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Effects of Several Organic Extracts on the Growth, Yield and Quality of *Anoectochilus Formosanus* Biomass

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Abstract *Anoectochilus formosanus* is a high economic value herbal remedy, the oldest in herbal medicine. Nowadays, *A. formosanus* has been applied in many products of caring human health. However, *A. formosanus* in nature have slow growth characteristics, restrictive adapting conditions and susceptible to fungal diseases. Application of plant tissue culture technology can help to produce a large amount of *A. formosanus* biomass in controllable conditions. To be able to use *A. formosanus* biomass for serving human, plant growth regulators should not be used because of their disadvantageous affected on production quality. This topic surveys the effects of some organic extracts, using traditional plant tissue culture, on growing ability, yield and quality of *A. formosanus*biomass. Some residual minerals which are useless for our health, such as NO_3^-, heavy metals (Cu, Zn, etc.) were also investigated for evaluating the quality of *A. formosanus*biomass. After 60 days of culture, media supplementing with coconut water, yeast extract or *Spirulina* extract separately influenced positively to the growth and development of *in-vitro A. formosanus*. Yeast extract with the concentration of 2 g/l obtained the highest results in all targets, average height of explant was 11.69 cm/explant, average fresh weight was 2.57 g/explant. Contents of NO_3^-, Cu^{2+}, Zn^{2+} are lower than the allowed threshold of FAO in all treatments. This study also examines the influence of culture density effecting on the yield capacity of *A. formosanus*. Results showed that, when cultured at a density of 10 explants per sigma box, their growth and development were better than others, achieved 251.44 g/L (total biomass/medium)

Keywords: *Anoectochilus formosanus*, coconut water, yeast extract, spirulina extract, liquid culture, micropropagation

Introduction

Anoectochilus formosanus is known as a high economic value medicinal plant, because it contains many biologically active substances, such as β-sitosterol, 4-hydroxycinnamic acid, β-D-glucopyranosyloxy and

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butanoidglucosides acid (Takatsuki *et al.*, 1992). In Oriental medicine, *A. formosanus* is used in many remedies to cure many disease, such as chest pain, stomachache, diabetes, nephritis, high fever, high blood pressure, importance, liver dysfunction, spleen dysfunction and chest throbbing pain (Lin, 2007). The genus *Anoectochilus*has 30 to 40 species and is widespread, among all those species *A. formosanus*Hayata is interested and application most recently. With its value, *A. formosanus* has been strongly exploited and used as materials for many human health care products.

However, in nature, *A. formosanus* has slow growth, low multiplication, its root and trunk are tender and sensitive to *Fusarium oxysporum*, which seriously affects its vitality. With the advantage of sciences and technology, micro-propagation method has been used on *A. formosanus* to create a large amount of seedlings. In 2007, Yoon *et al.* studied the biomass production of *A. formosanus*Hayata by bioreactor systems. Meng *et al.* (2008) studied the effects of plant regulators on the axillary bud of *Anoectochilus roxburghii*. Phung*et al.* (2010) studied for shoot multiplication of *in vitro Anoectochilus roxburghii* species. Shao *et al.* (2014) studied the effect of darkness on the characteristics of photosynthesis, chloroplasts structure and physiology of *Anoectochilus* genus. Lately, Vu *et al.* (2015) studied the effect of *in vitro* conditions on the process of breeding and quantity β-sitosterol activity of *Anoectochilus setaceus* Blume.

Organic extracts are non-compulsory ingredient of culture medium. Depending on each object, organic extracts can be supplemented into culture medium at different stages of micro-ropagation process. These extracts commonly have roles in the growth and development of explants. The present of some extracts also lead to decreasing plant growth regulators. In 2014, Paris *et al.*, studied the effects of coconut water and peptone on improving seed germination and protocorm like body formation of hybrid Phalaenopsis Supplementing organic extracts would stimulate the multiplication of *Cymbidium pendulum* (Kaur *et al.*, 2012). *A. formosanus* has been studied and produced for enhancing and protecting human health, motivating no plant growth regulators be used.

This report was performed for surveying the effects of some organic extracts on the growth and development of *A. formosanus* in non-plant growth regulators conditions to determine their roles, enhance the productivity and quality of their biomass. Besides, this report also evaluated the quality by defining the residual content of NO_3^-, Cu^{2+}, Zn^{2+}.

