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Effects of Different Concentrations of α-naphthaleneacetic Acid and 6-benzylaminopurine on Shoot Regeneration of *Vinca minor* L.

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ABSTRACT

Periwinkle (*Vinca minor* L.) is an evergreen perennial herbaceous plant that belongs to the family Apocynaceae. The aerial part of this plant contains certain alkaloids such as vincamine, isovincamine and vincine. The most important of these is vincamine that reduces blood pressure and promotes memory and the ability for mental concentration. Micropropagation could be a good alternative for the mass propagation of *Vinca minor*. For direct shoot regeneration, single node explants of field-grown *Vinca minor* cv. Budakalasz were aseptically cultured on a medium consisting of WPM salts, MS vitamins, 3% sucrose, 0.8% agar and 25 different combinations of BAP and NAA. After one month, explants were subcultured to the same medium. The number of shoots (taller than 3 mm), average shoot length, height of the longest shoot, callus amount, number of roots, and average root length per explant were measured two months after culture. The maximum shoot regeneration (5.6 shoots per explant) was obtained using 7.21 mg l^{-1} BAP and 0.0186 mg l^{-1} NAA.

Keywords: Callogenesis, *In vitro*, Shoot regeneration, *Vinca minor* L.

INTRODUCTION

Periwinkle (*Vinca minor* L.) is an evergreen perennial herbaceous plant that belongs to the family Apocynaceae (Bernath, 1993). Known since ancient times, this plant has been heralded for its properties as numerous as they are unproven, such as astringent, wound-healing, antidermatosic, antigalactic etc. (Bruneton, 1995). More than 40 alkaloids have been identified in the aerial part of *Vinca minor* such as vincamine, isovincamine and vincine. The most important of these is vincamine, an alkaloid frequently used in geriatric Medicine (Boyadzhiev and Yordanov, 2004; Bruneton, 1995; Omidbaigi, 2005). Vincamine reduces blood pressure (Omidbaigi, 2005; Tyler *et al*., 1988) and, owing to its vasodilatating effect which improves the cerebral blood circulation and extra the assimilation of oxygen by the brain tissue, vincamine promotes memory and the ability for mental concentration (Boyadzhiev and Yordanov, 2004). It is therefore included in numerous medical preparations and serves as a basis for several semisynthetic medicines with an enhanced or modified physiological effect (Boyadzhiev and Yordanov, 2004).

The growth of *Vinca minor* is comparatively slow (Tanaka *et al*., 1995). Moreover, this plant produces only a small amount of seeds which germinate rarely and so the plant is usually propagated from shoot cuttings rather than seeds (Bernath, 2000; Omidbaigi, 2005). Hence, micropropagation

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of *Vinca minor* could increase the propagation rate and provide enough plant materials for large-scale propagation. Thus, the technique of micropropagation could be an alternative method for mass propagation of this precious medicinal plant.

A limited number of studies has been carried out on micropropagation of *Vinca minor*. Stapfer and Heuser (1985) found that BA was the best cytokinin for shoot proliferation of *Vinca minor* cv. Bowlesii compared with kinetin or 2ip. They also reported that the addition of NAA to a medium containing 32 or 64 µM BA increased the number of shoots produced in comparison with a medium containing only BA. The greatest number of shoots (≥5 mm) was produced at 64 µM BA and 0.1 µM NAA (Stapfer and Heuser, 1985). In an other experiment, MS medium containing 0.02 mg l^{-1} NAA and 1 mg l^{-1} BAP and 2 - 4 mg l^{-1} gibberellic acid has been determined as the best medium for *Vinca minor* culture (Zhang and Yang, 1995). Tanaka *et al*., (1995) reported that, in a clone of *Vinca minor* Vm-101, the addition of 2.2 µM BA to the culture medium increased the number of shoots from hairy roots.

The aim of the present research was to study the effect of different concentrations of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) on shoot regeneration of *Vinca minor* cv. Budakalasz through the culture of single node explants.

