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Organogenesis from seedling derived leaf explants of primrose (*Primula heterochroma* Stapf.) as a scarce Iranian plant

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Abstract

A simple regeneration method is reported for *Primula heterochroma,* a rare plant, endemic to the North of Iran. The seedling derived leaf explants were cultured on several Murashige and Skoog (MS) basal media enriched with different concentrations of 6-benzylaminopurine (BA) or Thidiazuron (TDZ) in combination with different concentrations of 1-naphthaleneacetic acid (NAA). The results showed that the highest frequency of shoot regeneration was 93% and 94% on the media containing 2 $mg.l^{-1}$ BA + 2 $mg.l^{-1}$ NAA and 2 $mg.l^{-1}$ TDZ + 1 $mg.l^{-1}$ NAA, respectively, and the largest number of shoots per leaf also were induced in above-mentioned concentrations. Adventitious shoots were regenerated directly from leaf explants in all responsive explants. Regenerated shoots easily rooted without the necessity for transferring to an additional rooting medium. A method described in this paper may offer the potential of being applied to other endangered *Primula* spp.

Keywords: Endemic plant; *In vitro* culture; Plant growth regulators; Shoot regeneration; Thidiazuron.
Abbreviations: BA-6-benzylaminopurine; GA_3-Gibberellic acid; MS-Murashige and Skoog (1962) medium; NAA-1-naphthaleneacetic acid; PGR-Plant growth regulator; TDZ-Thidiazuron.

Introduction

The genus *Primula* L. is the most important one in the Primulaceae family. Considering the latest evaluations, it includes 500 species, mainly located in the temperate and cold regions of the northern hemisphere and in the tropical mountains (Hao et al., 2002). In the present study, we worked on the species *Primula heterochroma*, a native plant known in northern Iran (Parsakhoo et al., 2009; Poorbabaei and Poorrahmati, 2009). This plant, which produces white and purple flowers, is a cute ornamental specimen. Some of its natural habitats are endangered as a result of intensive grazing, and also sever winters, cause failure to the achieving of its maturity. Hence, the artificial propagation protocol is necessary to rescue the rare primrose and maintain the germplasm. There are many techniques available for the conservation of plant genetic resources of rare and endangered species. These include micropropagation, *in vitro* seed germination, direct and indirect regeneration, embryo rescue, micrografting and cryopreservation (González-Benito and Pérez, 1994; Chang et al., 2000). In the majority of *Primula* species, including *P. heterochroma*, there are well-known difficulties with seed germination under both greenhouse and field conditions (Coumans et al., 1979; Morozowska and Wesolowska, 2004). Direct and indirect adventitious shoot regeneration from leaf explants of some *Primula* species has been explained, previously. Shimada et al. (1997) reported that regeneration of adventitious shoots and somatic embryos from leaf explants of *P. cuneifolia var. hakusanensis* was stimulated by TDZ or zeatin. Yamamoto et al. (1999) obtained regenerated plants from young expanding leaves of *P. sieboldii* on media containing BA and NAA. Plant regeneration from leaf calli of *P. vulgaris* and *P. elatior* was stimulated by a high level of TDZ (Schween and Schwenkel, 2002 and 2003). Takihira et al. (2007) described the effect of different cytokinins (TDZ, BA and zeatin) on adventitious shoot regeneration from leaf explants of *Primula* × *pubescens* Jacq. However, there is no reliable regeneration protocol that could be applied to all major primrose cultivars.The direct shoot bud formation from appropriate explants is of great success for large-scale clonal multiplication of desired clone in commercial floriculture (Rout et al., 2006). Herein, we demonstrate the effect of different plant growth regulators (PGRs) on adventitious shoot regeneration from leaf explants of *P. heterochroma*, not described so far in any available literature.

Materials and methods

Plant material

The seeds for *in vitro* culture were collected from a western region of Guilan province, Rasht, Iran. Moreover, to avoid major damage to the populations themselves only a relatively small amount of seeds per plant were collected.

