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IN VITRO MULTIPLICATION OF *SOLANUM ANGUIVI* LAM, *PHYLLANTHUS AMARUS* SCHUM & THONN AND *JASMINUM CALOPHYLLUM* WALL - ETHANOMEDICINAL PLANTS

Johnson, M
Centre for Biodiversity and Biotechnology, St. Xavier's College (Autonomous) Palayamkottai, Tamil Nadu INDIA, Email: biojohnson2001@yahoo.com
Present Address: Department of Biology, Bahir Dar University, Bahir Dar, Ethiopia

Abstracts: *In vitro* multiplication of *Solanum anguivi* Lam, *Phyllanthus amarus* Schum & Thonn. and *Jasminum calophyllum* Wall are medicinal plants have been achieved on Murashige and Skoog's solid medium (1962) augmented with 6-benzylaminopurine. The maximum numbers of shoots (Solanum *anguivi*, 4.8 ± 0.17; *Phyllanthus amarus*, 16.3±0.45) are obtained in Murashige and Skoog's medium fortified with 6-Benzyl Amino Purine (4.44 µM), for *Jasminum calophyllum*, 4 .3 ± 0.45 are observed in MS medium fortified with 6-Benzyl Amino Purine (6.66 µM). The *in vitro* raised shoots were rooted on half strength Murashige and Skoog's solid medium. The maximum number of roots has been induced in half strength Murashige and Skoog's medium combinations with Naphthalene Acetic Acid (5.37 µM) and Indole-3-Acetic Acid (5.7 µM) for all three plants, subsequently hardened in poly cups after that transfered to greenhouse conditions and field conditions. Nearly 65% of plants are survived in the field.

Keywords: Micropropagation, *Solanum anguivi, Phyllanthus amarus, Jasminum calophyllum*

INTRODUCTION

Each and every human civilization on earth has been rooted in the biodiversity of nature. Biodiversity is the vast array of all species of plants, animals, insects and the microorganisms inhabitating the earth either in aquatic or the terrestrial habitats. Biodiversity provides humankind enormous direct benefits and indirect essential services through natural ecosystem function and stability. It comprises about 5 to more than 50 millions of species of which 2,70,000 plant species. The World Health Organization (WHO) estimates that up to 80% of the world's people rely on plants for their primary health care. Plants provide us food, clothes, medicines, timbers and fuel wood. Plants have contributed more than 7000 different compounds in use today as cardiac drugs, anticancer agents, hormones, antibiotics, laxatives, diuretics, analgesics, anaesthetics, ulcer treatments and antiparasitic compounds. Apart from medicine, they regulate climates, stabilise soil; and produce oxygen and make life on earth possible for humans and animals. World wide, tens of thousands species of higher plants and several hundreds of lower plants are used by

human beings for wide variety of purposes. In the tropics alone, it has been estimated that 25,000 - 30,000 species are in use and up to 25,000 species have been used in traditional medicines. In USA, 74% of drugs are based on plants (Singh, 2002).