MATERIALS AND METHODS

The nodal cuttings of *Vinca minor* with axillary buds were collected from Zardband research garden (elevation 1548 m above sea level, latitude 35°47′ North, longitude 51°37′ East) situated in the North of Tehran in May 2005. The nodal cuttings were carefully transferred to the laboratory after collection. Initially, cuttings were divided into small pieces of about 5 to 10 cm and their leaves were removed without separating the petioles. The explants were then washed under running tap water for 30 minutes and surface-sterilized by immersion in ethanol (96% v/v) for five seconds followed by 3% (w/v) sodium hypochlorite solution for 20 minutes. Subsequently, the plant materials were washed in sterile distilled water three times and, finally, the single node explants were prepared. The explants were cultured in test tubes (20×2.5 cm) containing 25 ml of medium. The medium consisted of WPM salts (Loyd and McCowan, 1980), MS vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar, and different concentrations and combinations of BAP and NAA. The pH of the medium was adjusted to 5.7 and autoclaved at 121°C for 20 minutes. All cultures were incubated at 25±2°C under a 16-h photoperiod provided by cool white fluorescent tubes with a light intensity of 3000 lux.

After one month, the explants were transferred to the same medium for a further additional month. The number of shoots (taller than 3 mm) per explant, average shoot length per explant, height of the longest shoot per explant, callus amount per explant, number of roots per explant, and average root length per explant were measured two months after culture.

In this study, 25 different hormonal combinations including different concentrations of BAP and NAA were studied in three separate experiments (Tables1, 2 and 4). The first experiment was carried out in a completely randomized design (CRD) with eight replications. Two factorial experiments based on a completely randomized design with two factors and eight replications were used in the second and third experiments. In the second experiment, BAP at two levels and NAA at seven levels, whereas in the third experiment BAP at five levels and NAA at three levels were applied. In all the experiments, each replication consisted of one test tube with one single node explant. Data were analyzed by one-way analysis of variance and the means were compared using Duncan's new multiple range test (DMRT) at the 5% level. In all cases, data analysis was carried out using SPSS Version 13 (SPSS Inc., Chicago, IL, USA).

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For acclimatization, the regenerated shoots, both with or without roots, were taken out and gently washed in tap water to remove all traces of the media. They were then planted in plastic cups (upper diameter 7.5 cm×length 8 cm, with a volume of 240 cm^3.) filled with a mixture of peat moss: perlite: sand (3:1:1) and kept in a growth chamber at 25±2°C under a 16-h photoperiod provided by cool white fluorescent tubes with a light intensity of 5000-6000 lux. To maintain humidity, the plantlets were covered with plastic caps, and then gradually opened during the one month acclimatization period. The pots were subirrigated by filling the saucers with N–P–K fertilizer (18–18–18). Acclimatized plantlets were transferred to the greenhouse and ultimately to field in natural condition.

RESULTS AND DISCUSSION

In the first experiment, the hormone treatment effect was statistically significant in the case of the number of shoots, average shoot length, height of the longest shoot, and callus amount per explant but the hormone treatments did not have any statistically significant effect on the number of roots produced and their average length per explant (Table 1). The medium containing 14.42 mg l^{-1} BAP along with 0, 0.186 or 0.0186 mg L^{-1} NAA, produced the maximum number of shoots, per explant (4.6, 4.2 and 4 shoots, respectively). However the number of shoots formed in these concentrations of NAA was not significantly different (Figure 1A). The present investigation using 14.42 mg l^{-1} BAP and 0.0186 mg l^{-1} NAA is in agreement with those of Stapfer and Heuser (1985) who reported the number of shoots (≥ 5mm) had a maximum of 0.0186 mg l^{-1} NAA in combination with 14.42 mg l^{-1} BAP.

Longer shoots were formed when the concentration of NAA increased from 0 to 0.2 mg l^{-1}. The highest average shoot length (2.09 cm) and the longest shoot (4.53 cm) were obtained when 14.42 mg l^{-1} BAP and 0.2 mg l^{-1} NAA were employed (Figure 1C).

