
Influence of agar concentration and liquid medium on *in vitro* propagation of *Ceropegia thwaitesii* Hook- an endemic species

Selvaraj Muthukrishnan¹, J.H. Franklin Benjamin² and M.V. Rao^{1*}

¹Department of Plant Science, Bharathidasan University, Tiruchirappalli- 620 024, Tamil Nadu, India, ²Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu, India.

Selvaraj Muthukrishnan, J.H. Franklin Benjamin and M.V. Rao (2013). Influence of agar concentration and liquid medium on *in vitro* propagation of *Ceropegia thwaitesii* Hook- an endemic species. International Journal of Agricultural Technology 9(4):1013-1022.

Abstract This study was aimed to improve the *in vitro* shoot multiplication and root induction efficiency of *Ceropegia thwaitesii*. Nodal explants were regenerated on Murashige & Skoog medium (MS) containing KIN (13.94 μM) + IAA (28.54 μM) and various concentrations of agar-agar. Reduced concentration of agar than normal and its complete elimination favoured both shoot multiplication and rooting during micropropagation of *Ceropegia thwaitesii*. Increasing the agar concentration affected various micropropagation parameters. The addition of agar concentration showed to be useful in improving the quality of regenerated shoots. Liquid medium devoid of agar proved to be the best for the micropropagation of *C. thwaitesii*.

Key words: *Ceropegia thwaitesii*, Chlorophyll content, *in vitro* rooting, micropropagation, liquid medium, hyperhydricity, Agar concentration

Introduction

Biotechnology involving tissue culture for crop improvement requires the ability to regenerate a great number of plants (Berrios *et al.*, 1999). The application of biotechnology for the improvement of *Ceropegia* has been limited due to difficulties associated with the lack of an efficient and reproducible method for plant regeneration. Interaction between *in vitro* raised plantlets with the gelling agent in culture medium is a dynamic process and the changes in gel consistency, affect the regeneration of plants or tissue (Scholten and Pierik, 1998). Traditionally, agar (0.6-0.8%) is added to the culture medium to increase its viscosity, as a result, the plant tissues and organs remain above the surface of the nutrient medium (Debergh, 1983). Increasing agar strength beyond a critical limit has been demonstrated to inhibit micropropagation and shoot growth and reduce the water availability to the cultures (Selby and Harvey, 1989). Casanova *et al.* (2008) suggested that low concentrations of

* **Corresponding author:** M.V. Rao; **e-mail:** mvrao_456@yahoo.co.in; herr.franklin@gmail.com

agar provide a poorly gelled medium that facilitates adequate and better diffusion of medium constituents resulting to better growth and subsequent rooting. The choice of gelling agent is also very important for plant *in vitro* regeneration (Kacar *et al.*, 2010). The medium must be firm enough to support explants, but if the rigidity is too high it may prevent adequate contact between the medium and the tissue (Berrios *et al.*, 1999). The objective of this study was to evaluate the regeneration capacity by using different agar concentrations. Methods of *in vitro* propagation in liquid medium have also been attempted where agar was completely omitted from the medium. By using liquid medium instead of gelled medium, propagation is accelerated, culture transfer frequencies may be decreased and cost of production is reduced.

Ceropegias are known to store starch, sugars, gum, albuminoid, fats, crude fibre and other valuable phytoconstituents which are routinely used in traditional Indian ayurvedic drugs (Kirtikar and Basu, 1935). The pharmacological importance of the genus *Ceropegia* is mainly due to the presence of a pyridine alkaloid called 'Ceropegin' (Sukumar *et al.*, 1995). In India, about 50 species are present and most of them are endemic to Western Ghats which is one of the centres of diversity of *Ceropegia* (Malpure *et al.*, 2006; Surveswaran *et al.*, 2009) and most of them are enlisted under endangered category (Nayar and Sastry, 1987). Most of the endemic species of *Ceropegia*, by virtue of being restricted only to a special habitat and narrow ecological niche, are highly vulnerable and merit special consideration in their conservation. Reasons for their decline are many, *e.g.*, destruction of forests, modifications of habitats, industrialization, pollution and introduction of exotic weeds (Yadav and Kamble, 2008).

