

Full Length Research Paper

Growth and development of *Phaius tankervilleae* (Banks) Blume when inoculated with orchid mycorrhizal fungi

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Phaius tankervilleae (Banks) Blume is the most attractive and most horticulturally valuable native *Phaius* species in Taiwan. Due to overharvesting in the wild, however, the plant is on the verge of extinction. Successful cultivation of *P. tankervilleae* has been achieved through *in vitro* seeding or tissue culture propagation, but plantlet survival rates are low upon transplantation. Previous studies demonstrated that inoculation with mycorrhizal fungi markedly improved plantlet survival rates, vegetative and reproductive growth in Orchidaceae plants. Four orchid mycorrhizal fungi (OMF), R01 (*Rhizoctonia solani*), R02 (*Rhizoctonia* sp., teleomorph = *Ceratobasidium* sp. AG-A), R15 (*Rhizoctonia* sp., teleomorph = *Ceratobasidium* sp. AG-Fb) and R19 (*Rhizoctonia* sp., teleomorph = *Ceratobasidium* sp. AG-G) were isolated from Taiwanese native orchid roots that were cultured for more than seven years in our laboratory. These fungi all proved to be non-pathogenic and were inoculated onto the root of *P. tankervilleae*. Inoculation with the appropriate OMF increased seedling survival rate 27 to 31% (R02; R15), plant height 6.3 cm (R15), leaf length 3.5 cm (R15), flower stalk length 10.2 cm (R19) and number of flowers per stalk (3.5) in *ex vitro*-grown *P. tankervilleae*. Inoculation with OMF (R19) promoted reproductive growth in *P. tankervilleae*, while OMF (R02) inoculation significantly increased photosynthetic rate and carbohydrate content. *P. tankervilleae* seedlings inoculated with the R15 isolate had a higher survival rate than with other treatments. Inoculations with *Rhizoctonia* spp. isolates R02, R15, or R19 also promoted reproductive growth in *P. tankervilleae*.

Key words: Pelotons, *Phaius tankervilleae*, reproductive growth, *Rhizoctonia* species, survival rate, vegetative growth.

INTRODUCTION

Phaius tankervilleae (Banks) Blume is a large terrestrial orchid native to Taiwan that is distributed from India, Sri Lanka and Southern China, through Thailand and Malaysia to Indonesia, Australia and the Pacific islands. In Taiwan, it grows in broad leaved forests at elevations

below 1000 m and throughout Lanyu Island. The orchid favors high temperatures and wet environments, and is not cold-resistant (Su, 2000). *P. tankervilleae* is a robust plant that blooms easily. Plant height 60 to 200 cm, without slender stems, fleshy pseudo bulbs ovoid-elliptic and internode very close, alternate leaves, ovate-lanceolate or broad lanceolate 3 to 5, 40 to 60 cm long, leaves papery, surface wrinkle. Flower stalk thick 60 to 100 cm long, formation from the side of pseudo bulb, 6 to 20 flowers of one inflorescence, flower diameter 8 to 10

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cm. Sepals white outside, white to yellowish white and tinged with red-brown inside, petals red-brown or purplish, lip yellowish white, tinged with red and decorated with dark purple spots (Bechtel et al., 1986; Su, 1990; Yeh et al., 2007). Horticulturally, the flowers are large and beautiful and flower from April to July. A single flower lasts about 20 days; the inflorescence itself lasts up to 45 days (Chang and Jian, 2010). *P. tankervilleae* has slender leaves, graceful greenery, and can be used as a potted plant, a garden plant, or as cut flowers or leaves (Mukherjee, 1979; Lee, 1989; Chang and Jian, 2010). *P. tankervilleae* has been overharvested in the wild and is on the verge of extinction (Su, 1989). The flower of *P. tankervilleae* is big and beautiful with great ornamental and economic value (Mukherjee, 1979; Lee, 1989; Chang and Jian, 2010). Only a small amount of *P. tankervilleae* could be found in the flower market of Taiwan. Su (1989) reported that the wild population of *P. tankervilleae* was gradually reduced (Su, 1989). It is urgent to preserve this endangered orchid.

