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Promoting role of an endophyte on the growth and contents of kinsenosides and flavonoids of *Anoectochilus formosanus* Hayata, a rare and threatened medicinal Orchidaceae plant*

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Abstract: *Anoectochilus formosanus*, commonly known as "Jewel Orchid", is a Chinese folk medicine used to treat hypertension, diabetes, and heart disease. The existence of *A. formosanus* is currently threatened by habitat loss, human and animal consumption, etc. The highly potent medicinal activity of *A. formosanus* is due to its secondary metabolites, especially kinsenosides and flavonoids. This orchid also has a unique mycorrhizal relationship. Most adult orchids rely on endophytes for mineral nutrition and have complex interactions with them, which are related to plant growth, yield and changes in secondary metabolites. This study investigated the promoting role of F-23 fungus (genus *Mycena*) on the biomass and contents of kinsenosides and flavonoids of *A. formosanus* in pot culture. The following were observed after 10 weeks of symbiotic cultivation: increased shoot height, shoot dry weight, and leaf numbers by 16.6%, 31.3%, and 22.5%, respectively; increased contents of kinsenosides, isorhamnetin-3-*O*-β-D-rutinoside, and isorhamnetin-3-*O*-β-D-glucopyranoside by 85.5%, 226.1%, and 196.0%, respectively; some hyphae in epidermal cells dyed red and/or reddish brown by safranine; and, significantly reduced number of starch grains in cortical cells. Moreover, F-23 fungus significantly improved the kinsenoside and flavonoid contents of *A. formosanus*. These findings supported the reports that endophytes can alter the production of secondary metabolites in their plant hosts, although further physiological, genetic and ecological analyses are warranted.

Key words: *Anoectochilus formosanus*, Endophyte, Secondary metabolite, Kinsenoside, Flavonoid
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1 Introduction

Anoectochilus formosanus Hayata, commonly known as "Jewel Orchid" (Cavestro, 1994) because of its beautiful foliage (Teuscher, 1978), is a terrestrial orchid widely distributed in Taiwan and Fujian Province of China, and Japan (State Administration of Traditional Chinese Medicine, 1999). It has been used as a Chinese folk medicine for many years (Fujian Institute of Traditional Chinese Medicine, 1982) to treat hypertension, diabetes, heart disease, lung and liver diseases, nephritis (Mak *et al*., 1990; Huang *et al*., 1991; Chiu and Chang, 1995; Zheng *et al*., 1996; Shih *et al*., 2002), and other diseases (Lin *et al*., 1993; Chan *et al*., 1994; Du *et al*., 2001; Shih *et al*., 2001).

The existence of *A. formosanus* is currently being threatened by its low seed germination rate, habitat loss and degradation, animal consumption, indiscriminate collection, and increasing demand by humans. Its wild sources are gradually decreasing (Zhang *et al*., 2010). Consequently, the prices of its

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fresh and dry states have increased to approximately CNY 320 and 3 200 per kilogram, respectively (Shiau *et al*., 2002), thereby limiting its clinical application. In a previous study, we used a micro-propagation technique for the large-scale production of *A. formosanus* to protect the wild population of this rare and threatened orchid species. The results demonstrate that *A. formosanus* maintains high genetic fidelity even after a 5-year propagation in vitro (Zhang *et al*., 2010).

The highly potent medicinal activity of *A. formosanus* is due to its secondary metabolites, especially kinsenosides. Kinsenosides were first isolated from *A. koshunensis* by Ito *et al*. (1993) and shown to have antihyperliposis and antihyperglycemic effects (Du *et al*., 2001; Zhang *et al*., 2007). Orchids also have a unique mycorrhizal relationship (Bernard, 1902). The mycobionts of most adult orchids have been mainly assigned to the form group Rhizoctonia (Roberts, 1999), as well as to other fungal groups such as Agaricales or Ascomycetes. The characteristic poorly developed root systems of most adult orchids are believed to rely on endophytes for mineral nutrition (Smith and Read, 1997) and have complex interactions with fungal endophytes. Despite all these interactions, fungal endophytes have been related to host plant growth, yield, fitness, and stress responses (Andrade-Linares *et al*., 2011).