NAA at higher concentrations above 0.2 mg l^{-1} decreased the average shoot length and height of the longest shoot (Table 1). Therefore, the addition of low concentrations of NAA to the medium increased the shoot height. The concentration of 14.42 mg l^{-1} BAP in combination with 2 mg l^{-1} NAA resulted in the highest number of roots per explant (1.8) and the combination of 14.42 mg l^{-1} BAP and 1 mg l^{-1} NAA gave the highest average root length (5 mm). The treatments with 0, 0.0186 or 0.186 mg l^{-1}

Table 1. Effect of different concentrations of BAP and NAA on number of shoots, average shoot length, height of the longest shoot, number of roots, average root length, and callus amount per explant of *Vinca minor.*

Treatment (BA+NAA)(mg l^{-1})	Shoot number	Average shoot length (cm)	Height of the longest shoot (cm)	Root number	Average root length (mm)	Callus amount (mm^3)
1 (14.42+0)	4.60^{a}	1.07^{b}	2.12^{bcd}	0.00^{b}	0.00^{b}	0.00^{b}
2 (14.42+0.0186)	4.00^{ab}	1.32^{b}	2.60^{bc}	0.67^{ab}	1.17^{ab}	0.00^{b}
3 (14.42+0.186)	4.20^{a}	1.39^{b}	3.56^{ab}	0.50^{ab}	2.25^{ab}	0.00^{b}
4 (14.42+0.2)	3.50^{ab}	2.09^{a}	4.53^{a}	0.50^{ab}	2.00^{ab}	12.5^{b}
5 (14.42+0.5)	3.33^{abc}	0.98^{b}	1.83^{cd}	1.00^{ab}	2.50^{ab}	3.17^{b}
6 (14.42+1)	2.60^{bc}	0.96^{b}	1.44^{cde}	1.40^{ab}	5.00^{a}	25.50^{b}
7 (14.42+2)	2.00^{cd}	1.08^{b}	0.96^{de}	1.80^{a}	2.50^{ab}	91.39^{a}
8 (14.42+3)	0.50^{e}	0.20^{c}	0.20^{e}	0.67^{ab}	2.00^{ab}	71.04^{a}
9 (14.42+4)	0.80^{de}	0.22^{c}	0.24^{e}	0.60^{ab}	2.00^{ab}	75.29^{a}

Means followed by different letters in same column are significantly different at p=0.05 (Duncan's multiple range test).

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NAA did not produce any callus while the highest amount of callus was obtained using 14.42 mg l^{-1} BAP in combination with 2, 3 or 4 mg l^{-1} NAA (91.39, 71.04 and 75.29 mm^3 respectively) (Table 1).

In the second experiment, the interaction effect of BAP with NAA was statistically significant in case of the number of shoots, average shoot length, height of the longest shoot, and callus amount per explant. Main effect of NAA or BAP and interaction effects of BAP with NAA were not statistically significant for the root number per explant. The main effect of BAP and interaction effect of BAP with NAA were not statistically significant for average root length, but the main effect of NAA was significant (Tables 2 and 3). The number of shoots per explant was the maximum at 7.21 mg l^{-1} BAP and 0.0186 mg l^{-1} NAA with 5.6 shoots. However, the media containing BAP alone at 14.42 or 7.21 mg l^{-1} produced 4.60 and 4.50 shoots per explant, respectively. Results obtained from the first treatment, i.e. 7.21 mg l^{-1} BAP and 0.0186 mgl^{-1} NAA is in concurrence with the findings of Stapfer and Heuser (1985). It was found that the addition of NAA to the medium containing 7.21 mg l^{-1} BAP increased the number of shoots.

