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EMBRYO CULTURE

Ex vitro rooting using a mini growth chamber increases root induction and accelerates acclimatization of Kopyor coconut (*Cocos nucifera* L.) embryo culture-derived seedlings

Sisunandar [1] · Alkhikmah [1] · Arief Husin [1] · Teguh Julianto [1] · Alice Yuniaty [2] · Alain Rival [3] · Steve W. Adkins [4]

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Abstract

Ex vitro rooting using a mini growth chamber to maintain relative humidity has been used to mass produce true-to-type Kopyor coconut (*Cocos nucifera* L.) seedlings through embryo culture. This new process was found to (1) improve the proportion of seedlings successfully transferred to soil (from 40 to 90% for seedlings with roots); (2) achieve this step in the shortest time possible (a reduction from 10 to 4 mo in *in vitro* culture); (3) improve root formation using indolebutyric acid (IBA; an improvement in the number of primary roots from 2 to 5); and (4) improve the vigor of seedlings *ex vitro* (improvements of fresh weight, shoot length, number of opened leaves, leaf thickness, amount of epicuticular wax, and stomatal density). The best *ex vitro* rooting and rapid acclimatization protocol was obtained when 4-mo-old seedlings with two opened leaves were kept in the mini growth chamber for 3 mo before being transferred into soil, and when the mini growth chamber was flooded with a quarter strength hybrid embryo culture (HEC) medium with 1 μM IBA but depleted of vitamins and sugar. This protocol was efficient in delivering high-value Kopyor seedlings to the field (90% success rate), with a decreased risk of contamination and lower labor cost. The improved process was found applicable to both tall and dwarf Kopyor and other coconut types.

Keywords Embryo culture · Coconut mutant · Morphological analysis · Indolebutyric acid

Introduction

The Kopyor coconut is of paramount interest, among Indonesian elite materials, as it is much sought after by customers for its soft and sweet endosperm flesh. In local markets, each fruit sells for almost 10 times the value of a normal coconut fruit. Such a high price is resulting from the considerable demand of this elite fruit that has a very limited production. Kopyor fruit formation is a result of a spontaneous recessive mutation (Nguyen et al. 2016) which appears rarely in coconut estates. Indeed, the Kopyor fruit has a soft and friable endosperm, which is unable to support the germination of the zygotic embryo; therefore, the true-to-type seedling production thought conventional seed route is not possible. The only alternative available for the production of true-to-type Kopyor seedlings is to use embryo rescue and *in vitro* cultivation.

Although a standard protocol for the embryo culture of coconut has been developed (Orense et al. 2011), the success rate of seedling, and then plant production, remains too low when it is applied to Kopyor seedling production. The low success rate of *in vitro* rooting (*ca.* 60–70%) and acclimatization (< 40%) steps remain the major two constrains for the application of the embryo culture protocol for the large scale production of Kopyor seedlings. Such limitations have also

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✉ Sisunandar
Sisunandar@ump.ac.id

[1] Coconut Research Center, Biology Education Department, The University of Muhammadiyah Purwokerto, Kampus Dukuhwaluh, Purwokerto 53182, Indonesia

[2] Faculty of Biology, The University of Jenderal Soedirman, Kampus Karangwangkal, Purwokerto 53123, Indonesia

[3] Cirad – DGDRS, Jakarta, Indonesia

[4] School of Agriculture and Food Sciences, Center for Plant Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD 4072, Australia

been described in several other woody species such as *Bactris gasipeas* Kunth (Steinmacher et al. 2011), and *Abrus precatorius* L. (Perveen et al. 2013).

Most approaches involve steps to improve the *in vitro* rooting initiation and proliferation followed by seedling acclimatization. Several approaches have been tested in order to improve *in vitro* rooting of normal embryo culture coconut seedlings such as increasing the concentration of sucrose in the medium (Karunaratne et al. 1985), prolonging exposure to plant growth regulators (Talavera et al. 2005), adding polyethylene glycol into the culture medium (Magdalita et al. 2010), or by removing the haustorium (Engelmann et al. 2011). However, if a prolonged step of *in vitro* rooting is used, it will result in a longer duration of *in vitro* culture and therefore in higher production costs. As a consequence, the price of true-to-type Kopyor seedlings now reaches US$ 70 per plant.

Moreover, a number of protocols have been developed in order to increase seedling vigor and survival during the acclimatization phase of embryo-derived coconut seedlings. Such improvements include using a plastic bag cover or a humidity tent to increase humidity around the seedling (Orense et al. 2011), using a wooden box chamber to achieve the same outcome (Magdalita et al. 2010), or by using a photoautotrophic system (Samosir and Adkins 2014). However, the survival rate of the *in vitro* produced Kopyor seedlings using these acclimatization processes was found to be very low in our conditions (< 30%).

Recently, several attempts have been made to create a more efficient protocol for micropropagation through the smart combination of *in vitro* rooting and acclimatization (Benmahioul et al. 2012; Clapa et al. 2013; Ranaweera et al. 2013; Cuenca et al. 2017). The seedlings which directly produced roots while acclimatizing resulted in a higher field survival rate as compared to those taken through just the *in vitro* rooting approach.

In addition, it has been shown that the labor cost of micropropagation can be reduced significantly (by 35 to 75%) when *ex vitro* rooting is combined with acclimatization (Hazarika 2003; Ranaweera et al. 2013; Wang et al. 2013; Mukhopadhyay et al. 2016). Up to now, there have been no successful reports of *ex vitro* rooting and acclimatization of Kopyor coconut seedlings derived from tissue culture. Therefore, this study focused on the development of an efficient and reliable protocol for the *ex vitro* rooting and acclimatization of Kopyor seedlings and assessing its performance through the morphological analysis of the produced seedlings.

