Direct organogenesis for mass propagation of *Peperomia pellucida* L. an epiphytic medicinal herb

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ABSTRACT

The direct organogenesis from nodal meristems of *Peperomia pellucida* L. was reported in this study. The explants were sterilized using 0.1% mercuric chloride. Since, the plant tissues are very soft and delicate 3 min treatment is optimum for surface sterilization of the explants. The adventitious shoots were induced on MS medium augmented with 2.0 mg/L BAP. Maximum 96% explants responded on this medium combination with 12.6 ±0.74 shoots per explant (4.2 ±0.44 cm average lengths). The shoots were further multiplied on MS medium + 0.5 mg/L each of BAP and Kin, resulted in maximum shoots formation (127.2 ±0.30 shoots per explant with 7.7 ±0.30 cm in length) after 4 week. The effects of auxins were tested for induction of in vitro roots from the cut ends of the shoots. The in vitro multiplied shoots were rooted on half strength MS medium fortified with 1.5 mg/L IBA. Maximum number of roots per shoot (10.8 ±0.39 roots with 6.8 ±0.33 cm length) was induced with IBA as compared to IAA. The rooted plantlets were carefully removed from the medium, washed properly and transferred to the paper cups contained soilrite. These were kept in the greenhouse for 4-6 weeks and moistened with aqueous solution of one-fourth strength MS salts. The hardened plantlets were finally transplanted in the field under natural conditions with 100% survival rate. The developed protocol can be used for mass multiplication of this medicinal plant which can help to fulfill the gap in the demand and supply of this epiphytic medicinal plant.

Keywords: *Peperomia pellucida*; organogenesis; in vitro propagation; rooting; hardening
1. INTRODUCTION

Peperomia pellucida L. is a rare epiphytic medicinal plant commonly known as pepper elder, little heart, shining bush and silver bush, belonging to one of the largest genera of the family Piperaceae (Mishra, 2012). This annual herbaceous species is native and endemic to the Caribbean countries, merits special conservation concern and listed as to be conserved in biodiversity profile of St. Kitts and Nevis, Washington (Horwith and Lindsay, 1999). It is mainly distributed in the Central and South America, Africa, South-East Asian countries, and Australia (Bayma et al., 2000; Arrigoni-Blank et al., 2004). It prefers to grow in loose, humid soils and a tropical to subtropical climate, where it can be found wild on slightly shaded and damp areas such as brooks, walls, yards and even roofs (Majumdar et al., 2011).

P. pellucida is known as Toyakandha and Varshabhoo in Indian systems of medicine. This herb is characterized by succulent, angular trailing stems, shiny, heart-shaped and fleshy bright green leaves, single seeded fruits on several fruiting spikes with a mustard-like odor, usually reaches maximum 15 to 45 cm in height. In rich habitats it grows as a shrubby creeper epiphyte. The aerial parts possess waxy surfaces (Ghani, 1998).

The bioactive compounds from the whole plant are reported as peperomins (mixture of secolignans, tetrahydrofuran lignans, methoxylated dihydronaphthalenone), sesamin, isoswertisin, flavonoids (acacetin, apigenin, isovitexin, and pellucidatin), phytosterols (campesterol and stigmasterol), arylpropanoids, pellucidin (Bayma et al., 2000). Leaves yield alkaloids, cardenolides, saponins and tannins and also rich source of crude fiber and carbohydrate content and minerals such as sodium, manganese, iron, zinc and copper (Xu et al., 2006; Ooi and Iqbal, 2012). The presence of saponins, terpenoids, phlobatannins, isoquinoline alkaloids, tannins, glycosides, tropane alkaloids and phenols, steroids and triterpenoids are reported by Gini and Jothi (2013).

Arylpropanoids isolated from this plant has been documented for antifungal activity, peperomins have cytotoxic and anticancer activity, dillapiole has insecticidal, antimicrobial anti-inflammatory, anticancer and gastroprotector activities (Wei et al., 2011; Parise-Filho et al., 2011; Maxia et al., 2012; Martinez et al., 2013).