The sexual reproduction of *P. tankervilleae* is accomplished through seed germination with fungal symbiosis and *in vitro* seed germination to obtain a seedling. In asexual reproduction, the top of the stem or flower stalk are used to culture an *in vitro* seedling, reproductive ramets and the flower stalk are buried in the soil and a seedling forms on the node of flower stalk (Lee, 1989; Wu 1991; Tsai, 2010). Previous studies demonstrated that inoculation with mycorrhizal fungi markedly improved vegetative and reproductive growth in Orchidaceae plants (Rasmussen et al., 1990; Chu, 2000; Takahashi et al., 2006; Chang, 2008; Smith et al., 2009; Wu et al., 2011). Orchid mycorrhizal fungi (OMF)-inoculated mother and daughter *Oncidium* sp. showed increased plant height and pseudo bulb length and width compared to the non-mycorrhizal control; leaf quantity, thickness, fresh weight, surface area, chlorophyll and soluble sugar content were also greater (Chu, 2000). Leaf length and fresh weight of *Paphiopedilum delenatii* Guillaumin orchids was greatly increased by OMF R04 and PaR2-inoculation (Tsai, 2003). *Phalaenopsis amabilis* (L.) Blume var. *Aphrodite* (Reichb. f.) Ames inoculated with R01 showed higher stalk length, and R02-inoculated plants showed increased floral diameter and higher bolting rates (Lan, 2001). *Doritaenopsis* Luchia Davis × *Doritaenopsis* Taisuco Firebird seedlings inoculated with R02 had a 40% flowering rate compared to 0% in the non-mycorrhizal control kept at the natural temperature in Taipei (Lan, 2001). *Haemaria discolor* var. *dawsoniana* showed greater stalk length and flower number when inoculated with R01 (Chou, 2004).

Orchidaceae *Phaius* species can be produced through *in vitro* seeding or tissue culture propagation, but plantlet survival rates decrease during the transplantation (personal communication). Since there is relatively little data on *P. tankervilleae*, identifying the effects that some OMF have on its growth and development was the main focus in selecting the appropriate fungi to improve its

survival rate during the transplant period, and on enhancing the vegetative and reproductive growth of *P. tankervilleae*. We also investigated the effects of OMF on orchid growth during the seedling stage, flowering period regulation and flower production promotion. This horticultural research can be useful for mass production of the seedling, regulating flowering periods, and improving the yield and quality of *P. tankervilleae* flowers. It can show the ornamental value and economic potential of this native Taiwanese orchid and promote its preservation, restoration and development as an ornamental plant.

MATERIALS AND METHODS

Mycorrhizal inocula

In this experiment, we isolated four strains of *Rhizoctonia* spp. including R01 (*Rhizoctonia solani*), R02 (*Rhizoctonia* sp., teleomorph = *Ceratobasidium* sp.; AG-A), R15 (*Rhizoctonia* sp., teleomorph = *Ceratobasidium* sp.; AG-Fb) and R19 (*Rhizoctonia* sp., teleomorph = *Ceratobasidium* sp.; AG-G), identified through DNA analysis, that were cultured in our laboratory for more than seven years. They all proved to be non-pathogenic (Chang, 2008). They were cultured on a medium prepared by mixing peat with 20% V8 juice to reach field capacity (Stevens, 1974). All *P. tankervilleae* plants used in this experiment were inoculated with a single fungal strain. All OMF used in this research are stored in the Bioresource Collection and Research Center at the Food Industry Research and Development Institute in Hsinchu, Taiwan.

Plant materials

The *P. tankervilleae* seedlings were provided by the Taoyuan District Agricultural Research and Extension Station Taiwan. *P. tankervilleae* seeds were sown and grown on agar medium for four months, subcultured, then subcultured again two months later. A sowing medium of 3 g L⁻¹ Hyponex 1 (7N-6P-19K), 150 ml L⁻¹ coconut water, 9 g L⁻¹ agar (Sigma, St. Louis, MO, USA), and 20 g L⁻¹ sucrose, was adjusted to pH 5.2. A subculture medium containing 2 g L⁻¹ NPK fertilizer (7N-6P-19K), 9 g L⁻¹ agar (Sigma, St. Louis, MO, USA), 20 g L⁻¹ sucrose, and activated carbon 1 g L⁻¹, was adjusted to pH 5.2. The seedlings were about 8 to 10 cm in height.

Medium and culturing method

The tissue culture container seedlings of *P. tankervilleae* were obtained and removed from glass containers, then planted in 7.5 cm-diameter black plastic pots with the mix media at a 1:1:1 (v:v:v) (volume) ratio of peat moss, coconut fiber and tree fern fiber. Samples were divided into five groups of 36 seedling each, including the non-mycorrhizal (NM) control and mycorrhizal treatment (*Rhizoctonia* isolates R01, R02, R15 and R19). Treatments were arranged in a completely randomized design (CRD). The roots of each seedling received 0.2 g of fungal inocula. As the plants became larger, they were transplanted with same medium into 18 cm-diameter plastic pots. Each pot initially received 2 g of Osmocote (14N-5.2P-11.6K) and was watered once every week. Water-soluble fertilizer (Peters, Scotts, Marysville, USA) was applied every two weeks, changing from 0.5 g L⁻¹ of 30N-4.3P-8.3K water-soluble fertilizer (Peters, Scotts, Marysville, USA) at the vegetative stage to 0.5 g L⁻¹ of 10N-12.9P-16.6K water-soluble fertilizer at the reproductive stage. The experimental site was a