Materials and Methods

Plant material and embryo culture methods Zygotic embryos from Kopyor coconut palms (*C. nucifera* L.) were isolated from 11-mo-old fruit collected from farms in three different areas of Indonesia, namely Pati (Central Java), Sumenep (Madura Island), and Lampung (Sumatera). Following harvest, intact fruit were packed into crates and sent to the Coconut Research Centre at The University of Muhammadiyah, Purwokerto. Embryo isolation and culture was undertaken following the method of Sisunandar et al. (2010) with minor modification. Soon after arriving, the nuts were dehusked and split, and then the soft endosperm containing the zygotic embryo was removed using a spoon. The endosperm tissue was then washed several times with tap water and quickly rinsed with ethanol (95%; *v/v*). Each embryo was aseptically isolated from the endosperm tissue in a laminar air low cabinet, surface sterilized using a calcium hypochlorite (6%; *w/v*) solution for 12 min, then dipped in a sterile liquid hybrid embryo culture (HEC) medium (Table S1, Rillo 2004) for 30 min.

The surface-sterilized zygotic embryos were then placed—haustorium down—onto a solid HEC medium added with sugar (60 g L^{-1}) and charcoal (2 g g L^{-1}) and incubated in the dark (27 ± 2°C) for 4 wk, during which time the embryos started to germinate. The seedlings were then transferred into a liquid HEC medium with lower sugar concentration (30 g L^{-1}) (Rillo 2004) and were incubated for a further 8 to 12 wk, under a 14-h photoperiod (25 ± 2 umol m^{-2} s^{-1}) at 27 ± 2°C until the seedlings had developed one to two open leaves.

Ex vitro rooting and acclimatization system A mini growth chamber (Fig. 1) was used for the *ex vitro* rooting and acclimatization of the three batches of Kopyor seedlings cv. Kopyor Brown Dwarf (KBD) derived from embryo culture. The chamber consisted of a glass box (70 × 40 × 40 cm; l/w/h) capable of acclimatizing 40 seedlings at a time. The chamber was partly filled with 12 L of a quarter strength macro- and micronutrient liquid HEC medium (Rillo 2004) deprived of vitamins and sucrose. To maintain the air exchange and relative humidity inside the chamber, the liquid medium was aerated (2 × 3 L min^{-1}) using an air pump (Amara AA 9904, China) and run throughout each experiment.

Effect of IBA on the success rate of ex vitro rooting and acclimatization Four- to 5-mo-old seedlings showing one to two open leaves (Fig. 2*A*, *B*) produced from the previously described *in vitro* system were divided into two groups based on the extent of their rooting system *viz.* (1) coconut shoots without any primary or secondary roots (NR; Fig. 2*A*) coconut seedlings with a well-developed root system (FR: two to three primary roots and with some secondary root growth (Fig. 2*B*).

The seedlings from each group were taken out of *in vitro* culture, washed gently with tap water to remove medium, and then dipped into a 2% (*w/v*) Dithane M-45 80 WP (Dow AgroSciences, India) fungicide solution for 20 min to avoid later fungal contamination.

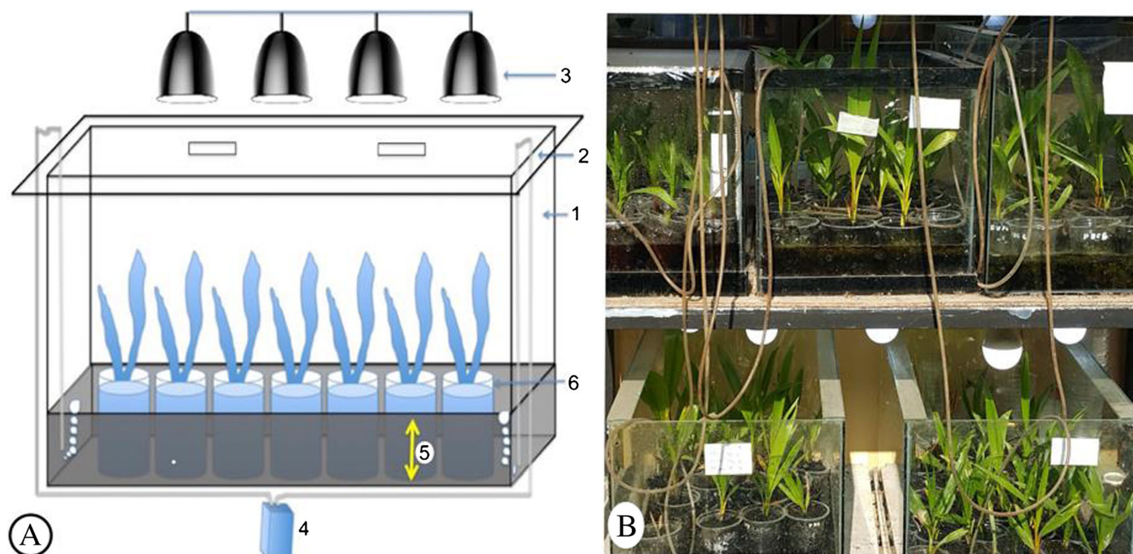

Figure 1. (*A*) A schematic diagram of the mini growth chamber design used for *ex vitro* rooting. The system consisted of a glass box (*1*) covered with lid (*2*) completed with an aerator (*4*) to maintain air humidity inside the box. The box was filled with a macro- and micronutrient of HEC medium contained IBA without added vitamin and sugar (*5*) in which the coconut seedlings were planted in plastic pots filled with coco peat and rice charcoal as a supporting substrate (*6*). The box were maintained under white light (*3*) with photon flux of 40 ± 2 μmol m^{-2} s^{-1} and 14 h photoperiod for 12 wk. (*B*) Examples of *ex vitro* rooting system of Kopyor coconut after 12 wk culture.

