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In vitro propagation of *Orthosiphon stamineus* Benth (Lamiaceae) an important medicinal plant using nodal and leaf explants

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Abstract
The present paper reports the *in vitro* propagation of the valuable medicinal plant, *Orthosiphon stamineus* Benth in various concentrations of plant growth regulators. Nodes and leaves were used as explants for the induction of multiple shoots and callus respectively. The explants were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations of 6-benzyl amino purine (BAP) and Kinetin (KIN) alone and BAP in combination with α- Naphthalene acetic acid (NAA) for multiple shoot induction. For callus induction leaf explants were cultured on MS medium supplemented with different concentrations of auxins like 2, 4-Dichlorophenoxyacetic acid (2, 4-D), NAA and Indole-3-butyric acid (IBA). The maximum number of shoots was produced in BAP 1.00mg/l after 8 weeks of culture. An average of 12.9 ± 0.10 shoots measuring 5.94 ± 0.13 mean length were produced from each explants. For root induction the shootlets were transferred to MS medium supplemented with different concentrations of IBA. Highest percentage of root induction was observed in 4.00 mg/L IBA. The rooted plantlets were hardened in the mixture of 1:1:1 ratio of soil, sand and vermicompost. 60-70% plants were successfully established in the field. Optimum callus induction was observed on MS medium supplemented with 2.00 mg/l 2, 4-D.The callus was subcultured on MS medium containing different combinations of BAP and NAA in various concentration. Though the callus showed greenish somatic embryoids, it failed to produce shoots even after 8 weeks of culture.

Keywords: *Orthosiphon stamineus Benth, nodal and leaf explants, MS medium, multiple shoots, callus*

1. Introduction
Orthosiphon stamineus Benth, a tropical, medicinal herb belonging to the family Lamiaceae, is native to South East Asia. *O. stamineus* commonly referred to as Cat's whiskers, Kidney tea plant or Java tea, is used in traditional folk medicine for centuries for the treatment of various human ailments. Leaves of *Orthosiphon stamineus* is extensively used as diuretic and for a wide range of conditions including rheumatism, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorders, gonorrhea, syphilis, renal calculus (kidney stones), gallstones, gout, edema, eruptive fever, hepatitis, and jaundice abdominal pain, kidney and bladder inflammation [1, 2, 3, 4]. Studies have revealed that leaves exhibit several pharmacological properties like antioxidant, hepatoprotective. [5] Antibacterial, [6] anti-inflammatory [7] cytotoxic [8] diuretic [9, 10] hypoglycemic [11] the diuretic property of *Orthosiphon stamineus* is due to its influence on the activity of adenosine A (1) receptor antagonists which can protect the kidney by increasing urine flow and sodium excretion [12] *O. stamineus* is conventionally propagated vegetatively by stem cutting. Delayed rooting of stem cuttings inhibited large scale propagation of this plant. To meet the market demand an alternative method –Tissue culture, serves for rapid multiplication and commercial supply of *O. stamineus*. The current study was carried out to propagate the important medicinal plant *O. stamineus in vitro* with various combinations or concentrations of plant growth regulators.

2. Materials and methods
2.1 Collection of plant
Healthy plants of *Orthosiphon stamineus* were collected from the Irula tribal woman`s welfare society, Thandurai, Chengalpattu, Tamil Nadu, identified by Dr. G. Jeya Jothi, Taxonomist from the Department of Plant Biology and Biotechnology, Loyola College, Chennai and grown in nursery green house. Young, healthy and disease free explants were used for *in vitro* studies.

2.2 Sterilization Procedures

Sterilization of inoculation and incubation room was achieved by fumigation of the room with formaldehyde for at least 12 hours. The excess formalin vapour was removed by placing liquid ammonia in a petridish in the fumigated area. The Laminar air flow chamber was swabbed with alcohol and exposed to germicidal UV (253.7 mm) lamp for half an hour before inoculation. The culture vials and the glasswares left in dilute sulphuric acid overnight and then washed using detergent with running water followed by rinsing with deionized distilled water. The vials and glasswares were allowed to dry for some time and dry sterilized in hot air oven at 180 °C for 2 hours or 360 °C for 1 hour. Forceps, blade, aluminium foil, blade holder, cotton, tissue paper, cutting board etc. were sterilized by wet sterilization using an autoclave. These tools were wrapped in aluminium foil and autoclaved at 15 - 20 Lbs for 15 minutes. The sterilized tools were immediately transferred to the laminar air flow chamber.