Ceropegia thwaitesii Hook, an endemic plant species, has a very restricted distribution in the Western Ghats of Tamil Nadu, India. It inhabits bare slopes of Pambar shola, and is highly vulnerable due to habitat destruction and over exploitation (Nayar and Sastry, 1988). Micropropagation protocol for its multiplication has been developed and reported earlier (Muthukrishnan *et al.*, 2012). The present study deals with the improvement of protocol by manipulating agar concentration in the medium and by using liquid medium.

Materials and methods

Effect of agar on shoot multiplication

In vitro shoot cultures were established using nodal explants as per protocol of Muthukrishnan *et al.*, (2012). *In vitro* multiplying shoot clusters harvested aseptically were used as the explants for further studies. In order to study the effect of different agar concentrations on shoot multiplication, the

standard shoot multiplication medium (MS Murashige & Skoog salts+ KIN+IAA) was modified using different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0% w/v) of agar (Hi Media Make). The medium was autoclaved at 121 °C, for 30 min. All the cultures were maintained in sterilized culture room at 26 ± 2 °C, under 16/8 h light regime provided by cool white fluorescent light (60 µmol⁻²s⁻¹ light intensity) and with 55 - 60% relative humidity. All the cultures were sub cultured on the fresh medium after 20-25 days.

Effect of agar on Rhizogenesis

To find out the effect of different concentrations of agar on *in vitro* rooting, its concentration was varied from 0 to 1.0% (w/v) in standard rooting medium containing IBA 2.46 µm. For this purpose, the shoots of uniform length were harvested aseptically from *in vitro* multiplying cultures and inoculated on the standard medium. In case of liquid medium filter paper bridges were used as support matrix.

Biochemical analysis

Biochemical characteristics such as, chlorophylls a and b, total chlorophyll and carotenoids were also recorded. Fresh leaves of same physiological age from *in vitro* derived explants were used. The leaf bits were weighed (50mg) using on an electronic balance and homogenized in 100% acetone using mortar and pestle. After homogenization, acetone was added and the entire mixture was transferred into a 25ml measuring cylinder. Care was taken so as not to spill while transferring. The homogenate was spun at 5000rpm for 5min. The pellet must be pale yellow or white in colour. The colour of the pellet indicates the complete extraction of pigments from the leaf sample, the supernatant was decanted in a clear test tube, and the absorbance was measured at 662, 645 and 470 nm using the Wellburn and Lichtenthalers (1984) formulae. The total amount of chlorophylls and carotenoid was quantified.

$$\text{Chlorophyll a (mg/l)} = 11.75 \times A_{662} - 2.35 \times A_{645}$$

$$\text{Chlorophyll b (mg/l)} = 18.61 \times A_{645} - 3.96 \times A_{662}$$

$$\text{Chlorophyll (a+b) (mg/l)} = 7.79 \times A_{662} + 16.26 \times A_{645}$$

$$\text{Carotenoids (mg/l)} = 1000 \times A_{470} - 2.27 \times \text{Chl a} - 81.4 \times \text{Chl b}/227$$

A_{662} , A_{645} , A_{470} are optical density at wavelength and 662, 645 and 470 nm

Experimental design and data collection

Observations of the cultures were made every week and the data were gathered after 35 and 45 days for shoot multiplication and rooting, respectively. All the experiments were conducted using complete randomized design. For each experiment, a minimum of 7 replicates were made and repeated thrice. Data related to shoot multiplication (frequency of response, number of shoots, number of node, number of leaves and shoot length) were also noted. Comparisons between treatments were made using Duncan's Multiple Range Test (DMRT) (Duncan, 1955).

