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Callogenesis and rhizogenesis of *Viola odorata* L.

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ABSTRACT
Viola odorata L. is a medicinal plant and source of different secondary metabolites. It has been proven that callus culture can be used as an effective method for multiplication of medicinal plants. The aim of this paper was to develop a protocol for callogenesis and rhizogenesis in *V. odorata*. Petiole and leaf were used as explants for callogenesis. Significant callus induction was observed on MS medium supplemented with 2 mg l^{-1} 2,4-D + 2 mg l^{-1} NAA+30 mg l^{-1} adenine sulphate. It was produced hard and embryogenic callus with white and greenish colour. The highest fresh weight of callus was obtained with 2.5 mg l^{-1} 2,4-D + 2.5 mg l^{-1} BAP (8.093 g) and the lowest with 0.5 mg l^{-1} BAP (0.295 g). Besides, the highest dry weight of callus was achieved with 2 mg l^{-1} 2,4-D +2 mg l^{-1} NAA+30 mg l^{-1} adenine sulphate (0.755 g) and the lowest with 0.5 mg l^{-1} TDZ (0.045 g). Roots from callus were induced using IAA and IBA. Maximum number of roots (10.60) and highest root length (2.92 cm) were recorded after 15 to 20 days in culture medium with 1.5 mg l^{-1} IBA. Present study reveals that this protocol could be used for *in vitro* callogenesis and rhizogenesis of *V. odorata*.

Keywords: auxins, callus, cytokinins, *in vitro*

Callogénesis y rizogénesis en *Viola odorata* L.

RESUMEN
Viola odorata L. es una planta medicinal y fuente de diferentes metabolitos secundarios. Se ha demostrado que el cultivo de callos se puede utilizar como un método eficaz para la multiplicación de plantas medicinales. El objetivo de este trabajo fue desarrollar un protocolo para desarrollar callogénesis y rizogénesis en *V. odorata*. Se utilizaron pecíolos y hojas como explantes para la callogénesis. Se observó una inducción significativa de callos en el medio de cultivo MS con 2 mg l^{-1} 2,4-D + 2 mg l^{-1} NAA + 30 mg l^{-1} adenina sulfato. Se produjeron callos duros y embriogénicos con color blanco y verdoso. La masa fresca más elevada de callo se obtuvo con 2.5 mg l^{-1} 2,4-D + 2.5 mg l^{-1} BAP (8.093 g) y la más baja con 0.5 mg l^{-1} BAP (0.295 g). Además, la masa seca más alta se obtuvo en medio de cultivo con 2 mg l^{-1} 2,4-D + 2 mg l^{-1} NAA + 30 mg l^{-1} de sulfato de adenina (0.755 g) y la más baja con 0.5 mg l^{-1} TDZ (0.045 g) . La inducción de raíces del callo se produjo con el uso de AIA e AIB. El número máximo de raíces y la mayor longitud de raíz (2.920 cm) se registró después de 15 a 20 días con 1.5 mg l^{-1} de AIB. El presente estudio revela que este protocolo podría utilizarse para la callogénesis y rizogénesis *in vitro* de *Viola odorata* L.

Palabras clave: auxinas, callo, citoquininas, *in vitro*

INTRODUCTION

Genus *Viola* include 400 species which commonly known as Sweet Violet. It belongs to family *Violaceae*. It is native from Europe and Asia and distributed in Kashmir and (Mabberley, 1987). *Viola odorata* L. is conventionally cultivated by rhizomes but for large propagation seeds are preferred. It is evergreen perennial herb growing about 10 to 15 cm. *Viola odorata* L. is well known for medicinal use in unani and Aurvedic

system. It is considered as rare and endemic parennial plant to Europe and Asia (Mabberley, 1987).

Viola odorata L. is medicinally important plant used in folk therapy for different diseases like diabetes, cancer, post-operative tumor, bronchitis and common digestive disorders. In traditional medicine it has been used in insomnia, low blood pressure (Duke *et al.,* 2002) and anxiety (Keville, 1991). Different groups of phytochemical compounds have been isolated from *Viola odorata* L. like glycosides, triterpenoid, flavonoid, cyclotides and alkaloids (Darwin, 2010).