Three different concentrations of IBA were then added into the liquid medium inside four identical mini growth chambers (Fig. 1): 0.0, 0.1, 1.0, and 10.0 μM. The seedlings were transferred individually into small plastic pots (7×10 cm; d x h) containing a combined coco peat and rice charcoal medium (1:1; *v*/*v*). Each IBA treatment was applied to 20 seedlings from each of the three batches, for both FR and NR, and with three replications. All pots were then placed into the mini growth chambers. The chambers were maintained under a 14-h photoperiod (40 ± 2 μmol m^{-2} s^{-1}) at room temperature (26–29°C). The chambers were closed with a glass lid, and the seedlings incubated for 8 wk. At this time, the chambers were gradually opened over the following 4 wk of culture (Fig. 1*B*).

Measurements on seedling morphology were taken before, and 12 wk after seedlings were placed in the chambers. To do this, all seedlings were cleaned carefully under running tap water and blotted dry on tissue paper. The following measurements were undertaken: fresh weight, shoot length (from shoot-root junction to tip of tallest leaf), number of opened leaves, and number of primary roots. The percentage of surviving seedlings after *ex vitro* rooting and acclimatization was also recorded.

Effect of cultivar Four Kopyor cultivars (Kopyor Brown Dwarf (KBD) and Kopyor Green Dwarf (KGD) collected from Pati, Central Java; Kopyor Kalianda Tall (KKT) collected from Lampung; and Kopyor Sumenep Tall (KST) collected from Madura Island, East Java) were tested for their ability to undergo *ex vitro* rooting and acclimatization. Each seedling from each cultivar was cultured individually, and then transferred into the growth chamber. The liquid medium added into the system was a quarter strength macro- and micronutrient liquid HEC medium supplemented with 1.0 μM IBA but without vitamins or sugar added. Sixty seedlings from each cultivar (in three replications) were then used to assess their ability to *ex vitro* rooting. The percentage of surviving seedlings after *ex vitro* rooting and acclimatization was also recorded after 12 wk in the mini growth chamber system.

Histological and biochemical analysis of seedlings surviving after acclimatization A two-step culture approach, firstly involving the transfer into small plastic pots (prenursery treatment; Fig. 3*A*), and then into bigger plastic pots (nursery treatment; Fig. 3*B*) was applied to batches of seedlings before field planting. The prenursery treatment was applied for 12 wk after the *ex vitro* rooting step, while the nursery treatment was applied for 12 wk after the prenursery treatment was completed. The seedlings surviving from *ex vitro* rooting were transferred individually onto plastic pots (15×10 cm; d × h) containing garden soil and combined with a compost (1:1; *v*/*v*), and grown in a glasshouse at a temperature of *ca.* 30 ± 3°C, a relative humidity of 60 to 95% under full sunlight for the prenursery treatment. The seedlings were then transferred into the bigger plastic pot ($20 \times 20 \times 20$ cm; l/w/h) containing the same growing media for the nursery treatment. The seedlings were grown in an open area for further 24 wk before being transferred to the field.

Leaf histology and biochemistry analysis were performed on 10 KGD seedlings and repeated at every step in the culture procedure, *viz.* before *ex vitro* rooting, 12 wk after *ex vitro* rooting had started, 12 wk after the prenursery

