Genetic Transformation of the Indonesian Black Orchid (*Coelogyne pandurata* Lindley) through *Agrobacterium tumefaciens* for Micropropagation

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ABSTRACT

The black orchid (*Coelogyne pandurata* Lindley) is an endemic orchid from East Kalimantan, that is characterized by the large size of flowers, with greenish sepals and petals, with a black labellum. Recently, the population of this orchid was decreased and its extinction threatened to be extinct because of overcollecting and/or habitat destruction. The mass propagation of this orchid is urgently needed. Micropropagation of the black orchid was carried out by introducing the Arabidopsis gene *Knotted1-like Arabidopsis thaliana* (*KNAT1*) using *Agrobacterium tumefaciens*. As the plant materials, four-month-old protocorms that were maintained on Vacin and Went (VW) medium were used with 3 replications. The experiment was carried out in three steps: 1) Obtaining the most suitable basic medium (Vacin and Went, VW; New Phalaenopsis, NP; Murashige dan Skoog, MS; and KNUDSON C, KC); 2) Selection of the best phase of developing protocorms for gene transfer; 3) and Genetic transformation of plasmid 35S-KNAT1 and pGreen (empty vector plasmid) using *A. tumefaciens* strain LBA4404 into orchid protocorms. The results show that the best medium for *in vitro* culture of the black orchid protocorm is a half strength of MS (1/2 MS) medium that could increase the growth rate of 9.6% protocorms up to phase 6 after 12-week cultivation. But after 12 weeks, the best medium for shoot induction to obtain plantlets is NP medium, so that 11.5% protocorms become plantlets with three leaves. The best condition for gene transfer is five-month-old protocorm. The frequency of genetic transformation using A. tumefaciens is 32.9% for pGreen vektor and 43.2% for p35S-KNAT1. This work is in progress.

Key words: Black orchids, *Coelogyne pandurata*, basic medium, protocorm, genetic transformation, *KNAT1, Agrobacterium tumefaciens*

INTRODUCTION

Black orchid (*Coelogyne pandurata* Lindley) is an endemic orchid of the Province of East Kalimantan, Indonesia, that is threatened by extinction. The over-collection, habitat destruction, and difficulties of cultivation through conventional methods are the chief problem of this orchid (Arditti, 1992). The uniqueness of this orchid is its very short (3-5 days) blooming period and difficult pollinazation (Arditti, 1992). Moreover, *in vitro* seed germination of this orchid needs special condition such as 3-4 months incubation in a dark prior germination (Wirakusumah, 2009, personal communication). For successful cultivation, *in vitro* seed germination is the key step (Arditti & Ernst, 1993). In order to obtain the optimal condition for *in vitro* seed germination of this orchid, some experiments using various culture media are needed to obtain the most suitable medium.

In orchid tissue culture, the various mediums for seed germination and shoot induction are Knudson C (KC), Vacin and Went (VW), and Murashige and Skoog (MS) with addition of some organic complexes such as coconut water (Arditti, 1993, Widiastoety dan Syafril, 1993; Demasabu *et al*., 1998; Untari *et al*., 2006). Islam *et al*. (1998) used *New phalaenopsis* (NP) medium for callus induction of Phalaenopsis. Semiarti *et al.* (2007) also used the NP medium for growing Phalaenopsis orchid before and after genetic transformation of the orchid using *A. tumefaciens.*

The results of our previous experiment (Semiarti et al., 2007) in which insertion of Arabidopsis *KNAT1* gene into Phalaenopsis orchid

protocorm resulted in multishoots production (about 31 shoots from one protocorm) will be useful for the micropropagation of black orchids. Multishoot occurrence in KNAT1 transgenic plants has also been reported by Chuck *et al.* (1996) in transgenic Arabidopsis plant and Nishimura *et al.* (2000) in Nicotiana. In Dendrobium "Madame Thong In" orchid, Yu *et al.* (2001) obtained the multishoots from callus that derived from cut off protocorms that transfered by *DOH1* gene *(KNAT1* homologous in Dendrobium*)*. Each shoot could be independently grown into a plantlet.

Here we report the genetic transformation of *KNAT1* gene under the control of *Cauliflower Mosaic Virus* (CaMV) in pGreen vektor using *Agrobacterium tumefaciens* strain LBA 4404 into protocorms of black orchids for micropropagation.

