# Regeneration of multiple shoots from petiole callus of *Viola serpens* Wall

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## ABSTRACT

Experiments were conducted to develop methodology for *in vitro* propagation and rapid multiplication of *Viola serpens* Wall. using petiole explant. The MS medium supplemented with 2, 4-D (6.78 µm) was found most suitable for callus induction in petiole explant. The best growth response and higher rate of shoot regeneration from petiole callus was observed on MS medium containing BAP (11.10 µm) as the average number of shoots could be increased to 36.4 on fourth successive subculturing. Higher rooting response with larger number of roots were observed in shoots inoculated on the half-strength MS medium supplemented with IBA (19.68 µm).

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**Key words:** Callus, growth regulators, multiple shoots, petiole, *Viola serpens*

## INTRODUCTION

*Viola serpens* Wall. belongs to family Violaceae and commonly known as "Banafsha." It is a small glabrous, perennial herb, which is found throughout India in moist woods and hilly districts. It is also found in China, Java, Ceylon, Philippines, and Thailand up to an altitude of 2000 m. In India, it is distributed in the Himalayan region, hills of Meghalaya, Nagaland, and Manipur.[1-4] It is also found in Ganjam hills of Orissa,[5-6] Himachal Pradesh, Tehri Garhwal in Uttar Pradesh,[7] Karnataka,[8] and Tamilnadu.[9]

The whole plant is medicinally useful. It is aperients, antiseptic, antipyretic, cooling, demulcent, diaphoretic, diuretic, emetic, emollient, expectorant, febrifuge, and purgative in action. It is useful in asthma, bleeding piles, cancer of throat, constipation, cough, fever, headache, and skin diseases.[10-11] This species also yields "Banafsha" of the bazaars and is considered to have medicinal properties similar to those of *Viola odorata* L. A decoction of flowers is administered for improving the complexion. It is also used in bilious and pulmonary affections. In Unani system of medicine, this plant is the main ingredient of "Joshanda" consisting mixture of drugs, used mainly for cough and cold in the form of decoction. A medicinal oil called "Raughan -e- banafsha", "Banafshadi-kwath," "Gulkand banafsha", and "Banafsha syrups" are prepared fromit.[12-13] The plant is propagated by divisions, cuttings or seeds. It grows well in a cool and moist climate, but exposure to heavy or frequent rains is fatal for its blooming. The soil and climatic conditions of Tehri-Garhwal (Uttar Pradesh), Shimla, Kullu, Kinnaur, Sirmour, and Chamba (Himachal Pradesh) are conducive for large-scale cultivation of this plant. However, commercial cultivation of this species has not been taken up so far. The localities traditionally known as rich are exploited heavily and this has adversely affected the natural regeneration, the result being that the plant is facing much depletion in nature. Further regular collection of the drug in an area without periodical resting time and lack of maintenance have increased their cost of collection. The quantity of *Viola serpens* Wall. exported from Himachal Pradesh has declined from 1991 to 1992.[14] Therefore, it is necessary to provide protection to these areas and to permit natural regeneration and simultaneously to bring out the plant under cultivation. Department of Indian Systems of Medicine and Homeopathy of the Ministry of Health and Family Welfare, Government of India has formulated a scheme for cultivation and development of medicinal plants wherein they have identified some species, *Viola serpens* Wall. being one of them, for promoting their

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cultivation in order to reduce pressure on their natural habitat and to meet the demand of the industry involved in producing the Indian Systems of Medicine.[15]

There is no report on tissue culture studies of *Viola serpens* Wall., though such studies on other species of *Viola* have been reported, such as *Viola odorata* L.,[16] *Viola patrinii* DC.,[17] and Viola wittrockiana.[18] Therefore, efforts were made to develop methodology for *in vitro* propagation of *Viola serpens* Wall.

