Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2011 Vol. 1 (2) April – June, pp. 81-89/Kaladhar et al.
Research Article

In Vitro Regeneration of the Medicinal Herb, *Evolvulus Nummularius* L. From Shoot Tip and Flower Explants

***DSVGK Kaladhar[1], S Harasreeramulu[2], Govinda Rao Duddukuri[3] and CH Surekha[4]**
[1,3,4]Department of Biochemistry and Bioinformatics, GIS, GITAM University, Visakhapatnam.
[2]Dept. of Biotechnology, Dr. V.S Krishna Govt. College, Visakhapatnam

****Author for Correspondence*:** E-mail: dkaladhar@gmail.com

ABSTRACT

Indirect shoot regeneration of *E. nummularius* was achieved by culturing terminal buds and flower on MS medium supplemented with various auxins (IAA and 2, 4-D) and Cytokinins (BA and Kn). Present protocol includes devising effective sterilization methods in treatment of explants. Weekly sub culturing to the media with same composition produced better results. Enormous amount of calli having regenerative potential were formed from the explants when cultured on MS medium supplemented with 1 mg/l 2, 4-D+1 mg/l BA. Different concentrations and combinations tested on MS medium containing 3 mg/l BA+ 0.5 mg/l IAA produced highest number of shoots per gram calli. Rooting was more at concentration of 1 mg/l IAA. The plantlets have shown eighty percent survival rate while transferring from *in vitro* to natural environment.

Key Words: *Evolvulus nummularius, In vitro,* Flower, Terminal bud

INTRODUCTION

Medicinal herbs are moving from fringe to mainstream use, as a greater number of people endeavor to opt for herbal formulations over the allopathic compounds, since these are devoid of side effects and cost effective (Dubey *et al.,* 2004). Popular demand and scientific interest in complementary or alternative medicine, particularly medicinal botanicals, has increased considerably in recent years (Borchers *et al.,* 1997). Screening and indexing of many plants, which have been used in traditional Indian and Chinese medicines since time immemorial, resulted in novel therapeutics, useful for the treatment of various ailments of man such as rheumatism, kapha, pitha, blood pressure, cancer etc. (Anonymous, 1948; Anonymous, 1959).

The traditional health care systems, including Ayurveda, were transmitted from generation to generation by 'Gurukula' and practice was dependent on the intimate knowledge which was passed on for many years by the Guru while he was treating each individual case to the disciple (Unnikrishnan, 2004). The earlier examples of western medicine had the influence of plant medicines used in ancient times. Current examples are the use of the cardiac glycosides from the purple foxglove, *Digitalis purpurea*, morphine and opiates from poppy, *Papaver somniferum*, reserpine from *Rauvolfia serpentina* and quinine from cinchona officinalis. Similarly vincristine from catharanthus roseus and taxol from taxus baccata were introduced as drugs for the treatment of cancer (Posey, 1998). Conservation of medicinal plants and capability to utilize them in a sustained manner are essential for the well being and continued survival of man (Ghimire *et al.,* 2004).

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes. *In-vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicine (Leena and Jaindra, 2003). The continuous use of plant derived products for health care problems has resulted in the establishment of various pharmacological laboratories (Fereidoon, 1999). The growing use of medicinal herbs and their practical utility has created new importance in opening of pharmacological and pharmocognosy laboratories that provide adequate information of diverse chemical substances and their usefulness in treating various ailments (Julia *et al.,* 2006).

First significant attempt of isolated plant cells on artificial nutrient medium was done by Haberlandt in 1902. Later, long term calli cultures were established from carrot cambium by Gautheret, 1939 and Nobecourt, 1939. Subsequently various culture media were reported to obtain cultured cells in diverse plant explants of several taxa by the work of Gautheret, 1940, Hildebrandt *et al.,* 1946, Nitsch 1951, Reinert and White, 1956, Murashige and Skoog, 1962, White, 1963, Gamborg *et al.,* 1968, Schenck and Hidebrandit, 1972 etc.

Investigations by Minocha, 1980, Bornman, 1983, Villalobos *et al.,* 1984 on culture conditions; White and Gilbey, 1966, Mulder-Krieger *et al.,* 1982 on nutrient requirements; Oka and Ohyama, 1975, Minocha, 1987, Nadel *et al.,* 1991 on hormonal requirements; MacRae and VanStaden, 1990, Pochet *et al.,* 1991 on gelling agents etc. revealed some other important aspects of culture.

