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New Cultivation Methods for *Anoectochilus formosanus* Hayata

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ABSTRACT

Anoectochilus formosanus Hayata is a beautiful orchid plant with ornamental and medicinal use in Taiwan. Growth of *A. formosanus* Hayata seedlings *in vitro* or *ex vitro* could be highly enhanced by the inoculation of orchid mycorrhizal fungi R02 or R04. R02 is a binucleate *Ceratobasidium* sp. AG-A while R04 is known as multinucleate *Rhizoctonia solani*, AG-6. In this orchid, we observed a tolypophagy-type of mycorrhizal infection with hyphae coil and pelotons formed in the cortex cells of roots. This orchid was very susceptible to *Fusarium*, *Pythium* spp. and mites during *ex vitro* growth. So how to grow this orchid without applying pesticides and insecticides became our prime concern. We developed a plastic bag cultivation method (PBCM) to grow *A. formosanus* plants in a 5 inch plastic pot with fermented bark compost as the growth medium, and every two of the 5 in. pots were covered with an OPP transparency plastic bag (9.2 × 7.5 × 30.0 cm) for protection of water loss, disease and mite infection. Thereafter, plants with PBCM were placed under benches in greenhouses or trees, until 6-8 months later for harvesting. No more water or fertilizer was needed and minimum labor was required during cultivation. This PBCM had been proved to be a very effective and low cost cultivation method for producing fungicide- and insecticide-free *A. formosanus* for medicinal use.

Keywords: labor saving, orchid mycorrhiza, plastic bag cultivation method (PBCM)

INTRODUCTION

Anoectochilus formosanus Hayata is a beautiful native terrestrial Jewel's orchid plant growing wild in the forests of Taiwan. It has been used for ornamental and medicinal purposes, such as to lower high blood pressure, to treat antihyperlipsis, diabetes, liver, heart and lung diseases and has antitumor and antivirus properties (Lin 2007; Wu 2007). So these plants are collected and sold at more than 300 US$/kg in fresh weight. If the plants were collected in the wild, which are mycorrhizal in nature, this price would be triple or more. Now they are mass propagated by micropropagation. Seeds of this orchid germinated well in Hyponex agar (Chou 2004) as well as in oat meal agar, in which orchid mycorrhizal fungi R02 or R04 was inoculated (Tsai l997; Chou and Chang 2004). But this orchid was highly susceptible to *Fusarium*, *Pythium* spp. and mites during *ex vitro* growth. So how to grow this orchid without applying pesticides and insecticides became our primary concern. Many reports have focused on the asymbiotic germination of *A. formosanus* Hayata (Tang *et al*. l996; Shiau *et al*. l998; Chang 1999), and several of my graduate students reported the effects of orchid mycorrhizal fungi (OMF) on this orchid (Chou 1997; Tsai l997; Lee 2001; Li 2001; Chou 2004).

This study attempts to enhance the growth of this orchid by the inoculation of R02, R04 in *in vitro* and *ex vitro* growth, and to prevent the infection of pathogens and mites during cultivation by using a plastic bag cultivation method (PBCM).

MATERIALS AND METHODS

Inoculum

OMF of *Rhizoctonia* spp. isolates, namely R02 and R04 OMF were produced on peat medium and supplemented with 20% of V-8 juice to field capacity level (Chang and Chou 2001) as the inoculum. Only one OMF isolate was inoculated for each treatment. The R02 was identified as a binucleate *Ceratobasidium* sp. AG-A, accession No. DQ102413, 99% match (Cheng, Department of Horticulture, National Taiwan University, pers. comm.) while R04 was known as multinucleate *Rhizoctonia solani*, AG-6 (Lee 2001).

Plant material

Micropropagated *A. formosanus* plantlets were bought from a commercial tissue culture company in Puli, Taiwan. The *A. formosanus* plantlets were cut and grown in agar medium for 8-10 months to develop shoots and roots, and only those plantlets with plant height ~10 cm or higher were chosen for *ex vitro* growth. In addition, the micropropagated plantlets in bottles were placed in the greenhouse for about one week to adapt to the new environment before transplanting. Micropropagated *A. formosanus* plantlets were named as either line C (Chiu) or T (Taitung) by the tissue culture supplier Mr. Chiu who had collected both lines in the wild of Taiwan.