Plant biodiversity and natural resources are reducing day by day because of over exploitation on the earth, especially medicinal plants for pharmaceutical purpose. So, to maintain the biodiversity, conservation of medicinal plants is required (Cragg and Newman, 2001). *Viola odorata* L. is one of them. By using the active constituents from *Viola odorata* L. herbal drug ´ODORATA´ has been formulated into syrup Hence, the development of tissue culture methods to achieve the production of true-to-type plants and to improve species using genetic engineering techniques has gained in importance. Tissue culture has now become a well-established technique for the conservation of rare and endangered medicinal plants (Neelima *et al*., 2010).

Callus induction of *Viola odorata* L. was studied by using MS media supplemented with different growth regulators. When MS media supplemented with 2,4-D (2.5 mg l^{-1}) + NAA (2 mg l^{-1}) highest callus frequency (80%) was observed on leaf explant. Also on the media containing 2,4-D (2.5 mg l^{-1}) + NAA (1 mg l^{-1}), 2,4-D (2.5 mg l^{-1}) + NAA (1.5 mg l^{-1}) and 2,4-D (2.5 mg l^{-1}) + NAA (3 mg l^{-1}) the frequency of callus induction was high (Mokhtari *et al.*, 2015). Callus induction was higher in leaves compared to stem and petiole. Young and green explant responded more than older explant. Besides, when half strength MS media supplemented with BA (2.5 mg l^{-1}) and 2, 4-D (0.15 mg l^{-1}) induced dark green, compact and larger callus within 40 days (Naeem *et al*., 2013). It has been proven that callus culture can be used as an effective method for multiplication of medicinal plants (Castillo and Jordan, 1997). The callus culture has several applications. Antioxidants play an important role to neutralize free radicals, prevention of cancer, neurodegenerative diseases and cardiovascular diseases. Medicinal plants are the source of antioxidants (Gerber *et al*., 2002; Di and Esposito, 2003). For instance, an assay of the antioxidant potential of the *in vitro* grown callus and the wild plant extract was determined by DPPH (α, α-diphenyl-β-picrylhydrazyl) method shown that the antioxidant activity of *in vitro* formed callus is higher than that of wild plant (Naeem, 2013).

Considering the importance of *V. odorata* as medicinal plant and source of different secondary metabolites which have medicinal values, the aim of this paper was to develop a protocol for callogenesis and rhizogenesis in *V. odorata*. In this regard there no prior studies about rhizogenesis from callus in this specie.

MATERIALS AND METHODS

Collection of plant material

Young and healthy plants of *Viola odorata* L. were collected from Sanjeevini vatika of Department of Horticulture, University of Agriculture Science, GKVK Bangalore - 65, India.

Preparation of explants

Young and healthy leaf and petiole of *V. odorata* were used as explants. The excised leaf and petioles were washed in running tap water for at least 20 minutes and later with distilled water. After that, petiole and leaf were separately treated with 0.3% Bavistin and one or two drops of Tween 20, during 15 min. Later, the explants were rinsed with sterile water thrice, each for 5 min and it were disinfected with 0.1% mercuric chloride solution for 5 min. Finally, the explants washed with sterile water twice for removing the traces of $HgCl_2$.

Callus formation and rooting

To induce callus formation from explants and its rooting, the MS media (Murashige and Skoog, 1962) was supplemented with cytokinins 6-Benzylaminopurine (BAP) and

Thidiazuron (TDZ) and auxins 2,4-Dichlorophenoxyacetic acid (2,4-D), Napthalene acetic acid (NAA), Indole butyric acid (IBA) and Indol acetic acid (IAA). pH of the medium was adjusted at 5.8 and prior to sterilization by autoclave and 0.6% agar agar was added for solidification (Himedia, Mumbai). Then, the media were autoclaving at 1.05 kg cm^{-2} (15 psi) of pressure for 30 min.