Results and discussion

Effect of agar in shoot multiplication

The effects of agar concentration as well as the interactions between plants and agar concentration were also significant for all the studied parameters. *In vitro* cultured shoots of *C.thwaitesii* grew faster on reduced agar or complete absence of agar in the medium (Fig. 3a). In the present investigation, the shoot clusters grown on standard shoot multiplication medium gelled with 0.8% agar (control), an average of 6.32 ± 0.26 shoots with 9.24 ± 1.14 leaves per cluster developed after 45 days (Fig. 1). Liquid medium (without agar) produced shoots with an average of 7.71 ± 0.75 , higher than the control and with an average of 18.57 ± 4.26 number of leaves. The average number of shoots at 0.2 (very weak gelled medium), 0.4 and 0.6% agar in the medium was 1.04 ± 0.45 . In the liquid medium, it produced more leaves compared to control (approx. 2 times higher than the control) with an average of 18.57 ± 4.26 . The number of leaves, nodes and shoot length were much less than in the control and liquid medium. The average shoot number of 1.18 ± 0.06 with 3.31 ± 0.17 shoot length was recorded in above and below the concentration of the control. According to Berthouly and Etienne (2005) and Escalona (2006) factors combined to allow an intermittent contact of culture medium with plant material, so as to make possible an efficient nutrient contribution and a periodical renewal of the internal atmosphere in culture flasks. They have been able to increase plant growing and sprout proliferation in some crops like *Coffea arabica*, *Ananas comosus*, and *Saccharum officinarum* in liquid medium. Likewise, Jova *et al.* (2011) obtained effective shoot multiplication in *Dioscorea alata*. Micropropagation on liquid medium is an alternative to agar (Adelberg and Fari, 2010; Adelberg *et al.*, 2012). Generally, plants propagated on liquid media multiply more rapidly, are larger in size, and grow more quickly in the greenhouse. Yet, hyperhydricity can

occur when plants are immersed in liquid medium but, here no symptoms of hyperhydric plants were observed. Agar concentration is important in determining culture response.

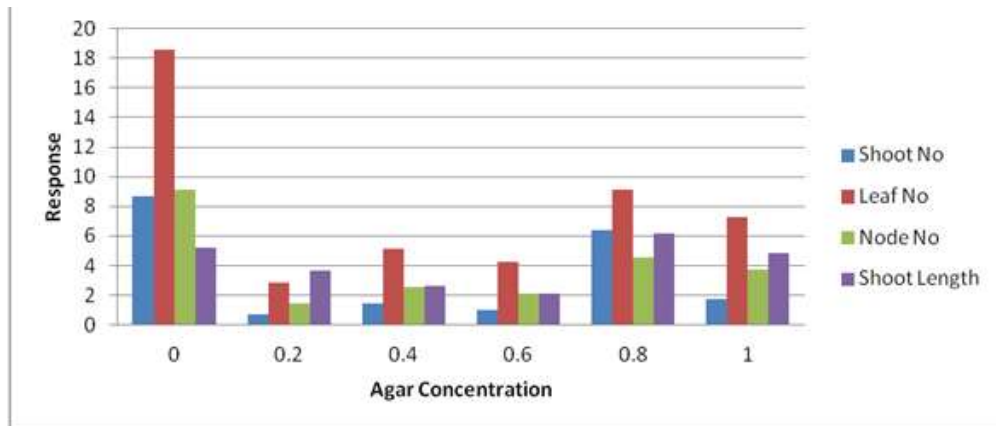


Fig. 1. Effect of Agar concentration on *in vitro* shoot multiplication of *C. thwaitesii* on MS medium containing KIN (13.94 μm) + IAA (28.54 μm)

