ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 7, pp. 12434-12439, July, 2016

International Journal of Recent Scientific Research

Research Article

IN VITRO ORGANOGENESIS AND RAPID MULTIPLICATION OF *OLDENLANDIA BIFLORA* L. – A LITTLE KNOWN MEDICINAL PLANT

Karthi C[1] and Velayutham P[2]*

Postgraduate and Research Department of Botany, Government Arts College (Autonomous), Karur 639005 – INDIA

ARTICLE INFO

Article History:

Received 06th April, 2015
Received in revised form 14th May, 2016
Accepted 23rd June, 2016
Published online 28th June, 2016

Key Words:

Oldenlandia biflora L., leaf explants, MS medium, auxins, cytokinins, acclimatization.

ABSTRACT

Oldenlandia biflora L. (Rubiaceae) is a small, straggling, delicate plant appearing in the rainy season on the banks of water courses. The whole plant is used in medicine. An efficient protocol for rapid multiplication of *O. biflora* was standardized in this study. The leaf explants were cultured on MS medium supplemented with different concentrations of IAA, IBA, NAA and 2,4-D for callus induction. All these auxins produced calli with a little variation in callus morphology. In combination with low concentration of BAP, green compact calli were developed. Shoots were initiated from the calli on MS medium containing cytokinins and low concentration of auxins. Of the two cytokinins, BAP was found to produce more number shoots when compared to KIN. In combination of these two cytokinins, number of shoots were increased. More than 40 shoots were obtained on MS medium containing 6 µM BAP+2 µM KIN+2 µM NAA. Basal medium alone is enough to initiate roots in this plant. However, the addition of auxins enhanced the rooting. The well rooted plantlets were transferred to root trainer for hardening. Subsequently these plantlets were established in the field through acclimatization. The highest rates of hardening and acclimatization were observed in this study with 95% and 90% respectively.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Medicinal plants have been used for centuries as remedies for human and animal diseases as they contain phytochemicals of therapeutic value. At present large number of drugs in use are derived from plants (Satyavati *et al.*, 1976). India is known to have rich repository of medicinal plants. The drugs are derived either from the whole plant or from different parts like leaves, stem, bark, root, flower and seed. Some drugs are prepared from excretory plant products such as gums, resins and latex (Kaido *et al.*, 1997). In traditional systems of medicine, the Indian Medicinal Plants have been used in successful management of various disease conditions like bronchial asthma, chronic fever, cold, cough, malaria, dysentery convulsions, diabetes, diarrhoea, arthritis, emetic syndrome, skin diseases, insect bite, etc. and in treatment of gastric, hepatic, cardiovascular and immunological disorders (Prakash and Gupta, 2005). Nowadays, people has awareness in cultivation of medicinal plants for their significant role in therapeutic value without any side effect.

The tissue culture techniques offer potential means not only for rapid mass multiplication of existing stock of fruit and forest trees but also for the conservation of important, elite and rare trees. Among the different techniques of biotechnology, plant tissue culture is the one being applied in crop improvement programme (Prakash *et al.*, 1994). Though the conventional breeding techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It overcomes the barriers in conventional vegetative propagation and fulfils the demand for large-scale cultivation in a short period by rapid mass multiplication. To date, we can speed up the production rate of the average plant by approximately 10,000 times and a large number of productive plants can be multiplied routinely (Rao *et al.*, 1996). The technique of plant tissue culture, therefore, holds great promise to plant breeders, pharmaceutical industries and others, besides helping in conservation of our precious natural wealth (Shekhawat *et al.*, 1998).

Plant cell and tissue cultures hold great promise for controlled production of a great number of useful secondary metabolites on demand. Large-scale plant tissue culture is found to be an attractive and alternative approach to traditional methods of plantation as it offers controlled supply of biochemical's independent of plant availability (Sajc *et al.* 2000). Discoveries of cell cultures capable of producing specific medicinal

*Corresponding author: **Velayutham P***
Postgraduate and Research Department of Botany, Government Arts College (Autonomous), Karur 639005 – INDIA

compounds at a rate similar or superior to that of intact plants have accelerated in the last few years (Vijaya *et al*., 2010).

Oldenlandia biflora is a small, weak, straggling delicate plant, appearing in the rainy season in fields and low ground on the banks of water courses. The entire plant is used in medicine, and is regarded as a valuable bitter tonic and febrifuge. It enters into the composition of numerous prescriptions for fever, diarrhoea, skin diseases, etc. From the ethnobotanical survey, it is found that this plant is used as folk medicine in treating dysentery, fever and also gastric ulcers. Pharmacological studies reveal that this plant possesses antibacterial (Sridhar *et al.*, 2012) and anticancer (Ding *et al.*, 2014) activities.

