SCIENCE AND TECHNOLOGY - Research Journal - Volume 3 - 1999
University of Mauritius, Réduit, Mauritius.

EFFECTS OF MEDIUM SUPPORT AND GELLING AGENT IN THE TISSUE CULTURE OF TOBACCO (*Nicotiana tabacum)*

by

D. PUCHOOA[*], P. N. PURSERAMEN *and* B. R. RUJBALLY

Department of Agriculture and Food Science, Faculty of Agriculture,

University of Mauritius, Réduit, Mauritius

(Received December 1998 - Accepted March 1999)

ABSTRACT

This study was designed to examine the effects of different media supports, brands of agar and gelling agents in the tissue culture of *Nicotiana tabacum*. There were significant differences ($P<0.05$) in terms of fresh weight, dry weight and number of shoots produced between the supports used. Best response was obtained with liquid agitated medium. However, there were no significant differences ($P<0.05$) between the gelling agents in terms of fresh weight, dry weight and number of shoots produced. Investigation in the response of two different cultivars of *Nicotiana tabacum*, *viz* Amarello and Virginia NC 95, to different support media, revealed significant differences ($P<0.05$), especially in their regeneration capability in liquid agitated medium. The gelling agents were also analyzed for their chemical properties. Large differences were observed between the gelling agents. The non-agar gel, gelrite, one of the best gelling agent, had a high content of ash, copper, iron, magnesium and zinc compared to the others.

Keywords : Media supports, gelling agents, agar brands, tissue culture, chemical analysis.

**For corrrespondence and reprints*

INTRODUCTION

Agar is the most fequently used solidifier in plant tissue culture media. It has the desirable characteristics of high gel clarity, stability and resistance to digestion by plant enzymes during use. Agar was also thought to be biologically inert but a number of reports on its adverse effects have been published (Romberger & Tabor, 1971; Debergh *et al*. 1981; Debergh, 1983), including batch-to-batch variability, inhibition of growth, presence of impurities and impartment and impairment of vitrification. The major criteria used in selecting the brand and the concentration of agar seem to be availability and price. Agar can be the most expensive component of plant tissue culture media. The cost of commercial micropropagation can be drastically reduced if cheap alternatives to the highly expensive purified agar could be used. A number of such substitutes have been tried including, methylcellulose and alginate (Adaoha Mbanaso & Roscoe, 1982), starches from barley, corn, potato, rice and wheat, gellan gum and potato starch (Bornman & Vogelmann, 1984; Calleberg *et al*. 1989), microcrystal cellulose (Gorinova *et al*. 1993), isubgol (Babbar, 1998), gelatin, pectin and a number of other support systems such as agitated liquid medium, filter paper, cotton wool, polyester fleece and glass beads. Differences in the performance of agars and gelled media have been attributed to limited diffusion of medium components and water (Romberger & Tabor, 1971; Stolz, 1971), impurities (Nairn *et al*. 1995) and to differences in gel strength (Debergh, 1983).

The use of liquid medium in tissue culture is often described as a means of reducing the cost of micropropagation (Alvard *et al*. 1993). The advantages include increased availability of water and dissolved substances to the explants and lower labour and production costs. Shoot production of *Rhododendron*, was ten-fold higher in liquid medium than on agar-solidified medium (Douglas, 1984) and with *Pinus caribaea* Skidmore *et al*. (1988) found that shoot incubated in liquid medium were longer than those cultured on solid medium. Although many positive results have been found with liquid cultures, hyperhydricity, a physiological disorder of tissue-cultured plants in which the organs or tissues, particularly leaves, have waterlogged, translucent and 'glassy' appearance, has been reported for many crops cultured directly in liquid media (Debergh *et al*. 1981; Ziv *et al*. 1983). A number of techniques to support plants over stationary liquid, and thus reduce vitrification, have been explored (Debergh & Maene, 1981; Conner & Meredith, 1984; Barve *et al*. 1986; Roberts & Smith, 1990) including use of rockwool, poly-urethane and cellulose plugs. Recently, the use of a two-phase (a liquid phase on a solid phase) medium has led to a high multiplication ratio combined with lower sensitivity to vitrification (Chu *et al*. 1993). However, higher labour requirements in the establishment and subculturing of both liquid cultures with support and the two-phase culture system have a direct effect on the price of producing plantlets.