Materials and methods

Materials

A. formosanus shoots with the height of 3 to 4 cm, the mass of 0.66 to 0.70 g culturing at Department of Plant cell technology, Institute of Tropical Biology, were used as the material for experiments.

The materials was cultured in airy sigma boxes, of which cover attached a 1 cm diameter filter membrane with the size of pore was 0.2 µm.

Culture media

SH medium (Schenk and Hildebrandt, 1972) supplemented with 30 g/l sucrose, 7.5 g/l agar, 0.4 g/l active coal and organic extracts depending on each treatment of experiments. Sterilized steam at 121^0C, 1 atm., in 20 minutes.

Culture conditions

The sigma boxes were placed under light intensity of 50 ± 5 µmol/m^2/s, lighting time of 12 hours/day, the average temperature of 25 ± 2^0C and humidity of 75 – 80%.

ffects of some organic extracts on the growth and development of in-vitro A. formosanus

A. formosanus shoots were cultured on medium supplemented with each organic extract with different concentrations depended on treatments as follows:-Coconut water: 50 ml/l, 100 ml/l, 150 ml/l, Spirulina extract (Earthrise, USA): 1 ml/l, 3 ml/l, 5 ml/l and Yeast extract (Hi Media Laboratories Pvt. Ltd): 1 g/l, 2 g/l, 3 g/l. Each treatment was repeated 3 times, observation targets were collected after 60 days of culture.

Effects of culture medium on the content of NO3-, Cu2+, Zn2+accumulating in A. formosanus culturing

A. formosanus shoots were culture on medium supplemented organic extracts individually, of which concentration was the best from previous experiment. After 60 days of culture, the completed explants of *A. formosanus* were collected, dried and treated by the Kjeldahl method. The sample were determined the accumulation of heavy metals Cu^{2+}, Zn^{2+} and NO_3^- by Spectro Direct spectrophotometer to assess the quality of *in vitro* explants.

Effects of culture density on yield and quality of in-vitro A. formosanus

The shoots were implanted into sigma boxes containing the best medium, which was the best from previous experiment, with 5 explants/box, 7 explants/box, 10 explants/box. Recording results of total biomass obtained respectively with 1 liter medium used for each density treatment, each one repeated 3 times.

Statistical analysis

The experiment was set in a completely random, the data are recorded and analyzed by Stat Graphics, ranking LSD at 0.05 level of significance.

Results

Effects of some organic extracts on the growth and development of in-vitro A. formosanus

Organic extract is known as an noncompulsory ingredient of plant cell culture medium. Depends on research objects, organic extracts can be added into culture medium at different stages of *in vitro* propagation process. These extracts play their roles in the growth and development of explants. The present of some extracts also help decreasing the plant growth regulators in *in vitro* culture. Research and products of *A. formosanus* with the aim is strengthen and protect human's health, so no plant growth regulators used has become more necessary. This paper surveyed the impact of coconut water, yeast extract and spirulina to the growth and development of *A. formosanus* shoots without using plant growth regulators.

After 60 days of culture, the recorded and analyzed result was showed in Table 1. According to that, treatments added organic extracts with different concentrations had positive effect which stimulated the growth and development of *A. formosanus*, as the result, almost their target observation was higher and significantly different compare to Control sample (Ctrl) added no organic extract.

Table 1. Effects of organic extracts on the growth and development of *in-vitro A. formosanus*

Treatments	Organic extracts	Concentration	Height of explant (cm)	Number of leaves/explant	FW/explant (g)	DW/explant (g)
Control	0	0	9.33g	4.92c	1.69e	0.16e
D50	Coconut water	50 ml/l	9.89f	5.57ab	2.00cd	0.19d
D100		100 ml/l	10.40e	5.85a	2.34ab	0.23ab
D150		150 ml/l	11.04cd	5.85a	2.35ab	0.23ab
N1	Yeast extract	1 g/l	11.15bc	5.5ab	2.19bc	0.21bc
N2		2 g/l	11.69a	6.0a	2.57a	0.25a
N3		3 g/l	10.30e	5.21bc	2.04cd	0.19cd
T1	Spirulina	1 ml/l	10.84d	5.92a	1.94d	0.19d
T3		3 ml/l	11.41ab	5.85a	2.34ab	0.24ab
T5		5 ml/l	10.42e	5.71ab	1.95d	0.18d

