In Vitro Regeneration and Somaclonal Variation of *Catharanthus roseus* Don. Using Leaf and Internodal Explants

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ABSTRACT

This work was done at the Tissue Culture Laboratory of the Floriculture Department, Faculty of Agriculture, Alexandria University, Egypt, during the period from 2010 to 2012, to determine the effect of cultivar (Rosea and Alba), explant (leaf and internode) and different concentrations of two growth regulators (PGR) (IAA and BA) on plant regeneration of *Catharanthus roseus*. The collected data were: callus induction, number of formed shoots, root induction and survival. Calluses developed from both leaf and intermodal explants of both cultivars when cultured on MS with PGRs. All the measured data were significantly influenced by the differences in cultivar, medium constituents and kind of explant. These differences were appeared in the interaction among them. Rosea was always superior to Alba. Internodal explant was better than leaf explant in all tested data. The medium which gave the highest average of callus induction was MS supplemented with 1.0 mg/l IAA+4.0 mg/l BA, while the medium without growth regulators did not produce any callus. The internodal explant of Rosea produced the highest mean value of shoot formation by MS medium supplemented with 0.0 mg/l IAA+4.0 mg/l BA. *In vitro* cuttings of regenerated *Catharanthus roseus* rooted easily. The highest rooting rate was recorded by MS supplemeted with 1.0 mg/l IAA+4.0 mg/l BA. 70% of the acclimatized plants survived and bloomed. Somaclonal variations were reported, show leaf variegation in color and morphology of some leaves. In this study different types of variants were obtained in leaf shape (28.5%).

Key words: *Catharanthus roseus*, shoot regeneration, plant growth regulators, somaclonal variation.

INTRODUCTION

Catharanthus roseus (L.) G. Don. (Commonly known as Periwinkle Family Apocynaceae) gained commercial importance because of its alkaloids contents in different plant parts. The alkaloids like antineoplastin, dimeric vinblastin and vincristine are mainly present in aerial parts, whereas, ajmalcine, vinceine, vincamine, raubasin and reserpine are present in roots and basal stem. *Catharanthus roseus* has more than 400 known alkaloids. Some are used by the pharmaceutical industry for the treatment of childhood leukemia, Hodgkin's disease, testicular cancer and cancerous tumors. *C. roseus* is an erect handsome herbaceous perennial plant which is a chief source of patented cancer and hypotensive drugs. It is one of the very few medicinal plants which have a long history of uses as diuretic, antidysenteric, hemorrhagic and antiseptic. It is known for use in the treatment of diabetes in Jamaica and India. Prevention of cancer, cancer treatment, reduction of high blood pressure, externally against nose bleeding, sore throat and mouth ulcers. Periwinkle organogenesis was first reported in the late 1970s by Dhruva *et al*. (1977) followed by Ramavat *et al*. (1978) and Abou-Mandour *et al.* (1979). However, the shoot regeneration rate was low. Krueger *et al*. (1982) established plant and leaf-organ cultures from seeds of *C. roseus* germinated aseptically on the MS medium supplemented with benzyladenine (BA). The culture showed abundant proliferation and produced vindoline and a complex variety of other alkaloids, including some possibly not produced by the intact plant. Furmanowa *et al*. (1994) regenerated *C.roseus* plantlets from shoot tips and axillary buds. About 200 rooting plantlets were obtained from one seedling. Swanberg and Dai (2008) used two periwinkle cultivars for the development of a plant regeneration system, using leaf and internodal explants plated onto woody plant medium (WPM) using a factorial arrangement of 6-benzyladeine (BA) and 1-naphthalene acetic acid (NAA). Shoots were successfully regenerated. Shoot production from leaf tissues was minimal for all cultivars, whereas internodal tissues showed variable rates of regeneration depending on the hormone combination. Faheem *et al*. (2011) used nodal segment explants cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of BAP, NAA and IBA. Multiple shoots were produced on all the concentrations of BAP, NAA and IBA; however BAP (0.5 mg/l) + NAA (1.0 mg/l) proved to be optimal for the production of maximum number of shoots. Best rooting response was observed on half strength MS containing 0.10 mg/l Indole-3-butyric acid (IBA). Mutation, breeding and tissue culture technique can be utilized for the improvement of ornamental characteristics (Hutchinson *et al*., 1992). The variability associated with tissue culture has provided a pool of variation upon which selection pressure has imposed to isolate unique forms of clones. This variation known as somaclonal variation has become an important tool for plant improvement (Skirvin *et al*., 1993). The objectives of this work were to determine the effect of cultivar, explant, and growth

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regulators on plant regeneration of *C.roseus*, and to search for valuable somaclonal variants of the plant.