India is one of the 18 megadiversity centres in the world. Indian flora is rich and diverse. India harbours about 48,000 species including about 17,000 angiosperms, 23,000 fungi, 2700 bryophytes, 5000 Algae, 1,600 lichens and 1132 pteridophytes (Nayar, 1996; Sarat Babu and Arora, 1999). Today diversity of plant life is facing serious threats, largely due to habitat loss, habitat degradation and increasing exploitation of natural resources. It has been estimated that globally 30% of the flora is threatened (Raven, 1999). The decline in the number and quality of the habitats is attributed to encroaching urbanization, growing industrialization, intensive farming and unsustainable harvesting of wild species. The current rate of tropical deforestation is estimated to be 0.8% per year. This is particularly so in tropical forests where, 2-5 species per hour is being lost. The current rates of species extinction are 1,000 - 10,000 times higher than the background rate of 10^{-7} species / species year inferred from fossil record. Under the current scenario, about 20% of all species are expected to be lost within 30 years and 50% or more by the end of 21st century. According to the World Resource Institute, India figures among 28 countries that are facing severe effects of increasing ecological imbalance if preservation is not taken on war footing. The IUCN report (1999) says that in India 7.7% of the plants are under threat. The establishment of plantations of cash crops like cardamom, coffee, rubber and tea is the main reason for the destruction of the evergreen forests and consequent demise of the ferns in the Western Ghats. There are four basic, often complementary strategies, for biodiversity conservation. They are *in situ* conservation, *ex situ* conservation, reduction of biotic pressure and rehabilitation. A combination of strategies and ingenious use of both *ex situ* and *in situ* methods have been advocated to achieve the conservation of rare and endangered plants (Wyse Jackson and Sutherland 2000). *Ex situ* conservation can be made by establishing botanical gardens, seed / spore banks, tissue culture repositories and cryopreservation facility. In India, attempts have been made in recent years for *ex situ* conservation of plants by cultivation and propagation in botanic gardens. Among the various biotechnological options, also reported in other agri-horticultural crops, micropropagation through tissue culture and *in vitro* spore germination are best applied and commercially exploited in plants (Fay, 1994). Application of this technology (*in vitro* multiplication) for large-scale

multiplication of certain species of plants from the Western Ghats has been demonstrated (Johnson, 1999; Andal, 1999; Johnson et al., 2002; Johnson and Manickam, 2003; Johnson et al., 2004; Johnson et al., 2005; Sudha and Seeni, 1994; Nair, 2000; Sivasubramanian, 2002; Manickam et al., 2000 and Beenni et al., 2003). That plant tissue culture as an effective tool to conserve plant genes and guarantee the survival of the endemic, endangered and over exploited genotypes is derived from the fact that, it makes use of small units (cells and tissues) without losing their mother plant, takes pressure off the warning wild populations and makes available large number of plants for reintroduction and commercial delivery. In a recent seminar held at Almora, emphasis was laid on mass propagation of economically important threatened species (Nandi *et al.*, 1999).

Based on this background, the present investigation was initiated to extend the good work already done in our laboratory to few other equally endangered and economically important ethanomedicinally important plants species viz. *Solanum anguivi* Lam, *Phyllanthus amarus* Schum & Thonn. and *Jasminum calophyllum* Wall. Because of the medicinal and commercial importance and potential there is a need propagate this plant in large scale. The conventional methods are not applicable for the large scale multiplication. So the plant tissue culture is a useful tool for conservation and rapid propagation of rare ethano medicinal plant. (Fay, 1994, Sudha and Seeni, 1994, Krishnan *et al.*, 1995, Manickam *et al.*, 2000, Johnson and Manickam, 2003 and Johnson *et al.*, 2004; Emmanuel et al., 2000; Elangomadhavan, 2003 and Nikhat, 2004 and Sonali, 2004). As *in vitro* culture of seeds of the species resulted in failure, the nodal segments were selected as the explants for the multiplication. The main objective of the present study was aimed to produce a protocol for the large scale multiplication of the medicinally important plants viz. *Solanum anguivi, Phyllanthus amarus* and *Jasminum calophyllum*.

MATERIALS AND METHODS

The plants [*Solanum anguivi, Phyllanthus amarus* and *Jasminum calophyllum*] were collected from the wild habitats and established in the green house attached to the Centre for Biodiversity and Biotechnology, St. Xavier's College campus. Young shoots were defoliated and the nodal segments (5cm length) were collected from the green house raised plant. The explants were washed in running tap water for 10 min. Surface decontamination of the explants consisted of passage through 0.1% (w/v) $HgCl_2$ for 1 to 3 min. (for *Solanum*

