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Original Article

Agrobacterium tumefaciens-mediated transformation of *Dendrobium lasianthera* J.J.Sm: An important medicinal orchid

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ABSTRACT

A protocol for genetic transformation mediated by *Agrobacterium tumefaciens* and production of transgenic *Dendrobium lasianthera* has been developed for the first time. The 8-week-old protocorm explants were used as target of transformation with *Agrobacterium tumefaciens* strain LBA4404 carrying plasmid pG35SKNAT1. Several parameters such as infection period, *Agrobacterium* density, concentration of acetosyringone, and co-cultivation period were evaluated for the transformation efficiency. The data were analyzed using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) with $p < 0.05$. Subsequently, KNAT1 gene expression was confirmed by polymerase chain reaction (PCR) analysis. The highest efficiency of transformation (70%) obtained from protocorm explants infected with *Agrobacterium* culture was at the OD_{600} concentration of 0.6 for 30 min, and co-cultivated with acetosyringone 100 μM for 5 days. The results of confirmation by PCR analysis show that the KNAT1 gene has been integrated and expressed in the genome of *Dendrobium lasianthera* transgenic.

1. Introduction

Currently, orchid has become a significantly commercial commodity in Indonesia. Despite being a major part of cut flower industry, orchid specifically genus *Dendrobium* has been known as traditional medicine. In fact, traditional medicines sourced from orchid have long been circulated in China [1]. Multiple bibenzyls secondary metabolite, fluorenones and gigantol have been isolated from *Dendrobium nobile* which has a higher antioxidant activity than vitamin C [2]. Extracts from leaf, stem, root and pseudobulb of *Dendrobium crumenatum* have an anti-microbial activity [3]. New compounds of dendroside D, dendroside E, dendroside F and dendroside G have been discovered in *Dendrobium nobile* and indicated immunomodulatory activity [4]. One of orchid's species in Indonesia that has anticancer activity is *Dendrobium lasianthera* J.J.Sm.

Three vegetative organs (root, stem and leaf) of *D. lasianthera* J.J.Sm, are toxic and have anticancer activity, however, the most toxic organ with the highest breast anticancer activity T47D is stem with LC50 (μg/mL) = 117 ± 6.35. Owing to its notable potential of becoming raw material for medicine and producing cut flowers, *Dendrobium lasianthera* is of high economic value and is promising to be cultivated.

The main problems in the development of orchid plant to be used as raw material for medicine are: the technique mass propagation is relatively difficult, too long vegetative phase in its life cycle (1–2 years), and genetic stability of the plant. To increase orchid production, genetic engineering is applied by inserting foreign gene into genome of *Dendrobium lasianthera* mediated by *Agrobacterium tumefaciens*.

The insertion of foreign genes into the genome of plants mediated by *Agrobacterium tumefaciens* is an effective and reproducible method and has been successfully applied to various plants such as *Artemisia carvifolia* [5], *Woodfordia fruticosa* [6], *Solanum trilobatum* [7], *Withania somnifera* [8], *Vanda kasem's* [9], and *Erycina pusilla* [10].

The genetic transformation by inserting *KNAT1 (KNOTTED1 like Arabidopsis thaliana)* gene into *Phalaenopsis amabilis* Blume has been done by Semiarti et al. which resulted in the formation of multiple shoots from one *protocorm* [11]. Recently, more success of genetic transformation in medicinal plants has been reported [7,12–13]. However, gene transformation of *KNAT1* into

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D. lasianthera protocorm mediated by *A. tumefaciens* has not been found yet.

KNAT1 is a group of first class KNOX gene which is successfully isolated and characterized from *Arabidopsis thaliana* and functions to organize formation, development, and maintenance of meristem in stem tip to keep the cells meristematic. Over-expression of *KNAT1* in *Arabidopsis* causes formation of adventitious shoots on both sides of leaf [14].

The success of genetic transformation mediated by *A. tumefaciens* was influenced by several factors. The factors are pre-incubation, *Agrobacterium* density, *Agrobacterium* strain, infection period, acetosyringone concentration, co-cultivation period, type and concentration of antibiotic to eliminate *Agrobacterium*.

In the present study, the effect of infection period, bacterial density, concentration of acetosyringone (AS), and co-cultivation period in the modified Vacin and Went [15] medium were examined for the transformation efficiency.

2. Materials and methods

2.1. Plant materials, Agrobacterium tumefaciens strain and construction used for transformation

Healthy 8-week-old *protocorm* (Fig. 5B) from *Dendrobium lasianthera* were used as the explants for *Agrobacterium-mediated* genetic transformation.