Most research work on *A. formosanus* focuses on the large-scale production using the micropropagation technique and the analyses of their chemical constituents, as well as the bioactivities and therapeutic applications. Little is known about the interaction between *A. formosanus* and fungus, especially the promoting role of fungus on the growth and contents of secondary metabolites of *A. formosanus*. A total of 69 strains isolated from *Anoectochilus*, *Dendrobium*, and *Cypripedium* were screened, among which 16 strains were selected for further analysis. Based on pot culture experiments under greenhouse conditions, one strain, namely F-23, was found to have the ability to increase the biomass of *A. formosanus*. Moreover, F-23 fungus was once identified and shown to be a mycorrhizal fungus of *Dendrobium officinale*, which might be of potential use to the mass cultivation of *D. officinale* under artificial conditions (Zhang *et al*., 2011). The present study was aimed to identify the growth-promoting endophytes (F-23) of *A. formosanus*. We investigated the promoting role of F-23 fungus on the biomass and contents of kinsenosides and flavonoids of *A. formosanus* in pot culture. The findings help elucidate the effect of endophytes on the production of secondary metabolites in their orchid host plants.

2 Materials and methods

2.1 Plant and fungal materials

A. formosanus Hayata belongs to *Anoectochilus* of Orchidaceae. After surface sterilization, aboveground parts of *A. formosanus* samples collected from Fujian Province, China, were cut into small pieces of stems with an axillary bud about 1 cm, and cultured in sterile micropropagation medium (MM) containing MS (Murashige and Skoog, 1962) basic medium and supplemented with 0.5 mg/L naphthaleneacetic acid (NAA), 1 mg/L 6-benzyl adenine (6BA), 1 g/L activated charcoal, and 30 g/L sucrose. The medium was solidified with 10 g/L agar and then incubated at (25±2) °C under a 12-h photoperiod and a photosynthetic photon flux density of 50 μmol/(m^2·s) (Zhang *et al*., 2010). After four months of micropropagation in vitro, the aseptic in vitro micropropagated plants of *A. formosanus* were transferred to fresh MM without NAA and 6BA and cultured for another two weeks. The above aseptic in vitro micropropagated plants were grown in a greenhouse for two weeks with a mean temperature, daily radiation, and humidity of 23.3 °C, 20.5 mol/m^2, and 62.5%, respectively. These in vitro micropropagated plants were used as plant materials for further experiments.

F-23 fungus isolated from the roots of *D. officinale* was collected from subtropical forests in Xishuangbanna, Yunnan Province, China. Phylogenetic analysis of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) indicated that F-23 fungus (GenBank accession No. FJ544251) belongs to the genus *Mycena* (Zhang *et al*., 2011). F-23 fungus was grown in 90-mm Petri dishes containing potato dextrose agar in the dark for two weeks at 25 °C. To prepare the inocula, the hyphae of F-23 were transplanted to culture bottles containing sterile substrate containing wheat bran and cottonseed husk (1/1), and grown in the dark for four weeks at 25 °C (Zhang *et al*., 2011). The mock inoculum had no hyphae and contained only wheat bran and cottonseed husk (1/1).

2.2 Plant inoculation

The aseptic in vitro micropropagated plants were transferred to 8 cm×8 cm×9 cm plastic pots filled with a humus soil/vermiculite mixture (HVM; 1/1), and then inoculated with the above F-23 inoculum or mock inoculum.

The plastic pots were initially filled with HVM to one-third of its capacity, and then a thin layer of oak leaves cut into 1 cm×1 cm pieces was evenly sprinkled on the HVM. Afterwards, a thin layer of raw sawdust of pine was sprinkled on the oak leaves. The inoculum (3 g) in small pieces was sprinkled on the sawdust, and *A. formosanus* plants were arranged on the inoculated sawdust. The roots were covered with HVM and buried 1 cm away from the pots' edge. Each pot was thoroughly drenched with tap water until excess water flowed out from the drainage holes. Control plants were added to the mock inoculum and subjected to the same inoculation procedure as above.

Each group consisted of 10 pots with 3–5 in vitro micropropagated plants in a pot. The pots were supplied with tap water every other day. All materials including the HVM, oak leaves, and sawdust were sterilized at 122 °C for 2.5 h prior to experiments.

2.3 Interaction studies

After 10 weeks of growth in the greenhouse, 20 plants from each treatment group were harvested. Endophyte strains were isolated from the roots of plant hosts, and all strains were subjected to further phylogenetic analysis of the ITS region of rDNA. The shoot height (namely the main stem height) and number of leaves were recorded, whereas stems (namely above-ground parts) were bagged and dried at 40 °C for dry mass determination. Fresh roots of the remaining plants were fixed in formalin-acetic acid-alcohol. All samples were observed and photographed (ZEISS Axiolmager A1, Germany) after a series of routine light microscope observations following the protocol of Feder and O′Brien (1968). This protocol involved dehydration in a graded ethanol series, embedding in paraffin, staining with safranine and fast green, and sealing with Canada gum.