Evolvulus nummularius is commonly called Kidney weed (Miller 1997) and locally known as Bichhamalia, Krishna ankaranti ; Jungi-ba, Tandi kode baha (Kambaska) Lakshmi krantha (**Fig. 1**). It is a perennial herb used as anthelmintic and in the treatment of bronchial asthma. Medicinally *E.nummularius* is highly useful in large quantities and hence *in vitro* cultivation is necessary in the present studies.

MATERIALS AND METHODS

Collection of Plant materials

Fresh plant material was collected at Visakhapatnam and the explants were sterilized within 4 hours.

Preparation of Culture Media

Glass double distilled water was used for the preparation of culture media. After addition of all constituents of media, the pH was adjusted to 5.8 using 0.1 N KOH or 0.1 N HCl. Gelling-agent (agar-agar) was added as per requirement and the medium was steamed to melt the gelling agent. It was then dispensed into test tubes (20 ml per tube) or Erlenmeyer flasks (100 ml per 250 ml flask) or screw capped bottles (50 ml per bottle) and was autoclaved at 121°C at a pressure of 15 lbs for 20 min. When no gelling agent was added, each tube contained liquid medium with a filter paper bridge of Whatman No. 1 filter paper. Heat labile constituents like hormones were filter-sterilized by passing through a Millipore membrane (0.22 µm pore size) ("Millipore Corporation", USA) and added aseptically to the autoclaved medium just before gelling. All the plant growth regulators used during the course of the present work were added before autoclaving the medium. As per the requirements, the medium was also poured in sterile glass petridishes (12 ml per 55 mm dish, 20 ml per 85 mm dish) in front of a laminar airflow hood. The composition of MS media used for culturing of explants.

Explants

Healthy explants such as shoot tips and young flowers of *Evolvulus nummularius* were selected for tissue culture

Surface Sterilization

The explants were washed thoroughly under running tap water, followed thrice (3X) with distilled water and submerged in 70% ethanol for 3 min. The explants were again rinsed 3X in sterile, double distilled water and are then submerged in 5% sodium hypochlorite for 10 minutes. Finally the explants were rinsed in 3X sterile, double distilled water. The explants were then treated with 0.5% mercuric chloride for 5 minutes and rinsed 3 times in sterile double distilled water. After the surface sterilization the explants were cultured on different nutrient media under aseptic conditions.

Control of Phenolic exudation

To control the phenolic exudation in the cultures of *Evolvulus nummularius* activated charcoal (1%) was used in culture media. Periodic subculturing (at weekly intervals) to fresh media with same compositions were also tested to overcome the problem.

Indirect Shoot Regeneration

Shoot regeneration potential via callus phase of different explants (shoot tips and flower) of Evolvulus *nummularius* were studied by culturing on MS medium fortified with different combinations of auxins and cytokinins.

Growth and Maintenance of Plant Tissue and Cell Cultures

The callus and shoot cultures thus obtained were subcultured at regular intervals of 1-2 weeks. Observations were recorded at every week.

Rooting of In Vitro Shoots

For root induction in *Evolvulus nummularius*, the shoots (size more than 3cm) were excised from primary cultures and cultured on semi solid MS medium supplemented with NAA (0.1-3mg/l) on IAA (0.1-3mg/l) individually.

Acclimatization and Transfer of Plantlets to Field

The plantlets, regenerated through various *in vitro* techniques, with healthy root and shoot systems were taken out from the culture medium and washed gently with sterile distilled water for removing all traces of medium from the plantlet. The washed plantlets were transferred to small plastic cups containing sterile sand. The pots were then covered with polythene bags to maintain high humidity and kept in plant growth chamber. The plantlets were moistened with water two times a day. After fifteen to twenty days, the polythene bags were removed and transferred to larger pots containing sterile sand and soil (1:1) and kept under shade in the net house for another two weeks before transferring to field.

Statistical Analysis

Depending upon the size and the availability of the explants, each experiment consisted of 20 replicates.

