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Influence of Genetic Transformation by *Agrobacterium tumefaciens* on Vincristine Production in Periwinkle Plant

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Abstract: *Catharanthus roseus* (L.) G. Don is one of medicinal plants produces many terpenoid indole alkaloids (TIAs). To improve the production of TIAs in *C. roseus*, the establishment of an efficient genetic transformation method is required. To develop a genetic transformation method for *C. roseus*, *Agrobacterium tumefaciens* strain LBA4404 was employed which harbors a binary vector pCAMBIA2301 containing a reporter β-glucuronidase (*GUS*) gene and a selectable marker neomycin phosphotransferase II gene (*NTPII*). Seeds were cultured on MS full strength medium supplemented with BAP at 1.0, 2.0 and 3.0 mg/l and Kin at 0.1, 0.2 and 0.3 mg/l and their interactions to produce shootlets. For callus induction, small pieces of leaves (leaf discs) were cultured on MS full strength medium containing 1, 2 or 3 mg/l 2, 4-D or NAA as well as their interaction. The influential factors were investigated systematically and the optimal transformation condition was achieved using micro-nodes or callus as explants, diluted solution 1: 100 of *A. tumefaciens,* infection times were 0, 30, 60 and 120 min and co-cultivation for 4 d on MS medium (half strength) containing 150?µM acetosyringone. With a series of selections of transformed callus and shoot using kanamycin-containing resistance medium, stable transgenic regenerated plantlets were successfully obtained. The expression of *GUS* and *NPT II* gene were confirmed by polymerase chain reaction (PCR) that exhibited two bands at 250 bp and 400 bp length (positive sample). To prove the efficiency of the established genetic transformation system, the cellular membrane stability in response to drought, salinity and heat stress, in addition to vincristine and proline content in *C. roseus* independent transgenic plants were investigated. In general, the results of metabolite analysis using high performance liquid chromatography (HPLC) showed that overexpression of *GUS* gene increased the yield of vincristine in transgenic plants 1.39 ug/ml compared with control.

Key words: *Catharanthus roseus* · *GUS* gene · Transformation · Vincristine · Cellular Membrane Stability (CMS)

INTRODUCTION

Cathranthus roseus G. Don is one of the best-studied medicinal plants [1, 2]. It is an erect, bushy, evergreen and ever blooming herb. *C. roseus* (L.) G. Don Apocyanaceae (Dog-bane family) is a diploid plant with a karyotype comprising of 16 chromosomes, eight bivalents are visualized at meiosis. Knowledge about its terpenoid indole alkaloids (TIAs) pathways is growing.

Since, *C. roseus* has somewhat small genome size, low chromosome number and self-compatibility, it has become a good model plant for transcriptomic, proteomic and metabolomic investigations on plant secondary metabolism [3, 2, 4].

The production of the most valuable dimeric indole alkaloids vinblastine and vincristine are extremely low in wild-type of *C. roseus* plant and hard to be improved using cell suspension and hairy root cultures systems [5, 6]. In addition, it is also difficult to synthesize TIAs by chemical methods due to their complicated structures [7]. The multi-step TIAs biosynthetic pathway is highly complex and strictly regulated through developmental,

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environmental, organo- and cell-specific controls [8,1,3, 9]. Therefore, production of indole alkaloids by *C. roseus* *in vitro* cultures is still one of the greatest interests and challenges that attract many researchers to explore the technologies to produce higher amount of dimeric antineoplastic drug molecules and their precursors. The anticancer alkaloids vinblastine and vincristine are derived from stem and leaf of *C. roseus* [10]. Under *in vitro* condition, different percentages of the methanolic crude extracts of *C. roseus* were found to show a significant anticancer activity against numerous cell types and especially greatest activity was found against the multidrug resistant tumor types [11, 12]. They are also used for treatment of leukemias, lymphomas and testicular cancer [13]. Widowati *et al*. [14] demonstrated that methanol extracts of aerial parts and roots of *C. roseus* reduced the proliferation of the human ductal breast epithelial tumor cell lines (T47D) with a mean IC50 of 2.8% by apoptosis.