## MATERIALS AND METHODS

Young plants of *Viola serpens* Wall. (Banafsha) were procured from Ranikhet and grown at Jawaharlal Nehru Ayurvedic Medicinal Plants Garden and Herbarium, Pune, in green house were used for *in vitro* propagation experiments. The excised leaf petioles of 1 cm length were washed in tap water for 15 min, then soaked in 5% Teepol solution for 5 min and washed again with tap water. The explants were then treated with 0.1% $HgCl_2$ solution for 1 min followed by several times washing with sterile water under aseptic conditions. These petiole explants were ruptured in the groove region by taking sharp cut with the surgical blade and inoculated on MS medium with varying concentrations of Kn, BAP, IAA, IBA, NAA and 2, 4-D, for induction of callus. The pH of medium was adjusted to 5.7 with 1N NaOH/1N HCl, before addition of 0.8% agar and autoclaved at 15 lb/inch$^2$ pressure and 121°C temperature for 20 min. The callus was subcultured on MS medium supplemented with BAP and Kn, singly, in varying concentrations for differentiation. Well-developed shoots (2-3 cm) from regenerating explants were excised and rooted on half- and full-strength MS plain medium and medium supplemented with IAA and IBA, singly, in different concentrations. The cultures were maintained at 25°C ± 2°C with 8/16-h (light/dark) photoperiod provided through white fluorescent tubes with light intensity of 3000 lux.

Observations were made every day. A mean of three replicates was taken for each explant, to see the callus growth under different nutritive conditions. The callus cultures were harvested and weighed after a growth period of 28 days. Dry weight was determined after 7 days drying in an incubator at 40°C. In differentiation studies, the number of shoots developed in a particular treatment was used as an index for comparison. Data obtained from the experiments were analyzed for mean and standard error using software SYSTAT 11(Stat Soft Inc.).

## RESULTS

### Effect of phytohormones on callus induction and growth

Petiole explants inoculated on MS plain medium and MS medium supplemented with Kn, BAP, IAA, IBA, and NAA, singly, in different concentrations, did not show any positive response toward callusing (data not given). However, the petiole explants inoculated on MS medium supplemented with 2, 4-D (6.78 and 9.05 μm) alone and in combination with BAP (6.66-15.53 μm) or Kn (6.97-16.26 μm) inducing the callus in petiole explants. Initially, petiole explants showed swelling after 4 days of inoculation [Figure 1]. After subculturing on the same medium, callus formation was observed within 7 days. The callus was initially developed at the ruptured portion in the groove and finally in the middle part of the explant. This callus was negligible, whitish and crystalline in nature [Figure 2]. On subsequent subculture on the same medium, callus grew in size [Figure 3]. MS medium with 2, 4-D (6.78 μm) was found best for callus induction from *in vitro* petiole explants with higher

**Figure 1:** Petiole explant showing swelling

**Figure 2:** Callus development in petiole

induction percentage (71%) [Table 1]. The quantity of dry callus (harvested after 28 days growth period) obtained on MS+2, 4-D (6.78 μm) and MS+2, 4-D (9.05 μm) was 142 mg and 127 mg per explant, respectively [Table 2]. Although the callus induction was observed in petiole explants inoculated on MS medium supplemented with 2, 4-D (6.78 μm) in combination with BAP (6.66-15.53 μm) or Kn (6.97-16.26 μm), the frequency of callus induction and dry weights of callus obtained decreased as compared to MS medium supplemented with 2, 4-D alone. It means 2, 4-D alone was very effective in inducing callus in petiole explants of *Viola serpens* Wall.

### Effect of phytohormones on callus differentiation

The whitish and crystalline callus obtained from aforesaid trials was subcultured on MS medium supplemented with BAP (6.66, 11.10, and 15.53 μm) and Kn (6.97, 11.61, and 16.26 μm), singly. The regeneration of multiple shoots from petiole callus was observed in all the concentrations tried. The callus subcultured on MS+BAP (6.66-15.53 μm) and MS+Kn (6.97-16.26 μm) increased in size and developed fibrous roots within 7 days, which turned greenish in color in next 9-10 days, and these roots did not show any growth further [Figure 4]. 100% callus explants showed development of fibrous roots. When these cultures were maintained further, formation of active green callus was observed after 2-3 weeks [Figure 5]. Subculturing the active callus on the same medium, i.e., MS+BAP (6.66-15.53 μm) and MS+Kn (6.97-16.26 μm), it increased in size and formation of shoot buds was observed, followed by regeneration of multiple shoots (4-6 per explant) explants within 7-10 days. The formation of shoot buds and regeneration of multiple shoots was observed from upper and lower surfaces of the callus [Figure 6]. When the cluster of multiple shoots with callus was subcultured on

