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In vitro regeneration and secondary metabolites of *Viola caspia* subsp. sylvestrioides Marcussen

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Abstract

Viola caspia subsp. *sylvestrioides*, a small important medicinal herb, belongs to the subsection *Rostratae* (sec. *Viola*) of genus *Viola* L. This study was performed to develop an efficient protocol for *in vitro* regeneration of *V. caspia* subsp. sylvestrioides as an *ex situ* technique for conservation of endangered plants and extraction of secondary metabolites. Leaf and petiole explants were cultured on half and full-strength Murashige and Skoog (MS) media supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP), isopentenyl adenine (2ip), thidiazuron (TDZ), and naphthalene acetic acid (NAA). Direct organogenesis was induced on half and full-strength MS media supplemented with TDZ (0.7 to 3.5 mg/l) from leaf and petiole explants. Indirect shoot organogenesis was induced on half-strength MS supplemented with 3.5 mg/l 2ip and 2.5 mg/l NAA followed by transferring the obtained callus onto a half-strength MS containing 0.5 mg/l BAP. The highest frequency of shoot organogenesis (100 and 86.66%) was noted for petiole and leaf explants, respectively, on half-strength MS medium supplemented with 2.8 mg/l TDZ (approx. 7.66 and 4.33 shoots per petiole and leaf explant, respectively). The induced shoots were used for root induction on a half-strength MS medium with 0.5 mg/l indole-3-butyric acid (IBA). Phytochemical constituents in leaf tincture were determined by gas chromatography/mass spectrometry (GC/MS) analysis. GC/MS results revealed that vitamin E was the main component in the tincture of *Viola* leaves. The present study provides a simple and rapid protocol for *in vitro* regeneration of *V. caspia* subsp. sylvestrioides plants which can be used for gene transfer and conservation purposes, and in pharmaceutical studies.

Key words: GC/MS analysis, organogenesis, thidiazuron, *Viola*, vitamin E

Introduction

Viola caspia (Rupr.) Freyn. is a perennial plant that requires cool, moist, shady habitats with humus-rich soil for growth. The species is octoploid with chromosome number $2n = 8x = 40$ (Marcussen and Borgen, 2011a). Plants develop insect-pollinated chasmogamous flowers which are replaced by seasonal cleistogamic flowers later in the season. Both flower types develop capsules fulfilled with seeds (Marcussen and Borgen, 2011a).

Based on the petal color, *Viola caspia* (Rupr.) Freyn is divided in two subspecies: *V. caspia* subsp. *sylvestrioides* Marcussen and *V. caspia* subsp. *caspia*. The *sylvestrioides* petal color is lavender blue (Fig. 1A) and it grows 1700–1800 m above the sea level in Ardebil, in the north-west of Iran (Yousefi et al., 2012). *V. caspia* subsp. *caspia* has whitish corollas and grows 300–400 m above the sea level in Lahijan, Guilan, in the north of Iran (Yousefi et al., 2012; Saeidi Mehrvarz et al., 2013).

Two taxa often grow in the same habitats in mixed populations, but there is no evidence of gene flow between these two subspecies. Intraspecific variation in *V. caspia,* based on allozyme analysis, showed that two corolla color morphs differ considerably in their allozyme multilocus profiles (Marcussen and Borgen, 2011a).

V. caspia subsp. *sylvestrioides* belongs to subsection *Rostratae* Kupffer W. Becker, of the genus *Viola* (*Viola*-

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ceae), that is commonly known as "Banafshe" in Persian and is an important small, ornamental, perennial medicinal herb restricted to northeastern Crimea, Turkey, Caucasus and the Caspian coast (Marcussen et al., 2011b). The whole plant is medicinally useful. The herbal tea of this plant is an expectorant, and the oil can be used for treating sinusitis, migraine, and headache (Zargari, 1997).

