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EFFECT OF 2,4-D AND CYTOKININS ON CALLUS INDUCTION IN DIFFERENT EXPLANTS OF *VIOLA CANESCENS* WALL. EX, ROXB.

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Abstract

Viola canescens Wall.ex, Roxb., is a perennial, soft pubescent herb of *Viola*ceae family, commonly known as Himalayan white violet/ Banfasa. One of the potent medicinal plant used in both codified and non codified system of traditional medicine. Potted plants of *Viola canescens* were used as mother source of explants. The explants (leaf & petiole) were inoculated on the agar solidified MS media fortified with different concentrations of 2,4-D alone or in combination with varying concentrations of cytokinins (BAP, Kn). Callus induction frequency differs significantly in both explants and high in leaf than in petiole. Present study reveals the CIF of both explants and the influence of different PGRs on the growth of calli.

Key words : Calli, culture medium, organogenesis, 2,4-D, BAP, Kn, *Viola canescens*.

Abbreviations : MS- Murashige and Skoog, **2,4-D**- 2,4 dichlorophenoxyacetic Acid, **BAP**- 6- Benzylamino purine, **Kn** - Kinetin, **v/v** - volume/ volume, **w/v** - weight/volume.

Introduction

Viola the largest genus of *Viola*ceae family with 525-600 species also known as pansies, which in horticulture means multi-coloured large flowered cultivars (Mann *et al*., 2016). *Viola canescens* commonly known as Banfasa, soft pubescent tufted perennial herb (plate A & B), has its high potential in ethnomedicinal preparations and used to cure cough, cold, fever, jaundice, malaria and also given as anti-cancerous drug. Plant also used for treating several nervous disorders (Adnan *et al*., 2010). Ethnomedicinal preparation are developed by communities across the different parts of world which accumulate this knowledge with the long experience of their life and passed as an important heritage from one generation to another generation (Bisht and Khajuria, 2014; Khajuria and Bisht, 2017). Phytochemicals from *Viola canescens* show presence of different groups of heterogeneous chemical compounds *viz* methyl salicylate, an alkaloid violin, saponins & glucosides (Rana *et al*., 2010), having antimalarial activity (Verma *et al*., 2011), antifungal activity (Rawal *et al*., 2015). Herb with Regional Himalaya as a center of origin is widely distributed between 1400-2400 AMSL in Himalaya and known by different names in different places i.e., Banfasha, Vanksha in Jammu and Kashmir; Ratmundi, Gugluphul & Banaksha in Himachal Pradesh. In Nepal, *Viola* locally called as Ghatteghaans. Due to high medicinal potency of *Viola canescens* it is being exploited unscientifically at high rate for medicinal purposes without any conservative means. Due to large heavy indiscriminate collection from wild, this plant is rapidly disappearing from their natural habitat as observed at Swat valley of Ajad Kashmir (Hamayun *et al*., 2006), Malam jabba valley of swat Pakistan etc.

Micropropagtion work on other species of *Viola viz Viola pilosa* (Soni *et al*., 2013), *Viola ordoata* (Naeem *et al*., 2013), *Viola uliginosa* (Slazak *et al*., 2015), *Viola patrinii* (Chalageri and Babu, 2012) has been reported. Hence there is need to carry out tissue culture work for mass multiplication and conservation of this plant to fill the gap between demand and supply for commercial use. Effective protocol for callus induction for generating indirect organogenesis seems to be a profitable alternative technique.

Materials and Methods

Plant material

Plants of *Viola canescens* were collected from its natural habitat (forest of Pauri Garhwal) and were

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maintained in earthen pots in department of Botany HNBGU Campus Pauri containing mixture of sand, soil and farmyard manure (1:1:1) v/v. The potted plants are then used as the mother source for obtaining explants for callus induction throughout the course of study

Establishment of aseptic cultures: The petiole and leaf part of plants were washed thoroughly with 1% (v/v) Labolene detergent for 10 min with continuous stirring and then in a gentle continuous flow of tap water for 15 min, followed by 0.1% w/v bavistin for 45 min and later surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) with two drops of Tween 20 per 100 ml of solution for 2 min. Following surface sterilization, explants were rinsed 4–5 times with sterile distilled water to remove all traces of $HgCl_2$ and were blotted on sterile filter paper discs.

Growth conditions : For all the experiments pH of the medium was adjusted to 5.86±0.1 prior to autoclaving at 15 lbs at 121°C for 15 min. The culture conditions (temperature 25±2°C, 16 h photoperiod using cool white fluorescent light, and 1500–2000 lux light intensity) were maintained throughout the course of study.