Only very few studies has been conducted so far to reveal the medicinal properties of this plant. The available literatures also show that no *in vitro* regeneration studies has been conducted so far. Based on this lime light, the present study is aimed for rapid and mass multiplication of plantlets from leaf explants through organogenesis.

MATERIALS AND METHODS

Plant Material and Establishment of Explants

In the present investigation, *Oldenlandia biflora* L. was selected for *in vitro* regeneration through callus culture from the leaf explants. The plant materials were collected from the betel vine filed of the Cauvery River banks near Velur, Tamil Nadu, India. The leaf explants were washed thoroughly under running tap water for 20 minutes followed by washed with surfactant (Teepol). They were rinsed with distilled water for 4-5 times. Then they were disinfected with 70% alcohol (v/v) for 45 seconds followed by 0.1% mercuric chloride (w/v) for 3 minutes. Finally, the explants were washed with sterile distilled water for 3-5 times to remove the traces of mercuric chloride.

Preparation of Culture Media

For the entire study MS medium (Murashige and Skoog, 1962) was used as basal medium with 3% sucrose and respective growth regulators for callus culture and regeneration. The medium was solidified with 0.8% agar and the pH of the medium was adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCl before autoclaving. The medium was autoclaved at 1.06 kg cm^{-3} at 121ºC under 15 lbs per sq. ft. pressure for about 20 min.

Callus culture

The leaf explants were inoculated on MS medium fortified with different concentrations of four auxins, viz., IAA, IBA, NAA and 2,4-D arranging from 2.5 µM to 12.5 µM alone or with low concentration of cytokinins (BAP or KIN) ranging from 1 µM - 5 µM for callus induction and callus proliferation. The suitable concentration of growth regulator was optimized for regeneration.

Regeneration of plantlets

The green compact calli were transferred to MS medium fortified with different concentrations of cytokinins, viz., BAP and KIN (3 µM - 12 µM) separately as well as in combination of these two cytokinins along with low concentration of auxin for shoot regeneration and mass propagation.

Rooting

The regenerated shoots of 3-5 cm long were transferred to MS basal medium alone and also with different concentrations of IAA, IBA and NAA ranging from 2-10 µM for rooting.

Culture maintenance and conditions

All cultures were maintained at 25±2°C in a culture room under cool white fluorescent lamps (Phillips, India) at intensity of 50 µmol m–2 s–1 with 16 hrs photoperiod.

Hardening and Acclimatization

Four to six week old plantlets with well established roots were removed from the culture tubes and washed the roots to remove the adherent medium. The plantlets were transferred to root trainer containing a mixture of coconut coir plus a mixture of red soil, sand and farm yard manure in the ratio of 2:1:1 and maintained inside the culture room at 25±2°C under 16 hr and 75-80% relative humidity. The plantlets were periodically irrigated with water for a period of two weeks. Subsequently these plantlets were established in the field through acclimatization.

Experimental Design, Data Collection and Statistical Analysis

The design of all the experiments was a complete randomized block and each experiment consisted of one explant per tubes and five replicates. The parameters recorded were frequency (number of cultures responding in terms of callus induction, multiple shoot proliferation and root development), number of shoots per callus, shoot length, number of roots per shoot, root length and survival rate (%). All of the experiments were repeated five times. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% level of significance (Gomez and Gomez, 1976).

RESULTS AND DISCUSSION

Callus induction

The leaf explants of *Oldenlandia biflora* were cultured on MS basal medium supplemented with different auxins, viz., IAA, IBA, NAA and 2,4-D at various concentrations ranging from 2.5-12.5 µM for callus induction. Callus initiation was observed 7 days after inoculation. The callus induction frequency and nature of callus were varied for different hormones and concentrations (Fig.1a,b; Table 1). Generally, the explants cultured on medium fortified with IAA produced light green calli, IBA produced whitish calli, NAA gave light green compact calli and 2,4-D formed yellowish green compact calli. Among the four auxins tested, maximum callus induction frequency was observed on medium containing 5 µM NAA. In general, the explants growing on medium having auxins alone produce whitish to greenish calli. When the explants cultured on medium supplemented with auxins in combination with low concentration of cytokinins produce greed compact callus. From this preliminary screening, 5 µM NAA was selected as suitable auxin for callus induction. Hence, low concentrations of BAP or KIN ranging from 1-5 µM were combined with a fixed concentration of 5 µM NAA (Fig.1a,b; Table 2). Of these

two cytokinins, BAP was found to produce green compact callus with higher frequency when compared to KIN. Of the different concentrations of BAP, the medium containing 5 µM NAA + 2 µM BAP was found to be the best in callus induction with 100% callus induction frequency.

