Journal of Plant Physiology and Breeding

2019, 9(1): 129-138 ISSN: 2008-5168

In vitro regeneration of periwinkle (*Catharanthus roseus* L.) and fidelity analysis of regenerated plants with ISSR Markers

Saleh Amiri[1], Reza Fotovat[1*], Ali Reza Tarinejad[2], Bahman Panahi[3] and Seyed Abolghasem Mohammadi[4]

Received: April 3, 2019 Accepted: May 14, 2019
[1]Department of Plant Production and Genetics, Faculty of Agriculture, University of Zanjan, Zanjan, Iran.
[2]Department of Plant Biotechnology, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran.
[3]Department of Genomics, Northwest and West Region Branch, Agricultural Biotechnology Research Institute, Tabriz, Iran.
[4]Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
*Corresponding Author; Email: r_fotovat@znu.ac.ir

Abstract
Catharanthus roseus is an important multipurpose medicinal plant. In this study, *in vitro* proliferation and root induction of periwinkle were optimized and regenerated plants were subsequently surveyed for genetic homogeneity using the inter simple sequence repeat (ISSR) markers. Shoot tips and nodal segments were cultured in Murashige and Skoog (MS) medium supplemented with different concentrations of benzylamino-purine (BAP), gibberellic acid (GA3), and indol-3-butyric acid (IBA) hormones. ISSR profiling of regenerated plants as well as the mother plant were surveyed with five primers. The highest establishment rate (80.67%) was obtained in the MS medium containing 1.0 mg L^{-1} GA3 and 1.0 mg L^{-1} BAP. Highest proliferation rate (5.20 shoots/explant) and average shoot length (6.30 cm) were observed in 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IBA. Moreover, the best rooting response (85.30%) was observed on half strength MS containing 1.0 mg L^{-1} IBA. Genetic fidelity analysis using ISSR markers showed the monomorphic banding pattern of the micro-propagated plants and the mother plant, which highlighted their genetic uniformity. This implies that periwinkle micropropagation through shoot tip is the most reliable method for true-to-type production of *C. roseus* in a large scale.

Key words: Anti-cancer; Axillary bud; Genetic fidelity; ISSR markers; Proliferation.

Citation: Amiri S, Fotovat R, Tarinejad AR, Panahi B and Mohammadi SA, 2019. *In vitro* regeneration of periwinkle (*Catharanthus roseus* L.) and fidelity analysis of regenerated plants with ISSR Markers. Journal of Plant Physiology and Breeding 9(1): 129-138.

Introduction

Madagascar periwinkle (*Catharanthus roseus* L.*)* is the most comprehensively assessed medicinal plant. Periwinkle produces several commercially valuable secondary metabolites, including the anti-cancer vinblastine, vincristine, and the anti-hypertensive alkaloids ajmalicine and serpentine. However, the most important drawback of the traditional cropping system is the low productivity of alkaloids from *Catharanthus* species and the increasing production costs (Maqsood and Abdul 2017). Thus, the application of alternative strategies and methods, such as *in vitro* techniques, is thought to be effective to address such concerns. Direct *in vitro* propagation by adventitious meristems and organs is a versatile tool to produce large numbers of homogeneous plant materials within a short time (Pietrosiuk *et al*. 2007; Pérez-Alonso *et al*. 2011). Moreover, organ culture is a fascinating alternative in the biosynthesis of plant secondary metabolites. The main advantage of this technique is that organ culture produces genetically more stable materials as compared with thecultivation of suspension cells and calli (Barrales-Cureno *et al*. 2017; Amiri *et al*. 2019b).

