight. Light and scanning electron microscopy showed that the infection of OMF on *A. formosanus* was a type of tolypophagy, with hyphal coils and pelotons (mycelial masses) formed in the cortical region of the roots. Mycorrhizal plants showed higher enzyme activities (superoxide dismutase; SOD in leaf; acid and alkaline phosphatases in roots) and markedly higher constituent contents, like ascorbic acid, polyphenols, and flavonoids, which made the mycorrhizal plants a better source of antioxidants, and of polysaccharides and phosphates indicating a more potent medicinal value. Mycorrhizal plants were also susceptible to diseases and mites, so a plastic bag cultivation method was applied and showed itself to be a very effective and labor saving way to grow fungicide- and pesticide-free *A. formosanus* plants.

Keywords: Inoculation; Pelotons; Plastic bag cultivation method; *Rhizoctonia* spp.; Tolypophagy.

Abbreviations: OMF, orchid mycorrhizal fungi; **R02**, *Rhizoctonia* sp.; **R04**, *Rhizoctonia solani*; **AG-6**, Anas-tomosis group 6.

INTRODUCTION

Anoectochilus formosanus (Orchidaceae) is a native perennial and terrestrial orchid plant grown in the forests of Taiwan. It is an orchid with beautiful netted-veins on the upper surface and purple-red color on the lower leaf surface. It has come to be known as a jewel orchid (Teuscher, 1978) and is regarded as the “king medicine” by the aboriginal population of Taiwan. It has been used to cure diseases, such as cancer, high blood pressure, diabetes, snake bite, and even liver, heart and lung diseases (Yen et al., 1996). It has been also used as a special feed for doves to prolong their stamina during long distance races (Shiau et al., 1995; Liu et al., 1987). In recent years, wild *Anoectochilus* plants have been intensively collected and have thus become scarce, resulting in a market value as high as 3-4 times the price of greenhouse cultivated plants. For the profit in the medicine market, many florists

have started to grow them on a large scale by using micropropagated seedlings (Shiau et al., 1995; Tang et al., 1996). Now, in 2006, the greenhouse-cultivated products are still around 120 USD/kg on a fresh weight basis. This is mainly due to the fact that the plants are highly susceptible to *Fusarium*, *Pythium* spp, and mites. Also the growing of this orchid has to be restricted to those areas with temperatures around 20°C or lower. Additionally, fungicides and pesticides must be applied intensively to assure good growth. Since this plant is used as an herb, minimizing the use of chemicals has been of great importance.

The germination and growth of this orchid could be stimulated by the inoculation of specific isolates of OMF (R01, R02 or R04) (Tsai, 1997; Chou and Chang, 2004). Differences in enzyme activities and in some component contents between the non-mycorrhizal control and the mycorrhizal plants of this orchid are lacking in the literature and were thus presented in this study. Our goal was to evaluate the benefits of using OMF in the commercial production of this orchid.

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MATERIALS AND METHODS

Usually 20 to 60 plants were grown for each experiment to assure that at least 12 replicates for each of the following treatments could be performed.

Inoculum of Orchid Mycorrhizal Fungi

Orchid mycorrhizal fungi (OMF) of R02 and R04 grown in crushed peatmoss of *Rhizoctonia* spp. were used as the so-called solid form of inoculum (Chang and Chou, 2001; Chou and Chang, 2004). R02 was binucleate and R04 (*Rhizoctonia solani*, Anastomosis group 6; Lee, 2001a) was multinucleate *Rhizoctonia* sp. About 0.2-0.3 g of fresh solid form inoculum was applied under the roots of seedlings *ex vitro* on Sunshine growth medium, which was a peat mixture.