2.4 Kinsenoside content

High-performance liquid chromatography (HPLC) was carried out on a Waters™ 600E controller (USA) with an evaporative light scattering detector (Waters 2420, USA) and an autoinjector (Waters 2707, USA). HPLC was performed under the following conditions: drift tube temperature, 45 °C; gas flow, 30 pounds per square inch (psi; 1 psi=6.895 kPa); YMC-Pack Polyamine II, 250 mm×4.6 mm i.d.; particle size, 5 μm; column temperature, 25 °C; and, 77.5% CH_3CN, 1 ml/min.

The dried powders of *A. formosanus* from each treatment were passed through a mesh sieve (250±9.9 μm), accurately weighed (100 mg), transferred into 5 ml of centrifuge tubes containing 2 ml of methanol, and marinated for one night. Given the unstable chemical nature of kinsenosides (i.e., they easily decompose at high temperatures), extraction was performed for 45 min by ultrasonication (HH-SY11-Ni, China) at low temperatures (4–15 °C). The extracts were centrifuged at 10000 r/min for 10 min. After removing the supernatant, 2 ml of deionized water was added to the residue and the extraction was performed again by ultrasonication for 45 min, followed by centrifugation at 10000 r/min for 10 min. The supernatant was collected and 2 ml of deionized water was added to the residue. The extraction and centrifugation procedures were repeated, and the supernatants were combined. The final solution was fixed at 10 ml and filtered through a 0.45-μm membrane. The injection volume of each sample solution was 20 μl. Three individual samples from each group (inoculated and non-inoculated with F-23) were extracted and analyzed by the proposed method. Two replicates of each extract were prepared. The relative standard deviation (RSD, presented as standard deviation (SD)/mean) was calculated as a measurement of method repeatability.

2.5 Flavonoid compound content

The HPLC system consisted of a Waters™ 600E controller, a photodiode array detector (Waters 2996, USA), an autoinjector (Waters 2707, USA), and a Venusil ASB C_{18} column (250 mm×4.6 mm i.d.; particle size, 5 μm). The mobile phase consisted of water containing 15% CH_3CN and 0.5% acetic acid (A) and CH_3CN (B). The gradient program was as follows: 0–15 min, 0–12% B; 15–25 min, 12%–18% B; 25–45 min, 18%–30% B, followed by column re-equilibration with 40% methanol for 15 min. The flow rate was 1 ml/min, the column temperature was 40 °C, and the detection wavelength was 365 nm.

Stock solutions of isorhamnetin-3-*O*-β-D-rutinoside (Bu-h, 1.64 mg/ml) and isorhamnetin-3-*O*-β-D-glucopyranoside (Bu-d, 0.53 mg/ml) were prepared in methanol. For calibration, working standard solutions were prepared by diluting the stock solution with appropriate quantities of methanol, and then stored at 4 °C and brought to room temperature before use.

The pretreatment of *A. formosanus* for each treatment was the same as that described in Section 2.3. Prior to the addition of methanol, all centrifuge tubes were cooled to room temperature to compensate for solvent loss. The conditions of centrifugation and filtration, as well as the injection volume and RSD calculation value were the same.

2.6 Data analysis

The experiment of plant inoculation was performed in a completely randomized block design and each group (inoculated and non-inoculated with F-23) consisted of 10 pots with 3–5 in vitro micropropagated plants in a pot. This experiment was repeated three times. Plant growth responses, including the shoot height, shoot dry weight, and leaf number, were analyzed with SPSS 16.0 software. Statistical comparisons between inoculated and non-inoculated plants were carried out by Tukey's post-hoc test and one way analysis of variance (ANOVA) according to the least significant difference ($P<0.05$).

3 Results

3.1 Effect of F-23 fungus on *A. formosanus* vegetative growth

All *A. formosanus* plants inoculated and non-inoculated with F-23 fungus were harvested 10 weeks after cultivation to determine the influence of the endophyte F-23 on the shoot growth of *A. formosanus* plants. Results showed that F-23 fungus significantly affected the *A. formosanus* in vitro micropropagated plants in pot (Table 1), and that F-23 plants appeared more vigorous than the controls (Fig. 1a). The shoot height and shoot dry weight of F-23-colonized *A. formosanus* plants increased by 16.6% and 31.3%, respectively, whereas the leaf numbers were 22.5% higher compared with controls (Table 1). Phylogenetic analysis of the ITS region of rDNA indicated that all strains isolated from the roots of plant hosts were F-23, which belongs to genus *Mycena*.