MATERIALIS AND METHODS

Plant material

Catharanthus roseus (L.) cvs. Rosea and Alba were obtained from the Floriculture Department Nursery, Faculty of Agriculture, El–Shatby, Alexandria. Internodes were cut to 5 to 6 mm long and leaves were cut into 10 to 12 mm long segments. All explants were first washed with detergent and distilled water, then disinfected with 15% bleach plus two drops of Tween 20 for 15 min, then placed in ethanol 70% for one minute and rinsed three times with autoclaved distilled water, to ensure that the internodes parts and leaf segments are totally sanitized.

Explants were cultured on solidified media containing 4.43 g/l MS with vitamins (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 7 g/l agar, the pH was adjusted at 5.8 ± 0.1. Three different concentrations of BA (0, 2.0 and 4.0 mg/l) and two different concentrations of IAA (0 and 1.0 mg/l) were added to the basal medium, poured in tubes (2.5 cm diameter × 15 cm height), Each tube with one explant considered as replication. Five replications were used for each explant. After culture the explants, tubes were sealed with parafilm and placed under a 16/8 h photoperiod at 21 ◦C. For all regeneration experiments, the number of explants producing callus were recorded after 1 month from the beginning of the experiment. The culli were subcultured on the fresh medium for shoot proliferation, the number of shoot formation were recorded after 1 month. Root formation was induced in a medium supplemented with different concentrations of PGR. Number of rooted cuttings were recorded. The culli produced from the two explants for both cultivars were subcultured for 6 times to search for any somaclonal variant and data of observation were recorded.

Acclimatization

Regenerated plants were washed with tap water to remove agar from the roots and were transplanted in pots 10 cm filled with peat moss and perlite (1:1 v/v). The pots were covered with plastic bags, which were removed gradually within two weeks. The pots were incubated under moist conditions in the greenhouse for acclimatization in the Floriculture Department Nursery, Faculty of Agriculture, El–Shatby, Alexandria.

Collected data callus induction, formed shoots, root induction, survival and observation of any variation.

Statistical analysis

Data were statistically analyzed as a 3-factors experiment cultivars (Rosea and Alba), explants (leaf and internode) and growth regulators (0, 1.0 mg/l IAA) and (0, 2.0 and 4.0 mg/l BA) in a completely randomized design with 5 replicates. Data were subjected to square root transformation prior to statistical analysis (Steel and Torrie,1980).Comparisons among means were made using the protected Least Significant Differences test (LSD) using SAS (1985) program (version 6).

RESULTS AND DISCUSSION

Analysis of variance indicated that factor of PGRs (IAA and BA) had a highly significant effect on all studied data (Table1). Significant interactions were obtained among the factors.

Data of the induction of callus, derived from leaf and internodal explants of two cultivars of *Catharanthus roseus*, were recorded after four weeks of incubation. Significant interactions were determined among cultivar, explant and BA as shown in (Table 2). Callus induction was influenced by cultivar, explant and medium constituents. Callus formation varied in both cultivars. The number of internodal explants, that developed calli ranged from 1.8 (Rosea) to 1.3 (Alba), however, the percentage of leaf explants, that developed calli ranged from 1.6 (Rosea) to 1.4 (Alba). The medium which gave the highest average of callus induction (1.8) across cultivars contained BA (4.0mg/l), while the medium devoid growth regulators gave the lowest callus induction with both cultivars .

The interaction between the two plant growth regulators (BA and IAA) on callus induction, presented in (Table 3), showed that the highest average of callus induction 1.6 was obtained from MS contained 4m/l BA + 1mg/l IAA.

Table 4 presents the interactions between IAA and explant source on callus induction. The highest number of callus induction (1.3) was obtained from internode explant with 1mg/l IAA.

