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In vitro direct multiplication of *Viola canescens* Wall. ex Roxb.: An important medicinal plant

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Abstract

The present study investigated the different sterilization procedures for seed explants of *Viola canescens* Wall. ex Roxb. collected from a wild-grown plant on Morni Hills, Panchkula and *in vitro* frequency of multiple shoot regeneration of cotyledonary node having both cotyledons. Maximum per cent aseptic germination of healthy seeds were obtained when seeds were treated with bavistin and streptomycin for 10 min followed by a quick immerse in 70% ethanol and finally sterilized with 1.0% $HgCl_2$ for 5 min and cultured on half-strength MS media containing 0.5 mg/l GA_3. Effect of three different cytokinins (KIN, BAP and TDZ) alone and in amalgamation with auxin (NAA) were evaluated to investigate the frequency of *in vitro* shoot multiplication from cotyledonary node having both cotyledons. The highest average number of shoots (10.6 ± 0.547) and maximum average shoot length (6.7 ± 0.101 cm) was obtained on MS media fortified with BAP (2.0 mg/l) + NAA (0.5 mg/l). Half strength MS medium augmented with IBA (2.0 mg/l) reported best for rooting of regenerated shoots with 86.66% efficiency. Finally, in the field setting, nearly 85 per cent of regenerated plantlets were able to thrive successfully.

1. Introduction

The Himalayas storehouse has incredible plant biodiversity, with many endemic, endangered, threatened and vulnerable plant herbs (Badola and Aitken, 2003). Many of these herbs have been well recognized to cure various diseases in traditional system of medicines (*i.e.*, Ayurveda, Unani, *etc.*) and shows better or comparable relief with allopathic medicines (Sagar *et al.*, 2018; Ansari *et al.*, 2019). *V. canescens* commonly known as Banafsha or Himalayan White Violet is one of the endangered species of Himalayas region (Masood *et al.*, 2014). It belongs to the Violaceae family, consisting of 20 genera and about 800 species (Chandra *et al.*, 2015). This species is spread worldwide in countries such as India, Pakistan, Bhutan and Nepal (Chandra *et al.*, 2015; Khajuria *et al.*, 2019). It is restricted mainly to the mountainous areas of the temperate and tropical zones (Singh *et al.*, 2005; Masood *et al.*, 2014). It is a perennial prostrate plant, which thrives especially in sciophile environments (Muhammad *et al.*, 2012; Mc Cauley and Jr., 2013).

In India, *V. canescens*, mainly prevalent in some cold arid deserts of northwest Himalaya, *i.e.*, Pangi valley of Chamba district in Himachal Pradesh (Trans Himalayan zone) and Garwal region of Uttarakhand at an altitude of 1600-2000 m (Rana *et al.*, 2014; Masood *et al.*, 2014). Some other regions of Uttarakhand (Nainital catchment area and Nanda Devi National Park) also exhibited the presence of *V. canescens* (Dua *et al.*, 2011; Shah *et al.*, 2014).

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Figure 1: Closeup view of *V. canescens* plant showing fruiting.

This plant is normally present in Pakistan in all temperate Himalayan ranges at an altitude of about 2000 m (Shinwari and Shinwari, 2010; Masood *et al.*, 2014; Kumar and Digvijay, 2014). *V. canescens* prefer to grow in the habitat of four other herbs such as *Euphorbia wallichii*, *Fragaria indica*, *Podophyllum emodi* with *Polygonum* (Saima *et al.*, 2009; Masood *et al.*, 2014). Another group affiliation investigated in Ayyubia National Park affirmed the foundation *of V. canescens* along side *Pinus wallichiana* and *Geranium nepalense* affiliation spread over the range 2282 meters and it runs up to 2300 meters AMSL or here and there marginally above at some different regions (Raja *et al.*, 2014; Masood *et al.*, 2014).