The *Violaceae* family includes species biosynthesizing cyclotides, flavonoids, alkaloids, saponins, tannins, coumarins, and salicylates. In traditional medicine viola is used to treat cough, asthma, and catarrhal and pulmonary problems (Pullaiah, 2006; Vohora, 1986). Metabolites of *Viola* have a wide range of biological activities and pharmacological applications, such as anti-cancer (Gerlach et al., 2010), anti-inflammatory (Koocheck et al., 2003), anti-viral (Hallock et al., 2000), antibacterial (Gruber et al., 2007), and antioxidant properties (Stojkovic et al., 2011), together with antifouling activities (Göransson et al., 2004), cytotoxic effects (Lindholm et al., 2002), and anti-bronchitis activity (Karnick, 1996).

These medicinal properties make *Viola* matebolites potential candidates for pharmaceutical and biotechnological studies. In recent years, there has been much progress in the understanding of the molecular regulation of the biosynthesis of secondary metabolites (Staniek et al., 2013). However, the lack of effective protocols for *in vitro* regeneration and genetic transformation of plants has limited the efforts of its regulation. The low seed germination percentage and a long dormancy period of viola limit its propagation by seeds. Moreover, in recent times *V. caspia* subsp. *sylvestrioides* has been found to be threatened in its habitats in Iran due to severe grazing and its excessive use (by people) in traditional medicine. Hence callus culture, plant regeneration, and micropropagation techniques could be considered as alternative methods for its conservation. Also, one of the benefits of *in vitro* regeneration is that both, the regenerated plants and the callus, can be used as a source of important bioactive metabolites. Therefore, an efficient protocol is necessary for successful tissue culture and plant regeneration of *Viola*. In recent years there have been some effective reports on plant regeneration of the *Viola* species (Chalageri and Babu, 2012; Kaloo et al., 2013; Vishwakarma et al., 2013; Slazak et al., 2015; Żabicki et al., 2019), but there is no report on tissue culture and plant regeneration of *V. caspia* subsp. *sylvestrioides*. In this study we aimed at obtaining an efficient shoot regeneration protocol of *V. caspia* subsp. *sylvestrioides* under *in vitro* conditions using leaf and petiole explants and assessing the secondary metabolites in leaf extracts by gas chromatography/mass spectrometry (GC/MS) analysis.

Materials and methods

Plant material and seed germination

Seeds of *V. caspia* subsp. *sylvestrioides* were collected from Almas road, Ardebil, Iran (the plants were identified by Dr. Shahryar Saeidi Mehrvarz, associate professor of botany, University of Guilan, Rasht, Iran). They were subsequently scarificated with 95% sulfuric acid for 8 min for breaking the dormancy, and after that, were sterilized with 10% sodium hypochlorite solution containing one drop of Tween-20 for 8 min and rinsed four times in sterile distilled water. For germination, the seeds were planted in glass bottles containing 30 ml of quarter-strength MS medium supplemented with 3% sucrose and 0.7% agar at a pH of 5.6–5.8 and were incubated with 16 h light photoperiod (35 $\mu mol\ m^{-2} s^{-1}$) inside a growth chamber at a temperature of 24–25 °C.

In vitro tissue culture and shoot organogenesis

The explants, including petiole segments (1 cm) and leaf blades (1 × 1 cm), were obtained from two-month old seedlings. For regeneration, two separate experiments were carried out. The first experiment was carried out to evaluate the effects of benzylaminopurine (BAP), isopentenyl adenine (2ip) (cytokinins), and, NAA (auxin) on callus induction and regeneration. Leaf and petiole explants were placed horizontally with the adaxial surface toward the media for callus and/or shoot induction. For this purpose, a half-strength MS medium was supplemented with BAP or 2ip at 2, 2.5, and 3 mg/l in combination with NAA (2, 2.5, and 3 mg/l). The rate of callus induction on explants was evaluated in the primary culture. Then the explants producing callus were subcultured in half-strength MS medium containing 0.5 mg/l BAP (for shoot induction) and after two successive passages, the induced shoots were cultivated on quarter-strength MS medium.

In the second experiment, petiole and leaf explants were prepared as described above. Explants were cultured in half and full-strength MS media solidified with

0.7% agar and supplemented with TDZ at concentrations of 0, 0.7, 1.4, 2.1, 2.8, or 3.5 mg/l. In both experiments, two parameters were evaluated: the frequency of regeneration and the number of shoots per explant.