**Table 1: Effect of phytohormones on petiole explant of *Viola serpens* wall**

| Medium | Callusing | Number of days | Percentage of explants responded±SE |
|---|---|---|---|
| MS plain | - | - | - |
| MS+2,4-D (2.26 μm) | - | - | - |
| MS+2,4-D (4.52 μm) | - | - | - |
| MS+2,4-D (6.78 μm) | + | 7 | 71±1.000 |
| MS+2,4-D (9.05 μm) | + | 7 | 70±1.154 |
| MS+2,4-D (6.78 μm)+ Kn (2.32 μm) | + | 22 | 36±0.333 |
| MS+2,4-D (6.78 μm)+ Kn (6.97 μm) | + | 17 | 57±2.309 |
| MS+2,4-D (6.78 μm)+ Kn (16.26 μm) | + | 17 | 57±2.333 |
| MS+2,4-D (6.78 μm)+ BAP (2.22 μm) | + | 22 | 40±1.732 |
| MS+2,4-D (6.78 μm)+ BAP (6.66 μm) | + | 18 | 70±2.517 |
| MS+2,4-D (6.78 μm)+ BAP (15.53 μm) | + | 18 | 70±2.081 |

Values are mean of three experiments, each with 100 replicates. SE=Standard error

**Table 2: Effect of phytohormones on growth of petiole callus of *Viola serpens* wall**

| Medium | Average fresh wt. of callus per explant in mg±SE | Average dry wt. of callus per explant in mg±SE |
|---|---|---|
| MS plain | - | - |
| MS+2,4-D (6.78 μm) | 2467±35.342 | 142.00±1.527 |
| MS+2,4-D (9.05 μm) | 2165±27.393 | 127.00±0.577 |
| MS+2,4-D (6.78 μm)+ BAP (2.22 μm) | 1400±45.575 | 94.33±0.879 |
| MS+2,4-D (6.78 μm)+ BAP (6.66 μm) | 1983±30.987 | 103.66±1.340 |
| MS+2,4-D (6.78 μm)+ BAP (15.53 μm) | 1863±22.503 | 109.33±4.259 |
| MS+2,4-D (6.78 μm)+ Kn (2.325 μm) | 1534±21.518 | 99.66±0.888 |
| MS+2,4-D (6.78 μm)+ Kn (6.97 μm) | 1565±28.185 | 100.66±0.330 |
| MS+2,4-D (6.78 μm)+ Kn (16.26 μm) | 1756±21.009 | 103.33±2.189 |

Values are mean of three replicates. SE=Standard error

**Figure 3:** Callus growth

**Figure 4:** Fibrous root formation

the same medium, the number of shoots were increased. Among different concentrations of BAP tried, BAP (11.10 μm) found highly effective in multiple shoot regeneration, as after fourth subsequent subculture, the average number of shoots (2.6 cm height) per explants increased up to 36.4 [Figure 7]. Kinetin was found less effective compared to BAP, since after fourth subsequent subculture the average number of shoots (1.7 cm height) could be increased up to 22, on MS medium supplemented with Kn (11.61 μm) [Table 3].

### Effect of phytohormones on *in vitro* rooting

To obtain the complete plantlet, *in vitro* grown shoots were separated and subcultured on full- and half-strength MS medium with or without auxins (IAA and IBA) in various concentrations. No rooting was observed in shoots inoculated on full strength and half- strength MS plain media. Rooting was observed in shoots inoculated on full strength as well as half strength MS media supplemented with IBA in all concentrations tried after 10-14 days, whereas on full strength MS medium it took 3 weeks [Figure 8]. Highest percentage of rooting (73.01%) was observed on half strength MS medium supplemented with 19.68 μm IBA, the average number of roots per shoot being 4.1. Whereas on full strength MS medium, maximum percentage was 17.44% in the medium containing 19.68 μm IBA with an average of two roots per shoot [Table 4]. All the shoots inoculated on half strength MS medium supplemented with IAA showed rooting, in all