The explants were cut into 1.0 cm small pieces, cultured on MS basal medium alone, and supplemented with different types of auxins (0.5, 1.5, 2.5 mg l^{-1} 2,4-D or 1.0, 2.0, 3.0 mg l^{-1} NAA) and cytokinins (0.5, 1.5, 2.5 mg l^{-1} BAP or 0.5, 1.5, 2.5 mg l^{-1} TDZ). Obtained callus was transferred to rooting MS media with IAA or IBA (0.5, 1.0, 1.5, 2.0 mg l^{-1} IAA or 0.5, 1.0, 1.5, 2.0 mg l^{-1} IBA).

The explant for callus formation and callus for rooting induction were incubated at 25 ± 2 °C and photoperiod of 16 h light. The observations were recorded after 60 days of culture (callus induction) and 40 days (root induction). The experiment consisted of 15 treatments. Each treatment included two explant per bottle and they was replicated 10 times. Details regarding color of callus, type, number of days for callus induction, fresh weight of callus (g) and dry weight of callus (g), number of days for rooting induction, number of root and root length (cm) were recorded.

Statistical analysis

The results were analyzed with one way analysis of variance (ANOVA) ($t \leq 0.05$) using completely randomized design (Panse and Sukhatme, 1985). The difference between treatments were compared with LSD (least significance different). The standard error of the mean and critical difference were calculated using the formula $SE(m) \pm = \sqrt{2SEM/r}$ and CD (0.05) = SE (d) x table value of t (0.05) at error degree of freedom.

RESULTS AND DISCUSSION

The study showed that addition of plant growth regulators (PGR) in the MS medium at different concentrations induced several responses in leaf and petiole explants. All the PGR induced calli formation with different characteristics but, different concentration of the same PGR induced callus with the same characteristics.

Different concentration of 2,4-D alone produced friable and translucent callus with creamish colour but in different days. The maximum (2.5 mg l^{-1}) caused the earliest callus induction (Figure 1, Table 1). In this sense, Narayani *et al.* (2017) found that half MS supplemented with 1.5 mg l^{-1} 2,4-D produced beige compact callus from petiole explant after 100 days of culture, while leaf explants produced beige friable callus within 120 days. In this work, the time for callus induction in all treatments was shorter.

On the other hand, the use of different concentration of BAP resulted in hard and brown callus. BAP at 1.5 mg l^{-1} induced callus earliest (Figure 1, Table 1). Different concentration of TDZ produced hard and embryogenic callus with green colour (Figure 1). The combination of PGR 2.5 mg l^{-1} 2,4-D + 2.5 mg l^{-1} BAP induced whitish brown compact nodular callus in 28-30 days. Naeem *et al.* (2013) reported that 2.5 mg l^{-1} BAP + 0.15 mg l^{-1} 2,4-D induced dark green compact callus in 40-45 days. White and greenish, hard and embryogenic callus was induced by 2.0 mg l^{-1} 2,4-D + 30 mg l^{-1} adenine sulphate with NAA (1.0, 2.0, 3.0 mg l^{-1}) (Figure 1, Table 1). In previous experiment, in *in vitro* regeneration of *Viola patrinii*, combination of 16.12 µM NAA with 13.33 µM BA resulted in maximum callus induction in terms of the percentage of calli (Gururaj and Uddagiri, 2012). Other authors demonstrated, after one month of culture on MS + 2 mg l^{-1} KIN + 2 mg l^{-1} 2,4-D callus induction medium, yellowish amorphous callus tissue proliferated on the cut edges of petiole explants and on the cut edges and surface of leaf explants but after two month of culture the response rate for both explant types was high, reaching 84.9% for petiole and 73.4% for leaf (Blazej *et al*., 2014).

Combined 2,4-D and BAP showed highest callus fresh weight (8.093 g) (2.5 mg l^{-1} 2,4-D+ 2.5 mg l^{-1} BAP) while lowest (0.613 g) was recorded with 2.5 mg l^{-1} 2,4-D+ 0.5 mg l^{-1} BAP (Table 2). In the case of dry weight, 2,4-D combined with BAP (2.5 mg l^{-1} 2,4-D+ 2.5 mg l^{-1} BAP) showed highest value (0.073 g) while lowest (0.051 g) was obtained with 2.5 mg l^{-1} 2,4-D+ 0.5 mg l^{-1} BAP (Table 2).

