



AXILLARY SHOOTS DERIVED FROM SHOOT TIPS IN *IN VITRO* MASS PROPAGATION OF *ANOECTOCHILUS FORMOSANUS* HAYATA

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ABSTRACT. Axillary shoot proliferation in *in vitro* mass propagation of *Anoectochilus formosanus* was successfully established via selection of different explant types, accessions and culture media to plantlet acclimatization. In the initiation stage, shoot tips and Murashige and Skoog (MS) medium containing 1.5 mg l⁻¹ N6-benzylaminopurine (BAP) and 0.25 mg l⁻¹ α-naphthalene acetic acid (NAA) were determined as high potential explant and medium for axillary shoot regeneration of A-1 and A-2 accessions of *A. formosanus* compared to others. High axillary shoots up to 7.0 shoots per explant with 1.0 cm shoot height and 9.8 leaves per explant derived from shoot tip explants of A-1 accession were significantly induced and proliferated in MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA. Higher root formation up to 2.4 roots per shoot and 1.0 cm root length of A-1 accession was easily prepared on Hyponex medium (2 g l⁻¹ 20N:20P:20K) containing 150 ml l⁻¹ coconut water (CW). While high survival rate of acclimatized plantlets as high as 90.4 % was successfully done by planting them in plastic boxes containing a mixture of burned rice-husk and organic manure (1:1, v/v) after 2 months. In the study, it was also revealed that in *in vitro* culture of A-1 accession of *A. formosanus* showed higher response compared to A-2 accession in all *in vitro* culture stages. The successful established protocol expected can be applied for preparing high-quality planting materials for commercial purposes and developing new route of *in vitro* mass propagation for other species of *A. formosanus*.

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Introduction

Anoectochilus is one of Orchidaceae family members and belong to a group of terrestrial orchids that are generally known as "Jewel Orchids" (Cavestro, 1994; Shiau *et al.*, 2002). The genus consists of 40 species widespread throughout Southeast Asia, New Caledonia and Hawaii (Ket *et al.*, 2004). *Anoectochilus formosanus* is one of jewel and terrestrial orchids grown primarily for its beautiful foliage as well as for medicine benefits (Tseng *et al.*, 2006; Wu *et al.*, 2007; Gutiérrez, 2010). The *A. formosanus*, normally grow at elevation range between 800 and 1.500 meter above sea level in Lanyu Island in Taiwan, Ryukyu Island in Japan and China's Fujian Province (Refish *et al.*, 2015), is an herbal plant widely used as dietary

supplement and folk remedy in Asia (Kuan *et al.*, 2011). For health purposes, it can be applied as hepatoprotection (Du *et al.*, 2003; Wu *et al.*, 2007; Fang *et al.*, 2008), anti-fatigue (Ikeuchi *et al.*, 2005), antioxidative (Wang *et al.*, 2002; Wang *et al.*, 2005), anti-hyperglycemia (Shih *et al.*, 2002; Ho *et al.*, 2018), anti-hyperliposis (Fang *et al.*, 2008), antiosteoporosis (Masuda *et al.*, 2008), anti-tumor (Shyur *et al.*, 2004; Tseng *et al.*, 2006), and immune modulation (Kuan *et al.*, 2011). The species also has high potential widely cultivated in several areas of Indonesia, however, developing the plant is constrained by availability of high-quality planting materials.

A. formosanus is a slow-growing perennial herb. Growth and development of its seedlings to reach maturity and bear seed take 2–3 years (Tsay, 2002). The plant is traditionally propagated by seeds, but their

germination rate is very low (Ket *et al.*, 2004). It flowers only once a year, especially in winter (October to December) (Tsay, 2002). Nowadays due to over-collection from natural resources, in one hand and less conservation efforts, this orchid is under the threat of extinction (Belitsky, Bersenev, 1999). Therefore, to overcome all these limitations, to maintain and prepare the availability of high quality of planting materials both for conservation and commercial purposes, propagation of the plants via tissue culture works is importantly addressed.

Several works in *in vitro* propagation on *Anoectochilus* were published previously. Several species of *Anoectochilus* developed via the *in vitro* cultures were *A. elatus* (Sherif *et al.*, 2012; Sherif *et al.*, 2016; Raja, 2017), *A. formosanus* (Chang, Chen, 1987; Shiau *et al.*, 2002; Tsay, 2002; Ket *et al.*, 2004; Refish *et al.*, 2015), *A. sikkimensis* and *A. regalis* (Gangaprasad *et al.*, 2000), *A. roxburghii* (Zhang *et al.*, 2015). These works generally used several explant sources such as nodes, internodes, leaves and shoot tips; Murashige and Skoog (1962; MS) medium used as basic medium; N6-benzyladenine (BA), N6-benzylaminopurine (BAP, thidiazuron (TDZ), kinetin (Kin), N6-(2-isopentenyl) adenine (2-iP), Zeatin (Zea), 2,4-dichlorophenoxyacetic acid (2.4-D), α -naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 4-amino-3,4,6-trichloro picolinic acid (Pic) applied as chosen hormones either individually or in combinations; and citric acid, trisodium citrate, peptone, coconut water, potato extract, and banana pulp utilized as potential additives applied in these cultures. Varied application and combination of them stimulated varied results, however, each *in vitro* culture stage needs specific treatment and condition.

Establishment of a new and reliable protocol route of *in vitro* propagation of *A. formosanus* as main objective of the study was successfully determined. The protocol was initiated by culturing shoot tips of A-1 accession as explant source for initiation and proliferation of axillary shoots, followed by plantlet preparation and its acclimatization. Detail and interesting finding in each step were discussed in this paper.