Culture measurement : Callus induction frequency for both explants was assessed during the period of 6 to 25 days after inoculation. Proliferation rate of calli were visually observed and scored as poor, good, better and best plate (F,G &H).

Table 1 : Effect of 2,4-D on callus induction from different explants.

S. no.	2,4-D (µM)	Petiole	Leaf
1.	0.00	16.67±0.38	26.67±0.47
2.	2.26	26.67±0.46	40.00±050
3.	4.52	40.00±0.51	43.33±0.52
4.	6.78	43.33±0.52	30.00±0.47
5.	9.04	43.33±0.51	23.33±0.41

Callus induction from petiole and leaf explants of *V. canescens* (values are mean ± S.D, $n = 10$, with each experiment repeated thrice).

Table 2 : Effect of Kn and BAP on callus induction from different explants.

S. no.	Kn (µM)	Petiole	Leaf	BAP (µM)	Petiole	Leaf
1.	4.64	0.00	0.00	4.44	0.00	0.00
2.	6.96	0.00	0.00	6.66	0.00	0.00
3.	9.28	0.00	0.00	8.88	0.00	06.67±0.21

Callus induction from petiole and leaf explants of *V. canescens* (values are mean ± S.D, $n = 10$, with each experiment repeated thrice).

Statistical analysis : Each treatment throughout the experiment consist of 10 explants and repeated thrice, S.D and correlation was calculated to justified results.

Results

Varying concentrations of Auxin (2,4-D) and cytokinins (Kn or BAP) alone or in combination were tried to induce calli in different explants (Leaf & petiole) and develop a reliable protocol for callus induction, which may be used for organogenesis, secondary metabolite production etc. The sterilized explants were inoculated on agar solidified MS medium with or without growth regulators. To analyze the effect of growth regulators on callus induction, different sets *i.e.*, MS medium without growth regulators, MS medium with 2,4-D, MS medium with BAP, MS medium with Kn, MS medium with 2,4-D and BAP and MS medium with 2,4-D and Kn were tried. Callus induction started from 6-13 days after inoculation in both explants plate (C, D & E). The explants first showed swelling at the cut ends of explants after that the swollen part burst to release calli mass. Some explants failed to respond and dried up or some time turned brown.

MS medium without growth regulators and with BAP alone did not show any callus induction, but MS medium fortified with Kn alone at 9.28 µM showed callus induction 6.67±0.21 with poor callus proliferation. When combinations of growth regulators was tried to induce callus, callus induction frequency increased, with maximum value 96.67±0.11 when MS medium fortified with 9.04 µM 2,4-D with 9.28 µM Kn for petiole explant and 96.67±0.11 when MS medium was fortified with 6.78 µM 2,4D with 6.96 µM Kn for leaf explant, leaf explant responded equally well when 6.78 µM 2,4D with 9.28 µM mg Kn was tried.

In case of petiole best calli proliferation was observed when media was fortified with 2,4-D and BAP in 6.78:8.88 µM combination but 9.04:6.66 µM, 9.04:8.88 µM & 11.3:4.44 µM concentrations also responded to be good while for leaf explants 2,4-D and BAP in 6.78:9.28 µM combination proved good for calli proliferation. When MS media was fortified with Kn, best calli proliferation was achieved in concentration 6.78:6.96 µM although 6.78:9.28 µM, 9.04:6.96 µM, 11.3:4.64 µM combinations were equally good for Petiole explants and 6.78:4.64 µM is the best concentration for calli proliferation in case of leaf explant while 6.78:6.96 µM, 6.78:9.28 µM & 9.04:6.96 µM concentrations responded equally good for calli proliferation in case of leaf explants.

Discussion

Callus induction is an initial step during indirect organogenesis and the obtained calli may be used for