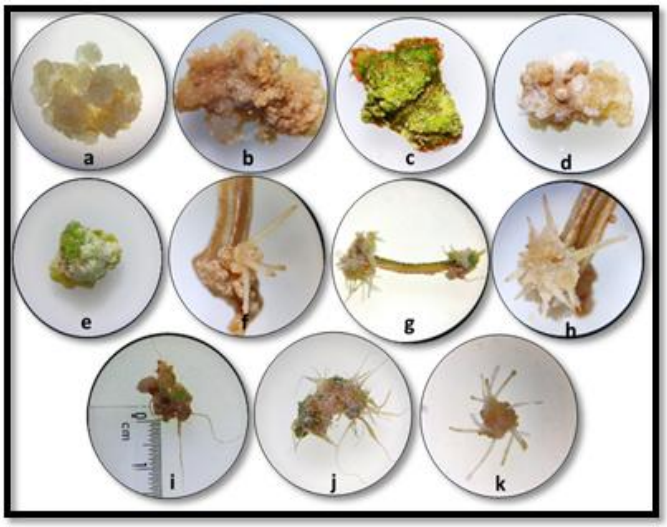

Figure 1. Callogenesis and rhizogenesis of *Viola odorata* L. (a) Creamish and translucent callus on 2.5 mg l^{-1} 2,4-D, (b) Brown callus on 2.5 mg l^{-1} BAP, (C) Green callus on 2.5 mg l^{-1} TDZ, (d) Brown whitish callus on 2.5 mg l^{-1} BAP and 2.5 mg l^{-1} 2,4-D, (e) Whitish green callus on 2 mg l^{-1} NAA and 2 mg l^{-1} 2,4-D + 30 mg l^{-1} adenine sulphate, (f) Induction of root on 0.5 mg l^{-1} IAA from callus of petiole, (g) Induction of root on 1.5 mg l^{-1} IBA from callus of petiole, (h) Induction of root on 1 mg l^{-1} IAA from callus of petiole, (i) Induction of root on 1.5 mg l^{-1} IAA from callus of leaf, (j) Induction of root on 1.5 mg l^{-1} IBA from callus of leaf, (k) Induction of root on 2 mg l^{-1} IBA from callus of petiole.

Table 1. Effect of BA, NAA, 2,4-D and TDZ on callus induction of *Viola odorata* L., after two months of *in vitro* culture in media with different concentration.

Treatment	Colour of callus	Type of callus	No. of days for callus induction
T_1 : 0.5 mg l^{-1} 2,4-D	Creamish	Friable and translucent	18-30
T_2 : 1.5 mg l^{-1} 2,4-D	Creamish	Friable and translucent	17-23
T_3 : 2.5 mg l^{-1} 2,4-D	Creamish	Friable and translucent	15-27
T_4 : 0.5 mg l^{-1} BAP	Brown	Hard	24-30
T_5 : 1.5 mg l^{-1} BAP	Brown	Hard	22-25
T_6 : 2.5 mg l^{-1} BAP	Brown	Hard	28-30
T_7 : 0.5 mg l^{-1} TDZ	Green	Hard and embryogenic	35-40
T_8 : 1.5 mg l^{-1}TDZ	Green	Hard and embryogenic	37-45
T_9 : 2.5 mg l^{-1}TDZ	Green	Hard and embryogenic	48-52
T_{10} : 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP	Whitish Brown	Compact and nodular	21-25
T_{11} : 2.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BAP	Whitish Brown	Compact and nodular	23-27
T_{12} : 2.5 mg l^{-1} 2,4-D + 2.5 mg l^{-1}BAP	Whitish Brown	Compact and nodular	28-30
T_{13} : 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA + 30 mg l^{-1} adenine sulphate	White and Greenish	Hard and embryogenic	17-25
T_{14} : 2.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} NAA + 30 mg l^{-1} adenine sulphate	White and Greenish	Hard and embryogenic	15-20
T_{15} : 2.0 mg l^{-1} 2,4-D + 3.0 mg l^{-1} NAA + 30 mg l^{-1} adenine sulphate	White and Greenish	Hard and embryogenic	18-27

Table 2. Fresh and dry weight of callus of *Viola odorata* L. (leaves and petioles), after 60 days of *in vitro* culture in media with different growth regulators.