In *C. roseus*, a number of transcription factors (TFs) were reported *i.e.* ORCA3, ORCA4 and ORCA5, which form a physical cluster, regulate a number of genes in the TIA pathway through overlapping yet distinct mechanisms [15, 16]. CrMYC2 activates ORCA3 by binding to a qualitative sequence in the promoter, whereas it indirectly activates ORCA4 and ORCA5 [17, 18]. In addition, CrMYC2 interacts with CrGBF1 and CrGBF2 to modulate TIA biosynthesis [19]. The CrMYC2–ORCA cascade has limited effects on expression of genes in the iridoid branch of TIA pathway. However, Schweizer *et al*. [20] reported that transient over expression of a de-repressed CrMYC2 (CrMYC2D126N) in *C. roseus* flower petals significantly activates expression of iridoid pathway genes. BIS1 and BIS2 are major regulators of the iridoid pathway [21, 22]. Transient overexpression of CrMYC2D126N , BIS1 and ORCA3 significantly induce the indole and iridoid pathway genes [20]. The bHLH TF RMT1 and JAZ proteins mediate crosstalk between iridoid and terpenoid pathways to balance TIA accumulation [23]. However, regulation of genes involved in the sequential conversion of tabersonine to vindoline in *C. roseus* leaves is limited. Combinatorial overexpression of wild-type or de-repressed CrMYC2 (CrMYC2D126N 96), along with BIS1 and/or ORCA3 does not induce the expression of vindoline pathway genes, suggesting that other TFs are likely involved in regulation of the vindoline pathway [20].

To date, genetic transformation of *C. roseus* has been mostly confined to hairy roots and suspension cells. *Agrobacterium rhizogenes*-mediated transformation involving productions of hairy (transgenic) roots in *C. roseus* had been reported [24-26]. However, the phenotypes of transgenic *C. roseus* plants transformed by *A. rhizogenes* were abnormal, such as shortened internodes, wrinkled leaves and abundant root mass [25]. Thus this kind of transgenic *C. roseus* plants is not suitable for the production of TIAs. Transgenic *C. roseus* cell suspension cultures transformed by either *Agrobacterium* infection or by particle bombardment had been established and studied intensively [24, 15, 27]. But these transgenic cell lines did not produce alkaloids in a stable manner and their ability to accumulate TIAs was gradually declined by long-term subculture [28]. Recently *A. tumefaciens* mediated transformation was employed in *C. roseus*, the transgenic callus and plants were obtained [29]. However, these transformation systems were not confirmed with other biochemical assays such as southern blot and high-performance liquid chromatography (HPLC). To address these issues, the present study aimed to develop an efficient transient system for producing transgenic and regenerated *C. roseus* plants. The stable of regenerated plants were successfully acquired to demonstrate this transformation system, *GUS* gene an essential gene marker that increase in TIAs biosynthetic pathway was overexpressed and the accumulation of vincristine in transgenic *C. roseus* plants was analyzed using HPLC.

MATERIALS AND METHODS

In vitro Shoot Proliferation

Plant Material: Seeds of periwinkle, *C. roseus* (L.) G. Don. (Apocyanaceae), were collected from the Farm of Agriculture Faculty, Cairo University. The seeds were used for *in vitro* micropropagation trials, after procedures of seeds disinfection.

The *in vitro* germinated seedling (shoots) with 2-3 leaves were explanted and used as a source of plant material for shootlets multiplication stage. Afterwards, the *in vitro* developed shootlets consisting of 3-5 leaves were used for callus production and regeneration trials.

Growth Conditions: Basal Murashige and Skoog (MS) nutrient medium [30] supplemented with 25 g/l sucrose and 7g/l agar was used. Plant growth regulators BAP and Kinetin, were used in different concentrations (Table 1) to find out suitable media combinations for the growth and/or proliferation of explants for vinca shootlets production. The pH of media was adjusted to 5.6-5.8 after adding plant growth regulators (PGR), then autoclaved at

Table 1: Media Composition used in shootlets proliferation of periwinkle tissue culture

Media Code	Treatment
M0	MS + 25 g/l sucrose + 7 g/l agar
M1	MS + 25 g/l sucrose + 7 g/l agar + 1.0 mg/l BAP
M2	MS + 25 g/l sucrose + 7 g/l agar + 2.0 mg/l BAP
M3	MS + 25 g/l sucrose + 7 g/l agar + 3.0 mg/l BAP
M4	MS + 25 g/l sucrose + 7 g/l agar + 0.1 mg/l Kin
M5	MS + 25 g/l sucrose + 7 g/l agar + 0.2 mg/l Kin
M6	MS + 25 g/l sucrose + 7 g/l agar + 0.3 mg/l Kin
M7	MS + 25 g/l sucrose + 7 g/l agar + BAP 1.0 + Kin 0.1
M8	MS + 25 g/l sucrose + 7 g/l agar + BAP 1.0 + Kin 0.2
M9	MS + 25 g/l sucrose + 7 g/l agar + BAP 1.0 + Kin 0.3
M10	MS + 25 g/l sucrose + 7 g/l agar + BAP 2.0 + Kin 0.1
M11	MS + 25 g/l sucrose + 7 g/l agar + BAP 2.0 + Kin 0.2
M12	MS + 25 g/l sucrose + 7 g/l agar + BAP 2.0 + Kin 0.3
M13	MS + 25 g/l sucrose + 7 g/l agar + BAP 3.0 + Kin 0.1
M14	MS + 25 g/l sucrose + 7 g/l agar + BAP 3.0 + Kin 0.2
M15	MS + 25 g/l sucrose + 7 g/l agar + BAP 3.0 + Kin 0.3