Table 1 Effect of auxins on callus induction from leaf explants of *Oldenlandia biflora* L.

Concentrations of auxins	Callus induction frequency (%)	Nature of callus
2.5 µM IAA	76	Light green friable
5.0 µM IAA	82	Light green compact
7.5 µM IAA	90	Light green compact
10.0 µM IAA	74	Yellowish green friable
12.5 µM IAA	66	Yellowish green friable
2.5 µM IBA	62	White friable
5.0 µM IBA	70	White friable
7.5 µM IBA	76	White slightly friable
10.0 µM IBA	86	White slightly friable
12.5 µM IBA	78	White friable
2.5 µM NAA	88	Light green friable
5.0 µM NAA	96	Light green compact
7.5 µM NAA	94	Light green compact
10.0 µM NAA	86	Light green slightly friable
12.5 µM NAA	80	Light green friable
2.5 µM 2,4-D	84	Yellowish green friable
5.0 µM 2,4-D	92	Yellowish green compact
7.5 µM 2,4-D	88	Pale green friable
10.0 µM 2,4-D	78	Brownish friable
12.5 µM 2,4-D	76	Brownish compact

Values are Mean of 5 replicates recorded after 30 days of culture.

Table 2. Combined effect of NAA and cytokinins on callus proliferation of *Oldenlandia biflora* L.

Concentrations of Plant Growth Regulators (µM)			Callus induction frequency (%)	Nature of callus
NAA	BAP	KIN		
5	1	---	92	Light green compact
5	2	---	100	Green compact
5	3	---	100	Green compact
5	4	---	94	Yellowish-green compact
5	5	---	90	Yellowish-green compact
5	---	1	80	Light green compact
5	---	2	84	Light green compact
5	---	3	92	Green compact
5	---	4	86	Green compact
5	---	5	80	Yellowish green compact

Values are Mean of 5 replicates recorded after 30 days of culture.

Generally auxin alone is enough for callus induction. However addition of low concentration of cytokinins produce green compact calli. Hence, in several studies low concentration of cytokinins are fortified along with auxins for callus culture (Velayutham and Ranjithakumari, 2003; Rehman *et al.*, 2003; Velayutham *et al.*, 2006, 2012; Naik *et al.,* 2014). In the present study also, whitish or light green calli were produced when they were cultured on medium containing auxins alone. But they turned into green when cultured on medium fortified with low concentration of cytokinin along with auxins. Of the four auxins tested, NAA was found to be the best hormone for callus induction in this study. Similar results were obtained in previous reports also (Wagner and Gailing, 1996; Velayutham and Ranjithakumari, 2003; Rehman *et al.*, 2003; Velayutham *et al.*, 2006, 2012). Variations in callus morphology according to the growth regulators and their concentrations were also observed in earlier studies (Baskaran and Jayabalan, 2006; Sen *et al.*, 2014; Senthilkumar and Nandi, 2015).

Shoot regeneration from callus

The green compact calli were transferred to regeneration medium containing different concentrations of cytokinins, BAP and KIN ranging from 3-12 µM. Of these two cytokinins, BAP was found to initiate more number of shoot when compared to KIN. Of the different concentrations, 6 µM BAP was found to be the best in shoot induction with 86% shoot induction frequency. When low concentration of KIN was combined with 6 µM BAP, the shoot induction frequency (96%) and more number of shoots were increased (Fig.1c,d; Table 3). However, addition of low concentration of auxins (IAA or NAA) produced 100% shoot induction frequency with maximum number of 46 shoots per callus and maximum shoot length of 7.7 cm within 30 days of subculture (Table 4).

Table 3. Effect of cytokinins on shoot regeneration and multiplication from the leaf derived callus of *Oldenlandia biflora* L.