MATERIALS AND METHODS

Plant materials and culture condition

Mature seeds and 4-month-old protocorms of black orchids were used as plant materials. The Protocorms were the generous gift of Mr. Wirakusumah (the owner of Edward and Frans Orchids Nursery, East Java). Seeds from fully ripening fruit (five-months-old fruit) were sown in various culture media: Knudson C (KC), Vacin & Went (VW), New Phalaenopsis (NP) (Islam *et al.*, 1998), and Murashige & Skoog (MS) in half and full strength concentration of macroelements with and without 150 $ml.l^{-1}$ coconut water. Protocorms were transferred into four kinds of orchid medium: VW, MS, NP and KC medium, each supplemented with 150 $mg.l^{-1}$ potato, 150 $mg.l^{-1}$ banana, 150 $ml.l^{-1}$ coconut water, and 1 ppm NAA. The in vitro cultures were incubated at 25°C with 1000 lux continuous light. The growth of protocorms, shoots and plantlets was examined every week.

Genetic Transformation

Genetic transformation of plasmid 35S::KNAT1 and pGreen vector into orchid was carried out according to the method of Semiarti *et al.* (2007), except that the liquid medium that was used to rinse the protocorm was half strength VW medium and 300 $mg.l^{-1}$ Cefotaxim. SIM (*Shoot Induction medium*; 0.15 µM NAA+ 5 µM 2iP) supplemented with 100 $mg.l^{-1}$ Kanamisin for selecting independent transformant. Frequency of transformation was decided by the ratio of the number of surviving protocorms per total number of transformed protocorms.

RESULTS AND DISCUSSION

Morphology of Black Orchid

The black orchid (*C. pandurata*) is an epiphytic sympodial orchid. Some pseudobulbs grow parallel with two leaves each. Five to seven flowers were arranged in a raceme, fragrance, each flower is 7-12 cm in diameter. Sepals and petals are green and the labellum (lip) is black. Seeds are microscopic in size, inside the fruit (Fig. 1).

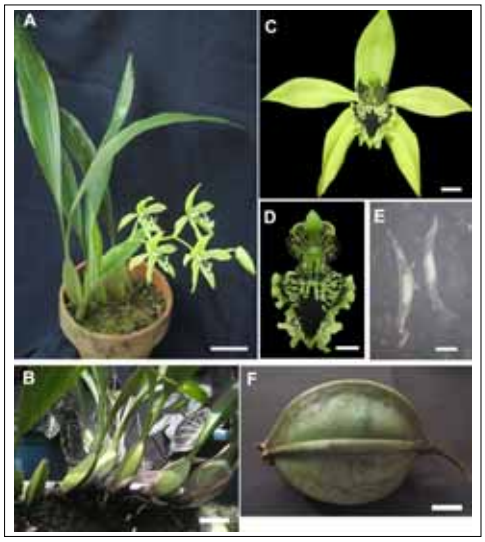

Fig. 1. Morphology of the Black Orchid. A. A Plant with flower; B. Plant at the base of pseudobulbs; C. Close up a flower; D. Black labellum; E. Mature seeds; F. Fruit. Bars: 5 cm in A and B; 1 cm in C, D, and F; 0,5 mm in E.

Developmental phases of black orchid

To analyze the growth rate of the black orchid embryo during seed germination, we classified the developmental period into six phases based on the growth phases, namely phase 1-6: phase 1/yellowish embryo, phase 2/green embryo, phase 3/bipolar embryo phase 4/first leaf formed embryo, phase 5/second leaf formed embryo and phase 6/third leaf formed embryo. The time course of embryo development observation showed that the color of the embryo started to change from yellowish (phase 1) into green (phase 2) three weeks after sowing. At four weeks, the green embryo formed a bipolar structure (phase 3), with one side darker than the other. The darker pole of the embryo changed into leaf primordia (phase 4) at the fifth week; protocorm with two leaves at seven weeks (phase 5) and protocorm with three leaves at eleven week (phase 6) (Fig. 2).

Twelve weeks after sowing, based on the growth rate of embryos, the data revealed that 1/2 MS medium is the best medium to support and accelerate the growth rate of black orchid embryos (Table I). This was indicated by 84.62% of the protocorms, which can grow up to phase 5 and 9.62% which grow up to phase 6 with the third leaf

Table 1. Growth of Black Orchid's Embryo in Various Medium at Twelve Weeks After Sowing

Variation of Medium	Number of Sowed Protocorm	Percentage of growing embryo at each phase						Death protocorm
		Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6	
1/2 KC	215	0.00%	0.00%	0.00%	57.21% (123)	27.91% (60)	0.00%	14.88% (32)
KC	174	0.00%	0.00%	0.00%	17.24% (30)	70.69% (123)	0.00%	12.07% (21)
KC+CW	175	0.00%	0.00%	0.00%	41.14% (72)	2.86% (5)	0.00%	56.00% (98)
1/2 VW	109	0.00%	0.00%	0.00%	59.63% (65)	40.37% (44)	0.00%	0.00%
VW	134	0.00%	0.00%	0.00%	7.46% (10)	40.30% (54)	0.00%	52.24% (70)
VW+CW	75	0.00%	0.00%	0.00%	9.33% (7)	25.33% (19)	0.00%	65.33% (49)
1/2 NP	193	0.00%	0.00%	0.00%	13.47% (26)	**86.53%** (167)	0.00%	0.00%
NP	112	0.00%	0.00%	0.00%	18.75% (21)	57.14% (64)	4.46% (5)	19.64% (22)
NP+CW	105	0.00%	0.00%	0.00%	1.90% (2)	72.38% (76)	8.57% (9)	17.14% (18)
1/2 MS	52	0.00%	0.00%	0.00%	5.77% (3)	84.62% (44)	**9.62%** (5)	0.00%
MS	262	55.73%	0.00%	0.76%	20.99% (55)	9.16% (24)	0.00%	13.36% (35)
MS+CW	73	0.00%	0.00%	0.00%	32.88% (24)	50.68% (37)	1.37% (1)	15.07% (11)