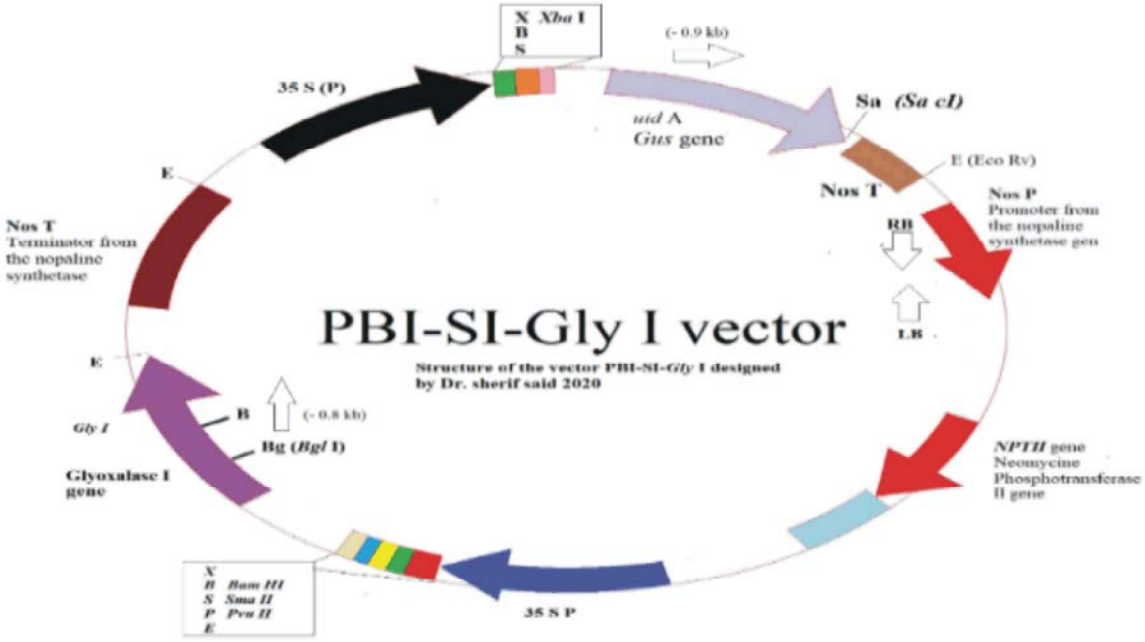

Fig. 1: Diagrammatic representation of the Agrobacterium binary vector

121°C and 1.5 lb for 15 min. Seeds were surface sterilized by dipping in 70% ethanol for 1 min, followed by immersion in 10% sodium hypochlorite for 10 min, followed by mercuric chloride at 0.1 % for the same time and then it was rinsed for three times in sterile distilled water [31].

Seeds were dried on sterilized Whatman filter papers. The sterilized seeds were germinated in jars containing full strength MS + 25 g/l sucrose + 7 g/l agar. The glass jars were incubated at 25±2°C under darkness for 48 h then transferred into a 16/8 h light/dark photoperiodic regime (1000 lux). The *in vitro* grown 15 days-old seedlings were used as a source of micro nodes explants and callus induction. For shootlets proliferation stage the seedling explants were cultured in glass jars with MS full strength media containing different concentrations and combinations of BA and Kin (Table 1).

For callus production, leaf discs were cultured on MS full strength medium supplemented with 1, 2 or 3 mg/l 2, 4-D or NAA at 1, 2 or 3 mg/l and their combinations were used.

Agrobacterium Mediated Transformation System of Periwinkle

***A. tumefaciens* Strain and the Used Vector:** For transformation, *A. tumefaciens* strain LBA4404 harboring callus of the binary pBI-S1 plasmid (kindly provided by Prof. Dr. Ahmed Bahieldin, Fac. of Agric., Ain Shams

Univ. and AGERI) was used. The vector contains a *GUS* reporter gene and a selection marker gene *NPTII* (neomycin phosphotransferase gene, conferring resistance to kanamycin which were inserted between the CaMV35S promoter and the *A. tumefaciens nos* terminator separately. An intron inside the coding sequence is included in the *GUS* reporter gene to ensure that expression of glucuroidase activity is derived from eukaryotic cells Fig. (1). To get fresh cells, a single colony of *A. tumefaciens* with Pb in vector was inculcated in liquid Luria Bertani (LB) medium containing 100 mg/l kanamycin and 100 mg/l streptomycin and grown at 28°C for 36 h with shaking (150 rpm[32]. The initial culture was diluted 1:100 with liquid LB medium and grown on a shaker (250 rpm) until the OD600 reached to 0.5, then the cells were centrifuged (2, 000 × g, 10 min) and the supernatant was removed. The bacterium was re-suspended in [32]. At last, the bacterium was shaken (100 rpm) again for 2 h in dark at 28°C.