Fig. 1: Mother plant of *Evolvulus nummularius*

Table 1: Effect of growth regulators (alone) on callus induction from various explants of *E.nummularius

Growth regulators(mg/l)	Explants	
	Node	**Immature flower**
IAA		
0.5	+	+
1.0	+	+
2.0	++	++
3.0	+++	++
2,4-D		
0.5	+	+
1.0	++	++
2.0	+++	+++
3.0	+++	+++
BA		
0.5	-	-
1.0	+	-
2.0	+	+
3.0	++	+
Kn		
0.5	-	-
1.0	-	-
2.0	-	-
3.0	+	-

Abbrev. *- = No callus; + = Little Callus; ++ = Moderate Callus; +++ = Profuse Callus*
**Data obtained from 20 replicates in each combination; In Vitro Culture period = 60 days*

Table 2: Effect of growth regulators (in combinations) on callus induction from various explants of *E. nummularius

Growth regulators(mg/l)		Explants	
		Node	Immature flower
2,4-D+BA			
0.5	0.5	+	+
1.0	0.5	++	+
0.5	1.0	+	+
1.0	1.0	+++	+++
2,4-D+kn			
0.5	0.5	+	+
1.0	0.5	++	+
0.5	1.0	+	+
1.0	1.0	++	++
IAA+kn			
0.5	0.5	-	-
1.0	0.5	+	-
0.5	1.0	-	-
1.0	1.0	+	+

Abbrev. *- = No callus; + = Little Callus; ++ = Moderate Callus; +++ = Profuse Callus*

**Data obtained from 20 replicates in each combination; In Vitro Culture period = 60 days*

Table 3: Effect of growth regulators on callus regeneration of *E.nummularius*

Growth regulator (mg/l)		% Response	Frequency of callus regeneration Mean±SE
BA			
0.5		70	1.85±0.34
1.0		80	1.85±0.29
2.0		85	2.50±0.38
3.0		80	2.05±0.30
IAA	Kn	No shoot formation was observed	
0.5	3.0		
BA	IAA		
0.5	1.0	65	1.85±0.38
1.0	0.5	75	2.35±0.39
2.0	0.5	80	1.90±0.32
3.0	0.5	80	2.60±0.37

Table 4: Effect of auxin (IAA) on *in vitro* rooting of *E. nummularius*

Growth regulator(mg/l)	% response	Frequency of rooting Mean±SE
IAA		
0.1	30	0.90±0.18
0.3	50	0.80±0.17
0.5	85	1.40±0.19
1.0	80	1.35±0.19
2.0	70	1.10±0.18
3.0	75	1.10±0.13

Arithmetic Means and Standard Errors of Means (S.E) were calculated in each experiment.

RESULTS

The explants from terminal buds and flowers of *E. nummularius* were induced to organogenesis through callus formation on different media supplemented with various hormonal combinations. Sterilization technique devised by us that included dipping the plant material into 70% [v/v] ethanol for 2-3 minutes, transferring it to a solution of 1% sodium hypochlorite containing 4 drops of Tween-60 for 20 minutes and placing it in 0.5% $HgCl_2$ for 5 minutes found to be most effective.

Addition of 1% activated charcoal was an effective method to overcome the problem of phenolic exudation but the addition retarded growth in cultures, hence it was not used in subsequent experiments. Weekly subculturing to the medium of same composition produced best results.

The shoot regeneration potential through callus phase from various explants via node and flower to various concentrations and combinations of growth regulators in MS medium was studied.

Callus induction

For callus induction, the explants were cultured on MS medium supplemented with IAA, 2, 4-D, BA, Kn either alone or in combinations (Tables 1 - 2).

1 Effect of IAA

MS medium supplemented with IAA at the range of 0.5 to 3.0 mg/l induced the callus formation in terminal buds and flower explants within 20 days. Higher concentrations of IAA (upto 3 mg/l) were effective in callus induction and beyond this concentration led to retarding effect in callus induction on both explants. The calli were greenish white and friable.

2 Effect of 2, 4-D

MS medium supplemented with 2, 4-D at the range of 0.5 to 3.0 mg/l induced the callus formation in terminal buds within 40 days and flower explants within 20 days. Higher concentrations of 2, 4-D ($\geq$ 3 mg/l) was effective for callus induction on terminal buds and flower explants. The calli were greenish white and friable. Leaving the calli in the medium turned brown and dried within 4 months.

3 Effect of BA

MS medium supplemented with BA in a range of 0.5 to 3.0 mg/l was tested for the induction of the callus formation in terminal buds and flower explants. Lesser concentrations of BA (upto 0.5 mg/l in terminal buds and upto 1 mg/l in flower) did not induce callus formation. Little or moderated induction of callus was observed upto 3 mg/l and did not show any progressive change in callus proliferation beyond 3 mg/l on terminal buds and flower explants. The calli were greenish white and friable.