Different letters a, b, c, etc. show the significant difference with $p \leq 0.05$ by ranking LSD

Surveyed effects of coconut water with different concentrations from 50 to 150 ml/l, the value of targets increase linearly with the increasing of coconut water concentration. Considering the height of shoot, treatments had significant difference and achieved the highest value at the treatment supplemented with 150 ml/l coconut water (the average height of shoot was 11.04 cm/explant). Number of leaf had no significant difference between treatments. Fresh weight (FW) and dry weight (DW) also increased as the increasing of coconut water concentrations, however, there was no difference between treatment supplemented 100 ml/l and 150 ml/l coconut water. Comparing treatments added yeast extracts and control, yeast extracts had positive effects on the growth of explants. Medium supplemented with yeast extracts obtained the highest results at all target observation compared with other surveyed extracts. Medium supplemented with 2 g/l yeast extract achieved the best result in all target observation. The average height of shoot was 11.69 cm, the average fresh weight was 2.57 g/explant, the average number of leaf was 6 leaves/explant. Observed explants after 60 days of culture, the explants of treatment N2 adding 2 g/l yeast extract were the biggest ones and leaves were dark (Fig. b2). Yeast extract contains amino acids (glycine, lysine and arginine) and vitamins,

especially inositol and thiamine (vitamin B1), which impulses the growth of explants. Increasing the concentration of yeast extract to 3 g/l, the growth of *A. formosanus*decreased clearly at all the targets. This showed that increasing the ingredients of yeast extract would lead to restricting the growth of cell. Tran Vu Ngoc Thi (2014) also published the similar consider when researched on *Curcuma zedoaria*Roscoe. The author believed that increasing concentration of yeast extract from 0.5 g/l to 4.0 g/l, it would restrict the accumulation of *C. zedoaria*Roscoe cell suspension biomass.

The results in Table 1 showed that adding spirulina extract motivate positive effects on the growth and development of *in vitro A. formosanus* (Fig. c2). Increasing concentration of spirulina extract, the targets also linearly increased. The best results were achieved of treatment T3 adding 3 ml/l spirulina extract, which occurred the average height of shoots was 11.41 cm/explant, the average number of leaf were 5.85 leaves/explant, FW and DW respectively were 2.34 and 0.24 g/explant. The ingredients of spirulina extract contains amino acids, vitamins such as vitamin A, vitamin B and vitamin E, helps the metabolism process, helps explants absorb nutrients, motivates their growth and development. The nutrient value of spirulina extract led to an advantage condition for explant to grow. However, increasing the concentration of spirulina extract to 5 ml/l, results of the targets decreased. Particularly, treatment T5 adding 5ml/l spirulina extract, motivated the average height of shoots was 10.42 cm/explant, the average number of leaf were 5.71 leaves/explant, FW and DW respectively were 1.95 g/explant and 0.18 g/explant. This showed higher concentrations of spirulina extract (5 ml/l) might restrict the growth of *A. formosanus*.

Therefore, the organic extracts such as coconut water, yeast extract and spirulina extract with different concentrations were surveyed in this research all showed positive effects on the growth and development of *A. formosanus* shoots. Especially the treatment supplementing with 2 g/l yeast extract achieved the best results.