Root induction and acclimatization

Four-week-old shoots (approx. 3 cm long) were transferred individually into a half-strength MS medium containing 0.5 mg/l IBA. *In vitro*-rooted shoots were taken out from the culture bottles and washed using sterile warm water to remove the solid media attached to the roots. The plantlets were transplanted into pots containing a mixture of soil, peat moss, and sand (4 : 3 : 3 w/w/w) autoclaved at 121 °C for 25 min. Individual potted plants were kept covered with transparent polyethylene bags, to maintain a relatively high humidity level during the initial stages of pre-hardening, at 3000 Lux and 16 h photoperiod at 24–25 °C in a growth chamber for two weeks. Then, the transparent polyethylene bags were gradually opened and removed between 14–18 days after the hardening was complete. After two months, at the beginning of spring, these plantlets were transferred to field conditions.

GC/MS analysis of V. caspia subsp. sylvestrioides tincture

For the GC/MS analysis, the plant leaves were thoroughly washed to remove dust particles and then air dried at room temperature. Immediately after drying, 2.5 g of leaves were powdered using an electric mixer-grinder and sieved through a BSS mesh (no. 85 sieve). The sieved powder was extracted with 50 ml ethanol. The tincture was filtered and kept at 4 °C before injecting into GC.

The tincture was analyzed by a GC/MS (Agilent 6890 GC), equipped with a Rtx-5 capillary column of 30 m length × 0.25 mm inner diameter (ID) and 0.25 μm stationary phase film thickness, with an Agilent 5973 mass selective detector. The column temperature range was 40 °C (holding time for 1 min) to 240 °C (holding time 5 min) varied at 3 °C min^{-1}. Helium was used as the carrier gas. For GC/MS detection, an electrospray ionization (ESI) system with an ionization energy of 70 eV was used. The injector temperature was 250 °C and injection volume was 1 μl. The components were identified by comparing their relative retention times with those of authentic samples on the HP-5 MS capillary column and also by comparing their mass spectra with those in the GC/MS library and literature (Adams, 2007).

Experimental design and statistical analysis

A factorial experiment, based on a completely randomized design with three replicates and 10 explants in each replicate, was used. The effect of different treatments was compared by Duncan's multiple range tests with a confidence level of $P \leq 0.01$. Statistical analysis and means comparison were done using the IBM SPSS Statistics 22.0 software.

Results

Tissue culture and plant regeneration

First experiment

In the preliminary experiments, *Viola* plants were growing well when their seeds were cultured on half, instead of a full-strength, MS medium. Therefore, for further experiments, all explants were cultivated on half-strength MS medium. Callus induction occurred along the edges of explants (petioles and leaves) within 3 weeks of culture on all the tested media (Fig. 2A, Fig. B). The use of 2ip in comparison with BAP resulted in a high percentage of callus-producing explants (Table 1). In the medium containing 2ip and NAA, the frequency of callus induction in the leaf explants was higher than in petiole explants (93.33 ± 5.77^{a} and 80.00 ± 10.00^{b}) respectively, for leaf and petiole explants in a medium containing 3mg/l 2ip and NAA). However, in the medium containing BAP and NAA, it was higher in the petiole explants (63.33 ± 5.77^{ab} and $46.66 \pm 5.77^{a,}$ respectively, for petiole and leaf explants in a medium containing 2.5 mg/l 2ip and 3 mg/l NAA). Shoot organogenesis was observed only on the medium containing 3 mg/l 2ip and 2.5 mg NAA after 2 months of culture (Fig. 2C). In this medium, the frequency of shoot organogenesis for leaf and petiole explants was 60% and 50%, respectively. Also, the frequency of shoot induction was 3.2 shoots per leaf and 2.7 shoots per petiole explant. Overall, with respect to shoot organogenesis and the frequency of shoot induction per explant, leaf explants are more productive than petiole explants. When explants were not cultured on a medium containing plant growth regulators, neither callus nor shoots were induced.