Concentrations of cytokinins (µM)		Shoot induction frequency (%)	Mean number of shoots	Mean Shoot length (cm)
BAP	KIN			
3	---	82	23.64 ± 0.72^{f}	5.71 ± 0.49^{de}
6	---	86	26.29 ± 0.77^{d}	6.27 ± 0.72^{bc}
9	---	74	21.89 ± 0.93^{h}	5.12 ± 0.81^{gh}
12	---	66	19.57 ± 0.66^{jk}	4.62 ± 1.04^{l}
15	---	62	16.91 ± 0.95^{mn}	3.63 ± 0.30^{mn}
---	3	54	13.21 ± 0.80^{o}	3.53 ± 0.78^{no}
---	6	62	18.47 ± 1.13^{kl}	4.91 ± 1.06^{jk}
---	9	68	20.72 ± 0.73^{ij}	5.06 ± 0.84^{hi}
---	12	78	23.51 ± 0.55^{fg}	5.48 ± 0.81^{f}
---	15	64	17.75 ± 0.83^{lm}	3.77 ± 0.68^{m}
6	1	92	31.60 ± 0.77^{b}	6.36 ± 0.53^{b}
6	2	96	35.70 ± 0.97^{a}	6.72 ± 0.65^{a}
6	3	88	28.16 ± 0.54^{c}	5.72 ± 0.77^{d}
6	4	86	26.19 ± 1.20^{de}	5.21 ± 0.73^{g}
6	5	80	21.75 ± 0.95^{hi}	4.92 ± 0.64^{ij}

Values are Mean of 5 replicates recorded after 30 days of culture.

Values in the last two columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at P<0.05 by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

Table 4. Combined effect of cytokinins and auxins on shoot multiplication and proliferation of *Oldenlandia biflora* L.

Concentrations of Plant Growth Regulators				Shoot induction frequency (%)	Mean number of shoots	Mean Shoot length (cm)
BAP	KIN	IAA	NAA			
6	2	1	---	92	33.14 ± 0.77^{gh}	6.67 ± 0.54^{e}
6	2	2	---	100	37.16 ± 0.59^{e}	7.11 ± 0.64^{cd}
6	2	3	---	100	39.51 ± 0.73^{c}	7.26 ± 0.46^{bc}
6	2	4	---	90	31.35 ± 1.11^{hi}	5.32 ± 0.79^{gh}
6	2	5	---	86	28.61 ± 0.78^{j}	4.82 ± 0.70^{j}
6	2	---	1	100	42.92 ± 0.45^{b}	7.47 ± 0.60^{ab}
6	2	---	2	100	46.75 ± 0.81^{a}	7.70 ± 0.65^{a}
6	2	---	3	100	39.30 ± 0.81^{cd}	6.37 ± 0.74^{ef}
6	2	---	4	96	36.93 ± 0.37^{ef}	5.52 ± 0.69^{g}
6	2	---	5	90	34.12 ± 0.72^{g}	5.20 ± 0.59^{hi}

Values are Mean of 5 replicates recorded after 30 days of culture.

Values in the last two columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at P<0.05 by Duncan's Multiple Range Test (same letter within the column of the respective growth

regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

Several growth regulators are available for shoot multiplication. However, BAP, KIN TDZ and 2-iP are widely used. In majority of studies the former two cytokinins are preferred for shoot induction and multiplication. In the present study also BAP and KIN were used alone or in combination of these two. As far as individual hormones concerned, BAP was found to be better in shoot induction in this plant similar to the earlier studies (Sahoo and Chand, 1998; Kadota and Niimi, 2003; Velayutham and Ranjithakumari, 2003; Vasudevan *et al.*, 2004; Velayutham *et al.*, 2006; Padmapriya *et al.*, 2011). However, the combination of these two cytokinins enhanced the multiplication rate. Several workers showed that the synergistic combination of two cytokinins was more effective for shoot differentiation (Velayutham *et al.*, 2006; Selvaraj *et al.*, 2006; Baskaran *et al.*, 2008; Ashraf *et al.*, 2014). Recent studies reveal that low concentration of auxins along with cytokinins enhance the rate of proliferation of shoots as in the present study (Izabella *et al.*, 2015; Purohit *et al.*, 2015; Senthilkumar and Nanhi, 2015).

Rooting and in vitro flowering

The isolated micro shoots of 3-5 cm length were transferred to half strength MS medium containing different concentrations of IAA, IBA and NAA and also on MS basal medium without supplementing auxins. The roots were initiated on basal medium itself. However duration of root initiation was two weeks and number of roots were low when compared to shoots growing on medium fortified with auxins where initiation of roots occurred within a week with more number of roots. Of the three auxins tested, 4 µM IBA was found to be the best in rooting with 100% root induction frequency, mean number of 19.03 roots per shoot and mean length of 4.60 cm. In higher concentration of auxins, instead of root basal callus was formed (Fig. 1e; Table 5).

Table 5 Effect of auxins on root induction from isolated shoots of *Oldenlandia biflora* L.