Treatments	Fresh weight of callus (g)	Dry weight of callus (g)
T_1 : 0.5 mg l^{-1} 2,4-D	0.385	0.083
T_2 : 1.5 mg l^{-1} 2,4-D	0.512	0.044
T_3 : 2.5 mg l^{-1} 2,4-D	1.028	0.149
T_4 : 0.5 mg l^{-1} BAP	0.295	0.041
T_5 : 1.5 mg l^{-1} BAP	0.548	0.050
T_6 : 2.5 mg l^{-1} BAP	0.725	0.068
T_7 : 0.5 mg l^{-1} TDZ	0.452	0.045
T_8 : 1.5 mg l^{-1} TDZ	0.441	0.040
T_9 : 2.5 mg l^{-1} TDZ	1.041	0.075
T_{10} : 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP	0.613	0.059
T_{11} : 2.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BAP	0.717	0.073
T_{12} : 2.5 mg l^{-1} 2,4-D + 2.5 mg l^{-1} BAP	8.093	0.071
T_{13} : 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA + 30 mg l^{-1} adenine sulphate	0.920	0.086
T_{14} : 2.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} NAA + 30 mg l^{-1} adenine sulphate	2.012	0.755
T_{15} : 2.0 mg l^{-1} 2,4-D + 3.0 mg l^{-1} NAA + 30 mg l^{-1} adenine sulphate	1.310	0.092
C.D.	0.428	0.201
SE(m)	0.151	0.071

CD: Critical Difference, SE (m): Standard error of mean

Table 3. Number of root, root length and number of days for root induction of *Viola odorata* L. from callus, after 40 days of *in vitro* culture in media with different growth regulators.

Treatments	No. of root per callus	Root length (cm)	No. of days for root induction
T_1 : 0.5 mg l^{-1} IAA	4.600	1.600	23-24
T_2 : 1.0 mg l^{-1} IAA	7.800	2.480	19-22
T_3 : 1.5 mg l^{-1} IAA	5.400	1.520	17-24
T_4 : 2.0 mg l^{-1} IAA	4.000	2.240	24-27
T_5 : 0.5 mg l^{-1} IBA	4.200	1.620	20-25
T_6 : 1.0 mg l^{-1} IBA	4.200	1.760	18-25
T_7 : 1.5 mg l^{-1} IBA	10.60	2.920	15-20
T_8 : 2.0 mg l^{-1} IBA	3.200	1.240	20-27
C.D.	2.297	N/A	
SE(m)	0.794	0.499	

CD: Critical Difference, SE (m): Standard error of mean

Among the different concentration of IAA, maximum number of root per callus (7.800) and root length (2.480 cm) were obtained with 1.0 mg l^{-1} after 19-20 days of culture (Figure 1). Also, different concentration of IBA induced roots in callus. Maximum number of roots (10.60) and root length (2.920 cm) were observed with 1.5 mg l^{-1} IBA (Figure 1, Table 3). The results was in accordance with previous reports in other *Viola* specie such as *V. serpens* Wall. Vishwakarma *et al.* (2013) inoculated petiole explants on full strength and half strength MS media supplemented with IAA and IBA. Both PGR, IAA and IBA induced callus rooting, but percentage of rooting was less in IAA.

This study reveals *in vitro* callogenesis and rhizogenesis of *Viola odorata* L. Experimental

results showed that among all treatments, 2,4-D used in higher concentration resulted in earliest callus induction. Besides, combination of 2.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} NAA is needed for production of callus with embryogenic structures within a short time period. TDZ also produced embryogenic callus but, it takes more time.