4 Effect of Kn

MS medium with various concentrations of Kn (0.5-3 mg/l) was found ineffective in inducing the callus formation from terminal buds and flower explants.

5 Effect of 2,4-D+BA

Studies on synergistic effects of growth regulators on terminal buds and flower were cultured with 2, 4-D (1.0 mg/l) and BA (1.0 mg/l) for callus induction which revealed enormous amount of calli from explants treated with 1mg/l of 2, 4-D and 1mg/l of BA. The calli were greenish white, friable and meristematic. These calli were used for regeneration on shoot induction medium.

6 Effect of 2, 4-D+Kn

MS media supplemented with 2, 4-D (0.5-1.0 mg/l) and Kn (0.5-1.0 mg/l)) on terminal buds and flower explants produced moderate callus induction in *E.nummularius*. The calli were greenish white, friable and meristematic.

7 Effect of IAA+Kn

MS medium containing the combinations of IAA and Kn yielded less effect in callus induction of *E. nummularius* explants. Little amount of callus was produced from explant cultured in medium supplemented with 1mg/l of 2, 4-D and BA each.

Callus regeneration

Calli obtained from terminal buds and flower explants on MS medium supplemented with 1mg/l of 2,4-D and 1mg/l of BA were selected for regeneration. It was observed that in MS+ BA (3.0 mg/l)+ IAA(0.5 mg/l) highest number of shoots (2-3 per gram callus) regenerated. Both Kn and BA were tested for shoot regenration from calli. Kn in MS medium proved ineffective in regeneration of shoots from the calli.

Only the callus induced growth regulators (BA+2, 4-D and BA) on MS medium were found to be regenerative on subsequent cultures. The regeneration capacity of various calli were tested good by subculturing on MS medium supplemented with BA (3 mg/l) and IAA (0.5 mg/l). The results are shown on **Table 3.** Morphogenic potential of the calli could be retained by weekly transfer of the callus to medium with the same composition (3 mg/l BA+0.5 mg/l IAA). Explants produced light green calli, which turned brown never showed regeneration.

Rooting *In Vitro*

Individual shoots with $\geq$3 cm were excised and cultured on MS medium supplemented with IAA (0.1-3.0 mg/l). Rooting was more at 1 mg/l concentration of IAA which subsequently decreased with increasing concentration of

IAA. Response percentage was also more at 0.5 mg/l of IAA and the results are given in Table 4.

Acclimatization and transfer of *in vitro* grown plants to field conditions

Healthy plantlets which were regenerated through *In vitro* technique were washed with sterile distilled water and transferred to small plastic pots containing sterile sand. The pots were covered with glass bottles/polythene bag. The plantlets were watered daily. Polythene bag/glass bottles were removed after 30 days. The plantlets producing new leaves within 10 days were transferred to large pots containing sand and soil in 1: 1 ratio. Morphologically there was no variation between the *In vivo* plants and *In vitro* grown plants. The plantlets showed eighty per cent survival rate after they were transferred to field environment.

DISCUSSION

To define and describe the future tasks of phytomedicinal research in the new millennium is an analysis, not only of the current state of development of phytomedicinal research but also of chemosynthetic pharmaceutical research. One advantage of phytotherapy is the availability of a wide group of medicinal drugs and preparations that have been used over the centuries almost exclusively on the basis of empirical evidence. A reservoir of around 3,00,000 plant species exists, of which only about 30 percent have been investigated scientifically, inclusively the herbs and preparations of Chinese, Indian, South American and African traditional medicines (Zohara and Uriel, 2005)

Tissue culture methods have been employed as an important aid to conventional methods of plant improvement. For the well establishment of good cultural conditions for the removal of external microorganisms and are able to grow readily culture media containing amino acid supplements, growth regulators, organic acids and vitamins (George, 1993)

Evolvulus nummularius (L.) is a low growing species, with creeping stems, small rounded leaves and bracteoles near or at the base of the flower stalk, which is wide spread in tropical America and also occurs in the Old World tropics, possibly through unintentional introduction. Linnaeus named it *convolvulus nummularius* in the Species Plantarum 1:157(1753) (William, 1972). Dash *et al.,* 2003 evaluated the aqueous and hydroalcoholic extracts of *E. nummularius* shown anthelmintic activity against adults of the Indian earthworm *Pheretima posthuma*.