Fig. 1. A) *Viola caspia* subsp. *sylvestrioides* in nature, B) *in vitro* germinated seedlings from seeds

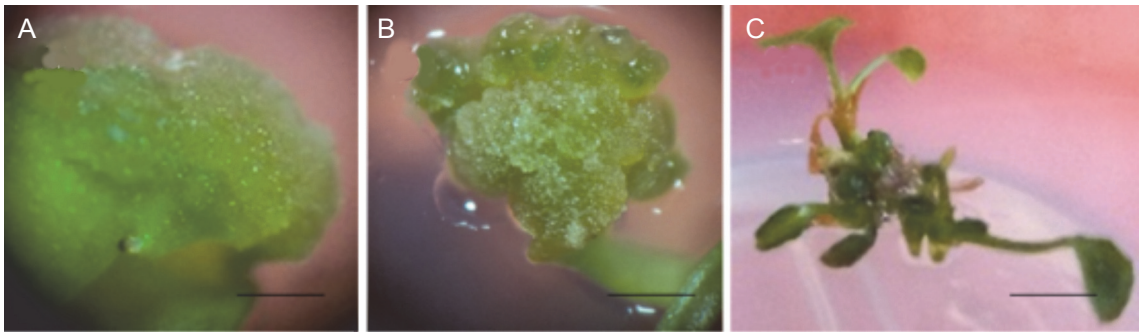

Fig. 2. Callogenesis and regeneration of *V. caspia* subsp. *sylvestrioides via* leaf and petiole explants on Murashige and Skoog (MS) medium containing isopentenyl adenine and naphthalene acetic acid; A) induction of callus from leaf, B) petiole, C) shoot regeneration from petiole explants; bars (a = 0.8 mm, b =1 mm, c = 2 mm)

Second experiment

During the second part of this study, shoots were developed directly from petiole and leaf explants (Fig. 3) on half and full-strength MS media, supplemented with TDZ alone in different concentrations, for a duration of 4 weeks. The half-strength MS medium supplemented with TDZ was more effective in promoting shoot development than the full-strength MS medium supplemented with the same concentration of TDZ. For leaf explants, there was no significant difference in shoot induction between the tested media with either full-or half-strength MS medium except for 2.8 mg/l (approximately 66.6% and 86.6%, respectively in full-and half-strength MS) and 2.1 mg/l (approximately 43.3% and 50%, respectively in full and half-strength MS). For petiole explants, there was a significant difference in shoot induction due to TDZ concentrations in full-strength MS medium (Fig. 4). The highest frequency of shoot organogenesis, 100% and 86.66%, was observed in petiole and leaf explants, respectively, on the half-strength MS medium supplemented with 2.8 mg/l TDZ, but this was not significantly different from shoots derived from petioles with 2.1 mg/l TDZ (Fig. 4A). With higher TDZ concentrations (3.5 mg/l), low frequencies of shoot organogenesis were observed in leaf and petiole explants (as compared to other media). For leaf explants, frequencies of shoot organogenesis were approximately 46.6% and 36.6%, respectively on full and half-strength MS media; however for petiole explants they were 36.6% and 50%, respectively, in full and half-strength MS media (Fig. 4A).

Frequency of shoot induction per explant

The number of shoots per leaf explant increased with TDZ concentration ranging from 0.7 to 2.8 mg/l, with either half-or a full-strength MS medium. The highest number of shoots (7.66 shoots) was obtained in leaf explants on the medium containing half-strength MS with 2.8 mg/l TDZ (Fig. 4B). For petiole explants, the highest number of shoots (4.33 shoots) was obtained on the medium containing half-strength MS with 2.8 mg/l TDZ (Fig. 4B). The induced shoots rooted on half-strength MS medium containing 0.5 mg/l IBA (Fig. 3F). All plants regenerated *in vitro* were acclimatized after two months

Table 1. Effect of isopentenyl adenine (2ip), benzylaminopurine (BAP), and naphthalene acetic acid (NAA) on the frequency of callus induction from petiole and leaf explants of *V. caspia* subsp. *sylvestrioides* on half-strength Murashige and Skoog (MS) medium