**Table 3: Effect of phytohormones on regeneration of multiple shoots from petiole callus of *Viola serpens* wall**

| Medium | Average height of shoots after 4 weeks in cm±SE | Number of shoots after fourth subculture*±SE |
|---|---|---|
| MS+Kn (6.97 μm) | 1.6±0.136 | 14.4±0.215 |
| MS+Kn (11.61 μm) | 1.7±0.129 | 22±0.717 |
| MS+Kn (16.26 μm) | 1.7±0.053 | 21.2±0.609 |
| MS+BAP (6.66 μm) | 0.7±0.064 | 22.8±0.645 |
| MS+BAP (11.10 μm) | 2.6±0.121 | 36.4±1.399 |
| MS+BAP (15.53 μm) | 1.5±0.147 | 19±0.358 |

*Subculture was carried out everytime after 7 days

**Figure 5:** Active green petiole callus

**Figure 6:** Shoot regeneration from petiole callus

**Figure 7:** Multiple shoots

**Figure 8:** Rooting of plantlet

concentrations used. Highest rooting percentage (30.15%) and average number of roots per shoot (4.3) were obtained on medium containing 22.83 μm IAA [Table 5].

## DISCUSSION

### Effect of phytohormones on callus induction and growth

The cytokinins, *i.e.*, BAP and Kn, and auxins, *i.e.*, IAA, IBA and NAA, did not show any effect on petiole explant when added in MS medium individually, but the BAP and Kn showed callus induction when added in combination with 2, 4-D (6.78 μm). The 2, 4-D (6.78 and 9.05 μm) alone also induced callusing in petiole explant of *Viola serpens* Wall. It means that addition of 2, 4-D in the medium is necessary to induce callusing. The best results in terms of callus induction and growth were obtained on MS+2, 4-D (6.78 μm) medium. On MS medium, there was a gradual increase in growth with 2, 4-D alone as well as in combination with BAP and Kn. However, MS medium supplemented with 2, 4-D alone showed better growth. In the present studies, supplementation of merely 2, 4-D (6.78 μm) in the medium gave satisfactory results. In *Trifolium pratense* L., callus induction was observed in petiole explant on B5 medium fortified with Kn in combination with NAA and 2, 4-D.[19] In *Viola wittrockiana*, callus induction in petiole explants was reported when inoculated on half-strength MS medium supplemented with 2, 4-D and BAP in combination. In *poplar* species, also callus initiation from the petioles took place on $N_6$ medium containing 2, 4-D.[20] It indicates that 2, 4-D is necessary to induced callusing. Murashige (1974) and Razdan (1993) had reported that 2, 4-D is a strong auxin for stimulating callus. This study supports their findings.[21-22]

### Effect of phytohormones on callus differentiation

It has been observed from earlier reports that *in vitro* propagation is possible through callus in *Viola* species. Regeneration of shoots from 5 years friable callus derived from petiole tissues of *Viola patrinii* DC occurred most effectively on MS medium containing two-folds diluted basal salts, $5 \times 10^{-6}$ M 1-naphthalene acetic acid and $10^{-6}$ Kinetin.[17] In *Viola wittrockiana* multiple shoot regeneration from petiole callus was observed on half-strength MS medium when supplemented with BAP in combination with TDZ and NAA.[18] In this study, it has been observed that multiple shoot regeneration is possible through petiole callus in *Viola serpens* Wall. on MS medium supplemented with BAP (6.66, 11.10, and 15.53 μm) and Kn (6.97, 11.61, and 16.26 μm), alone. In *Viola serpens* Wall., BAP was found more effective than Kn in shoot regeneration. Highest rate of shoot induction was observed with 11.10 μm BAP as compared to other concentrations. The superiority of BAP over Kn in multiple shoot induction was also reported in a number of medicinal plants such as *Centella asiatica* (L.) Urban,[23] *Bacopa monneri* L.,[2431, 32] *Psidum guajava* L.,[25] *Piper* sp.,[26] *Citrus* sp.,[27] *Zingiber officinale* Rosc.,[28] *Desmodium gangeticum* (L.) DC.,[29]and *Arachis hypogea* L.[30]