Genetic Transformation and Co-Cultivation: The explants micro-nodes containing two leaves of *C. roseus* were immersed in liquid MS medium with 150 µM acetosyringone in tissue culture tubes [32]. Then these tubes were wounding by scalpel to make wounds. After that, the explants were transferred into pre-sterilized flasks containing *A. tumefaciens* suspension and shaken gently for 0 (control), 30, 60 and 120 min at room temperature. Explants were then blot-dried with sterile paper towels and transferred onto petri dishes containing 1/2 MS medium (half strength) with 150 µM acetosyringone. The co-cultivation period was 4 days in the dark at 28°C.

Induced callus from leaf discs was used as a source of cells for transformation by using sonication device for 30 min at 25°C. The callus cells (0.5 g) were immersed in liquid media with diluted bacterial suspension (1:100) in tissue culture weatherman tubes and sonicated for 30 min at 25 °C. The callus was dried with sterile paper and re-cultureed on MS medium 150 µM acetosyringone for 4 days in dark at 28°C [32].

Molecular Characterization: DNA of putatively transformed micro nodes and callus were extracted according to the modified protocol of [33]. A total volume of reaction was 25 µl, consisted of template DNA, 1X Taq buffer, 0.4 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 µM primer and 1.5 U *Taq* polymerase. The *nptII* gene fragment was amplified using forward and reverse primers, 5′ GAGGCTATTCGGCTATGACTG 3′ and ATCGGGAGCGGCGATACCGTA 3′.

25 ng of plasmid DNA and 50 ng of DNA of untransformed explants and callus were taken as positive and negative controls, respectively. 50 ng of DNA of transformed explants and callus were taken to ascertain transgene integration. The cycling parameters were: denaturation cycle at 94°C (2 min) and 30 cycles at 94°C (1min), 56°C (1min) and 72°C (1min), followed by final cycle for extension at 72°C (7 min). PCR products were separated by electrophoresis on 1 % agarose gel using 100-1000 bp DNA ladder and visualized (after staining with ethidium bromide) under ultraviolet light at 312 nm using Highest Ultraviolet Intensity Spectroline (model TVC-312A), Variable Intensity Trans-illuminator, (USA).

Assessment of Vincristine Content in Transformed Plantlets

Extraction: *In vitro* micro nodes ranged (0.5 – 1.5 g) were extracted with 50 ml MeOH for 24 h by cold maceration. The filtered extracts were dried and residues were dissolved in 3 ml (1N) H_2SO_4 ; pH of each of the acidic extracts were adjusted to 9.0 with 20 % NH_4OH and shaken with 5 ml $CHCl_3$ and dried [34].

HPLC Analysis: The final residues were dissolved in 1 ml of a 1: 4 mixt. of 0.5 N H_2SO_4 and HPLC mobile phase, *i.e*. MeOH-Me_2CN-2.5 mM KPi buffer (pH 7.0) (48:20:32). The external standard used for quantitative analysis (vinblastine sulphate 1mg , produced by Mephacan.Com id-4936359) was also treated in the same way to eliminate any error in extraction. For HPLC analysis, a Merck LiChro CART C_{18} Column (125 x 4mm, 5µm) and the solvent system were used at a constant flow rate of 1 ml min^{-1} and 2500 Psi pressure A diode array detector was employed for the detection of the peaks, set at wave length of 255 nm and band width of 5 according to Aniruddha and Srivastava [34].

Cellular Membrane Stability (CMS)

Salinity Effect: The successful transformed explants (not callus) were cultured on MS medium containing 3000 ppm NaCl as a salinity stress.

Polyethylene Glycol (PEG) Effect: Leaf discs of treatment vials were submerged in 30 ml of polyethylene glycol (60% PEG-600) solution for 24 h at 10°C in the dark according to Blum [35], then the leaf pieces were washed with deionized distilled water and 30 ml of deionized distilled water were added.

Heat Stress Effect: Heat-treated vials were covered with plastic wrap and incubated in a water bath at 45°C for 1 h; control vials were maintained at room temperature (25°C) during the same period. Temperature treatment and duration were chosen after conducting preliminary experiments involving variations in water-bath temperature to determine the treatment conditions producing the greatest sensitivity in detecting genetic differences [36].