Medium	2ip [mg/l]	NAA [mg/l]	Rate of callus formation [%]	
			leaf	petiole
1	2.00	2.00	16.66 ± 5.77^{f}	13.33 ± 5.77^{d}
2	2.00	2.50	36.66 ± 5.77^{d}	23.33 ± 5.77^{cd}
3	2.00	3.00	53.33 ± 5.77^{c}	43.33 ± 5.77^{b}
4	2.50	2.00	23.33 ± 5.77^{ef}	16.66 ± 5.77^{d}
5	2.50	2.50	43.33 ± 5.77^{cd}	26.66 ± 5.77^{cd}
6	2.50	3.00	80.00 ± 10.00^{b}	73.33 ± 15.27^{a}
7	3.00	2.00	33.33 ± 5.77^{de}	20.00 ± 0.00^{cd}
8	3.00	2.50	53.33 ± 5.77^{c}	33.33 ± 5.77^{bc}
9	3.00	3.00	93.33 ± 5.77^{a}	80.00 ± 10.00^{a}
MS*	0.00	0.00	00.00 ± 0.00	00.00 ± 0.00
	BA [mg/l]	NAA [mg/l]	leaf	petiole
1	2.00	2.00	0.00 ± 0.00^{d}	10.00 ± 10.00^{f}
2	2.00	2.50	16.66 ± 5.77^{c}	46.66 ± 5.77^{d}
3	2.00	3.00	36.66 ± 5.77^{b}	56.33 ± 5.77^{abc}
4	2.50	2.00	3.33 ± 5.77^{d}	23.33 ± 5.77^{e}
5	2.50	2.50	23.33 ± 5.77^{c}	50.00 ± 0.00^{d}
6	2.50	3.00	46.66 ± 5.77^{a}	63.33 ± 5.77^{ab}
7	3.00	2.00	6.66 ± 5.77^{d}	33.33 ± 5.77^{e}
8	3.00	2.50	33.33 ± 5.77^{b}	53.33 ± 5.77^{cd}
9	3.00	3.00	53.33 ± 5.77^{a}	66.66 ± 5.77^{a}
MS*	0.00	0.00	00.00 ± 0.00	00.00 ± 0.00