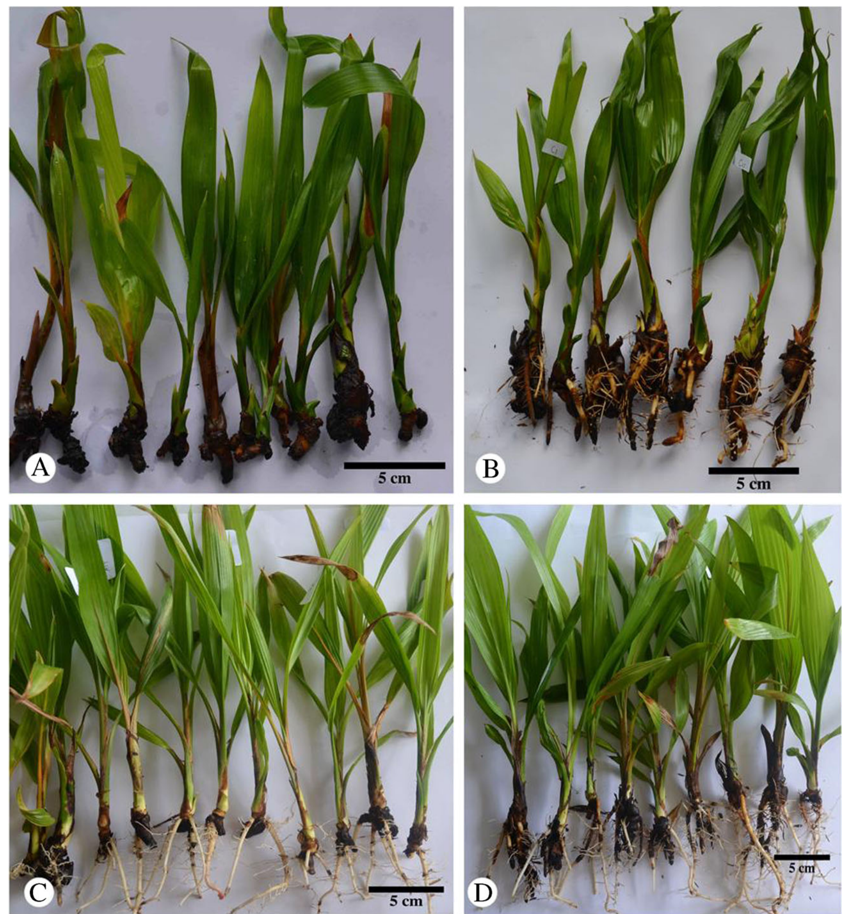

Figure 2. (*A*) Example of 4 mo-old Kopyor seedlings without rooting system induced during *in vitro* culture before being used for *ex vitro* rooting and acclimatization and (*B*) seedlings with 2–3 primary root and some secondary root during *in vitro* culture used. (*C*) Example of seedlings survived and produced primary and secondary root during *ex vitro* rooting for 12 wk, from seedlings without rooting system and (*D*) from seedlings with full rooting system.

treatment, and 24 wk after the nursery treatment. The measurements included stomatal density, morphometric analysis, leaf epicuticular wax thickness, and chlorophyll content. The stomatal density measurements were taken on both adaxial and abaxial surface of the unfolded second leaves (Samosir and Adkins 2014). The morphometric analysis was conducted on the second fully opened seedling leaf. Leaf samples (1 cm^2) were excised from these leaves and fixed in a formaldehyde: glacial acetic acid: and 70% ethanol; 5:5:90). Leaf sections were then dehydrated and embedded in paraffin wax. Transverse sections (8 μm thick) were performed using an automatic rotary microtome (Hestion ERM 4000, Germany). After washing with xylol, the sections were stained with safranin-fast green and mounted into slides using Canada balsam as described by Johansen (1940). Finally, the slides were viewed under an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan) at ×100 to ×200 magnification and photographed using an Olympus DP72 camera. Three slides were made from each of the 10 seedlings per culture step and selected for morphometric analysis. The thickness of adaxial epidermis, abaxial epidermis, palisade parenchyma, and spongy parenchyma were measured using ImajeJ Rel. 1.50e software (Schneider et al. 2012).

Leaf epicuticular waxes were measured according to the method of Preece and West (2006). The second fully open leaf was collected from 10 seedlings at each culture step, and from each leaf, a 10 × 15 mm section was cut then dipped into 100% chloroform (1.5 mL) three times for 10 s each time. The chloroform solution was then transferred into a preweighed 50-mm watch glass. The watch glass was then placed into a chemical fume hood overnight to evaporate the chloroform. The glass was then weighed again, and the difference between the final weight and the initial weight was taken as the amount of epicuticular wax from the leaf section.

Statistical analysis All data sets were statistically analyzed for variance using ANOVA, and statistical comparisons between culture steps were made using Duncan's Multiple Range Test (DMRT) using a $P = 0.05$ significance level. All analyses were performed using the Minitab (Release 15) statistical software package.

Results

Plant survival and root induction after ex vitro rooting After 12 wk of incubation in the mini growth chambers (Fig. 1),

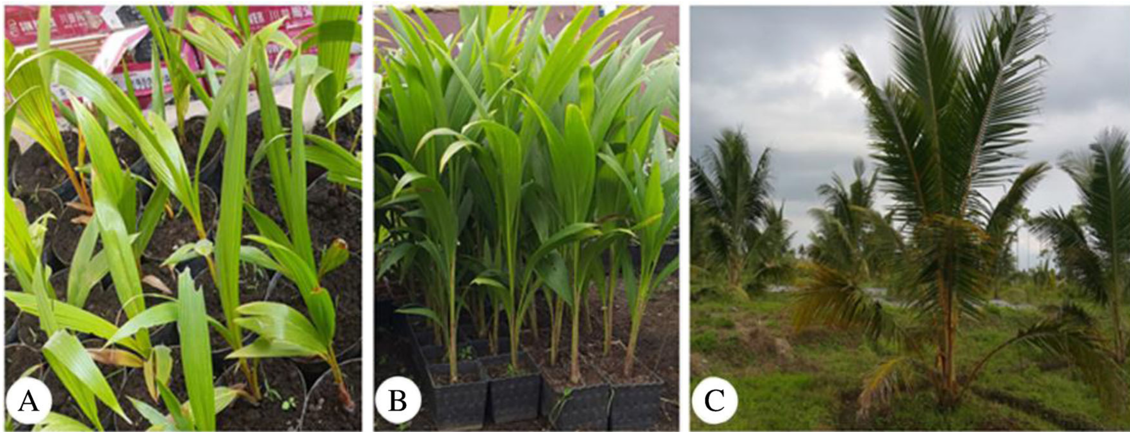

Figure 3. (*A*) Example 12-wk-old seedlings in the prenursery and (*B*) 24-wk-old seedlings in the nursery and ready for field planting. (*C*) Example of 2 yrs old Kopyor coconut in the field.

more than 56% of NR seedlings had survived *ex vitro* rooting, even though not all of the surviving seedlings produced roots during the same period (only 51% of seedlings produced root, Fig. 4). The percentage of seedlings surviving after *ex vitro* rooting and acclimatization increased to *ca.* 80% when the same procedure was applied to seedlings from the FR category. The *ex vitro* rooting technique produced healthy seedlings with several new leaves and new primary roots including many secondary roots (Fig. 2). All surviving seedlings were then transferred to the field successfully (Fig. 3).