Concentrations of Plant Growth Regulators	Root induction frequency (%)	Number of Roots per Shoot	Root length (cm)
MS basal	56	3.80 ± 0.57^{jk}	2.01 ± 0.49^{jk}
2 µM IAA	78	11.64 ± 0.94^{cd}	3.35 ± 0.34^{ef}
4 µM IAA	64	9.39 ± 0.66^{fg}	2.79 ± 0.60^{g}
6 µM IAA	60	5.13 ± 0.89^{j}	2.14 ± 0.51^{j}
8 µM IAA	basal callus	---	---
10 µM IAA	basal callus	---	---
2 µM IBA	96	15.94 ± 0.84^{b}	4.23 ± 0.47^{b}
4 µM IBA	100	19.03 ± 0.66^{a}	4.60 ± 0.68^{a}
6 µM IBA	88	10.48 ± 0.84^{de}	3.67 ± 0.79^{cd}
8 µM IBA	72	8.38 ± 0.43^{gh}	2.74 ± 0.55^{gh}
10 µM IBA	basal callus	---	---
2 µM NAA	84	13.14 ± 0.64^{c}	3.85 ± 0.50^{c}
4 µM NAA	72	9.57 ± 0.60^{ef}	3.45 ± 0.58^{de}
6 µM NAA	66	7.54 ± 0.60^{hi}	2.65 ± 0.73^{hi}
8 µM NAA	basal callus	---	---
10 µM NAA	basal callus	---	---

Values are Mean of 5 replicates recorded after 30 days of culture.

Values in the last two columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at P<0.05 by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

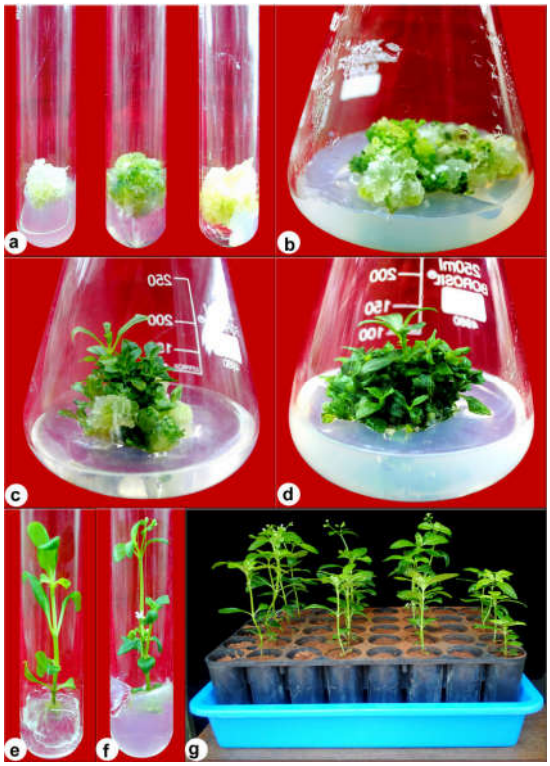

Fig. 1 *In vitro* Organogenesis and Rapid Multiplication of *Oldenlandia biflora* L. a. callus induction on MS medium; b. callus proliferation after 30 days; c,d. shoot induction and multiplication; e. root induction; f. *in vitro* flowering; g. hardening.

For root induction, IAA, IBA and NAA were used for several studies. Higher frequency of rooting was achieved by IBA in *Aristolochia indica,* (Manjula *et al.* 1997), *Gymnema sylvestris* (Komalavalli and Rao, 2000), Eclipta alba (Baskaran and Jayabalan, 2005), *Heliotropium indicum* (Senthilkumar and Rao, 2007), *Melothria maderaspatana* (Baskaran *et al.*, 2008), *Ruta graveolens* (Ahmad *et al.*, 2010), *Solanum nigrum* (Padmapriya, *et al.*, 2011) and *Astracantha longifolia* (Senthilkumar and Nandi, 2015). Whereas, NAA showed better root induction in *Cichorium intybus* (Velayutham and Ranjithakumari, 2003), *Viburnum odoratissimum* (Schoene and Yeager, 2005), *Plumbago zeylanica* (Velayutham *et al.* 2005), *Solanum nigrum* (Jabeen *et al.* 2005), *Hybanthus enneaspermus* (Velayutham *et al.*, 2012) and *Enicostemma littorale* (Nalini and Velayutham, 2013). In the present study, of the three auxins tested, IBA was found to induce more number of roots and NAA was observed next to IBA for rooting.