Data are from three independent experiments; * the medium was used as a control

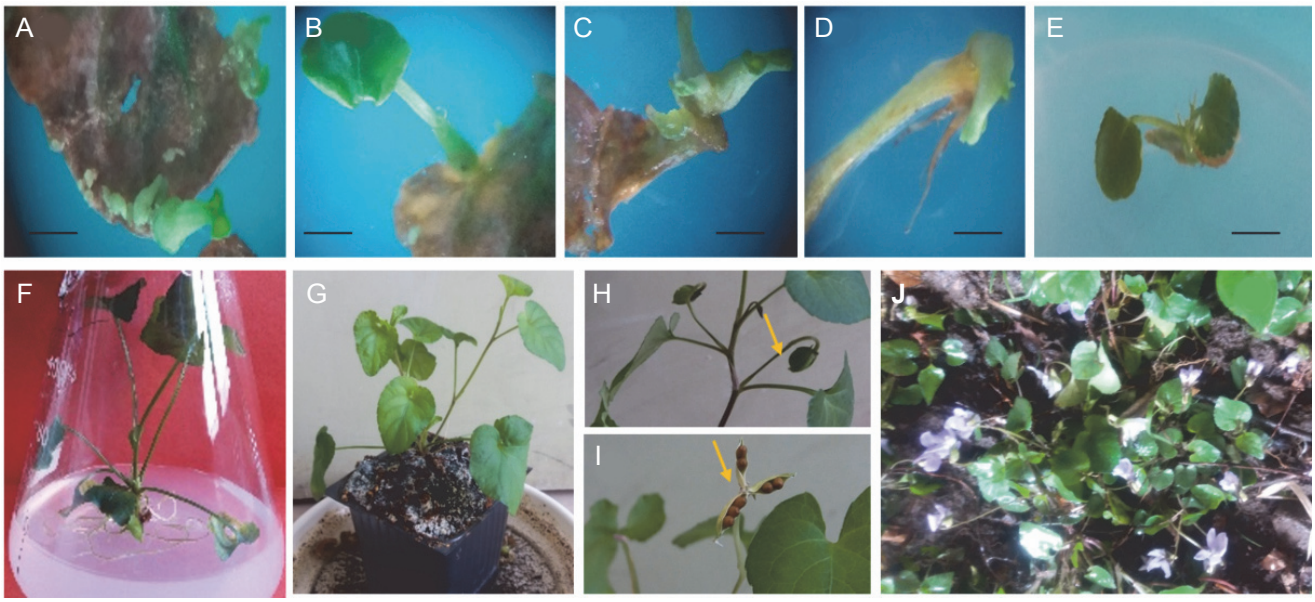

Fig. 3. Direct organogenesis of *V. caspia* subsp. *sylvestrioides*; A) and B) direct shoot formation from leaf lamina explants and C) and D) petiole explants; E) shoot formation from leaf explant after 3 weeks; F) rooted shoot after 2 weeks of culture on half-strength MS medium supplemented with 0.5 mg/l indole-3-butyric acid; G) acclimatized *in vitro* plant after 1 month of culture in the greenhouse; H) regenerated plantlets with capsules developed from cleistogamous flowers; I) opened, tripartite capsule, visible seeds; J) plants acclimatized to the field conditions after 1 year; bars (a = 1 mm, b = 2 mm, c = 1 mm, d = 2 mm, e = 5 mm)