Agrobacterium tumefaciens strain LBA4404 harboring a binary vector pG35SKNAT1 used for transformation was kindly given by Dr. Endang Semiarti from Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia. The T-DNA of pG35SKNAT1 contained neomycin phosphotransferase (NPTII) gene under the control of 35S cauliflower mosaic virus (CaMV) promoter (Fig. 1). Bacteria cultures were maintained at −80 °C for long term storage in 70% (v/v) glycerol.

2.2. Sensitivity test of protocorm to kanamycin

To identify the effective concentration of kanamycin as an agent of selection, *protocorms* were cultured on medium VW + 30 g/L sucrose + 2 g/L peptone + 0.5 mg/L benzyladenine + 1 mg/L thidiazuron containing different concentration of kanamycin (0, 25, 50, 75, 100 mg/L). Ten protocorms were used for each treatment, and the experiment was repeated three times. Cultures were incubated at 25 ± 2 °C under 16-h light/8-h dark photoperiod. *Protocorm* was sub-cultured to similar medium every three weeks for nine consecutive weeks. Observation was conducted in the ninth week to see *protocorm* sensitivity toward kanamycin. *Protocorm* was considered survived if the *protocorm* stayed green.

2.3. Suspension culture of A. Tumefaciens

One colony of *A. tumefaciens* strain LBA4404 harboring plasmid pG35SKNAT1 was inoculated into 10 mL of liquid LB medium with 100 mg/L kanamycin. The *A. tumefaciens cultures* were grown in shaking culture at 150 rpm for 18–20 h at 28 ± 2 °C. Two mL suspension of *A. tumefaciens* was measured for its optical density of 0.8 (OD_{600nm} = 0.8). Bacterial cells were collected using centrifugation at 6000 rpm for 5 min at 4 °C. Supernatant was removed, added 2 mL of VW medium, vortexed, and re-suspensed in 20 mL of VW medium.

2.4. Optimization the factors influencing the transformation efficiency

During the transformation of *D. lasianthera* mediated by *A. tumefaciens*, some factors influencing transformation efficiency were evaluated, they were bacterial density (OD_{600nm} at 0.2, 0.4, 0.6, 0.8, and 1.0), co-cultivation period (1, 2, 3, 4, and 5 days), acetosyringone concentration (0, 50, 100, 150, and 200 μM), and infection period (10, 20, 30, 40, and 50 min). In this study, factors that have been investigated and optimized through research and have showed the best results will be used in future research. First, we evaluated bacterial density with co-cultivation time on the third day, acetosyringone concentration 50 μM, and infection period at 20 min. Second, we evaluated co-cultivation period with bacterial density OD_{600nm} at 0.6, acetosyringone concentration 50 μM, and infection period at 20 min. Third, we evaluated acetosyringone concentration with bacterial density OD_{600nm} at 0.6, co-cultivation time at 5th day, and infection period 20 min. Finally, we evaluated infection period with bacterial density OD_{600} at 0.6, co-cultivation time on the fifth day, and acetosyringone concentration 100 μM. Tweenty five protocorms were used for each treatment, and the experiment was repeated four times. Kanamycin-resistant *protocorm* was collected after being cultured for 2 months. Transformation efficiency was determined by following formula: the amount of kanamycin-resistant *protocorm* is divided by the total amount of cultured *protocorm* x 100%.

2.5. Transformation and regeneration

2.5.1. Infection and co-cultivation

Protocorm was infected with 2 mL suspension of *A. tumefaciens* in 20 mL liquid IM medium of OD_{600nm} at 0.6 (Table 1) and shook at 100 rpm for 30 min. Next, *protocorm* was air dried in sterile filter paper to decrease bacterial suspension liquid. *Protocorm* was moved to 20 mL CCM medium (Table 1) in sterile 5 cm petridish. The plates were sealed with parafilm and kept in a dark room at 25 ± 1 °C for co-cultivation for 5 days.

