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Preface

On behalf of the conference organizing committee, we are happy to present the Proceedings of the Third International Conference on Mathematics and Natural Sciences (ICMNS 2010). The organizing committee of the ICMNS 2010 is highly pleased to have nearly two hundreds full papers submitted to the Conference. The ICMNS’s biannual event is organized jointly by the Faculty of Mathematics and Natural Sciences (FMIPA), the School of Life Sciences and Technology (SITH), and the School of Pharmacy (SF), Institut Teknologi Bandung. We are highly honored to host the event here in Bandung.

The aim of the ICMNS 2010 is to promote interdisciplinary researches in science and technology, to encourage the development of sciences and technologies for sustainable development, and to disseminate research in various fields of mathematics and natural sciences. The main theme of the ICMNS 2010 is “Science for Sustainable Development”. The conference deals with mathematics and natural sciences to fundamental and applied researches, including nine scopes and topics that are health sciences, biosciences and biotechnology, environmental science, pharmaceutical science, physical sciences, material science, mathematics, computer and computational science, and earth and space sciences.

Finally, we would like to express our gratitude to Dean of FMIPA, Dean of SF, Dean of SITH, PT Chevron, PT Biofarm, and Indonesian Journal of Physics (IJP) for the financial support and thank the invited speakers as well as participants for their contribution in making the conference a success. As general chairperson, I highly appreciate the great efforts of the members of the organizing committee whose hard work really made it possible to have this conference.

Bandung, April 30, 2011

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AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF INDONESIAN BLACK ORCHID

Coelogyne pandurata LINDLEY

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Abstract. Transgenic technology using Agrobacterium tumefaciens was conducted to improve micropropagation efficiency of Indonesian Black orchid (Coelogyne pandurata Lindley). The population of this endemic orchids in it's natural habitat in East Kalimantan was extremely decreased due to the overcollection and/or habitat destruction. Therefore, mass propagation of this orchid is urgently needed. The T-DNA was constructed in disarmed Ti plasmid containing the coding region of a neomycin phosphotransferase II gene as a selectable marker, an Arabidopsis gene Knotted1-like Arabidopsis thaliana (KNAT1) under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter. The pGreen plasmid was used as binary vector. As the plant materials, seeds of black orchid on New Phalaenopsis (NP) medium were used with 3 replications. Into preculture and cocultivation medium, 75 mg/l acetosyringone (AS), a wound response molecule was added. One-week old protocorms were cocultivated with Agrobacterium for gene transfer. Genetic transformation of plasmid 35S-KNAT1 and pGreen (empty vector) using A. tumefaciens strain LBA4404 were performed into orchid protocorms. The results show that germinated seeds on 1/2NP medium increased the growth rate of 86.5% protocorms up to phase 5 after 12-week cultivation. One-week old protocorms is the best explant for gene transfer. The frequency of genetic transformation into black orchid using A. tumefaciens is 66 % for pGreen vector and 61.6 % for p35S-KNAT1. The existance of transgene in the transforman genome was confirmed by PCR analysis using KNAT1 gene specific primers. The 35S::KNAT1 transformans generates shoot multiplication for about seven times higher than that of non-transformed plant(s).

Keywords: Black orchid, Coelogyne pandurata, shoot induction, genetic transformation, Agrobacterium tumefaciens.
1. Introduction

As an endemic orchid, the existence of black orchid (Coelogyne pandurata Lindley) in the Province of East Kalimantan, Indonesia is very important. It because of over-collection, habitat destruction, and difficulties of cultivation, the population of this orchid is highly decreased, recently [1]. The uniqueness of this orchid is on the very short period for blooming (3-5 days) and difficulties of pollination [1, 10]. According to Wirakusumah (2009, personal communication), in vitro seed germination of black orchid needs a special condition such as incubation in the dark prior to germination for 3-4 months. For successful cultivation, in vitro seed germination is the key step [2]. In order to obtain the optimal condition for in vitro seed germination of this orchid, some experiments using various culture media are needed.

