Improvement of Genetic Transformation Efficiency in *Vanda tricolor* Orchid Using Acetosyringone

Rindang Dwiyani[1*], Azis Purwantoro[1, 2] Ari Indrianto[1, 3], and Endang Semiarti[1, 3]

[1]Graduate school of Biotechnology, Universitas Gajah Mada, Yogyakarta, Indonesia
[2]Faculty of Agriculture, Universitas Gajah Mada, Yogyakarta, Indonesia
[3]Faculty of Biology, Universitas Gajah Mada, Yogyakarta, Indonesia

Abstract

Vanda tricolor Lindl. var. *suavis* is an Indonesian wild orchid which is now extremely rare in nature due to its habitat destruction. Development of an appropriate method for improving *Vanda* orchid through genetic modification could be valuable for horticulture and, indirectly, also for conservation. In this research, a method of *Agrobacterium*-mediated transformation of two *Vanda tricolor* obtained from Salak Mount, West Java and Merapi Mount, Yogyakarta in Indonesia protocorms was improved using acetosyringone (AS). Concentrations of 0 and 25 ppm AS were used in transformation of pG35S binary vector containing kanamycin resistance gene into *V. tricolor* protocorms. The result showed that 25 ppm AS was required on innoculation with *Agrobacterium* solution, without AS on cocultivation. Five weeks after treatment on the 300 ppm kanamicyn-containing medium, green protocorms were obtained, that was 11.01% for *V. tricolor* from Salak Mount with pre-culture treatment prior to innoculation, 9.39% for *V. tricolor* from Merapi Mount with pre-culture treatment prior to innoculation, and 1.37% for *V. tricolor* from Merapi Mount without pre-culture treatment prior to innoculation. The best condition to set high efficiency of transformation is pre-culture protocorms prior inoculation, soaking protocorm on 25 ppm AS for 30 minutes, then cocultivate its on AS-free callus induction medium.

Key words: *Vanda tricolor*, *Agrobacterium*, orchid protocorms, acetosyringone

---

*Corresponding author
Jl. Sekip Utara, Yogyakarta 55281, Indonesia
Tel. +62-274-564305; Fax.+62-274-563974
E-mail :rdwiyani@yahoo.com

Introduction

Vanda tricolor Lindl. var. *suavis* is an Indonesian wild orchid which is widespread in cultivation in its native regions of Java (West Java, East Java, and Central Java including Yogyakarta) and Bali in Indonesia (Gardiner, 2005). This species is now extremely rare in nature due to its habitat destruction. This species of orchid has white-fragrant flowers with brown spots and purple labellum and it is grown for commercial production of ornamental plant. Development of an appropriate method for improving *Vanda* orchid through genetic modification could be valuable for horticulture and, indirectly, also for conservation.

Agrobacterium-mediated transforma-tion on some orchid species has been succesfully done, such as on *Phalaenopsis* hybrid (Belarmino & Mii, 2000; Chai *et al*, 2002; Mishiba *et al*, 2005), *Dendrobium* (Yu *et al*, 2001; Men *et al*, 2003), *Phalaenopsis amabilis* (Semiarti *et al*, 2007), *Cymbidium* orchid (Chin *et al*, 2007), and Vanda orchid (Shresta *et al*, 2007).

Semiarti *et al* (2007) reported that the use of acetosyringone (AS) could improve transformation efficiency. Although Nan *et al* (1997) found that *Dendrobium* orchid contains coniferyl alcohol as inducer of *Agrobacterium* virulence genes, the effectiveness of AS on *Agrobacterium*-mediated transformation has been reported previously for *Phalaenopsis* orchid (Mishiba *et al*, 2005) and *Vanda* orchid (Shrestha *et al*, 2007). Increase of transformation efficiency with the use of AS in *Agrobacterium*-mediated transformation was also reported by Costa *et al* (2006) in almond (sub-family *Prunoideae*), in which transformation efficiency increase by 100 fold

with the use of 150 µM AS during the 21-days induction period compared to control.

Protocorms derived from seeds (self polinated) were used as target of transformation. The use of protocorms as target on *Agrobacterium*-mediated transformation has been successfully done for orchid by some researchers (Mishiba *et al*, 2005; Chin *et al*, 2007; Semiarti *et al*, 2007). Shresta *et al* (2007) used protocorm-like bodies (from somatic cells) of *Vanda* hybrid 'Tokyo blue' as target of transformation, but in this study we used protocorms from embryos of an wild orchid of *Vanda* (*ie. Vanda tricolor*) as target of transformation.