Light microscopy and SEM observation of orchid mycorrhiza

Root segments, surrounded by mycelia, were selected for mycorrhizal samples. After being stained with 0.05% of aniline blue in lactoglycerin for 15-20 min, they were examined by light microscopy (Koske and Gemma, 1989). For SEM observation, roots were collected and sterilized, cut and fixed by 3% glutaraldehyde for 1-2 days, then were dehydrated with acetone series, and critical point dried (CPD) by liquid carbon dioxide. Finally, the root segments were coated with gold for 90 s by an Biorad Ion Coater (Dawes, 1971). The SEM samples were examined by ABT-60 of SEM and digital images were recorded.

Growth of seedlings *in vitro* and *ex-vitro*

For symbiotic germination, seeds were collected in a laminar flow hood and sprinkled on the following growth media (Warcup, 1973): *In vitro* symbiotic growth seedlings were cultivated on oatmeal agar (OMA; 2.5 g l⁻¹ of oat meal and 11.5 g Difco agar). No-symbiotic growth seedlings were on: 1. Hyponex # 3 agar, containing 3 g of Hyponex # 3, 3 g tryptone, 30 g sucrose, and 8 g agar (modified from Kano, 1968); 2. WM medium (including 0.3 g NaNO₃, 0.2 g KH₂PO₄, 0.1 g MgSO₄·7H₂O, 0.1 g KCl, 10 g cellulose powder, 0.1 g malt extract and 8 g Sigma agar in 1 liter); and 3. WY medium (including 0.3 g NaNO₃, 0.2 g KHPO₄, 0.1 g MgSO₄·7H₂O, 0.1 g KCl, 10 g cellulose powder, 0.1 g yeast extract, and 8 g Sigma agar in 1 liter). One week after being sown, seeds were inoculated with OMF (R02 or R04 isolate). Only one isolate was inoculated for each treatment. Growth responses of mycorrhizal and non-mycorrhizal seedlings were compared 6 months after inoculation *in vitro*.

Ex vitro growth of seedlings were cultivated on Sunshine growth medium for 4 months in a greenhouse at Puli in central Taiwan. *Rhizoctonia* spp. of OMF, namely R02, R04 and a mixture of R02+R04 were used for the inoculation of OMF.

A plastic bag cultivation method for *A. formosanus* (Lee, 2001a) was applied to the cultivation of fungicide

and pesticide-free plants. By this method, polyethylene or polypropylene plastic bags were used to cover the container with the *ex vitro* grown *A. formosanus* plants as protection against diseases and mites. Field capacity water (i.e. squeezing the growth medium, yielded only a few drops of water) and slow-release fertilizer were applied to the growth medium. The plastic bag was then sealed with a paper clip.

Estimation of enzyme activities

For the activity of superoxide dismutase (SOD), the method of Hung (2002) was used. Sigma enzyme kits for both acid and alkaline phosphatases were obtained, and methods described in the Sigma technical bulletins # 386 (for acid phosphatase) and # 85 (for alkaline phosphatase) were applied. Naphthol AS-BI phosphoric acid and naphthol AS-BI phosphoric alkaline were used as the substrates for both enzymes, respectively.

Estimation of several component contents

Ascorbic acid, nitrate (Cheng, 1997) and phosphate were measured by a Merck, RQ flex spectrophotometer. Also flavonoids (Chang, 2000), polyphenols (Kujala et al., 2000) and polysaccharides (Lee, 2001b) were estimated and compared between non-mycorrhizal and mycorrhizal tissues in root, stem, and leaves.

RESULTS AND DISCUSSION

The morphology and flower of *A. formosanus* is shown in Figure 1A & B. This orchid has netted veins on the upper surface and red surfaces on its leaves and stems. The OMF inocula, namely R02 and R04, were *Rhizoctonia* spp. (Tsai, 1997), isolated from wild grown terrestrial orchid roots, including *A. formosanus* of Taiwan. The chosen isolates R02 and R04 enhanced the growth of this orchid more than R01 (Chou and Chang, 2004). Thus, R02 and R04 inocula were used for subsequent symbiotic germination and growth responses.

