Rapid and efficient plant regeneration from nodal explants of Orthosiphon spiralis Murr

Logeshbabu Mohanakrishnan1, Dhamotharan Ramasamy2 and Janarthanam Balasundaram1*†

1Poonga Biotech Research Centre, Plant Biotechnology Division, Choolaimedu, Chennai - 600 094. Tamil Nadu, India.
2PG & Research Department of Botany, Presidency College, Triplicane, Chennai 600 005. Tamil Nadu, India.

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A B S T R A C T

The aim of this study was to develop an efficient protocol for establishment of plant regeneration from nodal explants of Orthosiphon spiralis - an important medicinal plant. Nodal explants inoculated on MS medium supplemented with 4.44 μM 6- benzyl amino purine (BAP) and 1.34 μM α-naphthalene acetic acid (NAA) showed better growth response and produced 5.23 ± 2.1 shoots per explant with an average length of 6.43 ± 0.06 cm after 35 days. Roots were induced after transfer to half strength of MS supplemented with 2.46 μM indole -3- butyric acid (IBA) produced 7.3 ± 0.25 roots with an average height of 5.5 ± 0.2 cm after 30 days. The rooted plantlets were transferred for hardening, 80 per cent of plantlets survived and resumed growth in the mixture of soil, vermiculite and farmyard manure (1:1:1). This protocol imparts highly repeatable, successful and rapid technique that can be utilized for the commercial propagation and ex situ conservation of this medicinal plant. It is important to note that the morphology of the in vitro plantlets of Orthosiphon spiralis showed a true-to-type growth habit, both in vitro and when transferred to ex vitro growth conditions.

Introduction

Orthosiphon spiralis Murr is a herbaceous shrub, belonging to the family Lamiaceae, which grows to a height of 1.5 m and the leaves are arranged in opposite pairs. They are simple, green, and glabrous with a lanceolate leaf blade and a serrate margin. It is distributed mainly throughout South East Asia and tropical countries. O. spiralis is commonly called ‘Kidney Tea Plant’, is a medicinal plant widely used in the treatment of various kidney and urinary bladder diseases including nephrocrirrhosis and phosphaturia. The activity of the leaves is attributed to the presence of a bitter glycoside, reorthosiponin (Wealth of India, 1966). Orthosiphon stamineus and Orthosiphon aristatus are synonyms of Orthosiphon spiralis. Orthosiphon stamineus exhibits hypoglycemic activity (Mariam et al., 1996) and the plant is also used to treat hepatorenal syndrome and renal ischaemia (Shantanova et al., 1997; Nikolaev et al., 1996), Orthosiphon plants have extensively been exploited traditionally to treat several human ailments. Leaves of this plant have been used as diuretic, and to treat rheumatism, abdominal pain, kidney and bladder inflammation and hypertension (Hegnauer, 1966; Wangner, 1982; Eisai, 1995).

For commercial exploitation, the flowers and the floral buds of O. spiralis are usually removed to enhance the quantity of the active constituent in the leaves. This practice consequently hinders the seed setting and subsequent utilization of the plant for conventional method of propagation. In addition, poor germination potential of seeds also hinders the cultivation of this species. O. spiralis is variably distributed and very rare in the field and conservation of this species is needed to ensure its sustainable utilization (Rajendran et al., 2001). In vitro culture of O. spiralis has been attempted through organogenesis (Elangomathavan et al., 2003). The present study aims at developing a simple, rapid, economical, and high-frequency regeneration protocol from nodal explants of O. spiralis for potential application in large-scale propagation.