In traditional ethnomedicinal systems, *V. canescens* is used to medicate various conditions such as bronchitis, cold, cough, flu, cancer of the throat, respiratory tract problems, eczema, epilepsy, gastric acidity, pyrexia, dysentery, rheumatism *etc*,. (Hamayun *et al*., 2006; Abbasi *et al*., 2010; Hussain *et al*., 2011; Rani *et al*., 2013; Rana *et al*., 2014; Razzaq *et al*., 2015; Khajuria *et al*., 2019). The plant is used to treat various nervous problems (Adnanand Hlscher, 2010) and also reported to have astringent, antipyretic, carminative, demulcent, diaphoretic, anticancerous and purgative properties. Solvent extracts of *V. canescens*, exhibited hepatoprotective activity due to its antioxidants and membrane stabilization ability (Khan *et al*., 2017). Such therapeutic effects of plants have been linked to study over time owing to the existence of some secondary metabolites, such as saponins, glycosides and methyl salicylate (Barkatullah *et al*., 2012) and the presence of some cyclotides responsible for its antimicrobial properties (Burman *et al*., 2014).

Due to its high ethnomedicinal values, *V. canescens* was unsustainably overharvested from wild, bringing it into the criteria of being "vulnerable to extinction" (Abbasi *et al*., 2011). The survival of this species is at risk in the future, because of increasing need of this plant for medicinal use (Masood *et al*., 2014). The conservational status of *V. canescens* was found endangered in Battagram district of Pakistan (Haq, 2011; Masood *et al*., 2014), rare or vulnerable in Swat valley of Pakistan, vulnerable in Dir Kothistan valley (Jan *et al*., 2014) due to its over collection. Utilizing various conservation strategies and saving this valuable medicinal wealth from extinction is the need of the hour (Masood *et al*., 2014).

Micropropagation is an efficiently proven method for restoration of the traditional treasure of biodiversity. Conservation of other *Viola* species such as *V. baoshanensis* (Li *et al*., 2010), *V. patrinii* (Chalageri and Babu, 2012), *V. odorata* (Naeem *et al*., 2013; Mokhtari *et al*., 2016) and *V. pilosa* (Soni and Kaur, 2014) has been done with many mass multiplication tissue culture protocols. But, in *V. canescens*, only indirect organogenesis tissue culture work has been reported by Khajuria and Bisht (2018). Khajuria *et al*. (2019), but direct organogenesis for mass multiplication of *V. canescens* has not performed yet. However, the possibility of genetic variability, *i.e*., gene mutations or epigenetic changes (somaclonal variations) is theoretically more in callus mediated organogenesis as compared to direct organogenesis (Zayova *et al*., 2010; Krishna *et al*., 2016). Cotyledonary node is a young meristematic portion of seedling were removed aseptically and used as source of explant for *in vitro* plant regeneration through direct organogenesis (Gambley and Dodd, 1990; Venkatachalam *et al*., 2018). So, the present report contains skilled efforts for its conservation and cultivation of a large number of clones with maintaining elite genotypes selected for their superior characteristics. Therefore, an *in vitro* protocol that can effectively increase stock or commercial scale multiplication of *V. canescens* with less chance of somaclonal variations is urgently needed.

2. Materials and Methods

2.1 Plant material collection

Seeds of *V. canescens* were gathered from Morni hills Panchkula, Haryana in the month of June to July. All seeds were collected from different locations of Morni hills and stored at 4ºC temperature for up to 10 days from the date of collection. All seeds were hand graded to get the undamaged and healthy seeds followed by viability testing by flotation method (Ajiboye *et al*., 2011). Then, for 2 h the seeds were dried. Finally, good quality seeds was selected for further experiment.