Materials and Methods

Planting material and its preparation

Materials used in the study were two accessions of *A. formosanus* viz, A-1 and A-2 accessions collected by farmer at Jagakarsa, Jakarta. The harvested shoots with 4–5 leaves and 5–7 cm in length derived from the two accessions were explants sources utilized in the experiments. Before their sterilization, the explants were prepared by cutting leaves remaining petiole basal parts for 1–2 mm in length. The prepared explants were ready for sterilization stage.

Explant Sterilization and Preparation

All explants with short basal parts of petioles were pre-treated by placing them under running tap water for 60 minutes (min), followed by immersing them in 1% soap solution for 30 min, 1% pesticide solution (50%

benomil and 20% kanamycin sulphate) for 30 min with manual shaking and then rinsing 4–5 times (@ 3 min each) using distilled water until all disinfection materials totally removed. The explants were then brought to laminar air flow cabinet for sterilization. They were soaked in 0.05% mercury chloride (HgCl₂) solution added by 5 drops of Tween 20 for 5 min, followed by rinsing them with sterile distilled water (SDW) 2 times (@ 3 min each) with manual shaking. The remaining petiole basal parts were then removed carefully using tissue culture blade. The shoot tips with one short node and the third node were sliced and put in different 25 ml Erlenmeyers containing 5 ml SDW. After all explants successfully prepared, all shoot tip and node explants were further sterilized using 0.01% HgCl₂ for 3 min and rinsed with SDW 4–6 times (@ 3 min each) with the same treatment. The explants were then cultured in different initiation media.

Culture media

Basic media used in the study were full strength MS medium and half strength MS medium. All media components both macro, micro and vitamin were from Merck (Darmstadt, Germany), BAP and NAA from Sigma-Aldrich (Darmstadt, Germany), Swallow agar (Jakarta-Indonesia). Initiation, proliferation and rooting media were prepared by mixing stock solution of macro, micro, Fe chelate, and vitamin. The pH of media was adjusted to 5.8 using Model 420A pH meter of Thermo Orion (Beverly, USA) using 1N NaOH or HCl. After pH adjustment, the media were added by 30 g l⁻¹ sucrose (except as treatment) and 7 g l⁻¹ Swallow agar; mixed homogenously, boiled, poured in jam bottles (7 cm in diameter, 13 cm in height; 30 ml media per bottle) and sterilized for 20 min at 121°C and 15 kPa using Pressure Steam Sterilizer Vertical Cylindrical LS. 001, SMIC (Shanghai, China).

Culture incubation

Culture incubation applied in the study was light incubation. The light incubation was performed by placing explant cultures under cool fluorescent lamps for 12 h photoperiod with ~13 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Incubation period for all experiments was approximately 2 months.

Effect of explant types and initiation media on axillary shoot regeneration and proliferation

In the step, there were two types of explants tested *i.e.* shoot tips and nodes. While initiation media (IM) applied in the study were MS medium containing (1) 1.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA (IM-1), (2) 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (IM-2), (3) 1.5 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA (IM-3), (4) 1.5 mg l⁻¹ BAP and 0.05 mg l⁻¹ NAA (IM-4) and (5) 1.5 mg l⁻¹ BAP and 0.025 mg l⁻¹ NAA (IM-5). The experiment was arranged in a split plot design with three replications, where explant types of shoot tips and nodes were main plot; and 5 initiation media as subplot. Each treatment consisted of 3 bottles and each bottle was cultured 3 explants. Total explants used in the experiment were 270 explants. Two accessions of A-1 and A-2 of *A. formosanus* in the step were studied in different

experiments separately due to optimal handling and results expected.

Effect of proliferation media on axillary shoot production of two accessions of *A. formosanus*

In the stage, promising initiation medium established in the previous experiment *i.e.* MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA was optimized by reducing MS medium strength from full to half-strength; varying BAP concentration from 1.5 mg l⁻¹ increased to 1.75 mg l⁻¹ and reduced to 1.25 mg l⁻¹; and applying MS medium fortified by 1.5 mg l⁻¹ BAP, 0.25 mg l⁻¹ NAA and 60 mg l⁻¹ adenine sulphate (AS) promising medium from different study. Proliferation media (PM) tested in the stage were (1) MS medium fortified by 1.5 mg l⁻¹ BAP, 0.25 mg l⁻¹ NAA and 60 mg l⁻¹ AS (PM-1), (2) MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (PM-2; control), (3) MS medium supplemented with 1.75 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (PM-3), (4) MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (PM-4), (5) Half-strength MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (PM-5; control), (6) Half-strength MS medium supplemented with 1.75 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (PM-6), and (7) Half-strength MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA (PM-7). Shoot tips were better explant source shoot regeneration determined from the previous study were used to investigate response of A-1 and A-2 in the proliferation experiment. The experiment was arranged in a split plot design with three replications, where two accessions of A-1 and A-2 were applied as main plot; and 7 PM as subplot. Each treatment consisted of 3 bottles and each bottle was cultured 3 explants. Total explants used in the experiment were 378 explants.

