Advancement in protocol for in vitro seed germination, plant regeneration and cryopreservation of *Viola cornuta*

Milena Trajković¹ · Dragana Antonić¹ · Aleksandar Cingel¹ · Nabil Ghalawenji¹ · Angelina Subotić¹ · Slađana Jevremović¹

Received: 25 July 2018 / Accepted: 16 December 2018 / Published online: 2 January 2019
© King Abdulaziz City for Science and Technology 2019

Abstract

The aim of this study was to develop a fast, reliable and true-to-type protocol for in vitro plant regeneration and long-term storage of horned pansy (*Viola cornuta* L.). Seed germination over 60% was recorded after 12 weeks of growth at 10 °C or 4 °C. Calli formation and shoot induction were obtained in petiole and hypocotyl culture on half-strength MS mineral salts with full concentration of Na–FeEDTA and vitamins (½MS medium) with 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg/L) and 6-benzylaminopurine (BAP, 2.0 mg/L) and leaf culture on ½MS medium with thidiazuron (TDZ, 1.0 mg/L). The highest frequency of adventitious shoot induction (50%) with six shoots/explant was achieved in hypocotyl culture from top hypocotyl segments, close to epicotyl which was grown 8 weeks at 16 h light/8 h dark photoperiod. Subsequent shoot multiplication was achieved on ½MS medium with α -naphthaleneacetic acid (NAA, 0.1 or 0.5 mg/L) and BAP (1.0 mg/L). Rooting of shoots was obtained on ½MS medium with low concentration (0.1 mg/L) of auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or NAA, or without growth regulators. In vitro-derived plantlets were acclimatized under greenhouse conditions. All plants developed normally, bloomed and set seeds. Shoot tips were cryopreserved successfully using modified plant vitrification 3 (PVS3-based vitrification procedure). Cold acclimation for 2 weeks significantly improved shoot regrowth (64%) after thawing in comparison to non-acclimated shoots (39%). Clonal fidelity of regenerated plantlets at ploidy level was confirmed by chromosome counting. The presented protocol can be useful for mass propagation, genetic transformation studies and long-term storage of valuable *Viola* spp.

Keywords Horned pansy · Seed germination · Micropropagation · Hypocotyl culture · Cryopreservation

Introduction

Viola cornuta, also known as horned pansy, is a valuable perennial ornamental plant belonging to the Violaceae family. Horned pansy grows naturally in the high Pyrenees in Spain and France. There are more than 25 cultivars and hybrids that have been created by interspecific hybridization between *V. cornuta* with *V. wittrockiana* with flower colours ranging from yellow to violet. Along with their ornamental value, some cultivars represent an excellent model system to study the regulation of flower development and natural flower colour changes (Farzad et al. 2002, 2003, 2005).

These facts make *V. cornuta* a good candidate for investigating the possibilities for producing new cultivars using classical and modern biotechnology methods. In general, modern molecular approaches, such as genetic engineering, polyploidization or interspecific hybridization, provide great potential for the development of new traits such as novel flower colours that cannot be achieved with classical breeding (Chandler and Tanaka 2007; Chandler and Brugliera 2011; Jeknić et al. 2014). Most of the strategies for molecular breeding of ornamentals, especially *Agrobacterium*-mediated gene delivery, are performed under in vitro conditions and development of an efficient and true-to-type system for regenerating the entire plant is a prerequisite for these studies (Zhao et al. 2000; Karimi et al. 2009).

To date, several studies have been available about in vitro plant regeneration of ornamental or medicinally important *Viola* species such as: *V. baoshanensis* (Li et al. 2011), *V. tricolor* (Babber and Sharma 1991), *V. odorata* (Wijowska

✉ Slađana Jevremović
sladja@ibiss.bg.ac.rs

¹ Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”, University of Belgrade, Bulevar Despota Stefana 142, Belgrade 11060, Serbia

et al. 1999; Naeem et al. 2013), *V. patrini* (Sato et al. 1995; Chalageri and Babu 2012), *V. pilosa* (Soni and Kaur 2014), *V. serpens* (Vishwakarma et al. 2013), *V. uliginosa* (Slazak et al. 2015) and *V. wittrockiana* (Wang and Bao 2007). Different types of initial explants have been used for the establishment of callus cultures of *Viola* spp., including unfertilized ovules (Wijowska et al. 1999), seedling explants (Wang and Bao 2007; Li et al. 2011), or explants obtained ex vitro (Chalageri and Babu 2012; Naeem et al. 2013) or in vitro grown plants (Antonić et al. 2017). In the culture of seedling explants of *V. wittrockiana*, the presence of auxins in culture media is necessary for callus induction, and subsequent changes of several culture media are essential for shoot development. Most of the published protocols for shoot induction of *Viola* sp. require several changes of culture media (Wang and Bao 2007) and/or very long cultivation time for shoot induction (Slazak et al. 2015).