Proline Contents: Proline colorimetric determination was proceeded according to Bates *et al.* [37] and Marin *et al.* [38].

Data and Parameters: The cultures in this stage were incubated for one month and the determined parameters were: Number of shoot (calculated as the number of developed shoots /explant).

Shoot length (cm): measured as cm summation of shoot lengths/ number of shoots.

Number of leaves/shoots.

Callusing %: Number of callus formed from leaf disc /total leaf discs cultured X100

Statistical Analysis: Obtained data were statistically analyzed by using CRD design and mean of three replicates per treatments comparisons were based on LSD at 5% test, the software according to Maxwell and Delaney [39].

RESULTS AND DISCUSSION

***In vitro* Shootlets Induction Frequency:** Data concerning effects of plant growth regulators and their interaction on shoot induction frequency (Table 2 and Fig. 2), As shown in Table (2), revealed that there were significant differences between BAP and Kin effects on shootlet induction frequency. The highest percentage (7.44 shootlets/shoot), the tallest shootlets (10.58 cm) and the highest number of leaves (16.61 leaves/shoot) were noticed in MS medium containing 3 mg/l BAP. In this concern, Mondal *et al.* [40] found that aseptic culture of nodal segments of *Camellia sinensis* (L.) O. kuutze were initiated on half strength MS medium supplemented with 8.88 µM BAP in combination with 0.98 µM IBA. After 30 days, the sprouted buds were transferred to hormone-free MS medium for further multiplication, so as to allow the shoots to attain a height of 3.0 cm.

Moreover, combinations of auxins with cytokinins were found to be better for leaf callus induction and enhancement of alkaloid contents. The highest enhancement of total alkaloids content was found in 0.5 mg/l + 1 mg/l 2, 4-D + BA mg/l [41].

Also, Don *et al.* [42] found that shoot induction was obtained in 5 to 6 days at 3.0 mg/l BAP concentration in both MS and Gamborg media. However, it take 11 to 12 days for shoot regeneration at 0.5 mg/l BAP while no growth was observed in control culture. Number of shoots and shoot length were varied from 0.8 to 3.8 and 1.8cm to 4.0cm, respectively, in MS medium while in Gamborg medium to corresponding value were 0.6 to 3.0 shoots and 1.2 cm to 3.5 cm, respectively.

Sherif *et al.,* [31] mentioned that, MS media containing 3 mg/l BAP gave the best results for growth of vinca explants, while it increased the mean of shoot number to the maximum value (7.44), shootlets length (10.58 cm) and gave the highest number of leaves per shootlets (16.61).

Callus Induction: Data in Table (3) showed positive effects of all PGRs treatments (2, 4-D and NAA) on callus induction and using 2, 4-D at 3 mg/l was more efficient than other treatments where the callus was more friable, containing somatic embryos and no roots formation.

For callus formation, the previous researches showed that the leaf explants of *C. roseus* cultured on MS basal medium without plant growth regulators were only slightly expanded and no calli were observed [43]. Since phenols production caused necrosis and leaf explants turned brown and eventually died. First calli were formed out of the cut edges of the explants [44], they cultured on MS medium containing BAP in combination with 2, 4-D after three weeks. But, the production of enough calli for sub-culture was obtained after eight weeks. The maximum callus formation was obtained on MS medium containing 5: 2 mg/l 2, 4-D : BAP, minimum callus formation was obtained on MS medium containing 10: 2 mg/l 2, 4-D: BAP.

Optimization of Transformation Conditions

Morphological Characters: Data in Table (4) explained the effect of immersion duration time in bacterial suspension during transformation process on growth characters. To develop an efficient system for producing transgenic *C. roseus* plants using *A. tumefaciens*, the association parameters were systemically investigated

Fig. 2: Some growth characterization of *C. roseus* seedlings *in vitro,* as affected by growth regulators

Table 2: Effect of plant growth regulators on growth characterization of *C. roseus* seedling *in vitro*

PGRs Treatments	Shoot No./explant	Shoot Length (cm)	Leaves No/shootlet
M0	1.38	4.66	10.33
M1	2.72	5.01	8.80
M2	4.62	5.06	14.50
M3	7.44	10.58	16.61
M4	2.38	4.77	14.81
M5	2.77	5.58	12.05
M6	1.44	5.82	14.67
M7	2.33	7.06	13.17
M8	2.33	4.43	12.14
M9	2.22	6.28	13.97
M10	4.44	4.96	13.30
M11	2.22	5.69	11.72
M12	1.94	5.19	15.93
M13	2.22	5.80	10.37
M14	4.11	5.46	10.02
M15	3.16	3.77	15.43
L.S.D. 5 %	1.43	3.77	5.42

Table 3: Effect of plant growth regulators on callus induction of *C. roseus* leaves disc culture *in vitro*.