Development of a suitable protocol for large

scale regeneration of periwinkle is an important issue in production of secondary metabolites. Optimization of medium composition and the type of *in vitro* propagation technique is the first challenge in this regards. Direct organogenesis was first explained in the late 1970s by Dhruva *et al*. (1977) followed by Ramawat *et al*. (1978) and Abou-Mandour *et al*. (1979). Woody plant medium (WPM) containing 5 μM BAP and 5 μM α-naphthalene acetic acid (NAA) has been proposed as an efficient medium for direct micro propagation of *Sunstorm rose*, a close species of periwinkle (Swanberg and Dai 2008). Induced somatic embryos from callus was derived from *C. roseus* in MS medium containing either 2.5 μM thidiazuron (TDZ) or 5.3 μM NAA and 2.2 μM BAP (Dhandapani *et al*. 2008). Kumar *et al*. (2013) evaluated the effects of cytokinin and auxin types on regeneration of *C. roseus* L. They proposed that MS medium containing 1.0 mg L^{-1} of BAP and 0.2 mg L^{-1} NAA is the best medium for the initiation of shoots from axillary buds and shoot proliferation. The highest rooting rate, maximum number of roots per shoot and the highest root length were observed on MS supplemented with 1.0 mg L^{-1} indol-3-butyric acid (IBA) and 0.25 % charcoal. According to Bakrudeen *et al.* (2011), the highest number of shoots was observed 45 days after culturing MS, fortified with 4.0 mg L^{-1} NAA and BAP.

Genetic markers have been progressively used to address diverse questions in agriculture. There are variety of molecular techniques accessible to study genetic diversity in plants populations, such as random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP) and random amplified microsatellite polymorphisms (RAMP) (Panahi and Ghorbanzadeh Neghab 2013; Mahmoudi *et al*. 2014; Ghorbanzadeh Neghab and Panahi 2017; Panahi *et al*. 2019). ISSR markers are easy to use, characterized with greater reproducibility and efficiency, highly polymorphic with high sensitivity to low levels of genetic variation, and also have lower cost (Kumar *et al.* 2016; Tiwari *et al*. 2017).

Fidelity and homogeneity of regenerated plants of periwinkle, off-types and genetically true-to type of mother plant for stable production of secondary metabolites is an issue for large scale production of this species. Kumar *et al*. (2013) assessed the genetic fidelity of micropropagated *C. roseus* and indicated that all the plants derived from tissue culture were true-to-type and there were no somaclonal variations among these plants. Considering the importance of an efficient protocol, optimized for the large scale micropropagation of *C. roseus*, along with genetic homogeneity of regenerated plants for stable secondary metabolite production, this investigation was conducted to evaluate the effect of different plant growth regulators on periwinkle in *in vitro* direct regeneration, and subsequently, to assess the genetic fidelity by ISSR markers.

Materials and Methods

Plant material and culture conditions

In this research, healthy axillary buds and nodal segments with 2-3 cm long of *C. roseus* were obtained from the greenhouse of Azarbaijan Shahid Madani University, Iran. These explants

were exposed to sterilization procedures (Kumar *et al*. 2013). Agar 0.8% and 3% (w/v) sucrose was supplemented to the MS (Murashige and Skoog 1962) basal medium with the pH adjusted to 5.8 ± 1. Different plant hormones, GA3, BAP (as cytokinin) and IBA (as auxin) (Sigma, MO, USA), were used at different concentrations (mg L^{-1}) to determine the optimum concentration suitable for each stage of tissue culture.

Culture establishment and shoot multiplication

For shoot initiation, explants were cultured on MS medium supplemented with different concentrations of BAP and gibberellic acid (GA3) (Figure 1). Explants of approximately 2-3 cm long were cultured on fresh MS supplemented with BAP (0, 0.5, 1 and 1.5 mg L^{-1}) and IBA (0, 0.25 and 0.5 mg L^{-1}). The establishment percentage, number of shoots per regenerating explant and shoot length (cm) were recorded after four weeks of explant culture.

Rooting and hardening

The regenerated shoots were transferred to the root induction medium containing 0, 0.25, 0.5 and 1.0 mg L^{-1} IBA. Four-weeks-old rooted plantlets were washed thoroughly and planted into a mixture of peat moss and perlite (1:1 ratio), then transferred to the greenhouse for gradual hardening.