The aim of this reseach was to investigate the effects of acetosyringone addition on the percentage of transforman candidates in the *Agrobacterium*-mediated transformation of *V. tricolor* orchid. Prior to transformation, kanamycin test was performed to protocorms of *V. tricolor* orchid in various different ages. We also examined the need of pre-culture treatment before *Agrobacterium* infection.

Materials and Methods

Plant Materials. *V. tricolor* pods (6 months after self polination) collected from Salak Mount (West Java) and Merapi Mount (Yogyakarta) were used as seed sources. Orchid pod was washed, sterilized 3 times by dipping in 70% ethanol, firing, and put gently on sterillized petridish under asceptic condition. Seeds were then sown in New *Phalaenopsis* (NP) medium (Islam *et al*, 1998) enriched with 100 g/l tomato extract as used by Semiarti *et al* (2010). Five weeks after sowing, protocorms were collected and used as target of transformation.

Bacterial strain and plasmid vector. We used the same disarmed octopine type *Agrobacterium tumefaciens* LBA4404 harbours p35S binary vector which contains *Neophosphotransferase* gene (*NPTII* gene) as selectable marker (Semiarti *et al*, 2007), as shown in Figure 1.

Inoculation and Co-cultivation with *A. tumefaciens.* Three days before infection, eight week-old of germinated protocorm were transfered to fresh NP medium contains 1 ppm of 2,4D. *A. tumefaciens* was cultured overnight in LB liquid medium containing 200 mg/l of kanamycin. Inoculation of *A. tumefaciens* was performed by adding 2 ml of *A. tumefaciens* liquid culture into 8 ml of NP liquid medium (4× dilution), 0.01% of Tween 20, with or without 25 ppm of acetosyringone (AS). Precultured protocorms were then immersed in this diluted *Agrobacterium* liquid culture for 30 minutes, and immediately transfered onto sterilized filter paper for 60 minute-air drying. These protocorms then transferred into 0.2% (w/v) gellan gum-solidified NP medium added with 1 ppm of 2,4D with or without 25 ppm of AS for co-cultivation. After 3 days co-cultivation in this medium, protocorms were washed three times with ½NP liquid medium containing 10 mg/l of meropenem, and then transferred to 0.2% (w/v) of gellan gum-solidified NP nedium added with 5 µM of 2-*isopentenyladenine* , 0.15 µM of *Napthalene acetic acid* (Shoot Induction Medium or SIM) and 8 mg/l of meropenem to slow growth of *A. tumefaciens*. Protocorms were cultured in this medium for 7 days.

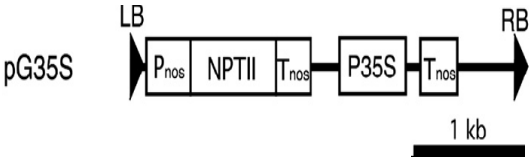

Figure 1. Structure of a binary vector pG35S harboured *NPTII* gene. Right border, RB; Left border, LB; Promoter of the nopaline synthase gene, Pnos; Polyadenylation site of the nopaline synthase gene, Tnos; Neomycin Phospotransferase gene, NPTII; 35S promoter of Cauliflower Mosaic Virus, P35S (Semiarti *et al*, 2007)

Elimination of *A. tumefaciens* and selection of transformant candidates. Seven days after maintaining the protocorm on bacterial-elimination medium, protocorms were then washed with ½NP liquid medium containing 10 mgl/l of meropenem for three times, then were transfrred in to selection medium, *ie.* SIM with 300 mgl/l of kanamycin and 8 mgl/l of meropenem. Protocorms maintaned in this medium for 5 weeks and subcultured every week or less in the case to eliminate of *A. tumefaciens*. Elimination of *A. tumefaciens* was performed by immersing protocorms with ½NP liquid medium containing 10 mgl/l of meropenem for 30 minutes, and then gently washed using the same medium for 2-3 times until free from *A. tumefaciens*. After 5 weeks

of selection, green protocorms were collected as candidates of transformants. Protocorms of *V. tricolor* orchid derivied from seeds required 20 weeks after sowing to perform plantlet.

Kanamycin test. Kanamycin test was performed twice on the protocorms of *V. tricolor* from Merapi Mount. First test was performed at 8 week-old protocorms and the second performed at 10 week-old protocorms. There was five variation of concentration of kanamycin applied on each age of protocorms. The concentration of 0, 100, 150, 200, and 250 mg/l were applied on eight week-old protocorms and the concentration of 0, 200, 300, 400, and 500 mg/l were for 10 week-old protocorms. The percentage of green (survive) and brown (dead) protocorms was observed after 5 weeks of application.