2.5.2. Selection and shoot induction

After co-cultivation, *protocorms* that have been infected were washed with sterilized aquadest three times, then air dried on sterile filter paper. *Protocorm* was cultured on selection medium (Table 1). The plates were then kept with a photoperiod of 16 h light/8h dark for 2 months. Next, protocorm was transferred to a sterile petridish which contained 20 mL of shoot induction medium and cultured for 3 months to distinguish kanamycin-resistant shoots. Culture was kept in the same condition as previously explained. The parameters of transformation was calculated

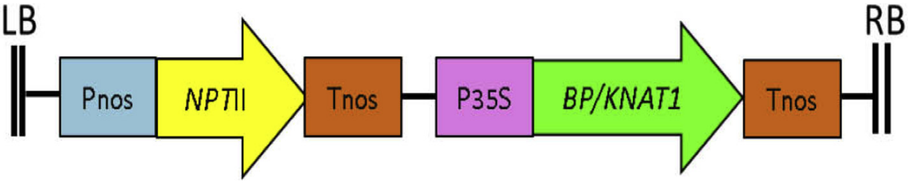

Fig. 1. Structure of the T-DNA pG35SKNAT1. BP/KNAT1 gene (1200 bp) under the control of the 35S promoter of cauliflower mosaic virus (CaMV); LB = Left Border; Pnos = promoter of the nopalin synthase gene; NPTII = neomycin phosphotransferase gene; Tnos = polyadenylation site of the nopaline synthase gene; P35S = 35S promoter of CaMV; RB = Right Border.

Table 1
List of medium used in the study.

Culture medium	Composition
Germination medium (GM)	VW medium + 3 g/L peptone[a] + 30 g/L sucrose[b]
Infection medium (IM)	VW medium + 100 μM acetosyringone[c]
Co-cultivation medium (CCM)	VW medium + 100 μM acetosyringone[c] + 30 g/L sucrose[b] + 0.5 mg/L thidiazuron[c] + 0.5 mg/L benzyladenine[c]
Selection medium (SM)	VW medium + 500 mg/L cefotaxime [c] + 100 mg/L kanamycin[d] + 30 g/L sucrose[b] + 0.5 mg/L thidiazuron[c] + 0.5 mg/L benzyladenine[c]
Shoot inductiom medium (SIM)	VW medium + 500 mg/L cefotaxime [c] + 100 mg/L kanamycin[d] + 30 g/L sucrose[b] + 0.5 mg/L thidiazuron[c] + 0.5 mg/L napthalene acetic acid[b] + 0.5 mg/L gibberelic acid[c]
Root induction medium (RIM)	VW medium + 100 mg/L kanamycin[d] + 30 g/L sucrose[b] + 0.5 mg/L indole acetic acid[d]

[a] HIMEDIA Laboratories, LBS Marg, Mumbai India.
[b] Merck, Darmstadt, Jermany.
[c] Phyto Technology Laboratories, Shawnee Mission, United States.
[d] Sigma-Aldrich, St. Louis, Missouri, United States.

Table 2
Summary of transformation mediated by *A. tumefaciens* of *D. lasianthera* in nine months.

Experiment	Total protocorms	No. of shoot ≥ 1 cm long	No. of transgenic plants[b]	Transformation of efficiency (%)[c]
Transformation	50	39	35	70
Wild type[a]	50	50	0	0

[a] Wild type: protocorms were not infected with *A. tumefaciens* and cultured on medium without kanamycin.
[b] Transgenic plants were confirmed by positive PCR.
[c] Transformation efficiency was calculated by number of no of transgenic divided by total protocorms × 100%.

as the percent protocorms showing shoot regenerating on selection medium (Table 2) and presence of transgene has been validated by polymerase chain reaction (PCR).

2.5.3. Root induction and plantlet acclimatization

Kanamycin-resistant shoots with ≥ 1 cm length were cultured individually on RIM medium (Table 1) for 3 months for root induction. All the cultures were maintained at 23 ± 1 °C under a 16 h-light and 8 h-dark photoperiod. Following *D. lasianthera* plantlets with 3–4 leaves, bearing 3–5 roots (approximately 2–4 cm in height) were removed from the culture tube and rinsed with tap water to wipe off the agar and transplanted to plastic pots loaded a mixture of coconut fiber and sphagnum moss (3:1 v/v). Potted plants were grown in the greenhouse under 30–40% natural light and sprayed two times a day for acclimatization.

2.6. Plant DNA isolation and confirmation of putative transgenic using polymerase chain reaction analysis

DNA of plant genom was isolated using DNA extraction kit (Genomic DNA Minikit Plant, Geneaid, United States), following manufacturer's protocol. Genomic DNA from the fresh shoots (100 mg) of putative transgenic and non-transgenic *D. lasianthera* plants were examined by PCR amplification for the presence of Knat1 gene. The oligonucleotide primers for Knat1 gene were "forward": 5′ CTT CCT AAA GAA GCA CGG CAG 3′ and "reverse" 5′ CCA GTG ACG CTT TCT TTG GTT 3′. These primers were expected to produce 1200 bp. PCR amplification was done using following program order: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 30 s, and followed by post extension for 7 min at 72 °C. The PCR products were analyzed by electrophoresis gel in 1% (w/v) agarose gels and viewed under UV transilluminator.