Genetic fidelity analysis

CTAB (Cetyl Trimethyl Ammonium Bromide) method was used for DNA extraction of seven randomly selected samples of both regenerated and mother plants. The extracted DNA quantified and qualified by using NanoDrop spectrophotometer (NanoDrop 1000, Thermo Scientific, USA) and electrophoresis in a 0.7% (w/v) agarose gel, respectively. ISSR profiling was conducted by using five inter-simple sequence repeats primers (Table 1). The thermal cycler program was adjusted as previously described (Amiri *et al*. 2019a) The PCR products were separated and visualized on 1.2% agarose gel electrophoresis marked with 7% ethidium bromide and photographed.

Experimental design and statistical analyses

All experiments were conducted as factorial based on completely randomized design with three replications. SAS ver. 9.1 (SAS Institute, NC, USA) was used to analyze the data and the means were compared by Duncan's multiple range test at $p \leq 0.05$.

Results

Establishment of explants

Germination of axillary bud explants on MS medium was initiated after four weeks (Figure 1A). The MS medium with 1.0 mg L^{-1} BAP and GA3 was the best medium for establishment of the explants (approximately 80.67%). The lowest establishment percentage (25%) belonged to the MS medium without growth regulators (Figure 2).

Shoots proliferation and elongation

Shoot initiation was best attained on the fortified MS medium containing different concentrations of BAP and GA3 (Figure 1B). The initial bud break began on the 14th day and followed by the progress of the apical bud, which later differentiated into leaves. The response of bud explants to shoot

induction on MS basal medium was satisfactory. In the MS containing BAP and IBA, the highest shoot multiplication was observed for the combination of 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA, with 5.20 shoots per explant. In addition, the highest shoot length (6.30 cm) was achieved in this medium. By contrast, the lowest shoot formation (1.22 shoots per explant) and the shortest shoot length (1.0 cm) were obtained in the hormone-free MS (Figure 3).

Rooting induction and hardening

Results showed that half strength MS supplemented with 1.0 mg L^{-1} IBA produced plantlets with numerous roots. The highest rooting percentage (85.30%) was achieved in half strength MS supplemented with 1.0 mg L^{-1} IBA, and the roots were shorter and thicker than those in the control. By contrast, the lowest rooting percentage (30%) was obtained in the auxin-free medium (Figure 4). The plantlets grown on rooting medium during the rooting phase expanded healthy shoot and root systems after 28 days of culture (Figure 4), and then, transferred into garden pots and covered with polythene bags to retain high humidity (Figure 1D). This work is the first to report on humidity regulation to improve the survival rate of *C. roseus* for commercial purposes.

Analysis of genetic uniformity by ISSR

Seven randomly *in vitro* regenerated plants together with the mother plant were subjected to ISSR analysis. In total 42 clear bands were scored ranging 150 to 1000 bp in size (Table 1 and Figure 5). The number of products for each primer ranged from 5 to 10, with a mean of 8 bands per primer. The ISSR profiling confirmed the genetic stability of regenerated plants.

Discussion

The best shoot initiation was recorded on the MS medium fortified with different concentrations of BAP and GA3. The positive relation between BAP and GA3 in increasing the establishment percentage was indicated. It has been reported that BAP is an effective determinant of the shoot proliferation (Kyozuka 2007; Ruzic and Vujovic 2008; Tank and Thaker 2014).

The combination of BAP with IBA (1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA) well increased shoots per explant as compared to the sole application of BAP, because direct shoot induction efficiency from stem explants increased when the cytokinin/auxin ratio was more than one. Additionally, BAP and IBA significantly influenced the number of shoots per explant and shoot length (Figure 3). Similar findings have been reported in previous studies (Singh *et al*. 2011; Kumer *et al*. 2013; Bagum and Mathur 2014; Amiri *et al*. 2019a). Generally, cultures of micro shoots on MS medium for four weeks showed optimum elongation, rendering them suitable for introduction to the root induction medium.

Root induction is a complex process that is determined by a wide range of growth regulators. The participation of auxin in root development has been well documented by different researchers (Vidoz *et al*. 2010; Pop *et al*. 2011). It has been also proposed that root induction is normally affected by medium strength, medium type and auxin treatment duration (Li *et al*. 2017). The diverse

impacts of auxins are often related to their effects on cell division and expansion by ARF (Auxin Response Factor) proteins mediation (Parry and Estelle 2006; Quint *et al*. 2009).