Callus induction treatments	Callusing percentage
Control	11.11 e
2, 4-D 1.0 mg/l	100.0 a
2, 4-D 2.0 mg/l	100.0 a
2, 4-D 3.0 mg/l	100.0 a
NAA 1.0 mg/l	88.89 ab
NAA 2.0 mg/l	100.0 a
NAA 3.0 mg/l	100.0 a
2, 4-D 1.0 + NAA 1.0	100.0 a
2, 4-D 1.0 + NAA 2.0	100.0 a
2, 4-D 1.0 + NAA 3.0	100.0 a
2, 4-D 2.0 + NAA 1.0	100.0 a
2, 4-D 2.0 + NAA 2.0	100.0 a
2, 4-D 2.0 + NAA 3.0	100.0 a
2, 4-D 3.0 + NAA 1.0	100.0 a
2, 4-D 3.0 + NAA 2.0	100.0 a
2, 4-D 3.0 + NAA 3.0	100.0 a
LSD 5 %	19.63

Table 4: Influence of transformation immersion duration time on the frequency of transient *GUS* expression (%) in explants *of C. roseus*

Bacterial suspension duration (min)	Shoot No	Transformation frequency %	Shoot length (cm)	Leaves No
0.0	1.1	0.00	0.66	3.5
30.0	1.2	0.00	0.90	4.5
60.0	1.2	75.0	1.40	4.2
120.0	1.2	50.0	1.40	4.1
L. S. D. 5 %	0.35	9.13	0.63	1.10

Table 5: HPLC chromatogram showed the quantity of vincristine in transgenic explants by *A. tumefaciens*, seeds and vincristine sulfate Vincosien standard

Source of explant produced vincristine	Retention time	Vincristine ug/ml
Transgenic explants *A. tumefaciens*	3.00	1.39
Seeds (open field plants)	3.00	0.025
Vincosien ® Standard	3.00	5.0

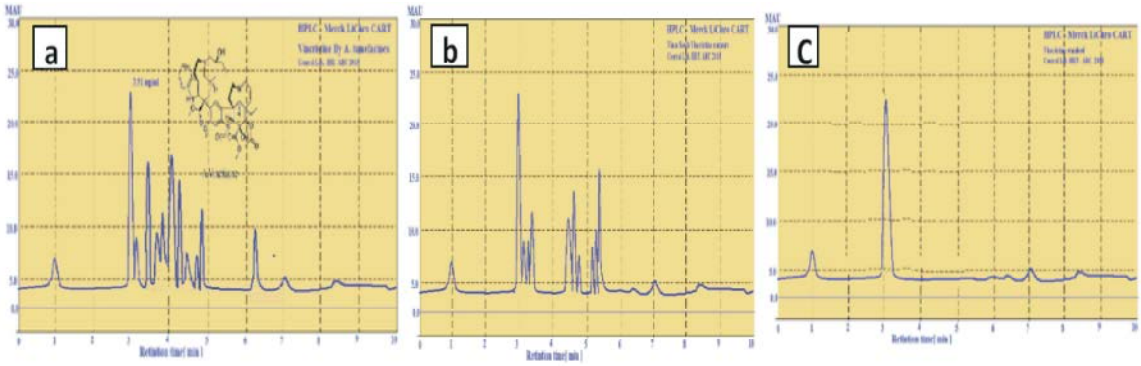

Fig. 3: HPLC analysis of periwinkle (a) seeds, (b) transformed explants and (c) HPLC vincristine sulfate Vincosien

and optimized, including the concentration of *Agrobacterium* (1:100), acetosyringone (150 uM) and co-cultivation duration. The detection was performed using *A. tumefaciens*-mediated *GUS* gene transient expression. The transformation frequency was calculated as the number of kanamycin resistance plants/number of explants ?×? 100 [29].

Among the duration time (0, 30, 60 and 120?min) the highest *GUS* transient expression (75%) and the more efficient growth for the explants (1.2 shootlet No., 1.4 cm shootlet length and 4.2 leaves No.) were achieved using the explants infection duration of 60 min (Table 4). In this concern, Wang, *et al.,* [32] found that the highest *GUS* transient expression (100%) and the relatively lower death rate for the explants (15%) were achieved using the optical density at 600 nm (OD_{600} = 0.8 of *A. tumefaciens*) and the explants infection time of 60 min. Also, they obtained the highest frequency of *GUS* transient expression (75 %) and lower death rate (25%) for the explants with 4 d co-cultivation and 150 µM of acetosyringone in co-culture medium (MS free) in dark.

