

In Vitro Growth and Shoot Multiplication in *Nicotiana tabacum* L. - Influence of Gelling Agent and Carbon Source

Neelu Joshi*

Department of Biotechnology and Bioinformatics, Padmashree Dr. D.Y. Patil University, Navi Mumbai – 400706 (India)

Correspondence: * jn772003@yahoo.co.in

ABSTRACT

The present study examined the influence of various gelling agents and carbon sources on *in vitro* growth and multiplication in *Nicotiana tabacum* L. cv. 'Havana 425' (Family: Solanaceae). Shoot multiplication was greatly favoured on medium gelled with reduced concentrations of agar. Complete absence of agar in the medium evoked a better response than that obtained on agar-containing medium. Replacement of agar by guar gum (at 2.0%, w/v) showed a two-fold improvement in *in vitro* growth and multiplication. On this medium ca. 12 elongated shoots were obtained. On medium gelled with Phytigel (0.1%), the rate of shoot multiplication was two times higher than that recorded on control. Incorporation of different carbon sources in the range of 1.5-4.0% (w/v) evoked varied responses in terms of shoot growth and multiplication. Glucose at 4.0% was the most effective carbon source where a maximum number of elongated shoots (>1.6 cm in height) were produced. Wet and dry weights of such shoots were also highest. The results emphasized the potential of guar gum as a gelling agent and glucose as a carbon source for increasing shoot multiplication and growth of *N. tabacum*.

Keywords: cost effectiveness, gel strength, propagation, rate of shoot multiplication

Abbreviations: BAP, benzylaminopurine; MS, Murashige and Skoog; NAA, naphthalene acetic acid; SM, standard shoot multiplication medium

INTRODUCTION

Nicotiana tabacum L. (Family: Solanaceae), commonly known as 'tobacco', is a native of America (Arslan and Okumus 2006) and is cultivated in all tropical countries. This plant is valued for its immense medicinal properties (contained in its leaves) and is of great commercial importance. Over 2500 compounds have been found in tobacco among which alkaloids and terpenoids are the major groups (Nugroho and Verpoorte 2002) having medicinal value. Alkaloids have important roles in addiction through recreational euphoria (Laviolette and Van der Kooy 2004), defensive function as insecticides (Kumar 2006; Lal and Verma 2006) and potential therapeutic use (Iqbal *et al.* 2006).

To meet the growing demand of this plant in the herbal drug industry, its large-scale cultivation is essential. In nature, this plant species propagates through seeds but poor seed viability and low germination frequency restricts its propagation on a large scale. Plant tissue culture and biotechnology offers an opportunity for rapid clonal propagation of desired species in order to provide superior identified planting material for commercial cultivation (Aitken-Christie and Connett 1992). Modern methods of plant tissue culture have been widely applied for conservation and cultivation of medicinal importance (Wawrosch 1999; Dave *et al.* 2003). In the case of *N. tabacum*, attempts have been made to multiply this plant by micropropagation through different pathways (Linsmaier and Skoog 1965; Tran Thanh Van *et al.* 1974; Mangat and Janjua 1987; Leuba and Tourneau 1990; Klems 2000; Babbar *et al.* 2005). These studies dealt mainly with the influence of plant growth regulators on *in vitro* shoot regeneration and very few studies have paid attention to the effect of other factors in *N. tabacum* like gelling agent on shoot regeneration (Lucyszyn *et al.* 2007, Ozel *et al.* 2008), source and size of explant (Nhut *et al.* 2003, Teixeira da Silva 2005), antibiotics (Teixeira da Silva and Fukai 2001, 2003), filter paper (Teixeira da Silva

2003), polyamines (Teixeira da Silva 2002) and organic nitrogen and phosphorous source on callus growth (Parc *et al.* 2007). One of the reasons may be that the use of somatic embryogenesis is more important than shoot regeneration. Very little information is available where the effect of these factors on shoot multiplication has been considered. A study of these factors is very important in order to optimize conditions for better rate of shoot multiplication and/or to provide a cheaper alternative for commercial cultivation. Also, since *N. tabacum* is used as a model system for production of recombinant proteins (Zhang *et al.* 2002, 2003; Kumar *et al.* 2006), an efficient *in vitro* propagation protocol for it is necessary. Therefore, the present investigation was undertaken to examine the effect of gelling agents and carbon sources on *in vitro* growth and shoot multiplication in *N. tabacum* in order to develop a highly reproducible and improved *in vitro* propagation protocol which would be useful in both large-scale multiplication and genetic transformation in this plant species.