Results and Discussion

Kanamycin test

Five weeks after kanamycin application, 95% of 8 week-old protocorms turned to brown after the treatment with kanamycin at concentration of 250 mgl/l. This indicated that selection medium should be performed at concentration of kanamycin more than 250 mgl/l (Figure 2). However, for 10 week-old protocorms, there were 20% of protocorms remained green after the treatment with kanamycin at concentration of 500 mgl/l (Figure 3). These data showed that older protocorms were more resistant to kanamycin than the younger protocorm. Based on these data, we used 8 week-old protocorms for the target of transformation and kanamycin at concentration of 300 mgl/l for selection. Beside that, our previous research (2009, unpublished) also found that more than 50% of *V. tricolor* embryos achieved its protocorm stage (yellow or green colour structure) at 8 week after germination.

Agrobacterium-mediated transforma-tion using young protocorms of *Phalaenopsis* orchid (at an early stage after germination) has been sucessfully done by some researchers (Mishiba *et al*, 2005; Semiarti *et al*, 2007).

Selection of Kanamycin-resistant Protocorms

Genetic transformation using *A. tumefaciens* LBA4404 strain harbored pG35S containing *NPTII* gene was conducted 3 times on the 8 week-old protocorms of *V. tricolor* orchid as the target of transformation The first transformation used *V. tricolor* from Salak Mount (West Java), the second and the third used *V. tricolor* from Merapi Mount. The difference beetween the second and the third was the use of pre-culture treatment on the second transformation, while the third was not used.

Five weeks after the protocorm grown on the selection medium containing 300 mg/l kanamycin, 100% of control protocorms (without *A. tumefaciens* infection) turned to brown in *V. tricolor* from Merapi Mount, but about 20% of those from Salak Mount (West Java) remained green (Figure 4). This indicated *V. tricolor* orchid from both origins having different resistance to kanamycin. These data also indicated that selection medium with kanamycin at concentration of 300 mgl/l was not appropriate for *V. tricolor* from Salak Mount (West Java). The failure of kanamycin inhibiting growth of explant have been reported previously for protocorm like bodies (plb) of *Dendrobium* orchid (Chia *et al*, 1994), *Cymbidium* orchid (Chin *et al*, 2007), and *Vanda* orchid (Shrestha *et al*, 2007), but kanamycin was successfully used as selectable marker in plant for protocorms of *P. amabilis* orchid (Semiarti *et al*, 2007).

Table 1 shows the percentage of green protocorm after 5 weeks on selection medium. Addition of AS on innoculation medium was required either with or without AS added on co-cultivation medium. The use of AS for *vir* induction are recommended in most of the monocot transformation protocols (Hiei *et al*, 1994; Ishida *et al*, 1996; Cheng *et al*, 1997; Tingay *et al*, 1997; Zhao *et al*, 2000), because in nature, the system of DNA transfer to plant cell by *Agrobacterium* works on dicot, but not on monocot. Effectiveness of AS on *Agrobacterium*-mediated transformation has been reported previously for *Phalaenopsis* hybrid (Mishiba *et al*, 2005). In this present study, addition of AS on innoculation increased greater percentage of protocorms surviving in selection medium rather than addition of AS on co-cultivation only. AS suplemented for transformation (inoculation) might stimulate higher concentration of AS

within tissue of the treated protocorm, thereby eliciting higher *vir*-gene-inducing activity in *Agrobacterium* (Nan *et al*, 1997). The presence of AS during transformation induced *vir*-genes activity and stimulated T-DNA transfer into plant cell (Zupan & Zambryski, 1995; Gelvin, 2003). The processing and transfer of T-DNA are mediated by products encoded by *vir* region (Zupan & Zambryski, 1995).

Pre-treatment culture was necessary for *Agrobacterium*-mediated transforma-tion on protocorms of *V. tricolor* orchid. This result was in line with Jacq *et al* (1993), Robischon *et al* (1995), and Mishiba *et al* (2005) who found that pre-culture of transformation target prior to infection resulted in higher transformation efficiency. The cell-cycle progression might be induced during pre culture (Mishiba *et al*, 2005), and T-DNA transfer is likely occur in cells with this condition (Villemont *et al*, 1997).

Further analysis need to be done to conclude that the protocol is improvement as claimed, such as PCR analysis and southern hybridization.