P. pellucida has a rich history of medicinal uses and long been used as a food (Loc et al., 2010). Herbal salad from aerial parts helps to relieve rheumatic pains and gout. The infusions and decoctions are effective for easing gastric ulcers, arthritis, wounds, high blood cholesterol, abdominal pains, constipation, kidney disorders and skin diseases (Arrigoni-Blank et al., 2004). Leaves have been used traditionally to treat headache, fever, eczema and convulsions (Gini and Jothi, 2013). It is used to lower cholesterol level (Bayma et al., 2000). In Philippines, a decoction from this plant is used to decrease uric acid levels so as to treat renal problems. It is used as an active ingredient in topical ointment for skin disorders such as acne, pimples and boils. Consumption of P. pellucida as green salads in the Philippines and as blanched vegetables in Thailand (Staples and Kristiansen, 1999) and herbal tea in Indonesia has been reported (Zakaria and Mohd, 1992).

In recent years this plant gained attention due to its potential in determination of naturally occurring radionuclides (238U, 234U, 232Th, 230Th, 226Ra, 228Ra and 210Pb) in surrounding soil (Sussa et al., 2011) and effecting prostaglandin synthesis (Aziba et al., 2001). P. pellucida have already contributed in transgenic research and in the production of heat labile enterotoxin B subunit through E. coli (Loc et al., 2010). The present study aims to
evaluate the effect of plant growth regulators and in vitro factors in mass multiplication of *P. pellucida* though direct organogenesis (Fig. 1 & 2).

**Fig. 1.** Range of occurrence *Peperomia pellucida* L.

**Fig. 2.** *Peperomia pellucida* L.
2. MATERIALS AND METHODS

2.1. Selection of explants and sterilization procedures

Two months old *P. pellucida* were collected from the South Western Ghats of India (Coastal Puducherry and Tamil Nadu), and grown in the greenhouse. The tender shoots obtained from freshly emerged herbs were dissected and brought to the laboratory. The shoot tips and nodal explants with one or two axillary buds were selected as explants. The explants were washed thoroughly with distilled water and soaked in 0.1% bavistin solution (systemic fungicide, BASF India Ltd.) for 8-10 minutes. They were surface sterilized with 0.1% mercuric chloride for 3-4 min, followed by 5-6 rinses with sterile distilled water in front of sprit lamp under laminar airflow cabinet. Proximal and distal end of surface sterilized explants were removed by sterilized scissors without disturbing nodal or apical meristems. The explants were inoculated horizontally or vertically on the culture medium. Different experiments were setup with various concentrations of growth regulators to select the best growth hormone for the response of shoot induction and proliferation of adventitious shoots *in vitro*.

2.2. Culture medium and *in vitro* environment

MS basal nutrient medium (Murashige and Skoog, 1962) was used for the experiments during this study for shoot proliferation, adventitious shoot regeneration and root induction. All media were supplemented with additives (50 mg/L ascorbic acid, 25 mg/L each of arginine, adenine sulphate and citric acid), 30 g/L sucrose and 8 g/L agar, finally the medium is dispensed into 15 × 150 mm culture tubes and 250 ml conical flasks. The pH of the medium was adjusted to 5.8 ±0.02 before autoclaving at 121 °C for 15 min. Cultures were maintained at 25 ±2 °C under a photoperiod of 12 h d^−1^ with a light intensity of 40-50 μmol m^−2^ s^−1^ SFPD, provided using cool white fluorescent lamps.

2.3. Establishment of culture initiation and shoot multiplication

The surface sterilized shoot apices and nodal explants were cultured on MS medium containing various concentration of cytokinins (BAP and Kin) ranging from 0.0-4.0 mg/L. The fresh shoots with explants were further multiplied using subsequent transfer on MS medium with different concentrations of cytokinins and auxins. *In vitro* produced axillary shoots (micro shoots with 2-3 nodes) were excised from mother explants and transferred to MS medium containing different concentrations of plant growth regulators and additives for better multiplication. Cultures were continuously subcultured on fresh medium after an interval of 4 week. The effect of plant growth regulators on bud breaking response, number and length of shoots, rate of multiplication on various media combinations were recorded.