MATERIALS AND METHODS

Plant material and culture establishment

In vitro shoot cultures were established using leaf explant taken from aseptically grown seedlings of *N. tabacum* L. cv. 'Havana 425'. Seeds (obtained from Dr. T. R. Ganapathi, BARC, Mumbai, India) were surface sterilized with 0.1% HgCl₂ (w/v) for 3 min under a Laminar flow bench (Microlift, Pune, India). After 3-4 thorough washes with sterile distilled water the seeds were aseptically inoculated on plain MS (Murashige and Skoog 1962) medium in culture tubes for their germination. Leaf discs (0.5 cm in diameter) obtained aseptically from 15 days-old *in vitro*-grown seedlings were cultured on MS medium containing 3.0% sucrose, 0.8% agar and supplemented with 2.0 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA for shoot bud induction (Kumar *et al.* 2006). Leaf explants of tobacco can form shoots *de novo* directly, depending on the

phytohormones in the medium. In it the shoot primordia arise from palisade mesophyll cells at the adaxial leaf surface (Dhaliwal *et al.* 2004).

Shoot multiplication

Shoot buds, after their initial proliferation on medium containing 2.0 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA, were subcultured along with the mother explant on the same fresh medium after 21 days for further shoot proliferation. This medium was designated as standard shoot multiplication medium (SM) in the present study as the shoot buds showed little multiplication on this medium (Table 1, Control). In an attempt to improve or refine the conditions for shoot multiplication, the influence of different gelling agents and carbon sources was examined. In all the subsequent experiments on shoot multiplication, clusters having 4-5 shoots were harvested from the proliferating shoot cultures and used as explants. In order to study the effect of gelling agents on shoot multiplication, the standard shoot multiplication medium was modified using different concentrations (as described in Table 1) of agar (Hi media), Guar gum (Satyam Guar gum Industries, Jodhpur, India) and Phytigel™ (Hi Media, Mumbai, India). Shoots were also inoculated on medium without gelling agent. In this case filter paper bridges were used to provide support to the growing shoots. In another experiment SM medium gelled with 0.8% agar was supplemented with different concentrations (1.5-4.0%, w/v) of carbon sources like sucrose (Hi Media), glucose (Hi Media) and jaggery (locally available; it is an unrefined sugar made by concentration of sugarcane juice without separation of the molasses and crystals). Shoots inoculated on medium containing 0.8% agar and 3.0% sucrose served as control for all the experiments.

The pH of the media was adjusted to 5.8 before autoclaving at 1.06 kg cm² for 15 min. All the cultures were maintained under controlled culture room conditions of temperature (28 ± 2°C), light (45 μmol m⁻² s⁻¹ for 16 h per day provided by white fluorescent tubes, Philips) and 50-60% relative air humidity. Shoot clusters were subcultured every two weeks without harvesting the shoots and were maintained for 42 days under the same incubation conditions.

The shoot cultures grown on medium containing different concentrations of various gelling agents and carbon sources were assessed and compared for their *in vitro* growth in terms of various growth parameters: average number of shoots, average shoot length, number of leaves per cluster, total wet and dry weights.

The wet and dry weights were determined by weighing shoot cultures on a Top Pan Electronic Balance (Contech) wet and after drying overnight at 60°C in a hot air oven, respectively.

Statistics

The experiments on the effect of gelling agent and carbon source were conducted in a completely randomized design (CRD). Each treatment consisted of six replicates and each experiment was repeated twice. The data were subjected to ANOVA using SPSS

for windows computer software (v. 14. SPSS Inc., USA). Differences between means were tested using the critical difference (CD) at the 5% level of significance.