Table 1 Effect of F-23 fungus on *A. formosanus* plants

Group	Shoot height (cm)	Shoot dry weight (g)	Leaf number
Control	8.66±1.64	0.067±0.009	2.36±0.63
F-23	10.1±0.96	0.088±0.005	2.89±0.78

Values are presented as the mean±SD, $n=20$. Control: plants not inoculated with F-23 fungus; F-23: plants infected with the fungal endophyte F-23. All $P<0.05$ vs. control

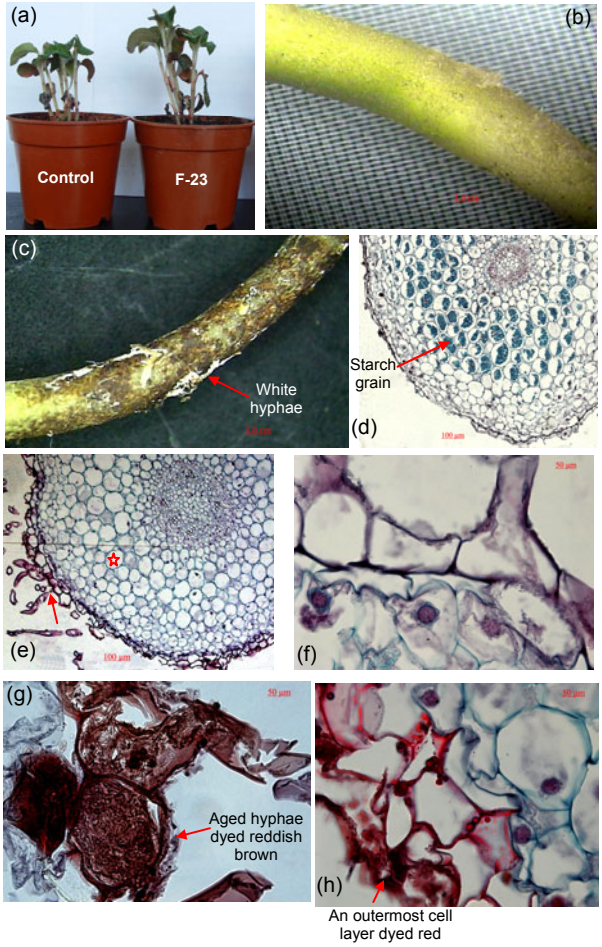

Fig. 1 Images of *A. formosanus* plants and roots

(a) *A. formosanus* plants inoculated with F-23 appeared more vigorous than the controls at the end of the incubation period in pots. (b, c) *A. formosanus* roots of the control group (b) and the F-23 group (c) viewed under a dissecting microscope after a 10-week inoculation. (d, e) Light micrographs of transverse section of roots showing starch grains in the control group (d) and root hairs and outermost layer cells dyed in red (arrow) by safranine and few starch grains (star) in the F-23 group (e). (f) Transverse section of the control showed that the root surface of the control had no hyphae and remained green. (g, h) Transverse section of *A. formosanus* roots infected with F-23 showing aged hyphae dyed reddish brown (g) and an outermost cell layer dyed red (h) (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3.2 Interaction of F-23 fungus with the root of *A. formosanus*

After 10 weeks of symbiotic cultivation, the plants infected with the fungal endophyte F-23 were more vigorous than the controls (Fig. 1a). The root surface of *A. formosanus* inoculated with F-23 fungus had a more remarkable change in color than the control. White hyphae were observed under a dissecting microscope on the surface of *A. formosanus* roots, which became khaki after 10 weeks of interaction between the endophyte F-23 and *A. formosanus* root (Fig. 1c), whereas the root surface of the control had no hyphae and remained green (Fig. 1b).

The cortical cells of the control roots of *A. formosanus* contained more starch grains than that of the transverse section of *A. formosanus* roots infected with F-23 (Fig. 1d). In addition, a transverse section of *A. formosanus* roots infected with F-23 show root hairs and outermost layer cells dyed in red by safranine (Fig. 1e). The transverse section showed that aged hyphae in root hairs and outermost layer cells were dyed reddish brown by safranine (Fig. 1g), and fresh hyphae in outermost layer cells were red (Fig. 1h), whereas the root hairs and outermost layer cells of the control had no change of color (Fig. 1f).