Effect of IBA on plant survival after ex vitro rooting The addition of IBA into the culture medium (Fig. 4) was found to increase the percentage of seedlings that survived *ex vitro* rooting. When the seedlings were cultivated in a liquid medium supplemented with 1.0 μM IBA for 12 wk, the percentage of

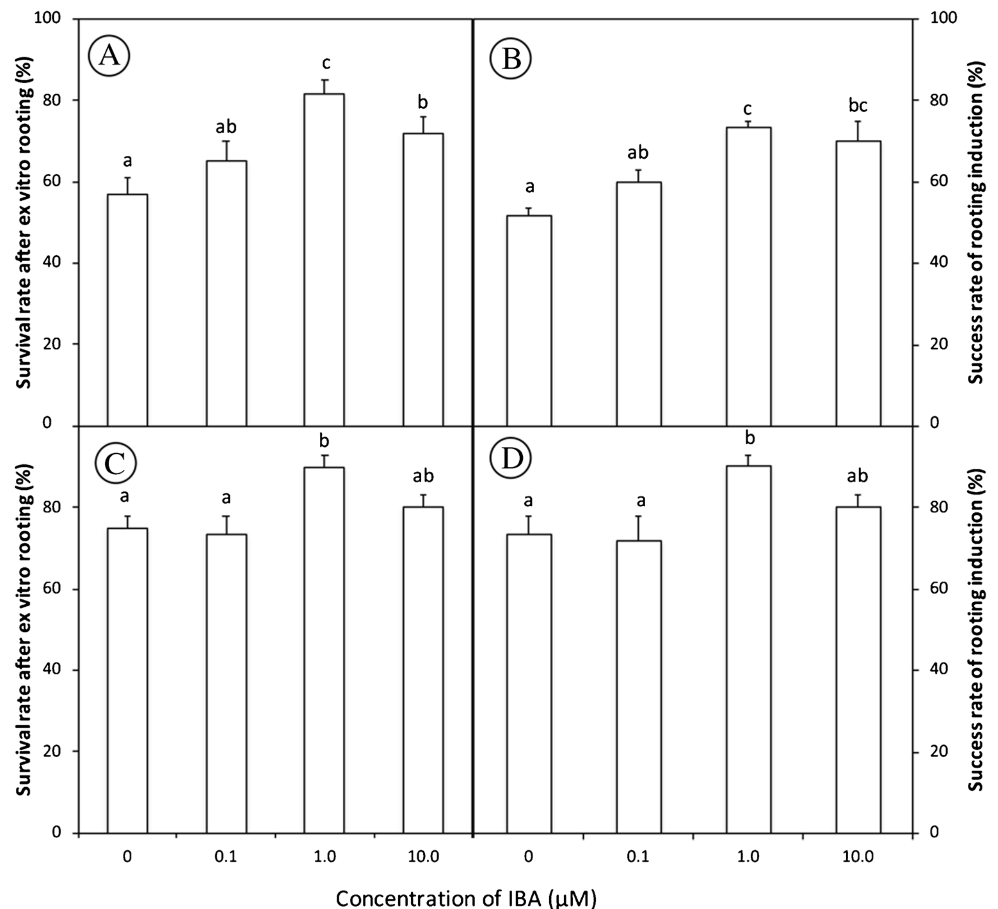

Figure 4. (*A*) The survival rate of seedlings after 12 wk of *ex vitro* rooting and (*B*) the success rates of rooting induction of seedlings without any root induced during *in vitro* culture and (*C*) survival, and (*D*) success of rooting of seedlings with 2–3 primary roots induced during *in vitro* culture. In each *bar chart*, treatments that are ascribed with different letters are significantly different at $P \leq 0.05$. The *bars* are means of three replications of 20 seedlings ± SE.

surviving NR and FR seedlings was increased up to *ca* 80 and 90%, respectively (Fig. 4). Although the seedlings fresh weight, shoot length, and number of new opened leaves did not increase significantly, the addition of IBA induced a sizeable increase in the number of primary roots (Fig. 5). Following IBA treatment, the number of primary root increased from 0 to 3 in NR seedlings and from 3 to 5 in FR seedlings.

Our results also showed that no significant differences could be observed between shoot length and number of opened leaves between NR and FR seedlings after 12 wk of *ex vitro* incubation. However, the FR seedlings did exhibit more vigorous characteristics such as higher fresh weight and higher number of primary roots when compared to their NR counterparts (Fig. 5).