RESULTS AND DISCUSSION

Tobacco (*N. tabacum* L.) has long been classified as a model species for *in vitro* organogenesis studies as its tissue cultures respond actively to plant growth regulators (PGRs) (Lucyszyn *et al.* 2007). Toldi *et al.* (2005) have reported the use of *N. tabacum* as a model to search for physiological parameters that provide an early indication of the morphogenetic response of leaf disc explants to different tissue culture-level manipulations in order to design an accelerated optimization process for this technology. Several authors have emphasized the optimization of PGRs for regeneration of tobacco plants from various explants like stem pith (Jr Meins *et al.* 1980; Klems *et al.* 2000), leaves (Klems *et al.* 2000; Dhaliwal *et al.* 2004), anthers (Evola, 1983) and isolated zygotes (He *et al.* 2008). Other factors like inorganic nitrogen requirement has been found to be important during shoot organogenesis of tobacco leaf discs (Ramage and Williams 2002). In another study, Srinivasan *et al.* (2007) reported the enhanced regeneration capacity of tobacco by heterologous expression of the *BABY BOOM* APZ/ERF transcription factor. The present study examined the influence of gelling agents and carbon sources on shoot multiplication in *N. tabacum*. The data presented here showed that leaf-derived shoot cultures of *N. tabacum* exhibited a significant differential response in terms of shoot multiplication and growth when inoculated on medium containing various concentrations of different gelling agents and carbon sources. Ozel *et al.* (2008) compared the effect of agar, isubgol and gelrite on *in vitro* shoot regeneration in tobacco and observed variable response of these gelling agents in terms of frequency of shoot regeneration, number of shoots and buds per explant and shoot length.

Gelling agents used to solidify plant growth medium can contain considerable quantities of mineral nutrients that have been reported to affect plant growth. In the present case the shoot cultures grown on SM gelled with 0.8% agar and 3.0% sucrose (control) produced an average of 6.7 shoots (Table 1). The shoots produced on this medium measured 1.7 cm in length with a total 8.92 g wet and 2.7 g dry weight.

Gel plant interaction is a dynamic process (Williams 1992) and changes in gel consistency may affect the regeneration of plants or tissues. Lowering the concentration of agar to 0.6 and 0.2% in the medium considerably improved the shoot multiplication in the present case. This may be due to the fact that at a lower concentrations of agar resistance to the diffusion of hormones and nutrients would be lower than at higher agar concentrations (Klimaszewska *et al.* 2000; Lucyszyn *et al.* 2007). Low agar levels have been

Table 1 Effect of different gelling agents on *in vitro* shoot multiplication in *N. tabacum* grown on MS medium containing 2.0 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA.

Type of gelling agent	Conc. (%)	Mean № of shoots	Mean shoot length (cm)	Mean № of leaves per shoot	Fresh weight (g) of shoot cluster	Dry weight (g) of shoot cluster
Agar	0.0	10.25 c	3.42 a	8.00 a	26.57 a	4.56 b
	0.2	8.70 d	3.00 a	6.00 b	16.33 d	4.30 b
	0.6	6.00 ef	2.60 ab	4.75 cd	9.32 f	2.31 d
	0.8 (control)	6.75 e	1.70 c	6.75 b	8.89 f	2.14 d
Phytigel™	0.1	15.00 a	1.60 c	3.75 d	26.30 d	5.31 a
	0.25	12.00 b	1.92 bc	3.25 d	23.21 b	3.24 c
	0.5	9.00 cd	1.99 bc	4.00 d	5.240 g	1.11 e
Guar gum	0.5	5.25 f	1.67 c	5.50 c	12.80 c	2.11 d
	1.0	9.50 cd	1.34 d	6.00 b	20.94 c	5.09 a
	2.0	12.25 b	1.92 bc	8.00 a	23.21 b	5.63
GM		9.47	2.11	5.60	17.28	3.58
SE		0.52	0.31	0.42	0.62	0.17
CD5%		1.50	0.91	1.23	1.79	0.51
CV		11.03	29.91	15.29	7.18	9.97

Values within column followed by different letters are significantly different at 5%



Fig. 1 Effect of different concentrations of agar on *in vitro* shoot multiplication in *N. tabacum*. (A) 0% agar, (B) 0.2%, (C) 0.6%, (D) 0.8%.