plastic greenhouse on the National Taiwan University campus (10 m above sea level), the average value of the photosynthetic photon flux (PPF) was 200 to 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at noon, and the average values of the highest and lowest temperatures were 32.21 and 15.21 °C respectively.

Carbohydrate content measurement

The analytical method for measuring carbohydrate content was modified from the method described in the study of Luchsinger and Cornesky (1962), Yoshida et al. (1972) and Lindsay (1973). The third and fourth leaves from the distal end and pseudo bulbs were taken from matured *P. tankervilleae* plants after 26 months of cultivation and the dried sample pulverized for testing. To evaluate starch content, the following steps were taken: (i) the sample was extracted with 80% EtOH and distilled water, (ii) 20 mM (pH 6.9) of sodium phosphate buffer was added to the precipitate, and then the sample was heated in a boiling water bath for 15 min. 0.5 ml α -amylase was then added and the sample placed in a 37°C water bath for 16 h. 1 ml crude extract was obtained to which was added 2 ml DNSA (3,5-dinitrosalicylic acid reagent) before returning to a boiling water bath for 10 min, cooled to room temperature and 2 ml distilled water was then added. Absorbance was then (iii) measured by a spectrophotometer (HITACHI, 2800 JP, Japan) at A_{570} wavelength and the value estimated. To evaluate total soluble sugar content, we took the following steps: (i) the sample was extracted twice with 80% EtOH; (ii) the extract was heated in the boiling water bath to remove ethanol, (iii) then chloroform and distilled water were added; (iv) anthrone solution was slowly added in an ice bath and then boiled in the water bath for 7 min 30 s before (v) measuring the absorbance by a spectrophotometer (HITACHI, 2800 JP, Japan) at A_{630} wavelength to estimate the value.

Photosynthetic rate estimation

The photosynthetic rate for matured *P. tankervilleae* plants after 26 months growth was detected on a 6 cm² leaf area using LI-6400 portable photosynthesis system (LI-COR Inc., Nebraska, U.S.A) (Jiang, 1996). The photosynthetic rate was measured at the second leaf of the daughter pseudo bulb from 5 to 10 cm above the pseudo bulb from 9 to 11 AM.

Plant growth and development

Plant height (leaf tip), number of leaves, leaf length and width, pseudo bulb diameter, number and length of flower stalks and number of flowers were measured for each *P. tankervilleae* plant. Each treatment involved 36 plants, 15 from each of these groups of 36 were randomly selected for statistical analyses, using ANOVA (analysis of variance) and LSD (least significant difference) test to compare the effects of treatments.

Anatomic observation of orchid mycorrhizal

We collected roots of *P. tankervilleae* with or without OMF inoculation and cleaned and stained them with aniline blue in acidic glycerol (Koske and Gemma, 1989) for optical microscopy (Olympus BH-2 USA). For scanning electron microscope (SEM) observations, the collected roots were dissected into sections, fixed with 2.5% glutaraldehyde for 10 to 12 h, dehydrated in an acetone series, critical point dried (SPI # 13200-AB Manual CPD) by liquid carbon dioxide, coated with gold for 90 s by a Biorad Ion Coater (Dawes, 1971), then examined and digitally recorded using the SEM Topcon-60.

RESULTS AND DISCUSSION

The survival rates (SR%) of *P. tankervilleae* seedlings were significantly increased for 22 months after inoculation with R02, R15, R19 when compared to those of the non-mycorrhizal (NM) control plants. The SR% of *P. tankervilleae* seedlings after treatments with the R15 and R02 isolates were 79 to 81% and 78 to 80%, respectively, a 27 to 31% higher SR% compared with 48 to 54% for the NM control plants (Figure 1). Plant height and leaf length were greatly increased in *P. tankervilleae* plants inoculated with R02 or R15 for 22 months. Plant height and leaf length were lower in R01-treated plants as compared with the non-mycorrhizal (NM) control plants (Table 1). The NM control, R02, and R15-inoculated plants were thus used to measure the carbohydrate content and photosynthetic rates. Results showed that when plants were inoculated with the R02 isolate, the rate of net CO₂ fixation increased significantly as compared with that for plants inoculated with R15 or NM control group (Figure 2).

