African Journal of Biotechnology Vol. 10(50), pp. 10095-10100, 5 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1310
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Mycorrhizal symbiosis enhances *Phalaenopsis* orchid's growth and resistance to *Erwinia chrysanthemi*

Po-Hung Wu[1], Ding-Ding Huang[2] and Doris C. N. Chang[1]*

[1]Department of Horticulture, National Taiwan University, No 1, Sec. 4, Roosevelt Rd., 10617, Taipei, Taiwan.
[2]Department of Biotechnology, Asia University, 500, Lioufeng Rd., Wufeng, Taichung 41354, Taiwan.

Accepted 13 July, 2011

Phalaenopsis **is the most important potted orchid genus in the world. However, the low seedling survival rate, long vegetative growth period and disease outbreak are problems in production. Orchid micorrhizal fungi (OMF) are their obligate partners in orchid physiology. Orchids use their symbionts to gain access to organic and mineral nutrients by increasing nutrient absorption and translocation to plants under natural conditions. The benefit of orchid mycorrhizal symbiosis using** ***Phalaenopsis*** **as model plants was conducted. We inoculated** ***in vitr*****o grown plantlets of** ***Doritaenopsis*** **Taisuco Wonder 'King Car Butterfly KC1111' and** ***Phalaenopsis*** **Tai Lin Redangel 'V31' with two OMF isolates,** ***Ceratobasidium*** **sp. AG-A (R02) and** ***Rizoctonia solani*** **AG-6 (R04). The effects of OMFs on orchid plant growth and** ***Erwinia*** **soft rot progression were examined after two months of** ***ex vitro*** **growth. The results showed that the presence of OMFs in** ***Phalaenopsis*** **roots significantly increased the growth and soft rot resistance of plants. Selectivity of cultivar type to different OMF was also observed. The relevance of this findings and future work are discussed.**

Key words: *Phalaenopsis,* orchid micorrhizal fungi, orchid, cultivar type.

INTRODUCTION

Orchids is the most important potting plant in the world with 30% annual market growing rate (Wang, 2004). *Phalaenopsis* is the most popular orchid species and is only sold in the retail market when in blossom (U. S. Dept. Agr. 2007; Vereniging van Bloemenveilingen, 2007). Taiwan is one of the largest orchid plant exporters. The total export value for 2007 was nearly US$50 million (Kras, 2008). Over the years, traditional breeding has generated a large pool of new hybrids and genetic variation. Great advances in tissue culture techniques also allow mass production of disease free orchid plantlets from seeds or vegetative tissues. *Phalaenopsis* is a monopodial epiphytic orchid having indeterminate inflorescence. The ability of *Phalaenopsis* to spike (bolt) and flower under inductive environmental conditions, such as low temperature and dim light, is highly correlated with leaf size as plants mature (Wang, 1995) but with a significant difference among various hybrids (Lee, 1991). The potential flower spikes usually emerge at the axils of the 3rd or/and the 4th basipetal mature leaf (Lin, 2002).

One of the major problems in orchid production is that seedlings require at least two years of vegetative growth before flowering. Orchid seedlings grown in flasks are first transferred to a community pot, then thumb pots, and then to a larger typical commercial pot. The duration of each transfer is about three to six months. *Phalaenopsis* spike and blossom can be controlled under proper temperature and light, and application of synthetic chemical and plant growth regulator, after plants have matured (Wang, 1995). Nevertheless, precocious flowering will affect flower size and number of flowers per spike. Poor seed germination and high mortality rates in young plantlets also affect orchid production in the nursery.

Another problem is disease outbreak during orchid cultivation. Diseases caused by bacteria, fungus or viruses can affect the quality of plants by leaving brown spots and scars if the disease is not controlled or eradicated, causing economic losses in orchid production.

*Corresponding author. E-mail: cymmvlsc@hotmail.com. Tel: +886-2-33664869. Fax: +886-2-23625542.