An in vitro system allows large-scale and continuous production of uniform plant material under control conditions as well as their conservation. This system is labour intensive, susceptible to contamination and errors with a high risk of possible loss of plant material. Therefore, the development of in vitro conservation methodologies is of great importance for short- or long-term maintenance of valuable plant material. Cryopreservation represents one of the main strategies for in vitro conservation, since plant material is stored in liquid nitrogen (LN) for a prolonged period of time requiring minimum space and low maintenance. In contrast to the continuous in vitro cultivation, the cryopreservation of plant germplasm material minimizes the occurrences of genetic or somaclonal variations (Kulus and Zalewska 2014). Recently, several cryopreservation techniques have been developed, which include direct immersion in LN after removal of intracellular water from plant tissue by physical evaporation or by exposure of the tissues to highly concentrated cryoprotective substances (vitrification). In vitrification-based cryopreservation protocols almost all freezable intracellular water is removed by dehydration to avoid unfavourable ice crystallization in cells. Many vitrification solutions have been developed in the last years and the most commonly used are glycerol-based plant vitrification solutions (PVS2 and PVS3, Sakai and Engelman 2007). The vitrification solution contains a high concentration of sucrose that, together with the other compounds, causes the desiccation of tissues prior to freezing, preventing the formation of ice crystals. There are not many reports about cryopreservation of *Viola* spp. Recently, Soni and Kaur (2014) published the results of their study on cryopreservation of medicinally important *V. pilosa* by PVS2-based vitrification technique that resulted in about 42% of successful shoots recovery after retrieval from LN.

The present study was undertaken with the goal of developing an efficient and reliable protocol for in vitro plant regeneration and long-term storage of germplasm material of

V. cornuta L. We evaluated the factors affecting in vitro seed germination, induction of plant regeneration using different seedling explants as primary explants, shoot multiplication, rooting and long-term storage of in vitro-grown shoots and evaluated the clonal fidelity of regenerated plants at ploidy level.

Materials and methods

Plant material

Seeds of *Viola cornuta* L. 'Lutea Splendens' were purchased from B & T World Seeds (<http://b-and-t-world-seeds.com>; Catalogue number 24421).

Surface sterilization and germination of seeds

Seeds were rinsed in tap water for 1 h before being soaked in a 20% commercial bleach solution containing 4% NaOCl for 20 min. The bleach solution was then decanted and 96% ethanol was added. After 1 min, ethanol was removed and the seeds were washed three times with sterile distilled water and placed in plastic Petri dishes (100 × 15 mm) filled with culture medium. Sterilized seeds were cultured on moist paper or culture medium supplemented with full-strength Murashige and Skoog (1962) mineral salts and vitamins, 20 g/L sucrose, 100 mg/L myo-inositol, 100 mg/L tyrosine, 80 mg/L adenine hemisulphate, gelled with 0.7% (w/v) agar (Torlak, Belgrade, Serbia) (MS medium), and the solid medium described above with half-strength MS mineral salts, full concentration of Na-FeEDTA and vitamins (½MS medium) for 4, 8 or 12 weeks. The pH of all culture media was adjusted to 5.8 using 1 M HCl and/or 1 M NaOH before autoclave sterilization. Twenty seeds were placed in each Petri dish with culture medium, sealed with Parafilm®, covered with aluminium foil and cultured in the dark in growth chambers set at 22 ± 2 °C, 10 °C or 4 °C. All treatments included four Petri dishes and experiments were repeated twice.

In vitro plant regeneration

The leaves, petioles and hypocotyl segments from well-developed seedlings (5–8 cm long) were cut into small pieces (5–10 mm long) and cultured in the dark in Petri dishes containing plant growth regulator (PGR)-free ½MS medium or ½MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg/L), and 6-benzylaminopurine (BAP, 2.0 mg/L) or thidiazuron (TDZ, 1.0 mg/L).

Additionally, the effect of light conditions and explant origin in the culture of hypocotyl explants was evaluated. Hypocotyl segments were cut into small pieces (5 mm

long), and cultured in Petri dishes containing 20 mL of $\frac{1}{2}$ MS medium supplemented with 2,4-D and BAP. Samples in three repetitions (Petri dishes with 25 explants) were cultured in the dark or 16 h light/8 h dark photoperiod with 80–100 $\mu\text{mol}/\text{m}^2/\text{s}$ PPFD light intensity at $22 \pm 2^\circ\text{C}$. The number of hypocotyl explants with developed calli and shoots was scored after 4 weeks of culture using a dissecting microscope (10 $\times$) and the experiment was repeated twice. To determine the effect of hypocotyl explant origin on shoot induction, we divided the seedling hypocotyl into three parts. Hypocotyl explants excised from the upper part of the hypocotyl were designed as h_1 section (top), between the top and bottom as h_2 section (middle) and the bottom part of the hypocotyl as the h_3 section. Explants were cultured on $\frac{1}{2}$ MS medium supplemented with 2,4-D and BAP in three repetitions (Petri dishes with 15 explants of the same hypocotyl segment type) and grown in a growth chamber at $22 \pm 2^\circ\text{C}$ at 16 h light/8 h dark photoperiod as described above.

Well-developed shoots from primary explants were transferred to 100 mL Erlenmeyer flasks containing 30 mL of solid $\frac{1}{2}$ MS medium supplemented with α -naphthaleneacetic acid (NAA, 0.1 or 0.5 mg/L) and BAP (1.0 mg/L) or PGR-free $\frac{1}{2}$ MS medium. All cultures were grown in a growth chamber under 16 h light/8 h dark photoperiod at $22 \pm 2^\circ\text{C}$. Stable shoot cultures were maintained by subculturing ten nodal segments on fresh culture medium containing NAA and BAP every 4 weeks.

For root induction, ~ 2 cm-long apical shoots from multiplication stage were cut and transferred to PGR-free $\frac{1}{2}$ MS medium or $\frac{1}{2}$ MS medium with low concentration (0.1 mg/L) of different auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or NAA. Ten shoots were cultured in 700 mL glass jars with 100 mL of culture media. After 8 weeks, several morphological characteristics were estimated: average number of developed branches/plant, plant height, number of nodules/plant, frequency of rooting, number of roots/shoot and average length of the longest root. The experiments were conducted in five replicates and repeated twice.