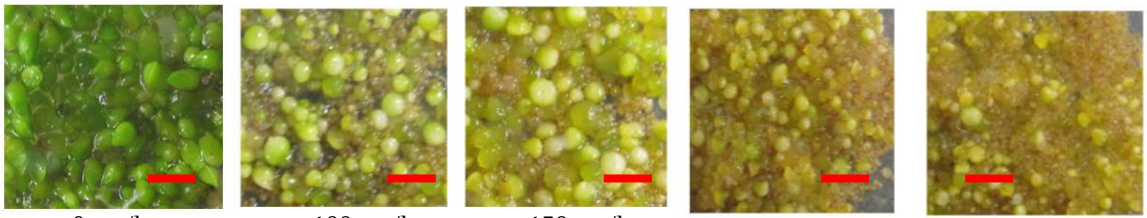

Figure 2. Kanamycin test on the 8 week-old protocorms of *V. tricolor* Merapi Mount. From left to the right, condition of protocorms after 5 weeks kanamycin application at concentration of 0, 100, 150, 200 and 250 mgl/l. Bar = 1500 µm.

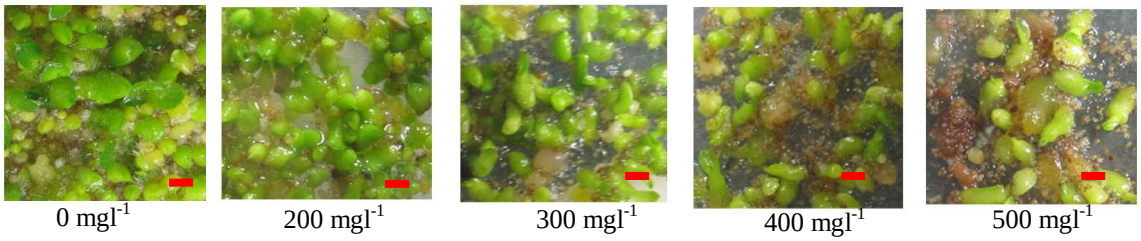

Figure 3. Kanamycin test on the 10 week-old protocorms of *V. tricolor* Merapi Mount. From left to the right, condition of protocorms after 5 weeks kanamycin application at concentration of 0, 200, 300, 400 and 500 mgl/l. Bar = 700µm.

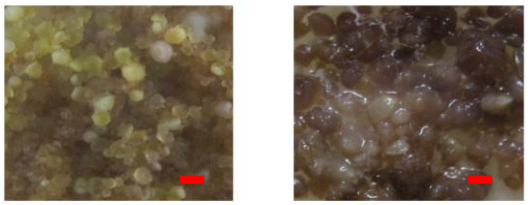

Figure 4. Control protocorms of *V.tricolor* after 5 weeks on medium containing 300 mg/l of kanamycin. Left, *V. tricolor* from Salak Mount (West Java), 20% of protocorms remained green; Right, *V. tircolor* from Merapi Mount, 100% of protocorms turned to brown. Bar = 300 µm

Conclusion

Resistance of *V. tricolor* protocorms to kanamycin varies depended on the development stage of protocorms and its origin. Older protocorms were more resistant than that of the younger protocorm. *V. tricolor* from Salak Mount (West Java) was more resistant to kanamycin than that from Merapi Mount (Yogyakarta). Addition of AS (especially at inoculation stage) and pre-culture treatment of protocorms prior to infection with *A. tumefaciens* was necessary to

be conducted in *Agrobacterium*-mediated- transformation of *V. tricolor* protocorms.

Table 1. The effects of Acetosyringone and pre-culture treatment on the transformation of 8 week-old protocorms of *V. tricolor* orchid.

Treatments		The percentage of green protocorms after 5 weeks on NP medium containing 300 mg/l of kanamycin		
Concentration of AS on Innoculation (ppm)	**Concentration of AS on co-cultivation medium (ppm)**	**Transformation 1 (*V. tricolor* West Java form with pre-culture treatment prior to innoculation) (%)**	**Transformation 2 (*V. tricolor* Merapi Mount with pre-culture treatment prior to innoculation) (%)**	**Transformation 3 (*V. tricolor* Merapi Mount without pre-culture treatment prior to innoculation) (%)**
0	0	2.56	0.78	0.35
0	25	2.10	*	0.50
25	0	11.01	9.39	1.37
25	25	4.21	4.00	0.97
Control protocorms		20	0	0

* Data missed due to contamination

Acknowledgements

We are grateful to The Ministry of National Education of Republic Indonesia who supported this research through the Program of 'Hibah Doktor '.

References

Belarmino MM & Mii M. 2000. *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. *Plant Cell Rep* 19: 435-442.