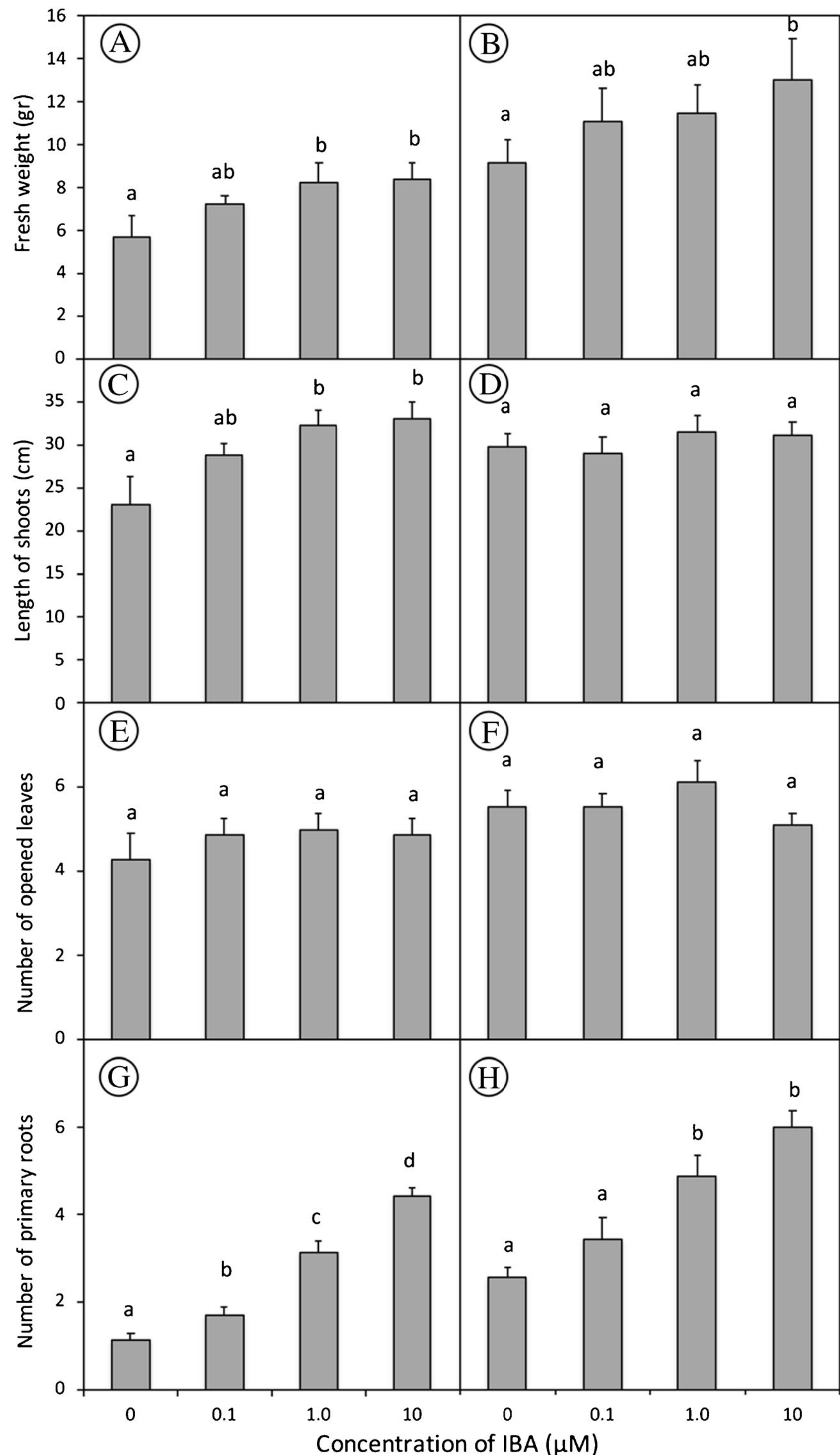

Figure 5. (*A*, *C*, *E*, *G*) Effect of concentration of IBA added into *ex vitro* rooting medium on morphological characteristics of seedlings without rooting system during *in vitro* culture and (*B*, *D*, *F*, *H*) seedlings with 2–3 roots after *ex vitro* rooting for 12 wk. In each *bar* chart, treatments that are ascribed with *different letters* are significantly different at $P \leq 0.05$. The *bars* are means of three replications of 20 seedlings ± SE.

Cultivar response to ex vitro rooting The response to *ex vitro* rooting treatment was not found to be cultivar-dependent. Indeed, the percentage of seedlings achieving *ex vitro* rooting was found to range between 81 and 85% in all four Kopyor cultivars under study (Fig. 6). Although NR-type seedlings originating from all the tested cultivars showed a lower survival rate and lower success rate of rooting induction compared to FR seedlings, differences were not found to be significant. Moreover, no significant difference could be observed in the percentage of root induction when tall and dwarf Kopyor ecotypes were compared.

Histological observations of leaves from in vitro and ex vitro grown seedlings The second opened leaf of the seedlings sampled before *ex vitro* rooting (*in vitro*), at 12 wk after *ex vitro* rooting, at 12 wk after prenursery, and 24 wk after nursery, showed significant changes in leaf thickness (Table 1). The thickness of leaves which developed during *ex vitro* rooting was found to reach 203 μm, resulting from a significant increase from only 142 μm in the leaves sampled on *in vitro* seedlings. The thickness of leaves grown for 24 wk in nursery almost doubled (289 μm). Our overall thickness measurements showed that all the tissues in the leaf developed during *ex vitro* rooting and nursery stages, while increasing the size of both adaxial and abaxial epidermis together with palisade and spongy parenchyma.

The amount of epicuticular waxes was also found to increase during *ex vitro* rooting and nursery stages (Table 1). The leaves which developed during *ex vitro* rooting step showed a wax content of 46.8 μg cm^{-2}, thus almost twice the concentration measured in *in vitro* grown seedlings (23 μg cm^{-2}). The measured wax concentration in leaves then increased during the following 36 wk in the nursery to reach 62.6 μg cm^{-2}.

The number of stomata per mm^2 was also found significantly different between leaves that developed under *in vitro* conditions when compared to those that developed during *ex vitro* rooting then during the nursery stages (Table 2). The leaves which developed during *ex vitro* rooting showed 62.2 stomata per mm^2 on the abaxial side. This number was found to increase significantly when compared to those developed during *in vitro* culture (only 47.6). The stomatal density of abaxial leaves then almost doubled during nursery stage to reach more than 112.2 stomata per mm^2. The number of stomata observed in adaxial leaves was found to be low at all development stage of seedlings, reaching no more than 7 stomata per mm^2.