The test on three surface sterilants (sodium hypochlorite, ethanol and mercuric chloride) was made to see the better method of sterilization of the explants. The sterilization was carried out by dipping the plant material into 70% [v/v] ethanol for 2-3 minutes, whereupon it was transferred to a solution of 1% sodium hypochlorite containing 4 drops of Tween-60 for 20 minutes and placing in 0.5% HgCl2 for 5 minutes was shown most effective. The efficiency of ethanol, sodium hypochlorite, Tween and mercuric chloride in surface sterilization was previously reported in many medicinal plants like - B*oesenbergia rotunda* (L.) (Tan *et al.,* 2005) and *Schisandra chinensis* (TURCZ.) BAILL (Kim *et al.*, 2005).

Addition of activated charcoal to overcome the problem of phenolic exudation was effective to get best results. Regular transfer of explants to fresh media with same hormonal composition also proved best for callus production. The shoot regeneration potential through callus phase from various explants via shoot tips and flower was studied. Various concentrations and combinations of growth regulators in MS medium were used for this study. For callus induction, the explants were cultured on MS medium supplemented with IAA, 2, 4-D, BA, Kn either alone or in combinations. Profused and higher amount of calli was produced from explants with 1mg/l of 2, 4-D and 1mg/l of BA.

Calli obtained from terminal bud and flower were selected for regeneration studies to find out optimum growth regulator combination needed in MS medium for callus regeneration. A combination of 3.0 mg/l of BA and 0.5 mg/l of IAA on MS medium produced highest number of shoots (2-3) per gram callus.

Rooting was more at IAA concentration of 1 mg/l. The plantlets showed 80% survival rate when transferred from *in vitro* to field condition.

Plant tissue culture is the need of the present situation. There is a high need to regenerate in vitro plants to preserve endangered/rare plants. The present experiment provided study of hormonal activity on *E. nummularius* tissues. Profused and higher amount of calli was produced from explants supplemented with 1mg/l of 2, 4-D and 1mg/l of BA. It was observed that in MS+ BA (3.0 mg/l) + IAA(0.5 mg/l) highest number of shoots (2-3 per gram callus) was produced. Rooting was more at 1 mg/l concentration of IAA. The plantlets showed eighty per cent survival rate after they were transferred to field environment.

ACKNOWLEDGMENT

Authors would like to thank management and staff of GITAM University, Visakhapatnam, India for their kind support in bringing out the above literature and providing lab facilities.

REFERENCES

Anonymous (1948). The Wealth of India. CSIR, New Delhi, India. **I** 62-63.

Anonymous (1959). The Wealth of India. CSIR, New Delhi, India. **V** 314-315.

Borchers AT, Hackman RM, Keen CL, Stern JS and Gershwin ME (1997). Complementary medicine: A review of immunomodulatory effects of Chinese herbal medicines. *American Journal of Clinical Nutrition* **66** 1303-1312.

Bornman CH (1983). Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro*. *Physiologia Plantarum* 57 5-16**.**

Dubey NK, Rajesh K and Pramila T (2004). Global promotion of herbal medicine: India's opportunity. *Current Science* **86** 37-41.

Fang R and George S (1995). Convolvulaceae. *Flora of China* **16** 271–325.

Fereidoon S (1999). Chemicals via higher plant Bioengineering. edited by *Fereidoon* Shahidi *et al*. Kluwer (Academic/*Plenum Publishers*,New york) 221-223.

Gamborg OL., Miller RA and Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50** 151--158.

Gautheret RJ (1939). Sur la possibilitite de realiser la culture indefinite des tissue de tubercules de carotte. *Compte Rendu Hebdomadaire des Séances de l'Académie des Sciences Paris* **208** 118-121.

Gautheret RJ (1940). Noveless rescherches sur le bourgeonnenieut du tissu cambial d'*Ulmus campestris* culture *in vitro*. *Compte Rendu Hebdomadaire des Séances de l'Académie des Sciences Paris* **210** 744-746.

George EF (1993). Plant propagation by tissue culture. Part 1, The Technology. 2nd edn. (*Exegetics Ltd*., England) 574.

Ghimire S, McKey D and Aumeeruddy TY (2004). Heterogeneity in ethnoecological knowledge and management of medicinal plants in the himalayas of nepal: implications for conservation. *Ecology and Society* **9** 6.

Haberlandt G (1902). Kulturversuche mit isolierten Pflanzenzellen. Sitzungsb. Akad. Wiss. Wien, *Math.-Natur*. **K1. 111** 69-72.