Light microscopy showed that the hyphae of OMF could infect the orchid roots either by root hairs or through root epidermal cells (Figure 1C) and formed pelotons in the cortical region of the roots (Figure 1D). SEM observation showed that typical orchid mycorrhiza were formed in the cortex region of *A. formosanus* roots (Figure 2). In mycorrhizal root, hypha could penetrate the cell wall (Figure 2A); also noted was the presence of young and old pelotons in the cortex cells of the roots (Figure 2B-D), indicating that it was a tolypophagy-type infection, in which definite layers of host cells and digestion cells occurred. This is a common relationship characteristic of the majority of orchids (Hadley, 1982). It should be noted that there were connections between the pelotons in the adjacent cells across cell walls (Figure 2C). The size of the connections showed that they were hyphae (H).

Growth of plantlets in either Murasgige & Skoog (MS) medium or other common micropropagated media would

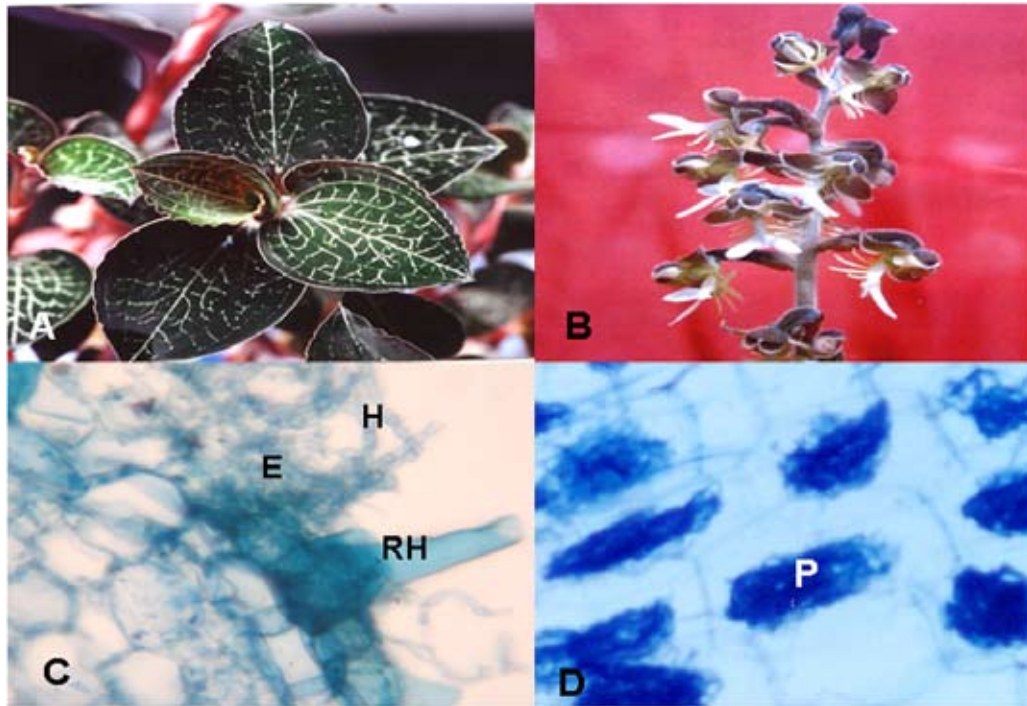


Figure 1. Morphology (A) and flower (B) of *Anoectochilus formosanus* Hayata. The *Rhizoctonia* hyphae (H) of orchid mycorrhizal fungi (C) could infect either through root hair (RH) or root epidermal cells (E) and then formed pelotons (P) in cortex (D) of root.