The present study also shows that higher concentrations of auxins lead to the formation of basal callus instead of rooting. More over, rooting occurs even in the absence of auxins in the basal medium. This shows the synthesis of endogenous auxin in this plant. The inhibition of rooting and production of callus in the presence of higher concentration of NAA was also shown in *Plumbago zeylanica* (Sivanesan, 2007).

While rooting takes place, along with shoot elongation, the plantlets attained sexual maturity and produced *in vitro* flowers without adding any flowering hormone in the medium. Being a

self-pollinating plant, *in vitro* seed setting also occurred as a result of *in vitro* pollination and *in vitro* fertilization (Fig. 1f).

Addition of GA3 on the rooting medium generally causes flowering in many plants (Stephen and Jayabalan, 1998; Tang, 2000). However, long photoperiod in culture room favours induction of flowers *in vitro* without adding flowering hormone (Demeulemeester and De Proft, 1999; Velayutham and Ranjithakumari, 2003; Velayutham *et al.,* 2006; Padmapriya *et al*., 2011). *In vitro* flowering and *in vitro* seed setting in this study also favoured by the presence of long photoperiod.

Hardening and Acclimatization

The well rooted plantlets were transferred to root trainer containing coconut coir and a mixture of red soil, sand and farm yard manured in the ratio of 2:1:1 for hardening. In the present study 95% of plantlets were survived in the root trainer (Fig. 1g). The well established plantlets were transferred to the field after 20 days. Survival rate of 90% was observed in the field in one month.

Many investigators used soil and sand (Singh and Tiwari, 2010; Thiagarajan and Venkatachalam, 2013) or soil, sand and farm yard manure for hardening in paper cups (Velayutham and Ranjithakumari, 2003; Bhosle *et al.*, 2005; Velayutham *et al.*, 2006, 2012; Nalini and Velayutham, 2013) or vermiculate soil (Karuppusamy and Kalimuthu, 2010; Sridhar and Naidu, 2011). In this work, coconut coir was used in addition to soil, sand and farm yard manure for easy penetration of tender roots. Since large number of plants are produced in the present study, root trainer was used for hardening.

References

Ahmad, N., Faisal, M., Anis, M. and Aref, I.M. (2010). *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L. *South African Jour. Bot.*, **76**:597-600.

Ashraf, M.F., Aziz, M.A., Kemat, N and, Ismail, I. (2014). Effect of cytokinin types, concentrations and their interactions on *in vitro* shoot regeneration of *Chlorophytum borivilianum* Sant. & Fernandez. *Electronic Jour. Biotech.*, 17 : 275-279.

Basakaran, P. and Jayabalan, N. (2006). *In vitro* mass propagation and diverse callus orientation on *Sesamum indicum* L. an important oil plant. *Jour. Agr. Tech.,* **2**(2):259-269.

Baskaran, P., Velayutham, P., Rajadurai, D.M. and Jayabalan, N. (2008). Micropropagation of *Melothria maderaspatana* (Linn.) Cogn. through apical buds derived from mature plants. *J. Biol. Res.-Thessalon*., **9**: 83 – 88

Baskaran. P. and Jayabalan, N. (2005). An efficient micropropagation system for *Eclipta alba* – a valuable medicinal herb. *In vitro* Cellular Develop. Biol. - Plant, **41**:532-539.

Bhosle, S.V., Thengane, R.J. and Thengane, S.R. (2005). *In vitro* multiple shoot regeneration and plant production in *Alysicarpus rugosus* DC. var. *heyneanus* Baker. *Indian J Exp Biol.,* **43**(10):916-20.

Demeulemeester, M.A.C., Proft, M.P.-de and De-Proft, M.P. (1999). *In vivo* and *in vitro* flowering response of chicory (*Cichorium intybus* L.): influence of plant age and vernalization. *Plant Cell Reports,* **18**:781-785.

Ding, X., Bai, D. and Qian, J. (2014). Novel cyclotides from *Hedyotis biflora* inhibit proliferation and migration of pancreatic cancer cell *in vitro* and *in vivo*. *Med. Chem. Res.*, 23(3):1406-1413.

Gomez, K. A. and Gomez, K. A. (1976). Statistical procedures for agricultural research with emphasis on Rice. Philippines International Rice Research Institute, Los Banos.

Izabela G.K., Lukasz K. and Halina W. (2015). The effect of cytokinins on shoot proliferation, secondary metabolite production and antioxidant potential in shoot cultures of *Scutellaria alpina*, *Plant Cell Tiss Organ Cult*, **122**:699–708.