In tissue culture of orchid, the various media used for seed germination and shoot induction are Knudson C (KC), Vacin and Went (VW), and Murashige-Skoog (MS) with addition of some organic complexes such as coconut water [2, 4, 9, 11]. Islam et al. (1998) used New phalaenopsis (NP) medium for callus induction of Phalaenopsis [5]. Semiarti et al. (2007) also used the NP medium for growing Phalaenopsis orchid before and after genetic transformation treatment of the orchid using A. tumefaciens [7]. Our previous experiment [7] indicated that insertion of KNAT1 gene into Phalaenopsis orchid protocorm induced multishoots production (about 31 shoots from one protocorm), and further results show the level of multiplication increased that become more than 90 shoots emerged from one embryo. It is very valuable and promising for micropropagation of black orchids. Multishoot occurrence in KNAT1 transgenic orchid plants has also been reported by Chuck et al. (1996) in transgenic Arabidopsis plant [3], and Nishimura et al. (2000) in Nicotiana [6]. In hybrid orchid Dendrobium "Madame Thong In", Yu et al. (2001) obtained the multishoots from callus that derived from cut off protocorms that transfered by DOH1 gene (KNAT1 homologous gene in Dendrobium) [12]. Each shoot could independently grew into plantlet.

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

2. Materials and Methods

2.1. Plant materials and culture condition

Mature seeds of black orchids were used as plant materials. Seeds from fully ripening fruit (five-months-old) were given by the late Mr. Wirakusumah (owner of Edward and Frans Orchids Nursery, East Java). The seeds were sown on New Phalaenopsis (NP) [5] in half strength and full strength NP medium with and without addition of 150 ml l⁻¹ coconut water. Developing orchid embryo (Protocorms) were transferred onto NP medium, 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 were examined every week.
2.2 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) [7], except that the liquid medium that was used to rinse the protocorm was half strength NP 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. Into each step 75 mg/l acetosyringone (AS) was added to improve the efficiency of T-DNA insertion [8].

\[ \text{Figure 1. Schematic Structure of 35S::KNAT1 containing T-DNA. LB, Left border; RB, Right Border, 35S: CaMV promoter; KNAT1 gene; HPT: Hygromycin phosphotransferase; Tnos: Nos terminal.} \]

Frequency of transformation was decided by the ratio of the number of surviving protocorms per total number of transformed protocorms.

3. Results and Discussion
3.1 Characteristics of Black Orchid
The black orchid (C. pandurata) is an epiphytic sympodial orchid, that characterized by a big size of greenish flower with a black labellum (Fig. 2). This orchid exhibits some pseudobulbs, that grow parallel with two leaves each. Five to seven flowers were arranged in a raceme and fragrance. Diameter of each flower is 7-12 cm. Sepals and petals are green and the labellum (lip) is black. Seeds are microscopic in size, and it located inside the fruit.

\[ \text{Figure 2. The Flower of Black Orchid. A. Close up flower; B. Labellum. Bars: 5 cm in A and 1 mm in B.} \]
3.2 Developmental phases of black orchid embryo

The development of black orchid embryo during seed germination was classified 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 embryo started to be changed from yellowish (phase 1) into green (phase 2) is one to two weeks after sowing. At three to 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 to twelve weeks (phase 6) (Fig. 3).

At twelve weeks after sowing, based on the growth rate of embryos, the data revealed that 1/2 NP medium is the best medium to support and accelerate the growth rate of black orchid embryos that resulting seed germination. About 86% of protocorms grew up to phase 5 (Table 1). The result shows that for embryo development during seed germination in black orchid, a half-strength concentration of complete elements containing medium is the best.

Table 1. Growth of Black Orchid’s Embryo on NP medium at twelve weeks after seed sowing

<table>
<thead>
<tr>
<th>Variation of Medium</th>
<th>No of seeds</th>
<th>Percentage of growing embryo at each phase</th>
<th>Death protocorm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phase 1</td>
<td>Phase 2</td>
</tr>
<tr>
<td>1/2 NP</td>
<td>193</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>NP</td>
<td>112</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>NP+CW</td>
<td>105</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

It might be the content of macro- and micro-elements in the half-strength medium have provided suitable concentration to promote the development of black orchid embryos. So that not necessary to use a full strength of the basic medium for black orchid’s seed germination. Tissue culture is an empirical science [2]. It is difficult to predict the type of explant, media, and conditions that would be suitable for a specific genus or species or clone. It is not possible to explain why a certain medium and culture conditions can lead to success while others fail. In black orchid, the half-strength NP medium may be the best for seed germination, in which the embryo will serve better response for genetic transformation treatment than that if we used full strength NP medium.
Agrobacterium-Mediated Genetic Transformation Of Indonesian Black Orchid