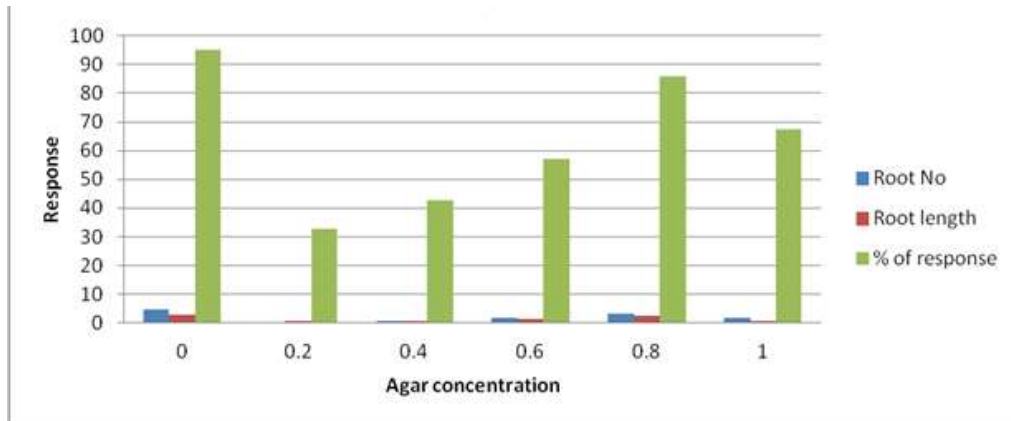


Fig. 2. Effect of agar on *in vitro* rooting of *C. thwaitesii* on MS medium containing IBA (2.46 μm)

Biochemical analysis

Low agar levels and liquid culture medium have been reported to promote shoot proliferation in several culture systems on account of faster uptake and better absorption of water by plants implanted on softer gels (Gurel and Gulsen 1998; Klimaszewska *et al.* 2000). Higher (1.93mg/g) total chlorophyll contents was observed on liquid medium in comparison to control (1.80mg/g). Likewise,

the carotenoid content in liquid medium derived plants was also higher (0.28mg/g) than control (0.20mg/g). In liquid medium the shoots and leaves were green, increasing concentration of agar to 0.2% stunted shoots with yellowish coloured leaves were observed. No significant differences in the chlorophyll contents were recorded at any other concentrations. Higher chlorophyll accumulation was reported in *Boswellia serrata* (Suthar *et al.* 2011), *Chlorophytum borivilianum*, *Terminalia bellerica*, *Feronia limonia* (Vyas *et al.* 2008) and *Dioscorea alata* (Jova *et al.* 2011) due to better growth performance of cultures in liquid medium. In our observation, increasing concentration of agar to 1% in the medium resulted to significant decrease in the total chlorophyll and carotenoid content of the plants and the shoot growth was significantly reduced (Table 1). The fresh and dry weights were also lesser than the control. Shoots and leaves were shorter and their color changed as compared to control and liquid medium (data not shown). Ghashghaie *et al.* (1991) reported that the number of shoots and fresh and dry weight decreased with increasing the agar concentration in rose. Due to reduced water availability, the fresh and dry weight decreases when increasing the agar concentration (Ruzic *et al.*, 1998; Suthar *et al.*, 2011).

Compared to greenhouse grown plants, *in vitro* liquid cultured plants showed a significant increase in chlorophyll a, b and total chlorophyll (Table 1 & Fig 3i). The levels of pigments in *in vitro* derived plants were also slightly higher numerically although no statistical significance was found. In micropropagated plants, the extent of increase was higher in chl *a* than chl *b*. As result of these changes, the chlorophyll *a/b* ratios were higher in plantlets of *in vitro* origin. In relation to the carotenoid content, a significant increase in this pigment was found only in green house grown plants. All the *in vitro* plantlets displayed higher pigment contents than in the corresponding *ex vitro* plant leaves.

Although Amancio *et al.* (1999) and Van Huylenbroeck *et al.* (2000) found a significant rise in chlorophylls in *ex vitro* leaves after transplantation, several earlier micropropagation studies showed an increased quantity of chlorophyll in *in vitro* grown leaves (Aoyagi and Bassham, 1986; Lee *et al.*, 1988; Premkumar *et al.*, 2001). In general, the fact that *in vitro* leaves resemble shade leaves in several aspects, the presence of exogenous carbohydrates (Sunitibala *et al.*, 1998; Ticha *et al.*, 1998), and the classical effect of exogenous cytokinins on chlorophyll content (Pospisilova *et al.*, 1993) could explain the increased chlorophyll.