In vitro seed germination

Seeds of *P. heterochroma* were kept in refrigerator (5 ^{o}C) until use. Seeds were surface sterilized by immersion in 70 % (V/V) ethanol for 30 sec followed by immersion in 2 % NaOCl solution containing two drops of Tween-20 per 100 ml, for 20 min, and then rinsed three times with sterilized distilled water. For seed germination, surface sterilized seeds

Table 1. Effect of different concentrations of PGRs on number of regenerated roots.

PGRs (mg.l^{-1})	NAA			
BA	0	0.2	1	2
0.2	0^{f}	1^{ef}	10^{a}	3^{cd}
1	0^{f}	0^{f}	2de	4^{bc}
2	0^{f}	0^{f}	5^{b}	4^{bc}
TDZ	0	0.2	1	2
0.2	0^{e}	0^{e}	3^{d}	7^{b}
1	0^{e}	0^{e}	2^{d}	0^{e}
2	0^{e}	0^{e}	10^{a}	5^{c}

Mean in each column followed by same letters are not significantly different at 1% level using Tukey's test.

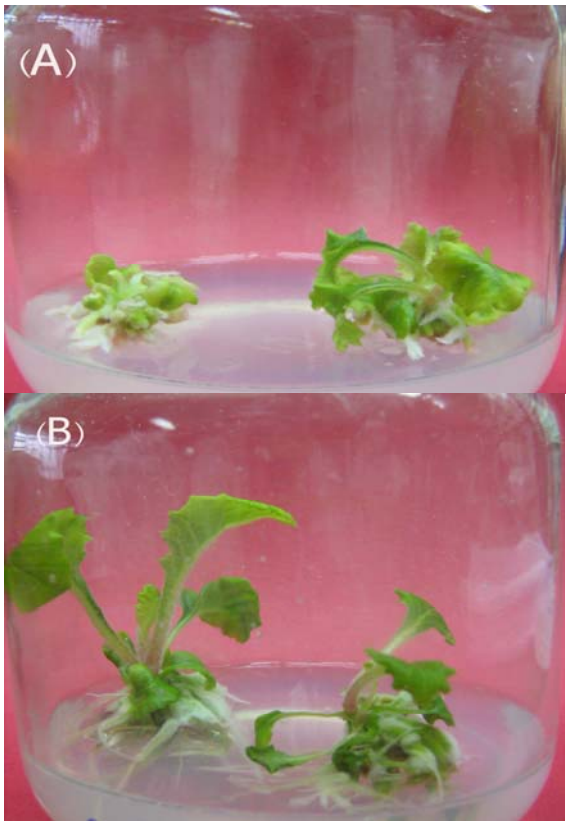

Fig 1. Plant regeneration from *in vitro* cultured leaves of *P. heterochroma*. (a) Shoot regeneration from leaves, after 27 days of cultivation on induction medium. (b) Shoot and root growth on leaves explants, after 45 days of cultivation on induction medium.

were incubated into sterile Petri dishes (9 Cm in diameter and 1.5 Cm in height) containing PGRs-free medium or medium supplemented with 1.5 mg.l^{-1} gibberellic acid (GA3). All media used in the present study consisted of full strength MS salts, MS vitamins, 100 mg.l^{-1} myoinositol and 3 % sucrose. The pH was adjusted to 5.7 and 7 g.l^{-1} agar (Duchefa, the Netherlands) were added prior to autoclaving at 121 ^{o}C for 20 min. The cultures were maintained in a growth chamber at 25 ± 2 ^{o}C, 70-80 % relative humidity and 16:8 photoperiod under cool white fluorescent light (32 μmol m^{-2} s^{-1}).

Leaf segments culture

The uniform and unicolour leaves were taken from 1-month-old *in vitro* seedlings. The pedicle and the apical parts of the leaves were removed. The remained parts of the leaves were divided into segments which were about 15 mm long. The leaf segments were wounded by three transverse cuts across the midrib and placed firmly on the medium surface with the adaxial side touching the medium. The media for culturing of the leaf explants were dispensed in 40 ml aliquots into (70 × 90 mm) glass jar after autoclaving.Experiments were as (I) Three levels of BA (0.2, 1 and 2 mg.l^{-1}) × four levels of NAA (0, 0.2, 1 and 2 mg.l^{-1}), (II) Three levels of TDZ (0.2, 1 and 2 mg.l^{-1}) × four levels of NAA (0, 0.2, 1 and 2 mg.l^{-1}). Cultures were observed at weekly intervals for up to 6 weeks. The frequency of shoot regeneration (percentage of responding explants), the number of regenerated shoots and roots were recorded at 45 days after culture initiation. Experiments were carried out in two factorial designs on the basis of completely randomized design in three replications.