2.4. Rooting of *in vitro* developed shoots

*In vitro* regenerated shoots with 5-6 nodes were allowed to undergo rooting phase on agar-gelled medium. The healthy shoots (4-5 cm long) from multiplication stage were separated and transplanted individually to full and half strength MS medium incorporated with 30 and 15 g/L sucrose, additives and various concentrations (0.0-3.0 mg/L) of auxins (IAA and IBA) to induce roots *in vitro*. The cultures were incubated on diffused light with high relative humidity for better rooting response.
2.5. Hardening and field transfer

The rooted plantlets were removed from the culture vessels and washed with distilled water to remove the remnants of medium and then planted in small paper cups filled with sterile soilrite® and moistened with 1/4th inorganic MS salts. The plants were covered by plastic membrane /transparent poly-cups and maintained in high humidity area of the greenhouse. After 4 weeks the plantlets were exposed to reduced humidity for 2 weeks and the covered poly-cups were gradually removed. The established plantlets were transferred to earthen pots filled with a mixture of garden soil, vermicompost and red soil in the ratio of 1:1:1. After attaining the morphogenic similarities of naturally growing plants, the micropropagated plantlets are exposed to grow in the field and the survival rate was recorded.

2.6. Experimental design, data collection and statistical analysis

All the experiments speculated in the present investigation were conducted with a minimum of 10 replicates per treatment. Each explant was taken as a replication and all the experiments were repeated thrice. Observations were recorded after the time period of 4 weeks interval. The results were expressed as the Mean ± Standard Deviation (SD) for all experiments.

3. RESULTS

3.1. Selection of explants and establishment of cultures

Among the two types of explants selected to test the effects of cytokinins in induction of shoots, nodal meristem responded better than the apical meristems. The explants were soft and brittle in nature, time duration of mercuric chloride treatment affects greatly in the survival of explants in culture medium. The surface sterilization of explants for 3 min responded better, less than 3 min caused microbial contamination and more than 3 min treatment dried the explants adversely. The affected ends of explants were excised by the sterile scissors before inoculation on to the culture medium. Nodal meristems responded well on MS medium incorporated with 2.0 mg/L BAP than Kin augmented medium. Maximum 96% explants responded on this combination of cytokinin and medium and the number of shoots were recorded as 12.6 ± 0.74 shoots per explant with 4.2 ± 0.44 cm length. Less number of shoots (4.8 ± 0.35 shoots and 4.8 ± 0.35 length) was observed on the same concentration of Kin with 80% response in present study (Fig. 3A and Table 1).

Table 1. Effects of cytokinin (BAP and Kin) on induction of shoots from the explants on MS medium.

<table>
<thead>
<tr>
<th>BAP (mg/L)</th>
<th>Kinetin (mg/L)</th>
<th>Response (%)</th>
<th>No. of shoots (Mean ± SD)</th>
<th>Length of shoots (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
<td>0.0 ± 0.00a</td>
<td>0.0 ± 0.00a</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>49</td>
<td>2.5 ± 0.29c</td>
<td>2.4 ± 0.15bc</td>
</tr>
<tr>
<td>1.00</td>
<td>-</td>
<td>60</td>
<td>3.2 ± 0.61de</td>
<td>2.7 ± 0.30d</td>
</tr>
<tr>
<td>Concentration</td>
<td>Ratio</td>
<td>Yield</td>
<td>BAP</td>
<td>Kin</td>
</tr>
<tr>
<td>---------------</td>
<td>-------</td>
<td>-------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>1.50</td>
<td>-</td>
<td>83</td>
<td>7.7 ± 0.40&lt;sup&gt;h&lt;/sup&gt;</td>
<td>3.5 ± 0.61&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.00</td>
<td>-</td>
<td>96</td>
<td>12.6 ± 0.74&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4.2 ± 0.44&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.50</td>
<td>-</td>
<td>81</td>
<td>5.0 ± 0.91&lt;sup&gt;h&lt;/sup&gt;</td>
<td>4.0 ± 0.19&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.00</td>
<td>-</td>
<td>76</td>
<td>4.6 ± 0.20&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>3.8 ± 0.26&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.50</td>
<td>-</td>
<td>60</td>
<td>4.1 ± 0.11&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.4 ± 0.10&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.00</td>
<td>-</td>
<td>45</td>
<td>3.0 ± 0.19&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.0 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>0.50</td>
<td>37</td>
<td>1.5 ± 0.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1 ± 0.25&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>1.00</td>
<td>43</td>
<td>2.9 ± 0.24&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.5 ± 0.61&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>1.50</td>
<td>65</td>
<td>3.6 ± 0.83&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.2 ± 0.36&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>2.00</td>
<td>80</td>
<td>4.8 ± 0.35&lt;sup&gt;h&lt;/sup&gt;</td>
<td>3.6 ± 0.23&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>2.50</td>
<td>71</td>
<td>5.0 ± 0.49&lt;sup&gt;g&lt;/sup&gt;</td>
<td>3.9 ± 0.40&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>3.00</td>
<td>59</td>
<td>4.2 ± 0.51&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.4 ± 0.37&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>3.50</td>
<td>44</td>
<td>3.6 ± 0.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.9 ± 0.22&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>-</td>
<td>4.00</td>
<td>25</td>
<td>2.5 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 0.24&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.05% level.