In the present study, the excised micro shoots cultured in full and half MS without plant hormones did not show any root induction even after four weeks of culture; however, root

Figure 1. Different stages of micropropagation of *C. roseus*. A: establishment of explants; B: proliferation of explants; C: rooting of explants; D: final adaptation stage in the greenhouse.

Table 1. ISSR primer sequences and the number of amplified fragments used in this study.

Primer	Primer sequence (5'- 3')	Temperature (°C)	No. of Amplified fragments
ISSR-1	(CA)8RG*	52	10
ISSR-2	(AG)8T	52	5
ISSR-3	(GA)8T	52	6
ISSR-4	(CA)8A	55	11
ISSR-5	CCC (GT)7	52	10

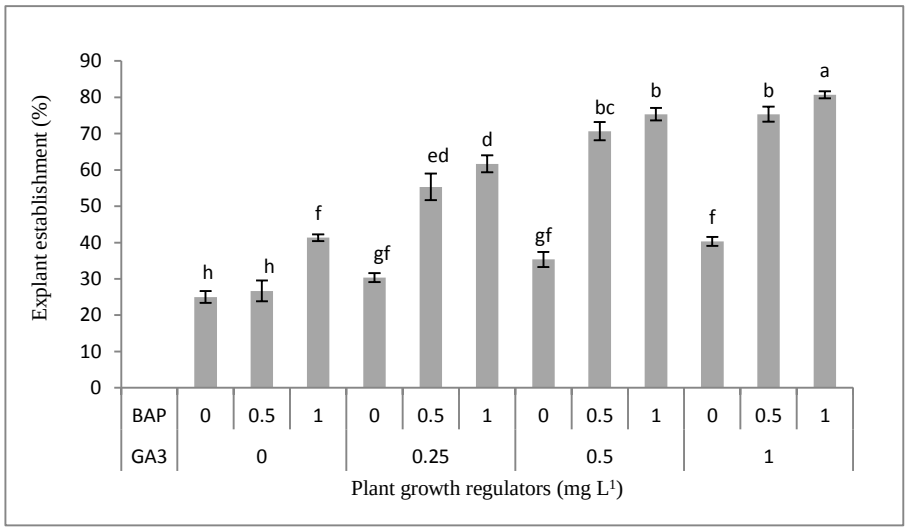

Figure 2. The effect of various concentrations of BAP and GA3 on the establishment of *C. roseus*

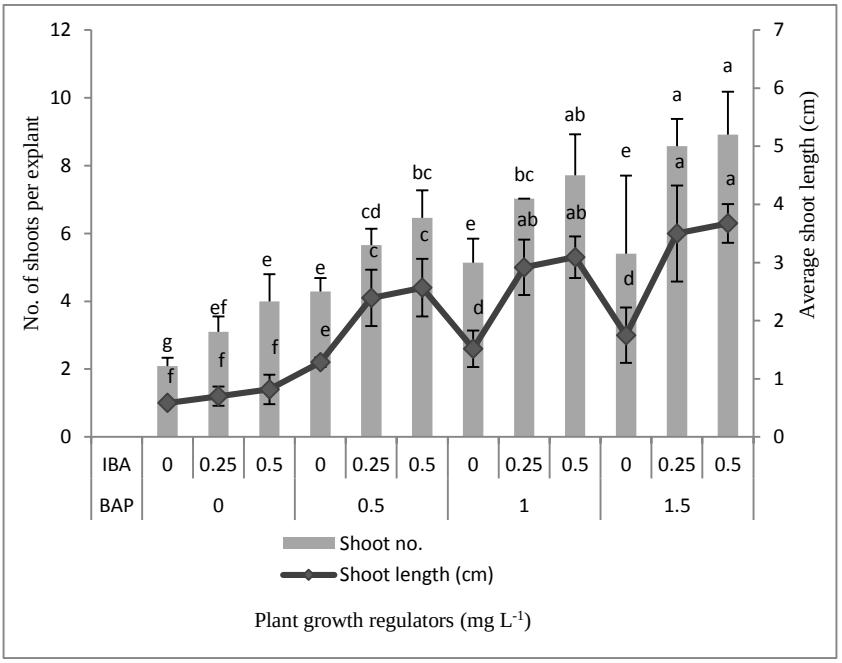

Figure 3. The effect of various concentrations of BAP and IBA on number of shoot per explant and shoot length of *C. roseus.*