Vincristine Contents: Data in Table 5 and Fig. 3 showed the indole alkaloid vincristine content in both transgenic explants by *A. tumefaciens* and seeds (open field plants) determined by HPLC device. Results revealed that, transgenic explants contained the highest value of vincristine 1.39 ug/ml (Fig. 3b) as compared with original seeds 0.025 ug/ml (Fig. 3a).

Taha *et al.* [45] assayed the total alkaloids (vinblastine and vincristine) of vinca transgenic calli and cell cultures and compared them to their relative percentage with those of the intact plant. The highest relative percentages 14.47, 13.62 and 11.5 of total alkaloids, (vinblastine and vincristine) were recorded with (CS7) compared with other selected cell lines. However, the non-transformed cell line (CS0) produced 9.48, 8.29 and 5.6 % for total alkaloids, vinblastine and vincristine, respectively. In current study, the total alkaloids, vinblastine and vincristine of the six transgenic calli lines were measured. The highest values 10.48, 8.3 and 6.19 % of total alkaloids, (vinblastine and vincristine) as a relative percentage with those of *C. roseus* intact plant were recorded in C4. However, the non-transformed calli line (C0) produced 8.78, 5.3 and 3, 83 (%) for total alkaloids, vinblastine and vincristine, respectively.

Cellular Membrane Stability (CMS): Membranes are the first targets of many plant stresses and maintenance of their integrity as a major component of drought and salinity tolerance in plants [46]. Therefore, in the present study, cellular membrane stability (CMS) index was used to assess salt (NaCl, 3000 ppm), drought (PEG, 60 %) and heat (45°C) tolerance amongst different lines, transgenic and non-transgenic (Table 6). At the end of 48 h of PEG mediated water stress, the value of CMS was reduced to 77 % in the transgenic explants overexpressing *GUS* and 67 % in the non-transgenic plants. In contrast to a rather delayed response to simulated drought stress, exposure of plants to salinity caused cellular membrane disruption soon after 6 h of treatment with 3000 ppm NaCl. However, non-transgenic plants were more susceptible to membrane injury and CMS was reduced to 28 % while, transgenic lines explants showed better membrane stability of 53 %.

Table 6: Effect of transformation on membrane stability and proline content

	CMS			
Type of explants	PEG 60%	Heat 45°C	NaCl 3000ppm	Proline mg/100 g FW
Transformed explants	77.00	33.00	53.00	9.19
Control	67.60	21.00	28.00	8.23
LSD 5%	9.77	8.23	12.22	0.77

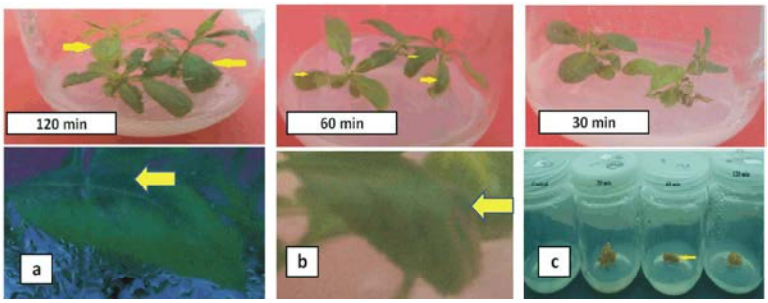

Fig. 4: (a and b) vinca explants positive GUS gene after treatments with acetosyringone, (C) callus positive of GUS gene, 30, 60 and 120 min explants after immersion treatments

Table 7: Based PCR of *Gus* gene and *NPTII* genes for duration infection by A. tumefaciens

Treatment	*GUS* PCR plantlets	*nptII* PCR plantlets	*GUS* PCR Callus	*nptII* PCR Callus	*Morphological Appearance for plantlets*	*Transformation frequency %*
Control	-	-	-	-	Greenish	0.0
30 min	+	+	+	+	Greenish	0.0
60 min	+	+	+	+	Blue/greenish	75.0
120 min	+	+	+	+	Blue/greenish	50.0

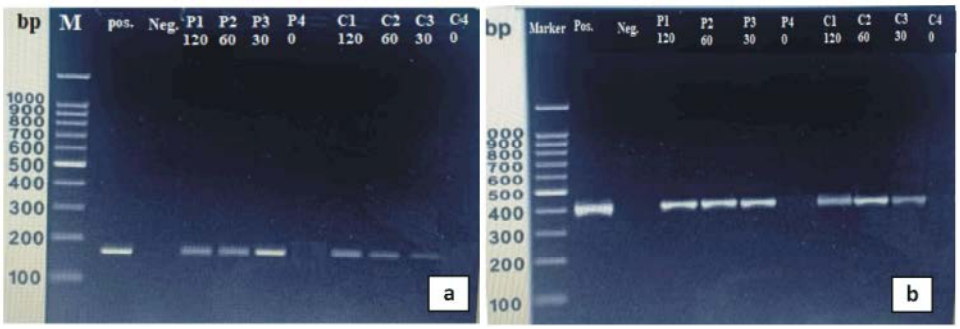

Fig. 5: (a) Based PCR of *GUS* and (b) *NPT II* genes for duration infection by *A. tumefaciens* P: Plantlets and C : Callus; 0 – 120 time of exposed to acetosyringone