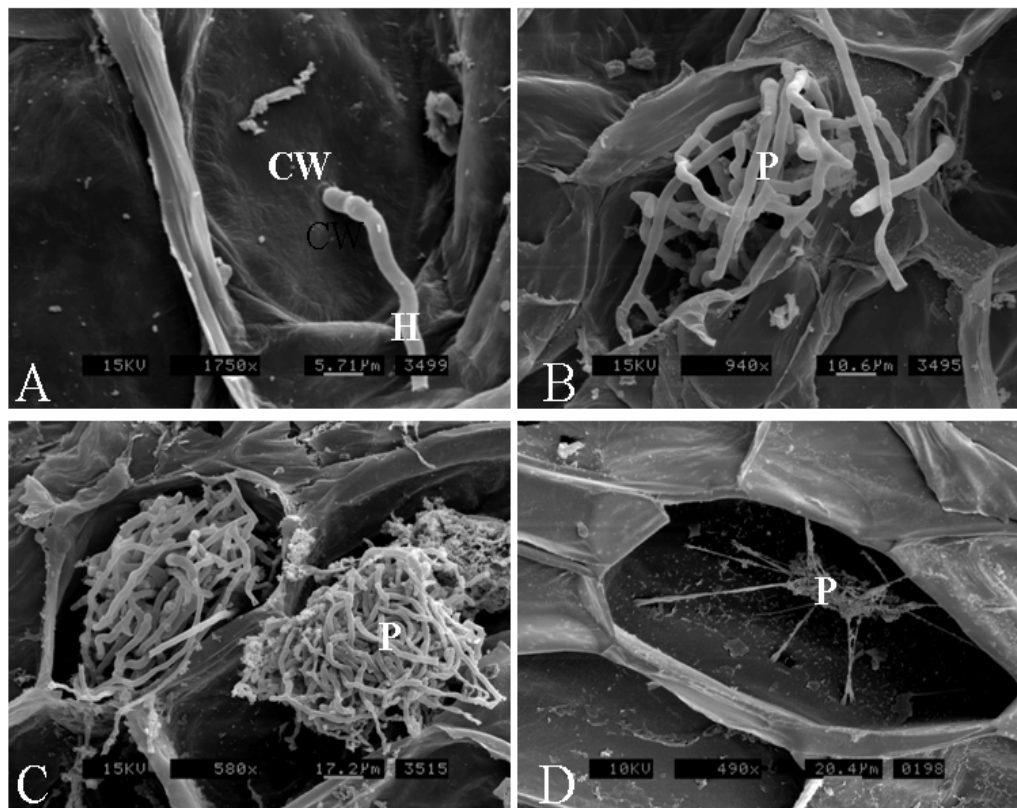


Figure 2. Orchid mycorrhizal development processes as revealed by scanning electron microscopy. Note the hypha (H) could pass through cell wall (CW) in A and form pelotons (P) in B-D in the cortex cells of orchid roots. Later the peloton in D would be digested.

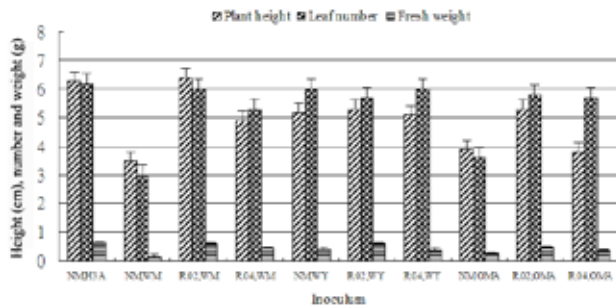


Figure 3. Growths of *Anoectochilus formosanus* Hayata in H3A, WM, WY and OMA media of non-mycorrhizal control (NM) and inoculated with orchid mycorrhizal fungi (R02 or R04) for 6 months *in vitro*.

cause the overgrowth of the OMF hyphae, and result in the death of seedlings or plantlets. Thus, OMA was chosen for the growth of mycorrhizal plantlets *in vitro* (Chou, 1997, 2004) while the growth of non-mycorrhizal plantlets was better in Hyponex # 3 agar (H3A) medium (Figure 3; Chou and Chang, 2004). After inoculation with *Rhizoctonia* sp. isolate and 4 months of *ex vitro* growth, results showed that both isolates (R02 and R04) could enhance plant height, number of leaves, root length, and fresh weight (Figure 4). The mixed inoculum of R02+R04 showed higher growth enhancement for strains L1 and P of *A. formosanus* than the single kind of inoculum (R02 or R04). Thus the mixed inoculum of *Rhizoctonia* spp. could be considered for practical use. The inoculation of R02 or R04 for plantlets *ex vitro*, was tested for more than 10,000