Hildebrandt AC, Riker AJ and Duggar BM (1946). The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. *American Journal of Botany* **33** 591-597.

Julia S. Whelan and Lana Dvorkin (2006). Plants from many healing landscapes: gathering information and teaching clinicians about the cultural use of medicinal herbs. *Journal of the Medical Library Association* **94** 223–226.

Kim TD, Ramesh AV and Park JI (2005). Somatic embryogenesis in *Schisandra chinensis* (TURCZ.) BAILL. *In Vitro Cellular and Developmental Biology – Plant* **41**(3) 253–257.

Leena T and Jaindra NT (2003). Role of biotechnology in medicinal plants. *Tropical Journal of Pharmaceutical Research* **2**(2) *243-253.*

MacRae S and VanStaden J (1990). *In vitro* culture of *Eucalyptus grandis*: Effect of gelling agents on propagation. *Journal of Plant Physiology*. **137** 249-251.

Minocha SC (1980). Callus and adventitious shoot formation in excised embryos of white pine (*Pinus strobus*). *Canadian Journal of Botany* **58** 366-370.

Minocha SC (1987). Growth Regulators and morphogenesis in cell and tissue culture of forest trees. In: Cell and Tissue Culture in Forestry, Vol. 1. General Principles and Biotechnology. edited by J. M. Bonga and D. J. Durzan, (*Martinus Nijhoff Publishers*, Dordrecht, The Netherlands) 50-66.

Mulder-Krieger TH, Verpoorte R and Baerheim SA (1982). The tissue culture of Cinchona pubescens: effect of media modifications on the growth. *Planta Medica* **44** 237-240.

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

Nadel BL, Altman A, Pleban S and Huttermann A (1991). *In-vitro* development of mature *Fagus sylvatica L*. buds. I. The effect of medium and plant regulators on bud growth and protein profiles. *Journal of Plant Physiology* **138** 596-601.

Nitsch JP (1951). Growth and development in *in vitro* of excised ovaries. *American Journal of Botany* **38** 566-577.

Nobecourt P (1939). Sur la perrennite et laugmentation de volume des cultures de tissues vegetaux. *Compte Rendu Hebdomadaire des Séances et Mémoires de la Société de Biologie* **130** 1270-1271.

Oka S and Ohyama K (1975). Effects of constituents on the growth of Broussonetia callus tissue. *Protocol* Crop *Science* Society *Japan* **44** 147-152.

Pochet B, Mestdagh M and André P (1991). Influence of agar gel properties on the *in vitro* micropropagation of different clones of *Thuja plicata. Plant Cell Reports **10** 406-409*.

Posey DA (1998). Biodiversity, Genetic Resources, and Indigenous Peoples in Amazonia: (Re) Discovering The Wealth of Traditional Resources of Native Amazonians. *In A. Hall (ed),* (Amazonia at the Crossroads, London) 188-204

Reinert J and White PR (1956). The cultivation of *in vitro* of tumour tissues and normal tissues of Pices glauca. *Physiologia Plantarum* **9** 177-189.

Schenck RU and Hildebrandt AC (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* **50** 199-204.

Tan SK, Pippen R, Yusof R, Ibrahim H, Rahman N and Khalid N (2005). Simple one-medium formulation regeneration of fingerroot Boesenbergia rotunda (L.) Mansf. Kulturpfl. via somatic embryogenesis. *In Vitro* Cellular & Developmental Biology Plant **41** 757-61.

William TS (1972). Typification of *Evolvulus nummularius, E. convolvuloides* and *E. alsinoides* (Convolvulaceae). *Taxon* **21**(5/6) 647-650.

White PR (1963). The cultivation of animal and plant cells. (*The Ronald Press Co*., New York).

White PR and **Gilbey SN (1966).** Sources. of nitrogen for spruce tissue cultures. *Physiologia Plantarum* **19** 177-186.

Unnikrishnan E (2004). Materia Medica of the Local Health Traditions of Payyannur. *Kerala Research Programme on Local Level Development*. Paper No. **80** 5.

Villalobos VM, Leung DWM and Thorpe TA (1984). Light-cytokinin interaction in shoot formation in cultured cotyledon explants of radiata pine. *Physiologia Plantarum* **61** 513-517.

Zohara Y and Uriel B (2005). Handbook of Medicinal Plants. (*Haworth Press*, Inc., New York) 3-4.