Statistical analysis

For statistical analysis of data, the SAS computer package (SAS 1985) was used and graphs were plotted using Microsoft Excel. Mean comparisons to identify significant differences among treatments were performed using Tukey's test (P<0.01).

Results and discussion

In vitro seedlings

Seeds from different natural habitats had no differences in germination capacity (data not shown). Result showed that seeds did not germinate within 70 days on PGR-free medium whereas, they were germinated (17%) after 49 days on medium enriched with 1.5 mg.l^{-1} GA_3. Morozowska and Wesolowska (2004) demonstrated that the best seed germination of *Primula veris* achieved when GA_3 and kinetin were applied together. Morozowska (2002) examined the influence of different GA_3 concentrations on germination of *P. veris* seeds and found that 0.9 and 1.8 × 10^{-3} M GA_3 promoted breaking of seed dormancy under laboratory conditions. The percentage of germinated seeds increased by 64.2-97.1% depending on the GA_3 concentrations.

Shoot regeneration

After 15 days of incubation, most leaf explants of *P. heterochroma* showed elongation and enlargement. Regenerated shoots appeared within 27 days of culture initiation and increased during 45 days (Fig 1). Shoot regeneration occurred mainly from the cuts across the midrib and the base of the leaf explants. The apical part of leaf did not show any regeneration and in most cases it became necrotic.All media induced shoot regeneration in responding explants via direct organogenesis. The primordia regenerated directly on leaf explants, without callus formation. The effects of TDZ, BA and NAA concentrations were highly significant on shoot formation. The number of shoots per leaf explant and leaf regeneration frequency showed significant differences (P<0.01) in all treatments. The interaction between cytokinins (BA and TDZ) with NAA concentrations for the number of shoots per leaf explant and leaf regeneration frequency was also significant (P<0.01). The optimum concentration of PGRs for adventitious shoot

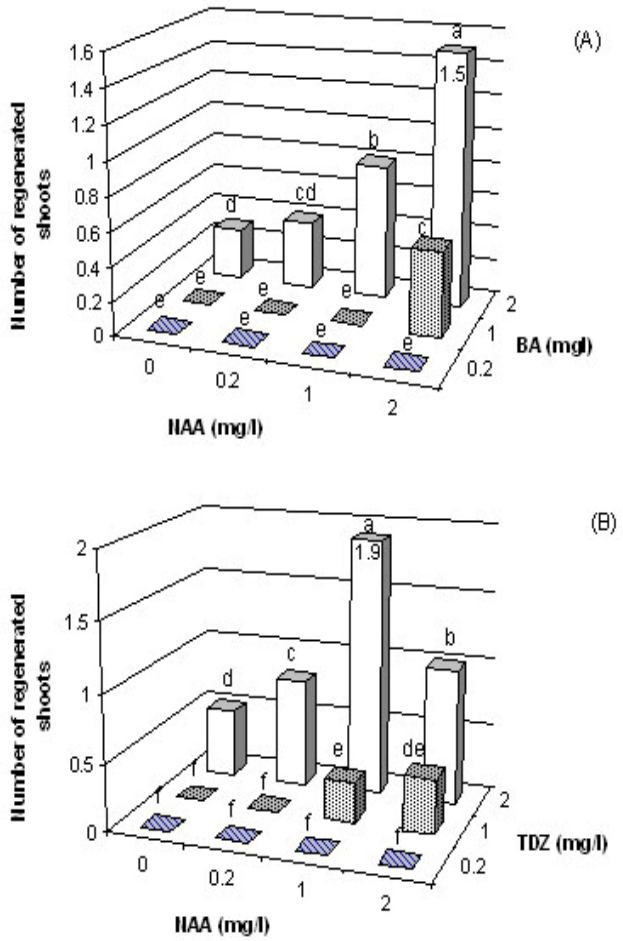

Fig 2. Effect of concentrations of (a) BA and NAA, and (b) TDZ and NAA on shoot regeneration from leaf explants of *P. heterochroma.* Mean in each column followed by same letters are not significantly different at 1% level using Tukey's test