Medium for asymbiotic and symbiotic growth of protocorms and seedlings

(a) Hyponex # 3 agar medium (H3; containing Hyponex #3 3 g , tryptone 3 g, sucrose 20 g and agar 7.8 g in 1 L of water) (Chang and Chou 2001) for asymbiotic growth, and (b) OMA (2.5 g/L) medium for symbiotic growth as inoculated with either R02 or R04 *ex vitro*. The *A. formosanus* was line C and/or T for protocorm and *ex vitro* growth.

Ex vitro containers, plant lines, growth medium, water and fertilizer and inoculation of OMF

Five inch plastic pots with a special hook for metal support were used as the growth container. The *A. formosanus* were lines C and T for the plastic bag cultivation test. Hyponex #3 fertilizer was diluted 1000X (1 g/L) as a fertilizer solution. The fertilizer solution was carefully mixed with the fermented bark growth medium until about field capacity, which was roughly estimated by squeezing the somewhat wet bark, in which a few drops of fertilizer solution would come out through spaces between fingers. 10 plants were placed per pot. The *A. formosanus* plantlets were removed from glass containers and the agar was discarded by shaking the roots in tap water. The wet plants should be put under shade until no more tap water is on them. Then we placed about

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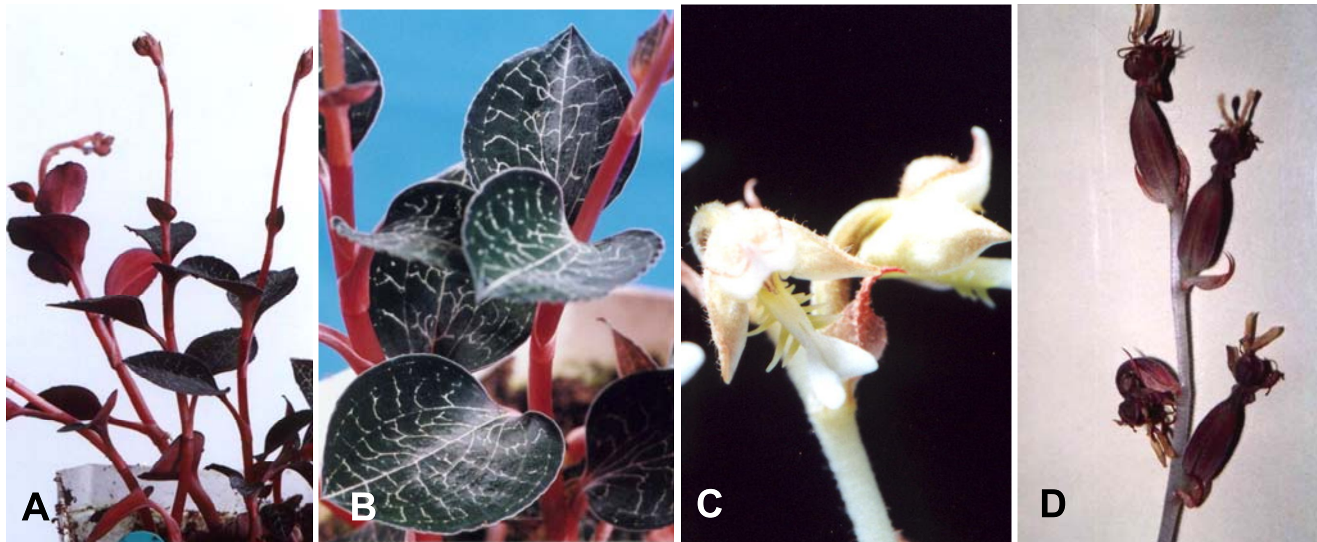

Fig. 1 Morphology of *Anoectochilus formosanus* plant (A, B), flower (C) and seed capsule (D). The whole plant was used as medicine for antitumor, lung and liver protection, etc.