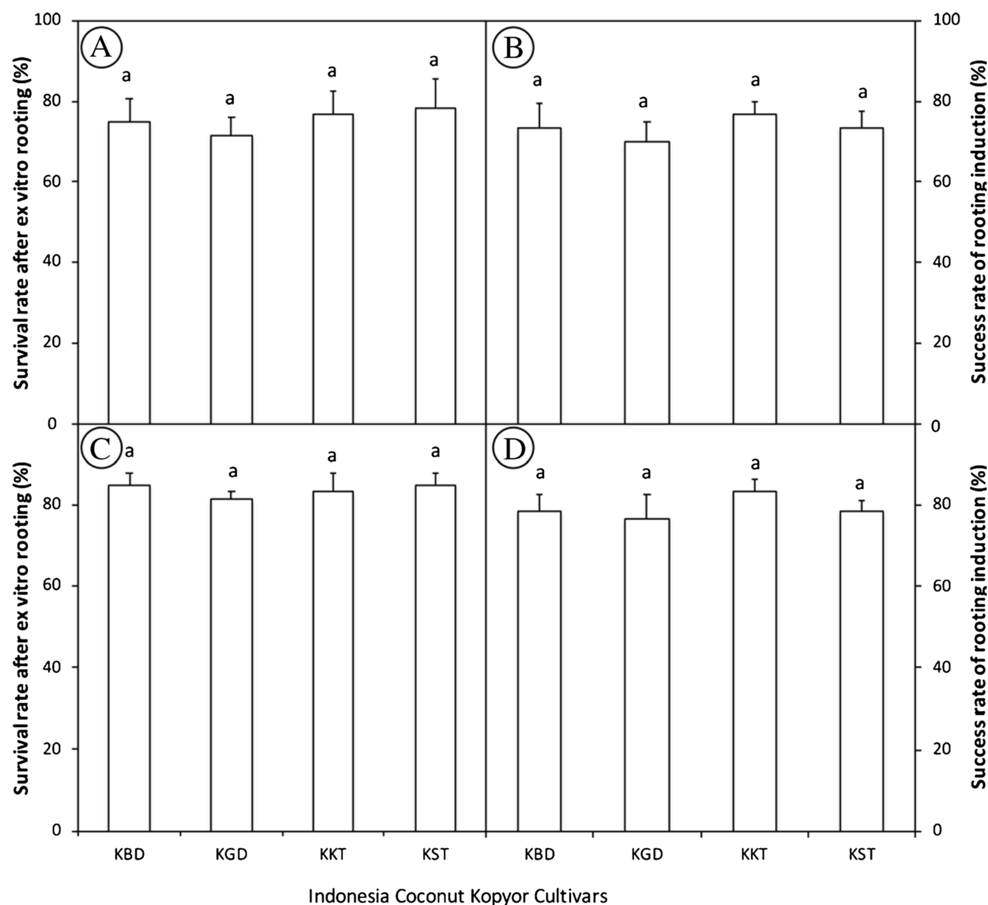

Figure 6. (*A*) Survival rate of seedlings after *ex vitro* rooting for 12 wk and (*B*) success rate of rooting induction in seedlings without any root induced during *in vitro* culture. (*C*) Survival rate and (*D*) success rate of rooting induction of seedlings with with 2–3 primary roots from cultivars Kopyor Brown Dwarf (*KBD*), Kopyor Green Dwarf (*KGD*), Kopyor Kalianda Tall (*KKT*), and Kopyor Sumenep Tall (*KST*). The *bars* are means of three replications of 20 seedlings ± SE. No significant different was observed from the treatments at $P \leq 0.05$.

Table 1. Histology analysis of leaves developed during four different steps of culture

Parameters	Steps of culture (duration of culture)			
	In vitro seedlings (4–5 mo)	*Ex vitro* rooting (12 wk)	Prenursery (12 wk)	Nursery (24 wk)
Total leaf thickness (μm)	141.7 ± 4.4a	202.7 ± 5.0b	206.6 ± 4.1 b	289.2 ± 5.1c
Adaxial epidermis thickness (μm)	18.9 ± 1.2a	27.5 ± 0.9b	29.7 ± 1.1bc	30.3 ± 2.3c
Abaxial epidermis thickness (μm)	11.1 ± 0.8a	16.8 ± 1.3b	17.5 ± 0.8b	19.4 ± 0.9b
Palisade parenchyma thickness (μm)	81.4 ± 6.1a	104.2 ± 3.2b	106.8 ± 3.2b	184.7 ± 2.8b
Spongy parenchyma thickness (μm)	30.2 ± 2.3a	54.3 ± 2.9b	52.6 ± 2.3b	54.9 ± 3.6b
Epicuticular wax (μg cm^{-2})	23.0 ± 1.0a	46.8 ± 2.6b	52.8 ± 2.5c	62.6 ± 3.2d

Within each parameter, the steps of culture treatment that was significantly different from others are denoted with *different letters* ($P < 0.05$). Data is presented as the mean ± SE of ten replicate seedlings

Discussion

There is an urgent need to improve embryo culture protocols for the large scale production of Kopyor planting material, as this is the only technique available to produce true-to-type plants. The embryo culture technique for coconut has been developed for many years. However, the success rate of embryo conversion into fully functional plant still remains low. The major bottleneck in Kopyor embryo culture is root induction and further acclimatization. Using the standard embryo culture protocol (Rillo 2004), the percentage of seedlings which were found unable to produce any roots during *in vitro* culture was high (30–40%) with a success rate of acclimatization lower than 30%. There is thus huge room for improvement before an efficient and robust protocol can be scaled-up to meet the growing demand emerging from national and regional markets in South East Asia.