Soft rot diseases caused by *Erwinina* spp. is the most devastating diseases in orchid production (Liau et al., 2003). Good husbandry by controlling water availability and quality, lighting, airflow, optimal temperature and preventing nutrient deficiency during cultivation is crucial in disease prevention. Development of transgenic orchids is an alternative method of disease control (Belarmino and Mii, 2000). Transfer of a sweet pepper ferredoxin-like protein and defensin into phalaenopsis was successful in the reduction of soft rot infection caused by *Erwinina* spp. (Liau et al., 2003; Chan et al., 2005).

Orchids are obligate that are dependent on orchid micorrhizal fungi (OMF) and use the symbionts to access organic and mineral nutrients by increasing absorption and translocation to plants (Smith and Read, 1997). Orchid seeds (OMF) inoculation of micropropagated plantlets or seedlings with mycorrhiza has improved the germination rate (Chang and Chou, 2001; Takahashi et al., 2001). It is also suggested that OMF seedlings increases *ex vitro* survival rates, enhances vegetative and reproductive growth, induces early flowering improves flower quality, and reduces disease infection (Chang, 2008). Burgeff (1959) observed that orchid seed germination was impossible in the absence of OMF under natural conditions. OMF inoculation was also reported to improve seed germination rates and protocorm development in various species (Chang and Chou, 2001; Takahoshi et al., 2001; Johnson et al., 2007). In this study, the effects of OMF inoculation on plant growth and development of soft rot disease (*Erwinia chrysanthemi*) are described in *Phalaenopsis* orchids.

MATERIALS AND METHODS

Phalaenopsis Dtps. Taisuco Wonder 'King Car Butterfly' (KC1111) and *Phalaenopsis* Tai Lin Redangel 'V31' (V31) were used in this study. *In vitro* established orchid plants were used as initial plant materials. Orchids were kept in a growth chamber under a 12 h light/12 h dark photoperiod with average day/night temperatures of 28/23°C. Relative humidity was 70% with lighting of 3000 Lux. Plants were fertilized once weekly, alternating between 0.2 and 0.5 $g \cdot L^{-1}$ of a Peters water soluble fertilizer (20N-8.6P-16.6K; Scotts, Marysville, Ohio) at EC value of 0.6 TO 0.8 ds/m. Chilean sphagnum moss *Sphagnum Magellanicum* was used as the potting medium.

Orchid micorrhizal fungi culture and plant inoculation

Two OMF isolates R02 and R04 were used. R02 is *Ceratobasidium* sp. AG-A whereas R04 is *Rizoctonia solani* AG-6 (Chang, unpublished data). R02 and R04 inoculums were prepared as described previously (Chang and Chou, 2007). About 0.1 g of inoculum was applied around the roots of KC1111 and V31 cultivars while transplanting *in vitro* plants to pots. OMF growth in root was verified using florescent light microscopy based on a method developed by Chang's laboratory. Briefly, OMFs were found intracellularly in the cells of the cortex and they were confined to the roots. OMF infection forms a tightly coiled hyphal structure termed a peloton in the cells. Each florescent dot indicates a peloton in the cell by immunofluorescence assay (Smith and Read, 1997; Chang and Chou, 2007). OMF inoculated plants, mycorrhizal KC1111 (inoculated with R02 or R04) and mycorrhizal V31 (inoculated with R02 or R04), were used for growth assessment and *E. chrysanthemi* inoculation after two months.

E. chrysanthemi culture and plant inoculation

E. chrysanthemi was obtained from Dr. Hao-ren Huang at the National Cheng-Kung University, Tainan, Taiwan. A single colony of *E. chrysanthemi* was grown overnight in 5 ml of YEP medium. The bacterial culture was then adjusted to OD_{600} = 1.0 and used for inoculation with a series of dilutions (1×10^{-1} to 10^{-7}).

Fully grown leaves (2nd leaf from the top) from OMF infected orchid plants were used for inoculation. Leaf inoculation was carried out using a syringe needle 2 cm apart. 10 µl each of series diluted bacterial inoculum was applied directly onto the wounding site with a micropipette. Inoculated orchid plants were placed in sealed plastic bags and kept in the growth chamber. The area of soft rot infection was characterized by forming a water clear zone after 24 h of inoculation. The radius of the infection zone was measured and calculated using least significant difference (LSD). Mean of 10 replicates for each treatment was presented.