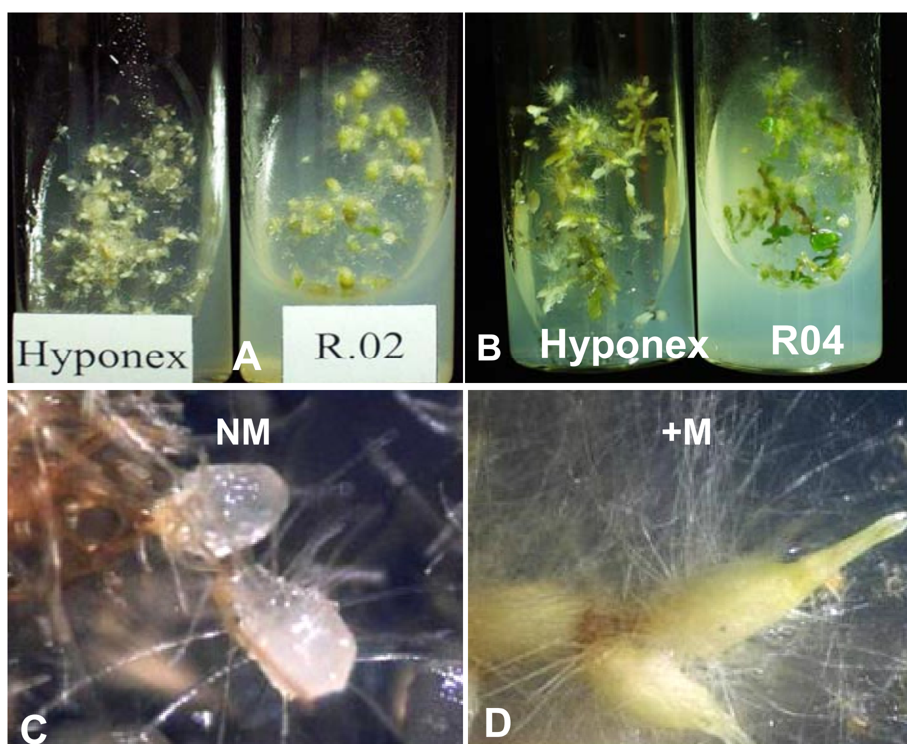

Fig. 2 Asymbiotic (NM) and symbiotic germinations of *Anoectochilus formosanus* Hayata seeds on Hyponex medium for non-mycorrhizal control (NM) and oat meal agar for mycorrhiza plants (R02, R04) for 2 months (A, B). Note that the growth of the mycorrhizal seedlings (**A**, R02; **B**, R04; **D**) was faster than those of the non-mycorrhizal control (NM in **C**).

1/2 volume of Bai-Chu-Hai growth medium – which is a fermented bark compost, containing NPK=0.4: 0.2: 0.2 (%) – into pots. About 1-2 g of OMF inoculum was applied evenly on the bark for the inoculation of OMF for 10 seedlings, onto which the orchids were then placed. Only one isolate of R02 or R04 was inoculated under the roots of the plants in each treatment. More bark was then added to the plants as usual.

Plastic bags, metal supporting and sealing

Commercially available transparent polyethylene (PE) or oriented polypropylene (OPP) plastic bags (9.2 X 7.5 X 30.0 cm) were used. After the plants were transplanted, and the OMF inoculation was completed in the pots, a metal support for each pot was fixed by side hooks on the pots to prevent the collapse of the plastic bag and then to seal the bag by rolling the top edges down twice and then tightening it with binder or paper clips. From that point onwards no more water and fertilizer were supplied for 6 months or even longer, until the plants were harvested.

Observation of structural changes by light microscopy

Roots of *A. formosanus* with or without inoculation of the OMF were collected. Cleared roots were stained with aniline blue in acidic glycerol (Koske and Gemma l989) for light microscopy.

Measurement of net CO_2 uptake rate and titratable acidity

Net CO_2 uptake rate was measured in those plants grown by PBCM or without a plastic bag by using a photosynthetic apparatus LI-6400 (LI-COR, Lincoln, Neb., USA). The titratable acid was measured by taking the third leaf on top of each plant with 2 hour intervals (Chu *et al*. 1990).

Statistical analyses

At least 10-15 plants were used per replication, and means and standard errors were calculated from three replicates. Data was subjected to analysis of variance (ANOVA) with mean separation (p = 0.05) by Duncan's multiple range test (DMRT) and used for

the tables and the graphs which were plotted by Excel or Sigmaplot.

RESULTS AND DISCUSSION

Recently R02 was identified as a *Ceratobasidium* sp. AG-A, accession No. DQ102413, 99% match by DNA method, while R04 is known as *Rhizoctonia solani*, AG-6 (Lee 2001).

The morphology of *A. formosanus* plants is shown in **Fig. 1**. It is a beautiful jewel's orchid with red stems (**Fig. 1A**) and clear veins (**Fig. 1B**), and the lower side of leaves is red in color (**Fig. 1A**). *A. formosanus* has multiple functions as a medicinal plant, and was considered as the 'medicine-king' by the aborigines of Taiwan for centuries.