Phase 1 Phase 2 Phase 3 Phase 4 Phase 5 Phase 6

Fig. 2. Developmental phases of The Black Orchid's embryo

emerging from the shoot tip. These results indicate that half-strength concentration of complete elemens containing medium is needed for black orchid seed germination.

When we started to use 4-month-old protocorms as plant materials, based on the criteria above, the best medium for the maintenance of the developmental process of orchid protocorms and shoots is NP medium. Three out of 26 protocorms reached phase 5 within two months of subculture on NP medium (Table II). This is consistent with the results of Islam *et al.* (1998), who obtained the best condition for callus induction on NP medium. It because of the nitrogen and phosphate content in the NP medium are higher than the others.

Fig. 3. Shoots of transforman (s). (A), Non transforman; B. pGreen transformed shoots; C. pKNAT1 transformed shoots.

Table II. Growth of black orchid protocorms from 4 months in vitro culture, after 2 months transfered into various culture media.

Medium	Number of transfered protocorms	Number of protocorms reach developmental phase				
		Phase 1	**Phase 2**	**Phase 3**	**Phase 4**	**Phase 5**
KC	15	0	7	8	0	0
VW	15	0	2	13	0	0
MS	23	0	3	18	2	0
NP*	26	0	8	13	2	3

*the best medium

Table III. Frequency of transformation of the black orchid (*C. pandurata* Lindley) using *Agrobacterium tumefaciens*.

	Replication	**Number of transformed protocorms**	**Number of survival protocorms**
Non-transformant	3	-	45.97% (24/62)
pGreen (Vector)	3	54	32.96% (18/54)
P35S::KNAT1	3	35	43.25% (15/35)

Frequency of genetic transformation of black orchid using A. tumefaciens

The transformation result of p35SKNAT1 and pGreen vector show that the frequency of transformation is relatively high, about 43.25% (15 of 35 protocorms are kanamycin-resistant), and 32.96% for pGreen vector (18 of 54 protocorms are kanamycin-resistant) (Fig. 3 and Table 3). PCR analysis to confirm the insertion of *KNAT1* gene is still in progess. The expression of *KNAT1* gene in the black orchid transformants might improve the totipontency of the orchid to form shoots as has occurred in another natural orchid, *Phalaenopsis amabilis* (Semiarti *et al.*, 2007). The results will support both the conservation effort and orchid farmers.

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アグロバクテリウム遺伝子導入法を用いたインドネシア産 *Coelogyne pandurata* のマイクロプロパゲーション法の開発

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摘要

ブラックオーキッド（Coelogyne pandurata Lindley）はインドネシア東カリマンタン産のランであり、花が大きくガク片と花弁は緑色で唇弁は黒色である。最近、このランは、過剰採取と生息地の破壊によって絶滅の危機に瀕している。このランの人工増殖は緊急の課題である。シロイヌナズナの *Knotted1-like Arabidopsis thaliana* (*KNAT1*)遺伝子をアグロバクテリウム法により導入したブラックオーキッドを用いてクローン増殖を試みた。植物材料として、Vacin and Went（VW）培地で 4 ヶ月培養したプロトコームを用いた。シュート形成の条件検討を 3 点について行った。第一に、最適培地（VW、New Phalaenopsis (NP)、Murashige・Skoog（MS）、Knudson C（KC））の検討、第二に、遺伝子導入に最適のプロトコームの生育段階の検討、第三に、*A. tumefaciens* LBA4404 を用いてプロトコームへのプラスミド 35S-KNAT1 と pGreen ベクターの導入を行った。ブラックオーキッドのプロトコーム培養の最適培地は 1/2 MS であった。12 週間の培養で 9.6 %のプロトコームがフェイズ 6 以上になった。しかし、12 週間以降の幼葉殖物の生育には NP 培地が最適であり、11.5 %のプロトコームが 3 葉を形成した。遺伝子導入に最適なプロトコームは 5 ヶ月培養したものであった。遺伝子導入効率は pGreen ベクターについては 32.9%、p35S-KNAT1 については 43.2 %であった。