There are many commercial grades of gelling agents. The importance of using pure grade gelling agents in experimental tissue culture work is widely acknowledged, however, their use in commercial micropropagation is not necessary. Less pure grade gelling agents may contain various ions, sulphonated polysaccharides and long fatty acids but their effect on plant growth *in vitro* is unknown (George & Sherrington, 1984). There are a number of published reports on the chemical and physical characteristics of agars and gelled media (Debergh, 1983; Scholten & Pierik, 1998) but what makes an optimum agar for plant *in vitro* culture work, is not known.
This report presents the chemical analyses of five commercially available gelling agents and discusses their performance in the tissue culture of *Nicotiana tabacum*. A comparison between two cultivars of *Nicotiana tabacum* to different types of support is also presented.

MATERIALS AND METHODS

Plant material and medium

Two cultivars of *Nicotiana tabacum viz*. Amarello and Virginia NC 95 were used as source of explants. The plants were grown under field conditions at Richelieu Experimental Station. Amarello was grown from May to September while Virginia NC 95 from September to December. All plants were fertilized monthly with, either 225 Kg/arp for Virginia NC 95 or 300 Kg/arp for Amarello, complete fertilizer 6:18:24. Disease and pest control for the two cultivars are shown in Table 1.

Young unfolded leaves from plants in the pre-flowering stage were used as explant materials. They were washed in soapy water followed by rinsing under tap water. As the leaves were too large, they were cut into $5cm^2$ sections and soaked, with stirring, in 0.6 gL^{-1} Benlate solution for 15min. After rinsing under running tap water, the leaf sections were sterilized by soaking in a solution containing 0.5% sodium hypochlorite and 2 drops of Tween 20 (polyoxyethylene sorbitan monolaurate)/100ml. After 20 min of gentle agitation, the leaves were rinsed four times in sterile distilled water (10 min in each rinse), cut into $1cm^2$ sections, avoiding large veins and borders, and inoculated into the culture medium, adaxially in the case of gelled media. Murashige & Skoog's (1962) culture medium was used throughout.

Support matrix

The different types of matrix used for support were: (i) gelled medium, (ii) static liquid medium, (iii) agitated liquid medium, and (iv) static liquid medium with beads and filter paper as support. The latter was prepared by placing 10 glass beads (15mm diameter) per 250ml conical flask and layered with a filter paper. The beads were soaked overnight in bleach and washed with distilled water before use.

Gelling agents

The gelling agents used in the experiments were:

(i) Gelrite gellan gum (Phytagel, Sigma), a fermentation derived, non-agarophytic heteropolysaccharide, which gels at lower concentrations than agarophytic agar,

(ii) Agar A (Extra Pure, Hi-Media),

(iii) Agar B (imported from Japan and distributed by Li Wan Po & Co. Ltd.),

(iv) Agar C (imported from E.C and distributed by Ip Min Wah & Co. Ltd.) and

(v) Foodgrade cornstarch (Mammy).

The last three gelling agents are available at most local supermarkets

Table 1. Disease and pest control for *Nicotiana tabacum*.

Pest / Disease	Symptoms	Control
Tobacco budworm (*Heliothis spp*)	Small larvae feed in the vegetative bud causing a ragged appearance as the leaves develop	1.2 gL^{-1} Lannate 90 / 0.5gL^{-1} Decis (in alternate)
Leaf spots	Irregular, angular and dark coloured lesions	2.0 gL^{-1} Dithane / 0.5 gL^{-1} Benlate (in alternate)

Preparation of culture media

For solid media, Difco Bacto-agar at 0.8% w/v was used as a control in all experiments. Due to unavailability of a penetrometer to determine gel strength (firmness), solidity was assessed by touching. A concentration of 8.0 gL^{-1} was found to be necessary for Agar A, B and C. For Gelrite, 2.5 gL^{-1} was used as