Jabeen, F.T.Z., Venugopal, R.B., Kiran, G., Kaviraj, C.P. and Rao, S. (2005). Plant regeneration and *in vitro* flowering from leaf and nodal explants of *Solanum nigrum* (L). - An important medicinal plant. *Plant Cell Biotechnology and Molecular Biology*, **6**(1&2):17-22.

Kadota, M. and Niimi, Y. 2003. Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in *in vitro* pear cultivar shoots. *Plant Cell, Tissue and Organ Culture*, **72**:261–265.

Kaido TL, Veale, DJH, Havlik I, Rama DBK, (1997). Curent and Future status of Herbal medicnes, *Ethnopharmacol*, **55**:185-191.

Karuppusamy, S. and Kalimuthu, K. (2010). Rapid *in vitro* multiplication and plant regeneration from nodal explants of *Andrographis neesiana*: a valuable endemic medicinal plant. *Andvances in Biol. Res.,* **4**(4):211-216.

Komalavalli, N. and Rao, M.V. (2000). *In vitro* micropropagation of *Gymnema sylvestre*-a multipurpose medicinal plant. *Plant Cell, Tissue and Organ Culture*, **61**: 97-105.

Manjula, S., Anita, T., Benny, D. and Nair, C.M., (1997). *In vitro* plant regeneration of *Aristolachia indica* through axillary shoot multiplication and organogenesis. *Plant Cell Tiss. Org. Cult.*, **51**:145-148.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum,* **15**:473-497.

Naik, A.B., Nazneen, F.H. and Shankar, C.P. (2014). Effect of different plant growth regulators on callus induction in *Caesalpinia sappan* L. A medicinal plant. *Int. J. of Recent Sci. Res*., **5**(11): 1991-1994.

Nalini, P. and Velayutham, P. (2013). *In vitro* Mass Propagation of *Enicostemma littorale* Blume from Shoot tip Explants. *Journal of Biology, Agriculture and Healthcare*, **3**(5):72-79.

Padmapriya, H., Karthikeyan, A.V.P, Jahir Hussain, G., Karthi, C. and Velayutham, P. (2011). An efficient protocol for *in vitro* propagation of *Solanum nigrum* L. from nodal explants. *Journal of Agricultural Technology,* **7**(4):1063-1073.

Prakash, A.H., Vajranabhaiah, S.N. and Chandrasekara Reddy, (1994). Changing patterns in water relation and solute contents during callus development under drought stress in sunflower (*Helianthus annus*). *Adv. Pl. Sci.,* **7**: 58-63.

Prakash, P. and Gupta, N. (2005). Therapeutic uses of *Ocimum sanctum* Linn. (Tulsi) with a note on eugenol and its pharmacological actions. a short review. *Indian Jour. Physiol. Pharmacol.,* **49**(2):125-131.

Purohit, S., Vivek R., Arun, K. Jugran, Rahul V. Singh, Indra D. Bhatt. and Shyamal K. Nandi. (2015). Micropropagation and genetic fidelity analysis in *Valeriana jatamansi* Jones. *Jour. Applied Res. Med. Arom. Plants,* **2**:15–20.

Rao, P.S., Supresanna, P. and Ganapathi, T. (1996). Plant biotechnology and Agriculture: Prospects for crop improvement and increasing productivity. *Science and Culture,* **62**:185-191.

Rehman, R.U., Israr, M., Srivastava, P.S., Bansal, K.C. and Abdin, M.Z. (2003). *In vitro* regeneration of witloof chicory (*Cichorium intybus* L.) from leaf explants and accumulation of esculin. *In vitro Cellular Developmental Biology - Plant*, **39**: 142-146.

Sahoo, Y. and Chand, P.K. (1998). Micropropagation of *Vitex negundo* L. a woody aromatic medicinal shrub, through high frequency axillary shoot proliferation. *Plant Cell Reports*, **18**:301-307.

Sajc, L.D., Grubisic, G. and Vunjak, N. (2000). Bioreactors for plant engineering: an outlook for further research. *Biochem Eng J.*. **4**:89–99.

Satyavati, G.V., Raina, M.K. and Sharma, M. (1976). Medicinal Plants of India (published by ICMR, New Delhi).

Schoene, G. and Yeager, T. (2005). Micropropagation of sweet viburnum (*Viburnum odoratissimum*). *Plant Cell, Tissue and Organ Culture*, **83**:271–277.

Selvaraj, N., Vasudevan, A., Manickavasagam, M. and Ganapathi, A. (2006). *In vitro* organogenesis and plant formation in cucumber. *Biol. Plant.,* **50**:123–126.