2.3 Media preparation

The MS basal medium (Murashige and Skoog, 1962) [13] was used in this study. The culture medium consists of all micro and macro nutrients, vitamins and aminoacids. All the required chemicals for the preparation of MS medium were weighed with the help of Shimadzu electronic monopan balance. The stock solution of 500 times strength of MS medium was prepared and stored in the refrigerator. A stable standard stock solution of various PGRs were prepared by weighing 10 milligram (mg) each of PGRs and dissolved in 10 millilitre (ml) of distilled water and stored at 5 ^{0}C. The medium was supplemented with various concentrations of Cytokinins namely BAP (6-benzyl amino purine and KIN(kinetin) alone or in combinations of BAP and NAA for multiple shoot formation and various concentration of Auxins namely 2,4-D, IBA, NAA at 1mg/l concentration for callus induction. The plant growth regulators were either used alone or in combinations. Required proportion of the components of the medium, namely micro and macro, vitamins and growth regulators were added in a conical flask with help of measuring cylinder and sterile syringe. The medium was made up to the required volume by adding deionised sterile distilled water prior to the addition of 3%(w/v) sucrose and 0.8%(w/v)Agar. The PH of the medium was measured and adjusted to 5.8 using 0.1N NaOH and if necessary with 0.1 N HCL. Sucrose was added as a carbon source. After the addition of agar the medium was warmed up till the agar melted. The liquid medium was poured equally in the culture vials (10 ml in each vials) and was closed with cotton plugs immediately. The medium was autoclaved at 121 ^{0}C for 15 psi for 15 minutes. The mouth of the culture vials were sealed tightly after autoclaved, undisturbed to cool. The labeling of the medium was done with labels and the culture vials were kept under sterile condition.

2.4 Isolation and surface sterilization of explants

Healthy and young explants, nodal axillary buds and leaf discs of about 1-1.5 cm were selected for sterilization. The excised explants were thoroughly washed in running tap water for about 15-20 minutes and in 5% (w/v) detergent solution of Tween 20 for 10 minutes to remove adhering particles of living and non-living. After thorough rinsing in sterile distilled water The explants were surface sterilised with 70% ethanol for 30 seconds and then in 0.02% (w/v) aqueous $HgCl_2$ for 3-5 minutes under aseptic conditions. Sterilised explants were transferred to the inoculation chamber, where they were washed with double distilled sterile water for at least 7 times to remove any trace of surface sterilizing chemicals present on the surface of the explants.

2.5 Inoculation

Prior to inoculation all the implements and the vials were kept inside the laminar air flow chamber in the presence of UV light for about 30 minutes. The explants were first kept on sterile tissue paper in order to remove water from the surface. The explants were transferred to the cutting board and the explants were further trimmed to 0.5-1cm and planted on the culture medium. The mouth of the culture vials was held close to the flame and sealed immediately after inoculation with cotton plug and labeled. The inoculated culture vials were incubated in dark and light conditions at 24+2 ^{0}C. Light condition were provided by cool-white fluorescent tubes (polylux XL) of 4000 lux.

2.6 Shoot tip culture

For multiple shoot induction, nodal explants were inoculated on MS medium supplemented with hormones such as BAP and KIN (0.5, 1.0, 2.0 and 3.0 mg/l) individually and BAP in combination with NAA (0.1, 0.25 and 0.5 mg/L). The culture were maintained for 4 weeks and were subcultured on the same combinations of MS medium for further proliferation and elongation. Each treatment consisted of 10 replicates and was repeated twice. Total number of shoots per explant and length of the shoots were measured after 8 weeks of culture.

Shootlets 3-5 cm in lengh were separated, exised and transferred to rooting medium supplemented with various concentrations (1.0, 2.0, 3.0 and 4.0 mg/L) of IBA individually. After 4 weeks of culture, the total number of roots produced per shoot and length of the roots were measured.

Rooted plants (4-5 cm) were carefully removed from the test tubes and washed with sterile water to remove traces of agar adhering to the roots. The plantlets were transferred to paper cups filled with mixtures of sterilized sand, soil and vermicompost in the ratio 1:1:1. The acclimatized plantlets were transferred and established in the field.