An *ex vitro* rooting protocol was assessed in the present study in the aim of simplifying the procedure and reduce production costs. We found that almost all seedlings (*ca.* 90%) which underwent *ex vitro* rooting survived and showed a high percentage of root induction (*ca.* 80%; Fig. 4). This method has been successfully applied in a number of species including several horticultural plants (Clapa et al. 2013), *Pistacia vera* L. (Benmahioul et al. 2012), *Camellia sinensis* (L.) Kuntze (Ranaweera et al. 2013), *Caralluma edulis* (Edgew.) Benth. Ex Hook.f. (Patel et al. 2014), and *Castanea sativa* Mill (Cuenca et al. 2017).

Our improved *ex vitro* rooting protocol combines root induction with acclimatization in a single step. This new protocol improved the success rate of the acclimatization step when compared to standard protocols involving classical *in vitro* rooting followed by acclimatization (Talavera et al. 2005; Magdalita et al. 2010). It was also found to generate the same success rate as the photoautotrophic protocol developed for coconut by Samosir and Adkins (2014).

Both NR and FR-type Kopyor seedlings did show better growth parameters (higher fresh weight, number of opened leaves, and number of primary roots) when 1.0 μM IBA was added to the liquid medium during *ex vitro* rooting (Fig. 5). The improvement of rooting induction and survival after acclimatization with IBA treatment has been observed for the *ex vitro* rooting of several plants species such as *Gypsophila paniculata* L. (Wang et al. 2013), *P. vera* L. (Benmahioul et al. 2012), and *C. sativa* Mill. (Cuenca et al. 2017). In coconut, this is the first report on the successful supplementation of IBA during *ex vitro* rooting.

The *ex vitro* rooting protocol under study was also found applicable to four different Kopyor cultivars (Fig. 6). The high success rate obtained during *ex vitro* rooting and acclimatization of coconut using mini growth chamber was of same range to that described for other horticultural plants (Clapa et al. 2013), and *C. sinensis* (L.) Kuntze (Mukhopadhyay et al. 2016). Several

Table 2. Comparison of the stomatal density during four different steps of culture

Parameters	Steps of culture (duration of culture)			
	In vitro seedlings (4–5 mo)	*Ex vitro* rooting (12 wk)	Prenursery (12 wk)	Nursery (24 wk)
Stomatal density in adaxial surface (mm^2)	6.6 ± 0.2c	6.5 ± 0.4c	2.8 ± 1.0b	0.4 ± 0.1a
Stomatal density in abaxial surface (mm^2)	47.6 ± 2.1a	62.2 ± 2.4b	101.7 ± 2.5c	112.2 ± 2.2d

Within each parameter, the steps of culture treatment that was significantly different from others are denoted with *different letters*. Data is presented as the mean ± SE of ten replicate seedlings

authors showed that success rates in root induction were cultivar-dependent in *Prunus armeniaca* L. (Cati et al. 2014) and *Cornus alba* L. (Ilczuk and Jacygrad 2016).

The high success rate of *ex vitro* rooting was paralleled by leaf histology parameters. Indeed, the leaves which developed during *ex vitro* rooting were thicker with larger epidermis and parenchyma tissues and stronger epicuticular wax (Table 1). By using mini growth chamber, the seedling environment was kept at high relative humidity (almost 100%) for 8 wk followed by a gradual decrease for the following 4 wk. Improved ventilation using air pump and increased light intensity are also key factors for successful *ex vitro* rooting in coconut. Such factors have proven efficient in increasing leaves thickness and synthesis of epicuticular wax during *ex vitro* rooting and acclimatization in several plant species (Pospisilova et al. 1998; Hazarika 2003; Pospisilova et al. 2007; Clapa et al. 2013; Samosir and Adkins 2014).

Thicker leaves are important, as they enable better photosynthetic capacity and metabolic activity in coconut (Samosir and Adkins 2014) as in other species such as *Hibiscus moscheutos* L. (Preece and West 2006), *Aralia elata* (Miq,) Seem. (Yokota et al. 2007), and *Phellodedron amurense* Rupr. (Yokota et al. 2007). Meanwhile, increasing epicuticular wax of leaves which developed during *ex vitro* rooting was found to decrease transpiration rate (Pospisilova et al. 1998; Hazarika 2003). This is a key parameter for plant adaptation during transfer to *ex vitro* conditions. Similar results have been found in other species such as *H. moscheutos* L. (Preece and West 2006), *Photinia* x *fraseri* Dress (Larraburu et al. 2010).

The stomatal density of leaves produced during *ex vitro* rooting was found to be species-dependent. Indeed, in *H. moscheutos* L. (Preece and West 2006) and *G. paniculata* L. (Wang et al. 2013), leaves which developed during *in vitro* culture showed a higher stomatal density when compared to the ones which developed under *ex vitro* conditions. However, our experiment showed that coconut leaves developing under *ex vitro* conditions did show a higher number of stomata (Table 2). The same result was also reported in normal (non-Kopyor) coconut by Samosir and Adkins (2014) and for other species such as *Nicotiana tabacum* L. (Pospisilova et al. 1998).

The *ex vitro* rooting using mini growth chamber can be applied for rooting induction and acclimatization for all types of Kopyor coconut seedlings, with or without rooting system (Fig. 3). This means that no rooting step under sterile conditions was required. As a result, the duration of *in vitro* cultivation of plantlets could be reduced down to 4 mo, as compared to 10 mo when a standard protocol (Talavera et al. 2005) or from 6 mo using photoautotrophic cultivation process (Samosir and Adkins 2014).

In conclusion, the production cost of coconut plants derived from embryo culture can be decreased significantly by applying *ex vitro* rooting. Most importantly, the *ex vitro* rooting using mini growth chamber is not cultivar-dependent, so it can be applied to almost all coconut cultivars or even probably other palm species such as the date palm or the oil palm.

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