RESULTS AND DISCUSSION

Effect of OMF inoculation on growth of orchid plants

Growth of two KC1111 and V31 plants were evaluated after two months of OMF inoculation. OMF isolates R02 and R04 were used for inoculation of mycorrhizal plants. Mycorrhizal KC1111 (R02 and R04) had larger leaf size than the control (Figure 1A and B). Similar results were also found in mycorrhizal V31 plants inoculated with R02 but not with R04 (Figure 1C and D). Mycorrhizal V31 (R04) showed no enhanced growth which may be due to no or low level of R04 colonization in the roots because of host selectivity. Growth stimulation was confirmed by weighing the plants. The fresh weights of orchid plants were increased significantly as indicated by their appearances as shown in Figure 2. The relationship between orchids with OMF is ubiquitous in the plant kingdom. Orchids use their symbionts to gain access to organic and mineral nutrients by increasing absorption and translocation to plants (Smith and Read, 1997). The fungi form hyphae and penetrate the soil around the infected organ which help to break down complex organic materials and tap unusual substrates for nutrients. OMFs can also transfer carbon from neighboring trees to orchids and increase the nutrient absorption surface of host plant root systems (Dearnaley, 2007). Alexander and Hadley (1984) found that plant growth and the uptake of phosphorus and nitrogen were enhanced in mycorrhizal *Goodyera repens* plants. The rate of 32^{P} uptake was up to 100 times greater than that of non-mycorrhizal plants. They also found a reduction in root/shoot ratio in mycorrhizal plants and plantlets. Decrease in root/shoot ratio is often associated with increased nutrient availability. It is not clear whether nutrients was translocated across a living interface between plants and