2.2 Surface sterilization regimes for seeds and *in vitro* culturing

Pretreatment was given similar to all sets, after washing with flowing tap water for half an hour to eliminate all adhering dust particles, followed by washing with Tween 20 solution (*i.e.*, 2-3 drops in 200 ml DDW). After rinsing 5-6 times with double distilled water (DDW), seeds were dipped in 0.1% solution of bavistin and streptomycin for 10 min, then again rinsed 5 times with DDW. Afterward, explants quickly dipped in 70% ethanol and washed 3 times with sterilized DDW, followed by three independent sterilization experiments performed under aseptic conditions with hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$), at various strength of solutions such as 0.1%, 0.5%, 1.0% (w/v) for 5 min (Table 1.). Finally, seeds were rinsed with autoclaved DDW for 4-5 times to remove all adhering traces of H_2O_2, NaOCl and $HgCl_2$. Then, the treated seeds were aseptically inoculated to petri plates (BOROSIL) containing half strength MS media supplemented with (0.5 mg/l GA_3) for germination.

2.3 Culture media and conditions

Half strength or full strength MS (Murashige and Skoog, 1962) media fortified with or without various concentration of plant growth regulators, 3.0% (w/v) sucrose and solidified using 0.8% (w/v) agar (HI Media labs, India) as shown in Table-1, Table-2 and Table-3 were used in present experiment. The pH of the medium was adjusted between 5.79-5.81 using 0.1N NaOH or 0.1N HCl former to autoclaving at **15 psi** for 20 min. Cultures were maintained in dark for initial 3 days in culture room sustained at temperature 25 ± 2°C and then transferred back to culture room at same temperature, under an 8/16 hour light/dark photoperiod cycle provided with a photosynthetic photon flux density (PPFD) of 70 µ mol $m^{-2}s^{-1}$ and about 60-80% humidity. All aseptic manipulations were carried out underaseptic environment of laminar airflow chamber.

2.4 Explant preparation and shoot multiplication

Seed germination began after 1 weeks of inoculation and per cent aseptic seeds germinated and per cent contaminated were recorded after 3rd weeks. Cotyledonary nodes (with both cotyledons) as an explant were aseptically excised from the seedlings between 3-4 week after inoculation and cultured on MS medium augmented with various strength solution of plant growth regulators (Table 2). MS nutrient media devoid of any plant growth regulator was taken as control. Per cent germination, average number of days required for bud break, average number of shoots, average shoot length and comparative response of culture was recorded (Table 2) upto 5^{th} week of culture.

2.5 *In vitro* rooting of micropropagated shoots

The regenerated *in vitro* multiple shoots were withdrawn and relocated to a medium of half and full strength MS augmented with or without various concentrations (1.0, 2.0 and 3.0 mg / l) of rooting hormones (IAA, IBA) to stimulate *in vitro* root induction. Cultures were initially maintained in dark for 3 days after inoculation and then moved back to culture room at temperature 25 ± 2°C, 8/16 h light/dark photoperiod cycle, PPFD of 70 µ mol m^{-2} s^{-1} and about 60-80% humidity. The per cent root regeneration, average number of days required for root induction, Mean number of roots produced

per shoot, mean length of root and culture response was recorded up to four week of culture.

2.6 Data analysis

Data was analysed using software SPSS 16.0. The impact of various treatments was evaluated using a one-way variance study (ANOVA) and the disparity between their thresholds was measured using the post-hoc Duncan Multiple Range Test (DMRT) at $p<0.05$ (Duncan, 1955). Values have same letters are non-significant.

3. Results

3.1 Effect of different concentration of different sterilants (NaOCl, H_2O_2 and $HgCl_2$) with various exposer time on seed explants

H_2O_2, NaOCl and $HgCl_2$ were the three sterilants at various concentrations (0.1%, 0.5% and 1.0%), were investigated to standardize for the best protocol for higher *in vitro* aseptic germination (Table 1). The maximum per cent aseptic germination (80.0 ± 02.54) and higher comparative culture response was reported with T9 treatment, *i.e.*, 1.0 % $HgCl_2$ for 5 min with lowest (07.3 ± 0.816) per cent contamination among all the tested treatments. T1 treatment (0.1 % H_2O_2) reported the lowest (03.3 ± 0.408) per cent aseptic germination and highest per cent contamination (91.6 ± 01.08). Progressive increases in per cent aseptic germination (or progressive decrease in per cent contamination) was reported with increase of respective concentrations (0.1% to 1.0%) of all the three sterilants tested. However, comparative among the three different chemical sterilants tested (H_2O_2, NaOCl and $HgCl_2$), the H_2O_2 reported higher per cent aseptic germination followed by NaOCl and lowest with H_2O_2).