Oncidium orchids typically have large pseudo bulbs that store the carbohydrates necessary for flowering (Yong and Hew, 1995; Chang and Lee, 1999). *Cymbidium* orchids only produce flowers when the amount of carbohydrates stored in their pseudo bulbs is sufficient (Komori, 1986). Since *P. tankervilleae* plants also have large pseudo bulbs (Tsai and Chang, 2009), we wanted to determine their carbohydrate content during the early stage of flower bud differentiation. Analytical results indicated that 80% EtOH-soluble sugars in leaves and starch content in pseudo bulbs of plants at 26 months after inoculation with R02 were higher than those in NM control plants (Table 2). Total carbohydrate content in leaves and pseudo bulbs of OMF (R02)-inoculated plants was also higher than in NM control plants.

Furthermore, *P. tankervilleae* plants inoculated with *Rhizoctonia* spp. flowered early (Figure 3), and plants inoculated with R02 or R19 isolates flowered 5 days earlier than did NM control plants (Table 3 and Figure 3). Other than greater height as compared to the NM control, no other vegetative growth differences, such as number of leaves and pseudo bulbs, occurred between the R01-inoculated plants and the control. Compared to the NM control plants, plants inoculated with R01, R02, R15 or R19 isolates for 28.5 months had significantly increased flower stalk length, with R19 isolates also significantly increasing the number of flowers (Table 4 and Figure 3).

The NM *P. tankervilleae* plants did not have any pelotons in their root cortex cells (Figure 4A), while plants infected with R02 had pelotons built by a number of mycelia (Figure 4B). Although, SEM showed no pelotons in the cortex cells of control group roots (Figure 5A), they were present in the R02-infected plant roots (Figure 5B).

Figure 5C gives details on the infected cells that formed pelotons while Figure 5D shows digested hyphae. The mycelia extended into the surrounding non-infected cells after the pelotons had degraded into amorphous clumps,

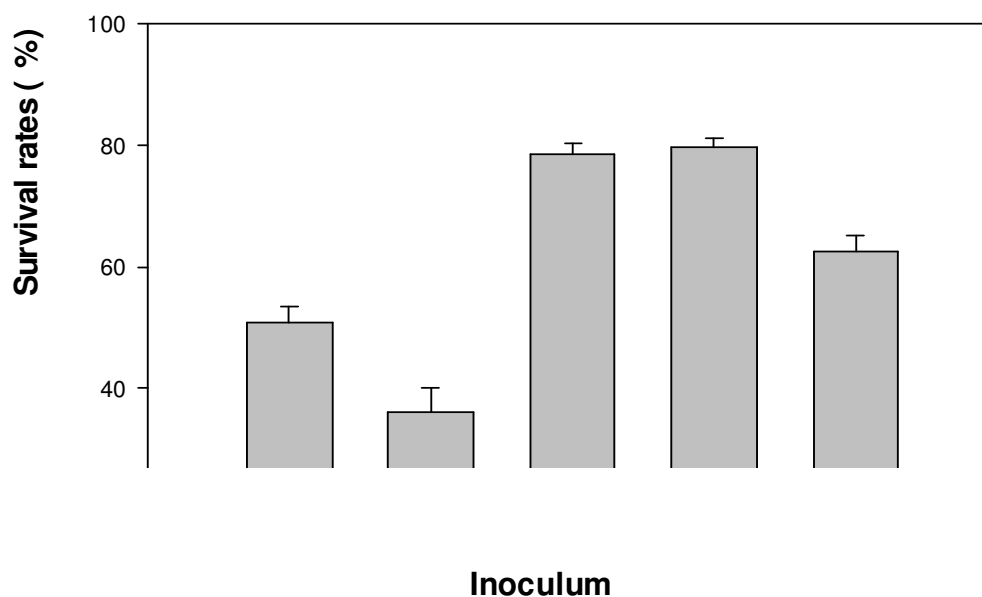


Figure 1. Survival rates (%) of *Phaius tankervilleae* seedlings inoculated with *Rhizoctonia* spp. (R01, R02, R15, or R19) of orchid mycorrhizal fungi after 22 months of *ex vitro* growth. NM indicates non-mycorrhizal control.

Table 1. Vegetative growth for seedlings of *Phaius tankervilleae* after inoculation with various *Rhizoctonia* spp. of orchid mycorrhizal fungi for 22 months.