Table 1. Effect of Agar in biochemical content of *in vitro* raised *C.thwaitesii*

Agar concentration (%)	Chlorophyll content (mg/g fresh tissue)			
	Chl a	Chl b	Total chl	Carotenoids
Liquid medium	1.580±0.07a	0.389±0.07a	1.938±0.07a	0.280±0.07a
0.2	0.482±0.00fg	0.006±0.01e	0.489±0.01d	0.179±0.01e
0.4	0.455±0.01e	0.002±0.01e	0.453±0.01d	0.192±0.01d
0.6	0.432±0.01g	0.102±0.00d	0.430±0.00d	0.185±0.01e
0.8	1.445±0.02b	0.359±0.03b	1.804±0.03b	0.209±0.05c
1.0	1.225±0.02d	0.346±0.02b	1.703±0.02c	0.189±0.02d
<i>Ex vitro</i>	1.421±0.02c	0.322±0.02c	1.744±0.02bc	0.323±0.02b



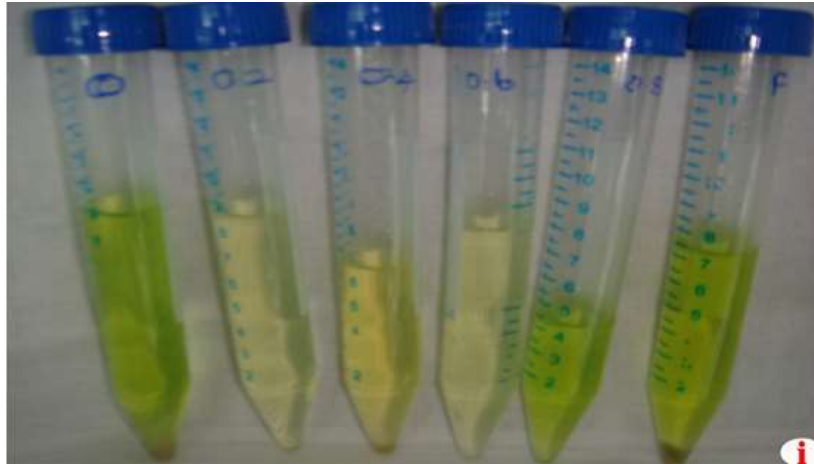


Fig. 3. Effect of agar on micropropagation of *C.thwaitesii*. Shoot multiplication: (a) liquid Medium (b) 0.4% agar (c) 0.8% agar (d) 1.0% agar. Rooting: (e) liquid medium (f) 0.2% agar (g) 0.4% (agar h) 0.8% agar (i) Biochemical analysis.

Effect of agar in Rhizogenesis

Rhizogenesis, the formation of roots, is a crucial step in micropropagation for the formation of complete plantlets. When shoots were rooted on standard root induction medium gelled with 0.8% agar (control) the average root was 3.42 ± 0.52 per shoot with an average root length of 2.37 ± 0.18 cm (Fig. 2 & Fig. 3h). Below and above the control concentration of agar, the root number (1.75 ± 0.36) and root length (0.75 ± 0.18) was approximately more or less even (Fig 3f & g).

However, the best rooting response was obtained when shoots were rooted on medium without agar (liquid medium) with a maximum of 4.85 ± 0.40 roots per shoot with average length of 2.87 ± 0.22 cm (Fig. 3e). Similarly, liquid medium has been used successfully to obtain high rooting frequency in *Helianthus annuus* (Pearson *et al.*, 2011), *Musa sapientum* (Akbar and Roy, 2006), *Boswellia serrata* (Suthar *et al.*, 2011) and *Aloe barbadensis* (Adelberg *et al.*, 2012). In conclusion, the use of liquid media protocol for the micropropagation of *C.thwaitesii* is a good alternative.

References

- Adelberg, J. and Adelberg, J.N. (2012). Effects of Cytokinin on Multiplication and Rooting of *Aloe barbadensis* during Micropropagation on Agar and Liquid Medium. *Journal of Medicinally Active Plants* 1(1):1-5.
- Adelberg, J. and Fari, M. (2010). Applied physiology and practical bioreactor designs for micropropagation of ornamental plants. *Propagation of Ornamental Plants* 10:205-219.