The asymbiotic protocorm growth results showed that H3 had a somewhat higher germination rate than those of mycorrhizal seedlings inoculated with R02 in oat meal agar medium for 2 months (**Fig. 2A, 2B**). But the growth of the mycorrhizal seedlings (**Fig. 2A, 2B, 2D**) was faster than the non-mycorrhizal control (**Fig. 2A, 2B**, hyponex and **2C**), while the inoculation of binucleate (R02) and multinucleate (R04) OMF could highly enhance the growth rates of *A. formosanus* (**Fig. 2A-D**). These results confirmed again that R02 and R04 were effective isolates (Tsai l997). Mass propagation of *A. formosanus* seedlings or plantlets could be easily achieved. But for *ex vitro* growth, the plants were highly susceptible to root rot (*Fusarium* spp.), stem rot (*Pythium* spp.) and mites infection. Commercial growers of this orchid have to frequently apply pesticides and insecticides. This big problem should be urgently resolved because this orchid is consumed as a herbal medicine. Therefore, the plastic bag cultivation method (PBCM) was developed by using special designed plastic pots (**Fig. 3A-D**). PBCM not only can protect the plants from the infection by diseases, mites and insects as well as 2.7 L glass jars (**Fig. 4B**), but can also be grown in greenhouses (**Fig. 4C**) or under trees or forests in the open air (**Fig. 4D**). And the cost of the plastic bag (**Fig. 4A**) is only about 1/40 of the big glass jar (**Fig. 4B**). The plants could bear flowers and seed capsules if hand pollination was used. Normal roots formed (**Fig. 5A**). The fungal hyphae could penetrate roots through the epidermis or even root hairs (**Fig. 5B**). The pelotons formed in the cortex of *A. formosanus* roots were stained with aniline blue and observed by light microscopy (**Fig. 5C, 5D**). The results of structural change indicated that the infection of OMF on *A. formosanus* was a type of tolypophagy (**Fig. 5**), thus, hyphal coils (**Fig. 5C**) and pelotons formed (**Fig. 5C, 5D**) in the cortical region of the roots (Hadley 1982).

Fig. 3 Five inch plastic pot with hook (arrow) on both sides for metal support (MS) was the container using for plastic bag cultivation of *Anoectochilus formosanus* Hayata.

The growth comparison of non-mycorrhizal and mycorrhizal protocorms (**Table 1**) *in vitro* and *ex vitro* seedlings (**Table 2**) of *A. formosanus* showed significant differences. The growth of plants with and without plastic bags for 4 months is shown in **Table 3**. If plants grew longer, more fresh weight could be achieved by the PBCM treatment, more than the control (**Fig. 6**). Since this plant is sold by fresh weight, so an increase in fresh weight would be very

Fig. 4 Plastic bag cultivation method (PBCM) for *Anoectochilus formosanus* Hayata (A). The price of plastic bag was only 1/40 of the 2.7 L glassware (**B**). The plants by PBCM could be put under benches in greenhouse (**C**) or trees (**D**) until harvest.