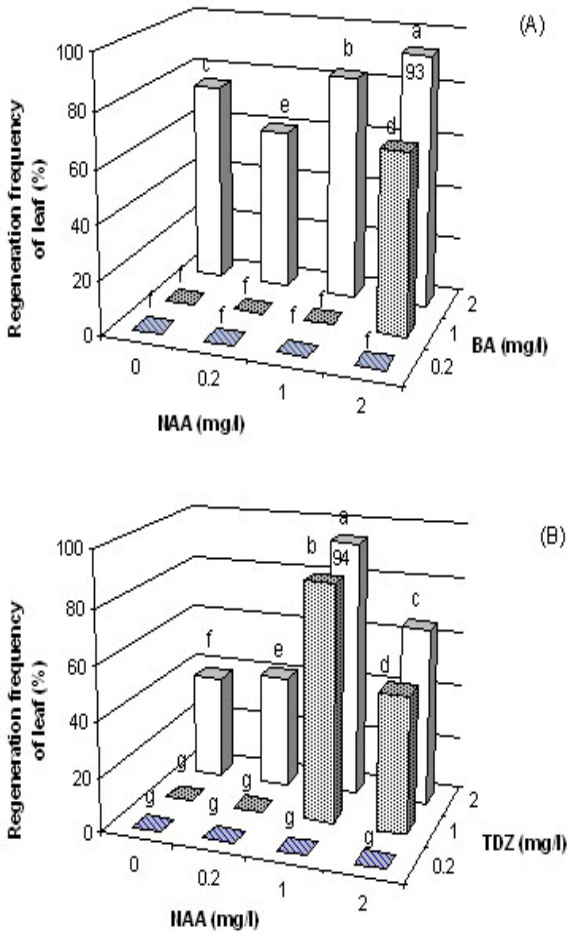

Fig 3. Effect of concentrations of (a) BA and NAA, and (b) TDZ and NAA on frequency of regeneration from leaf explants of *P. heterochroma.* Mean in each column followed by same letters are not significantly different at 1% level using Tukey's test

regeneration varied among treatments. The most suitable medium for shoot regeneration of *P. heterochroma*, in the first (I) experiment, was the MS medium supplemented with 2 mg.l^{-1} BA + 2 mg.l^{-1} NAA, on which an average of 1.5 new shoots were obtained from each explants (Fig 2a). In the second (II) experiment, the highest shoot regeneration (1.9 shoots) was obtained on MS medium supplemented with 2 mg.l^{-1} TDZ + 1 mg.l^{-1} NAA (Fig 2b). The regeneration frequency ranged from 0 % to 93 % in the first experiment (Fig 3a) and from 0 % to 94 % in the second experiment (Fig 3b). Treatments giving higher regeneration frequency also tended to produce more shoots per leaf explant.An auxin/cytokinin combination was also a prerequisite for the efficient leaf regeneration in many other species like *Gentiana* (Hosokawa et al. 1996), *Paulownia* spp. (Dimps Rao et al. 1996; Lo et al. 1997) and *Gypsophila* (Zucker et al. 1997). Mamidala and Nanna (2009) and Jafari Najaf-Abadi and Hamidoghli (2009) have shown that use of PGRs, particularly cytokinin in culture medium is the most important factors for shoot proliferation tomato and *Rubus*, respectively. In the presence of cytokinins alone (2 mg.l^{-1} BA or 2 mg.l^{-1} TDZ), the means number of regenerated shoots per leaf explants were 0.3 and 0.5, respectively. Coumans et al. (1979) obtained vegetative shoot production on fragments of *P. obconica* L. The same authors found that flower buds were produced at higher BA concentrations (1 to10 mg.l^{-1}). They observed that when the BA concentration was low (1 mg.l^{-1}), vegetative buds were produced instead of flower buds. They also observed that on MS medium with low BA (1 mg.l^{-1}) and NAA (0.1 mg.l^{-1}), numerous vegetative buds were produced on inflorescence tips. Borodulina et al. (2001) obtained the highest multiplication coefficient on B5 medium (Gamborg, 1968) enriched with 2.25 mg.l^{-1} BA, 0.01 mg.l^{-1} NAA and 1.7 mg.l^{-1} GA_3. The presence of TDZ has been shown to have a powerful ability to stimulate shoot induction on many woody plants (Lane et al., 1998) and ornamental species (Lin et al., 1997). The effect of TDZ on adventitious shoot regeneration from cultured cells and tissues have already reported in some *Primula* species, *P. cuneifolia* var. *hakusanensis* (Shimada et al., 1997), *P. vulgaris* and *P. elatior* (Schween and Schwenkel, 2002 and 2003) and *Primula* × *pubescens* (Takihira et al., 2007). The best concentration of TDZ (2 mg.l^{-1}) reported by Schween and Schwenkel (2002) is similar to the present study.