**Table 4: Effect of IBA on *in vitro* rooting in *Viola serpens* wall**

| Medium | Rooting %±SE | Average number of roots/shoot±SE | Average length of roots/shoot in cm±SE | Number of days required for rooting |
|---|---|---|---|---|
| 1/2MS plain | - | - | - | - |
| 1/2MS+IBA (4.90 μm) | 28.56±2.751 | 2.3±0.100 | 0.8±0.057 | 14 |
| 1/2MS+IBA (9.84 μm) | 34.91±1.586 | 2.6±0.200 | 0.8±0.058 | 14 |
| 1/2MS+IBA (14.76 μm) | 41.26±1.586 | 2.6±0.115 | 0.4±0.057 | 11 |
| 1/2MS+IBA (19.68 μm) | 73.01±3.176 | 4.1±0.208 | 1.3±0.100 | 10 |
| 1/2MS+IBA (24.60 μm) | 58.72±4.198 | 3.6±0.116 | 0.8±0.100 | 14 |
| MS plain | - | - | - | - |
| MS+IBA (4.90 μm) | 4.76±0.000 | 1±0.100 | 1.5±0.200 | 21 |
| MS+IBA (9.84 μm) | 7.93±1.586 | 1±0.058 | 1.7±0.100 | 21 |
| MS+IBA (14.76 μm) | 15.85±1.593 | 1±0.116 | 1.9±0.153 | 21 |
| MS+IBA (19.68 μm) | 17.44±1.593 | 2±0.145 | 2.0±0.208 | 21 |

Values are mean of three experiments each with 21 replicates. SE, Standard error, IBA=Indole-3-butyric acid

**Table 5: Effect of IAA on *in vitro* rooting in *Viola serpens* wall**

| Medium | Rooting %±SE | Average number of roots/shoot±SE | Average length of roots/shoot in cm±SE | Number of days required |
|---|---|---|---|---|
| 1/2MS plain | - | - | - | - |
| 1/2MS+IAA (5.71 μm) | 7.93±1.586 | 1.5±0.058 | 1.0±0.173 | 14 |
| 1/2MS+IAA (11.42 μm) | 8.11±4.196 | 2.0±0.153 | 1.7±0.200 | 14 |
| 1/2MS+IAA (17.13 μm) | 12.80±1.586 | 2.4±0.173 | 2.3±0.116 | 16 |
| 1/2MS+IAA (22.83 μm) | 30.15±1.586 | 4.3±0.200 | 2.3 ±0.100 | 16 |

Values are mean of three experiments with 21 replicates. SE, Standard error, IAA=Indole-3-acetic acid

### Effect of phytohormones on *in vitro* rooting

Root initiation was observed in shoots of *Viola serpens* Wall. inoculated on full-strength and half-strength MS media supplemented with IAA and IBA. However, the half-strength MS medium supplemented with IBA (19.68 μm) was found better for rooting, it gave 73.01% rooting response with 3.6 average number of roots having average length of 1.3 cm. Although IAA also induced rooting, percentage of rooting was less as compared to that obtained with IBA.

## CONCLUSION

The cytokinins and auxins failed to induce callusing in petiole explant, when added to MS medium singly except 2, 4-D (6.78 and 9.05 μm). The MS medium supplemented with 2, 4-D (6.78 μm) was found most suitable for callus induction and growth. The hormone free medium and medium supplemented with BAP, Kn, IAA, IBA, and NAA singly did not support the induction of callus. However, the BAP and Kn showed callus induction when added in combination with 2, 4-D (6.78 μm). Thus, the results show that the presence of 2, 4-D in the medium is necessary for callus induction from the petiole of *Viola serpens* Wall. Although the formation of active green callus and regeneration of multiple shoots were observed on MS+BAP (BAP; 6.66, 11.10, and15.53 μm) and (Kn; 6.97, 11.61, and 16.26 μm), media, the best growth response, and higher rate of shoot multiplication were observed on MS medium containing BAP (11.10 μm) as the average number of shoots could be increased to 36.4 on fourth successive subculturing and shoots attained 2.6 cm average height after 3-4 weeks of the growth period. Although root initiation was observed in shoots inoculated on both full-strength and half-strength MS medium supplemented with IAA and IBA, the half-strength MS medium supplemented with IBA (19.68 μm) was found better as there was higher rooting response with larger number of roots.

This efficient and simple protocol reported herein could be useful for rapid multiplication of this medicinally important plant.

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