Materials and Methods

Healthy plants of O. spiralis (Fig. 1A) collected from Azhiyar (Coimbatore), Tamil Nadu, India and were raised in pots containing soil and farm yard manure (1:1) under green house conditions at Poonga Biotech Research Centre, Chennai. Nodal segments were collected from potted plants, brought to the laboratory and processed. For surface sterilization, the explant were cleaned thoroughly under running tap water for 20 minutes;
Table – 1: Effect of different concentration of cytokinin (BA) and auxin (NAA) in MS medium on in vitro shoot multiplication from nodal explants of *O. spiralis*

<table>
<thead>
<tr>
<th>Plant growth regulator (μM)</th>
<th>Shoot induction (%)</th>
<th>Number of shoot per explant</th>
<th>Shoot length (cm)</th>
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</thead>
<tbody>
<tr>
<td><strong>BA</strong></td>
<td><strong>NAA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.11</td>
<td>0.54</td>
<td>25.00 ±5.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00 ±1.00&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.34</td>
<td>0.54</td>
<td>36.67 ±2.89&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.67 ±0.58&lt;sup&gt;abcdef&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.69</td>
<td>0.54</td>
<td>40.00 ±0.00&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>4.33 ±1.15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.40</td>
<td>0.54</td>
<td>25.00 ±5.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.67 ±0.58&lt;sup&gt;abcdef&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.22</td>
<td>0.54</td>
<td>40.00 ±0.00&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.00 ±1.00&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.34</td>
<td>46.67 ±2.89&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>3.67 ±0.58&lt;sup&gt;abcdef&lt;/sup&gt;</td>
<td>2.93 ±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.69</td>
<td>41.67 ±5.77&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.00 ±0.00&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.00 ±0.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.40</td>
<td>30.00 ±1.00&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>1.67 ±1.15&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.03 ±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.44</td>
<td>0.54</td>
<td>31.67 ±2.89&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.67 ±0.58&lt;sup&gt;abcdef&lt;/sup&gt;</td>
</tr>
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</table>

Data were recorded after 35 days of culture. Results represent mean ± SD of six replicated experiments. Values denoted by different letters differ significantly at *p* < 0.05 level using Duncan’s multiple range test (DMRT).

Table - 2: Rooting proliferation from in vitro shootlets of *Orthosiphon spiralis*

<table>
<thead>
<tr>
<th>PGRs Concentration IBA (μM)</th>
<th>% response</th>
<th>Roots/shoot</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.49</td>
<td>30.0 ± 5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.98</td>
<td>41.7 ± 7.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.46</td>
<td>100 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.92</td>
<td>55.0 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12.30</td>
<td>36.6 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data were recorded after 30 days of culture. Results represent mean ± SD of six replicated experiments. Values denoted by different letters differ significantly at *p* < 0.05 level using Duncan’s multiple range test (DMRT).
Figure 1(A – H): Plant regeneration from nodal explants of *Orthosiphon spiralis*

A) Mother plant of *Orthosiphon spiralis*; B) Nodal explants inoculated on MS medium supplemented with BA 4.44 μM and 1.34 μM NAA μM after two weeks of culture; C) Initiation of shoot from nodal explants after three weeks of culture; D) Proliferation of multiple shoots from internodal explants at 35 days of cultured on MS medium containing BA 4.44 μM and 1.34 μM NAA μM; E) Healthy *in vitro* shootlets inoculated on half strength MS medium containing 2.46 μM IBA; F) A well established plant; G) Well established and hardened *in vitro* plants successfully transferred to the paper cups; H) Hardened plants transferred to external environment condition showing luxurious growth.
washed with a solution of Tween 20 (2 drops in 100 ml of water) for 1 min, and again washed with sterile distilled water. The cleaned explants were finally treated with 0.1% (w/v) mercuric chloride (HgCl₂) for 4 minutes under aseptic conditions and washed 5 times with sterile distilled water to remove traces of HgCl₂.

After surface sterilization, explants were trimmed to 0.8 – 1.0 cm and inoculated on MS (Murashige and Skoog, 1962) basal medium supplemented with concentrations and combinations of BA (1.11, 2.22, 4.44, 6.66 and 8.88 μM) and NAA (0.54, 1.34, 2.69 and 5.36 μM) for shoot multiplication. At the end of the experiment, percentage of shooting, shoot length and the number of shoots per explant were recorded after 35 days in culture. The proliferated shootlets (6.0 cm in length) were excised from cultures and transferred to half strength MS medium supplemented with IBA (0.49, 0.98, 2.46, 4.92 and 12.30 μM) for in vitro rooting. Root number and length were recorded after 30 days in culture. Well developed plantlets were rinsed thoroughly with sterile water to remove residuals and potted with a mixture of red soil, vermiculite and farmyard manure (1:1:1), covered with transparent polyethylene bags to ensure high humidity. After 15 days, the fully acclimatized plantlets were transplanted to plastic pots (80 mm diameter).