Results indicate that, once the concentration of BAP is 14.42 mg l^{-1}, the number of shoots per explant decreases when NAA concentration increases (Table 2). The highest average shoot length (4.15 cm) and the longest shoot produced (6.71 cm) were obtained from 7.21 mg l^{-1} BAP in combination with 0.2 mg l^{-1} NAA. The medium containing 14.42 mg l^{-1} BAP and 2 mg l^{-1} NAA resulted in the highest number of roots (1.8 per explant). The highest average root length (5 mm) was produced by using 7.21 mg l^{-1} BAP+0.2 mg l^{-1} NAA as well as 14.42 mg l^{-1} BAP+1 mg l^{-1} NAA. Explants cultured on the medium supplemented with the combination of 7.21 mg l^{-1} BAP and 2 mg l^{-1} NAA produced almost no shoots per explant. These explants formed the greatest amount of callus. The medium containing only BAP (14.42 or 7.21 mg l^{-1}) did not produce any callus.

Table 3 shows main effect of NAA on average root length. The concentration of 1 mg l^{-1} NAA resulted in the highest average root length (3.85 mm).

Table 2. Interaction effects of BAP at 2 level and NAA at 7 levels on number of shoots, average shoot length, height of the longest shoot, number of roots, average root length, and callus amount per explant of *Vinca minor*.

Treatments			Traits					
	BAP (mg l^{-1})	NAA (mg l^{-1})	Shoot number	Average shoot length (cm)	Height of the longest shoot (cm)	Root number	Average root length (mm)	Callus amount (mm^3)
T_1	14.42	0	4.60^{ab}	1.07^{bcde}	2.12^{cd}	0.00^{b}	0.00^{b}	0.00^{c}
T_2	14.42	0.0186	4.00^{bc}	1.32^{bc}	2.60^{bc}	0.50^{ab}	0.88^{b}	0.00^{c}
T_3	14.42	0.2	3.67^{bcd}	2.09^{b}	4.53^{b}	0.33^{ab}	1.33^{b}	12.50^{c}
T_4	14.42	0.5	3.33^{bcde}	0.98^{cde}	1.83^{cd}	1.00^{ab}	2.50^{ab}	3.17^{c}
T_5	14.42	1	2.60^{de}	0.96^{cde}	1.44^{cd}	1.40^{ab}	5.00^{a}	25.50^{c}
T_6	14.42	2	2.00^{e}	1.08^{bcde}	0.96^{cd}	1.80^{a}	2.50^{ab}	91.39^{b}
T_7	14.42	3	0.50^{f}	0.20^{de}	0.20^{d}	0.00^{b}	0.00^{b}	71.04^{b}
T_8	7.21	0	4.50^{ab}	1.16^{bcd}	2.58^{bc}	0.00^{b}	0.00^{b}	0.00^{c}
T_9	7.21	0.0186	5.6^{a}	1.36^{bc}	4.30^{b}	0.40^{ab}	1.10^{b}	0.50^{c}
T_{10}	7.21	0.2	2.14^{e}	4.15^{a}	6.71^{a}	1.33^{ab}	5.00^{a}	1.80^{c}
T_{11}	7.21	0.5	2.75^{cde}	1.30^{bc}	1.75^{cd}	0.40^{ab}	3.00^{ab}	83.10^{b}
T_{12}	7.21	1	0.00^{f}	0.00^{e}	0.00^{d}	1.00^{ab}	2.70^{ab}	96.82^{b}
T_{13}	7.21	2	0.20^{f}	0.12^{de}	0.12^{d}	0.50^{ab}	1.13^{b}	129.75^{a}
T_{14}	7.21	3	0.00^{f}	0.00^{e}	0.00^{d}	0.00^{b}	0.00^{b}	67.43^{b}

Means followed by different letters in same column are significantly different at p=0.05 (Duncan's multiple range test).

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Table 3. Main effect of NAA on average root length.

NAA (mg l^{-1})	Average root length (mm)
0	0.00^{c}
0.0186	1.00^{bc}
0.2	3.17^{ab}
0.5	2.73^{ab}
1	3.85^{a}
2	1.89^{abc}
3	0.00^{c}

Means followed by different letters in same column are significantly different at p=0.05 (Duncan's multiple range test).