Cryopreservation procedure

Nodal segments of in vitro-grown shoots were cultured for 3 weeks on $\frac{1}{2}$ MS medium with NAA and BAP for development of axillary shoot. Developed apical shoots (1 cm) were cut and cultured on $\frac{1}{2}$ MS medium supplemented with 0.1 mg/L BAP for 2 weeks at 22 or 4°C for cold acclimation. Shoot tips (1–2 mm) were isolated and cultured on $\frac{1}{2}$ MS medium with 0.3 M sucrose for 1 day before immersion in loading solution (2M glycerol, 0.4 M sucrose) (Nishizawa et al. 1993) filled in cryovials for 30 min. Osmotic dehydration with PVS2 solution (30% w/v glycerol, 15% w/v ethylene glycol and 15% v/w DMSO in liquid $\frac{1}{2}$ MS

medium with 0.4 M sucrose) (Sakai et al. 1990) was tested at 0°C or 22°C for 20 min. Osmotic dehydration with PVS3 (50% v/w sucrose, 50% v/w glycerol in liquid $\frac{1}{2}$ MS medium) (Nishizawa et al. 1993) was tested at 22°C for 45 min before direct immersion in LN. Cryovials with shoot tips remained at least 1 day or several months immersed in LN. Re-warming was performed in a 42°C water bath for 1–2 min. After re-warming, the PVS solutions were replaced with unloading solution containing $\frac{1}{2}$ MS with 1.2 M sucrose for 20 min. Re-warmed shoot tips were cultured on $\frac{1}{2}$ MS medium with 0.1 mg/L BAP. Survival was estimated 1 week after thawing as the number of green shoot tips, while the regrowth was measured as full recovery of shoots and development of new well-formed leaves after 4 weeks of culture.

Plant acclimatization

Rooted plants were washed with water to remove adhering agar and planted in a soil mix of peat and perlite (3:1) in plastic pots (30 cm $\times$ 20 cm). Plastic pots with plantlets were covered with transparent foil during the first 2 weeks of acclimatization. Afterward, plantlets were grown uncovered in greenhouse conditions till flowering.

Chromosome analysis

For chromosome counting, root tips were collected from seedlings and in vitro-derived plantlets (before and after cryopreservation) rooted on medium supplemented with 0.1 mg/L NAA. Root tips were pre-treated with 2 mM 8-hydroxiqinoline at room temperature for 4–5 h, fixed in Carnoy's solution (ethanol:glacial acetic acid 3:1) for 24 h at 4°C and stored dehydrated in 70% ethanol before analysis. Dehydrated roots were hydrolysed in 1 N HCl for 15 min at 60°C and subsequently stained with 1% (w/v) aceto-orcin for 10 min. Stained root tips (1–2 mm) were squashed in a drop of 45% acetic acid. Ten plantlets per sample and about 30 cells per plant were used for chromosome analysis using light microscopy (Carl Zeiss AxioVision microscope, Zeiss, Germany).

Data analysis

Data analysis was performed using the Statistica 8 statistical package. The significance of culture media and temperature on seed germination was determined using two-way analysis of variance (ANOVA). All other data were analysed using one-way ANOVA. The differences between treatment means were tested using Fisher's LSD test ($p \leq 0.05$).

Results and discussion

In vitro seed germination

The germination rate of *V. cornuta* on moist filter paper was low. Only 10% of *V. cornuta* seeds germinated after 4 weeks of cultivation at 22 °C. For the same period of time, 32–36% of seeds grown on culture medium germinated under standard growth condition (22 °C) regardless of the concentration of mineral salts in the culture medium (Table 1). In vitro seed germination of *V. cornuta* was significantly improved by increasing the duration of culture and lowering of incubation temperature (Table 1). The best seed germination (63.7–71.2%) was obtained in seeds cultured for 12 weeks at lower temperature (10 or 4 °C), compared with 45% of seed germination obtained at 22 °C for the same period of time. Also, chilling of seeds for longer than 8 weeks significantly promoted seed germination regardless of the medium composition (Table 1). Analysis of variance showed that the temperature of seed cultivation was the most important factor affecting seed germination of *V. cornuta* L. ‘Lutea Splendens’. Seeds of other *Viola* spp. are not easy to germinate and showed remarkable variation in germination requirements during cultivation in culture in vitro (Mitchell et al. 2000; Li et al. 2011; Mokhtari et al. 2016). Mitchell et al. (2000) reported that *V. cornuta* ‘Perfection’ seeds germinated at high rates on moist filter paper under a broad range of temperatures (20–25 °C) and chilling did not have a positive effect on seed germination. We obtained seed germination over 70% after prolonged culture of seeds and lowering the temperature of cultivation to 10 °C. Higher

germination rates of horned pansy seeds at 10 °C are not unexpected findings, since this temperature regime is usually present during autumn and spring in the regions where this species is naturally found. Similar results of enhanced seed germination after cultivation at 10 °C were found for many other plant species from the Mediterranean region (El Aou-ouad et al. 2014). Although there are reports about very low seed germination of *Viola* spp. on culture media with decreased concentration of mineral salts (Li et al. 2011), it was not a significant factor for in vitro seed germination of *V. cornuta*.

In vitro plant propagation and acclimatization

The concentration of mineral salts in culture medium was an important factor for induction, further development and multiplication of *V. cornuta*. The best results were achieved on media with half-strength MS mineral salts containing full-strength Na–FeEDTA and vitamins. These findings are in agreement with those of Sato et al. (1995) and Mokhtari et al. (2016), who revealed that dilution of basal salts of MS medium was important for successful shoot induction and plantlet regeneration of other violas and pansies.