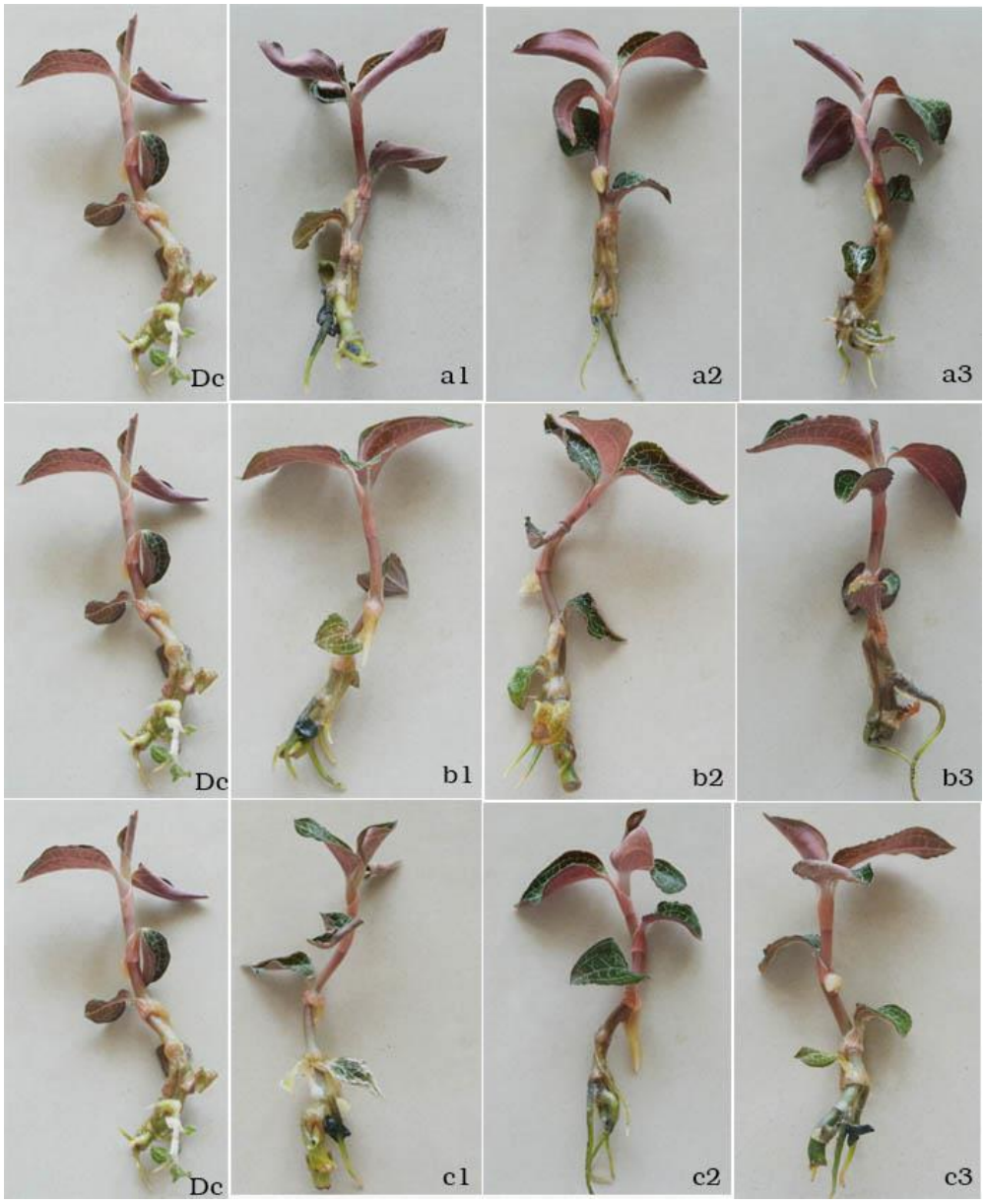

Figure 1. Effects of some organic extracts on the growth and development of *in vitro A. formosanus*

a1: 50 ml/l coconut water; a2: 100 ml/l coconut water; a3: 150 ml/l coconut water; b1: 1 g/l yeast extract; b2: 2 g/l yeast extract; b3: 3 g/l yeast extract; c1: 1 ml/l spirulina extract; c2: 3 ml/l spirulina extract; c3: 5 ml/l spirulina extract.