<i>Rhizoctonia</i> inoculum	Plant height (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)
NM*(CK)	25.2 ^{cd}	4.0 ^a	19.4 ^b	4.7 ^{ab}
R01	21.8 ^d	3.3 ^b	16.4 ^c	4.3 ^b
R02	30.6 ^{ab}	3.9 ^{ab}	22.9 ^a	5.2 ^a
R15	31.5 ^a	4.2 ^a	22.9 ^a	5.0 ^a
R19	28.4 ^{abc}	3.7 ^{ab}	20.5 ^{ab}	4.8 ^{ab}
ANOVA F values 2.04 (df=4,70)	8.8	2.2	7.2	2.9

*NM = non-mycorrhizal control; R01, R02, R15, R19 = orchid mycorrhizal fungi. Each treatment had 15 replicates. The effects of mycorrhizal infection of *Phaius tankervilleae* were tested by ANOVA. The F values of ANOVA are presented in the table. Means in each column followed by the different letters were significantly different (P=0.05) as determined by LSD test.

Table 2. Carbohydrate content of leaf and pseudo bulb for seedlings of *Phaius tankervilleae* after inoculation with orchid mycorrhizal fungi (R02, R15) *ex vitro* for 26 months.

<i>Rhizoctonia</i> spp of inoculum	Leaf		Pseudo bulb	
	80% EtOH soluble sugars (mg·g ⁻¹)	Total carbohydrate (mg·g ⁻¹)	Starch (mg·g ⁻¹)	Total carbohydrate (mg·g ⁻¹)
NM*(CK)	73.8 ^b	98.3 ^b	235.4 ^b	321.2 ^b
R02	95.5 ^a	122.8 ^a	321.5 ^a	412.1 ^a
R15	91.3 ^{ab}	110.0 ^{ab}	313.6 ^a	389.5 ^a
ANOVA F values 2.53 (df=2,26)	6.3	8.1	23.7	24.1

*NM = non-mycorrhizal control; R02, R15 = orchid mycorrhizal fungi. Each treatment had 9 replicates. The effect of mycorrhizal infection of *Phaius tankervilleae* was tested by ANOVA. The F values of ANOVA are presented in the table. Means in each column followed by different letters were significantly different (P=0.05) as determined by LSD test.

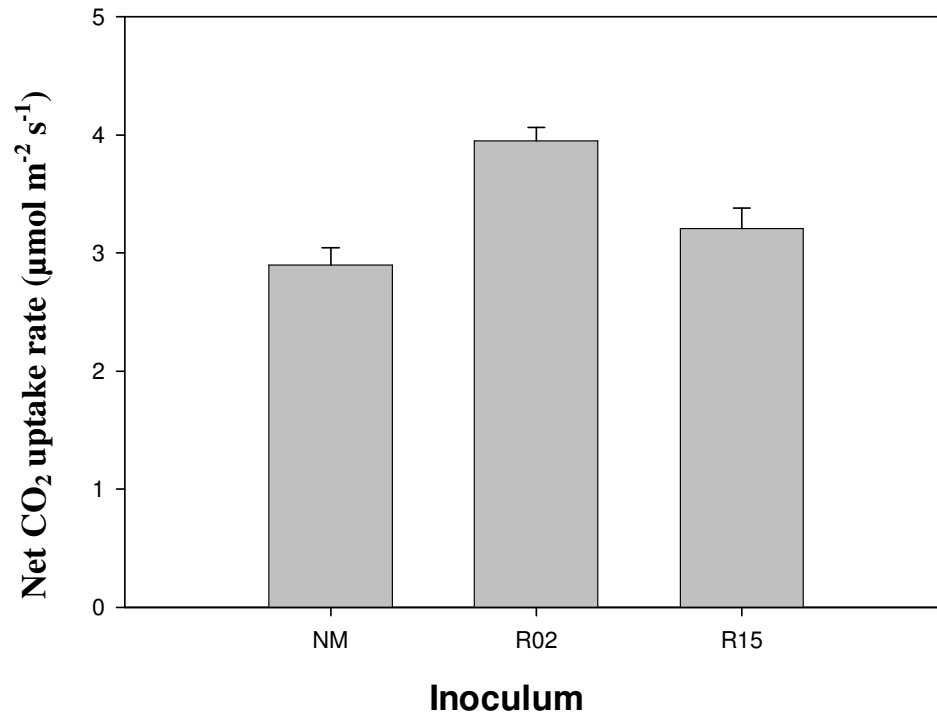


Figure 2. Net CO₂ uptake rate for *Phaius tankervilleae* seedlings after inoculation with orchid mycorrhizal fungi for 26 months. NM indicates non-mycorrhizal control.