Shoot rooting

Preparing plantlets for acclimatization purpose were carried out by culturing shoots with 2–3 leaves and ± 2.5 cm in height on different rooting media. The rooting media (RM) tested in the stage were (1) MS medium supplemented with 60 g l⁻¹ sucrose (RM-1), (2) MS medium supplemented with 60 g l⁻¹ sucrose and 1.5 g l⁻¹ activated charcoal (AC) (RM-2), (3) Half-strength MS medium with full vitamin (RM-3), (4) Half-strength MS medium with full vitamin and 1.5 g l⁻¹ AC (RM-4), (5) MS medium containing 0.2 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA (RM-5), (6) MS medium supplemented with 0.25 mg l⁻¹ BAP and 1.5 g l⁻¹ AC (RM-6), and (7) Hyponex medium (2 g l⁻¹ 20N:20P:20K) containing 150 ml l⁻¹ coconut water (CW) and 1.5 g l⁻¹ AC. The experiment was arranged in a split plot design with three replications, where two accessions of A-1 and A-2 were applied as main plot; and 7 RM as subplot. Each treatment consisted of 3 bottles and each bottle was cultured 5 explants. Total explants used in the experiment were 210 shoots.

Plantlet acclimatization

Plantlets of A-1 and A-2 accessions with 5–8 leaves, 3–5 cm in height and 2–3 roots derived from the previous experiment were used for acclimatization step. The plantlets were then pulled out from culture bottles gently using blunt forceps. The roots of plantlet were put under running tap water to remove remains of agar attaching them. The plantlet roots were then immersed in 1% pesticide solution (50% benomil and 20% kanamycin sulphate) for 3 min, air-dried them on paper for a while, then cultured on plastic boxes containing mixture of burned-rice husk and organic manure (1:1, v/v) watered sufficiently. The plastic boxes were then covered by plastic transparent for 30 days. Each week, the transparent plastic was opened, then the acclimatized plantlets were sprayed by 1 g l⁻¹ Growmore high N solution then covered again. Each plastic box was planted ± 40 plantlets for two replications. The experiment was arranged in complete randomized design (CRD) with 8 replications. Each treatment consisted of 20 plantlets. Total plantlets acclimatized in the step were 320 plantlets.

Variables

Variables observed in the study were (1) Number of shoots per explant, (2) height of shoot (cm), (3) number of leaves per explant, (4) number of roots per shoot, (5) length of roots (cm), (6) percentage of survivability (%), and (7) Number of survival plantlets and Quality of plants. Periodical observation in each experiment was carried out to know and observe response and alteration of explant during incubation period. Final observation and variables measurement were recorded ± 2.0 months after culture.

Analysis of Data

Collected data generated from these experiments were carried out by analysis of variance (ANOVA) using SAS Release Windows 9.12. If there were significant differences between means, the mean values were further analyzed using Tukey test, P = 0.05 (Mattjik, Sumertajaya, 2006).

Results

Effect of explant types and initiation media on axillary shoot regeneration of A-1 and A-2 accessions

Under periodical observation it was clearly known that development of new shoots and initial proliferated shoots was noted 7–10 days after culture, while initial new leaves were observed 10–15 days after culture. The initial proliferated shoots and leaves grew continually and increased in number and size of shoots following incubation time. In the end of experiment, number of shoots was in range of 1–3 shoots per explant with 0.5–2.3 cm shoot height and 1–4 leaves per shoot.

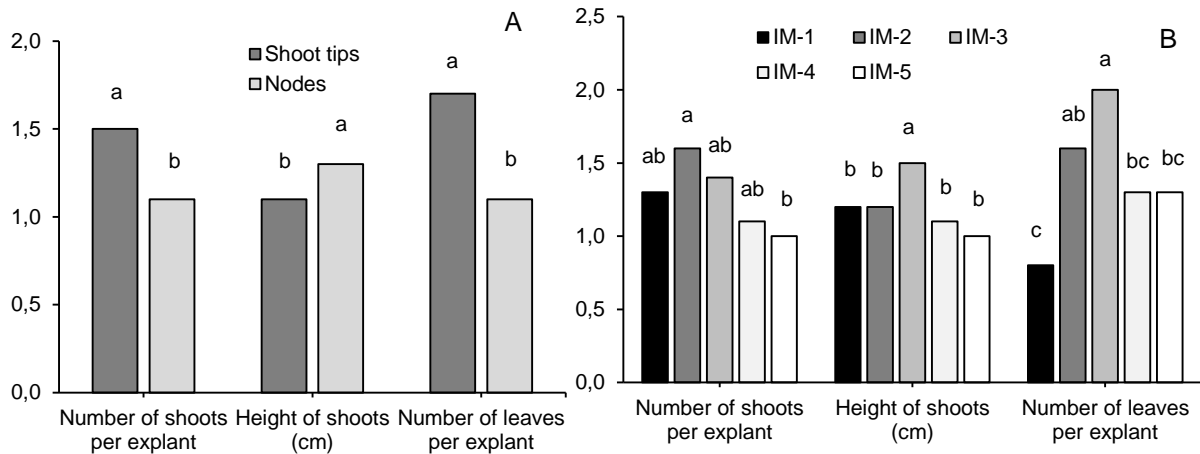


Figure 1. Effect of explant types and initiation media on axillary shoot regeneration. IM-1, MS medium containing 1.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA, IM-2, MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, IM-3, MS medium with 1.5 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA, IM-4, MS medium fortified by 1.5 mg l⁻¹ BAP and 0.05 mg l⁻¹ NAA, and IM-5, MS medium augmented with 1.5 mg l⁻¹ BAP and 0.025 mg l⁻¹ NAA. A. Effect of explant types on axillary shoot regeneration, B. Effect of culture media on axillary shoot regeneration. Vertical histogram bars followed by the same letter in the same cluster are not significantly different based on Tukey test, P = 0.05.