Coelogyne pandurata Lindley

Figure 3. Developmental phases of the Black Orchid Embryo (Phase 1-6)

Table 2. Frequency of transformation by pGreen and pKNAT1 on regeneration medium, two-months after selection on kanamicyn containing medium

<table>
<thead>
<tr>
<th>No.</th>
<th>Transformant (NT)</th>
<th>Kan300 mg/l (+/-)</th>
<th>No of protocorms</th>
<th>Percentage of Kan resistance plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Non</td>
<td>-</td>
<td>100</td>
<td>Survive: 98 %</td>
</tr>
<tr>
<td>2.</td>
<td>NT</td>
<td>+</td>
<td>237</td>
<td>Survive: 37.6 %</td>
</tr>
<tr>
<td>3.</td>
<td>pGreen</td>
<td>+</td>
<td>707</td>
<td>Survive: 66.0 %</td>
</tr>
<tr>
<td>4.</td>
<td>p3SS: :KNAT1</td>
<td>+</td>
<td>701</td>
<td>Survive: 61.6 %</td>
</tr>
</tbody>
</table>

The expression of KNAT1 gene in the black orchid transformants could be judged from the ability of transformants to form multishoots as the same as in another natural orchid, Phalaenopsis amabilis [7]. The phenomenon of multishoot occurrence in KNAT1 transgenic plants has also been reported by Chuck et al. (1996) in transgenic Arabidopsis, and Nishimura et al. (2000) in Nicotiana [3, 6]. Yu et al. (2001) obtained multishoots of a hybrid orchid from callus protocorms that transferred by DOH1 gene, in which each shoot could independently grow into plantlet [12]. PCR analysis has confirmed the existence of KNAT1 gene in the genome of survive plant on antibiotic containing medium of black orchid transformants was done for 10 plants each. For other transformant candidates, confirmation of the transgene is still in progress. Fig. 4B and C show that non-transformant plant produces only one shoot from embryo, but transformant produces seven shoots from one protocorm. Addition of cytokinin as phytohormon such as Kinetin or 2- penthenyl adenine (2-iP) that combined with NAA (synthetic auxin) in medium might induce and increase the number of multishoot production.
Figure 4. Black orchid transformants. A. Two-months old candidate of transformants grow on kanamycin antibiotic containing medium in selection plate; B. A shoot grows from a non-transformant protocorm; C. Multishoots grow from one 35S::KNAT1 transformant protocorm; D. Electrophoregraph of PCR product of transformants using transgene specific primers KNAT1F1-R1: Lane 1, Lambda/Styl DNA marker, Lane 2: plasmid 35S::KNAT1. Lane 3: Non transformant plant, Lane 4-7: Transformant #1-4, amplified 1.2 kb DNA fragment of KNAT1 transgene. Bars: 1 cm in A, 0.5 cm in A-C.
from transformant. Further result of transformation with *P. amabilis* indicated that the maintenance of transformant on NP medium + 3 µM 2-iP+ 0.15 µM NAA induced high level of multishoot production up to 91 shoots per embryo. It is worth to try in black orchid. The high capability of shoot production will strongly support both conservation and agribusiness of orchid. It will serves as a new approach for orchid micropropagation for *in vitro* shoot production.

**Table 3.** Shoot Multiplication of transformant on (1/2NP +NAA 0.15 µM) medium

<table>
<thead>
<tr>
<th>No</th>
<th>Medium</th>
<th>No of transformant observed</th>
<th>Average No of shoot emerged from explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild type (NT)</td>
<td>½ NP</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>NT</td>
<td>½ NP SIM Kan</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>p35S::KNA T1</td>
<td>¼NP SIM Kan</td>
<td>8</td>
</tr>
</tbody>
</table>
4. Conclusion

Seeds of the Black orchid Coelogyne pandurata Lindley germinated optimally on 1/2NP medium, that 86.5% protocorms grow fast up to phase 5 after 12-week cultivation. One-week old protocorms is the best condition as target plant material for gene transfer using Agrobacterium tumefaciens. The frequency of genetic transformation into black orchid using A. tumefaciens is 66 % for pGreen vector and 61.6 % for p3SS-KNAT1. The multishoot was produced in 3SSno-KNAT1 containing transformants, but not in non-transformant.
Acknowledgement

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