Correct selection of primary explants for establishing an efficient protocol for in vitro plant regeneration is a prerequisite for further transformation studies for *Viola* sp. and many other plant species (Alimohammadi and Bagherieh-Najjar 2009). In our study, the formation of first shoot primordia was observed after 4 weeks in leaf culture on medium supplemented with TDZ (Fig. 1a) and in petiole (Fig. 1b) and hypocotyl culture (Fig. 1c) on medium supplemented with 2,4-D and BAP (Table 2). Our results showed that petioles are the best explants for shoot induction in *V. cornuta*. This is in accordance with results obtained for not only seedling explants, but also other ex vitro or in vitro grown shoots of different *Viola* spp. (Sato et al. 1995; Wang and Bao 2007; Vishwakarma et al. 2013; Antonić et al. 2017). Seedling explants (petiole, leaf or root segments) as an initial source of primary explants for establishing a protocol for *Viola* spp. in vitro regeneration have been used for *V. wittrockiana* ‘Caidie’ (Wang and Bao 2007) and *V. baoshanensis* (Li et al. 2011). Wang and Bao (2007) observed only callus induction on petiole and leaf explants of *V. wittrockiana* ‘Caidie’ after 4 weeks of culture on media supplemented with 2,4-D and BAP. Successful shoot regeneration was possible only on petiole-derived calli after subsequent change of several different media. In contrast to previous study, Li et al. (2011) reported high callus (93%) and shoot regeneration (60%) from leaf explants of *V. baoshanensis* after 6 weeks of culture on media supplemented with NAA and BAP. However, there is no information about the effect of TDZ as the sole PGR in the first step of morphogenesis induction in *Viola* sp. Slazak

Table 1 Effect of culture media and incubation temperature on seed germination of *V. cornuta* L. ‘Lutea Splendens’

Culture		Seed germination (%)		
Medium	Temperature (°C)	4 weeks	8 weeks	12 weeks
MS	4	0.00 ± 0.00 ^{a**}	38.75 ± 8.00 ^a	63.75 ± 5.15 ^b
	10	15.00 ± 5.40 ^b	53.75 ± 8.75 ^{a,b}	63.75 ± 5.54 ^b
	22	32.50 ± 3.23 ^c	40.00 ± 2.04 ^a	45.00 ± 3.53 ^a
½MS	4	0.00 ± 0.00 ^a	41.25 ± 3.75 ^a	68.75 ± 7.74 ^b
	10	17.50 ± 4.33 ^b	60.00 ± 2.89 ^b	71.25 ± 4.27 ^b
	22	36.25 ± 6.57 ^c	55.00 ± 4.56 ^{a,b}	56.25 ± 5.54 ^{a,b}

*Seeds were cultured on full-strength Murashige and Skoog mineral salts and vitamins (MS) or half strength MS mineral salts and full strength Na–FeEDTA and vitamins in dark

**Values represent mean ± SE. Within the column, values with different letters are significantly different using Fisher’s LSD test ($p \leq 0.05$)

et al. (2015) reported that shoot induction in culture of *V. uliginosa* leaf and petiole explants (6% and 10%, respectively) occurred 6 months after transfer of calli induced on medium with 2,4-D and kinetin (KIN) to medium with TDZ (1.0 mg/L). In our study, we observed significant potential of TDZ (1.0 mg/L) to induce adventitious shoots after only 4 weeks of culture of the initial *V. cornuta* leaf explants. The activity of TDZ is usually explained by induction of cytokinin accumulation as well as by accumulation of auxin in primary explants cultured on media with TDZ (Murthy et al. 1998; Murch and Saxena 2001).

The review of literature showed that there was no information about in vitro regeneration of violas and pansies, from hypocotyl segments as primary explants. Different factors can influence the morphogenic potential of initial explants in culture in vitro such as culture conditions, plant growth regulators, genotype, explant position on mother plant and physiological status of mother plant or age of seedlings (Sharma et al. 2011; Gomes and Garcia 2013). We found the best morphogenic response in hypocotyl explants grown on medium supplemented with 2,4-D and BAP where most of the explants first developed callus (>97%), and shoots developed only sporadically (2%, Table 2). Morphogenetic response of hypocotyl explants was increased in segments grown under 16 h light/8 h night photoperiod achieving 32% of shoot induction after 4 weeks of culture. Furthermore, the induction of adventitious shoots in hypocotyl explants of horned pansy depended on which part of the hypocotyl was used (Fig. 1d). Hypocotyl segments close to the epicotyl showed the best shoot induction potential (Table 3). After 4 weeks of culture, the highest shoot induction response was obtained in the proximal h_1 section, which was significantly higher than that in other hypocotyl segments (h_2 and h_3). More than five times higher shoot induction response was achieved in h_1 (45%) than in the h_3 hypocotyl segment (8%). After prolonged culture (8 weeks), half of the initial h_1 hypocotyl explants developed shoots and two times more adventitious shoots were developed in h_1 than in other hypocotyl sections (Table 3). Furthermore, we observed that the morphogenetic potential of different hypocotyl explants greatly varied among different seeds. Figure 1e–g presents the morphogenetic response of the top (Fig. 1e), middle (Fig. 1f) and bottom (Fig. 1g) hypocotyl segments derived from five seeds. We noticed that all hypocotyl segments from the same seeds, irrespective of their position, showed capability to develop shoots (Fig. 1e–g, fifth in a row), while those from others produced no shoots (Fig. 1e–g, second in row), indicating genotype dependency of this process in *V. cornuta*. The phenomenon of the varied morphogenetic response of different hypocotyl segments is described in many regeneration systems. It ranged from the highly responsive proximal part where hypocotyl sections adjacent to the cotyledon gave the highest