2.7. Experimental design and statistical analysis

The experiment was arranged in Completely Randomized Design (CRD). The data was analyzed by one way analysis of variance (ANOVA) with SPSS (Version 20), and means of differences among treatment were examined using Duncan's multiple range test (DMRT) at $p < 0.05$ [16].

3. Results

3.1. Kanamycin sensitivity

Sensitivity test of *protocorm* toward kanamycin as an agent of selection in this study had been done with concentration of 0, 25, 50, 75, and 100 mg/L. The experimental results (Fig. 2) showed that kanamycin presence in medium causes significant toxicity to *protocorm* and declining survival response. A survival response of 90% was noticed on medium without kanamycin (control), higher kanamycin concentration caused a more significant decrease toward survival response that was 60% of survival response on kanamycin 25 mg/L and 34% of survival response on kanamycin 50 mg/L. The cultured protocorm on medium which contained kanamycin 75 mg/L and 100 mg/L produced dead *protocorm*, hence the survival response was 0%. This indicated that in those concentrations *protocorm* could not develop.

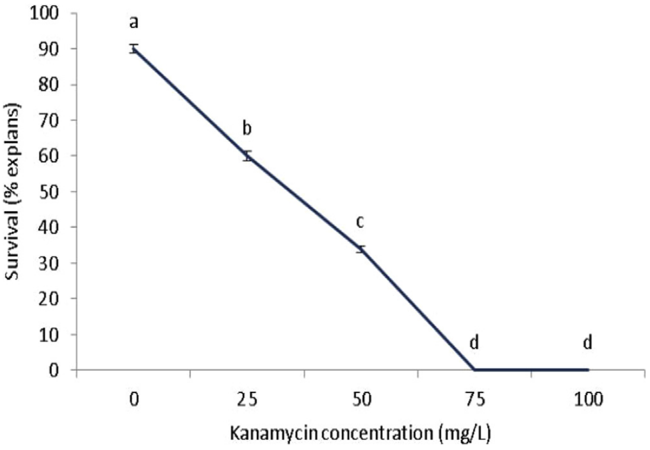

Fig. 2. The influence of kanamycin toward VW medium on survival *protocorm* explants. The data was recorded after 9 weeks of culture.

3.2. Optimization of factors influencing transformation efficiency

3.2.1. Effects of bacterial density on the transformation efficiency of D. Lasianthera

In this study, we evaluated the influence of different bacterial density to transformation efficiency. The suspension culture of the *Agrobacterium* with OD_{600nm} at 0.6 produced the highest transformation efficiency (67% ± 1.2), followed by 0.8 (59% ± 0.7), 0.4 (55% ± 2.1), 1.0 (52% ± 1.5), and 0.2 (31% ± 1.2) respectively (Fig. 3A). Results showed that the transformation efficiency increased steadily in accordance with the bacterial density and reached the highest transformation efficiency at OD_{600} 0.6, however the bacterial density was higher than that resulted in lower transformation efficiency.

3.2.2. Effect of co-cultivation period on the transformation efficiency of D. Lasianthera

Co-cultivation is one of important steps in transformation mediated by *Agrobacterium*. After being infected by *Agrobacterium tumefaciens*, the explants are usually co-cultivated first in regeneration medium. During co-cultivation period, *Agrobacterium tumefaciens* will transfer T-DNA which bring certain gene into plants genom. In the study, we selected five different durations for co-cultivation 1–5 days. Co-cultivation period of a day produced efficiency of transformation (25.2% ± 0.7), 2 days (40% ± 2.0), 3 days (45% ± 1.2), 4 days (60% ± 1.5). The highest efficiency of transformation (65% ± 1.0) was obtained when *protocorm* and *Agrobacterium tumefaciens* had been co-cultivated for 5 days period and the lowest efficiency of transformation (25.2% ± 0.7) was obtained when *protocorm* had been co-cultivated with *Agrobacterium tumefaciens* for a day only (Fig. 3B).