Different types of explants and culture media tested in the first experiment, in fact, gave significant effect on axillary shoot regeneration statistically, P = 0.05. Shoot tips were the most appropriate explant source for obtaining higher axillary shoot regeneration than that of the node explants. The explants induced 1.5 shoots per explant with 1.1 cm shoot height and 1.7 leaves per explant (Figure 1A). While IM-2, MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, was the suitable medium for the experiment purpose. The medium successfully stimulated 1.6 shoots per explant with 1.2 cm in height of shoots and 1.6 leaves per explant (Figure 1B). The IM-3 medium was the second best medium, while the lowest results were performed by IM-5 medium.

Investigating effect of explant types and culture media was also gave significant interaction effect in all variables observed, where explant types exhibited higher effect than culture media. Shoot tip explants cultured on IM-2 medium were better combination treatment in obtaining high axillary shoot regeneration than other combinations. The combination successfully induced axillary shoots per explant up to 2.0 shoots (Table 1) with 1.2 cm shoot height (Table 2) and 2.1 leaves per explant (Table 3). The second best treatment was performed by culturing of the explant on the IM-3 medium. While the lowest results were indicated by culturing the similar explant on the IM-5 medium. Node explants in combination with all media tested generally regenerated lower number of shoots and leaves per explant, however, they generally produced higher shoot performances (Table 1, 2 and 3).

In the second experiment, testing explant types and culture media for A-2 accession almost gave similar results as performed by A-1 accession. Shoot tips were better explant type for inducing axillary shoots than the nodes.

Table 1. Interaction effect of type of explants and initiation media on number of shoots per explant

| Type of medium | Type of explant | |
|----------------|-----------------|-------|
| | Shoot tips | Nodes |
| IM-1 | 1.6 ab | 1.0 a |
| IM-2 | 2.0 a | 1.2 a |
| IM-3 | 1.7 ab | 1.1 a |
| IM-4 | 1.3 ab | 1.0 a |
| IM-5 | 1.0 b | 1.0 a |
| CV, % | 23.94 | 16.21 |

Notes: IM-1, MS medium containing 1.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA, IM-2, MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, IM-3, MS medium with 1.5 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA, IM-4, MS medium fortified by 1.5 mg l⁻¹ BAP and 0.05 mg l⁻¹ NAA, and IM-5, MS medium augmented with 1.5 mg l⁻¹ BAP and 0.025 mg l⁻¹ NAA. Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Table 2. Interaction effect of type of explants and initiation media on height of shoots (cm)

| Type of medium | Type of explant | |
|----------------|-----------------|-------|
| | Shoot tips | Nodes |
| IM-1 | 1.2 ab | 1.2 a |
| IM-2 | 1.2 ab | 1.2 a |
| IM-3 | 1.4 a | 1.6 a |
| IM-4 | 1.1 ab | 1.2 a |
| IM-5 | 1.0 b | 1.4 a |
| CV, % | 12.80 | 10.85 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Table 3. Interaction effect of type of explants and initiation media on number of leaves per explant

| Type of medium | Type of explant | |
|----------------|-----------------|--------|
| | Shoot tips | Nodes |
| IM-1 | 1.5 ab | 0.0 b |
| IM-2 | 2.1 a | 1.0 ab |
| IM-3 | 2.2 a | 1.9 a |
| IM-4 | 1.6 ab | 1.0 ab |
| IM-5 | 0.9 b | 1.6 a |
| CV, % | 10.26 | 14.41 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

The explant produced the shoots up to 1.7 shoots per explant with 1.1 cm in height of shoots and 2.0 leaves per explant (Table 4). IM-2 medium was also the appropriate medium that successfully stimulated 2.2 shoots per explant with 1.2 cm shoot height and 2.0 leaves per explant (Table 5). Though the explant types and culture media showed significant effect on axillary shoot formation statistically, there was no interaction effect of the two treatments.

Table 4. Effect of explant types on axillary shoot regeneration of A-2 accession

| Type of explant | Number of shoots per explant | Height of shoots (cm) | Number of leaves per explant |
|-----------------|------------------------------|-----------------------|------------------------------|
| Shoot tips | 1.7 a | 1.1 b | 2.0 a |
| Nodes | 1.4 b | 1.3 a | 1.8 b |
| CV, % | 12.46 | 21.17 | 19.41 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, $P = 0.05$.

Table 5. Effect of explant types on axillary shoot regeneration of A-2 accession

| Type of medium | Number of shoots per explant | Height of shoots (cm) | Number of leaves per explant |
|----------------|------------------------------|-----------------------|------------------------------|
| IM-1 | 1.6 ab | 1.1 a | 2.0 a |
| IM-2 | 2.2 a | 1.2 b | 2.0 a |
| IM-3 | 1.5 b | 1.3 a | 2.0 a |
| IM-4 | 1.4 b | 1.2 a | 1.9 a |
| IM-5 | 1.3 b | 1.3 a | 1.8 a |
| CV, % | 12.46 | 21.17 | 19.41 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, $P = 0.05$.

Improvement of axillary shoot production of A-1 and A-2 accessions on different proliferation media

Based on periodical observation, initial axillary shoot and leaf formation was almost similar noted as the previous experiments with different values on number of shoots produced per explant, height of shoots and

number of leaves per explant. In the stage, two *A. formosanus* accessions and proliferation media generated significant effect on axillary shoot production statistically, $P = 0.05$. A-1 accession kept the most responsive accession on axillary shoot production with 4.9 shoots per explant, 1.1 cm in height of shoots and 9.2 leaves per explant. While PM-7, MS medium fortified by 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, was the most optimal medium and produced 4.3 shoots per explant with 1.1 cm shoot height and 7.0 leaves per explant (Figure 2B). PM-1 medium was the second best medium, but PM-6 stimulated the lowest results compared to others. In the study, it was also revealed that improving axillary shoot production was established by lowering BAP concentration from 1.5 to 1.25 mg l⁻¹.