Third Experiment

In the third experiment, the interaction effect of BAP with NAA was statistically significant in all six traits studied (Table 4). The maximum number of shoots per explant was obtained by using 14.42 mg l^{-1} or 7.21 mg l^{-1} BAP but without NAA (4.60 and 4.5 shoots, respectively). The highest average shoot length (6.37 cm) and the longest shoot (9.46 cm) as well as the highest root number per explant (4.67) were obtained from 0.9 mg l^{-1} BAP in combination with 0.2 mg l^{-1} NAA (Figure 1D). The superior length of shoot is a desirable trait since, by dividing it into smaller pieces, a large number of propagules could be provided.

The average root length was highest at 0.2 mg l^{-1} NAA+1.8 mg l^{-1} BAP (10.16 mm) and 0.2 mg l^{-1} NAA+0.9 mg l^{-1} BAP (9.33 mm). Explants cultured on the medium without NAA and with different concentrations of BAP did not produce any callus, and only a few roots were formed, if any at all. The greatest amount of callus was obtained using 7.21 mg l^{-1} BAP and 0.5 mg l^{-1}NAA.

The results of this experiment showed that the hormone treatments used could not increase the number of shoots, in comparison with the earlier experiments.

Acclimatization

Among plantlets that were produced from different hormone treatments, above 80%

Table 4. Interaction effect of BAP at 5 level and NAA at 3 levels on number of shoots, average shoot length, height of the longest shoot, number of roots, average root length, and callus amount per explant of *Vinca minor.*

Treatments			Traits					
	BAP (mg l^{-1})	NAA (mg l^{-1})	Shoot number	Average shoot length (cm)	Height of the longest shoot (cm)	Root number	Average root length (mm)	Callus amount (mm^3)
T_1	14.42	0	4.60^{a}	1.07^{ef}	2.12^{efg}	0.00^{d}	0.00^{c}	0.00^{c}
T_2	14.42	0.2	3.50^{abc}	2.14^{de}	4.53^{cd}	0.33^{d}	1.33^{bc}	12.50^{c}
T_3	14.42	0.5	3.33^{abcd}	0.98^{ef}	1.83^{efg}	1.00^{cd}	2.50^{bc}	2.60^{c}
T_4	7.21	0	4.50^{a}	1.16^{ef}	2.58^{ef}	0.00^{d}	0.00^{c}	0.00^{c}
T_5	7.21	0.2	2.14^{d}	4.15^{bc}	6.71^{bc}	1.33^{cd}	5.00^{b}	1.13^{c}
T_6	7.21	0.5	2.75^{bcd}	1.30^{ef}	1.75^{efg}	0.40^{d}	3.00^{bc}	83.10^{a}
T_7	3.6	0	3.83^{ab}	1.48^{ef}	3.22^{de}	0.00^{d}	0.00^{c}	0.00^{c}
T_8	3.6	0.2	2.33^{cd}	2.92^{cd}	3.33^{de}	2.00^{bc}	5.00^{b}	0.00^{c}
T_9	3.6	0.5	0.80^{e}	0.38^{f}	0.38^{fg}	0.60^{d}	2.60^{bc}	39.35^{b}
T_{10}	1.8	0	2.75^{bcd}	2.44^{de}	4.06^{de}	0.20^{d}	2.20^{bc}	0.00^{c}
T_{11}	1.8	0.2	2.17^{d}	4.73^{b}	8.57^{ab}	2.60^{b}	10.16^{a}	2.60^{c}
T_{12}	1.8	0.5	0.33^{e}	0.32^{f}	0.32^{fg}	0.25^{d}	1.13^{bc}	39.21^{b}
T_{13}	0.9	0	2.43^{cd}	3.01^{cd}	4.71^{cd}	0.25^{d}	1.50^{bc}	0.00^{c}
T_{14}	0.9	0.2	2.00^{d}	6.37^{a}	9.46^{a}	4.67^{a}	9.33^{a}	17.26^{c}
T_{15}	0.9	0.5	0.00^{e}	0.00^{f}	0.00^{g}	0.00^{d}	0.00^{c}	40.82^{b}

Means followed by different letters in same column are significantly different at p=0.05 (Duncan's multiple range test).

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Figure 1. *In vitro* shoot regeneration from single node explant of *Vinca minor* and their transferring to *ex vitro* conditions.