2.7 Callus culture

For callus induction the sterilized leaf explants were cultured on MS medium supplemented with various concentration of Auxins like 2, 4-D, NAA and IBA alone (1.00, 2.00, 3.00, 4.00 mg/l). The *calli* formed were periodically subcultured every two weeks on MS medium containing BAP(1mg/L) +NAA(0.5mg/l); BAP(1mg/l)+2,4-D(0.5 mg/l); BAP (1 mg/l)+IBA (0.5 MG/L); BAP (2 mg/l) +NAA(0.5mg/l); BAP(3mg/l) +NAA(0.5mg/l) for multiple shoot induction.

3. Results

3.1 Induction and proliferation of multiple shoots

Initiation of shoots in nodal explants occurred within 5-8 days of inoculation while it took more than 10 days in basal medium. Among the various cytokinins tested, BAP (1 mg/l) produced maximum number of multiple shoots (12.9 ± 0.10) per explants with 86% of response after 8 weeks of inoculation, followed by kinetin producing 8.6 ± 2.11 multiple shoots with 68% response. Maximum height of shoots (5.94 ± 0.13 cm) was observed at 1 mg/l BAP followed by 4.16 ± 0.19 cm in kinetin 2 mg/l [Table1, Fig 1]

After 4 weeks the shootlets were excised and further

transformed to the same concentration of medium for mass propagation. In subsequent subculturing high frequency of bud break and multiple shoot induction was observed in BAP1 mg/l (Fig 1-c), followed by KIN 2 mg/l. The induction of multiple shoot decreased gradually in all other hormones tested.

The addition of NAA to BAP in the MS medium, decreased the proliferation of axillary bud at very low concentration (0.50 BAP mg/l+0.10 NAA mg/l; 0.50 BAP mg/l +0.20 NAA mg/l; 1.00 BAP mg/l +0.20 NAA mg/l). However multiple shoot were induced in higher concentrations (1.00 BAP mg/l+0.50 NAA mg/l; 2.00 BAP mg/l+ 0.20 NAA mg/l; 2.00 BAP+0.50 NAA mg/l). Morover the addition of NAA to BAP in the MS medium induced the formation of callus at the base of the nodal explants there by decreasing the induction of multiple shoots (Fig 1–e). Only single shoots with roots were produced in lower concentration of BAP and NAA (Fig 1-d). Multiple shoots could only be generated by combining low concentrations of auxin and cytokinin with BAP (2.0 mg/L) and NAA (0.5 mg/L), being the most suitable.

Table 1: Effect of plant growth regulators on multiple shoot induction from nodal explants of *O. Stamineus* after 8 weeks of culture.

Hormone Concentration (mg/l)	Explants responded (%)	Average number of shoots /explants a	Mean length of shoots(cm)a
BAP			
0.50	69%	11.8± 1.32	3.28 ± 0.16
1.00	86%	12.9 ± 0.10	5.94 ± 0.13
2.00	79%	8.70 ± 0.95	4.56 ± 0.17
3.00	64%	6.40 ± 1.43	3.28 ±0.14
KIN			
0.50	68%	8.6 ± 2.11	3.53 ± 0.28
1.00	54%	5.6 ± 0.52	2.32 ± 0.06
2.00	82%	7.3 ± 1.49	4.16 ± 0.19
3.00	60%	3.1 ± 0.74	3.18 ± 0.09
BAP +NAA			
0.50 +0.10	-		
0.50 +0.20	-		
1.00 +0.20	-		
1.00+0.50	40%	2.6±0.54	1.38 ± 0.51
2.00 +0.20	40%	3.17±0.75	2.97±0.18
2.00 +0.50	72%	3.73 ± 1.35	3.34±0.29

[a] Mean ± Standard Deviation
NO multiple shoot formation.