In the study, it was also revealed that two *A. formosanus* accessions and seven proliferation media had significant interaction effect in all variables observed, where the accessions gave higher effect than the proliferation media. Shoot tip explants of A-1 accession cultured on PM-7 were the best combination treatment in resulting high axillary shoots compared to others. The combination treatment successfully regenerated axillary shoots per explant as high as 7.0 shoots (Table 6) with lower shoot height down to 1.0 cm (Table 7) and higher number of leaves per explant up to 9.8 leaves (Table 8) than other combinations. The second best combination was recorded on the shoot tips of A-1 accession with PM-2 medium, while the lowest combination results were determined on the explant and accession with PM-6 medium. Whereas the shoot tip explants of the A-2 accession indicated higher results when they cultured on the PM-1 medium. Culturing them in other media reduced the axillary production results significantly. The results also revealed that each explant source and genotype had specific behavior in tissue culture condition.

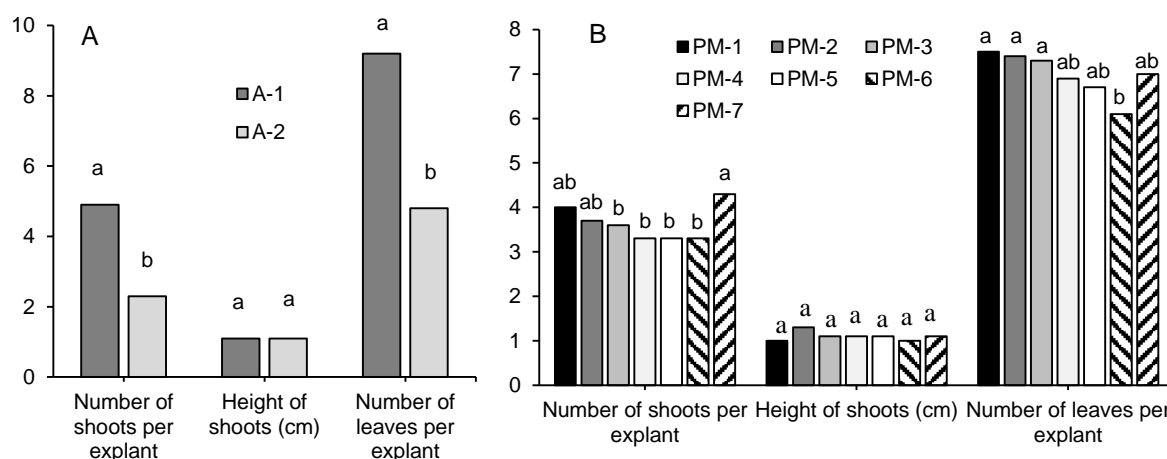


Figure 2. Effect of different *A. formosanus* accessions and proliferation media on axillary shoot production. PM-1, MS medium fortified by 1.5 mg l⁻¹ BAP, 0.25 mg l⁻¹ NAA and 60 mg l⁻¹ AS, PM-2, MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-3MS medium supplemented with 1.75 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-4, MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-5, Half-strength MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-6Half-strength MS medium supplemented with 1.75 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-7, Half-strength MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA. A. Response of two *A. formosanus* accessions on axillary shoot production, B. Effect of proliferation media on axillary shoot production. Vertical histogram bars followed by the same letter in the same cluster are not significantly different based on Tukey test, $P = 0.05$

Table 6. Interaction effect of type of explants and initiation media on number of shoots per explant

| Type of medium | Type of accession | |
|----------------|-------------------|--------|
| | A-1 | A-2 |
| PM-1 | 5.2 ab | 2.8 ab |
| PM-2 | 5.7 ab | 1.8 ab |
| PM-3 | 4.3 b | 3.0 a |
| PM-4 | 4.3 b | 2.3 ab |
| PM-5 | 4.4 b | 2.1 ab |
| PM-6 | 3.8 b | 2.8 ab |
| PM-7 | 7.0 a | 1.6 b |
| CV, % | 19.17 | 25.63 |

Notes: PM-1, MS medium fortified by 1.5 mg l⁻¹ BAP, 0.25 mg l⁻¹ NAA and 60 mg l⁻¹ AS, PM-2, MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-3MS medium supplemented with 1.75 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-4, MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-4, Half-strength MS medium supplemented with 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-6Half-strength MS medium supplemented with 1.75 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA, PM-7, Half-strength MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA. Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Shoot Rooting

Culturing shoots derived from A-1 and A-2 accessions on different rooting media also exhibited varied responses on root formation. Under periodical observation it was known that initial roots immersing in basal parts of intercalary meristem areas of nodes were clearly observed 6–10 days after culture. The initial roots grew continually and developed in number, size and length following the incubation time. In the end of experiment, number of roots per shoot was varied from 1–4 roots with 0.5–2.0 cm root length.