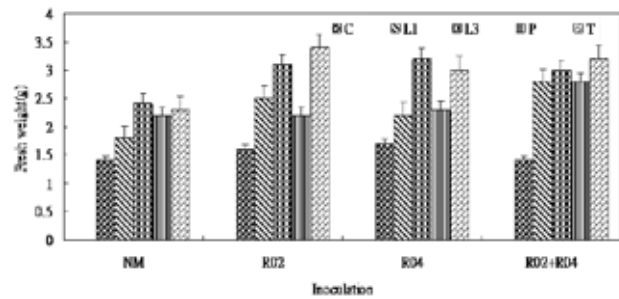


Figure 4. Growth of five *Anoectochilus formosanus* Hayata lines (C, L1, L3, P & T) in greenhouse as cultivated in plastic bag and inoculated with *Rhizoctonia* sp. of orchid mycorrhizal fungi (R02, R04) for 4 months *ex vitro*.

plantlets both in Taipei and at Puli (central Taiwan), and all showed enhanced growth. Many growers asked for a continuous supply of OMF inoculum in their commercial production of *A. formosanus*. Thus R02 or R04 isolates are recommended for such production of *A. formosanus ex vitro*. A commercial supply of pathogen-free OMF inoculum would become a practical trend in the near future. Also the plastic bag cultivation method had proven to be an effective and labor-saving method of cultivating fungicide and pesticide-free plants against disease- and mite-susceptible *A. formosanus* plants grown *ex vitro*. If field capacity water and a small amount of slow release orchid fertilizer were applied before sealing the bag with a paper clip, then watering or applying fertilizer to the plants in the plastic bag was unnecessary for 6-8 months.