anguivi, 2 min; for *Phyllanthus amarus* 1 min. and for *Jasminum calophyllum* 1.5 min) and then rinsed thrice in sterilized distilled water. Then single node (1cm length) was dissected out and inoculated aseptically onto Murashige and Skoog's solid medium (1962) vertically in 25 mm x 150 mm culture tubes. The medium was supplemented with various concentrations and combinations of plant growth regulators viz BAP, NAA, IAA and IBA. The pH of the medium was adjusted to 5.8 before adding 0.5% agar and autoclaved at 121°C for 15 min in Astell scientific autoclave (U.K). All the cultures were incubated at 25°C under 16 h of illumination provided by cool white fluorescent tubes of approximately 1500-2000 lux. Experiments were performed with a minimum of 20 replicates for shoot, 10 replicates for root and repeated twice. Thirty day old rooted plants were washed thoroughly in small polycups containing mixture of sterilized sand and garden soil (1:1) and irrigated with 1/10 diluted liquid MS medium and covered with polythene bags for *in vitro* hardening. Such plants were then subsequently transferred to pots containing garden soil sand and compost (2:1:1) and kept under mist chamber for wearing. (After the hardening the plantlets were transferred to field establishment rate was tabulated.)

RESULTS AND DISCUSSION

***Solanum anguivi* Lam**

After 3 days from inoculation, the axillary buds in the nodal explants were started to breakdown to initiate shooting. The BAP inoculated nodal explants showed their first response by enlarging and bursting of axillary buds as reported by Zaman *et al.*, (1996), Manjula *et al.*, (1997), and Anand and Molly (1997). After 4 weeks of culture, the multiple shoots were formed on MS medium fortified with BAP different concentrations (Table: 1). Shooting response of explants ranged from 35.3 ±0.32% to 75.3 ±0.36.

Table 1: Effect of Plant Growth Regulators on the formation of multiple shoots from *Solanum anguivi* nodal segments cultured on MS medium.

Plant Growth Regulators (μM)		% of shooting response ± S.E	No. of shootlets per plant ± S.E	No. of nodes per shoot let ± S.E	Shootlet length (mm) ± S.E
BAP	NAA				
0.44	0	35.3 ±0.32	3.8 ± 0.12	4.2 ± 0.32	55.5 ± 1.4
2.22	0	61.0 ± 0.23	4.1 ± 0.12	5.4 ± 0.36	65.7 ± 1.0
4.44	0	75.3 ± 0.36	4.8 ± 0.17	6.8 ± 0.56	75.2 ± 0.79
8.88	0	70.3 ± 0.45	4.4 ± 0.28	5.1 ± 0.45	85.3 ± 1.02
13.32	0	60.8± 0.25	2.3 ± 0.21	3.3 ± 0.38	40.6 ± 0.88
22.22	0	56.3 ± 0.34	1.1 ± 0.08	2.4 ± 0.23	29.8 ± 0.92
2.22	2.685	58.3 ±0.32	2.3 ± 0.16	3.8 ± 0.56	45.7 ± 0.57
8.88	2.685	58.3 ±0.81	1.9 ± 0.27	2.8 ± 0.63	30.5 ± 0.96

The maximum number of shoots (4.8 ± 0.17) per explant was obtained in MS Solid medium fortified with BAP (4.44 μM) (Table 1). At higher concentrations of BAP (13.32 & 22.22 μM) the number of shoots per explant was reduced (2.3±0.21& 1.1 ± 0.08. respectively).As far as the shoot length was concerned, maximum length (85.3 ± 1.02 & 75.2 mm ± 0.79) was obtained at 8.88 μM & 4.44 μM of BAP supplemented medium respectively. The same response was observed in *Moreinga pteryosperma* (Vandhanamohan *et al.*, 1995) *Citrus sincensis* (Tapati *et al.*, 1995) and *Eustoma grandifloruna* (Semeniuk and Griesbach, 1987) whereas the culture medium devoid of growth regulators failed to stimulate bud break in all the nodal explants. The result indicates that the presence of cytokinin in the medium is required by the explants to respond which is in conformity with the result of Loh and Rao (1989), Amin and Jaiswal (1987) and Amin (1991). The mean length of shoot was very much reduced in medium augmented with BAP (8.88 μM) combination with NAA (2.685 μM). The same result was reported in *Adhatoda beddomei* (Sudha and Seeni, 1994). The shoots produced in the presence of BAP and NAA had not only basal callus response but also short internodes. BAP was found to be effective when compared with kinetin and auxin for induction and multiplication of shoots in *Actimidia* (Shen *et al.*, 1990 and Marino and Bartazza, 1990). At the final stage of development, the *in vitro*-grown shoots were rooted in ½ MS medium augmented with various concentrations and combinations of auxins. After 2 weeks of dark incubation the roots were formed at the base of *in vitro*