recommended by the manufacturers and for cornstarch (Mammy), 90 gL^{-1} was found to be necessary to give a firm gel. The cornstarch was first made into a slurry and added to the media before adjusting the volume and pH which was 5.8 for both liquid and gelled medium. All media were autoclaved at 121^0C for 15 min and cooled at room temperature. The only plant growth regulator added to either the liquid or gelled media was N^6 - benzyladenine (BA, 5µM). For the experiment to compare the response of two cultivars of *N. tabacum* to the support matrix, 250ml conical flasks with 80ml of medium per flask were used while for the experiment to investigate the response of *N. tabacum* to different gelling agents, 250mL baby food jars with 50mL of medium per jar were used.
Gelled cultures, static liquid cultures and static liquid cultures with beads and filter paper as support were maintained in a growth room while agitated liquid cultures were placed in an incubator shaker (Model G25, New Brunswick Scientific Co.). In all cases, artificial light was provided by parallel fluorescent tubes installed above the cultures. The luminous intensity was 1000lux (The equivalent of 15.7 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetically active radiation) and all cultures were subjected to a 16 / 8h light / dark photoperiod. The ambient temperature was 24 ± 2^0C. The speed of the shaker was maintained at 90 ± 10rpm. Humidity was not controlled.

Chemical analysis

Concentrations of elements were determined by atomic absorption spectrometry (Unicam 929) or flame photometer (Corning 410). Nitrogen was determined by the Macro-Kjeldahl technique using an automated nitrogen determination system (Gerhardt Kjeldatherm). Concentrations of elements in dry agar were determined in duplicate.

Experimental design

For the comparison of the response of the two cultivars of *N. tabacum* to the matrix support, a completely randomized design with 4 x 2 factorial distribution, that is, four treatments (Bacto-agar gelled medium, gelrite gelled medium, liquid agitated medium and static liquid medium with glass beads as support) and two cultivars, was used. For the investigation of the response of *N. tabacum* (Amarello) to different gelling agents, a completely randomized design was again used but with six treatments. All treatments consisted of 10 replicates and each replicate contained two explants. The fresh weight, dry weight and number of shoots (> 0.8cm) were recorded after 32d in culture. The mean was calculated. Significant differences between treatment means were determined using either the F-test or the t-test.

RESULTS

Effect of different gelling agents

Results (Table 2) show that there were no significant differences ($P<0.05$) between the gelling agents in terms of fresh weight, dry weight and the number of shoots produced after 32d in culture. Best results were obtained when using gelrite as the gelling agent. Although the lowest number of shoots were obtained when using cornstarch, leaf development was more pronounced than the other five gelling agents.

Medium matrix and cultivars

Apart for the cultivar Virginia NC95 in static liquid medium, an increase in weight was noted in all treatments after 32d in culture. For both cultivars, lowest fresh weight, dry weight and mean number of shoots were obtained in liquid static medium while the highest fresh weight and dry weight were obtained in agitated liquid medium. Liquid agitated medium also gave the highest number of shoots but for the cultivar Amarello only. There were significant differences in terms of fresh weight, dry weight and number of shoots produced after 32d in culture (1) between solid and liquid media, (2) between the treatments, (3) between the cultivars and (4) between different methods of culture (treatments) and cultivars at 5% level of significance (Tables 3, 4 and 5).

Table 2. The effects of different gelling agents on fresh weight, dry weight and number of shoots of 'Amarello' tobacco produced after 32d in culture

Gelling agent	Amount (gL^{-1})	Fresh wt (g)	Dry wt (g)	No of shoots
Bacto-agar	8.0	4.75^{a}	0.36^{b}	3.21^{c}
Gelrite	2.5	4.98^{a}	0.38^{b}	3.41^{c}
Agar A	8.0	3.48^{a}	0.26^{b}	2.99^{c}
Agar B	8.0	4.14^{a}	0.31^{b}	3.01^{c}
Agar C	8.0	2.38^{a}	0.17^{b}	2.82^{c}
Cornstarch	90	2.77^{a}	0.20^{b}	1.80^{c}

For a particular parameter, means followed by the same letter do not differ significantly at 5% level of significance (F-test).

Table 3. Mean fresh weight (g) of the two cultivars of tobacco leaf explants after 32 d in culture

Treatments	Amarello	Virginia NC 95
Difco Bacto-agar solidified medium	13.07[d]	12.51[d]
Gelrite solididified medium	12.30[d]	12.48[d]
Liquid static medium	2.37[b]	0.22[a]
Liquid medium with beads and filter paper support	3.62[c]	4.33[c]
Agitated liquid medium	15.72[e]	15.46[e]

Means followed by the same letter are not significantly different at 5% level of significance with a t-test. Fisher's Least Significant Difference (LSD) = 0.975

Table 4. Mean dry weight (g) of the two cultivars of tobacco leaf explants after 32 d in culture

Treatments	Amarello	Virginia NC 95
Difco Bacto-agar solidified medium	1.13[ef]	0.98[d]
Gelrite solidified medium	1.06[de]	0.98[d]
Liquid static medium	0.20[b]	0.02[a]
Liquid medium with beads and filter paper as support	0.40[c]	0.38[c]
Agitated liquid medium	1.25[fg]	1.18[fg]

Means followed by the same letter are not significantly different at 5% level of significance with a t-test. Fisher's Least Significant Difference (LSD) = 0.146.

