



ISSN: 0975-833X

## RESEARCH ARTICLE

### RAPID AND EFFICIENT PLANT REGENERATION FROM SHOOT TIP EXPLANTS OF *Orthosiphon spiralis* MURR- AN IMPORTANT MEDICINAL PLANT

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#### ARTICLE INFO

##### Article History:

Received 20<sup>th</sup> July, 2013  
Received in revised form  
25<sup>th</sup> August, 2013  
Accepted 04<sup>th</sup> September 2013  
Published online 10<sup>th</sup> October, 2013

##### Key words:

*Orthosiphon spiralis*,  
Mass propagation.  
Shoot tip explant.

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#### ABSTRACT

An efficient plant regeneration protocol was developed for *Orthosiphon spiralis* - an important medicinal plant. Shoot tip explants inoculated on Murashige and Skoog (MS) medium supplemented with 1.0 mg l<sup>-1</sup> 6- Benzyl adenine (BAP) and 0.25 mg l<sup>-1</sup> NAA showed better growth response and produced 52.3 ± 2.1 shoots per explant with an average length of 6.43 ± 0.06 cm after 30 days. Rooting of shoots was achieved on growth regulator free half strength MS medium produced 7.3 ± 0.25 cm roots with an average height of 5.4 ± 0.2 cm after 28 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).

#### 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, simple, green, glabrous with a lanceolate leaf blade and a serrate margin. It is distributed mainly throughout South East Asia and tropical countries (Shobana *et al.*, 2011). *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 nephrocirrhosis and phosphaturia. The activity of the leaves is attributed to the presence of a bitter glycoside, orthosiponin (Wealth of India, 1966). *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 (Alshawsh *et al.*, 2011; Kannappan *et al.*, 2010). 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, the seeds of *Orthosiphon spiralis* show a very low germination percentage and propagation by seed does not produce homogenous populations, resulting in great variability in potent metabolites (Shobana *et al.*, 2011). Earlier *in vitro* propagation of *O. spiralis* has been attempted through organogenesis (Elangomathavan *et al.*, 2003). *In vitro* plant regeneration from shoot tip explants has not been reported so far. The present study aims at developing a simple, rapid, economical, and high frequency regeneration protocol from shoot tip explants of *O. spiralis* for potential application in large-scale propagation.

#### MATERIALS AND METHODS

**Plant material:** *Orthosiphon spiralis* an erect, perennial shrub with coarsely toothed leaves and white flowers in cluster were collected during the middle of October 2010 from Azhiyar hill (Coimbatore), Tamil Nadu, India and were raised in pots containing soil and farm yard manure (1:1) under green house conditions at Department of Biotechnology, D.G. Vaishnav College, University of Madras, Arumbakkam, Chennai – 600106, Tamil Nadu, India.



Plant regeneration from shoot tip explants of *O. spiralis*

**Explant preparation:** Shoot tip explants were collected from potted plants, brought to the laboratory and processed. For surface sterilization, the explants were cleaned thoroughly under running tap water for 20 minutes; 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)

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mercuric chloride (HgCl<sub>2</sub>) for 4 minutes under aseptic conditions and washed 5 times with sterile distilled water to remove traces of HgCl<sub>2</sub>.

**Shoot regeneration:** After surface sterilization, explants were trimmed to 0.6 – 0.8 cm and inoculated on MS (Murashige and Skoog, 1962) basal medium supplemented with different concentrations and combinations of BAP (0.25, 0.5, 1.0 and 2.0 mg l<sup>-1</sup>) and NAA (0.1, 0.25, 0.5 and 1.0 mg l<sup>-1</sup>) 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.

**Rooting and acclimatization:** The proliferated shootlets (6.0 cm in length) were excised from cultures and transferred to half strength MS medium supplemented with no growth regulator for root development. Root number and length were recorded after 30 days of culture. Well developed plantlets were rinsed thoroughly with sterile water to remove residuals and potted with a mixture of red soil, vermiculite and farm yard 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).

**Culture medium and conditions:** 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 with a pressure of 1.5 kg cm<sup>-2</sup> for 15 min. Cultures were maintained at 25 ± 1°C under 16h photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol m<sup>-2</sup>s<sup>-2</sup> provided by cool white fluorescent lamps (Phillips, India) and with 60 – 65 % relative humidity. The plant growth regulators were filter-sterilized using 0.2 μm 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 using the Duncan's multiple range test (DMRT) using SPSS (SPSS version 16.0) at 5% level of significance (p < 0.05).

## RESULTS AND DISCUSSION

Multiple shoots developed from shoot tip explants cultured on MS medium supplemented with BAP (0.25 - 2.0 mg l<sup>-1</sup>) and NAA (0.1 – 1.0 mg l<sup>-1</sup>) showed differential response according to the hormonal concentration used (Table 1).