number of shoots (Fári and Czako 1981; Shang et al. 2006; Sharma et al. 2011; Wang et al. 2011) to the hypocotyl culture where the bottom parts of the hypocotyl (closer to the root) produced more shoots (Nagori and Purihit 2004; Chen et al. 2008). The different responses of hypocotyl segments may be due to variation in the endogenous concentration of PGRs in the mother plants of different plant species (George 1993; Ju et al. 2012). Also, germination conditions of seeds such as temperature and light can significantly influence the subsequent morphogenetic response in culture in vitro. For instance, Muktadir et al. (2016) found that the response of hypocotyl segments (top, middle and bottom sections) was influenced by seed germination and culturing light pre-treatment.

The necessity of frequent changes of several different culture media during in vitro plant regeneration of *Viola* spp. was pointed out in several reports (Wang and Bao 2007; Naeem et al. 2013). Here, we showed very fast and efficient shoot induction from different seedling explants after 4 weeks of culture. All de novo formed shoots were further cultivated on $\frac{1}{2}$ MS PGR-free medium for elongation or $\frac{1}{2}$ MS medium supplemented with NAA and BAP for multiplication and two to three new shoots developed after 4 weeks of subculturing. There were no significant differences in shoot multiplication of *V. cornuta* between two used culture media. The PGR combinations NAA/BAP in a ratio 1:5 or 1:10 proved to be very efficient in shoot multiplication of *Viola* sp. (Wang and Bao 2007).

Regenerated shoots of *V. cornuta* can be easily rooted on $\frac{1}{2}$ MS culture media without PGRs or supplemented with low concentration (0.1 mg/L) of different auxins (IAA, IBA and NAA, Fig. 2). After 8 weeks in culture, all derived plantlets were able to branch (Fig. 1i) regardless of the culture media (Fig. 2d). Significantly higher rooting frequency (Fig. 2a), average root number/plant (Fig. 2b) and length of the longest root/shoot (Fig. 2c) were observed in plantlets grown on medium supplemented with NAA. Also, a significantly higher number of nodal segments per shoot were recorded (Fig. 2e) and the highest length of shoots (Fig. 2f) was observed on this medium in comparison to other culture media. According to an analysis of all these parameters, we conclude that the best medium for rooting *V. cornuta* 'Lutea Splendens' shoots was $\frac{1}{2}$ MS medium supplemented with a low concentration of NAA. According to literature data, *Viola* sp. shoots can spontaneously develop roots on culture medium for shoot multiplication (Wang and Bao 2007) or be easily rooted after transfer to PGR-free medium (Li et al. 2011) or medium supplemented with IBA (Chalageri and Babu 2012; Vishwakarma et al. 2013). Our results are in accordance with these data, since horned pansy shoots can be easily rooted by 8 week culture on PGR-free medium. Auxins are considered as main PGRs responsible for root induction in plants (Jarvis and Yasmin 1987). We find that

supplementation of rooting media with low concentration of NAA can significantly improve the rooting of shoots in horned pansy culture.

In vitro-derived plantlets were planted during July and were successfully acclimatized (96%) to greenhouse conditions and flowered in the next spring. According to

Fig. 1 Plant regeneration of horned pansy *V. cornuta* ‘Lutea Splendens’ using seedlings explants. **a** Leaf explants cultured on $\frac{1}{2}$ MS medium with TDZ (1.0 mg/L). **b, c** Four-week-old petiole (**b**) and hypocotyl (**c**) explants cultured on $\frac{1}{2}$ MS medium with 2,4-D (0.1 mg/L) and BAP (2.0 mg/L). **d** Position of hypocotyl sections on seedling used in this study: top (h_1), middle (h_2) and bottom (h_3) section. **e–g** Callus and adventitious shoot formation on hypocotyl explants: h_1 (**e**), h_2 (**f**) and h_3 (**g**). **h** Shoot regrowth 2 weeks after thawing from LN. **i** Rooted plantlets ready for planting. **j** Flowered in vitro-derived horned pansy plants. **k** Fruit with developed seeds. Scale bars **a–c** 1 mm; **d–g, k** 5 mm; **h–j** 10 mm

the morphological characteristics of flowers, all in vitro-derived plants resembled control plants grown from seeds directly. The plantlets achieved full physiological maturity, flowered (Fig. 1j) and set seeds (Fig. 1k).