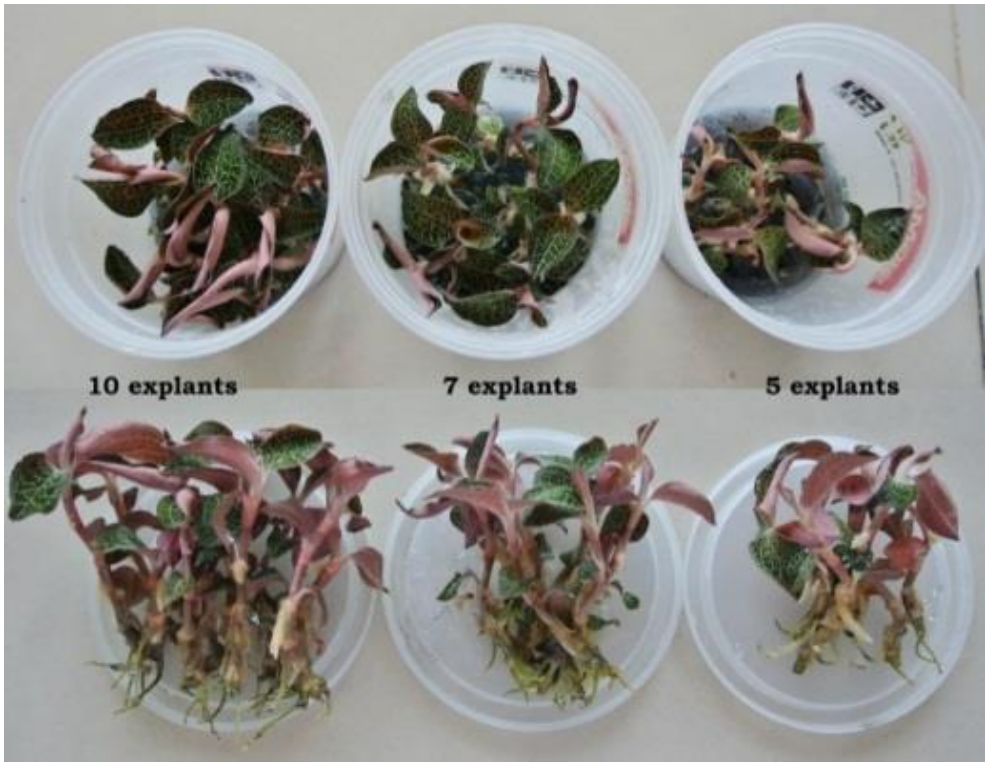

Figure 2. Effects of culture density on the yield of *in vitro A. formosanus*
10 explants/box; 7 explants/box; 5 explants/box, respectively from the left.

Effects of culture medium to the content of NO3-, Cu2+, Zn2+ accumulating in in-vitro A. formosanus

Nowadays, food safety has been an essential issue. With the aim is to produce biomass for pharmaceutical, the norm of food safety, ingredients of non-eliminated accumulation matter need to be concerned. Especially the accumulation of heavy metals (Cu, Zn, Fe …). The high content of NO_3^- also impacts negatively to human's health. For estimating the quality of *A. formosanus*, we performed to evaluate some targets: the content of NO_3^-, Cu^{2+}, Zn^{2+} accumulated in *in vitro A. formosanus* biomass.

The explants were cultured on nutrient medium supplementing with different organic extracts. The concentrations of these extracts were the best results of previous experiment. After 60 days of culture, obtaining, analyzing and evaluating the targets of explants. Analyzed the content of NO_3^- in *A. formosanus*, the result showed all explants on surveyed media containing NO_3^- below than the allowed threshold (followed Decision number 867/1998/QĐ-BYT of Department of health). The results of Table 2 also

showed the content of heavy metals such as Cu^{2+}, Zn^{2+} was very low and below than the allowed threshold of FAO. Therefore, SH medium supplemented with coconut water, yeast extract or spirulina extract separately not only promote the growth and development of explants, but guarantee the quality of *in vitro* explants, safety about residual heavy metal like Cu^{2+}, Zn^{2+} and the content of NO_3^- for human.

Table 2. Effects of nutrient medium to several quality targets of *in vitro Anoectochilus formosanus*

Treatments	The content of NO_3^- (mg/kg FW)	The content of heavy metals (mg/kg FW)	
		Cu^{2+}	Zn^{2+}
Coconut water	77.2	0.80	0.02
Spirulina extract	129.92	1.08	0.01
Yeast extract	92.12	0.68	0.01

Effects of culture density on the yield of in-vitro A. formosanus

Culture density is an important request to growing naturally. Density impacts much to crop plant yield. On the same cultivated area, the higher growing but still suitable for the growth and development of crop plants, the higher income profit. On laboratory size, culture density also has its important effects on the growth and development of explants. As growing naturally, in laboratory condition with the same medium volume, the same cultured area that may product and still guarantee the quality will bring to us much value, especially in *in vitro* multiplication. This report aims to improve the process of producing a large amount of *A. formosanus* biomass in laboratory conditions to supply medicinal product for health care productions. Therefore, determining the appropriate density is necessary to improve the yield of *in vitro A. formosanus*.