3.2.3. Effect of acetosyringone concentrations on the transformation efficiency of D. Lasianthera

The genetic transformation mediated by *Agrobacterium* needs to transfer a single stranded T-DNA from *Agrobacterium* to plant cell, including vir genes induction. Acetosyringone has a significant role in increasing vir genes induction which causes activation of vir genes to transfer the T-DNA into plant genom. To investigate the effect of acetosyringone on transformation efficiency, different concentrations of acetosyringone (0, 50, 100, 150, and 200 μM) in the co-cultivation medium were tested. The results (Fig. 3C) showed that the lowest transformation efficiency (15% ± 1.0) was obtained for explants without acetosyringone treatment. Transformation efficiency increased as the increase of acetosyringone concentration up to 100 μM and maximum transformation efficiency (65% ± 1.5) was observed at 100 μM.

3.2.4. Effect of infection period on the transformation efficiency of D. Lasianthera

The infection period of *Agrobacterium* determined the success of transformation. In the study, we selected five different infection period (10, 20, 30, 40, and 50 min). The efficiency of transformation was 35% ± 1.4, 42% ± 2.1, 70% ± 2.3, 66% ± 1.8, and 52% ± 2.2 when the infection period was 10, 20, 30, 40, and 50 min, respectively. The result of this study (Fig. 3D) showed that an infection period of 30 min produced the highest efficiency of transformation (70% ± 2.3) compared to infection period of 10 min (35% ± 1.4), 20 min (42% ± 2.1), 40 min (66% ± 1.8), and 50 min (52% ± 2.2).

3.3. Detection of putative transgenic using PCR analysis

Lane 3–7 contained the PCR products from shoots transformed with *A. tumefaciens* strain LBA4404 carrying *Knat1* gene. A single