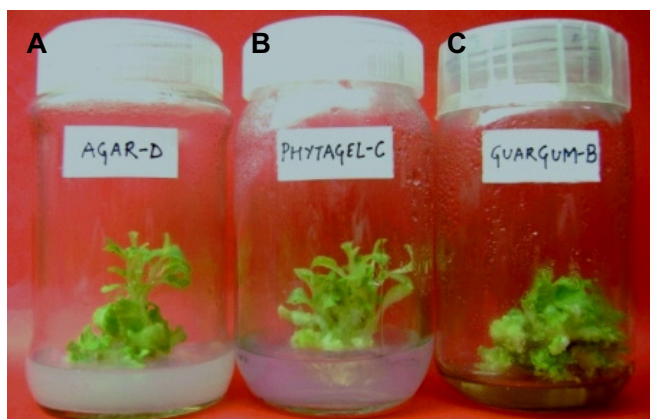


Fig. 2 Effect of different gelling agents on *in vitro* shoot multiplication in *N. tabacum*. (A) 0.8% agar, (B) 0.1% Phytigel™, (C) 2.0% guar gum.

reported to promote shoot proliferation in turmeric (Salvi *et al.* 2002), *Chlorophytum borivilianum* (Joshi 2005) and carnation (Casanova *et al.* 2008). Decreasing agar concentration in the medium has been reported to increase wet and dry weight in the shoots of amelanther (Brand 1993). Similar results were obtained in the present case. Vyas *et al.* (2008) reported the stimulatory effect of liquid medium on *in vitro* growth of a few medicinal plants. Similar to that, in this study also complete elimination of agar from the medium evoked the best response in terms of shoot multiplication and growth (Fig. 1). On this medium, 10.0 shoots measuring 2.25 cm in length were obtained (Table 1). Such shoots had an average wet weight of 8.92 g. Also the number of leaves per shoot was maximum (7) in this case. Such shoots did not show hyperhydric symptoms and required filter paper bridges as mechanical support for better absorption and growth. This type of support system has earlier been used by Misra (2002) for shoot bud differentiation of *Cajanus cajan* inoculated on liquid medium. Gangopadhyay *et al.* (2002) have reported enhanced rate of multiplication in *N. tabacum* through the use of coir in liquid culture medium.

During the present investigation, the response obtained on control medium was improved when agar was replaced with Phytigel™. Phytigel™, when incorporated at 0.1% (w/v) produced maximum (15) number of shoots (Table 1). Shoot length, however, was less than that recorded on control. The beneficial role of Phytigel™ in tissue culture has been reported in *Bacopa monnieri* (Shrivastav and Rajani 1999) and *Celastrus paniculatus* (Rao and Purohit 2008). Although the number and length of shoots obtained on medium gelled with Phytigel™ was higher than the control, the average number of leaves per shoot was less and such shoots were thin and hence, were not suitable for rooting.

In the present study, addition of cheaper gelling agent like guar gum (2.0%) in place of agar showed a significant improvement in shoot proliferation and growth as compared to control (Fig. 2). At this concentration, the average num-

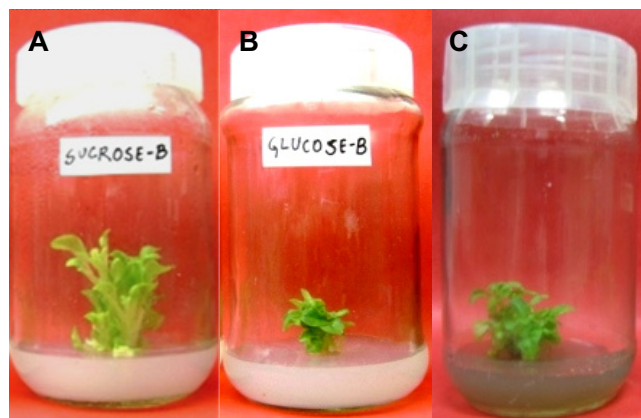


Fig. 3 Effect of different carbon sources on *in vitro* shoot multiplication in *N. tabacum*. (A) 3.0% sucrose, (B) 4.0% glucose, (C) 4.0% jaggery.

ber of shoots was two times higher than that obtained in the control. Such shoots measured 1.9 cm in length and were lush green with a total of 26.18 g wet weight and 3.22 g dry weight. The average number of leaves per shoot was 7.0 at this concentration. A decrease in concentration of guar gum to 1.0 and 0.5% resulted in a decline in shoot number, shoot length, wet and dry weights. The promotive effect of Guar gum on *in vitro* shoot proliferation has been reported in *T. bellerica* (Rathore 2004), strawberry (Lucyszyn *et al.* 2006) and tobacco (Lucyszyn *et al.* 2007). Suitability of guar gum on *in vitro* androgenesis in anther cultures of *N. tabacum* has been demonstrated by Babbar *et al.* (2005). In the present study, although the average number of shoots obtained on 2.0% guar gum was equal to and a little less than that obtained on 0.25% and 0.1% Phytigel™ respectively, the average shoot length was higher on guar gum.