Shoot formation

In general, shoots were generated during the second month when calli were subcultured on the new medium (Fig.1). Shoot regeneration was found to be PGR and explant-dependent in *Catharantus roseus* (Table 5).

Results presented in Table 5 show the interactions between 2 PGRs and 2 explant sources.It showed that the internodal explant produced the highest mean value of shoot formation (2.5). The medium which contained the highest cytokinin concentration and no auxin (0.0 IAA+4.0 BA) showed the greatest potential for shoot formation across explant source and it was significantly superior to all other medium constituents.

Table 1. Analysis of variance of number of callus induction, shoot regeneration and root induction for two cultivars of *Catharanthus roseus* (rosea and alba)., (Transformed scale)

Source of variation	DF	Callus induction	Shoot regeneration	Root induction
		MS	MS	MS
Cultivar	1	0.01	0.3	0.1959
BA	2	5.87**	12.4**	3.72**
IAA	1	0.49**	1.4**	4.81**
Explant	1	0.09	0.00004	0.45*
Cultivar xBA	2	0.11	0.5**	0.052
Cultivar x IAA	1	0.01	0.15	0.036
Cultivar x explant	1	0.04	0.05	0.034
BA x IAA	2	0.33**	0.74**	1.24**
BA x explant	2	0.82**	1.7**	0.12
Explant x IAA	1	0.54**	1.94**	1.71**
cultivar x BA x IAA	2	0.04	0.07	0.036
cultivar x BA x explant	2	0.19**	0.22*	0.052
Cultivar x IAA x explant	1	0.04	0.009	0.067
BAx IAA x explant	2	0.08	0.61**	0.498*
Cultivar xBAx IAAxexplant	2	0.11	0.09	0.0402
Error	92	0.0376	0.0688	0.108

*,** significante at 0.05 and 0.01 level of probability, respectively.
BA= 6-benzyladenine.(0, 2.0 and 4.0 mg/l BA).
IAA= Indole acetic acid.(0,1.0mg/l).
Cultivars: *C.rosea* and *C.alba*
Explants: leaf and internode

Table 2. Effect of cultivar, explant and (BA mg/l) on number of callus induction of *Catharanthus roseus.*

BA (mg/l)	cultivars			
	Rosea		Alba	
	Leaf	internode	Leaf	internode
0	0.7	0.7	0.7	0.8
2	1.2	1.1	1.3	1.2
4	1.6	1.8	1.4	1.3

Data were subjected to square root transformation.
$L.S.D_{0\ .05}$ (genotype x BA x Explant) = 0.17.

Table 3. Effect of plant growth regulators mg/l (BA and IAA) on number of callus induction of *Catharanthus roseus*

IAA (mg/l)	BA/mgl			Mean
	0	2	4	
0	0.7	1.08	1.4	1.09
1	0.7	1.34	1.6	1.2
Mean	0.75	1.21	1.5	

Data were subjected to square root transformation.
$L.S.D_{0\ .05}$ (BA x IAA) = 0.12.

Table 4. Effect of explant and IAA on number of callus induction of *Catharanthus roseus,* after 4 weeks from incubation

IAA (mg/l)	Explant		Mean
	Leaf	internode	
0	1.05	1.13	1.09
1	1.23	1.31	1.22
Mean	1.18	1.13	

Data were subjected to square root transformation.
$L.S.D_{0.05}$ (IAAmg/l x Explant) = 0.10.

Table 5. Effect of explant and plant growth regulator (BA and IAA) on number of shoot formationof *Catharanthus roseus.*

BA (mg/l)	IAA mg/l			
	0		1	
	Leaf	internode	Leaf	internode
0	0.7	0.7	0.8	0.7
2	1.0	1.6	1.5	1.8
4	1.6	2.5	1.7	1.5

Data were subjected to square root transformation.
L.S.D (BA x IAA x explant) = 0.23.

In general,the results showed that a significantly higher shoot formation was obtained from the internodal explants than that of leaf explants.

Table 6 presents the interactions between BA, cultivar and explant source. Data showed that the highest level of BA (4 mg/l) with nodal explant produced the highest mean value (2.1).