3.2 *In vitro* establishment and multiplication

The cotyledonary nodes with cotyledons, excised from 3-4 week old seedlings of *V. canescens* and cultured on MS media with or without growth regulators revealed various responses (Table 2). Cotyledonary node did not show any morphogenic response on medium rid of growth regulators (Control). Medium supplemented with BAP, KIN and TDZ promoted bud differentiation from the cotyledonary node but medium supplemented with BAP was found more efficient for initiation and subsequent shoot proliferation (76.66 %) as compared to KIN and TDZ. Initially, a small callus knob was formed at the base of the cotyledonary node. Soon after small protuberances arise in the cotyledonary nodes which further developed into elongated slender shoots. Protuberances kept on emerging upto the 5th week of culture. The frequency of shoot bud development and the number of shoots per explant increased with increasing concentration of KIN, BAP and TDZ, respectively (Table 2). High concentration of BAP (2.0 mg/l) showed maximum response (76.66%) of bud regeneration from cotyledonary nodes among all the plant growth regulators tested individually. TDZ showed less response towards shoot regeneration as compare to KIN and BAP. However, comparative among the three plant growth regulators tested individually (KIN, BAP and TDZ), the BAP reported higher per cent regeneration in lesser number of average days required for bud break with highest number of average shoots as well as longer average shoot length followed by KIN and then TDZ.

Table 1: Effect of various surface sterilization treatments, being followed for disinfection of seeds of *V. canescens* and inoculatedon half strength MS medium augmented with (0.5 mg/l GA_3) for germination

Chemicals	Treatments	Concentration (%)	% Contamination	% Aseptic Germination	Response
H_2O_2	T 1	0.1	91.6 ± 01.08^{i}	03.3 ± 0.408^{h}	+
	T 2	0.5	88.3 ± 02.04^{h}	10.0 ± 0.707^{g}	+
	T 3	1.0	71.6 ± 02.04^{f}	15.0 ± 0.707^{f}	++
NaOCl	T 4	0.1	79.3 ± 0.816^{g}	11.6 ± 0.408^{g}	+
	T 5	0.5	42.3 ± 0.408^{e}	22.3 ± 0.408^{e}	++
	T 6	1.0	23.3 ± 0.816^{c}	44.6 ± 0.408^{c}	+++
$HgCl_2$	T 7	0.1	28.0 ± 0.707^{d}	37.0 ± 01.22^{d}	+++
	T 8	0.5	15.6 ± 01.08^{b}	57.3 ± 0.408^{b}	+++
	T 9	1.0	07.3 ± 0.816^{a}	80.0 ± 02.54^{a}	++++

Data generated from three independent experiments (n=100), where, "±" values represent mean standard deviation (SE).

A combinatory effect of auxin and cytokinin was also observed for regeneration potential and multiple shoot induction (Table 2). Augmentation of NAA (0.5 mg/l) to MS medium in combination with 2.0 mg/l of BAP, TDZ and KIN significantly increased the shoot multiplication response. Per cent explant regeneration (83.33%), response, average number of shoots (10.6 ± 0.547) and average shoot length (6.7 ± 0.101) was reported highest in media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA among all the medias (Table 2), observed after 5th week of culturing. On visual observation, it was noticed that cultures in MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA produce healthy shoots and larger expanded leaf areas as compared to basal medium augmented with BAP, TDZ and KIN individually. Increasing the concentration of TDZ and BAP reduced the average shoot number (data not mentioned) and showed vitrification symptoms. However, comparative among the combination of 0.5 mg/l of NAA with (2.0 mg/l of KIN, BAP and TDZ), the BAP reported BAP + NAA reported best results followed by KIN+NAA and then TDZ+NAA.