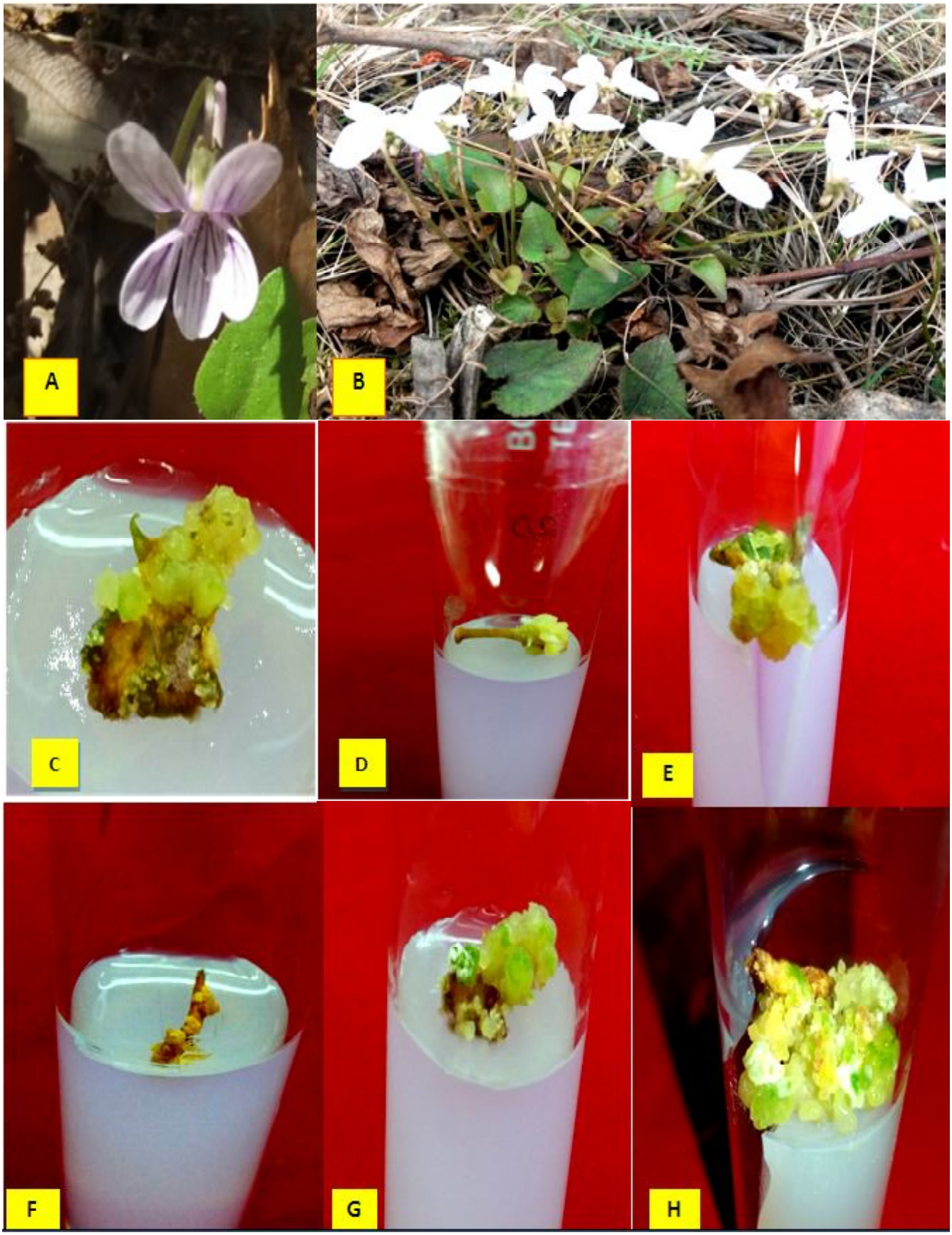

Plate : *Viola canescens* Wall ex Roxb.

Table 3 : Effect of Auxin and cytokinins on callus induction from Petiole explants.

2,4-D(µM)	BAP(µM)				Kn(µM)		
	0.00	4.44	6.66	8.88	4.64	6.96	9.28
0.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
2.26	16.67±0.38	36.67±0.51	53.33±0.50	56.67±0.49	36.67±0.51	43.33±0.49	56.67±0.49
4.52	26.67±0.46	56.67±0.51	60.00±0.51	70.00±0.47	46.67±0.50	56.67±0.51	66.67±0.48
6.78	40.00±0.51	63.33±0.48	73.33±0.45	93.33±0.14	63.33±0.48	93.33±0.14	93.33±0.14
9.04	43.33±0.52	73.33±0.43	86.66±0.35	90.00±0.25	83.33±0.30	93.33±0.21	96.67±0.11
11.3	43.33±0.51	76.67±0.44	86.33±0.27	86.66±0.27	83.33±0.37	90.00±0.25	90.00±0.25

Callus Induction from Petiole explants of *V. canescens* (values are mean ± S.D, $n = 10$, with each experiment repeated thrice).

Table 4 : Effect of auxin and cytokinins on proliferation of petiole explants.