The influence of type and quantity of carbon sources (sugars) on morphogenesis has been reviewed extensively (Thorpe and Biondi 1981; Friends *et al.* 1994). In the present case, sucrose, jaggery and glucose differentially stimulated *in vitro* shoot multiplication and growth (Fig. 3). This differential response could probably be due to their differential role in vascular differentiation, differences in the endogenous content of reducing sugar in cultured tissue (Romano *et al.* 1995) and differential sensitivity of the tissues to the breakdown products such as furfural and hydroxyfural formed during autoclaving (Hsiao and Bornman 1989). In the present case highest concentration of sucrose (4.0%) promoted maximum shoot multiplication and growth. At this concentration, the number of shoots was higher than that obtained on control but the shoot length however, was less. Large amounts of available sugars in the culture medium can speed up cell division, increasing the volume and weight of tissues cultured (Gurel and Gulsen 1998). In contrast to this, Rao and Purohit (2008) reported the inhibitory effect of higher concentrations of sucrose on shoot organogenesis in *Celastrus paniculatus*. A decrease in sucrose concentration to 1.5% showed a decrease in all the growth parameters in the present study. Such shoots were stunted and yellow.

Addition of glucose in place of sucrose in the medium could not evoke a better response than the control except at 4.0% (Table 2). At this concentration an average of 10.0 shoots measuring 1.6 cm in height were obtained. A decrease in the concentration of glucose resulted in a decrease in shoot growth and multiplication. Incorporation of glucose at 3.0% promoted shoot multiplication and elongation as equal as observed on medium containing 4.0% sucrose. Glucose proved to be an efficient carbon source for tissue culture of *Quercus suber* (Romano *et al.* 1995), and beech (Cuenca and Vieitez 2000). In the present case jaggery failed to evoke a better response than the control even at its highest concentrations. In contrast to this Rao and Purohit

Table 2 Effect of different carbon sources on *in vitro* shoot multiplication in *N. tabacum* grown on MS medium containing 2.0 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA.

Type of gelling agent	Conc. (%)	Mean № of shoots	Mean shoot length (cm)	Mean № of leaves per shoot	Fresh weight (g) of shoot cluster	Dry weight (g) of shoot cluster
Sucrose	1.5	3.75 e	0.90 c	3.75 de	2.56 e	0.87 d
	3.0 (control)	6.25 c	1.95 a	4.25 cde	11.72 c	2.64 b
	4.0	7.75 b	1.43 ab	5.25 bc	13.67 b	3.12 b
Glucose	1.5	3.00 ef	0.87 c	4.75 bcd	11.28 c	1.96 c
	3.0	7.50 b	1.17 bc	3.25 e	12.43 c	2.68 b
	4.0	10.25 a	1.60 abc	5.00 bc	20.64 a	4.76 a
Jaggery	1.5	2.25 f	1.37 ab	5.25 bc	2.15 e	0.41 d
	3.0	3.00 ef	1.83 ab	7.00 a	2.75 de	0.76 d
	4.0	5.00 d	1.99 a	5.75 b	3.85 d	0.82 d
GM		5.41	1.45	4.91	9.00	2.00
SE		0.34	0.24	0.37	0.40	0.20
CD5%		0.99	0.70	1.09	1.17	0.59
CV		12.69	33.55	15.29	8.96	20.61

Values within column followed by different letters are significantly different at 5%

(2008) reported the promotive effect of jaggery on shoot elongation in tissue culture of *Celastrus paniculatus*. In the present study it was therefore, concluded that shoots of *N. tabacum* multiplied much better on a reduced concentration (0.2%) of agar or a complete lacks thereof. It was also concluded that agar could be replaced by guar gum and sucrose by glucose for efficient shoot multiplication of *N. tabacum*. This would contribute in making the production process cost-effective. A high multiplication rate is one of the important determinants for cost-effective production of tissue culture plants (Vasil 1991) as it reduces the number of sub-cultures required in mass cloning and thus cuts labour cost. A high multiplication rate can also partly compensate for the losses on account of contamination and also during the process of rooting, hardening and acclimatization.