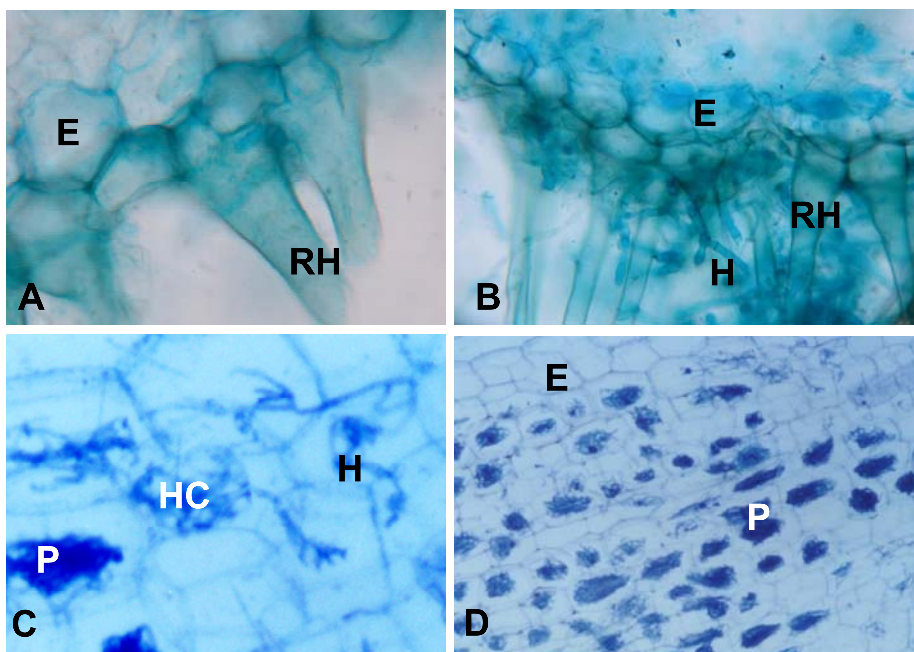

Fig. 5 Light microscopic observation of aniline blue stained *Anoectochilus formosanus* roots. Note that non-mycorrhizal control with root hair (RH in **A**) and epidermis (E). Mycorrhizal root cells with root epidermis (E), and the presence of orchid mycorrhiza fungal hyphae (H) and root hair (RH) in **B**. Hyphal coil (HC) and peloton (P) formed in the mycorrhizal roots in **C** and **D**.

Table 1 Protocorm growth of *Anoectochilus formosanus* Hayata after the inoculation of orchid mycorrhizal fungi *in vitro* for 4 months.

Medium, treatment	Plant height (cm)	Leaf No.	Leaf length (mm)	Leaf width (mm)	Root No.	Fresh weight (mg)
Hyponex #3 NM	1.6 b	1.0 a	3.0 b	2.0 b	2.0 a	50.1 b
OMA, NM	1.2 c	0.0 b	0.0 c	0.0 c	0.0 b	15.5 c
OMA, R02	2.3 a	2.1 a	7.0 a	4.0 a	3.0 a	67.3 a
OMA, R04	2.0 a	2.0 a	6.0 a	3.5 a	2.5 a	65.1 a

15 replicates were tested for each treatment.
NM: non-mycorrhizal control. OMA: oat meal agar medium. R02, R04: orchid mycorrhizal fungi.
P=0.05.

Table 2 *Ex vitro* growth of *Anoectochilus formosanus* Hayata seedling after the inoculation of orchid mycorrhizal fungi for 4 months.

Treatment	Plant height (cm)	Leaf No.	Root No.	Node No.	Fresh weight (mg)
NM	2.4 b	4.0 b	3.0 b	4.0 a	71.8 b
R02	4.0 a	5.8 a	4.9 a	4.8 a	256.0 a
R04	4.1 a	6.0 a	5.0 a	4.5 a	265.5 a

15 replicates were tested for each treatment.
NM: non-mycorrhizal control. R02, R04: orchid mycorrhizal fungi.
P=0.05.

Table 3 Growth of *Anoectochilus formosanus* Hayata for 4 months by control or plastic bag cultivation methods.

Cultivation method	Plant height (cm)	Leaf No.	Leaf length (mm)	Leaf width (mm)	Root No.	Fresh weight (mg)
Control	6.3 b	5.0 a	2.0 a	1.6 a	3.7 a	1.6 a
Plastic bag cultivation	8.1 a	5.0 a	2.0 a	1.6 a	3.8 a	1.8 a

15 replicates were tested for each treatment.
Means in each column followed by the different letters were significantly different (P=0.05) as determined by Duncan's multiple Range Test.

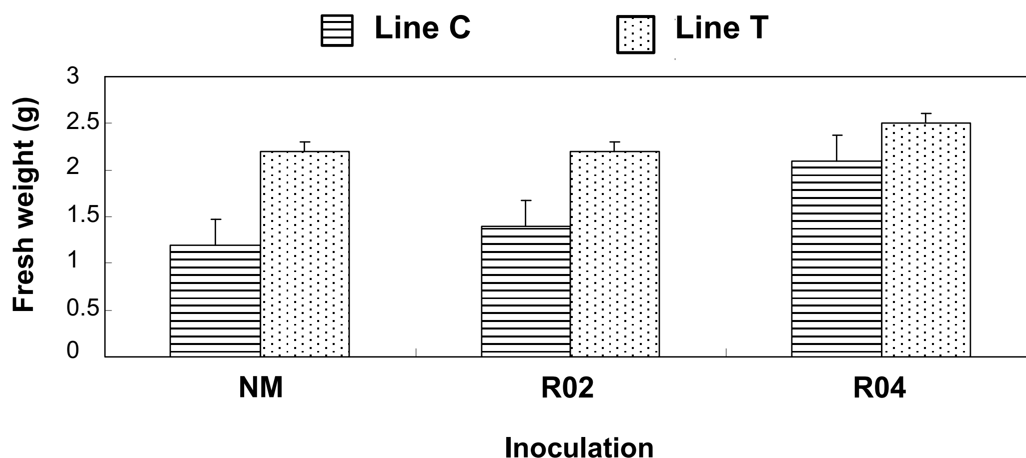

Fig. 6 Fresh weight (g) of *Anoectochilus formosanus* Hayata plants in greenhouse of NTU when cultivated in plastic bag for 4 months.