3.3 *In vitro* rooting

Regenerated shoots were excised and transferred to the half and full-strength MS medium with or without fortification with auxin (IAA and IBA) at different concentrations for root induction after 5th week of shoot regeneration culture (Table 3). Root started emerging in

inoculated shoots after one week of inoculation depending on the auxin type (IBA and IAA) and concentration supplemented to the medium. The concentration of auxin played a vital role in root induction. Medium devoid of auxin did not show any root induction. Highest rooting response was found in ½ MS medium augmented with 2.0 mg/l IBA, where about 86.66% culture responded with on an average root number of 5.8 ± 1.095 per shoot and an average root length of 2.42 ± 0.074 cm with very good culture response. Half strength MS medium proved best for root induction as compared to full strength MS medium. In contrast, shoots cultured in medium enriched in IBA and IAA showed healthy root elongation without intervening callus stage.

Table 2: Effect of various concentrations of growth regulators and their combination, fortified in basal MS medium on shoot organogenesis and multiplications from cotyledonary node explant of *V. canescens*.

Medium + plant growth regulators (mg/l)	Regeneration %	Average no. of days required for first bud break	Average No. of shoots ±SE	Average shoot length ±SE (cm)	Response
Control (MS)	0^{k}	0^{o}	0.00^{i}	0.00^{h}	-
MS+KIN (0.5)	60.00^{gh}	35^{k}	2.8 ± 0.447^{fg}	2.7 ± 0.450^{fg}	+
MS+KIN (1.0)	56.66^{hi}	30^{j}	3.4 ± 0.547^{ef}	3.7 ± 0.482^{d}	++
MS+KIN (1.5)	70.00^{de}	28^{i}	4.0 ± 0.707^{de}	4.6 ± 0.151^{c}	+++
MS+KIN (2.0)	73.33^{cd}	25^{g}	5.4 ± 0.547^{c}	5.5 ± 0.151^{b}	+++
MS+BAP (0.5)	60.00^{gh}	26^{h}	3.4 ± 0.547^{ef}	2.8 ± 0.387^{f}	++
MS+BAP (1.0)	66.66^{ef}	24^{f}	3.8 ± 0.447^{de}	3.2 ± 0.355^{e}	++
MS+BAP (1.5)	73.33^{cd}	19^{d}	4.2 ± 1.09^{de}	3.9 ± 0.326^{d}	+++
MS+BAP (2.0)	76.66^{bc}	17^{c}	6.0 ± 0.707^{bc}	4.5 ± 0.135^{c}	+++
MS+TDZ (0.5)	33.33^{j}	45^{n}	1.0 ± 1.000^{h}	2.3 ± 0.204^{g}	+
MS+TDZ (1.0)	50.00^{i}	42^{m}	2.2 ± 0.447^{g}	2.6 ± 0.178^{fg}	+
MS+TDZ (1.5)	53.33^{hi}	40^{l}	2.8 ± 0.836^{fg}	3.2 ± 0.167^{e}	+
MS+TDZ (2.0)	63.33^{fg}	35^{k}	3.6 ± 0.547^{def}	3.6 ± 0.238^{de}	++
MS+KIN (2.0) +NAA (0.5)	80.00^{ab}	15^{b}	6.4 ± 0.547^{b}	5.7 ± 0.132^{a}	+++
MS+BAP (2.0) +NAA (0.5)	83.33^{a}	7^{a}	10.6 ± 0.547^{a}	6.7 ± 0.101^{b}	++++
MS+TDZ (2.0) +NAA(0.5)	76.66^{bc}	20^{e}	4.4 ± 0.894^{d}	4.4 ± 0.241^{c}	+++

Response: - (No response), + (Very poor), ++ (Poor), +++ (Good) and ++++ (Very good).
Data generated from three independent experiments (n=30) and "±" values represent standard deviation (SE).