The present study also underlies the importance of cost-effective guar gum as a gelling agent in plant tissue culture using tobacco as an experimental model. Elimination of expensive gelling agents will reduce the cost of tissue culture considerably (Özel *et al.* 2008). Also, guar gum, like agar, is biodegradable and does not pose any threat to the environment when dispensed of after use (Jain and Babbar 2002).

ACKNOWLEDGEMENTS

Laboratory Facilities provided by Prof. D. A. Bhiwgade, Head, Department of Biotechnology and Bioinformatics, Padmashree Dr. D. Y. Patil University, Navi Mumbai, is gratefully acknowledged. The author also thanks Prof. Nitin Desai of the same department for providing references during preparation of the manuscript.

REFERENCES

- Aitken-Christie J, Connett M (1992) Micropropagation of forest trees. In: Kurata K, Kozai T (Eds) *Transplant Production Systems*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 163-194
- Arslan B, Okumus A (2006) Genetic and geographic polymorphism of cultivated tobaccos (*Nicotiana tabacum* L.) in Turkey. *Russian Journal of Genetics* **42**, 667-671
- Brand MH (1993) Influence of agar and ammonium nitrate on tissue nitrogen, culture growth, and vitrification. *Horticulture Science* **28**, 443-591
- Babbar SB, Jain R, Walia N (2005) Guar gum as a gelling agent for plant tissue culture media. *In Vitro Cellular and Developmental Biology – Plant* **41**, 258-261
- Casanova E, Moysset L, Trillas MI (2008) Effect of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. *Biologia Plantarum* **52**, 1-8
- Cuenca B, Vieitez AM (2000) Influence of carbon source on shoot multiplication and adventitious shoot bud regeneration in *in vitro* beech cultures. *Plant Growth Regulation* **32**, 1-12
- Dave A, Bilochi G, Purohit SD (2003) Scaling-up production and field performance of micropropagated medicinal herb 'Safed Musli' (*Chlorophytum borivilianum*). *In Vitro Cellular and Developmental Biology – Plant* **39**, 419-424
- Dhaliwal HS, Yeung EC, Thorpe TA (2004) TIBA inhibition of *in vitro* organogenesis in excised tobacco leaf explants. *In Vitro Cellular and Developmental Biology – Plant* **40**, 235-238
- Evola SV (1983) Chlorate-resistant variants of *Nicotiana tabacum* L. *Molecular and General Genetics* **189**, 447-454
- Friends AL, Coleman MD, Isebrands JG (1994) Carbon allocation to root and shoot system of woody plants. In: Davis TD, Haissing BE (Eds) *Biology of Adventitious Root Formation*, Plenum Press, New York, pp 245-274
- Gangopadhyay G, Das S, Mitra SK, Poddar R, Modak BK, Mukherjee KK (2002) Enhanced rate of multiplication and rooting in *Nicotiana tabacum* through the use of coir in aseptic liquid culture media. *Plant Cell, Tissue and Organ Culture* **68**, 301-310
- Gurel S, Gulsen Y (1998) The effects of different sucrose, agar and pH levels on *in vitro* shoot production of almond (*Amygdalus communis* L.). *Tropical Journal of Botany* **22**, 363-373