Table 5. Mean number of shoots
for the two tobacco cultivars after 32d in culture

Treatments	Amarello	Virginia NC 95
Difco Bacto-agar solidified medium	68.0[e]	53.8[d]
Gelrite solidified medium	67.1[e]	52.6[d]
Liquid static medium	1.0[b]	0.0[a]
Liquid medium with beads and filter paper as support	9.6[c]	9.9[c]
Agitated liquid medium	82.5[f]	0.0[a]

Means followed by the same letter are not significantly different at 5% level of significance with a t-test. Fisher's Least Significant Difference (LSD) = 4.62.

When either Difco Bacto agar or gelrite-solidified media were used, for both cultivars, nodules appeared along the cut edges of the explants. These nodules formed shoots and eventually plantlets. In cases where static liquid medium or liquid medium with beads and filter paper were used, very little shoot formation occurred, especially in Virginia NC 95, which became necrotic after a few days in culture. Here again shoot formation arose mainly along the cut edges. On the other hand, using agitated liquid medium, the cultivar Amarello showed extensive shoot proliferation, both along cut edges and on the entire surface of the explants. However, the same treatment for cultivar Virginia NC 95 did not produce any shoots after 32d in culture. Instead, extensive callusing was observed. Some shoots formed in liquid media showed signs of hyperhydricity. (The differences in fresh weight, dry weight and number of shoots produced for the cultivar Amarello using the same gelling agents in the two experiments are due to using different containers and different amounts of medium).

Chemical analysis

The concentration of elements in the different gelling agents are given in Table 6.

Gelrite had a high ash, nitrogen, copper, iron, magnesium, zinc and calcium content compared to the other gelling agents. Cornstarch on the other hand had a low content of most elements analysed. Apart from the high ash, iron and calcium contents of Agar C, there were striking similarities in the concentrations of elements in Difco Bacto agar, Agar A, B and C.

Cost analysis of the gelling agents

The cost analysis of the different gelling agents (Table 7) showed gelrite to be the most expensive and cornstarch the cheapest.

Table 6. Concentration of elements in the gelling agents

	Gelling Agent					
Component	Difco Bacto agar	Gelrite	Agar A	Agar B	Agar C	Cornstarch
Ash (%)	2.20	7.09	2.95	1.30	7.58	0.16
Nitrogen (%)	0.02	0.07	0.02	0.04	0.03	0.09
Magnesium (%)	0.24	0.40	0.02	0.06	0.02	0.02
Calcium (%)	0.45	0.89	0.07	0.23	0.92	nd
Copper (ppm)	2.00	6.81	3.27	3.09	2.13	1.95
Iron (ppm)	2.87	8.63	2.90	1.94	5.07	1.26
Zinc (ppm)	4.60	9.18	4.35	3.60	1.24	0.13

[nd] not detected

Table 7. Cost analysis of the gelling agents

Gelling Agent	Price / kg (Rs)	Cost / L medium (Rs)
Difco Bacto agar	3,000	24.00
Gelrite	13,000	32.50
Agar A	2,200	17.60
Agar B	810	6.48
Agar C	432	3.46
Cornstarch	16.25	1.46

DISCUSSION

The present study indicated that plantlet production from leaf explants of tobacco *in vitro* can be influenced by the support medium. Differences in the response of different cultivars of tobacco to the support medium were also noted. The fresh weight, dry weight and the number of shoots produced for the cultivar Amarello after 32d in culture was higher in liquid agitated medium than on solidified medium. For the same treatment, the cultivar Virginia NC 95 did not produce any shoots.