Table 3: Effect of IAA and IBA on *in vitro* rooting of *V. canescens*

Medium + plant growth regulators (mg/l)	Root regeneration (%)	Average no. of days	Mean no. of roots/shoot ± SE	Mean length of Root ± SE (cm)	Response
1/2MS	0.00^{j}	0^{i}	0^{f}	0^{h}	-
1/2MS+IAA (1.0)	16.66^{hi}	21^{e}	1.8 ± 0.447^{de}	1.48 ± 0.148^{d}	+
1/2MS+IAA (2.0)	43.33^{fg}	14^{c}	2.8 ± 0.836^{cd}	2.16 ± 0.114^{b}	+
1/2MS+IAA (3.0)	50.00^{ef}	14^{c}	2.6 ± 0.894^{cd}	2.04 ± 0.134^{b}	++
1/2MS+IBA (1.0)	73.33^{b}	7^{b}	5.2 ± 0.836^{ab}	1.74 ± 0.167^{c}	++++
1/2MS+IBA (2.0)	86.66^{a}	6^{a}	5.8 ± 1.095^{a}	2.42 ± 0.074^{a}	++++
1/2MS+IBA (3.0)	73.33^{b}	6^{a}	3.4 ± 0.547^{c}	2.42 ± 0.132^{a}	+++
MS	0.00^{j}	0^{i}	0^{f}	0^{h}	-
MS+IAA (1.0)	10.00^{i}	27^{h}	1.2 ± 0.447^{e}	0.42 ± 0.083^{g}	+
MS+IAA (2.0)	20.00^{h}	25^{f}	2.4 ± 1.140^{cd}	1.08 ± 0.258^{e}	+
MS+IAA (3.0)	36.66^{g}	26^{g}	2.0 ± 0.707^{de}	1.18 ± 0.192^{e}	+
MS+IBA (1.0)	53.33^{de}	16^{d}	2.8 ± 0.836^{cd}	0.64 ± 0.167^{f}	++
MS+IBA (2.0)	66.66^{bc}	16^{d}	4.6 ± 1.516^{b}	1.56 ± 0.181^{cd}	+++
MS+IBA (3.0)	60.00^{cd}	14^{c}	2.6 ± 0.547^{cd}	1.52 ± 0.192^{d}	++

Response: - (No response), + (Very poor), ++ (Poor), +++ (Good) and ++++ (Very good).
Data generated from three independent experiments (n=30) and "±" values represent standard deviation (SE).

Figure 1: High-frequency plant regeneration from the cotyledonary node of *Viola canescens* Wall. ex Roxb. (a) Shoot bud initiation from a cotyledonary node after 3 weeks in culture on MS basal medium augmented with BAP (2.0) + NAA (0.5). (b.) Shoot regeneration with small callus after the 4th week of inoculation in BAP (2.0) + NAA (0.5). (c.) High frequency shoot proliferation after 5th week on MS + BAP (2.0) + NAA (0.5). (d.) *In vitro* rooting in ½ MS + IBA (2.0 mg/l) after 3 week in culture. (e.) Acclimatized plantlet in the plastic pot after 4 weeks of transfer. (f.) Well acclimatized plant showing flowering.

3.4 Acclimatization and hardening

Regenerated plantlets having healthy roots were extracted out of rooting media and cleaned properly to eliminate adhering medium and transferred to pots containing pre-soaked vermiculite and ½ MS solution. Plantlets were preserved for 2 weeks inside the mist chamber to maintain high (80%) humidity under diffused sunlight through greenhouse sheet of mist chamber in temperature 27 ± 3°C. After two weeks, pots were carried out of the mist chamber and placed outside under 80% shaded place for acclimatization to the natural environment. About 85% of regenerated plants survived in the field.