- He Y, Sun M, Yang H (2008) Regeneration of fertile plants from isolated tobacco zygotes by *in vitro* culture. *Chinese Science Bulletin* **49**, 810-814
- Hsiao KC, Bornman CH (1989) Cyanide induced oxygen consumption in autoclaved culture medium containing sugars. *Plant Cell Reports* **8**, 90
- Iqbal Z, Lateef M, Jabbar A, Ghayur MN, Gilani AH (2006) *In vitro* and *in vivo* anthelmintic activity of *N. tabacum* L. leaves against gastrointestinal nematode of sheep. *Phytotherapy Research* **20**, 46-48
- Jain N, Babbar SB (2002) Gumkatira – a cheap gelling agent for plant tissue culture media. *Plant Cell, Tissue and Organ Culture* **71**, 223-229
- Joshi N (2005) *In vitro* scaling-up production, molecular characterization and evaluation of 'Safed Musli' (*Chlorophytum borivilianum* Sant et. Fernand). PhD thesis, Mohanlal Sukhadia University, Udaipur, India, 85 pp
- Klems M, Ball J, Machackowa I, Eder J, Prochazka S (2000) The uptake and metabolism of 3H-benzylaminopurine in tobacco (*Nicotiana tabacum* L.) and cucumber (*Cucumis sativum* L.) explants. *Plant Growth Regulation* **31**, 135-142
- Klimaszewska K, Bernier-Cardou M, Cyr D, 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 and Developmental Biology – Plant* **36**, 279-289
- Kumar N (2006) Medicinal plants: Future source of pesticides. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences* **8**, 57-59
- Kumar GB, Srinivas L, Ganapathi TR, Bapat VA (2006) Hepatitis B surface antigen expression in transgenic tobacco (*N. tabacum*) plants using four different expression cassettes. *Plant Cell, Tissue and Organ Culture* **84**, 315-323
- Lal C, Verma LR (2006) Use of certain bioproducts for insect-pest control. *Indian Journal of Traditional Knowledge* **5**, 79-82
- Laviolette SR, van der Kooy D (2004) The neurobiology of nicotine addiction: bridging the gap between molecules to behaviour. *National Review of Neurosciences* **5**, 55-56
- Leuba V, Tourneau DL (1990) Auxin activity of phenylacetic acid in tissue culture. *Journal of Plant Growth Regulation* **9**, 71-76
- Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. *Physiologia Plantarum* **18**, 100-127
- Lucyszyn M, Quorin M, Homma MM, Sierakowski MR (2007) Agar/galactomannan gels applied to shoot regeneration from tobacco leaves. *Biologia Plantarum* **51**, 173-176
- Lucyszyn N, Quorin M, Kochler HS, Reicher F, Sierakowski MR (2006) Agar/galactomannan blends for strawberry (*Fragaria x ananassa* Duchesne) cv. Pelican micropropagation. *Scientia Horticulturae* **107**, 358-364
- Jr Meins F, Lutz J, Foster R (1980) Factors influencing the incidence of habituation for cytokinin of tobacco pith tissue in culture. *Planta* **150**, 264-268
- Mangat BS, Janjua S (1987) Cyclic nucleotides and *in vitro* plant cultures: I. Induction of organogenesis in tobacco (*Nicotiana tabacum*) callus cultures. *Journal of Experimental Botany* **38**, 2059-2067
- Misra P (2002) Direct differentiation of shoot buds from leaf explants of *Cajanus cajan* L. *Biologia Plantarum* **45**, 347-351
- Nhut DT, Aswath CR, Teixeira da Silva JA, Le BV, Thorpe T, Tran Thanh