Adventitious root regeneration

In the course of our study, adventitious root regeneration by direct organogenesis was easily achieved in all responsive explants in compare with shoot regeneration. These results

indicated that root regeneration was significantly ($P<0.01$) affected by concentrations of PGRs. In the first experiment, the maximum root regeneration observed on medium supplemented with 0.2 $mg.l^{-1}$ BA + 1 $mg.l^{-1}$ NAA and in the second experiment, on medium supplemented with 2 $mg.l^{-1}$ TDZ + 1 $mg.l^{-1}$ NAA (Table 1). In the present study, number of regenerated roots was enhanced with the increasing of NAA concentrations. Borodulina et al. (2001) and Mizuhiro et al. (2001a) reported on the rooting of several *Primula* species on half-MS medium without growth regulators. According to Coumans et al. (1979), Borodulina et al. (2001), Schween and Schwenkel (2002) and Takihira et al. (2007), the use of auxins (IBA or NAA) promote normal rooting of different *Primula* species, and this is supported by our results for *P. heterochroma*.At the end of this experiment, several rooted plantlets were transplanted to pots containing perlite, sand and soil (1:1:1) mixture and acclimatized under high humidity conditions at room temperature. Overall, this paper reports an efficient and simple methodological procedure for organogenesis from leaves of *P. heterochroma,* a rare plant endemic to Northern Iran. In this experiment, the shoots always obtained directly from leaves without callus phase, and all media induced shoot regeneration in responsive explants via direct organogenesis. This result was in agreement with Takihira et al. (2007) who reported similar results for the auricula (*Primula* × *pubescens* Jacq). In spite of difficulties in seed germination, we established different types of culture of *P. heterochroma*, and a successful organogenesis procedure has been set up. We developed a protocol for adventitious shoot regeneration from leaf explants of *P. heterochroma*. The protocol developed in the present study may be useful for micropropagation of other endangered *Primula* spp.

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References

Benson EE, Danaher JE, Pimbley IM, Anderson CT, Wake JE, Daley S, Adams LK (2000) *In vitro* micropropagation of *Primula scotica*: a rare Scottish plant. Biodivers Conserv 9: 711–726

Borodulina ID, Vechernina NA, Dolganova ZV (2001) *In vitro* propagation of two hybrid *Primula* L. species. Rastitel'nye Resursy 4: 114–122

Chang C, Chen CT, Tsai YC, Chang WC (2000) A tissue culture for protocol for propagation of a rare plant, *Lilium speciosum* Thunb. var. *gloriosoides* Baker Bot Bull Acad Sin 41: 139-142

Coumans M, Coumans-Gillès MF, Delhez J, Gaspar T (1979) Mass propagation of *Primula obconica*. Acta Hortic 91: 287–293

Dimps Rao C, Chong-Jin G, Prakash P, Kumar (1996) High frequency adventitious shoot regeneration from exiced leaves of *Paulownia* spp. cultured *in vitro*. Plant Cell Rep 16: 204–209

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspensions cultures of soybean root cells. Exp Cell Res 50: 151–158

González-Benito ME, Pérez C (1994) Studies on the cryopreservation of nodal explants of *Centarium rigualii* Esteve, an endemic threatened species through vitrification. Botanic Gardens Micropropagation News 1: 82–84