2,4-D(µM)	BAP(µM)				Kn(µM)		
	0.00	4.44	6.66	8.88	4.64	6.96	9.28
0.00	-	-	-	-	-	-	-
2.26	+	++	++	++	++	++	++
4.52	+	++	+++	+++	++	+++	+++
6.78	+	+++	+++	++++	+++	++++	++++
9.04	++	+++	++++	++++	+++	++++	+++
11.3	++	++++	+++	+++	++++	+++	+++

- No callus, + Poor, ++ Good, +++ Better, ++++ Best

Callus proliferation from Petiole explants of *V. canescens* (with each experiment repeated thrice).

Table 5 : Effect of auxin and cytokinins on callus induction from leaf explants.

2,4-D(µM)	BAP(µM)				Kn(µM)		
	0.00	4.44	6.66	8.88	4.64	6.96	9.28
0.00	0.00	0.00	0.00	0.00	0.00	6.67±0.21	0.00
2.26	26.67±0.47	36.66±0.51	36.67±0.49	56.67±0.51	43.33±0.52	46.67±0.49	63.33±0.48
4.52	40.00±050	56.67±0.50	73.33±0.44	76.67±0.42	73.33±0.46	70.00±0.46	83.33±0.28
6.78	43.33±0.52	80.00±0.41	93.33±0.21	90.00±0.32	86.67±0.35	96.67±0.11	96.67±0.11
9.04	30.00±0.47	63.33±0.51	66.67±0.45	60.00±0.51	60.00±0.51	56.67±0.51	63.33±0.46
11.3	23.33±0.41	56.66±0.49	53.33±0.51	53.33±0.50	56.67±0.52	63.33±0.49	56.67±0.51

Callus induction from Leaf explants of *V. canescens* (values are mean ± SD, $n = 10$, with each experiment repeated thrice).

Table 6 : Effect of Auxin and cytokinins on callus proliferation from leaf explants.

2,4-D(µM)	Kn(µM)				BAP(µM)		
	0.00	4.64	6.96	9.28	4.44	6.66	8.88
0.00	-	-	-	-	-	+	-
2.26	+	++	+++	++	++	++	++
4.52	+	++	+++	+++	++	++	+++
6.78	+	++++	++++	++++	+++	+++	++++
9.04	++	+++	++++	+++	++++	++++	++++
11.3	+	+++	+++	+++	+++	+++	++++

- No callus, + Poor, ++ Good, +++ Better, ++++ Best.

Callus proliferation from Leaf explants of *V. canescens* (with each experiment repeated thrice).

Table 7 : Correlations among different growth regulators.

	Conc A* Cytokinins	Conc B* 2,4-D	BAP+2,4-D (Petiole)	Kn+2,4-D (Petiole)	BAP+2,4-D (Leaf)	Kn+2,4-D (Leaf)
Conc A*	1					
Conc B*	0.000	1				
BAP+2,4-D (Petiole)	0.467	.808**	1			
Kn+2,4-D (Petiole)	0.218	0.145	0.425	1		
BAP+2,4-D (Leaf)	0.363	.830**	.946**	0.481	1	
Kn+2,4-D (Leaf)	0.221	0.000	0.302	.925**	0.310	1

** Correlation is significant at the 0.01 level (2-tailed).

* Concentration A(BAP 4.44 µM, 6.66 µM & 8.88 µM and Kn 4.64 µM, 6.96 µM & 9.28 µM), Concentration B (2,4-D 2.26 µM-11.3 µM as mentioned in these tables).

production of secondary metabolite and to study mineral composition (Saric *et al*., 1997) etc. Callus induction frequency in *Viola canescens* is under the great influence of growth regulators without which no calli is observed. It is evident that 2,4-D alone is able to induce calli in both explants with maximum callus induction frequency 43.33± 0.52 for petiole explant and when MS medium fortified with 6.78 µM & 9.04 µM respectively. Obtained calli were studied for some morphological features, produced calli was cream, yellow, yellow-green, fluorescent green in colour with compact, nodular and fibril morphology, which changes its colour to brown and then finally black by the end of 120 days. From this study, it is evident that with the increase in Auxin concentration from 2.26-9.04 µM in case of petiole explant and 2.26-6.78 µM in case of leaf explant there is an increase in callus induction frequency which later decreases with further increase in Auxin concentration in both explants these finding are in agreement with earlier finding of Malik *et al*. (2004). Thus in present study, it was concluded that lower concentration of 2,4-D alone or combination with cytokinin is very promising for callus induction but at high concentration 2,4-D has retarding effect on callus induction from explants of *V. canescens*, leaf showed more sensitivity or retarding effect than petiole for callus induction when exposed to higher concentration.

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