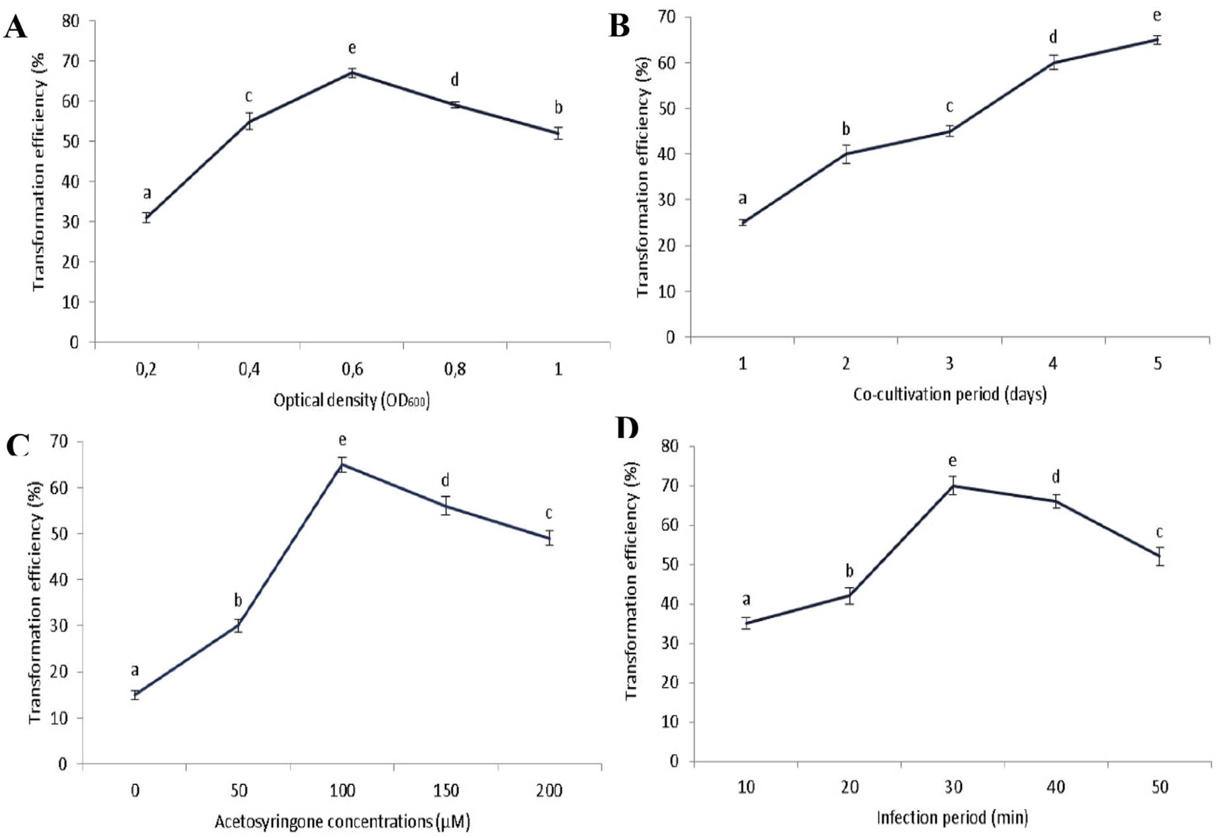

Fig. 3. Effects of various factors affecting transformation effeciency of *D. lasianthera*. A bacterial density, B co-cultivation period, C acetosyringone concentrations, D infection period.

band of 1200 bp was observed on lanes 3–7 containing PCR products from putative transformer. The presence of *Knat1* gene in putative transformer confirmed the successful transformation event and supported the observation that the transformed protocorm survived on the selection media containing kanamycin.

3.4. Regeneration of putative transgenic plants

Putative transgenic shoot having a length more than 1 cm was sub-cultured on RIM medium and root appeared after 3 weeks of culture (Fig. 5E).

4. Discussion

4.1. Determination of kanamycin sensitivity

Sensitivity test of target tissue toward antibiotic is an important step in transformation [17–19]. Non-transformer tissue sensitivity test toward antibiotic was done first on regeneration medium which contained various antibiotic concentration. The lowest antibiotic concentration that can inhibit or turn out the target tissue can be used as an agent to select transformer tissue. Based on this study (Fig. 2), we found that kanamycin 75 mg/L was the lowest concentration which was able to kill a non-transgenic *protocorm* and the best concentration for transformer selection. For our further studies, we used 75 mg/L kanamycin as the selection agent. Several authors have been successful in using kanamycin 75 mg/L as a selection agent of transformation on different plants that are *Withania somnifera* [12] and transgenic *Urochloa brizantha* [20]. However, Mu et al. and Aggarwal et al. reported that kanamycin with lower concentration 15 mg/L and 50 mg/L were suitable for use in *Cerasus humilis* and *Eucalyptus tereticornis* [21–22].

4.2. Optimization of factors influencing transformation efficiency

Several factors such as bacterial density, co-cultivation period, acetosyringone concentrations, and infection period that influenced efficiency of transformation are illustrated in [Fig. 3].

The bacterial density in suspension may influence efficiency of transformation [23–25]. The transformation efficiency described in (Fig. 3A) was obtained from 5 treatments, each treatment showed significant result (DMRT, $p < 0.05$). The lowest transformation efficiency (31% ± 1.2) was obtained from treatment OD_{600nm} 0.2. This could be the result of inadequate number of *Agrobacterium tumefaciens* cells to infect and transfer T-DNA to protocorm cells. This claim was supported by An et al. stating that OD_{600nm} 0.2 was too low, hence there was a few of *A. tumefaciens* that would transfer the T-DNAs to target cells and cause low transformation efficiency [26]. *Protocorm* treated with *Agrobacterium tumefaciens* on OD_{600nm} 0.2, 0.4, 0.6 produced transformation efficiency that steadily increased, following after, the transformation efficiency decreased on OD_{600nm} 0.8 and 1.0. The highest efficiency of transformation (67%±1.2) was reached on treatment with OD_{600nm} 0.6. The same result has been reported by Subramaniam et al. Shrestha et al. and Zhang et al. that bacterial density of OD_{600nm} at 0.6 yielded the highest transformation efficiency on *Dendrobium* Savin white, *Vanda*, and *Cattleya* [27–29]. Therefore, OD_{600nm} 0.6 was used for transformation of *D. lasianthera*.

Co-cultivation period was started from 1 day until 7 days [30–33]. The results of observation (Fig. 3B) depicted that the longer co-cultivation, the more efficient the transformation and it clearly showed significant results among 5 different treatments (DMRT, $p < 0.05$). Shorter co-cultivation period (1–3 days) generated low efficiency of transformation, it could be stated that co-cultivation period of 1–3 days lacked of time for *A. tumefaciens* to transfer T-DNA into *protocorm* cells of *D. lasianthera*. The co-cultivated *protocorm* for 5 days produced the highest efficiency of transformation (65%±1.0), but it also resulted in a high bacterial overgrowth and necrosis of explants. Therefore, a 4-day co-cultivated period was used for transformation system for *D. lasianthera*. Similar results were reported by Gnasekaran et al. that 4-day co-cultivation period was suitable for use in transformation of *Vanda* kasem's [9]. However, co-cultivation for longer time (15 days) was used in *Helianthus annuus* [34].