In the study, different accession of *A. formosanus* and rooting media gave significant effect of root formation statistically, P = 0.05. A-1 accession indicated higher responses than A-2 accession. The accession induced 1.8 roots per shoot and 0.9 cm root length (Figure 3A). Furthermore, though higher number of roots per shoot

determined of RM-4 and RM-5 (Figure 3B), there were no significant differences compared to others. While optimal root formation of A-1 accession shoots was established on RM-7, Hyponex medium (2 g l⁻¹ 20N:20P:20K) containing 150 ml l⁻¹ CW and 1.5 g l⁻¹ AC. The combination treatment stimulated 2.4 roots per shoot (Table 9) and 1.0 cm root length (Table 10). Whereas maximal root formation of A-2 accession shoots was determined on RM-4, half-strength MS medium with full vitamin and 1.5 g l⁻¹ AC and RM-5, MS medium containing 0.2 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA.

Table 7. Interaction effect of type of explants and initiation media on height of shoots (cm)

| Type of medium | Type of accession | |
|----------------|-------------------|-------|
| | A-1 | A-2 |
| PM-1 | 1.1 b | 1.0 a |
| PM-2 | 1.4 a | 1.2 a |
| PM-3 | 1.1 b | 1.1 a |
| PM-4 | 1.2 ab | 1.0 a |
| PM-5 | 1.0 b | 1.0 a |
| PM-6 | 1.1 b | 1.0 a |
| PM-7 | 1.0 b | 1.2 a |
| CV, % | 10.24 | 11.25 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Table 8. Interaction effect of type of explants and initiation media on number of leaves per explant

| Type of medium | Type of accession | |
|----------------|-------------------|--------|
| | A-1 | A-2 |
| PM-1 | 9.3 a | 5.7 a |
| PM-2 | 9.8 a | 5.0 ab |
| PM-3 | 9.5 ab | 5.1 ab |
| PM-4 | 9.3 ab | 4.7 ab |
| PM-5 | 9.1 ab | 4.2 b |
| PM-6 | 7.4 b | 4.7 ab |
| PM-7 | 9.8 a | 4.3 ab |
| CV, % | 14.01 | 18.48 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

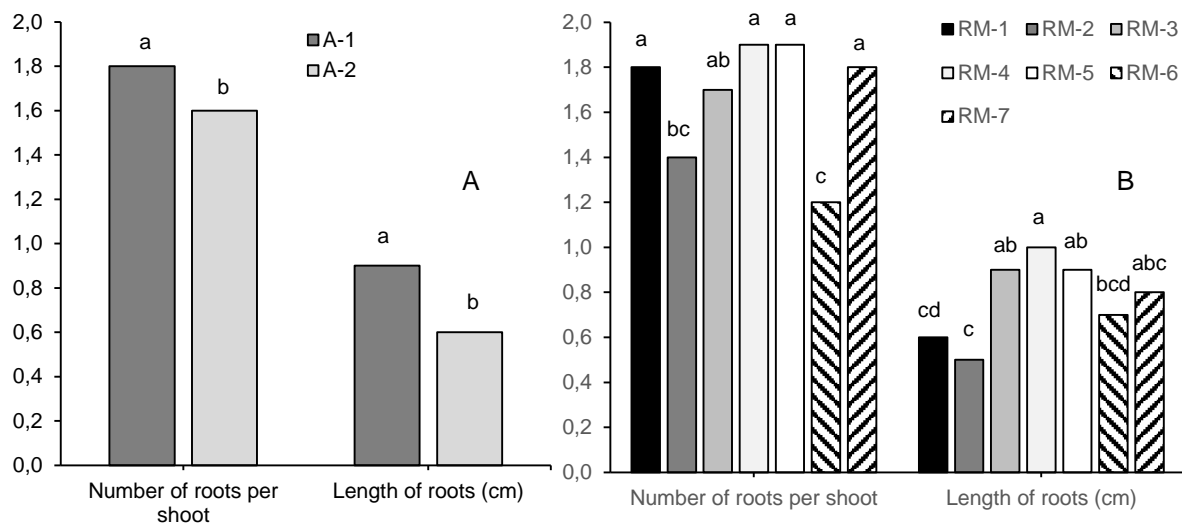


Figure 3. Response of two *A. formosanus* accessions and effect of seven rooting media on root formation. RM-1, MS medium supplemented with 60 g l⁻¹ sucrose, RM-2, MS medium supplemented with 60 g l⁻¹ sucrose and 1.5 g l⁻¹ AC, RM-3, half-strength MS medium with full vitamin, RM-4, half-strength MS medium with full vitamin and 1.5 g l⁻¹ AC, RM-5, MS medium containing 0.2 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA, RM-6, MS medium supplemented with 0.25 mg l⁻¹ BAP and 1.5 g l⁻¹ AC, and RM-7, Hyponex medium (2 g l⁻¹ 20N:20P:20K) containing 150 ml l⁻¹ CW and 1.5 g l⁻¹ AC. Vertical histogram bars followed by the same letter in the same cluster are not significantly different based on Tukey test, P = 0.05



Figure 4. *In vitro* propagation process of *A. formosanus* based on selection of explant types, accessions and culture media. A – Harvested shoots of A-1 accession accepted from Jagakarsa farmer. B – Prepared explants of A-1 accession ready for sterilization step. C – Shoot tips and nodes used as explant sources used in the initiation experiments. D – Initial regenerated shoots derived from shoot tips of A-1 accession on MS medium containing 1.25 mg l^{-1} BAP and 0.25 mg l^{-1} NAA ± 30 days after culture. E – Regenerated shoots derived from shoot tips of A-1 accession on the same medium ± 2 months after culture. F – Regenerated shoots derived from nodes of A-1 accession on the same medium ± 2 months after culture. G – Plantlets prepared on Hyponex medium (2 g l^{-1} 20N:20P:20K) containing 150 ml l^{-1} CW and 1.5 g l^{-1} AC ± 30 days after culture. H – Immersing of plantlet roots in 1% pesticide solution (50% benomil and 20% kanamycin sulphate) for 3 min. I – Plantlets of A-1 accession planted in plastic box containing a mixture of burned-rice husk and organic manure (1:1, v/v). J – Covering plantlets of A-1 accession planted in plastic box containing with transparent plastic for one month. K – Morphological performances of A-1 accession ± 2 months after acclimatization. L – Morphological performances of A-2 accession ± 2 months after acclimatization