shootlet with basal callusing response. Maximum number of roots (7.2 ± 0.18) was obtained in ½ MS medium fortified with 5.7 µM of IAA and 5.37 µM of NAA. The basal callus was formed in all the concentrations (Table - 4) IBA alone or in combination with NAA or IAA produced 2.9 ± 0.13 mm and 4.1 ± 0.12 mm roots per shoot respectively. When the length of the roots reduced in IBA (4.92 mm) combined with IAA (5.7 µM) and NAA (5.37 µM). The maximum root length (35.3 ± 0.93 µM) was obtained in IAA (5.7 µM) combined with NAA (5.37µM) (Table – 4). These results are in consonance with that of Anand *et al.*, (1997) and Usha and Swamy, (1998), who worked on *Kampfrenia rotunda* and *Artemisia annua* respectively. The results indicated that IAA was found be suitable for root induction.

Phyllanthus amraus Schum & Thonn.

The MS medium supplemented with different concentrations and combinations of BAP inoculated nodal segments are started to initiate the multiple shooltlets after 3 days. The present results directly coincided with Nikhat, 2004: Sonali, 2004 and Usha, 2004 observations, they also observed the multiple shoot formations on BAP supplemented medium inoculated tubes other plant growth regulators failed to produce shoots. The highest percentage, maximum number of shoots and maximum length of shoots obtained in MS medium augmented with BAP 4.44 µM (65.6±0.85, 16.3±0.45 and 12.3±0.54). The maximum nodes per shoots also observed in the very same concentrations (8.7±0.56/ shoots) (Table 2). The results of Johnson and Manickam, 2003 in *Baliospermum montanum*, Johnson et al., 2004 in *Rhincánthus nasutus* were directly coincided with the present observation.

Table2: Effect of Plant Growth Regulators on the formation of multiple shoots from *Phyllanthus amarus* nodal segments cultured on MS medium.

Plant Growth Regulators (μM) BAP	% of shooting response ± S.E	No. of shootlets per plant ± S.E	No. of nodes per shoot let ± S.E	Shootlet length (cms) ± S.E
0.44	0.00	00±0.00	0.00	0.00±0.00
2.22	35.8±0.87	5.3±0.56	6.3±0.46	3.8±0.34
4.44	65.6±0.85	16.3±0.45	8.7±0.56	12.3±0.54
8.88	21.3±0.56	6.7±0.56	3.3±0.32	1.8±0.34
13.32	11.1±0.32	3.3±0.45	1.7±0.56	1.3±0.38

The in vitro raised shootlets were separated individually and inoculated into ½ MS medium supplemented with different concentration and combinations of auxins for rooting. After 7 days the roots were observed on the root inducing medium inoculated tubes. The formation rooting is varied from 45.1± 0.83 to 86.5 ± 0.56, the maximum percentage (86.5 ± 0.56), maximum number of roots per shoot (5.9 ± 0.13) observed in the ½ MS medium fortified with IAA 9.84 μM in combination with NAA 5.37μM. The maximum length of rootlets also observed in the very same concentrations (Table 4). The present study results were directly linked with previous reports on *Muccuna* species (Elangomadhavan, 2003; *Passiflora mollissima* (Nikhat, 2004) and *Vitex negundo* (Sonali, 2004).

***Jasminum calophyllum* Wall**

After 5 days the nodal segments inoculated tubes were started to proliferate the shoots with different percentage based on the concentrations of plant growth regulators. Each and every concentrated tube is varying the percentage of shoot formations. The maximum percentage of shoot formation (85.3 ± 0.45), maximum length of shoots (88.3 ± 1.02) and number of nodes per shoot (5.1 ± 0.45) also maximum in MS medium augmented with BAP 6.66 μM. The number of shoots per node (5.4 ± 0.28) also observed in the very same concentration (Table -3).