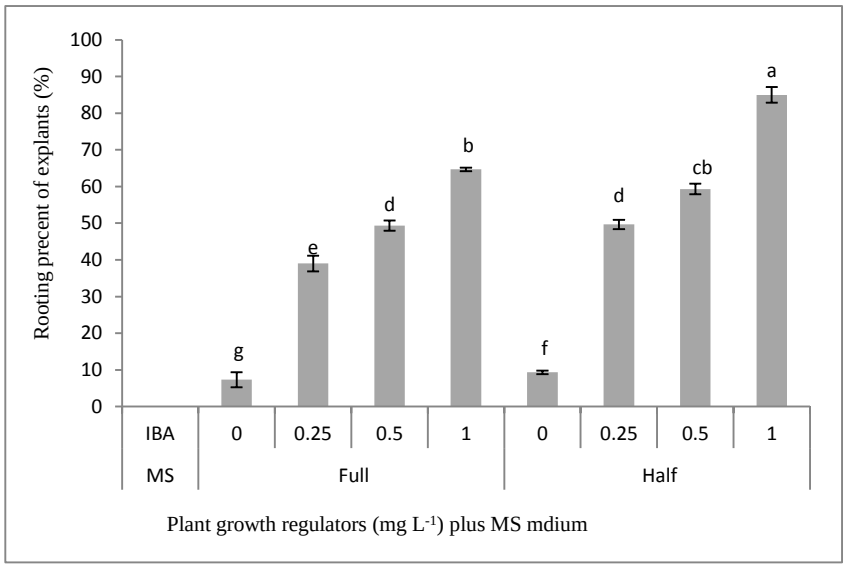

Figure 4. The effect of various concentrations of IBA and two types of MS medium (full and half) on rooting of *C. roseus.*

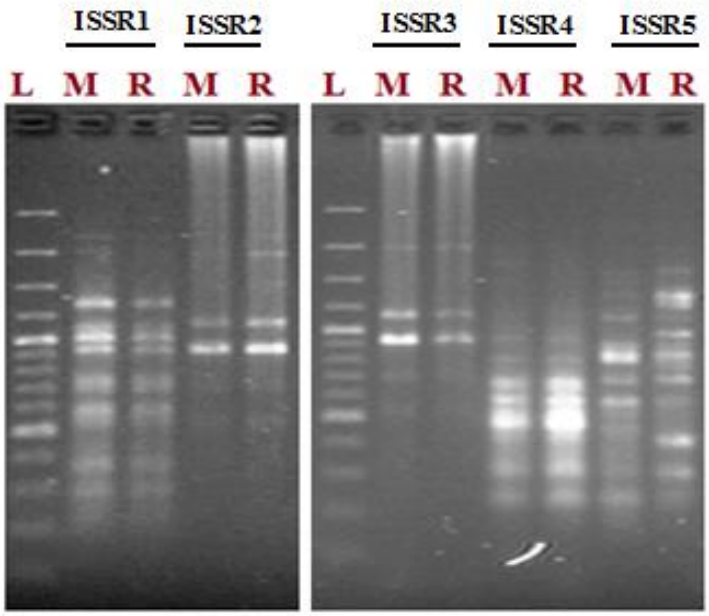

Figure 5. ISSR amplification pattern obtained for the randomly selected mother (M) and *in vitro* regenerated (R) plants produced by five ISSR primers. L: DNA marker (100 Kbp).

induction initiated in the MS medium containing different concentrations of auxin. Similar observations have been reported in other plant species (Pop *et al*. 2011; Bakrudeen *et al.* 2011; Kumer *et al*. 2013).