The probable reasons of differences in *in vitro* morphogenetic response may be attributed to (a) genetic differences among the genotypes used, (b) differences in the growth regulators Prasad *et al*., (1983) reported that the rate of shoot multiplication is genotypic dependent. Rademaker (1990) reported that cultivar and explant type had a greater effect on regeneration than the type of medium. Addition of exogenous growth regulators to the medium was necessary for plant regeneration of periwinkle has been found in other plant species such as chokecherry, black cherry, and gerbera (Dai *et al*., 2004; Kumar *et al*., 2004; Espinosa *et al*., 2006) and in periwinkle (Swanberg and Dai, 2008).

The results of the present study demonstrate that BA is important for shoot initiation. Growth regulators induce the competence of tissue to respond to further developmental signals (Christianson and Warnick, 1985 and 1988). Cells often respond differently at different developmental stages, and interactions between auxin and cytokinin signaling pathways may occur (Shi *et al*., 1994). The inclusion of cytokinin in the culture media enables callus to be induce from a wide range of plant species. More importantly cytokinin allows initiation of multicellular meristematic regions capable of differentiation into organized structures (Lowe *et al.*, 1996).

Rooting and acclimatization:

Table 7 shows that the medium contained the highest concentration of IAA gave the highest mean value for root induction as compared with the other medium constituents. Roots were healthy and strong in appearance (Fig.1A). This result correlates with a previous study that found internodal tissues to be best suited for the regeneration of *Catharanthus* (Mollers and Sarkar, 1989 and Swanberg and Dai, 2008).

Survival rate

The pots were incubated under moist conditions in the greenhouse for plantlets acclimatization in the Floriculture Department Nursery, Faculty of Agriculture, El– Shatby, Alexandria. Plantlets were checked daily for six months till reaching the flowering stage. 70% of plants were survived and bloomed.

Somaclonal variation

Somaclonal variation often occurred on regenerators from callus and was used for mutation breeding without mutagenic treatment in some crop species (Larkin and Scowcroft, 1981).

Leaf variation

Leaves of seventy plants growing in the greenhouse were inspected for morphological variation. The results are shown in Table 8. The leaf shape of the original plant (normal) was ovate. From the observation, there were abnormalities in leaves. Some plants produced variegated leaves, others produced a wide range of leaf deformities (28.5%). 8 plants produced deformed leaves, 7 plants produced wavy margins and 5 plants produced variegated leaves (Fig.2).

The results demonstrated that new phenotypes from periwinkle could be produced by somaclonal variation through *in vitro* regeneration. Variation in leaf morphology was obtained by Bouman and De klerk (1996) in some ornamental plants. Plant growth regulators affect the rate of somaclonal variation indirectly by increasing the multiplication rate and inducing adventitious shoots (Bairu *et al*., 2006). In conclusion, the finding of this work could be useful in further study. More investigation could be done to improve the regeneration of *Catharanthus roseus*.

Table 6. Effect of cultivar, BA and explant on number of shoot formation of *Catharanthus roseus*

BA (mg/l)	cultivar			
	Rosea		Alba	
	Leaf	internode	Leaf	internode
0	0.7	0.7	0.7	0.9
2	1.5	1.1	1.8	1.5
4	1.9	2.1	1.8	1.5

Data were subjected to square root transformation.
L.S.D (genotype x BA x explant) = 0.23.

Table7. Effect of explant and plant growth regulators (BA and IAA) on number of roots induction of *Catharanthus roseus*.

BA (mg/l)	IAA (mg/l)			
	0		1	
	Leaf	internode	Leaf	internode
0	0.7	0.7	0.7	0.7
2	0.8	1.1	0.7	1.5
4	1.1	1.3	0.9	1.9

Data were subjected to square root transformation.
L.S.D (explant x BA x IAA) = 0.29

Table 8. Number of leaf forms of *Catharanthus* plants developed *in vitro*

Total	Leaf shape			
	(deformed)	Wavy margins	Variegated leaves	Original ovate
70	8	7	5	50