Nodal explants responded better as compared to the apical shoot on MS medium augmented with BAP. Special care has been taken to sterilize the explants by cutting the both ends of explants after sterilization because of the softness of the explants. The positive role of BAP than other cytokinins on shoot bud induction as well as regeneration capacity of nodal meristems was reported by several researchers on different species (Phulwaria et al., 2013; Patel et al., 2014; Sreeranjini and Siril, 2014; Shekhawat et al., 2015). Cytokinins enhance the rate of cell divisions which ultimately resulted in shoot bud breaking from the nodal meristems of the explants. The success in culture induction and multiplication primarily relays on the type of explants selection. The selection of axillary buds and its proliferation on MS medium administered with BAP and Kin have been reported in several species (Bhat et al., 2010; Padmapriya et al., 2011; Shekhawat et al., 2014).

3.2. Multiplication of in vitro regenerated shoots

Shoot multiplication reported on MS medium fortified with different concentrations of BAP and Kin. Among the various concentrations and combinations of growth hormones tested to multiply the shoots in vitro, frequent response in multiplication and the development of juvenile shoots from the mother explants was observed when Kin was augmented with BAP, the rate of multiplication was less impressive in the experiments when the BAP alone
was used (without Kin). It was observed that half strength MS medium supplemented with 0.5 mg/L each of BAP and Kin with additives resulted in maximum shoots formation (127.2 ± 0.30 shoots with 7.7 ±0.30 cm in length) in multiplication experiments (Fig. 3B, 3C and Table 2). Stunted growth or dwarf shoot clumps were obtained on full strength MS medium with same hormonal concentrations and half strength MS medium was used for further elongation of shoots (Fig. 3D and 3E). The frequency of shoot multiplication in one-fourth MS medium in the presence of cytokinins

![Fig. 3A. Induction of multiple shoots from the nodal explants](image)

![Fig. 3B and 3C. Multiple shoots with stunted growth of shoots on full strength MS medium](image)

![Fig. 3D and 3E: Elongated multiple shoots on half strength MS medium](image)

![Fig. 3F: In vitro rooted shoots](image)

![Fig. 3G: Plantlets in soilrite in paper cups in greenhouse](image)

![Fig. 3H and 3I: Hardened plantlets](image)

![Fig. 3J and 3K: Field transferred plants under natural conditions](image)
Table 2. Effects of cytokinin (BAP and Kin) and their concentrations on multiplication of shoots on half strength MS medium.

<table>
<thead>
<tr>
<th>BAP (mg/L)</th>
<th>Kinetin (mg/L)</th>
<th>No. of shoots (Mean ± SD)</th>
<th>Shoot length (cm) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.10</td>
<td>66.4 ± 0.47(^a)</td>
<td>5.3 ± 0.24(^b)</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>127.2 ± 0.30(^c)</td>
<td>7.7 ± 0.30(^d)</td>
</tr>
<tr>
<td>1.00</td>
<td>1.00</td>
<td>85.9 ± 0.24(^b)</td>
<td>6.9 ± 0.27(^c)</td>
</tr>
<tr>
<td>1.50</td>
<td>1.50</td>
<td>70.4 ± 0.36(^a)</td>
<td>4.7 ± 0.19(^a)</td>
</tr>
<tr>
<td>2.00</td>
<td>2.00</td>
<td>64.7 ± 0.41(^a)</td>
<td>4.2 ± 0.22(^a)</td>
</tr>
</tbody>
</table>

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance variation between the concentrations was studied using DMRT at 0.05% level.