The success of transformation mediated by *A. tumefaciens* was interfered by the presence of acetosyringone in co-cultivation medium. Various acetosytingone concentrations 0–400 μM had been used for genetic transformation [35–37]. The result (Fig. 3C) illustrated that there were significant differences among five treatments (DMRT, $p < 0.05$). The highest efficiency of transformation (65%±1.5) was reached on co-cultivation medium given 100 μM of acetosyringone. Higher concentrations of acetosyringone resulted in decreasing of transformation efficiency. The same result have been reported by Kartikeyan et al. Duan et al. Hosein et al. and Afolabi et al. It was reported that acetosyringone concentration of 100 μM yielded the highest transformation efficiency on *Rice*,

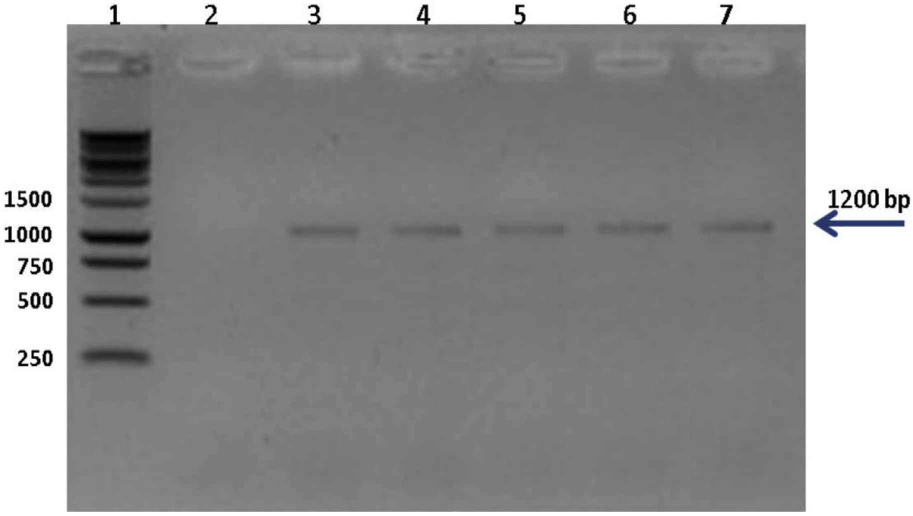

Fig. 4. Polymerase chain reaction analysis of transgenic *D. lasianthera* using Knat1 primers. 1 = marker, 2 = wild type, 3–7 = transgenic plants (arrow = Knat1 amplified size 1200 bp).