Shoot tip cryopreservation

Shoot tips of *V. cornuta* are susceptible to PVS2 solution. Very low survival (21–24%) was achieved when shoot tips were dehydrated with PVS2 at room temperature or 4 °C prior immersion in LN. Further regrowth of shoots was

blocked and only callus formation was observed. On the other hand, successful shoot regrowth and full recovery without callus interphase were achieved with PVS3-based cryopreservation (Fig. 1h). The main difference between PVS2 and PVS3 solutions lies in their composition. PVS2 contains ethylene glycol and DMSO which penetrate the cell walls and membranes and cause thinning and spontaneous pore formation of the bilayer, increasing diffusion across the membrane (Hughes et al. 2013). In our study, a higher regrowth percentage was observed after PVS3 dehydration, mainly as a result of the osmotic effect of PVS3, because the cryoprotectants in this solution did not penetrate the cells (Hughes and Mancera 2014). Cold acclimation prior to cryopreservation with PVS3 solution significantly improved the regrowth of cryopreserved *V. cornuta* shoot tips (Table 4). The preculture of shoots at 4 °C for a month prior to the vitrification procedure improved the post-thawing recovery of *V. pilosa* (Soni and Kaur 2014). Cold acclimation was found to improve post-cryopreservation recovery in many plant species (Reed 1990; Kulus and Zalewska 2014). It increases the freezing tolerance in plants (Folgado et al. 2015) and can be a very useful step in the development of an efficient protocol for

Table 2 Effect of explants type (petiole, hypocotyl, leaf segments) and the culture media on frequency of callus formation (%) and shoot induction (%) of *V. cornuta* ‘Lutea Splendens’ in culture of seedlings explants

Culture media*	Explant type	Callus formation (%)	Shoot induction (%)
$\frac{1}{2}$ MS	Leaf	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^{a**}
	Petiole	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a
	Hypocotyl	0.00 $\pm$ 0.00 ^a	0.00 $\pm$ 0.00 ^a
$\frac{1}{2}$ MS + 0.1 2,4-D + 2.0 BAP	Leaf	64.25 $\pm$ 1.90 ^e	0.00 $\pm$ 0.00 ^a
	Petiole	35.24 $\pm$ 1.14 ^c	12.85 $\pm$ 5.16 ^b
	Hypocotyl	97.87 $\pm$ 1.98 ^f	2.01 $\pm$ 1.93 ^a
$\frac{1}{2}$ MS + 1.0 TDZ	Leaf	59.17 $\pm$ 1.32 ^{de}	19.32 $\pm$ 0.22 ^b
	Petiole	42.97 $\pm$ 1.34 ^{cd}	0.00 $\pm$ 0.00 ^a
	Hypocotyl	13.30 $\pm$ 0.12 ^b	0.00 $\pm$ 0.00 ^a

*Explants were cultured on half-strength MS mineral salts with full-strength Na–FeEDTA and vitamins ($\frac{1}{2}$ MS medium). Plant regulators are in mg/L

**Values are mean $\pm$ SE. Within a column, values with different letters are significantly different using Fisher’s LSD test ($p \leq 0.05$)

Table 3 Influence of segment position on shoot induction in *V. cornuta* ‘Lutea Splendens’ hypocotyl culture

Hypocotyl segment position	Shoot induction (%)		No. of shoot/segment	
	4 weeks	8 weeks	4 weeks	8 weeks
h_1 *	45.00 $\pm$ 6.31 ^{b**}	50.00 $\pm$ 6.94 ^b	4.22 $\pm$ 0.49 ^b	6.07 $\pm$ 0.66 ^b
h_2	11.67 $\pm$ 3.19 ^a	25.00 $\pm$ 9.95 ^{ab}	2.86 $\pm$ 0.80 ^{ab}	2.93 $\pm$ 0.65 ^a
h_3	8.33 $\pm$ 6.31 ^a	8.33 $\pm$ 6.31 ^a	2.00 $\pm$ 0.69 ^a	3.10 $\pm$ 0.83 ^a

*The position of the hypocotyl segments is illustrated in Fig. 1c. The upper part of the hypocotyl, close to the epicotyl was designed as the h_1 section, the middle as the h_2 section and the bottom part as the h_3 section. Hypocotyl sections were cultured on ($\frac{1}{2}$ MS medium) with 2,4-D (0.1 mg/L) and BAP (2.0 mg/L)

**Values represent mean $\pm$ SE. Within a column, values with different letters are significantly different using Fisher’s LSD test ($p \leq 0.05$)

Fig. 2 Effect of different auxins on rooting ability and several morphological characteristics of in vitro regenerated shoots of *V. cornuta* 'Lutea Splendens' cultured on half-strength MS mineral salts with full-strength Na-FeEDTA and vitamins with 0.1 mg/L of each auxin (IAA, IBA, NAA) or without auxin. **a** Rooting frequency (%).

b Average number of roots/shoot. **c** Average length of the longest root/shoot. **d** Average number of developed branches/plant. **e** Average number of nodes/plant. **f** Average plant height. Values represent mean $\pm$ SE. Histograms with different letters are significantly different using Fisher's LSD test ($p \leq 0.05$)

cryopreservation of violas and pansies. According to our procedure, 2 weeks of cold acclimation is sufficient for increasing the dehydration tolerance; on following the PVS3-based cryopreservation procedure, more than 60% of shoots recovered 1 month after retrieval from LN.

Clonal fidelity of regenerated plants

The presented protocol for shoot induction, multiplication and cryopreservation of horned pansy shoots provides a high

Table 4 The effect of cold acclimation on survival and regrowth of *V. cornuta* 'Lutea Splendens' shoot tips cryopreserved with the PVS3 vitrification procedure

Cold acclimation	Survival (%)		Regrowth (%)	
	-LN	+LN	-LN	+LN
–	100	43.33 $\pm$ 12.01 ^a	100 ^a	39.72 $\pm$ 1.21 ^a
2 weeks	100	86.67 $\pm$ 6.67 ^b	95.83 $\pm$ 4.16 ^a	64.08 $\pm$ 3.02 ^b

**Values are mean $\pm$ SE. Within a column, values with different letters are significantly different using Fisher's LSD test ($p \leq 0.05$)