**Table 1. Effect of different concentrations of BAP and NAA on MS on *in vitro* propagation from shoot tip explants of *Orthosiphon spiralis***

Plant growth regulator (mg l <sup>-1</sup> )		Shoot induction (%)	Number of shoot per explant	Shoot length (cm)
BAP	NAA			
0.25	0.1	25.00 ± 5.00 <sup>a</sup>	2.00 ± 1.00 <sup>abc</sup>	2.07 ± 0.38 <sup>bc</sup>
	0.25	36.67 ± 2.89 <sup>bc</sup>	2.67 ± 0.58 <sup>abcd</sup>	1.90 ± 0.17 <sup>b</sup>
	0.5	40.00 ± 0.00 <sup>cd</sup>	4.33 ± 1.15 <sup>d</sup>	3.33 ± 0.15 <sup>f</sup>
	1.0	25.00 ± 5.00 <sup>a</sup>	2.67 ± 0.58 <sup>abcd</sup>	1.93 ± 0.12 <sup>b</sup>
0.5	0.1	40.00 ± 0.00 <sup>cd</sup>	2.00 ± 1.00 <sup>abc</sup>	1.90 ± 0.10 <sup>b</sup>
	0.25	46.67 ± 2.89 <sup>de</sup>	3.67 ± 0.58 <sup>cd</sup>	2.93 ± 0.06 <sup>e</sup>
	0.5	41.67 ± 5.77 <sup>d</sup>	2.23 ± 0.00 <sup>abc</sup>	2.00 ± 0.50 <sup>b</sup>
	1.0	30.00 ± 10.00 <sup>ab</sup>	1.67 ± 1.15 <sup>ab</sup>	2.03 ± 0.23 <sup>b</sup>
1.0	0.1	31.67 ± 2.89 <sup>ab</sup>	2.67 ± 0.58 <sup>abcd</sup>	2.67 ± 0.29 <sup>de</sup>
	0.25	85.00 ± 0.50 <sup>f</sup>	52.3 ± 2.1 <sup>e</sup>	6.43 ± 0.06 <sup>h</sup>
	0.5	50.00 ± 5.00 <sup>e</sup>	4.00 ± 1.00 <sup>d</sup>	3.87 ± 0.21 <sup>g</sup>
	1.0	40.00 ± 5.00 <sup>cd</sup>	3.33 ± 1.53 <sup>abcd</sup>	2.43 ± 0.06 <sup>cd</sup>
2.0	0.1	25.00 ± 5.00 <sup>a</sup>	2.67 ± 1.15 <sup>abcd</sup>	1.93 ± 0.06 <sup>b</sup>
	0.25	26.67 ± 2.89 <sup>a</sup>	2.67 ± 0.58 <sup>abcd</sup>	2.23 ± 0.31 <sup>bc</sup>
	0.5	35.00 ± 5.00 <sup>bc</sup>	3.00 ± 1.73 <sup>abcd</sup>	1.93 ± 0.1 <sup>b</sup>
	1.0	30.00 ± 0.00 <sup>ab</sup>	1.00 ± 0.00 <sup>a</sup>	1.47 ± 0.06 <sup>a</sup>

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)

In the present study, BAP along with NAA found to optimal levels individual multiple shoots in *O. spiralis*. Initiation of multiple shoots in most of the treatments was observed within 3 weeks of culture. Maximum number of multiple shoots developed in MS medium containing 1.0 mg l<sup>-1</sup> BAP and 0.25 mg l<sup>-1</sup> 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. 1 A,B,C). 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 at higher concentration of 2.0 mg l<sup>-1</sup> BAP along with 1.0 mg l<sup>-1</sup> NAA gradual fall in the number of shoots per explants. Earlier studies reported, nodal explants of *O. spiralis* on MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP produced 32.25 ± 1.06 shootlet per explants (Elangomathavan *et al.*, 2003). In our study, shoot tip explants inoculated on MS medium supplemented with synergetic combination of 1.0 mg l<sup>-1</sup> BAP and 0.25 mg l<sup>-1</sup> NAA showed better growth response (85 %) and produced upto 52.3 ± 2.1 shoots per explant.



**Fig. 1. (a – g) –Plant regeneration from shoot tip explants of *Orthosiphon spiralis***

(a) & (b) Initiation of shoots from shoot tip explant after three weeks of culture on MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP and 0.25 mg l<sup>-1</sup> NAA, (c) Proliferation of multiple shoots (52.3 ± 2.1) from shoot tip explants at 35 days of culture on MS medium containing 1.0 mg l<sup>-1</sup> BAP and 0.25 mg l<sup>-1</sup> NAA, (d) Rooted plantlets after 30 days of culture on ½ strength MS medium without growth regulator, (e) A well established plant, (f) Well established and hardened *in vitro* plants successfully transferred to the paper cups, (g) Hardened plants transferred to external environment condition showing luxurious growth

### Plant regeneration from shoot tip explants of *Orthosiphon spiralis*

The regenerated shoots with 4-5 leaves were rooted on the growth regulator free half strength MS medium. The root induction was initiated after two weeks of culture, and after four weeks, the root system was well developed (Fig. 1D, E). The percentage of rooting was 100 % and 7.3 ± 0.25 roots per shoot explant (Table 2). The high number of root per shoot produced on half strength growth regulator free medium in *O. spiralis*. In earlier report wherein higher concentration of IBA (4.9 μM and 2.95 μM) for effective rooting in *O. spiralis* and *Caesalpinia bonduc* (L.) Roxb were used (Elangomathavan *et al.*, 2003; Santosh Kumar *et al.*, 2012).

**Table 2. Rooting response on *in vitro* shoots of *Orthosiphon spiralis* on growth regulator free half strength MS medium**

Medium	% response	Number of roots / shoot	Root length (cm)
½ MS	100 ± 0.0	7.3 ± 0.25	5.4 ± 0.2

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)

Hardening of the regenerated *Orthosiphon spiralis* in red soil, vermiculite and farmyard manure (1:1:1) for 3 weeks, showed 90 % survival. 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. 1 F,G). In conclusion, the results showed the ability of the shoot tip 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.

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