Plantlet regeneration via adventitious shoot bud proliferation from leaf explants in *Potentilla fulgens* Wall. ex Hook.—A plant possessing hypoglycemic activity

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A method for adventitious shoot bud regeneration from leaves of *Potentilla fulgens* has been developed. Leaves were obtained from plants growing in the natural habitat. Explant browning, a major hurdle in the establishment of cultures, was overcome by treating leaves with a combination of antioxidants (100 mg l⁻¹ ascorbic acid, 100 mg l⁻¹ citric acid and 20 mg l⁻¹ L-cysteine HCl). Influence of the growth regulators BAP (6-benzylaminopurine) and NAA (α-naphthaleneacetic acid) on adventitious bud differentiation and shoot regeneration was observed on modified Murashige and Skoog’s (MMS) agar medium. The most effective treatment was MMS with 0.1 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA, which gave 80% bud induction frequency with 38.4 BFC (Bud Forming Capacity) index and 48 shoots per explant of 3.5 cm length. Rooting was induced on MS basal medium. The regenerated plants had 70% survival rate.

**Keywords:** adventitious shoot regeneration, explant browning, *Potentilla fulgens*

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**Introduction**

*Potentilla fulgens* Wall. ex Hook., found at higher altitudes (1500-2000 m) of Khasi Hills, Meghalaya, India, has been used as folk remedy against diarrhoea¹⁻³ and toothache⁴. It has also been found to have hypoglycemic effect in diabetic mice⁵. Tubers of the plant have been widely used by the local populace as an additive in betel-leaf, called *Kwai*. Since planned cultivation of *P. fulgens* does not exist, the plant is threatened to over-harvesting from its natural habitat to meet the high demand. Appreciating the threat, work on *in vitro* propagation of this popular medicinal plant was initiated. The present communication reports the salient findings of the study.

**Materials and Methods**

The plants of *P. fulgens* grow on acidic (pH 6.0 ± 0.5), reddish-brown loamy soils in the natural habitat of Mawphlang area (1818 m altitude) at East Khasi Hill district of Meghalaya. The leaves, collected from the 2nd to the 7th nodes, were brought to the laboratory and washed in running water for 1 hr. To control microbial contamination various anti-microbial treatments were tested: surface sterilization with 0.1% (w/v) HgCl₂ for 3, 5, 8 or 10 min; surface sterilization with 5, 7, 8 or 9% (w/v) calcium hypochlorite for 15, 20, 25 and 30 min; spraying of explant donors with 0.4% (w/v) Bavistin (Cabendazim) for 30 days at 7-day intervals followed by surface sterilization with either 0.1% (w/v) HgCl₂ for 5 min or 9% (w/v) calcium hypochlorite for 30 min. After each treatment, explants were rinsed 5-6 times with sterile distilled water.

To find out the combinations that could control the browning of explants, the explants were soaked in antioxidant solutions containing ascorbic acid, citric acid (each 25, 50 or 100 mg l⁻¹) and L-cysteine-HCl at (5, 10 or 20 mg l⁻¹) for 2, 4, 8, 10, 12, 18 or 24 hr. At the end of the antioxidant treatment, intact leaves were inoculated on modified Murashige and Skoog’s (MMS) medium constituted of MS⁶ basal salts, 100 mg l⁻¹ inositol, 2 mg l⁻¹ glycine, 2 mg l⁻¹ pyridoxine-HCl, 2 mg l⁻¹ nicotinic acid, 1 mg l⁻¹ thiamine-HCl, and 30 g l⁻¹ (w/v) sucrose. Two cytokinins, 6-benzylaminopurine (BAP) and kinetin (Kn), and two auxins, α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA), each at 0.1, 0.25 or 0.5 mg l⁻¹

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concentrations were also supplemented into the MMS medium alone and in different combinations (cytokinin + auxin). All media were gelled with 0.75% (w/v) agar. The pH of the media was adjusted to 5.6 ± 2 prior to autoclaving at 15 p.s.i. for 15 min. Cultures were maintained at 22±1°C under fluorescent light (30 μmol m⁻² s⁻¹) with 12 hr light/dark cycle. Observation on frequency of adventitious bud formation was taken 7 days after the initiation of adventitious buds, and then cultures were transferred to fresh medium of the same composition on the same day. Shoots were separated from clusters 30 days after the subculture and data on number of shoots per explant and length of shoots were recorded. The regenerated shoots were inoculated on MS basal medium for root induction. The frequency of rooting was recorded 15 days after root induction. Regenerated plantlets having well developed root system were transferred to plastic cups containing sterile soil and sand (2:1) and, 30 days later, transferred to earthen pots containing the same ratio of soil and sand.