Chai ML, Xu CJ, Senthil KK, Kim JY, & Kim DH. 2002. Stable transformation of protocorm like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens. Hort Sci* 96: 213-224.

Cheng M, Fry JE, Pang SZ, Zhou HP, Hironaka CM, Duncan DR, Conner W, & Wan YC. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol* 115: 971-980.

Chia TF, Chan YS, & Chua NH. 1994. The firefly luciferace gene as a non-invasive reporter for *Dendrobium* transformation. *Plant J* 6: 441-446.

Chin DP, Mishiba K, & Mii M. *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. 2007. *Plant Cell Rep* 26: 735-743.

Costa MS, Miguel C, & Oliveira M. 2006. An improved selection strategy and the use of acetosyringone in shoot induction medium increase almond transformation efficiency by 100-fold. *Plant Cell, Tissue and Organ Culture* 85: 2005-2009.

Gardiner LM. 2005. *Vanda tricolor*, conservation in Java, Indonesia: Genetic and geographic structure and history. A paper in 3rd International Orchid Conservation Congress 2005.

Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the Biology behind the "Gene-Jockeying" Tool. *Microb. and Mol Biol Reviews* 1: 16-37.

Hiei Y, Ohta S, Komari T, & Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6: 271-282.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, & Kumashiro T. 1996. High efficiency of transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat Biotechnol* 14: 745-750.

Islam MO, Ichihasi S, & Matsui S. 1998. Control of growth and development of protocorm like body derived from callus by carbon sources in *Phalaenopsis*. *Plant Biotechnol* 15: 183-187.

Jacq B, Lesobre O, Sangwan RS, & Sangwan-Norreel BS. 1993. Factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugarbeet. *Plant Cell Rep* 12: 621-624.

Men S, Ming X, Liu, R, Wei C, & Li Y. 2003. *Agrobacterium*-mediated genetic transformation of a *Dendrobium* orchid. *Plant Cell, Tissue and Organ Culture* 75: 63-71.

Mishiba K, Chin DP, & Mii M. 2005. *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. *Plant Cell Rep* 24: 297-303.

Nan GL, Tang CS, Kuchnle AR, & Kado CI. 1997. *Dendrobium* orchid contain an inducer of *Agrobacterium* virulence genes. *Phisiol Mol Plant Pathol* 51: 391-399.

Robichon MP, Renou JP, & Jalouzot R. 1995. Genetic transformation of *Pelargonium x hortorum*. *Plant Cell Rep* 15: 63-67.

Semiarti E, Indrianto A, Purwantoro A, Isminingsih S, Suseno N, Ishikawa T,

Yoshioka Y, Machida Y, & Machida C. 2007. *Agrobacterium*-mediated transformation of the wild orchid species *Phalaenopsis amabilis*. *Plant Biotechnol* 24: 265-272.

Semiarti E, Indrianto A, Purwantoro A, Martiwi INA, Feroniasanti YML, Nadifah F, Mercuriana IS, Dwiyani R, Iwakawa H, Yoshioka Y, Machida Y, & Machida C. 2010. High-frequency genetic transformation of *Phalaenopsis amabilis* using tomato extract-enriched medium for the pre-culture of protocorm. *J Hort Sci & Biotechnol* 3: 205-210.

Shrestha BR, Chin DP, Tokuhara K, & Mii M. 2007. Efficient production of transgenic plants of *Vanda* through sonication-assisted *Agrobacterium*-mediated transformation of protocorm-like bodies. *Plant Biotechnol* 24: 429-434.

Tingay S, Mc. Elroy D, Kalla R, Fieg S, Wang M, & Brettel R. 1997. Agrobacterium-mediated barley transformation. *Plant J* 2: 1369-1376.

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, & Sangwan-Norreel BS. 1997. Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. *Planta* 201: 160-172.

Yu H, Yang SH, & Goh CJ. 2001. *Agrobacterium*-mediated transformation of a *Dendrobiu*m orchid with the class 1 knox gene *DOH1*. *Plant Cell Rep* 20: 301-305.

Zhao ZY, Gu W, Chai T, Tagliani L, Miller M, Wang N, Pang H, Rudert M, Schroeder S, Hondred D, Seltzer J, & Piercce D. 2000. *Agrobacterium*-mediated shorgum trans-formation. *Plant Mol Biol* 44: 789-798.

Zupan JR, & Zambryski 1995. Transfer of T-DNA from *Agrobacterium* to the plant celll. *Plant Physiol* 107: 1041-1047.