The combined effect of BAP and Kin was reported significantly better in multiple shoots regeneration. These results are also supported and presented by various researchers (Karthikeyan et al., 2009; Phulwaria et al., 2011; Shekhawat and Shekhawat, 2011) while working on different plant species like *Centella asiatica*, *Salvadora persica*, *Arnebia hispidissima* and *Morinda coreia*. It was observed that incorporation of BAP greatly affects the initiation as well as multiplication of shoots *in vitro* in present.

3. 3. *In vitro* rooting of the shoots

Table 3. Effects of Auxins (IBA and IAA) on induction of roots from *in vitro* generated shoots on half strength MS medium

<table>
<thead>
<tr>
<th>IBA (mg/L)</th>
<th>IAA (mg/L)</th>
<th>No. of roots (Mean ± SD)</th>
<th>Root length (cm) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>0.0 ± 0.00(^a)</td>
<td>0.0 ± 0.00(^a)</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>8.2 ± 0.56(^g)</td>
<td>3.8 ± 0.45(^f)</td>
</tr>
<tr>
<td>1.00</td>
<td>-</td>
<td>9.7 ± 0.83(^j)</td>
<td>4.2 ± 0.60(^f)</td>
</tr>
<tr>
<td>1.50</td>
<td>-</td>
<td>10.8 ± 0.39(^i)</td>
<td>6.8 ± 0.33(^e)</td>
</tr>
<tr>
<td>2.00</td>
<td>-</td>
<td>9.3 ± 0.23(^h)</td>
<td>5.6 ± 0.41(^b)</td>
</tr>
<tr>
<td>2.50</td>
<td>-</td>
<td>8.8 ± 0.68(^e)</td>
<td>3.2 ± 0.38(^d)</td>
</tr>
<tr>
<td>3.00</td>
<td>-</td>
<td>6.5 ± 0.73(^c)</td>
<td>2.7 ± 0.31(^b)</td>
</tr>
</tbody>
</table>
In *vitro* multiplied shoots of different size were excised and transferred/inoculated in auxin containing MS medium. Out of various concentrations considered for *in vitro* rooting experiments, MS medium containing IBA were reported effective in roots induction. The shoots with the length around 6.0-7.0 cm were responded well in rooting experiments, maximum number of roots (10.8 ±0.39) with 6.8 ±0.33 cm length were obtained on half strength MS medium fortified with 1.5 mg/L IBA (Fig. 3F). Comparatively weak, fragile and less number of roots was recorded with the same concentration of IAA (Table 3). The longer shoots were responded well in rooting experiments due to their sturdy nature. The positive response of IBA over IAA is also supports by earlier works in *Ceropegia bulbosa* (Phulwaria *et al.*, 2013), *Leptadenia reticulate* (Patel *et al.*, 2014), *Morinda coreia* (Shekhawat *et al.*, 2015) etc.

3.4. Hardening and acclimatization of plantlets

The remnants of medium from rooted plantlets were cautiously removed with soft brush. The complete plantlets, thus formed were hardened in eco-friendly paper cups containing sterile soilrite® moistened with one-fourth MS salts. After one month plantlets were firmly fixed their roots in the soilrite® (Fig. 3G). These were further transferred to soil, vermiculite and sand (1:1:1) mixture in earthen pots (Fig. 3H and 3I). Shoots rooted under *in vitro* conditions are partially autotrophs, having poor development of epicuticular wax and weak regulation of stomata. Therefore, gradual hardening is essential for high rate of survival. The hardened plantlets were finally transferred to the garden (Fig. 3J and 3K), where 100% plants survived successfully. It has been observed in this study that the soilrite® plays crucial role in the hardening process of plantlets by providing physical support, retention of moisture, good aeration to *in vitro* regenerated plantlets.

4. CONCLUSION

The cytokinins and auxins were tested for effective initiation, multiplication and rooting of the shoots in *P. pellucid* in present study. *In vitro* requirement is an efficient means of *ex situ* conservation of plant diversity and it assists in sustainable maintenance of the present day...
dwindling germplasm on long term basis, especially for medicinal plant. The study established the role of plant growth hormones in vitro propagation system of P. pellucid, higher proliferation rate was achieved with uniform and vigorous growth. This rate could be maintained even at the level of survival of plantlets in the field conditions.

Conflict of interest

The authors report that there is no conflict of interests regarding the publication of this article.

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