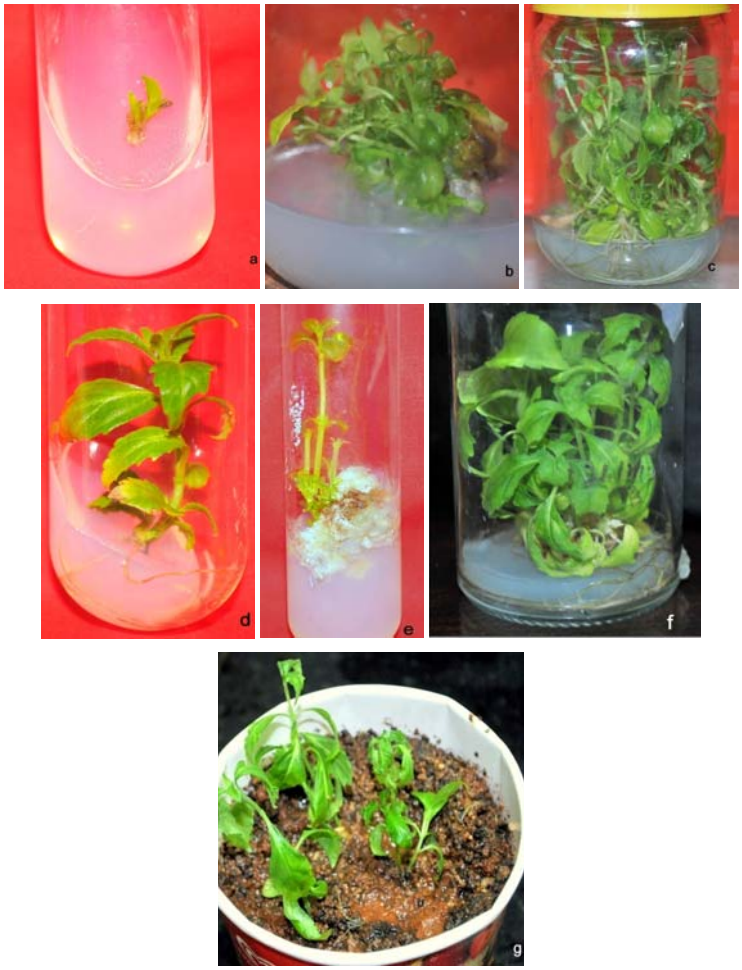

Fig 1: *In vitro* multiplication of *Orthosiphon stamineus.* a-Shoot tip explant after 7 days of culture inoculated on MS medium containing 1mg/l BAP; b- Multiple shoots formed after 2 weeks ; c- shoots formed after subculturing shootlets on MS medium containing 1mg/l BAP showing root formation ; d- Single shoot proliferation on MS medium containing lower concentration of BAP and NAA; e Callus formation at the base of explants in the presence of BAP and NAA; f- *In vitro* root formation on MS medium with 4 mg/l IBA after 4 weeks of culture; g- *In vitro* acclimatized plantlets in plastic cups containing soil, sand and vermicompost in the ratio 1:1:1 for hardening.

3.2 Rooting and hardening

Rooting was done In MS medium supplemented with different concentration of IBA. It was observed that the % of response, average number of rootlets and mean length of rootlets increased with increase in concentration of IBA (Table 2 Fig 1). Highest % of response (100%) and number of rootlets (9.2 ± 4.08) with root length (5.97 ± 0.66) was observed in 4 mg/L. During hardening 60-70% of plants were established when transferred to polycups containing soil, sand and vermicompost in the ratio of 1:1:1

Table 2: Effect of plant growth regulators on Root induction from the shoot lets of *O. stamineus* cultured on MS medium

Hormone of Concentration (mg/l)	Explants responded (%)	Average number of roots /explants [a]	Mean length roots(cm)[a]
0.5	80%	3.8±1.30	2.43 ± 0.39
1.00	100%	7.8±0.84	4.36 ± 0.50
2.00	78%	8.38± 1.10	5.93 ± 1.90
4.00	100%	9.2 ± 4.08	5.97 ± 0.66

3.3 Induction of callus

Callus induction began by curling of leaves and callus appeared on the cut end of the leaf explants within 8-10 days of inoculation. Highest fresh weight callus was obtained from the leaf explants inoculated in 2.00 mg/l 2, 4-D within 2 weeks of culture followed by 4.00 mg/l 2, 4-D (Table 3 Fig 2).The callus was subcultured in 2, 4-D for proliferation and maintenance. The callus initially appeared light creamy greenish, later light brown and eventually turning to dark brown after 8 weeks of culture. The combinations of all MS medium containing BAP with 2,4-D,NAA and IBA failed to produce multiple shoots, but greenish somatic embryoids appeared on callus after 8 weeks of culture on higher concentration of BAP(2mg /l)+NAA (0.5 mg/l) and BAP (3 mg/l)+NAA(0.5 mg/l) (Fig 2-f).

Table 3: Effect of plant growth regulators on callus induction from leaf explants of *O. Stamineus* after 4 weeks of culture.