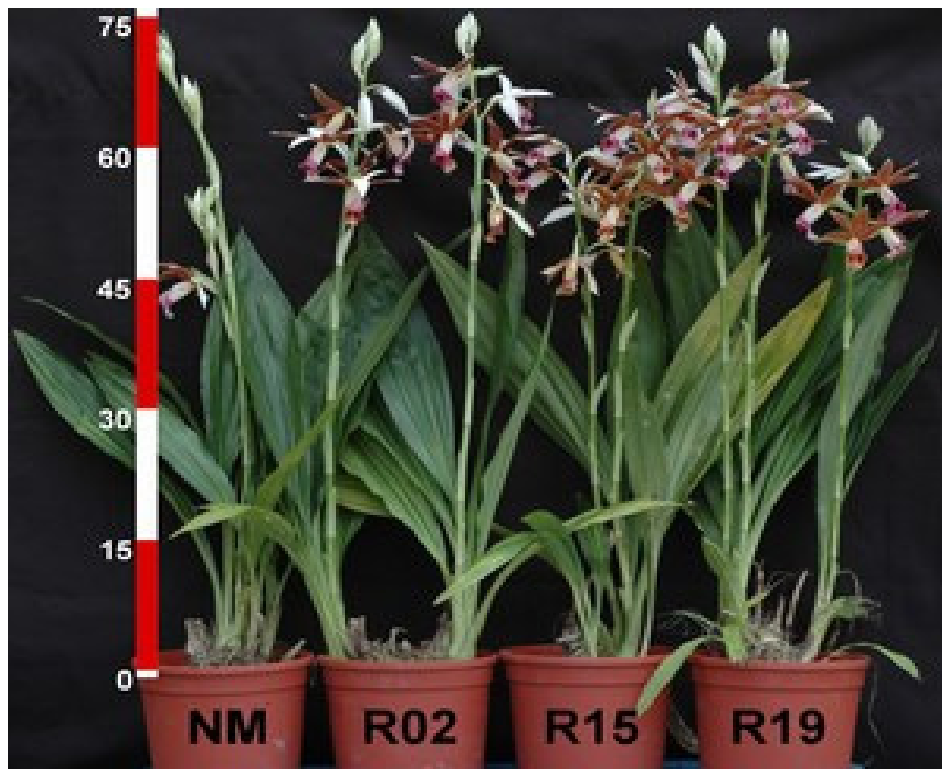


Figure 3. Vegetative and reproductive growth of *Phaius tankervilleae* (Banks) Blume seedlings after inoculation with various *Rhizoctonia* spp. of orchid mycorrhizal fungi for 30.5 months. NM indicates non-mycorrhizal control.

Table 3. Days before flowering in *Rhizoctonia* spp.-inoculated *Phaius tankervilleae*.

<i>Rhizoctonia</i> spp of inoculum	Days to flower after OMF inoculation
NM*(CK)	918.8 ^a
R01	916.1 ^{ab}
R02	914.0 ^b
R15	915.6 ^{ab}
R19	914.4 ^b
ANOVA F values 2.04 (df=4,70)	4.1

*NM = non-mycorrhizal control; R01, R02, R15, R19 = orchid mycorrhizal fungi. Each treatment had 15 replicates. The effects of mycorrhizal infection of *Phaius tankervilleae* were tested by ANOVA. The F values of ANOVA are presented in the table. Means in each column followed by different letters were significantly different (P=0.05) as determined by LSD test.

Table 4. Comparison of growth and development of *Rhizoctonia* spp-inoculated *Phaius tankervilleae* seedlings at 28.5 months.

<i>Rhizoctonia</i> spp of inoculum	Plant height (cm)	Flower stalk length (cm)	No. of flowers
NM*	46.2 ^b	53.9 ^c	11.9 ^b
R01	54.2 ^a	62.6 ^{ab}	11.6 ^b
R02	41.6 ^b	59.6 ^{ab}	14.2 ^{ab}
R15	41.7 ^b	60.2 ^{ab}	14.2 ^{ab}
R19	45.0 ^b	64.1 ^a	15.4 ^a
ANOVA F values 2.04 (df=4,70)	10.5	9.2	7.7

*NM = non-mycorrhizal control; R01, R02, R15, R19 = orchid mycorrhizal fungi. Each treatment had 15 replicates. The effects of mycorrhizal infection of *Phaius tankervilleae* were tested by ANOVA. The F values of ANOVA are presented in the table. Means in each column followed by different letters were significantly different (P=0.05) as determined by LSD test.