Moreover, several root hairs spread on the root surface of the control, which was not inoculated with hyphae (Fig. 1b). The outermost cell layer of the roots collapsed because of the infection with hyphae, and some hyphae were observed in the outermost cell layer and its adjacent cells (Figs. 1g and 1h).

3.3 Effects of F-23 fungus on the kinsenoside content of *A. formosanus*

The correlation coefficient of kinsenoside was R^2=0.9995, and the linear injection volume ranged from 5.78 to 57.8 μg. The chromatographic retention time (t_R) of kinsenoside was 3.89 min, whereas the t_R values of the control and F-23 groups were 3.873 and 3.877 min, respectively. The kinsenoside content of F-23 group was 85.5% higher than that of the control (Table 2). The RSD values of kinsenosides in these samples were less than 5.0% (Table 2), which showed acceptable variation within the measurement of the method.

Table 2 Chromatographic retention time (t_R) and content of kinsenosides

Group	t_R (min)	Kinsenosides content (mg/g)	RSD* (%)
Control	3.873	73.709	3.02
F-23	3.877	136.759	1.87

* Values of RSD are presented as SD/mean (n=3). Growths of *A. formosanus* non-inoculated and inoculated with F-23 fungus are as control and F-23, respectively

3.4 Effects of F-23 fungus on flavonoid compound content of *A. formosanus*

The t_R values of Bu-h, control, and F-23 groups were 19.209, 19.167, and 19.233 min, respectively. The t_R values of Bu-d, control, and F-23 groups were 20.567, 20.550, and 20.583 min, respectively. The Bu-h content of the F-23 group was increased by 226.1%, whereas the Bu-d content was 196.0% higher than that of the control (Table 3). The RSD of the kinsenoside in these samples was less than 5.1%, which showed acceptable variation within the measurement of the method.

Table 3 Chromatographic retention time (t_R) and contents of isorhamnetin-3-*O*-β-D-rutinoside (Bu-h) and isorhamnetin-3-*O*-β-D-glucopyranoside (Bu-d)

Group	Bu-h		Bu-d		RSD* (%)
	t_R (min)	Content (μg/g)	t_R (min)	Content (μg/g)	
Control	19.167	47.425	20.550	166.647	2.37
F-23	19.233	154.639	20.583	493.336	2.83

* Values of RSD are presented as SD/mean (n=3). Growths of *A. formosanus* non-inoculated and inoculated with F-23 fungus are as control and F-23, respectively

Chromatographic data also showed that some unknown flavonoids apart from Bu-h and Bu-d existed in both samples, especially in the F-23 group (Table 4). The F-23 group had one unknown flavonoid at t_R=10–15 min, whereas the control had two unknown flavonoids at t_R=10–15 and 25–30 min. In addition, with the exception of the newly found unknown flavonoid in the F-23 group at 21.317 min, the chromatographic peak areas all increased by different percentages as follows (compared with the control at the same t_R values): 40.6% at 10–15 min, 182.2% at 15–20 min, 215.5% and 193.5% at 20–25 min, 60.3% and 1179.2% at 30–35 min, and 57.2% at 35–40 min (Table 4).

Table 4 Chromatographic data of unknown flavonoids in the samples

Group	10–15 min		15–20 min		20–25 min		25–30 min		30–35 min		35–40 min	
	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area
Control	10.800	3082	16.633	17518	20.850	8239	21.267	15979	31.800	14599	38.883	19928
	11.467	7813			22.517	2627	23.617	3832	32.883	3234		
F-23	10.917	4334	16.700	49438	20.883	25994	NAP	NAP	31.917	23397	38.967	31318
	NAP	NAP			21.317	24298	NAP	NAP	32.933	41368		
					22.533	7709						

t_R: chromatographic retention time; NAP: no absorption peak at 365 nm. Growths of *A. formosanus* non-inoculated and inoculated with F-23 fungus are as control and F-23, respectively