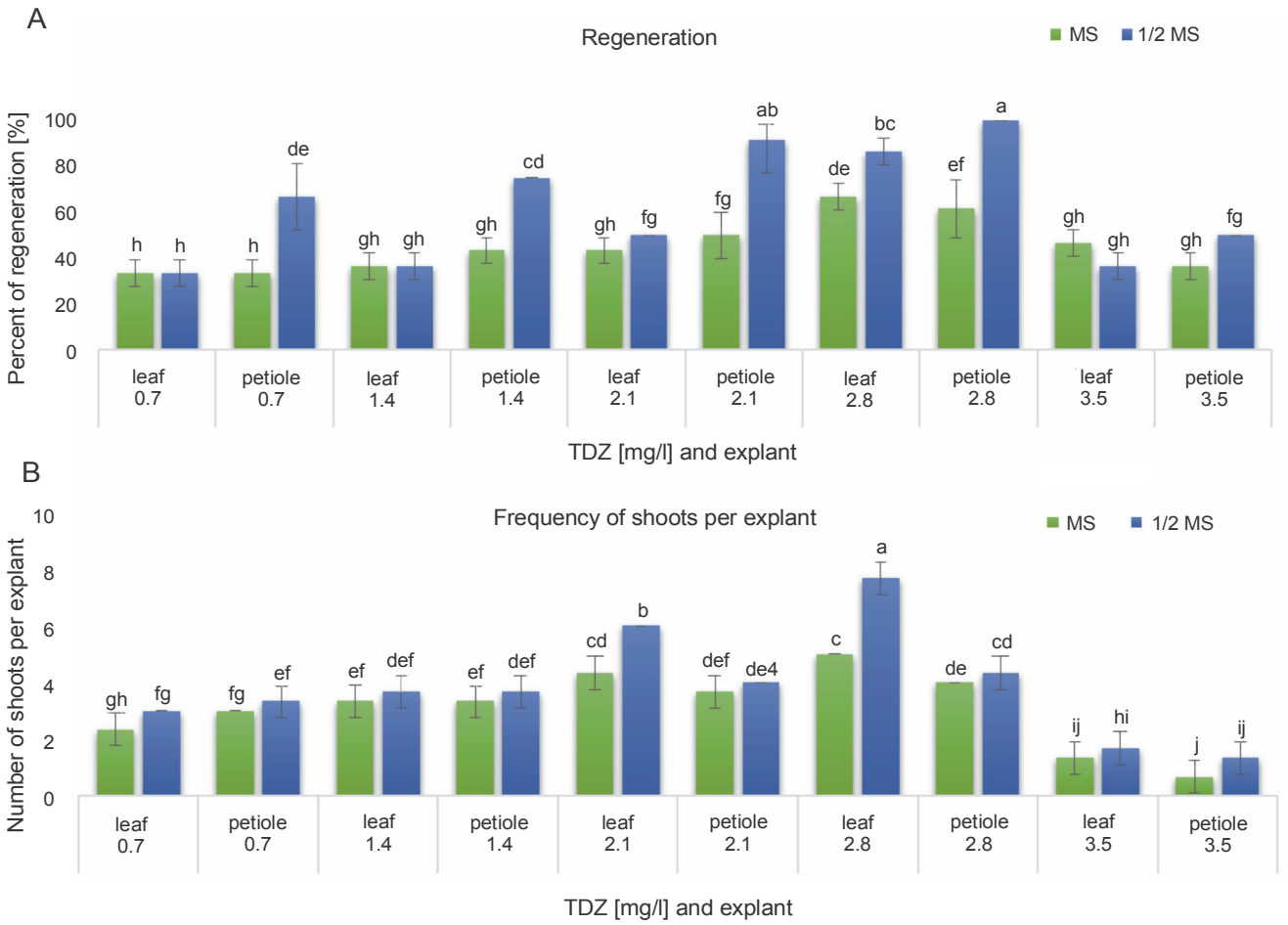

Fig. 4. Effect of thidiazuron concentration, explant type and MS strength on shoot organogenesis and the frequency of shoot induction per leaf and petiole explant of *V. caspia* subsp. *sylvestrioides*. A) shoot organogenesis frequency; B) frequency of shoot induction per explant

Table 2. Chemical composition and percentage of compounds in leaf tincture of *V. caspia* subsp. *sylvestrioides*

Peak	Compounds	Retention time [min]	Percent of total [%]
1	Ethyl iso-allocholate	30.135	1.289
2	1,2-benzenedicarboxylic acid, dinonyl ester	31.347	24.19
3	Vitamin E	35.028	35.257
4	Phthalic acid, bis(7-methyloctyl) ester	42.406	17.232
5	2,4,6-decatrienoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,7,9-trimethyl-1-	42.850	16.402
6	Docosanoic acid, 1,2,3-propanetriyl ester	43.851	3.116
7	D-homo-24-nor-17-oxachola-1,20,22-triene-3,16-dione,7-(acetyloxy)-14,15:21,23-diepoxy-4,4,8-trimethyl-,	44.084	2.514

in the greenhouse (Fig. 3G). In the greenhouse and under field conditions, they produced chasmogamous and clestogamous flowers and capsules filled with seeds (Fig. 3I, Fig. 3J).

GC/MS analysis of the leaf tinctures

The GC/MS analysis of the leaf tinctures resulted in the identification of 7 different compounds (Table 2). The identified constituents of the tincture are listed in

Table 2 and are presented in order of their retention times. The main compounds found were vitamin E (35.257%) and 1,2-benzenedicarboxylic acid, dinonyl ester (24.19%), while ethyl iso-allocholate and D-homo-24-nor-17-oxachola-1,20,22-triene-3,16-dione,7-(acetyloxy)-14,15:21,23-diepoxy-4,4,8-trimethyl-, were found in small quantities (1.289%, and 2. 514%, respectively).

Discussion

In recent years the use of different cytokinins including BAP, kinetin (KIN) and TDZ has been reported for the regeneration and micropropagation of *Viola* plants (Slazak et al., 2015, Żabicki et al., 2019). Our results show that petiole and leaf explants regenerated with 2ip at 3 mg/l and NAA at 2.5 mg/l (Fig. 2C). The results (also) revealed that a higher concentration of 2ip (3 mg/l) was more effective in callus proliferation and the regeneration of explants. Similar observations have also been made by Chaudhuri et al. (2004) in *Tylophora indica*.