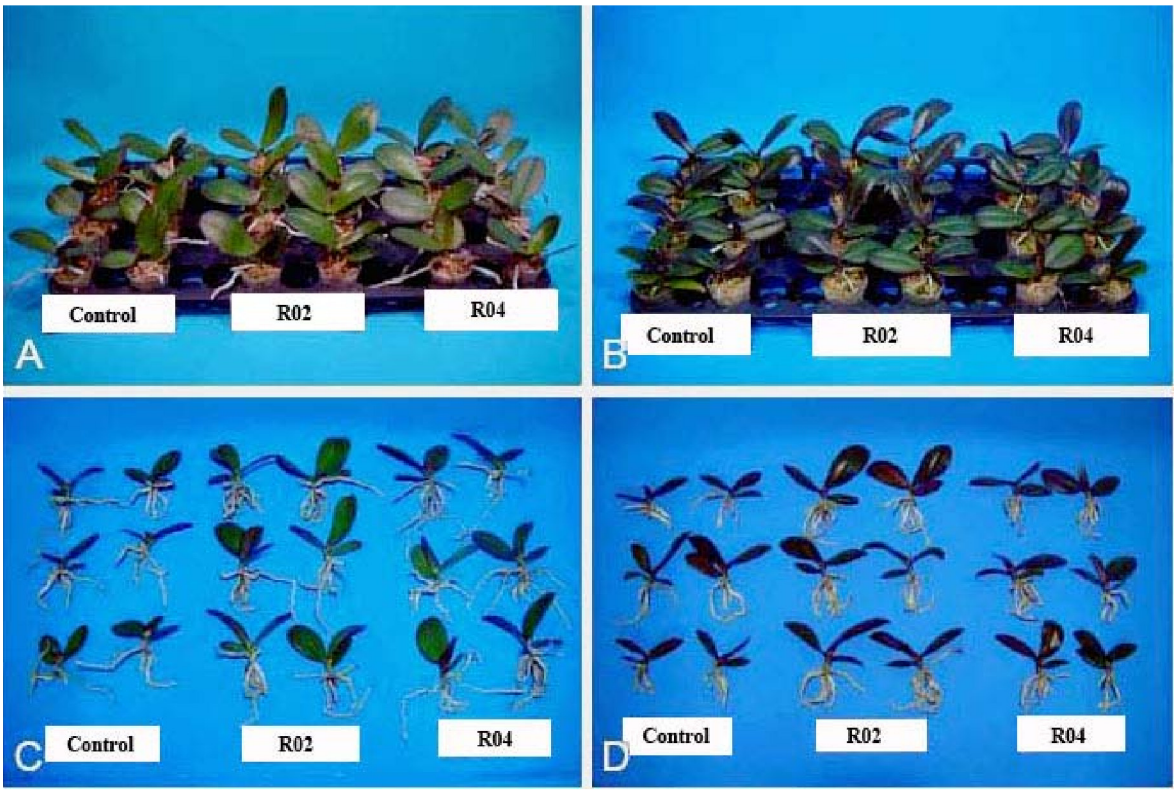

Figure 1. Plant growth of *Doritaenopsis* Taisuco Wonder 'King Car Butterfly' (KC1111) and *Phalaenopsis* Tai Lin 'Redangel 'V31' (V31) inoculated with orchid micorrhizal fungi. (A) and (C) KC111 orchid plants inoculated with *Rizoctonia* isolates R02 and R04, respectively; (B) and (D) V31 orchid plants inoculated with *Rizoctonia* isolates R02 and R04, respectively. Growth stimulation is characterized by orchid plants having a larger leaf span and better root growth than control after 4 months of inoculation.

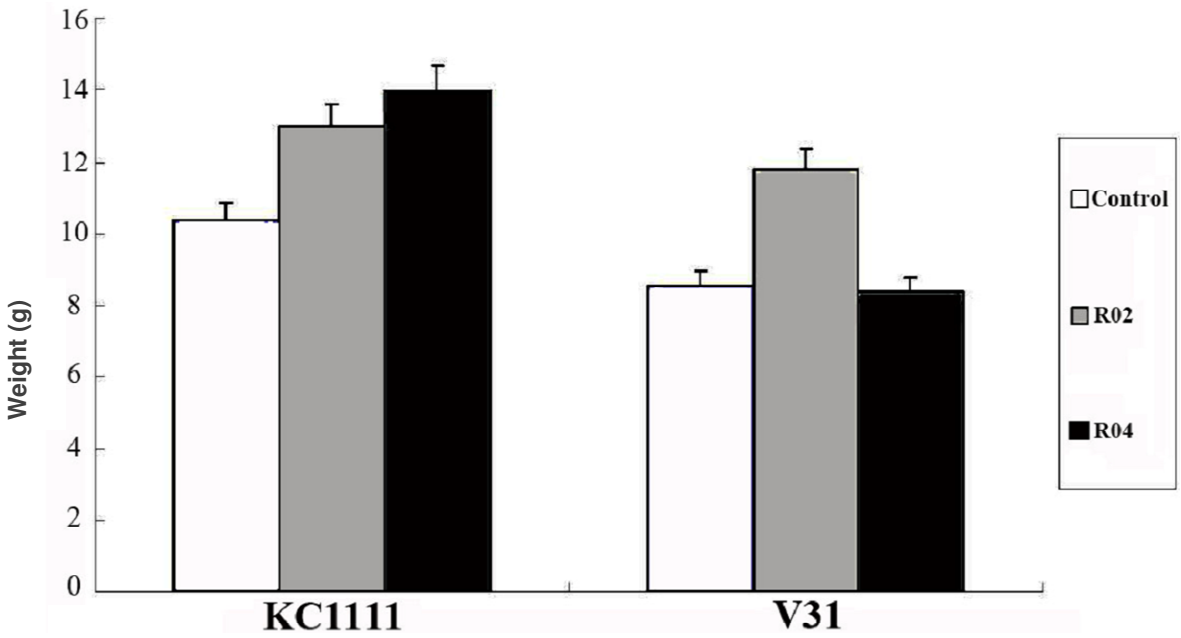

Figure 2. Fresh weights of four months old *Doritaenopsis* Taisuco Wonder 'King Car Butterfly' (KC1111) and *Phalaenopsis* Tai Lin 'Redangel 'V31' (V31) plants after been inoculated with Rizoctonia isolates R02 and R04 respectively. Mean total with a common letter was not different ($P \leq 0.05$) by least significant difference and are expressed in grams.