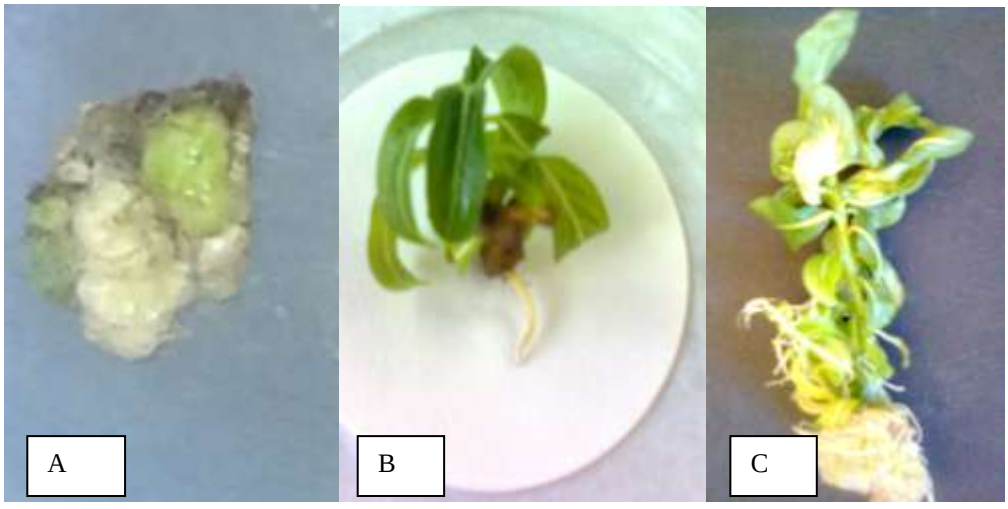

Fig.1. A, B and C: Callus induction, shoots regeneration, and root formation from explant of *Catharanthus roseus*.

Fig. 2. Somaclonal variation in p *Catharanthus roseus* plants.
A: Varigated leaf

Fig. 2. Somaclonal variation in *Catharanthus roseus* plants.
B: Normal and deformed leaf

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الملخص العربي

الإكثار المعملى وإحداث تغيرات لنبات الونكا بإستخدام منفصلين نباتيين الأوراق والسلاميات

هدي السيد المقدم

أجريت هذه التجربة بمعمل الزهور ونباتات الزينة لزراعة الأنسجة فى كلية الزراعة جامعة الإسكندرية فى خلال الفترة من 2010-2012 لتحديد الظروف المثلى لإكثار صنفين من أصناف الونكا معمليا وهى Rosea وAlba. وإستخدم منفصلان نباتيان مختلفان هما الأوراق leaves والسلاميات internodes وتركيزات مختلفة من منظمات النمو وهى IAAو BA وكانت النتائج كالتالى:

كان هناك تفاعلا بين الأصناف والجزء المستخدم من كل صنف وكذلك بينهما وبين منظمات النمو المستخدمة.

فى جميع الصفات التى درست وهى القدرة على إنتاج الكالس و عدد الفروع الخضرية و القدرة على تكوين جذور كان الصنف Rosea متفوقا دائما على الصنف Alba.

كذلك كان المنفصل النباتى أو internode متفوق دائما على الأوراق. وكانت التركيزات التى أعطت أفضل نتائج للكالس هى:

1.0 mg/l IAA+ 4.0 mg/l BA بينما إنتاج المحاميع الخضرية بأعلى معدل كان مع البيئة المحتوية على 4.0 mg/l BA فقط.

فى حين أن البيئة المحتوية على 1.0 mg/l IAA + 4.0 mg/l BA أعطت أعلى معدل لتكوين الجذور.

أظهرت النتائج أيضا حدوث بعض التغيرات المورفولوجية فى لون أوراق بعض النباتات حيث ظهرت مبرقشة باللونين الأخضر والأبيض الكريمى وكذلك فى طبيعة نمو بعض الأوراق حيث ظهرت أيضا بعض الإختلافات فى شكل الورقة بنسبة (28.5%)

من هذه الدراسة يمكن إستنتاج أن: يمكن إنتاج نبات الونكا معمليا بإستخدام internode وإستخدام بيئة نمو محتوية على 1.0 mg/l IAA+ 4.0 mg/l BA.

70 % من النباتات التى تم أقلمتها وصلت إلى مرحلة الأزهار. وعلى ذلك فإن إستمرار تجارب زراعة الأنسجة وإستخدام طرق أخرى فعالة فى إستحداث الطفرات قد نتمكن من الحصول على تغيرات فى لون وشكل الأزهار لنبات الونكا وأيضا الحصول على صفات مرغوبة تجاريا.