- Akbar, M.A. and Roy, S.K. (2006). Effects of Liquid Medium on Rooting and Acclimation of Regenerated Microshoots of Banana (*Musa sapientum* L.) cv. Sagar. *Plant Tissue Culture and Biotechnology* 16(1):11-18.
- Amancio, S., Rebordao, J.P. and Chaves, M.M. (1999). Improvement of acclimatization of micropropagated grapevine: Photosynthetic competence and carbon allocation. *Plant Cell Tissue and Organ Culture* 58:31-37.
- Aoyagi, K. and Bassham, J.A. (1986). Appearance and accumulation of C4 carbon pathway enzymes in developing maize leaves and differentiating maize A188 callus. *Plant Physiology* 80:322-333.
- Berrios, E.F., Gentzittel, L., Serieys, H., Alibert, G., Sarrafi, A. (1999). Influence of genotype and gelling agents on in vitro regeneration by organogenesis in sunflower. *Plant Cell Tissue and Organ Culture* 59: 65-69.
- Berthouly, M. and Etienne, H. (2005). Temporary immersion systems: a new concept for use liquid medium in mass propagation. In: Hvoslef-Eide A.K. & Preil W., eds. *Liquid culture systems for in vitro plant propagation*. Dordrecht, The Netherlands: Springer, 165-195.
- Casanova, E., Moysset, L. and Trillas, M.I. (2008). Effect of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots, *Biologia Plantarum* 52:1-8.
- Debergh, P. (1983). Effects of agar brand and concentration on tissue culture medium, *Physiologia Plantarum* 59:270-276.
- Duncan, D.B. (1955). Multiple range and multiple F test. *Biometrics* 11:1-42.
- Escalona, M. (2006). Temporary immersion beats traditional techniques on all fronts. In: *Prophyta*. The annual 2006. Roelofarendsveen, The Netherlands: Prophyta Foundation; Velsen-Zuid, The Netherlands: Blue Bird Publishers pp. 48-49.
- Ghashghaie, J., Brrenckmann, F. and Saugier, B. (1991). Effects of agar concentration on water status and growth of rose plants cultured in vitro, *Physiologia Plantarum* 82:73-78.
- Gurel, S. and Gulsen, Y. (1998). The effects of different sucrose, agar and pH levels on in vitro shoot production of almond (*Amygdalus communis* L.), *Turkish Journal of Botany* 22: 363-373.
- Jova, M.C., Kosky, R.G. and Cuellar, E.E. (2011). Effect of liquid media culture systems on yam plant growth (*Dioscorea alata* L. 'Pacala Duclos'). *Biotechnology, Agronomy, Society and Environment* 15(4):515-521.
- Kacar, Y.A., Biçen, B., Varol, I., Mendi, Y.Y., Serçe, S. and Çetiner, S. (2010). Gelling agents and culture vessels affect in vitro multiplication of banana plantlets. *Genetics and Molecular Research* 9(1):416-424.
- Kirtikar, K.R. and Basu, B.D. (1935). *Indian medicinal plants* Vol. 3. M/s Bishen Singh Mahendrapal Singh, New Delhi, India pp. 1638.
- Klimaszewska, K., Bernier-Cardou, M., Cyr, D. and Sutton, B. (2000). Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L., *In vitro cellular & Developmental Biology- Plant* 36:279-289.
- Lee, N., Wetzstein, H.Y. and Sommer, H.E. (1988). Quantum flux density effects on the anatomy and surface morphology of *in vitro*-developed sweetgum leaves. *Journal of the American Society for Horticultural Science* 113:167-171.
- Malpure, N.V., Kamble, M.Y. and Yadav, S.R. (2006). A new variety of *Ceropegia oculata* Hook. (Apocynaceae: Asclepiadoideae) from Satpuda hill ranges of Maharashtra, India. *Current Science* 91:1140-1142.