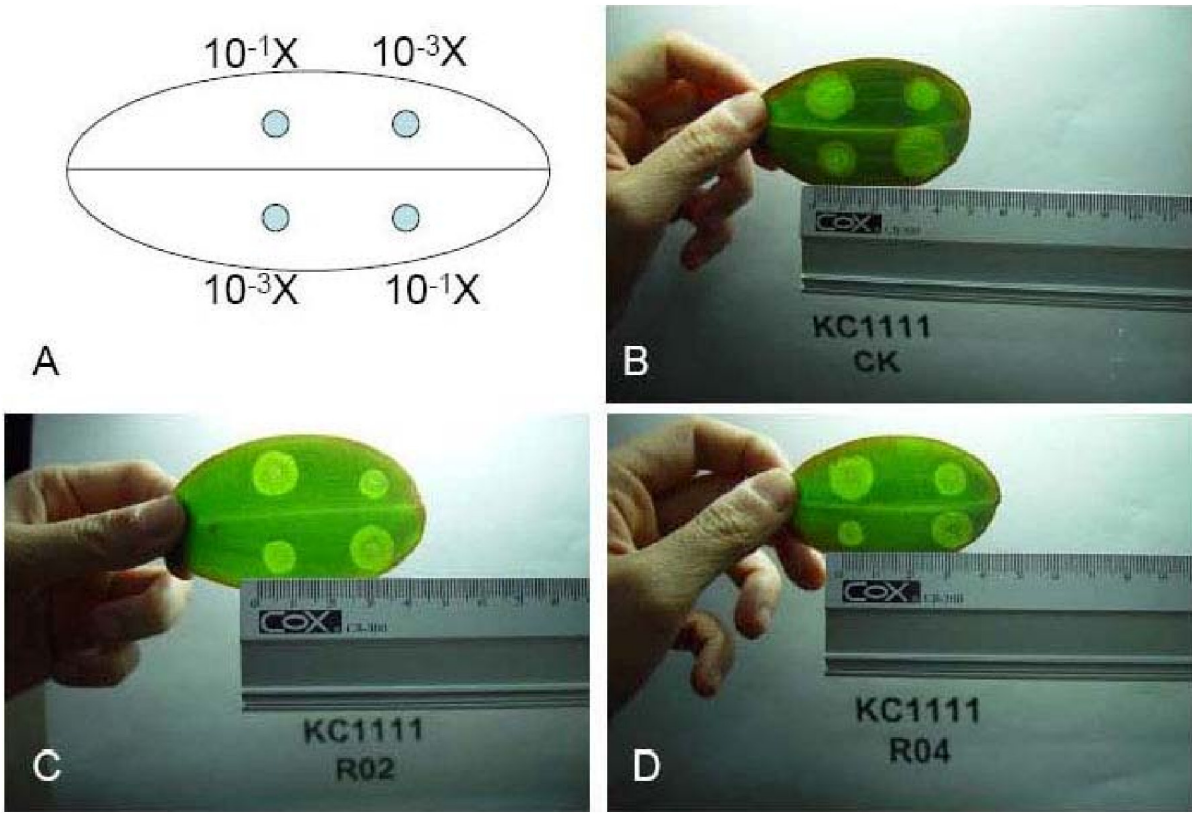

Figure 3. Soft rot development on leaves of *Doritaenopsis* Taisuco Wonder 'King Car Butterfly' (KC1111) inoculated with *E. chrysanthemi* after 24 h of inoculation. (A) 10 µl each of series diluted *E. chrysanthemi* inoculum was applied directly onto the wounding site with a micropipette; (B) non-micorrhizal fungi infected control; (C) *Rizoctonia* isolates R02 infected; (D) *Rizoctonia* isolates R04 infected.

mycorrhiza or released upon digestion of the fungus. It has been suggested that nutrients are possibly derived from the digestion of the fungi. However, it is still not known if the enzymes involved in the digestion are produced by plant or OMF. OMFs inoculation was also reported to improve growth and development of other orchid plants in *Haemaria discolor* (Chang and Chou, 2001) and *Anoectochilus formosanus* Hayata (Chang and Chou, 2007).