Fig. 3 Root tip meristematic cells with chromosomes of *Viola cornuta* 'Lutea Splendens' plants. **a** Cell with chromosomes of seedling. **b** Cells of in vitro regenerated plantlets before cryopreservation. **c** Cells of plantlets regenerated after cryopreservation

level of necessary preconditions for true-to-typeness at the ploidy level of the regenerated plants. We confirmed the stability of ploidy level in regenerated *V. cornuta* plants by chromosome counting in root tip meristematic cells showing the diploid set of chromosomes ($2n = 2x = 22$), the same as in seedlings (Fig. 3a). Slazak et al. (2015) reported significant somaclonal variations induced by culture conditions and the type of organogenesis of *V. uliginosa*. Long-term indirect organogenesis (several months) on medium supplemented with 2,4-D gave the highest level of polyploidization. Slazak et al. (2015) suggested that more direct and fast methods for shoot induction should be preferred for micropropagation proposes even when the process is less effective. In our protocol, shoots were regenerated by indirect shoot organogenesis after short exposure (4 weeks) of petiole and hypocotyl explants to 2,4-D. Also, a high potential for shoot induction was achieved on medium supplemented with TDZ as a sole PGR in leaf culture without a long callus phase during shoot induction. Further cultivation and shoot multiplication were done by activation of axillary branching on medium without 2,4-D which increased the opportunity for high fidelity of regenerated plants (Fig. 1b). Chromosome number remained stable in plants regenerated after cryopreservation (Fig. 3c). According to the presented protocol for cryopreservation using the PVS3-based vitrification procedure, shoot regrowth of horned pansy was obtained without intermediate callus formation which implicates high clonal fidelity of regenerated plantlets after long-term storage. These findings might be useful for cryopreservation of valuable clones, mutants or obtained transgenic lines which can be stored long term without somaclonal variations. In vitro-derived horned pansy plantlets were acclimatized in greenhouse conditions and grown to new flowering season to get seeds. No variations in plant, flower or seed morphology were noticed in a population of in vitro-derived plants in comparison to plants derived from seeds. The presented

study might be very useful for micropropagation of valuable cultivars and varieties, in genetic engineering studies and in long-term storage of this species and other *Viola* spp.

Acknowledgements We thank Dr. Zoran Jeknić (Oregon State University, USA) for helpful discussions and critical reading of the manuscript. This research was sponsored by the Ministry for Education, Science and Technological Research, Serbia (Project TR31019).

Compliance with ethical standards

Conflict of interest The authors declare that there was no conflict of interest.

References

- Alimohammadi M, Bagherieh-Najjar MB (2009) *Agrobacterium*-mediated transformation of plants: basic principles and influencing factors. *Afr J Biotechnol* 8:5142–5148
- Antonić D, Trajković M, Cingel A, Subotić A, Jevremović S (2017) Plant regeneration from *in vitro*-derived leaf and petiole explants of *Viola cornuta* L. 'Lutea Splendens'. *Propag Ornament Plants* 17(3):95–102
- Babber S, Sharma K (1991) Study of anatomy of vitrified structure in *Viola tricolor* L. *Ann Biol Hissar* 7:93–96
- Chalageri G, Babu UV (2012) *In vitro* plant regeneration via petiole callus of *Viola patrinii* and genetic fidelity assessment using RAPD markers. *Turk J Bot* 36:358–368
- Chandler S, Brugliera F (2011) Genetic modification in floriculture. *Biotech Lett* 33:207–214
- Chandler SF, Tanaka Y (2007) Genetic modification in floriculture. *Crit Rev Plant Sci* 26:169–197
- Chen R, Nanimatsu S, Nakadozono Y, Bamba T, Nakazawa Gyokusen K (2008) Efficient regeneration of *Eucommia ulmoides* from hypocotyl explant. *Biol Plant* 52(4):713–717
- El Aou-ouad H, Medrano H, Lamarti A, Gullías J (2014) Seed germination at different temperatures and seedling emergence at different depths of *Rhamnus* spp. *Cent Eur J Biol* 9(5):569–578
- Fári M, Czako M (1981) Relationship between position and morphogenetic response of pepper hypocotyl explants cultured *in vitro*. *Sci Hort* 15:207–213