CONCLUSIONS

Viola odorata L. is important medicinal and ornamental herb used in Unani medicine. It can be propagated by tissue culture techniques. The use of leaf and petiole explants for callus induction and rhizogenesis in culture medium supplemented with several combinations of growth regulators achieve callus and roots. For producing large amount of callus, 2.5 mg l^{-1} 2,4-D+ 2.5 mg l^{-1} BAP is recommended. Present study reveals that this protocol could be used for *in vitro* callogenesis and rhizogenesis of *V. odorata*.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Conceptualization KVH and SRB, Methodology KVH, writing-original draft KVH, writing –review & editing KVH and SRB.

REFERENCES

Blazej S, Elwira S, Marta S, Micha R, Justyna B, Aneta S, Ulf G, Elzbieta K (2014) Micropropagation of *Viola uliginosa* (Violaceae) for endangered species conservation and for somaclonal variation-enhanced cyclotide biosynthesis. Plant Cell Tiss Organ Cult 120: 179–190

Castillo PD, Jordan A (1997) Thidiazuron induced high frequency shoot organogenesis from leaf derived callus of medicinal climbers, *Tylophora indica* L. (Burm F.) Merrill. *In Vitro* Cell Dev Biol Plant 41: 124-128

Cragg GM, Newman DJ (2001) Natural product drug discovery in the next millennium. Pharm Biol 39 Suppl 1: 8-17

Darwin DM (2010) Anticancer and chemosensitizing abilities of cycloviolacin 02 from *Viola odorata* L. and psyle cyclotides from *Psychotria leptothyrsa*. Biopoly 40: 617-25

Di MV, Esposito E (2003) Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Current Drug Target CNS and Neurological Disorder 2: 95-107

Duke JA, Bogenschutz-Godwin MJ, Ducelliar J, Duke PAK (2002) Sweet Violet (*Viola odorata* L.). Handbook of Medicinal Herbs, 2nd edition. CRC Press, Boca Raton

Gerber M, Boutron M, Hercberg S, Riboli E, Scalbert A, Siess M (2002) Food and cancer. State of the art about the protective effect of fruits and vegetables. Bull Cancer 89: 293-312

Gururaj C, Uddagiri V (2012) *In vitro* plant regeneration via petiole callus of *Viola patrinii* and genetic fidelity assessment using RAPD markers. Turk J Bot 36: 358-368

Keville K (1991) *Viola odorata* L. En: Rosart S (Ed). Illustrated Herb Encyclopedia, pp. 207. Michael Friedman publishing group Inc, New York

Mabberley D (1987) The Plant Book A portable dictionary of the vascular plants. Camb Univ Press, Cambridge; ISBN: 0521414210

Mokhtari A, Otroshy M, Barekat T (2015) Plant regeneration through callus induction on medicinal herb *Viola odorata* - role of plant growth regulators and explants. Agriculture and Forestry 61(3): 161-170; doi: 10.17707/ AgricultForest.61.3.16

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-97

Naeem M, Naveed I, Muhammad S, Naqvi S (2013) Standardization of tissue culture

conditions and estimation of free scavenging activity in *Viola Odorata* L.. Pak J Bot 45(1): 197-202

Narayani M, Chadha A, Srivastava S (2017) Callus and cell suspension culture of *Viola odorata* as *in vitro* production platforms of known and novel cyclotides. Plant cell, Tissue and Organ culture 130(2): 289-299

Neelima R, Barkha K, Dipika P, Baljinder K, Sarita A (2010) Conservation of biodiversity of highly important medicinal plants of India through tissue culture technology - a review. Agric Biol J N Am 1(5): 827-833

Panse VG, Sukhatme PV (1985) Statistical methods for agricultural workers II[nd] enlarged. Ed ICAR, New Delhi

Vishwakarma UR, Gurav AM, Sharma PC (2013) Regeneration of multiple shoots from petiole callus of *Viola serpens* Wall. Pharmacognosy Res 5: 86-92

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