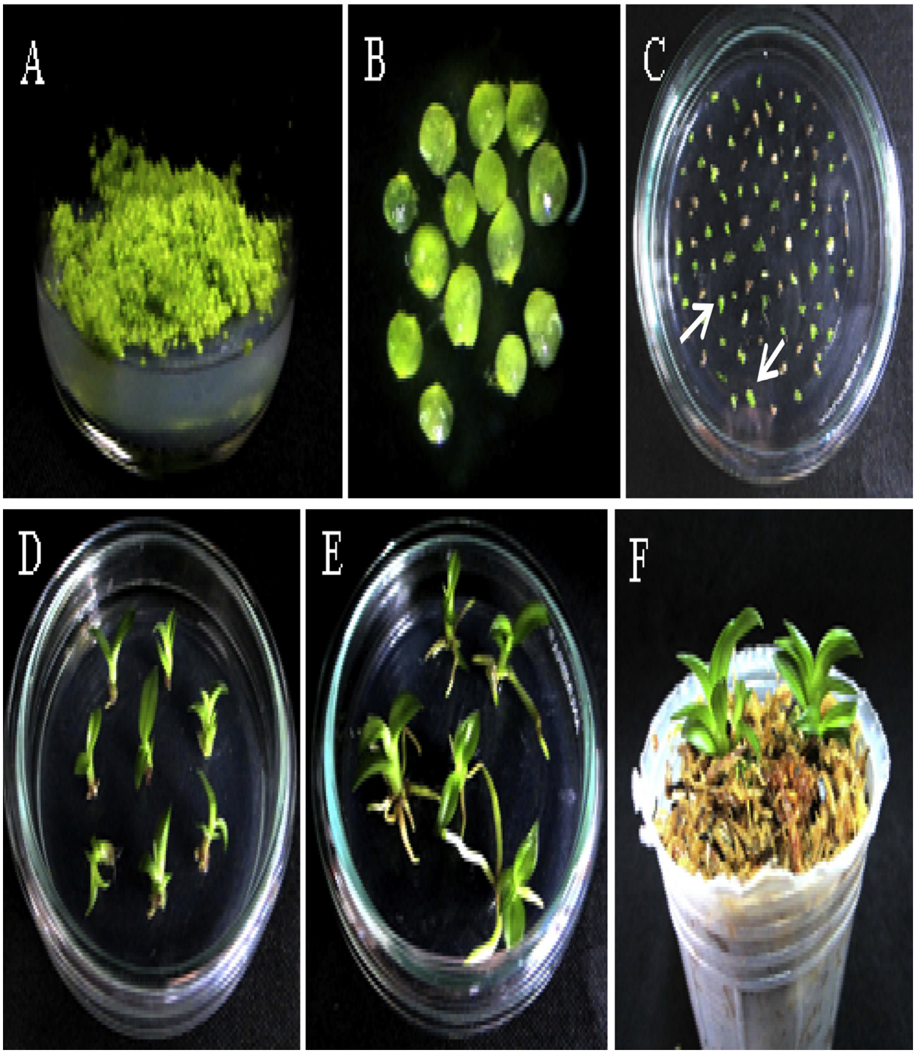

Fig. 5. Seed germination and regeneration of *protocorm Dendrobium lasianthera*. (A) Seed germination on VW medium + 3 g/L peptone + 30 g/L sucrose. (B) *Protocorms* were used as target of transformation, (C) Transgenic protocorms were cultured on SM medium (Arrow indicated of transformed *protocorms*), (D) Well developed shoots from *protocorms* were cultured on SIM medium, (E) Rooted plantlet were cultured on RIM medium, (F) Transgenic plant grew on mixture of coconut fiber and sphagnum moss.

Nicotiana, *Anthurium*, and *Cotton* [38–41]. Therefore, acetosyringone concentration of 100 μM was further used in the transformation of *D. lasianthera*. Our result is contrastive to Rashid et al. and Suratman et al. which added acetosyringone in higher concentration (150 μM and 200 μM) and produced the increase of transformation efficiency on *Wheat* and *Citrulus vulgaris* [35,37]. The differences between the results might due to genotype variation.

Any results of transformations from previous research indicated that infection period varied from few minutes to few hours, 5 min on *Artemisia carvifolia* [5]; 30 min on *Oncidium* Gower Ramsey, *Crambe abyssinica*, and *Dendrobium chrysotoxum* Lindl [42–44]; 40 min on *Cordyline fruticosa* [45]; an hour on *Helianthus tuberosus* [46]; 4 h on *Erycina pusilla* [10]. The results of observation (Fig. 3D) indicated that infection period 30 min was optimum for transforming *D. lasianthera protocorm*. Since there were significant differences (DMRT, $p < 0.05$) among treatments, 30 min was chosen as the infection period in order to get the highest efficiency of transformation. Men et al. stated that 30 min of infection period on *Dendrobium nobile* generated a higher efficiency of transformation (18%) rather than infection period of 45 min and 60 min [47]. The results of the study also indicated that infection period of 10 min and 20 min shorter generated lower efficiency of transformation that were 35% ± 1.4 and 42% ± 2.1. An infection period of 40 min and 50 min longer also yielded reduction of transformation efficiency 66% ± 1.8 and 52% ± 2.2, and overgrowth of *Agrobacterium* on the surface of *protocorm* led to necrosis.

4.3. Molecular analysis of the putative transformer

The results of PCR analysis (Fig. 4) revealed that 1200 bp *Knat1* transgene had been successfully amplified from putative transformer kanamycin resistant. Non-transformer plant (wild) was used as control, and it showed no band amplified from them in PCR analysis. This proved that *protocorm D. lasianthera* had been successfully transformed mediated by *Agrobacterium tumefaciens* strain LBA4404 to express *Knat1* gene.

5. Conclusion

In conclusion, a simple and optimized *Agrobacterium*-mediated genetic transformation protocol has been established for *Dendrobium lasianthera* using protocorms explants and has been demonstrated molecularly from the integration of transgene into the genome of orchids. Transgenic plantlets were successfully regenerated. Thus, this protocol has the potential to be applied for transformation of other medicinal orchids.

Conflict of interest statement

We declare that we have no conflict of interest.

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