Similarly, for heat stress, transgenic plants differed significantly over non-transgenic plants after 1 h of heating stress showing superiority over non-transgenic plants. Proline content in transformed explants cultured on MS medium supplemented with 3000 ppm of NaCl, increased to 9.19 mg/100 g FW compared with 8.23 mg/100 g FW of control. In this concern, Shalini, *et al*. [47] used cellular membrane stability (CMS) index to assess salt (NaCl, 400 mM) and drought (PEG, 2%) tolerance amongst different lines. At the end of 48 h of PEG mediated water stress CMS was reduced to 70% in the transgenic lines ST31, ST11 and ST8 overexpressing *hva1* gene and 50% in the non-transgenic plants.

Results shown in Table (7) demonstrate that the transformation of *C. roseus* was improved in the presence of acetosyringone, since most of the initial explants produced KANR plants when 150 mM of acetosyringone was added to the co-culture (MS free) medium. The results showed that the highest *GUS* transient expression (75 %) with the explants infection of 60 min compared with 30, 120 min infection duration (Table 4). The co-cultivation with *A. tumefaciens* between 1 to 3 d and the acetosyringone concentration (150 mM) on T-DNA delivery were tested. The results showed that the highest frequency of *GUS* transient expression (75%) and relatively lower death rate (25 %) were recorded for the

explants with 4 d co-cultivation and 150 mM of acetosyringone in co-culture medium incubated in dark, the explants were more greenish with few blue/green spots on the infection wounding zone (Fig. 4, yellow arrows and Table 7).

The data in Fig. 5a showed positive and negative presented of *Gus* gene for the explants treated with acetocyringone at 150 uM for 30, 60 and 120 min for each explants and callus. Also, *NPTII* gene was presented in both callus and explants treated with acetocyringone at 150 uM (Fig. 5b).

Secondly, the effects of co-cultivation duration and acetosyringone concentration on transformation frequency were optimized separately. Using micro nodes as explants, callus induction, shoot initiation and root initiation media containing kanamycin were employed in sequence. After *Agrobacterium* mediated transformation and 2 d co-cultivation in dark, transgenic calli were induced by growing the explants for 10 d in selective medium (MS) with kanamycin (data not shown). Then the induced transgenic calli were subjected to additional selection medium (MS) containing higher levels of kanamycin (Fig. 4c), to eliminate false positive plants. It was found that most of explants became brown gradually and died except the putative transformants. The green shoots of putative transformants that appeared in 40 d (Fig. 4a) were transferred onto shootlets induction medium (Fig. 4C).

In the Alkaloids pathway, tabersonine is converted into different compounds, especially vindoline, a chemical precursor to vinblastine by deacetylvindoline- 4-O acetyltransferase (DAT) [48]. Finally, vinblastine and vincristine are synthesized by the compression of catharanthine and vindoline metabolites [49]. Production of diverse types of TIAs pathway metabolites is controlled at transcriptional, translational and post-translational levels and is fluctuated by many internal and external elicitors [50]. For instance, phytohormones including salicylic acid (SA) and ethylene (ET) stimulate the production rate of catharanthine, vindoline and vinblastine in the *C. roseus*, whereas abscisic acid (ABA) and gibberellic acid (GA) tend to decrease the production rate of TIAs [48]. However, the signal transduction pathway mechanisms explaining how these growth hormones regulate TIAs biosynthesis are largely unknown. The LEA gene (Late embryogenesis abundant protein) was first studied in cotton, whose expression occurred in the maturing seeds and anhydrous plants [51, 52]. LEA proteins are important systems in plants, as low molecular weight proteins that are involved in protecting plants from damage caused by environmental stresses, especially drought [53].

CONCLUSION

All the obtained results showed that the *C. roseus* transformation protocols developed in this work has a great potential to be used in the discovery of gene functions in TIAs biosynthetic pathway to improve their production. An *Agrobacterium*-mediated transformation and regeneration system for *C. roseus* were established in the current study. The parameters influencing the transformation frequency are systematically investigated, including the concentration of *Agrobacterium* and acetosyringone, co-cultivation duration and selection pressure of kanamycin. The results showed that the transformation frequency arrived at 75%. The HPLC results revealed that the production of vincristine was enhanced in transgenic plants with *GUS* overexpression.

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