For all the above studies, MS basal medium supplemented with 3% (w/v) sucrose was used for all in vitro culture studies. The pH of the medium was adjusted to 5.6 ± 0.2 prior to adding 0.9% (w/v) agar, and autoclaved at 121 °C at 0.6 kg cm⁻² for 15 min. Cultures were maintained at 25 ± 1°C under 16h photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol m⁻² s⁻¹ provided by cool white fluorescent lamps (Phillips, India) and with 60 – 65 % relative humidity. The plant growth regulators were filter-sterilized using 0.2 mm filter (Minisart®; Sartorius) prior to addition to culture media.

Statistical Analysis
Each experiment was repeated three times and each treatment had six replicates. The data were analysed using analysis of variance (ANOVA), and means were compared the Duncan’s multiple range test (DMRT) at 5% level of significance (p < 0.05).

Results and Discussion
Multiple shoots developed from nodal explants cultured on MS medium supplemented with BA (1.11 - 8.88 μM) and NAA (0.54 - 5.40 μM) showed differential response according to the hormonal concentration used (Table 1). In the present study, BA along with NAA seems to influence the induction of shoots in O. spiralis. Initiation of multiple shoots in most of the treatments was observed within 3 weeks of culture. High number of multiple shoots developed in MS medium containing BA 4.44 μM and 1.34 μM NAA showed better growth response (85 %) and produced 52.3 ± 2.1 shoots per explant with an average length of 6.43 ± 0.06 cm after 35 days (Table 1; Fig. 1B-D). This synergistic combination of auxin and cytokinin on organogenic differentiation has been well explained in plant tissue culture (Baskaran and Jayabalan, 2005; Gururaj et al., 2007; Janarthanam and Seshadri, 2008; Janarthanam and Sumathi, 2010). Whereas higher concentration of BA 8.88 μM along with NAA 5.40 μM gradually fall in the number of shoots per explants. However, an earlier report available on the production of in vitro shootlets from nodal explants of O. spiralis on MS medium supplemented with BA 2.22 μM produced 32.25 ± 1.06 shootlet per explants. In our study, nodal explants inoculated on MS medium supplemented with synergetic combination of BA 4.44 μM and 1.34 μM NAA showed better growth response (85 %) and produced 52.3 ± 2.1 shoots per explant.

Individual shoots from a multiple shoot complex were separated after 28 days of culture and transferred to half strength MS medium supplemented with IBA (0.49 – 12.30 μM). The root induction was initiated after two weeks of culture, and after four weeks, the root system was well developed (Fig. 1E, F). The maximum rooting response (100 %) was achieved on medium supplemented with IBA (2.46 μM), with an average of 7.3 ± 0.25 roots per shoot explant (Table 2). Earlier studies reported the use of higher concentration of IBA (4.9 μM) for effective rooting in O. spiralis (Elangomathavan et al., 2003). In the present study root induction was obtained with lower concentration of IBA.

Ninety percent plantlet survival was seen after hardening of the regenerated Orthosiphon spiralis in red soil, vermiculite and farmyard manure (1:1:1) for 3 weeks. However, the rate decreased as some plants died over the next 4 - 5 weeks after transfer to soil. It was observed that very gradual acclimatization of in vitro grown plants to the external environment is most essential to Orthosiphon spiralis. Eighty percent of the plants transferred to pots survived and resumed growth (Fig. 1G-H).

In conclusion, the results showed the ability of the node explants to produce higher number of shootlets without any intervening callus phase, where all the plantlets were uniform in height and growth. Hence, we propose this protocol a simple, economical, rapid and highly reproducible to obtain more plantlets within a short period of time.

References