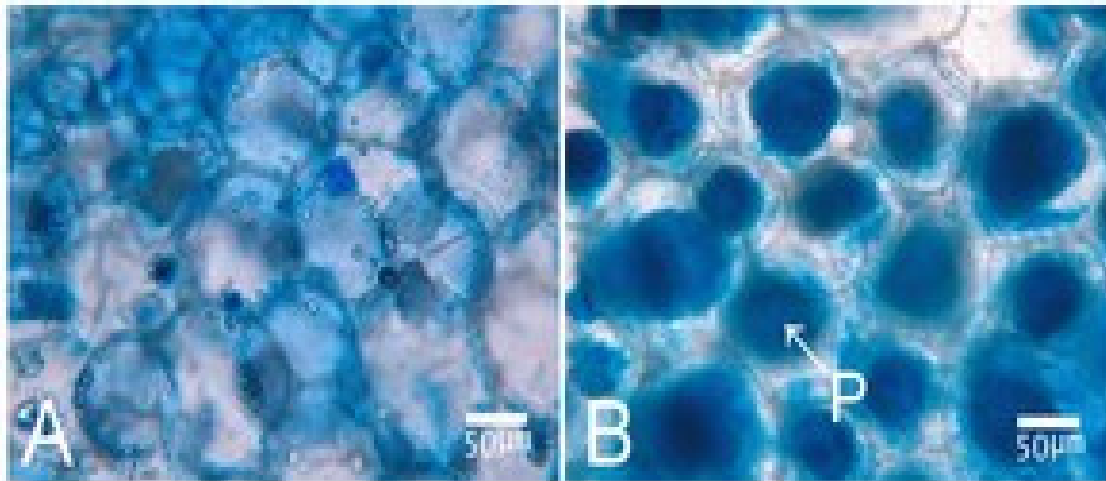


Figure 4. Optical microscopic observation of *Phaius tankervilleae* (Banks) Blume roots inoculated with *Rhizoctonia* sp. of orchid mycorrhizal fungus (R02) for 22 months and stained with aniline blue (0.05%). A, Control. B, Root cortex cells of *P. tankervilleae* (Banks) as inoculated with R02.

after which pelotons formed until the entire root was infected. This particular infection process is known as tolypophagy. Plants infected with R15 or R19 also produced pelotons, the digested amorphous clumps

also can be observed (Figure 5E and F). The Mycorrhizal structures formed by different *Rhizoctonia* species in *P. tankervilleae* roots were all similar.