- Van K** (2003) Tobacco thin cell layer morphogenesis. In: Nhut DT, Tran Thanh Van K, Le BV, Thorpe T (Eds) *Thin Cell Layer Culture System: Regeneration and Transformation Applications*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 17-64
- Nugroho LH, Verpoorte R** (2002) Secondary metabolism in tobacco. *Plant Cell Tissue and Organ Culture* **68**, 105-125
- Ozel CA, Khawar KM, Arslan O** (2008) A comparison of the gelling of isubgol, agar and gelrite on *in vitro* shoot regeneration and rooting of variety Samsun of tobacco *Nicotiana tabacum* L.). *Scientia Horticulturae* **117**, 174-181
- Parc G, Rembur J, Rech P, Chriqui D** (2007) *In vitro* culture of tobacco callus on medium containing peptone and phytate leads to growth improvement and higher genetic stability. *Plant Cell Reports* **26**, 145-152
- Ramage CM, Williams RR** (2002) Inorganic nitrogen requirements during shoot organogenesis in tobacco leaf discs. *Journal of Experimental Botany* **53**, 1437-1443
- Rao MS, Purohit SD** (2008) *In vitro* regeneration in *Celastrus paniculatus* Willd. Influence of explant, gelling agent and carbon source. *Proceedings of the National Academy of Sciences India* **74**, 161-166
- Rathore P** (2004) Further studies on micropropagation of some medicinal plants of *Aravallis* of Rajasthan. PhD thesis, M.L. Sukhadia University, Udaipur, India, 103 pp
- Romano A, Noronha C, Martins-Loução MA** (1995) Role of carbohydrates in micropropagation of cork oak. *Plant Cell, Tissue and Organ Culture* **40**, 159-167
- Salvi ND, George L, Eapen S** (2002) Micropropagation and field evaluation of micropropagated plants of turmeric. *Plant Cell, Tissue and Organ Culture* **68**, 143-151
- Shrivastava N, Rajani M** (1999) Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. *Plant Cell Reports* **18**, 919-923
- Srinivasan C, Liu Z, Heidmann I, Supena EDJ, Fukuoka H, Joosen R, Lambalk J, Angenent G, Scorza R, Custers JBM, Boutilier K** (2007) Heterologous expression of the *BABY BOOM* APZ/ERF transcription factor enhances the regeneration capacity of tobacco (*Nicotiana tabacum* L.). *Planta* **225**, 341-351
- Teixeira da Silva JA** (2005) Simple multiplication and effective genetic transformation (4 methods) of *in vitro*-grown tobacco by stem thin cell layers. *Plant Science* **169**, 1046-1058
- Teixeira da Silva JA** (2003) Filter paper significantly affects the morphogenic programmes, and buffers the phytotoxic effect of antibiotics in chrysanthemum and tobacco thin cell layer *in vitro* culture. *HortScience* **38**, 1403-1407
- Teixeira da Silva JA** (2002) Polyamines in the regulation of chrysanthemum and tobacco *in vitro* morphogenic pathways. *Propagation of Ornamental Plants* **2**, 9-15
- Teixeira da Silva JA, Fukai S** (2003) Effect of aminoglycoside antibiotics on *in vitro* morphogenesis from cultured cells of chrysanthemum and tobacco. *Journal of Plant Biology* **46**, 71-82
- Teixeira da Silva JA, Fukai S** (2001) The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. *Journal of Applied Horticulture* **3**, 3-12
- Thorpe TA, Biondi S** (1981) Regulation of plant organogenesis. *Advances in Cell Culture* **1**, 213-239
- Toldi O, Ahanen K, Kovacs G, Sorvari S, Toth S, Dulai S, Scott P** (2005) Integrated applications of physiological and molecular methods to forecast determinative morphogenetic events in tissue cultured tobacco (*Nicotiana tabacum* L. cv Samsun) leaf discs. *Plant Growth Regulation* **47**, 59-64
- Tran Thanh Van M, Thi Dien N, Chlyah A** (1974) Regulation of organogenesis in small explants of superficial tissue of *Nicotiana tabacum* L. *Planta* **119**, 149-159
- Vyas S, Rao MS, Suthar R, Purohit SD** (2008) Liquid culture stimulates *in vitro* growth and multiplication in four medicinally important plants. *Medicinal and Aromatic Plant Science and Biotechnology* **2**, 96-100
- Vasil IK** (1991) Rationale for the scale-up and automation of plant propagation. In: Vasil IK (Ed) *Cell Culture and Somatic Cell Genetics of Plants (Vol 8) Scale-up and Automation in Plant Propagation*, Academic Press, Orlando, pp 1-6
- Wawrosch C, Maskay N, Kopp B** (1999) Micropropagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch. Ham Ex Wall. *Plant Cell Reports* **18**, 997-1001
- Williams RR** (1992) Towards a model of mineral nutrition *in vitro*. In: Kurata K, Kozai T (Eds) *Transplant Production Systems*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 213-230
- Zhang B, Yang Y, Lin Y, Rao Q, Zheng G, Wu K** (2003) Expression and production of bioactive human interleukin-18 in transgenic tobacco plants. *Biotechnology Letters* **25**, 1629-1635
- Zhang GG, Rodrigues L, Rouinski B, White KA** (2002) Production of HIV-1-p24 protein in transgenic tobacco plants. *Molecular Biotechnology* **20**, 131-136