Erwinia soft rot progression on leaves of OMF symbiotic orchid

The second leaves from the top, were used for *E. chrysanthemi* inoculation. A series dilution between 1 × 10^{-1} and 10^{-7} was tested for effectiveness of inoculation (data not shown). Soft rot development was characterized by forming a clear circle zone on the leaves (Figures 3 and 4). The disease progressing on the leaves of mycorrhizal KC1111 (inoculated with R02 or R04) and mycorrhizal V31 (inoculated with R02 or R04) were compared using two bacterial dilutions (1 × 10^{-1} and 10^{-3}). The mean of 10 replicates for each treatment is presented in Table 1. The development of soft rot on mycorrhizal KC1111 (inoculated with R04 at highest inoculum) leaves was significantly reduced. Nevertheless, the reduction was more evident on mycorrhizal KC1111 (inoculated with R04) leaves. Mycorrhizal V31 inoculated with R02 significantly reduced soft rot development but not with R04. Disease symptom reduction is correlated to the better plant growth.

It is not known if the reduction in soft rot development is due to the fact that plants grow better, making plants have higher resistance, or that plants are capable of producing antibacterial reagents from mycorrhizal symbiosis. It was found that the activities of acid and alkaline phosphatases in roots and superoxide dismutase in leaves, contents of polysaccharides, polyphenols, flavonoids, ascorbic acid and phosphate were significantly higher in the OMF infected *Anoectochilus formosanus* tissues than in the non-mycorrhizal control (Chang and Chou, 2007). Watkinson et al. (2005) also found that the level of trehalose increased when the orchid *Cypripedium parviflorum* was inoculated with *Thanatephorus pennatus*. It is suggested that trehalose plays a protective role against abiotic stress (Penna, 2003). Genes induced in orchids during mycorrhizal