True-to-type clonal fidelity is considered as one of the important pre-requisites for the large scale micro-propagation of crop and medicinal plant species. The occurrence of cryptic genetic defects in the regenerates can be a major problem for the extensive utility of the micro-propagation system. Some molecular markers could be applied to evaluate the clonal fidelity of tissue cultured plants. However, a single marker analysis is unable to completely guarantee the clonal fidelity of the regenerated plants (Salvi *et al*. 2001). Many researchers have documented the ability of ISSR markers for true-to-type clonal fidelity analysis in relation to *in vitro* micropropagated plants (e.g. Werner *et al*. 2015; Khatun *et al*. 2018). The monomorphic banding pattern of micro-propagated plants and the mother plant confirmed the genetic homogeneity of the plants produced in *in vitro* conditions and indicated the reliability of in vitro propagation system used in this research. Monomorphic products were obtained by all tested primers between the regenerated and mother plants which highlighted the true-to type of the mother plant and possibly the constant production of secondary metabolites in the *in vitro* regenerated plants.

Conclusion

In general, *C. roseus* can be propagated from the nodal explant in MS medium, supplemented with 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA. Moreover, half strength MS medium supplemented with 1.0 mg L^{-1} IBA is possibly ideal for *in vitro* rooting.

The present experiment described a proficient micro-propagation protocol for the rapid and genetically homogeneous propagation of *C. roseus* species.

Acknowledgment

The financial supports of the Zanjan University and Azarbaijan Shahid Madani University are gratefully acknowledged.

References

Abou-Mandour AA, Fischer S and Czygan FC, 1979. Regeneration of intact plants from haploid and diploid callus cells of *Catharanthus roseus*. Zeitschrift für Pflanzenphysiologie 91(1): 83-88.

Amiri S, Fotovat R, Tarinejhad A, Panahi B and Mohammadi SA, 2019a. Optimization of hormonal combinations for in vitro regeneration of lesser periwinkle (*Vinca minor* L.) and assessment of genetic homogeneity. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences doi.org/10.1007/s40011-019-01141-6.

Amiri S, Panahi B, Mohammadi R and Fattahi F, 2019b. Effect of plant growth regulator combination on direct in vitro regeneration of Persian lilac (*Melia azedarach* L.). Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. doi: 10.1007/s40011-019-01099-5.

Bagum T and Mathur M, 2014. *In vitro* regeneration of *Catharanthus roseus* and *Bacopa monnieri* and their survey around Jaipur district. International Journal of Pure and Applied Bioscience 2(4): 210-221.

Bakrudeen AAA, Subha Shanthi G, Kavitha MS and Rao MV, 2011. In vitro micropropagation of *Catharanthus roseus* – an anticancer medicinal plant. Acta Botanica Hungarica 53(1-2): 197-209.

Barrales-Cureño HJ, Andrade-Hoyos P, Luna-Cruz A, Reyes-Reyes C, Chávez-Salinas S and López-Valdez LG, 2017. In Vitro Biotechnological Production and Pharmacological Studies of Antileukemic Alkaloids of *Catharanthus roseus*. In: Naeem M, Aftab T and Khan M (eds.) *Catharanthus roseus.* Pp. 17-34. Springer, Cham, Switzerland.

Dhandapani M, Kim DH and Hong SB, 2008. Efficient plant regeneration via somatic embryogenesis and organogenesis from the explants of *Catharanthus roseus.* In Vitro Cellular & Developmental Biology - Plant 44: 18-25.

Dhruva B, Ramakrishnan T and Vaidyanathan C, 1977. Studies in *Catharanthus roseus* callus cultures, callus initiation and differentiation. Current Science 46: 364-365.

Ghorbanzadeh Neghab M and Panahi B, 2017. Molecular characterization of Iranian black cumin (*Nigella sativa* L.) accessions using RAPD marker. BioTechnologia 98(2): 97-102.