The advantages of using mycorrhizal *P. tankervilleae*

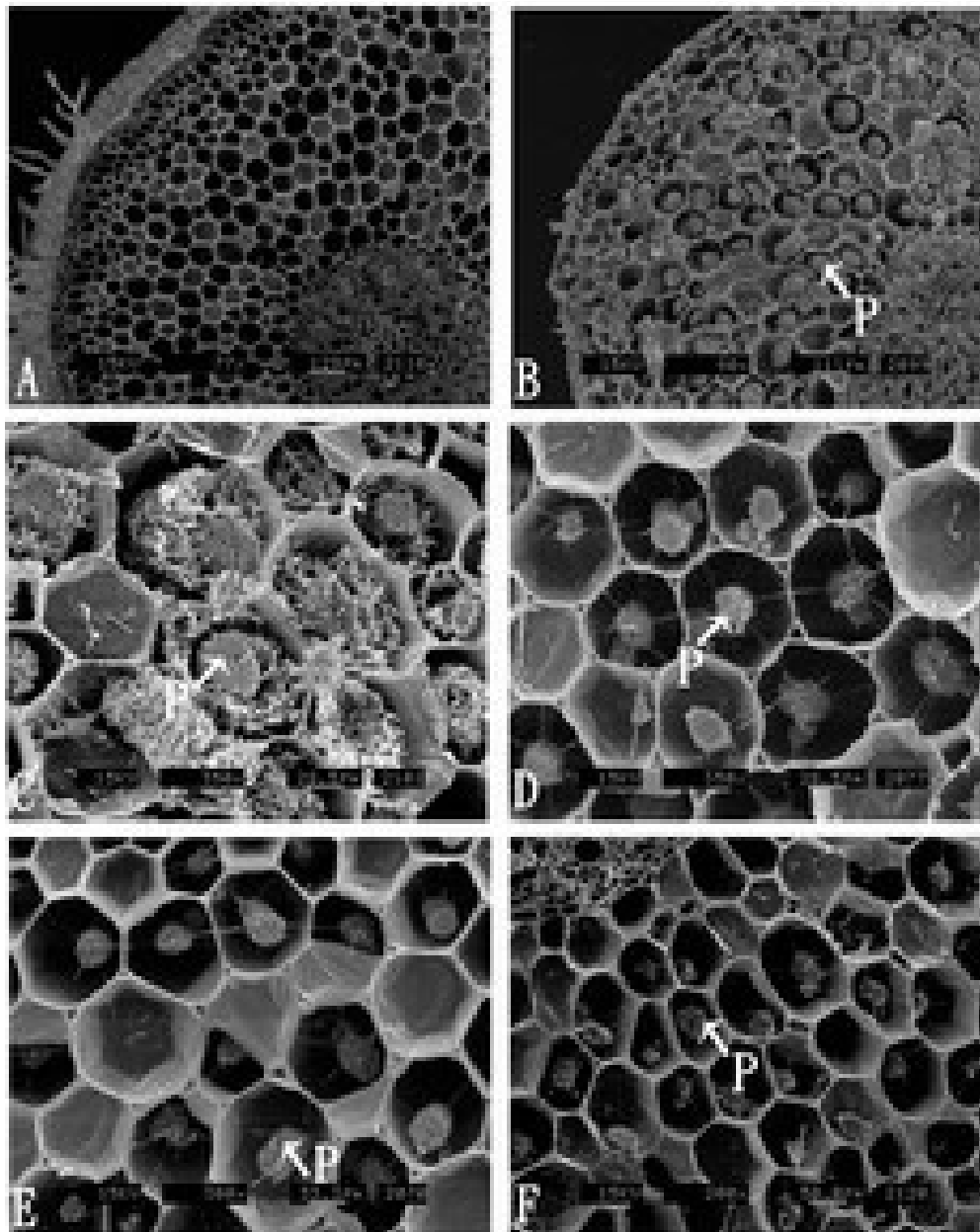


Figure 5. SEM observation of structural changes by hyphal infection in the cortex cells of *Phaius tankervilleae* (Banks) Blume roots after inoculation with *Rhizoctonia* spp. of orchid mycorrhizal fungi R02 (A-D), R15 (E), or R19 (F) for 22 months. A, Control. B, Root cortex cells of *P. tankervilleae* (Banks) Blume as inoculated with R02. C, Root cortex cells of *P. tankervilleae* (Banks) Blume as inoculated with R02 and the formation of peloton (P). D, Later stages of pelotons (P), which were digested in the roots of *P. tankervilleae* (Banks) Blume as inoculated with R02. E, Later stages of pelotons (P), which were digested in the root of *P. tankervilleae* (Banks) Blume as inoculated with R15. F, Later stages of pelotons (P), which were digested in the root of *P. tankervilleae* (Banks) Blume as inoculated with R19.

include an increased transplant survival rate for seedlings grown in glassware. Tsai (2003), Jin et al. (2009) and Smith et al. (2009) had similar results with other Orchidaceae species. Inoculation with an appropriate OMF (R19) was thus beneficial for *P. tankervilleae*

reproductive growth, earlier flowering, increased number of flower stalks, and increased flower count. Similar results were obtained by Lee et al. (1997), Chu (2000), Takahashi et al. (2006) and Smith et al. (2009) with other Orchidaceae plants. Plants inoculated with the R02 isolate

of *Rhizoctonia* sp. had higher total carbohydrate content and more flower stalk length than did NM control plants; those inoculated with R02 also had a higher CO₂ fixation rate (higher rate of photosynthesis). Inoculation with orchid mycorrhizal fungi increased chlorophyll content (Chu, 2000; Chou, 2004). The fungi also help turn plant cellulose into carbohydrates that are easily absorbed, and can act as a carbon source for orchids (Smith, 1966). Furthermore, OMF provides minerals, vitamins, and other nutrients for plants (Bernard, 1911; Hadley, 1984; Alexander and Hadley, 1985), promotes the absorption of nitrogen, phosphorus and potassium for seedlings (Alexander and Hadley, 1984; Zhao et al., 1999), and promotes plant growth and development, which finally leads to better reproductive growth than in NM control plants. These results lead to the conclusion that the symbiotic relationship between OMF and *P. tankervilleae* enhances the photosynthetic rate, seedling viability, and reproduction growth. Many researches focused on the Orchidaceae symbiosis with mycorrhizal for restoration of orchids (Batty et al., 2001; Beltran-Nambo et al., 2010). Further application of OMF may preserve and restore the endangered orchid species for future commercial production.

Conclusion

In conclusion, the inoculation of OMF was done with just a small amount (about 0.3 g/plant) of inoculum under the roots of orchid; hence, it was very simple. However, this treatment could highly increase the survival rate, growth and development of this orchid. Hopefully, this practice will help to preserve and restore more of the endangered orchid species.

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