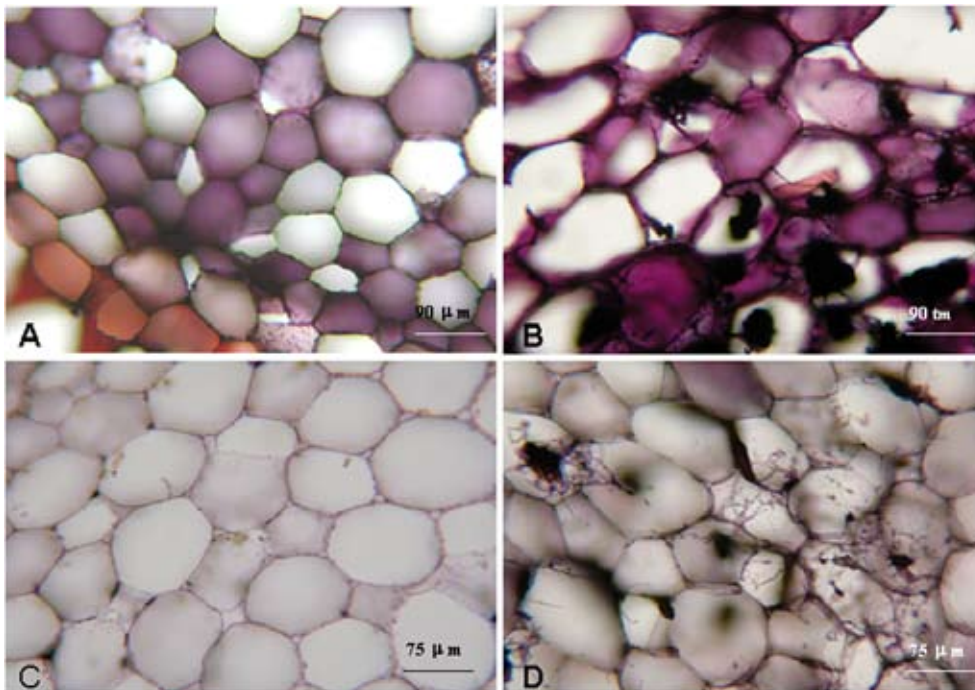


Figure 5. Acid (A & B) and alkaline (C & D) phosphatase enzymatic histochemistry in non-mycorrhizal (A & C) and orchid mycorrhizal roots (B & D) of *Anoectochilus formosanus* Hayata. Mycorrhizal roots (B & D) showed higher enzyme activities than the non-mycorrhizal control (A & C). Note that the alkaline phosphatase activity was only shown in fungal structures (D).

Changes in SOD activity and acid and alkaline phosphatases as well as some component contents in non-mycorrhizal and mycorrhizal tissues were estimated. Results showed that in root: acid and alkaline phosphatases activities (Figure 5); in leaf: SOD activity and ascorbic acid, flavonoid, polyphenol (Figure 6), phosphate and polysaccharide contents (Figure 7), in stem: polyphenol and polysaccharide contents and meanwhile ascorbic acid, polyphenol and polysaccharides contents (Figures 6 & 7) all increased significantly more in the mycorrhizal tissues than in the non-mycorrhizal control. The only exception was the nitrate content, which showed no difference between non-mycorrhizal and mycorrhizal tissues (Figure 7). The alkaline phosphatase activity was present only in the fungal structures (Figure 5-D). This meant that this enzyme was brought in by the OMF, indicating a more effective way for phosphate metabolism. The results indicated that mycorrhizal tissues were better antioxidants than the non-mycorrhizal control, with higher SOD activity and more ascorbic acid, polyphenol, and flavonoids (Figure 6). Thus, as a medicine, mycorrhizal plants would become more effective.

There are six important findings in this paper: 1. An investigation of the mycorrhizal literature indicates that this is the first paper to report on the application of *Rhizoctonia* spp. to the successful commercial-scale cultivation for *A.*

formosonus orchids; 2. Previously we were told that only bi-nucleated *Rhizoctonia* spp. were useful in enhancing the growth of orchids. Our data clearly indicated that both bi-nucleate (R02) and multi-nucleate (R04) *Rhizoctonia* isolates can enhance the germination (Chou and Chang, 2004) and growth of *A. formosonus* (Chou, 2004); 3. The Hyponex #3 agar medium was very easy to prepare and resulted in the highest *A. formosonus* growth rate in our study. Previously some scientists used only Hyponex #1 agar medium (Kano, 1968; Liu et al., 2001) while others used more complex agar media, such as MS medium for orchids; 4. Our data clearly demonstrated the applicability of single or mixed isolates of *Rhizoctonia* spp. to the practical horticulture production of orchids, especially for the inoculation of outplanted seedlings *ex vitro*; 5. This is the first report to compare the differences between non-mycorrhizal and mycorrhizal tissues for their enzyme activities and component contents, and to prove that the mycorrhizal tissues of *A. formosonus* become more effective as a medicine; 6. Before disease and mite-resistant *A. formosonus* strains are found, the plastic bag cultivation method developed by our group, has proved to be a very effective and labor saving way to grow fungicide- and pesticide-free *A. formosonus* plants.

To date, we have tested more than five commercial strains of *A. formosonus* and concluded that both R02 and R04 (*R. solani*, Anatomosis group 6) definitely enhance their growth. However, mycorrhizal plants were also susceptible to diseases and mites. Thus the plastic bag cultivation method was developed to cultivate fungicide- and pesticide-free *A. formosonus* plants.