Table 3. Effects of culture density on the yield of *in vitro A. formosanus*

Treatments	Number of explants/box	Height of explants (cm)	Number of leaves/explant	FW/explant (g)	Sum of FW/liter of medium (g)
C5	5	10.43c	5.78	1.93b	112.63c
C7	7	11.53b	5.90	2.60a	198.98b
C10	10	11.95a	6.20	2.59a	251.44a

Different letters a, b, c, .etc. show the significant difference with $p \leq 0.05$ by ranking LSD

The results showed that different culture density also effected on the growth and development of explants (Table 3). At the height target, after 60 days of culture, treatment with the highest culture density were 10 explants/box that achieved the average height of explants was 11.95 cm. Increasing culture density would reduce the number of failed explants, helps retain them straight. Moreover, plants have light-guide characteristic, so they would develop vertically to get light downwardly. Increasing density would lead to stimulating the growth of shoots to catch the light. That can explain why the height of plants is linear with culture density.

In the same nutrient medium, the explants of different treatments showed no statistical difference in the target of the number of leaves. But in FW target, there was significant difference. The explants of the treatment with 5 explants/box got the lowest FW (1.93 g g/explant). Two other treatments got no significantly different FW. This showed that, the culture density and surveyed time, culture medium still supplied fully nutrient for explants, so all explants got the height and FW higher.

The total amount of weight collected correlatively to 1 liter medium was an important indicator to evaluate the effects of *A. formosanus* multiplication in each treatment. This target would show a brief assessment of economic efficiency in the use of culture media. When compared between treatments, there are significant differences in different culture density. Culture productivity increased linearly with the culture density. Particularly, treatments C5 (5 explants/box) obtaining a total number of FW was 112.63 g, correlatively with 1 liter culture medium. Treatment with culture density of 7 explants/box got 198.98 g/liter of medium, almost two-fold comparing to the C5 treatment. Keeping on increasing the culture density to 10 explants/box, the total FW obtained 251.44 g/liter of medium, this was the highest result comparing to other treatments. Therefore, the culture density of 10 explants/box not only help the explant growing and developing well, but has its effective on multiplication *A. formosanus*.

Discussion

The research finding wasthe Fresh weight and dry weight increased coconut water concentration, there was no difference between treatment supplemented 100 to 150 ml/l coconut water. As similar to George *et al.* (2008) who reported that coconut water contained amino acids, nitrogen component, inorganic component, organic acids, carbohydrate source, vitamins and had the regulation ability of the growth, as cytokinin and auxin. Coconut water contained 94% water that impulse plant growth process (Yong *et al.*, 2009).

Result showed coconut water played the role in the growth of *in vitro A. formosanus*. Yeast extract contained amino acids, vitamins, inositol and thiamine (vitamin B1), which impulses the growth of explants (Zoltán Molnár *et al.*, 2011).

This ressut showed that increasing the ingredients of yeast extract would lead to restricting the growth of cell. Tran Vu Ngoc Thi (2014) reported as the similar consider when researched on *Curcuma zedoaria*Roscoe. The author believed that increasing concentration of yeast extract from 0.5 g/l to 4.0 g/l, it would restrict the accumulation of *C. zedoaria*Roscoe cell suspension biomass.

The ingredients of spirulina extract contains amino acids, vitamins such as vitamin A, vitamin B and vitamin E, helps the metabolism process, helps explants absorb nutrients, motivates their growth and development (Dillon *et al.*, 1995).

Conclusion

The organic extracts such as coconut water, yeast extract or spirulina extract supplementing individually into SH medium have a positive impact to stimulating the growth and development of *in vitro A. formosanus*. Explants culturing on those medium has ingredients of accumulative heavy metals Cu^{2+}, Zn^{2+} and content of NO_3^- lower than the allowed threshold of FAO, safety for pharmaceuticals. Culture medium supplementing with 2 g/l yeast extract is the best one for the growth of explants. The appropriate culture density is 10 explants/sigma box for enhancing the yield of *A. formosanus*.

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