Khatun MM, Tanny T, Yesmin S, Salimullah MD and Alam I, 2018. Evaluation of genetic fidelity of *in vitro*-propagated *Aloe vera* plants using DNA-based markers. ScienceAsia 44: 87-91.

Kumar A, Mishra P, Baskaran K, Shukla AK, Shasany AK and Sundaresan V, 2016. Higher efficiency of ISSR markers over plastid *psbA-trnH* region in resolving taxonomical status of genus *Ocimum* L. Ecology and Evolution 6(21): 7671-7682.

Kumar A, Prakash K, Sinha RK and Kumar N, 2013. In vitro plant propagation of *Catharanthus roseus* and assessment of genetic fidelity of micropropagated plants by RAPD marker assay. Applied Biochemistry and Biotechnology 169(3): 894-900.

Kyozuka J, 2007. Control of shoot and root meristem function by cytokinin. Current Opinion in Plant Biology 10(5): 442-446.

Li Sh, Huang P, Ding G, Zhou L, Tang P, Sun M, Zheng Y and Lin S, 2017. Optimization of hormone combinations for root growth and bud germination in Chinese fir (*Cunninghamia lanceolata*) clone leaf cuttings. Scientific Reports 7: 5046. doi:10.1038/s41598-017-05295-z.

Mahmoudi B, Panahi B, Mohammadi SA, Daliri M and Babayev MSh, 2014. Microsatellite based phylogeny and bottleneck studies of Iranian indigenous goat populations. Animal Biotechnology, 25(3): 210-222.

Maqsood M and Abdul M, 2017. Yeast extract elicitation increases vinblastine and vincristine yield in protoplast derived tissues and plantlets in *Catharanthus roseus*. Brazilian Journal of Pharmacognosy 27(5): 549-556.

Murashige T and Skoog F, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15(3): 473-497.

Panahi B and Ghorbanzadeh Neghab M, 2013. Genetic characterization of Iranian safflower (*Carthamus tinctorius*) using inters simple sequence repeats (ISSR) markers. Physiology and Molecular Biology of Plants 19(2): 239-243.

Panahi B, Mohammadi SA and Doulati-Baneh H, 2019. Characterization of Iranian grapevine cultivars using machine learning models. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences doi.org/10.1007/s40011-019-01131-8.

Parry G and Estelle M, 2006. Auxin receptors: a new role for F-box proteins. Current Opinion in Cell Biology 18(2): 152-156.

Pérez-Alonso N and Jiménez E, 2011. Produccion de metabolitos secundarios de plantas mediante el cultivo *in vitro*. Biotecnología Vegetal 11(4): 195-211.

Pietrosiuk A, Furmanowa M and Łata B, 2007. *Catharanthus roseus*: micropropagation and in vitro techniques. Phytochemistry Reviews 6: 459-473.

Pop TI, Pamfil DC and Bellini C, 2011. Auxin control in the formation of adventitious roots. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 39(1): 307-316.

Quint M, Barkawi LS, Fan KT, Cohen JD and Gray WM, 2009. Arabidopsis *IAR4* modulates auxin response by regulating auxin homeostasis. Plant Physiology 150(2): 748-758.

Ramawat KG, Bhansali RR and Arya HC, 1978. Shoot formation in *Catharanthus roseus* (L.) G. Don. callus cultures. Current Science 47: 93-94.

Ruzic DV and Vujovic T, 2008. The effects of cytokinin types and their concentration on *in vitro* multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). HortScience 35(1): 12-21.

Salvi ND, George L and Eapen S, 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant Cell, Tissue and Organ Cultre 66: 113-119.

Singh R, Kharb P and Rani K, 2011. Rapid micropropagation and callus induction of *Catharanthus roseus in vitro* using different explants. World Journal of Agricultural Sciences 76: 609-704.

Swanberg A and Dai W, 2008. Plant regeneration of periwinkle (*Catharanthus roseus*) via organogenesis. HortScience 43(3): 832-836.