- Farzad M, Griesbach R, Weiss MR (2002) Floral color change in *Viola cornuta* L. (Violaceae): a model system to study regulation of anthocyanin production. *Plant Sci* 162:225–231
- Farzad M, Griesbach R, Hammond J, Weiss MR, Elmendorf HG (2003) Differential expression of three key anthocyanin biosynthetic genes in a color-changing flower, *Viola cornuta* cv. Yesterday, today and tomorrow. *Plant Sci* 165:1333–1342
- Farzad M, Soria-Hernanz DF, Altura M, Hamilton MB, Weiss MR, Elmendorf HG (2005) Molecular evolution of the chalcone synthase gene family and identification of the expressed copy in flower petal tissue of *Viola cornuta*. *Plant Sci* 168:1127–1134
- Folgado R, Panis B, Sergeant K, Renaut J, Swennen R, Hausman JF (2015) Unravelling the effect of sucrose and cold pretreatment on cryopreservation of potato through sugar analysis and proteomics. *Cryobiology* 71:432–441
- George F (1993) Plant propagation by tissue culture, part I: the technology. Exegnetics, Edington, 1–574
- Gomes MP, Garcia QS (2013) Reactive oxygen species and seed germination. *Biologia* 68(3):351–357
- Hughes ZE, Mancera RL (2014) Molecular mechanism of the synergistic effects of vitrification solutions on stability of phospholipid bilayers. *Biophys J* 106:2617–2624
- Hughes ZE, Malajczuk CJ, Mancera RL (2013) The effects of cryosolvents on DOPC- β -sitosterol bilayers determined from molecular dynamics simulations. *J Phys Chem B* 117:3362–3375
- Jarvis BC, Yasmin S (1987) Plant growth regulators and adventitious root development in relation to auxins. *Biol Plant* 29:189–198
- Jeknić Z, Jeknić S, Jevremović S, Subotić A, Chen CHH (2014) Alteration of flower color in *Iris germanica* L. ‘Fire Bride’ through ectopic expression of phytoene synthase gene (*crtB*) from *Pantoea agglomerans*. *Plant Cell Rep* 33:1307–1321
- Ju HJ, Eck JV, Gray SM (2012) Factors influencing plant regeneration from seedlings explants of hairy night shade (*Solanum sar-rachoides*). *Plant Cell Tissue Org Cult* 108:121–128
- Karimi O, Esna-Ashari M, Karimi Kudistani G, Aghavaissi B (2009) *Agrobacterium*-mediated genetic transformation of plants: the role of host. *Biol Plant* 53:201–212
- Kulus D, Zalewska M (2014) Cryopreservation as a tool used in long term storage of ornamental species—a review. *Sci Hortic* 168:88–107
- Li JT, Deng DM, Peng GT, Deng JC, Zhang J, Liao B (2011) Successful micropropagation of the cadmium hyperaccumulator *Viola baoshanensis* (Violaceae). *Int J Phytoremediat* 12:761–771
- Mitchell WC, Cornetto TM, Nunez M, Patel M (2000) Analysis of horned violet, *Viola cornuta* seed germination. *Bull N J Acad Sci* 45:1–5
- Mokhtari A, Zarei M, Samsamzadeh B, Moradi K (2016) Interactive effects of plant growth regulators and explants on direct shoot regeneration of *Viola odorata*. *Biol Tech* 97(1):33–39
- Muktadir MA, Habib MA, Mian MAK, Akhond MAY (2016) Regeneration efficiency based on genotype, culture conditions and growth regulators of eggplant (*Solanum melongena* L.). *Agric Natl Res* 50:38–42
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Murch SJ, Saxena PK (2001) Molecular fate of thidiazuron and its effects on auxin transport in hypocotyl tissues of *Pelargonium × hortorum* Bailey. *Plant Growth Regul* 35:269–275
- Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. *In Vitro Cell Dev Biol Plant* 34:267–275
- Naeem M, Naveed I, Naqvi SMS, Mahmood T (2013) Standardization of tissue culture conditions and estimation of free scavenging activity in *Viola odorata* L. *Pak J Bot* 45:197–202
- Nagori R, Purihit SD (2004) *In vitro* plantlet regeneration in *Annonasquamosa* through direct shoot bud differentiation on hypocotyl segments. *Sci Hort* 99:89–98
- Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. *Plant Sci* 99(1):67–73
- Reed BM (1990) Survival of *in vitro*-grown apical meristem of *Pyrus* following cryopreservation. *Hort Sci* 25:111–113
- Sakai A, Engelman F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *Cryo Lett* 28:151–172
- Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Rep* 9(1):30–33
- Sato T, Kwon O, Miyake H, Taniguchi T, Maeda E (1995) Regeneration of plantlets from petiole callus of wild viola (*Viola patrinii* DC). *Plant Cell Rep* 14:768–772
- Shang AQ, Cai I, Yan XJ, Hu HZ, Zhao LJ (2006) Plant regeneration from *in vitro* cultured hypocotyl explants of *Euonymus japonicus* Cu Zhi. *Agric Sci Chin* 5(3):196–201
- Sharma S, Kumar N, Reddy MP (2011) Regeneration in *Jatropha curcas*: factors affecting the efficiency of *in vitro* regeneration. *Ind Crop Prod* 24:943–951
- Slazak B, Sliwanska E, Saluga M, Ronikier M, Bujak J, Słomka A, Göransson U, Kuta E (2015) Micropropagation of *Viola uliginosa* (Violaceae) for endangered species conservation and for somaclonal variation-enhanced cyclotide biosynthesis. *Plant Cell Tissue Org Cult* 120:179–190
- Soni M, Kaur R (2014) Rapid *in vitro* propagation, conservation and analysis of genetic stability of *Viola pilosa*. *Physiol Mol Biol Plant* 20:95–101
- Vishwakarma UR, Garav AM, Sharma PC (2013) Regeneration of multiple shoots from petiole callus of *Viola serpens* Wall. *Pharm Res* 5:86–92
- Wang J, Bao MZ (2007) Plant regeneration of pansy (*Viola wittrockiana*) ‘Caidie’ via petiole-derived callus. *Sci Hort* 111:266–270
- Wang XL, Yhou JX, Yu MD, Li ZG, Jin XY, Li QY (2011) Highly efficient plant regeneration and *in vitro* polyploidy induction using hypocotyl explants from diploid mulberry (*Morus multicaulis* Poir.). *In Vitro Cell Dev Biol Plant* 47:434–440
- Wijowska M, Kota E, Przywara L (1999) *In vitro* culture of unfertilized ovules of *Viola odorata* L. *Acta Biol Cracov Bot* 41:95–101
- Zhao Z, Cai T, Miller M, Wang N, Pang H, Rudert M, Shroeder R, Hondred D, Seltzer J, Pierce D (2000) *Agrobacterium*-mediated sorghum transformation. *Plant Mol Biol* 44:789–798