Table 3: Effect of Plant Growth Regulators on the formation of multiple shoots from *Jasminum calophyllum* nodal segments cultured on MS medium.

Plant Growth Regulators (μM)		% of shooting response ± S.E	No. of shootlets per plant ± S.E	No. of nodes per shoot let ± S.E	Shootlet length (mm) ± S.E
BAP	NAA				
0.44	0	15.3 ±0.32	0.8 ± 0.12	1.2 ± 0.32	25.5 ± 1.4
2.22	0	41.0 ± 0.23	4.1 ± 0.12	2.4 ± 0.86	45.7 ± 1.0
4.44	0	45.3 ± 0.36	4.8 ± 0.17	3.8 ± 0.76	65.2 ± 0.79
6.66	0	85.3 ± 0.45	5.4 ± 0.28	5.1 ± 0.45	88.3 ± 1.02
13.32	0	60.8± 0.25	2.3 ± 0.21	3.3 ± 0.38	40.6 ± 0.88
22.22	0	36.3 ± 0.34	1.1 ± 0.08	1.4 ± 0.23	29.8 ± 0.92

The *in vitro* raised shootlets were transfered to root inducing medium aseptically. The maximum percentage (65.4 ± 0.48) rootlets formation obtained in ½ MS medium supplemented with IAA 9.84 μM in combination with NAA 5.37μM (Table - 4).

The matured plantlets were transferred to polycups containing vermiculate for hardening. Adopting the *in vitro* hardening procedure described in materials and methods could increase the rate of survival. Subsequently the hardened plants were shifted to green house. The *in vitro* multiplication of *S. anguivi, P.amarus and J. calophyllum* as described in the present work is expected to increase commercial cultivation of the species. The present study protocol is good one for the large scale multiplication at the same time we can use techniques for the morphogenesis, plant breeding / plant transformation and physiological studies.

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Table 4: Effect of plant Growth Regulators on rooting response of *in vitro* raised shoots of *Solanum anguivi, Phyllanthus amarus* and *Jasminum calophyllum* cultured on ½ MS medium.

Plant Growth Regulators (µM)			*Solanum anguivi* Lam			*Phyllanthus amarus*			*Jasminum calophyllum* L		
IAA	IBA	NAA	% of rooting response ± S.E	No. of rootlets per Shootlets ± S.E	Length of root lets (mm) ± S.E	% of rooting response ± S.E	No. of rootlets per Shootlets ± S.E	Length of root lets (mm) ± S.E	% of rooting response ± S.E	No. of rootlets per Shootlets ± S.E	Length of root lets (mm) ± S.E
9.84	0	0	60.3 ± 0.38	4.1± 0.12	23.4±0.37	60.3 ± 0.38	4.1± 0.12	53.4±0.41	52.4 ± 0.41	3.8± 0.36	43.4±0.34
4.92	5.7	0	55.8 ± 0.42	3.1±0.14	19.8±0.4	45.1 ± 0.83	3.3 ±0.14	49.8±0.38	41.3± 0.23	3.1±0.17	39.8±0.28
9.84	**0**	**5.37**	70.4 ± 0.33	3.9±0.13	25.6±0.34	86.5 ± 0.56	**5.9±0.13**	**75.6±0.36**	66.4 ± 0.48	**4.7±0.35**	**55.6±0.27**
4.92	0	5.37	65.8 ± 0.32	2.9±0.13	20.8±0.8	70.3 ± 0.38	2.7±0.13	60.8±0.78	44.8 ± 0.24	2.8±0.16	31.8±0.42
0.00	5.7	**5.37**	60.3 ± 0.41	**7.2±0.18**	**35.6±0.93**	53.3 ± 0.52	4.2±0.18	45.6±0.66	52.3 ± 0.38	3.2±0.82	46.6±0.23

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