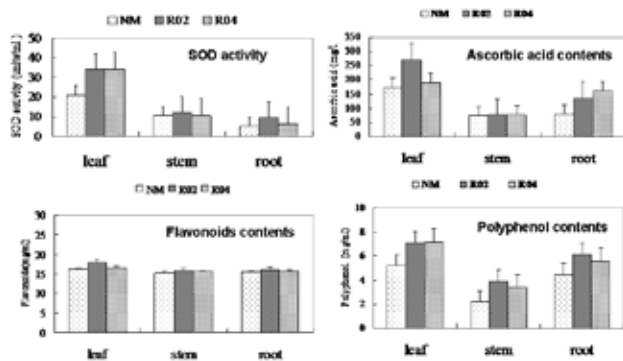


Figure 6. Superoxide dismutase (SOD) activity and chemical components of non-mycorrhizal (NM) and mycorrhizal (R02, R04) *Anoectochilus formosana* Hayata.

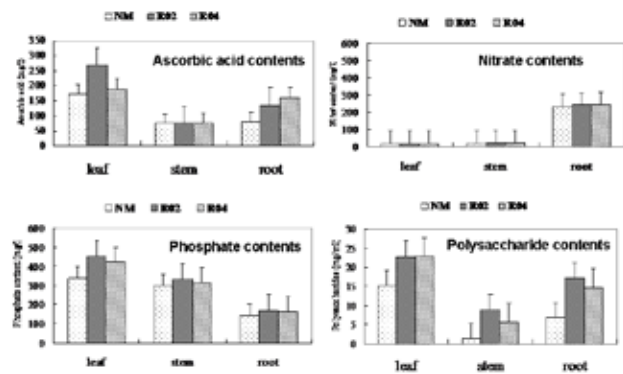


Figure 7. Chemical components of non-mycorrhizal (NM) and mycorrhizal (R02, R04) *Anoectochilus formosana* Hayata.

CONCLUSIONS

It is highly recommended that H3A medium be used for the asymbiotic growth and OMA for mycorrhizal plantlets of *A. formosonus in vitro*. The inoculation of R02 or R04 enhances the growth of *A. formosonus in vitro* and *ex vitro*. We also strongly recommend inoculating OMF (R02 or R04) during outplanting of the plantlets for practical production of *A. formosonus*. The mycorrhizal *A. formosonus* plants would be a better antioxidants than the non-mycorrhizal control plants. The plastic bag cultivation method is a very effective and labor saving way to grow fungicide- and pesticide-free *A. formosonus* plants.

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台灣金線連受絲核菌屬蘭花菌根影響之 生長反應、酵素活性及成分變化

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由台灣野生地生蘭根部，包括金線連，所分離之絲核菌屬雙核 (R02) 及多核 (R04) 蘭共生菌 (OMF)，簡稱蘭菌，可顯著促進玻璃瓶內及移出瓶外台灣金線連 (*Anoectochilus formosanus* Hayata) 之生長。R02 或 R04 蘭菌在瓶內接種必須使用燕麥培養基，菌根植株可長得比在花寶三號洋菜培養基的植株為大。金線連移出瓶外之生長，則受絲核菌屬蘭菌 (R02, R04) 單一或混合接種的顯著促進，例如株高、葉數、根長及鮮重。光學及掃描式電子顯微鏡的觀察顯示，蘭菌的感染屬於菌球消化型，即會在根部皮層細胞內形成菌絲團。菌根植株有較高的酵素活性（如葉片中的超氧化歧化酶，及根中的酸性與鹼性磷酸酶），及顯著為高的成分含量，例如抗壞血酸，多酚類及類黃酮，這些成分使菌根金線連植株，成為較佳的抗氧化物，同時它又含有較高的多醣類及磷酸含量，都使它們成為較有效的藥用植物。菌根植株仍會遭受疾病或紅蜘蛛的侵襲，故採用本研究室所發展的金線連塑膠袋栽培法，證明它是目前一種很有效，且省工的栽培法，可以有效生產無農藥台灣金線連植株。

關鍵詞：絲核菌屬；接種培養基；菌球消化；菌絲團；塑膠袋栽培法。