Table 9. Interaction effect of accession types and rooting media on number of roots per shoot

| Type of medium | Type of accession | |
|----------------|-------------------|-------|
| | A-1 | A-2 |
| RM-1 | 2.0 b | 1.7 b |
| RM-2 | 1.4 cd | 1.3 b |
| RM-3 | 2.1 b | 1.3 b |
| RM-4 | 1.6 c | 2.2 a |
| RM-5 | 1.7 c | 2.2 a |
| RM-6 | 1.2 d | 1.2 b |
| RM-7 | 2.4 a | 1.3 b |
| CV, % | 10.61 | 19.79 |

Notes: RM-1, MS medium supplemented with 60 g l⁻¹ sucrose, RM-2, MS medium supplemented with 60 g l⁻¹ sucrose and 1.5 g l⁻¹ AC, RM-3, half-strength MS medium with full vitamin, RM-4, half-strength MS medium with full vitamin and 1.5 g l⁻¹ AC, RM-5, MS medium containing 0.2 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA, RM-6, MS medium supplemented with 0.25 mg l⁻¹ BAP and 1.5 g l⁻¹ AC, and RM-7, Hyponex medium (2 g l⁻¹ 20N:20P: 20K) containing 150 ml l⁻¹ CW and 1.5 g l⁻¹ AC. Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Table 10. Interaction effect of accession types and rooting media on number of root length (cm)

| Type of medium | Type of accession | |
|----------------|-------------------|--------|
| | A-1 | A-2 |
| RM-1 | 0.7 d | 0.5 b |
| RM-2 | 0.5 e | 0.5 b |
| RM-3 | 1.2 b | 0.7 ab |
| RM-4 | 1.4 a | 0.6 ab |
| RM-5 | 1.1 b | 0.8 a |
| RM-6 | 0.8 cd | 0.6 ab |
| RM-7 | 1.0 bc | 0.7 ab |
| CV, % | 17.86 | 26.05 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Plantlet acclimatization

Acclimatization plantlets, though in some plant tissue culture works to be a critical point, in the study plantlets were easily transferred to *ex vitro* condition successfully under gradual process as described in materials and methods. Percentage of survivability was varied from 80 to 95% or 16–19 survival plantlets in a mixture of burned-rice husk and organic manure (1:1, v/v) were noted in each replication. A-1 accession kept resulting in higher responses than A-2 accession with percentage of survivability up to 90.4% and 18.1 survival plantlets in average (Table 11). Plantlets acclimatized derived from A-1 accession generally had healthy and vigour growth compared to the A-2 accession.

Table 11. Different responses of *A. formosanus* accessions in acclimatization

| Type of accession | Percentage of survivability (%) | Number of survival plantlets |
|-------------------|---------------------------------|------------------------------|
| A-1 | 90.4 a | 18.1 a |
| A-2 | 85.0 b | 17.0 b |
| CV, % | 2.51 | 2.39 |

Means followed by the same letter in the same column are not significant difference based on Tukey test, P = 0.05.

Discussion

In vitro propagation protocol initiating from axillary shoot regeneration, proliferation, shoot rooting and acclimatization of plantlet on *A. formosanus* was successfully established. The new findings can be used as a new alternative method in preparing high qualified

planting materials in developing the plant commercial-ly in large scale. The protocol can also complete and improve other studies published previously (Chang, Chen, 1987; Shiao *et al.*, 2002; Ket *et al.*, 2004; Refish *et al.*, 2015). Results of the study can also be utilized as comparison method and improving ideas for other *Anoectochilus in vitro* studies of *A. sikkimensis* and *A. regalis* (Gangaprasad *et al.*, 2000), *A. roxburgii* (Zhang *et al.*, 2015), *A. elatus* (Sherif *et al.*, 2012; Sherif *et al.*, 2016; Raja, 2017).

Successful initiation stage in *in vitro* culture of plant will lead to high potential in achieving the next step. Ket *et al.* (2004) recorded that high axillary shoots derived from shoot tip explants up to 11.2 shoots per explant with 3.8 cm shoot length of *A. formosanus* was established on Hyponex medium supplemented with 2 mg l⁻¹ TDZ and 1.0 g l⁻¹ AC, while lower results down to 5.0 – 7.8 shoots were recorded on MS medium containing 3 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA (Chang, Chen, 1987). In the study, MS medium containing 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA as potential medium was improved by decreasing concentration of BAP from 1.5 to 1.25 mg l⁻¹ to produce 7.0 axillary shoots per explant with 1.0 cm shoot height and 9.8 leaves per explant derived from shoot tips of A-1 accession of *A. formosanus*. While in other studies, 5.6 shoots per explant of *A. regalis* and 4.5 shoot per explant of *A. sikkimensis* were successfully regenerated on Woody Plant Medium supplemented with 0.5 mg l⁻¹ BAP (Gangaprasad *et al.*, 2000), 2.7 shoots per explant with 2.2 cm shoot length of *A. elatus* were recorded on MS medium fortified by 1.0 mg l⁻¹ BA (Sherif *et al.*, 2012), 7 shoots per explant with 6.2 cm shoot length on MS medium 2.5 mg l⁻¹ TDZ and 5% CW (Raja, 2017); 1–10 shoots per explant and 0.5–4.0 cm shoot length of *A. roxburghii* was noted on MS medium fortified by 0.2 mg l⁻¹ 2,4-D, 0.9 mg l⁻¹ NAA, 1 mg l⁻¹ BA, 0.25 mg l⁻¹ Zeatin, 0.6% agar and 4.5% sucrose (Refish *et al.*, 2015), high proliferation rate of shoots up to 4.33 on half-strength MS medium fortified by 3.0 mg l⁻¹ BA, 1.0 mg l⁻¹ Kinetin, 0.5 mg l⁻¹ NAA and additives (Zhang *et al.*, 2015).