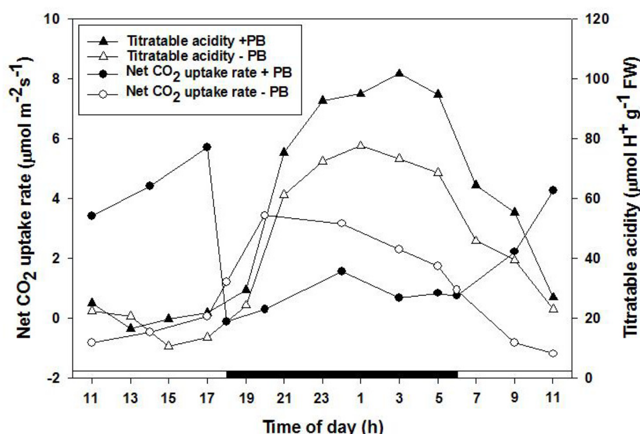

Fig. 7 Changes of CO_2 uptake rate and of titratable acidity of leaves of *Anoectochilus formosanus* plants grown in plastic bag (+PB) or without (–PB). Note that this orchid is a CAM plant in nature, and changes its CO_2 uptake pattern as influenced by the PBCM.

beneficial to growers.

After the plants were sealed in plastic bags for up to 12 months, even with no additional supply of water and fertilizer, plants still grew and remained healthy. For those without protection by plastic bags, most plants died after 4 months, due to the serious infection of *Fusarium* spp. and mites. PBCM thus proved to be an effective and extremely labor-saving way of growing *A. formosanus*. Some of the plantlets transplanted from tissue culture flasks would die even when cultured by PBCM. If healthy micropropagated plantlets and sterilized growth medium were used, or some fungicides were applied into water for removing the agar on the roots, then the death of the plants would be much more reduced. For those plants grown by PBCM, none have ever been infected by mites.

The CO_2 fixation pattern for *A. formosanus* and net CO_2 uptake rate and titratable acidity (**Fig. 7**) for those plants grown with or without plastic bags changed. There was not much difference in fluctuation pattern for the titratable acid of the leaf between PBCM or ordinary pot cultivation method (OPCM). But the acid contents in PBCM were much higher than those grown without bags. The results showed that the acid contents increased sharply during the dark period, but decreased soon during the day (**Fig. 7**), which is typical for CAM plants. But those plants in PBCM take up more CO_2 during the day. The results indicated that *A. formosanus* was a CAM plant in nature (without bag), but would shift from CAM to a C3-like in plastic bags (**Fig. 7**). This finding could help to explain why the plant grew faster by PBCM. It was suggested that in PBCM, the *A. formosanus* plants became facultative CAM plants.

Fig. 8 Growth of *Anoectochilus formosanus* by control method (left) and plastic bag cultivation method (right).

CONCLUSIONS

The inoculation of binucleate (R02) and multinucleate (R04) OMF could highly enhance the protocorm growth *in vitro* (**Fig. 2; Table 1**) as well as the growth of *A. formosanus ex vitro* (**Table 2; Fig. 6**). The use of plastic bag cultivation method (PBCM; **Fig. 8**) not only could protect the plants from the infection of diseases and insects, but also could highly increase the growth of this orchid. For CO_2 uptake, this plant can change from CAM (without bag) to a C3-like plant (with bag) by the PBCM, and hence enhance the growth of *A. formosanus*. Before disease and mite resistant lines of *A. formosanus* are found, the PBCM is an effective and extremely labor-saving way to prevent the infection of *Fusarium* spp., *Pythium* spp. and mites. The *A. formosanus* plants produced by PBCM are safe for use as a herbal medicine. Now this method is applied by several orchid growers in Taiwan, and has proved to be very useful for growing this medicinal kind of orchid. It is suggested to use a bio-degradable plastic bag that can last for one year for PBC of *A. formosanus* in practical use.

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