4 Discussion

Orchids belong to the largest plant family, which was estimated to comprise more than 25000 species worldwide (Jones, 2006). Various orchid species are economically important, including terrestrial species, epiphytes, and lithophytes. Some orchids are used as food and drink flavoring, and others are used as important natural medicines. Orchids also have a huge horticultural market worth 100 million dollars annually in the USA alone (Griesbach, 2002). Although terrestrial species account for only one-third of orchids, almost half of extinct orchid species are terrestrial herbaceous perennials according to the International Union for Conservation of Nature. These perennials are most likely to be at a greater extinction risk because of increased threats to their existence, especially under the current climatic change scenarios (Swarts and Dixon, 2009). However, research on the unique mycorrhizal relationships of orchids (Bernard, 1902) considerably lags behind research on other important mycorrhizal types, particularly the arbuscular mycorrhiza. Accordingly, in this study we observed the growth and contents of secondary metabolites of *A. formosanus* inoculated with F-23 fungus in open pot cultures.

As described by Imhof *et al*. (2013), mycoheterotrophic plants lack chlorophyll and depend on an intimate association between their underground organs and fungi. These fungi were considered to be connected to photosynthetic plants for carbon compounds and some of them involved may also increase access to soil-derived nutrients. Our results showed that F-23 fungus significantly affected *A. formosanus* in vitro micropropagated plants in pots. Tang and Guo (2004) demonstrated that an endophyte of *Epulorhiza* sp. can increase the survival rate, fresh weight, dry weight, and activities of four enzymes (chitinase, β-1,3-glucanase, polyphenol oxidase, and phenylalanine ammonia-lyase) of *A. formosanus* in open pot cultures. The contents of fructose, sucrose, polysaccharide, and total soluble sugar were also enhanced when *Epulorhiza* sp. was present but not when no inoculum was added (Tang *et al*., 2008). Therefore, we hypothesized that the increase in *A. formosanus* biomass may be due to F-23 fungus by increasing the enzyme activities and photosynthesis of the host plant.

It is becoming increasingly clear that soil microbial communities comprised of a vast array of bacteria, fungi, nematodes, and other organisms, are not passively determined but actively regulated by plants (Sugiyama *et al*., 2012). Light micrographs of the interactions between *A. formosanus* root and F-23 fungus showed that the number of starch grains was significantly reduced in cortical cells, which was reported for the first time. Some reddish brown aged hyphae were observed in the outermost layer of cells and adjacent cells. Several reasons have been suggested for the decomposition of starch grains in cortical cells. First, the decomposition of starch grains may provide carbon for endophyte growth. Second, it may enable continued fungal colonization because N, P, and water continue to flow from the fungal partner, although carbon exchange is essentially reversed with photosynthate in adult photosynthetic orchids (Dearnaley, 2007). Third, under the promoting role of the endophyte, the decomposition of starch grains may provide the carbon required for the growth of the host plant *A. formosanus* itself. In addition, all the abovementioned reasons may simultaneously occur because nutrient exchange in photosynthetic orchids is highly complex (Selosse *et al*., 2004; Cameron *et al*., 2006).

Plants secrete both high- and low-molecular weight compounds from their roots, whose exudates function not only as nutrients for soil microbes but also as signal molecules in plant-microbe interactions (Sugiyama and Yazaki, 2012). Studies of the changes in secondary host metabolites as an effect of orchid mycorrhizal relationships are limited. Nevertheless, reports that endophytes can alter the production of secondary metabolites in their plant hosts are increasing. Chen *et al*. (2006) found that the production of alkaloids in *Dendrobium candidum* protocorms can be induced by a fungal elicitor. Our study showed a significant increase in kinsenosides and flavonoids in *A. formosanus* when co-cultured with F-23 fungus. Sugiyama and Yazaki (2012) demonstrated that flavonoids and strigolactones in root exudates serve as signal molecules to establish the symbiotic interactions between legume plants and rhizobia or/and arbuscular mycorrhizal fungi, while root exudates also function to acidify surrounding soils to acquire phosphate. Moreover, Demain and Fang (2000) demonstrated that secondary metabolites, including a heterogeneous group of chemically different natural products, may be related to survival functions for the producing organism. These survival functions include symbiosis, differentiation, metal transport, especially competition against other micro- and macro-organisms, etc. The mechanisms of changes of some secondary metabolites require further physiological, genetic and ecological analyses. Our results contributed to this interesting subject and may facilitate the discovery of novel compounds. The findings can also enable the development of strategies for enhancing the production of secondary metabolites in other natural plants using suitable endophytes.

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Compliance with ethics guidelines

Fu-sheng ZHANG, Ya-li LV, Yue ZHAO, and Shun-xing GUO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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