A. Shoot regeneration on medium containing 14.42 mg l^{-1} BAP+0 mg l^{-1} NAA
B. Shoot regeneration on medium containing 7.21 mg l^{-1} BAP+0.0186 mg l^{-1} NAA
C. Shoot regeneration on medium containing 14.42 mg l^{-1} BAP+0.2 mg l^{-1} NAA
D. Shoot regeneration on medium containing 0.9 mg l^{-1} BAP+0.2 mg l^{-1} NAA
E. Regenerated shoots (plantlets) transferred to the *ex vitro* condition.
F. Hardened plant in the filed (40 days after transferring to the filed).

survived through acclimatization and grew well. These plants continued growth after transferring to soil (Figures 1E and F).

CONCLUSION

Among 25 BAP + NAA combinations used in this study, the explants produced shoots without forming any roots or callus in three combinations, and produced shoots (number of shoots≥2) along with roots in 14 combinations. In the other eight combinations, however, the number of shoots produced was less than one (<1.00), although these combinations produced callus and, in some cases, roots.

Optimum shoot regeneration was obtained when a combination of 7.21 mg l^{-1} BAP and 0.0186 mg l^{-1} NAA were employed (Figure

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1B). This treatment produced 5.6 shoots per explant. The average shoot length was 1.36 cm and some of the explants produced roots.

According to the present study, the presence of root along with the shoot significantly improved the survival frequency. Therefore, producing root would be effective on further establishment under *ex vitro* conditions. Moreover, little callus formation was observed in this treatment which is desirable for a successful micropropagation. Direct shoot regeneration was the main target of the present work, and it was necessary to find ideal conditions for explants to develop with minimum callus because callus production may cause somaclonal variation.

According to this study, the concentration of 0.0186 mg l^{-1} NAA in combination with 14 or 7 mg l^{-1} BAP significantly increased the number of shoots per explant (Table 2). As a result, a higher shoot number may be produced by modifying these hormone treatments. It is suggested therefore, that 0.0186 mg l^{-1} NAA concentration or less, in combination with concentrations less than 7 mg l^{-1} BAP could be experimented.

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آثار غلظتهای مختلف نفتالن استیک اسید و بنزیل آمینوپورین بر باززایی نوساقه در گیاه دارویی پروانش صغیر(*Vinca minor* L.)

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چکیده

پروانش صغیر، گیاهی همیشه سبز، علفی و چندساله متعلق به خانواده خرزهره می‌باشد که قسمتهای هوایی آن حاوی آلکالوئیدهایی نظیر وینکامین، ایزووینکامین و وینسین است. مهمترین آنها وینکامین است که موجب کاهش فشار خون و افزایش حافظه و توانایی تمرکز ذهنی می شود. به منظور باززایی مستقیم نوساقه در این گیاه ، ریزنمونه‌های تک‌گره‌ای از گیاهان رقم بوداکالاز رشد یافته در مزرعه تهیه شدند و در شرایط استریل روی محیط کشت حاوی نمکهای محیط WPM، ویتامینهای محیط MS ،۳٪ ساکارز، ۰/۸٪ آگار و ۲۵ ترکیب مختلف بنزیل آمینوپورین و نفتالن استیک اسید کشت شدند. بعد از گذشت یک ماه، ریزنمونه‌ها روی همان محیط کشت، زیرکشت شدند و در پایان دومین ماه، تعداد نوساقه‌ها (بلندتر از ۳ میلی‌متر)، میانگین طول نوساقه‌ها، ارتفاع بلندترین نوساقه، مقدار کالوس، تعداد ریشه‌ها و میانگین طول ریشه‌ها اندازه‌گیری شدند. تیمار حاوی ۷/۲۱ میلی‌گرم در لیتر بنزیل آمینو پورین و ۰/۰۱۸۶ میلی‌گرم در لیترنفتالن استیک با تولید ۵/۶ نوساقه در هر ریزنمونه، بیشترین میزان باززایی نوساقه را داشتند.

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