Plant growth Regulator (mg/l)	Explants inducing callus (%)	Nature of the callus	
		Texture	colour
2, 4 –D			
Control	20%	F	LB
1.00	78%	F	LB
2.00	95%	F	LB
3.00	60%	F	LB
4.00	80%	F	LB
NAA			
1.0	25%	F	LB

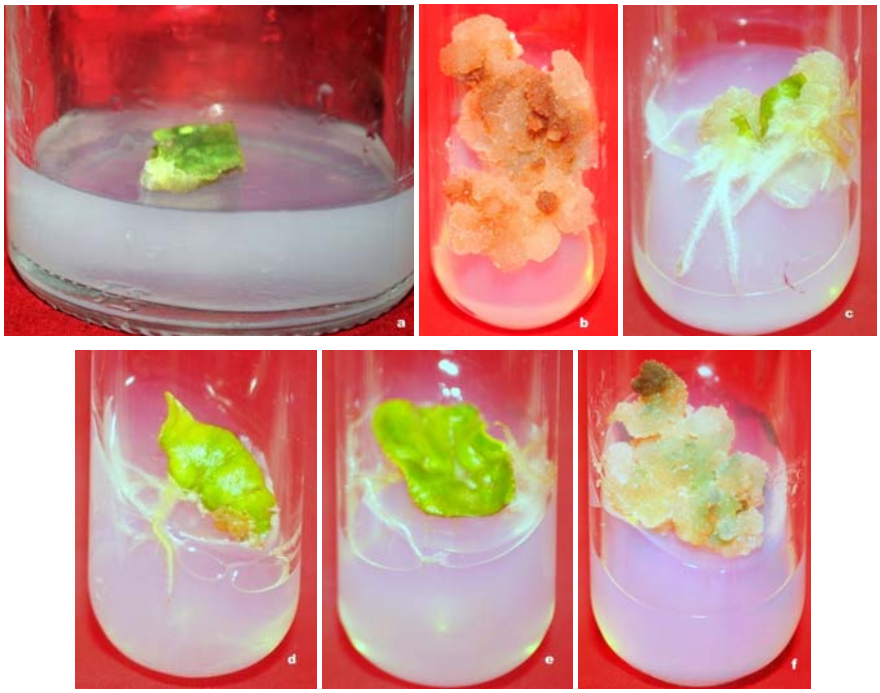

Fig 2: Somatic embryogenesis of *Orthosiphon stamineus* using leaf explant. a- Callus proliferation on MS medium containing 2 mg/l 2, 4 –D after 1 week of culture. b- Friable brown callus after 8 weeks of culture. C-callus proliferation in NAA 1 mg/l MS medium with rootlets. dand e lack of callus proliferation in higher concentration of NAA and IBA respectively. Only rootlets can be seen. f- greenish somatic embryos on MS medium containing BAP (3 mg/l) +NAA (0.5 mg/l).

4. Discussion

The described surface sterilization methods of nodal and leaf explants were able to produce 80-85% aseptic explants. The best effective plant growth regulator for multiple shoot induction and proliferation from nodal explants of *O. stamineus* is BAP 1.0 mg/l followed by KIN 2 mg/l. Remarkable decrease in % response was observed at lower and higher level of BAP. Similar results were also reported in medicinal plants like *Paederia foetida* and *Codonopsis pilosula*. [14, 15]. In a previous study nodal segments of *O. stamineus* produced 82% of shoots when cultured in 0.5 mg/LBAP [16] and in another study 1.5 mg/L BAP produced higher no of shoots [17]. The difference in response to hormones combination could be due to factors such as plant genotype and physiological states of the explants that influence shoot regeneration of a plant [18]. Our findings showed that high BAP: NAA promotes shoot formation but low BAP: NAA ratio induces root formation and callus

induction in *O. stamineus*. Further, combination of BAP and NAA into media did not have much effect in the induction and proliferation of multiple shoots. This study clearly shows that, BAP alone is effective for rapid shoot multiplication. The stimulatory effect of BAP in multiple shoot induction has been reported in medicinal plants like *Lippia alba* and *Vinca rosea*. [19, 20]. For root induction IBA 4.00mg/l was found to be best growth hormones. IBA was found to be an effective hormone in rooting in other members of Lamiaceae like *plectranthus bourneae* and *Ocimum gratissimum* [21, 22]. The degree of callus induction from leaf explants varied in different plant growth regulators (Fig 2; b). Similar results were also reported in other lamiaceae members like *Coleus vettivereoids* [23] and Ocimum basilicum [24].

The present study reports a rapid propagation of shoots and callus induction. However further studies has to be carried out to identify suitable growth regulators which can induce shoots from callus.

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