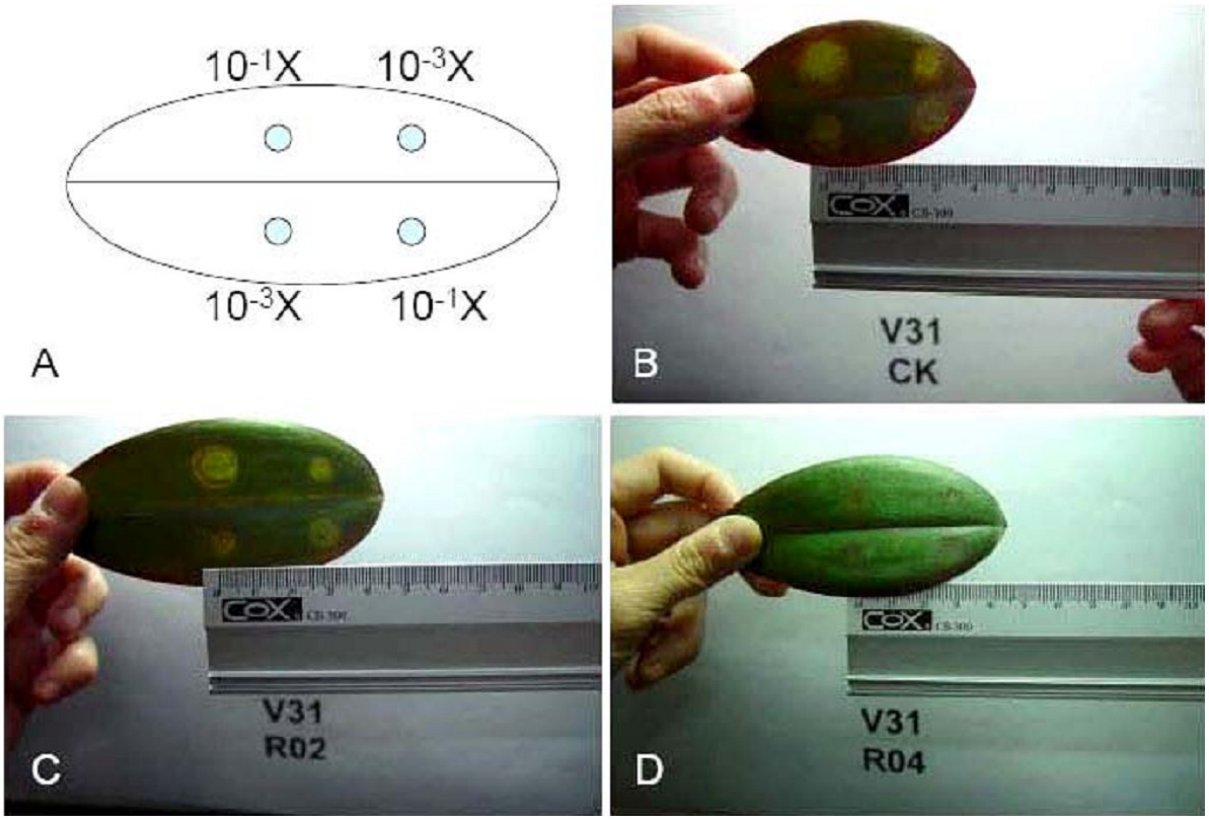

Figure 4. Soft rot development on leaves of *Phalaenopsis* Tai Lin 'Redangel 'V31' (V31) inoculated with *E. chrysanthemi* after 24 h of inoculation. (A) 10 µl each of series diluted *E. chrysanthemi* inoculum was applied directly onto the wounding site with a micropipette; (B) non-micorrhizal fungi infected control; (C) *Rizoctonia* isolates R02 infected; (D) *Rizoctonia* isolates R04 infected.

Table 1. Radius of the soft rot development in orchid micorrhizal fungi (*Rizoctonia* isolates R02 and R04) inoculated *Doritaenopsis* Taisuco Wonder 'King Car Butterfly' (KC1111) and *Phalaenopsis* Tai Lin 'Redangel 'V31' (V31), respectively. Soft rot development was characterized by the formation of a clear circle zone.

Orchid cultivar	Treatment	Radius of soft rot tissue (mm)	
		1×10^{-1} dilution	1×10^{-3} dilution
KC1111	Control	6.9[a]	3.9[a]
	R02	6.4[ab]	4.2[a]
	R04	5.7[b]	3.2[a]
V31	Control	7.0[a]	4.1[a]
	R02	4.3[b]	2.2[b]
	R04	6.0[a]	3.2[a]

Means in each column followed by different letter at 5% significant level are significant as determined by least significant difference (LSD) test.

symbiosis are associated with the initiation of OMF infection and spread, nutrient translocation, digestion of OMF fungi and promoting plant growth. Induction of biotic and abiotic stress related genes were also reported during arbuscular mycorrhiza development in *Medicago truncatula* (Wulf et al., 2003). It is suggested that flavonoids and phenolic compounds may be the signal for molecules produced in plants to induce spore germination and direct mycelium growth to a suitable host in legumes during symbiosis (Hirsch and Kapulnik, 1998; Martin et al., 1999).

The interaction between plant and fungus is suggested

to be highly specific and regulated by the plant to prevent parasitism by the fungus (Hirsch and Kapulnik, 1998; Martin et al., 1999). The fungus recolonises the plant each year and continues to benefit the host. It is therefore assumed that the plant exudes a powerful attractant (Smith and Read, 1997). Orchinol is a phytoalexin produced by the orchid plants and may play such a function to control the development of the mycorrhiza, both in space and time (Beyrle et al., 1995). Thus, the mechanism of increasing soft rot disease resistance by mycorrhiza needs further investigation.

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