TDZ, a diphenyl urea derivative, is involved in the biosynthesis of auxin with increasing levels of indole-3-acetic acid (IAA) and its precursor (tryptophan) (Murthy et al., 1998). In many plant species, TDZ appeared as a potent bioregulant in cell and tissue cultures (Yancheva et al., 2003; Matand and Prakash, 2007). Depending on the genotype of plants and the type of explants, TDZ induces different regeneration responses (Passey et al., 2003). TDZ, with a high cytokinin-like activity (Huetteman and Preece, 1993), is widely used to promote shoot proliferation in many plant species, including the members of *Violaceae* (Sato et al., 1995; Slazak et al., 2015; Żabicki et al., 2019). The study by Slazak et al. (2015) revealed that TDZ had the potential to induce direct and indirect organogenesis in leaf and petiole explants of *Viola uliginosa* Besser (Slazak et al., 2015). The authors also reported that organogenesis was induced in leaf and petiole fragments with a combination of KIN and 2,4-D, followed by a callus transfer on TDZ (Slazak et al., 2015). In the current study, the different concentrations of TDZ, the type of explants and the strength of the MS medium proved to be important factors affecting the frequency of shoot regeneration. Petiole explants and half-strength MS medium (2.8 mg/l) were more productive in efficient shoot organogenesis in *Viola caspia* subsp. *sylvestrioides*, in agreement with previously reported results on *Viola wittrockiana* (Sato et al., 1995), where it was shown that multiple shoot organogenesis could be achieved from petiole callus using a half-strength MS medium supplemented with BAP in combination with NAA. The number of regenerated shoots formed on leaf explants was greater than the number of shoots obtained on petiole explants (approx. 7.6 and 4.3 shoots per explant, respectively, for leaf and petiole explants on a medium containing 2.8 mg/l TDZ), which was consistent with the results obtained by Slazak et al. (2015).

Over the past decades, efforts have been made to extract secondary metabolites of medicinal plants, elucidate their structure and evaluate their biological activity. When natural resources are limited or a chemical synthesis is impossible, plant cell tissue culture techniques appear to be eco-friendly, alternative methods for the production of secondary metabolites. Also, in many instances it is difficult to cultivate plant species with traditional methods, or it takes a long time, frequently, several years (Greger, 2017). The excessive exploitation of medicinal plants from wild populations in their natural habitats for medical and industrial purposes and insufficient cultivation fields have led to the reduction of biodiversity and depletion of natural populations of these plants. Therefore, various sets of biotechnical approaches, such as *in situ* and *ex situ* conservation methods (Huang, 2011; Liu et al., 2011), should be applied to improve the yields of medicinal plants.

Herb preparation and extraction methods are the main steps in bioactivity testing and chemical identification of biologically active ingredients of medicinal herbs. In traditional medicine, *Viola* tinctures are used for the treatment of colds and bronchitis. Recently Hellinger et al. (2014) reported that the tincture of *Viola tricolor* inhibited the proliferation of activated lymphocytes. Also, it negatively affected other hyper-responsive immune functions, like the effector function, by reducing IFN-γ and producing TNF-α (Hellinger et al., 2014). GC/MS results of *V. caspia* subsp. *sylvestrioides* tincture showed that it contained a high amount of biologically active compounds such as vitamin E and 1,2-benzenedicarboxylic acid and dinonyl ester. Vitamin E is one of the lipid-soluble components in the antioxidant defense system of cells. It carries out numerous, significant biological activities in the body due to its role in the preven-

tion of oxidative stress (Burton et al., 1983), protection of cell membranes (Howard et al., 2011), regulation of platelet aggregation and protein kinase c activation (Li et al., 2001), and prevention of diseases such as cardiovascular (McAnally et al., 2007) and Alzheimer's diseases (Mangialasche et al., 2013), and cancer (Wells et al., 2010). The presence of high amounts of vitamin E in the *V. caspia* subsp. *sylvestrioides* tinctures indicates that viola species may be are useful in the therapy of most disorders. This regeneration protocol can produce large populations of these plants which will be useful for the extraction of these pharmaceutical compounds.

Conclusion

This study has developed an efficient, effective, and reproducible protocol for direct and indirect shoot organogenesis of *V. caspia* subsp. *sylvestrioides*. In addition, the tincture obtained from *V. caspia* subsp. *sylvestrioides* leaves contains high amounts of biologically active compounds such as vitamin E. To conclude, the present study can contribute to the mass propagation of *V. caspia* subsp. *sylvestrioides* for *ex situ* conservation purposes, gene transformation goals, production of large amounts of pharmaceutical compounds and commercial cultivation. The acclimatized *in vitro*-obtained plants can also be used as alternatives to wild populations of *V. caspia* subsp. *sylvestrioides*.

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