Tiwari AK, Kumar G, Tiwari B, Kadam GB and Saha TN, 2017. Genetic diversity among turf grasses by ISSR markers. Indian Journal of Agricultural Sciences 87(2): 251-256.

Tank JG and Thaker VS, 2014. Systemic control of cell division and endoreduplication by NAA and BAP by modulating CDKs in root tip cells of *Allium cepa*. BioMed Research International doi.org/10.1155/2014/453707.

Vidoz ML, Loreti E, Mensuali A, Alpi A and Perata P, 2010. Hormonal interplay during adventitious root formation in flooded tomato plants. Plant Journal 63(4): 551-562.

Werner ET, Soares TCB, Gontijo ABPL, Souza Neto, JD and do Amaral JAT, 2015. Genetic stability of micropropagated plants of *Crambe abyssinica* Hochst using ISSR markers. Genetics and Molecular Research 14(4): 16450-16460.

باززایی درون شیشه‌ای گیاه پروانش (*Catharanthus roseus* L.) و بررسی پایداری ژنتیک گیاهچه‌های حاصل از کشت بافت با استفاده از نشانگرهای ISSR

صالح امیری[۱]، رضا فتوت[۱*]، علیرضا تاری نژاد[۲]، بهمن پناهی[۳] و سید ابولقاسم محمدی[۴]

۱- گروه مهندسی تولید و ژنتیک گیاهی، دانشکده کشاورزی، دانشگاه زنجان، زنجان.
۲- گروه بیوتکنولوژی گیاهی، دانشکده کشاورزی، دانشگاه شهید مدنی آذربایجان، تبریز.
۳- گروه ژنومیکس، پژوهشکده بیوتکنولوژی کشاورزی شمالغرب و غرب کشور، تبریز.
۴- گروه به نژادی و بیوتکنولوژی گیاهی، دانشکده کشاورزی، دانشگاه تبریز، تبریز.
*مسئول مکاتبه؛ Email: r_fotovat@znu.ac.ir

چکیده

پروانش با نام علمی *Catharanthus roseus* به عنوان یک گیاه دارویی مهم و پرکاربرد از نظر خواص دارویی می‌باشد. در این تحقیق، پس از بهینه‌سازی شاخه‌زایی و ریشه‌زایی گیاه پروانش، یکنواختی ژنتیکی گیاهان باززایی شده با استفاده از پنج جفت نشانگر ISSR مورد ارزیابی قرار گرفت. ریزنمونه‌ها پس از استریل روی محیط کشت موراشیک و اسکوگ (MS) حاوی غلظت‌های متفاوت از هورمون‌های گیاهی بنزیل آمینو پورین (BAP) و اسید ژیبرلیک (GA3) و ایندول بوتیریک اسید (IBA) کشت شدند. تجزیه داده‌ها نشان داد که بیشترین میزان استقرار (۸۰/۶۷ درصد) در یک میلی‌گرم در لیتر BAP و GA3 حاصل شد. بیشترین ضریب شاخه‌زایی (۵/۲۰ شاخه تولید شده بر حسب ریزنمونه) و میانگین طول شاخه (۶/۳۰ سانتی‌متر) در تیمار حاوی ۱/۵ میلی‌گرم در لیتر BAP + ۰/۵ میلی‌گرم در لیتر IBA حاصل شد. همچنین، بهترین پاسخ ریشه‌زایی(۸۰/۳۰ درصد) در محیط کشت ۱/۲MS حاوی یک میلی‌گرم در لیتر IBA به دست آمد. برای بررسی یکنواختی ژنتیکی گیاهان باززایی شده در مقایسه با گیاه مادری از نشانگر ISSR استفاده شد و نتایج نشان داد که ریزازدیادی گیاه پروانش از طریق نوک شاخه‌ها یک روش قابل اطمینان برای تولید مقادیر زیادی گیاهچه با میزان تولید یکنواخت از متابولیت‌های ثانویه می‌باشد.

واژه‌های کلیدی: جوانه جانبی؛ شاخه‌زایی؛ ضد سرطان؛ نشانگرهای ISSR؛ یکنواختی ژنتیکی.