- Muthukrishnan, S., Franklin, B.J.H., Muthukumar, M., Sherif, N.A., Senthilkumar, T. and Rao, M.V. (2012). In vitro propagation of *Ceropegia thwaitesii* Hook- an endemic species of Western Ghats of Tamil Nadu, India. African Journal of Biotechnology 11(59):12277-12285.
- Nayar, M.P. and Sastry, A.R.K. (1988). Red data book of Indian plants, Vol 1& 2. Botanical Survey of India, Kolkata.
- Nayar, M.P. and Sastry, A.R.K. (1987). Red Data Book of Indian Plants. Botanical Survey of India. Calcutta pp. 58.
- Pearson, C.H., Cornish, K., McMahan, C.M., Whalen, M., Rath, D.J., Dong, N. and Wong, S. (2011). Reprinted from: Issues in new crops and new uses. 2007. J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria, VA. Using Peat Pellets in Liquid Media to Root Sunflower Tissue Culture Plants.
- Pospisilova, J., Catsk'ý, J., Synková, H., Maháčkova, I. and Solárová, J. (1993). Gas exchange and *in vivo* chlorophyll fluorescence in potato and tobacco plantlets in vitro as affected by various concentrations of 6-benzylaminopurine. Photosynthetica 29:1-12.
- Premkumar, A., Mercado, J.A. and Quesada, M.A. (2001). Effects of *in vitro* tissue culture conditions and acclimatization on the contents of Rubisco, leaf soluble proteins, photosynthetic pigments, and C/N ratio. Journal of Plant Physiology 158:835-840.
- Ruzic, D.J. and Cerovic, R. (1991). Influence of agar brands and concentration on in vitro shoot multiplication of the cherry rootstock Gisela 5, Acta Horticulturae 468:209-216.
- Scholten, H.J. and Pierik, R.L.M. (1998). Agar as gelling agent: Differential biological effects in vitro, Scientia Horticulturae 77:109-116.
- Selby, C., Lee, R. and Harvey, B.M.R. (1989). The effects of culture medium rigidity on adventitious bud production and tissue vitrification in needle cultures of sitka spruce (*Picea sitchensis* (Bong.) Carr.), New phytologist 113:203-210.
- Sukumar, E., Gopal, H. and Rao, R.B. (1995). Pharmacological actions of cerpegin, a novel pyridine alkaloid from *Ceropegia juncea*. Fitoterapia 66:403-406.
- Surveswaran, S., Kamble, M.Y., Yadav, S.R. and Sun, M. (2009). Molecular phylogeny of *Ceropegia* (Asclepiadoideae, Apocynaceae) from Indian Western Ghats. Plant Systematics and Evolution 281:51-63.
- Suthar, R.k., Habibi, N. and Purohit, S.D. (2011). Influence of agar concentration and liquid medium on in vitro propagation of *Boswellia serrata* Roxb. Indian journal of Biotechnology 10:224-227.
- Sunitibala, Y., Gupta, S. and Mukherjee, B.B. (1998). Effect of sucrose on growth and chlorophyll synthesis of teak shoots in mixotrophic culture. Journal Plant Biochemistry and Biotechnology 7:57-59.
- Ticha, I., Cáp, F., Pacovská D., Hofman, P., Haisel, D., Capková V. and Schäfer, C. (1998). Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown in vitro. Physiologia Plantarum 102:155-162.
- Van Huylbroeck, J.M., Piqueras, A. and Debergh, P.C. (2000). The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated *Calathea* plants. Plant Science 155:59-66.
- Vyas, S., Rao, M.S., Suthar, R.k. and Purohit, S.D. (2008). Liquid culture system stimulates in vitro growth and shoots multiplication in four medicinally important plants, Medicinal Aromatic Plant Science and Biotechnology 2:96-100.
- Wellburn, A.R. and Lichtenthaler, H. (1984). Formulae and program to determine total carotenoids and chlorophyll a and b of leaf extracts in different solvents. In: (Sybesma, ed.) Advances in photosynthesis research. Vol. 2, Kluwer. The Hague pp. 9-12.
- Yadav, S.R. and Mayur Y Kamble, (2008). Threatened Ceropegias of the Western Ghats and Strategies for their Conservation. In: Rawat, G.S. (Ed.). 2008. Special Habitats and

International Journal of Agricultural Technology 2013, Vol. 9(4): 1013-1022

Threatened Plants of India. ENVIS Bulletin: Wildlife and Protected Areas, 11(1).
Wildlife Institute of India, Dehradun, India pp. 239.

(Received 27 November 2012; accepted 30 June 2013)