4. Discussion

The challenging task or main obstacle of plant tissue culture is using a nature grown plant which is highly prone to contamination as a source of explant for aseptic *in vitro* culturing (Sarasan *et al*., 2006). The first step of present investigation was carried out to standardized a protocol for seed sterilization using different sterilants (H_2O_2, NaOCl and $HgCl_2$). Out of three sterilants used, $HgCl_2$ was found better in correlation to NaOCl and H_2O_2. A similar observation is described by Xu *et al*. (2005) for explant sterilization of *Pinellia ternata* (Thunb.) Breit, Maina *et al*. (2010) in *Arachis hypogeal*; Groach and Singh (2013) in *Saussurea lappa*. While H_2O_2 showed poor results with the maximum percentage of contamination and minimum percentage of survived explant as reported by Farooq *et al*. (2002) for surface sterilization of *Annona squamosa*.

In vitro establishment of cotyledonary node and shoot multiplication has been developed using basal MS media fortified with varied type of plant growth regulators, *i.e*., BAP, KIN and TDZ with different concentrations alone or in combination with NAA. The role of cytokinin is well known for induction and shoot proliferation (Kaloo *et al*., 2013; Groach *et al*., 2014). Seed germination potential was directly affected by the seeds' storage time after collection as well as

sterilization treatments. Seeds stored for longer duration did not show any germination even after soaking in lukewarm water for 48 h (Goel *et al*., 2009). Freshly, collected seeds cultured within one week on fresh basal medium after pre-treatment with 1.0% $HgCl_2$ for 5 min (T9) showed a maximum survival rate and minimum percentage of contamination.

BAP found strongest in terms of the effect on shoot elongation and shoot number per explant, followed by KIN and TDZ at concentration 2.0 mg/l. In an earlier study, MS medium supplemented with 2.0 mg/l BAP were found significant for shoot multiplication in *Andrographis paniculata* (Shailaja *et al*., 2020). Equal concentrations of BAP and KIN were found optimum for effective shoots regeneration in *Rubia cordifolia* (Sharma *et al*., 2021). Adding a small concentration of NAA (0.5 mg/l) along with a high concentration of cytokinin significantly enhances explant regeneration, induction and shoot multiplication regeneration. Synergic result of using cytokinin with auxin has been found most promising for direct regeneration in many medicinal plant species like *Brassica juncea* (Guo *et al*., 2005), *Mucuna pruriens* (Faisal *et al*., 2006) and *Vitex trifolia* (Ahmad *et al*., 2013). On increasing concentration of cytokinin leads to vitrification, *i.e*., wilting and yellowing in color (Martin *et al*., 2006; Bhatia *et al*., 2008; Soni and Kaur, 2014). Overdose of auxin increased callus induction at the base of the cotyledonary node.

Half and full strength basal medium along with most frequently used auxins (IAA and IBA) were evaluated at different concentrations (1.0 - 3.0 mg/l) for optimizing rooting response in *in vitro* raised shoots of *V. canescens* for complete plant regeneration. Half strength MS medium fortified with IBA at concentration 2.0 mg/l found to be good for regeneration of healthy and elongated roots as compare to IAA on the same concentration. The effectiveness of IBA for rooting have been documented in many plant species such as *Tylophora indica* (Faisal, Anis 2003), *Aegle marmelos* (Nayak and Behera, 2007), *Streblus asper* (Gadidasu *et al*., 2011), *Viola patrinii* (Chalageri and Babu, 2012) and *Viola serpens* (Jha *et al*., 2020). Present research became successful to acclimatize about 90% regenerated plants back to the environment.

5. Conclusion

A sterilization and direct *in vitro* propagation technique is standardized from cotyledonary node explants of *V. canescens*. We suggest the usage of $HgCl_2$ for five minutes of seed sterilization and MS media augmented with 2.0 mg/l of BAP and 0.5 mg/l of NAA for multiple shoots regeneration from cotyledonary node. The present report contains skilled process for the conservation and direct *in vitro* propagation of a large number of *V. canescens* plants supposed with less somaclonal variation compared with indirect method.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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