Hao G, Hu CM, Lee NS (2002) Circumscriptions and phylogenetic relationships of *Primula* Sects. *Auganthus* and *Ranunculoides*: evidence from nrDNA ITS sequences. Acta Bot Sin 44: 72–75

Hosokawa K, Nakano M, Oikawa Y, Yamamura S (1996) Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of Gentiana. Plant Cell Rep 15: 578–581

Jafari Najaf-Abadi A, Hamidoghli Y (2009) Micropropagation of thornless trailing blackberry (*Rubus* sp.) by axillary bud explants. Aust J Crop Sci 3 (4): 191-104.

Lane DW, Iketani H, Hayashi, T (1998) Shoot regeneration from cultured leaves of Japanese pear (*Pyrus pyrifolia*). Plant Cell Tiss Organ Cult 54: 9–14

Lin SH, De Jeu MJ, Jacobsen E (1997) Direct shoot regeneration from excised leaf explants of *in vitro* grown seedlings of *Alstroemeria* L. Plant Cell Rep 16: 770–774

Lo KH, Giles KL, Sawhney (1997) Acquisition of competence for shoot regeneration in leaf discs of *Saintpaulia ionantha* × *confusa* hybrids (African violet) cultured *in vitro*. Plant Cell Rep 16: 416–420

Mamidala P, Nanna RS (2009) Efficient *in vitro* plant regeneration, flowering and fruiting of dwarf Tomato cv. Micro-Msk. Plant Omics Journal 2 (3): 98-102.

Mizuhiro M, Yamada K, Ito K, Kadowaki S, Ohashi H, Mii M (2001a) Plant regeneration from cell suspension-derived protoplasts of *Primula malacoides* and *Primula obconica*. Plant Sci 160: 1221–1228

Morozowska M (2002) Some factors affecting viability, dormancy and germination of *Primula veris* (L.) seeds. Folia Hort 14/1: 243–253.

Morozowska M, Wesołowska M (2004) *In vitro* clonal propagation of *Primula veris* L. and preliminary phytochemical analysis. Acta Biol Cracov Bot 46: 169–175

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay of tobacco tissue cultures. Physiol Plant 15:473–497

Parsakhoo A, Hosseini SA. Pourmajidian MR (2009) Plants canopy coverage at the edge of main communications network in Hyrcanian forests. J Ecol Nat Environ Vol 1(2), pp 037-044

Poorbabaei H, Poorrahmati G (2009) Plant species diversity in loblolly pine (*Pinus taeda* L.) and sugi (*Cryptomeria japonica* D. Don.) plantations in the western Guilan, Iran. Int J Biodvers Conserv Vol 1(2), pp 038-044

Rout GR, Mohapatra A, Mohan Jain S (2006) Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. Biotechnology Adv 24: 531–560

S.A.S. (1985) Statistical Analysis System User's Guide: Statistics. S.A.S. Inc., Cary, North Carolina

Schween G, Schwenkel HG (2002) *In vitro* regeneration in *Primula* ssp. via organogenesis. Plant Cell Rep 20: 1006–1010

Schween G, Schwenkel HG (2003) Effect of genotypes on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in the greenhouse of *Primula* ssp. Plant Cell Tiss Organ Cult 72: 53–61

Shimada T, Matsushita T, Otani M (1997) Plant regeneration from leaf explants of *Primula cuneifolia* var. hakusanensis, "Hakusankozakura". Plant Biotech 14: 47–50

Takihira M, Otani M, Tsuchiya S, Shimada T (2007) Plant regeneration from leaf explants of auricula cultivars (*Primula* × *pubescens* Jacq.). Plant Biotech 24: 425–427

Yamamoto T, Magaya Y, Maruyama Y (1999) Mass propagation of *Primula sieborldii* E. Morr. Through leaf segment culture. Bull Minami-Kyusyu Univ 29 (A): 9–14

Zucker A, Ahroni A, Shejtman H, Vainstein A (1997) Adventitious shoot regeneration from leaf explants of *Gipsophila paniculata* L. Plant Cell Rep 16: 775–778