Shoot rooting was important step in preparing high successful acclimatization of plantlets. In the study, high root formation with 2.4 roots per shoot and 1.0 cm root length of A-1 accession of *A. formosanus* was established on Hyponex medium (2 g l⁻¹ 20N:20P:20K) containing 150 ml l⁻¹ CW. Almost similar results with 2.3 roots per shoot and 2.5 cm root length were noted on Hyponex medium with 2% sucrose AC free (Ket *et al.*, 2004). In other *Anoectochilus* species, 4.3 roots per shoot and 2.2 cm in length of roots of *A. elatus* were recorded on MS medium containing 0.3 g l⁻¹ AC (Sherif *et al.*, 2012), 3.2 roots per shoot with 2.1 cm root length on Mitra medium amended with 1.0 mg l⁻¹ AgNO₃ (Sherif *et al.*, 2016), 86.6% root formation and 4.4 cm root length on MS medium fortified by 7.0 mg l⁻¹ IBA (Raja, 2017).

Well plantlet preparation will be nothing and in *in vitro* culture works when the prepared plantlets were

fail to be acclimatized. High percentage of plantlets survivability of *A. formosanus* around 90% was established on mixture medium of peat moss and vermiculite after 2 months (Shiau *et al.*, 2002), while in the study, percentage of survivability up to 90.4% and 18.1 survival plantlets was determined on a mixture of burned-rice husk and organic manure (1:1, v/v) after 2 months. In other *Anoectochilus* species, 95% survival plantlets of *A. sikkimensis* and 70% for *A. regalis* were recorded at loose humic rich soil after 12 months (Gangaprasad *et al.*, 2000), 100% survivability was proved in greenhouse after 4 weeks (Ket *et al.*, 2004); 80% survivability of *A. elatus* acclimatized plantlets was noted on coconut choir, AC and commercial fertilizers (3:1:1, v/v/v) (Sherif *et al.*, 2012), 72.3% survival rate was noted at a mixture of garden soil, sand, vermicompost and tea waste (8:4:2:1) (Sherif *et al.*, 2016). 88% survival plantlets were observed in Kulivalavu regions of Kolli Hill after 60 days (Raja, 2017). Whereas high survival rate up to 90.2% of *A. roxburghii* plantlets was noted on plastic cups containing sterile sand and peat soil mixture in a ratio of 1:2 that their surfaces were covered by live moss (Zhang *et al.*, 2015).

Specific behaviour of each explant and genotype on different types of culture media in *in vitro* culture of *A. formosanus* was also successfully revealed in the study. Shoot tip was more responsive than node explant; A-1 accession better than A-2 accession. The two explants and accessions of *A. formosanus* indicated different growth and performances in each step of culture. Ket *et al.* (2004) also found the similar results in *A. formosanus* that shoot tip explants produced higher axillary shoots than the node explants. In *A. elatus*, node explant was more productive than shoot tip explants on shoot regeneration (Sherif *et al.*, 2012), internode > node > leaf explants on callus proliferation and regeneration (Sherif *et al.*, 2016). Gangaprasad *et al.* (2000) recorded that node explant of *A. regalis* had high number and faster growth of shoots than node derived from *A. sikkimensis*.

Conclusions

The serial experiments carried out in the study were successfully established an *in vitro* propagation protocol for *A. formosanus* via axillary shoot regeneration and proliferation. In the initial step, shoot tips as explant sources and MS medium containing 1.5 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA were determined as high potential explant and medium for axillary shoot regeneration for A-1 and A-2 accessions of *A. formosanus*. High axillary shoot production up to 7.0 shoots per explant with 1.0 cm shoot height and 9.8 leaves per explant derived from shoot tip explants of A-1 accession was resulted in MS medium supplemented with 1.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA. The regenerated shoots were easily rooted on Hyponex medium (2 g l⁻¹ 20N:20P:20K) containing 150 ml l⁻¹ CW with 2.4 roots per shoot and 1.0 cm root length of A-1 accession. The well growth plantlets were successfully acclimatized on

plastic boxes containing a mixture of burned rice-husk and organic manure (1:1, v/v) with survival rate as high as 90.4 %. In all step of *in vitro* culture of *A. formosanus* it was also revealed that A-1 accession showed higher response compared to A-2 accession.

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Conflict of interest

We declare that there is no conflict of interest dealing with Jagakarsa farmer who supplied research materials, authors and Indonesian Ornamental Crops Research Institute and Central Java Assessment Institute for Agriculture Technology that facilitated and funded the research activities.

Author contributions

All authors play important roles equally in designing, carrying out and analysing all data regenerating from the research till writing, editing and approving the final manuscript.

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