The experimental design was randomized complete block with twenty replicates. For experiments to determine the best antioxidant treatment, a replicate consisted of an Erlenmeyer flask containing 10 leaf explants soaked in 100 ml of antioxidant solution. For experiments to determine the best decontamination method and the best medium formulation, a replicate consisted of a culture tube containing one leaf explant. Analysis of variance (ANOVA) and separation of means was carried out using Duncan’s Multiple Range Test (DMRT) at 5% level of significance. To appreciate better the impact of the culture media, the Bud Forming Capacity (BFC) index was calculated according to Martinez-Pudilo et al⁷:

\[
\text{BFC index} = \left( \frac{\text{% explant forming buds}}{\text{mean number of shoots per explant}} \right) \times 100
\]

Results and Discussion

Microbial contamination and browning of explants were the major hurdles in establishing *P. fulgens* cultures *in vitro*. The results of antimicrobial treatments are presented in Fig. 1. Explants obtained from 0.4% (w/v) Bavistin sprayed plants followed by surface sterilization with 0.1% (w/v) HgCl₂ was the best decontamination method. The combination of fungicide treatment and surface sterilization with 9% (w/v) calcium hypochlorite also resulted in significant control of contaminants but calcium hypochlorite caused damage to the explants.

Browning was observed in untreated explants of all ages. Leaves turned brown within 24 hr of inoculation. However, 5-7 day old leaves (1.2-1.5 cm long leaves collected from the 2nd and 3rd nodes) when exposed to Tr 4 (100 mg l⁻¹ ascorbic acid, 100 mg l⁻¹ citric acid and 20 mg l⁻¹ L-cysteine-HCl) for 24 hrs, browning was significantly reduced (Table 1). It was observed that injury of leaves before or after antioxidant treatments caused death due to browning. Hence, care was taken to use intact leaves as explants. The factors that controlled explant death due to

![Fig. 1—Effect of various anti-microbial treatments on decontamination of leaf explants. (Means with same letters are not significantly different according to DMRT at \( p = 0.05 \); Vertical bars = ± Standard error)](image)

<table>
<thead>
<tr>
<th>Treatment (24 hr)</th>
<th>Concentration (mg l⁻¹)</th>
<th>% of explant survival*</th>
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<tr>
<td></td>
<td>Ascorbic acid</td>
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<td>Tr 0</td>
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<td>Tr 4</td>
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*Means ± S.E. followed by the same letters are not significantly different according to Duncan’s Multiple Range Test at \( p = 0.05 \)
browning are the age of explant and the appropriate antioxidant treatment.

Browning of explants and subsequent death is a common problem in tissue culture of plant species containing phenolic compounds and extra steps are usually incorporated in the culture protocol to overcome this problem. Potentilla species contain phenolic compounds. To overcome the damage due to browning of explants, several workers have used various combinations of antioxidants and absorbents. In the present study on P. fulgens, a similar strategy was followed to control browning of explants.

Positive response for shoot bud regeneration was observed from explants cultured on MMS media having both BAP and NAA (Table 2). However, media containing BAP or NAA alone (MMS1 and MMS2) failed to induce differentiation and shoot growth. Leaf explants cultured on media supplemented with Kn or IAA alone and in various combinations also did not respond to bud initiation. The medium MMS4 (0.1 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA) gave the best results for adventitious shoot bud induction frequency (80%), number of shoots (48) per explant and shoot length (3.5 cm) (Table 2). However, the MMS3 (0.25 mg l⁻¹ BAP and 0.25 mg l⁻¹ NAA) and MMS5 (0.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA) showed substantially inferior results. Although, the time required for bud induction and bud break were the same but MMS3 and MMS5 had substantially less BFC index (3.18 and 1.15) as compared to MMS4 (38.4). Thus, MMS medium containing 0.1 mg l⁻¹ BAP along with 0.1 mg l⁻¹ NAA (MMS4) was the most effective in supporting adventitious shoot regeneration from P. fulgens leaves. Influence of BAP and NAA on adventitious shoot regeneration was also reported in several other plant species.

Adventitious shoot bud initiation was observed 20 days after inoculation of explants on MMS4 medium. The shoot buds differentiated all over the leaf surface (Fig. 2a). The buds regenerated to shoots (Fig. 2b) 7 days after transfer to fresh medium. Clusters of shoots (Fig. 2c) developed during the next 10 days. The

![Image](image-url)
regenerated shoots were then transferred to MS basal medium for rooting. Well-formed roots (Fig. 2d) developed 15 days after the shoot transfer and had 90% root induction frequency. The rooted plantlets were acclimatized (Fig. 2e) and transferred to the field. The plants had 70% survival rate.

It is relevant to mention here that limited callusing (Fig. 2b) was observed from leaf margins of explants cultured on all the induction media. However, these calli did not differentiate further to shoots or roots.

Shoot organogenesis from leaf explants is a frequently used route of in vitro propagation of plant species. It has been used recently in case of Dianthus chinensis, Saintpaulia ionantha and Platanus acerifolia. The present report on shoot organogenesis from leaf explants of P. fulgens can be used for rapid clonal propagation of this important medicinal plant endemic to the temperate Himalayas.

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References