Sen, M.K., Nasrin, S., Rahman, S. and Jamal, A.H.M. (2014). *In vitro* callus induction and plantlet regeneration of *Achyranthes aspera* L. a high value medicinal plant. *Asian Pac. Jour. Trop. Biomed.*, **4**(1):42-46.

Senthilkumar, M. and Nandi, S.C. (2015). High frequency plant regeneration with histological analysis of organogenic callus from internodal explants of *Astracantha longifolia* Nees. *Jour. Gen. Eng. Biotech.*, **13**:31-38.

Senthilkumar, M. and Rao, M.V. (2007). *In vitro* micropropagation of *Heliotropium indicum* L. in an Ayurvedic Herb. *Ind. Jour. Biotech.*, **6**: 245-249.

Shekhawat, N. S., Mughal, M. H., Johri, B.M., and Srivastava, P.S. (1998)**.** Indian contribution to plant tissue and organ culture- pp-751-752.

Singh, J. and Tiwari, K.N. (2010). High frequency *in vitro* multiplication system for commercial propagation of pharmaceutically important *Clitoria ternatea* L. a valuable medicinal plant. *Industrial Crops and Products*, **32**:534-538.

Sivanesan, I. (2007). Shoot regeneration and somaclonal variation from leaf callus cultures or *Plumbago zeylanica* L. *Asian Jour. Plant Sci.*, **6**(1):83-86.

Sridhar, N., Pradeep Kumar, N.V.S., Sasidhar, D., Kiran Venkatesh, A. and Kanthal, L.K. (2012). Antibacterial and Phytochemical Evaluation of *Oldenlandia biflora* L. and *Pergularia daemia*, *Int. J. Drug Dev. & Res.*, **4**(2): 148-152.

Sridhar, T.M. and Naidu, C.V. (2011). An efficient callus induction and plant regeneration of *Solanum nigrum* L. an important anti-ulcer medicinal plant. *Jour. Phytol.*, **3**(5):23-29.

Stephan, R. and Jayabalan, N. (1998). *In vitro* flowering and seed setting formation of coriander (*Coriandrum sativum* L.). *Curr. Sci.,* **74**:195-197.

Tang, W. (2000). High frequency plant regeneration via Somatic embryogenesis and organogenesis and *in vitro* flowering of regenerated plantlets in *Panax ginseng*. *Plant Cell Rep*. 19:727-732.

Thiagarajan, M. and Venkatachalam, P. (2013). A reproducible and high frequency plant regeneration from mature axillary node explants of *Gymnema sylvestre* (Gurmur) an important antidiabetic endangered medicinal plant. *Industrial Crops and Products*, **50** : 517-524.

Vasudevan, A., Selvaraj, N., Ganapathi, A., Kasthurirengan, S., Ramesh Anbazhagan, V. and Manickavasagam, M. (2004). Glutamine: a suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus*. *Biol. Plant,* **48**:125–128.

Velayutham, P. and Ranjitha Kumari, B.D. (2003). Direct shoot regeneration from leaf explants of chicory (*Cichorium intybus* L.). *Plant Cell Biotech. Mol. Bio.*, **4**(3&4): 125-130

Velayutham, P., Jahir Hussain, G. and Baskaran, P. (2005). *In vitro* plantlet formation from nodal explants of *Plumbago zeylanica* L. – an important medicinal plant. *J. Swamy Bot. Cl.* **22**: 117-120.

Velayutham, P., Karthi, C., Nalini, P. and Jahirhussain, G. (2012). *In vitro* Regeneration and Mass Propagation of *Hybanthus enneaspermus* (L.) F. Muell. from the stem explants through callus culture. *Jour. Agri. Tech*., **8**(3): 1119-1128

Velayutham, P., Ranjithakumari, B.D., and Baskaran, P. (2006). An effeicient *in vitro* plant regeneration system for *Cichorium intybus* L. an important medicinal plant. *Jour. Agri. Tech*. **2**(2) : 287-298.

Vijaya, S.N., Udayasri, P.V., Aswani, K.Y., Ravi, B.B., Phani, K.Y. and Vijay, V.M. (2010). Advancements in the production of secondary metabolites. *Jour. Nat. Prod.*, **3**:112–123.

Wagner, G.M. and Gailing, O. (1996). The influence of different carbon sources on callus formation and shoot proliferation of chicory (*Cichorium intybus* L. var. *sativum*). Landbauforschung *Volkenrode,* **46**:14-15.

****