There were major differences in the response of *N. tabacum* leaf explants when they were subjected to different types of liquid cultures. Explants of both cultivars produced almost no shoots in static liquid culture. This was most likely due to asphyxiation of the explants as a result of their complete immersion in the liquid medium. The use of a support to the explants affords a better aeration. However, there were significant differences in terms of fresh weight, dry weight and the number of shoots produced between explants inoculated on a medium solidified with a gelling agent and using beads / filter paper as support. However, Macleod & Nowak (1990) found no differences in the regeneration capability of white clover using either agar solidified medium or small solid glass beads as matrix. According to the same authors, a 60% saving on media components can thus be made by substituting agar with beads. In our work, further investigations need to be carried out to see whether different results are obtained by culturing tobacco leaf explants directly on the beads, without the filter paper.

Explants inoculated in liquid medium with continuous agitation at 90rpm gave the best response, for both cultivars, in all parameters tested. This result is likely due to the agitation promoting gaseous exchange and at the same time eliminating gravitational effects and the formation of nutrients and growth regulators gradients in the medium. Bhagyalakshmi & Singh (1995) reported similar findings in the *in vitro* culture of banana. Flow in an agitated flask is generally divided into two regions - a 'stagnant' region where flow speed is relatively low and a 'bulk flow' region (Kurata & Mita, 1996).
Growth and organ differentiation were dependent on the medium support and differed between cultivars. The cultivar Amarello produced shoots in both agitated liquid medium and solid medium but with significant differences. Virginia NC 95, on the other hand, produced shoots on solid medium but massive callus on agitated liquid medium. This indicates that the response of growth performance and organ differentiation *in vitro* is highly dependent on the genotype. Our findings are in agreement with that of Budimir & Vujieie (1992). Shoot regeneration on solid medium, for both cultivars, however, occurred *via* nodule formation, a phase where tissues become competent (Attfield & Evans, 1991). Root formation, in parallel or just after shoot regeneration indicated that exogenous plant growth regulators are not required.

Hyperhydricity, a serious physiological disorder common to liquid cultures and characterized by thick, elongated, brittle and translucent leaves, was observed in a number of liquid cultures where regeneration occurred. This has been reported by various workers (Debergh *et al*. 1992; Sulluman & Maziere, 1992; Roche, 1996). A number of factors have been listed as being responsible for hyperhydricity such as, the level of cytokinin, low light, high temperature, type of culture vessels, length and number of subcultures and damage during disinfestation. However, many of these factors will only act to induce or evoke hyperhydricity when other conditions in the culture system are not optimized. For example, Debergh (1983) found that benzyladenine induced hyperhydricity when cultures were stressed by high water retention capacity in the head space of the container. Hyperhydricity can be overcome by a number of ways including use of solidified media with a higher concentration of gelling agent or a gelling agent with a higher gel strength (Von Arnold & Eriksson, 1984; Debergh *et al*. 1981; Ziv *et al*. 1983), lowering the cytokinin concentration (Leshem *et al*. 1988; Paques & Boxus, 1987), avoiding the use of benzyladenine, increasing Ca^{2+} and / or decreasing NH_4^+ (Ziv *et al*. 1987). Other techniques including use of pectin or hydrolyzed agar, phloridzin or phloroglucinol, $CoCl_2$, Ni^{2+}, fructose or galactose as a carbon source, methionine, silver nitrate and growth retardants have been investigated (Debergh *et al*. 1992).

Considerable differences may exist in the performance of batches of the same brand of gelling agents. For this study, a large batch of all gelling agents were purchased and freshly prepared gels were used for all the experiments. The performance of the gelling agents in bioassays showed gelrite to be the best. Agar A and B were comparable to Difco Bacto agar, used as a control. Agar C was of bad quality while cornstarch can be classified as moderate. Possible explanations for the differences between gelling agents include limited diffusion of nutrients (Romberger & Tabor, 1971), lateral diffusion of water (Stolz, 1971) and impurities (Nairn *et al*. 1995).

Gel strength is often regarded as an important criterion for agar quality (Debergh, 1983). Media gelled with 2.5 gL^{-1} gelrite, 8.0 gL^{-1} agar A, B or C appeared to have the same gel firmness as media gelled with 8.0 gL^{-1} Difco Bacto agar. A potential difficulty noted with cornstarch was the softness of the medium. In our study, 90 gL^{-1} of cornstarch was found to be necessary to give a firm gel. At this level, there was no problem with solidification of the media. Further investigations need to be carried out in the use of acid-modified starch which seems to produce better gels (Henderson & Kinnersley, 1988).

Gelling agent is a major source of unknown variation and a rich source of impurities. The significance of the contribution of contaminants of a gelling agent to the final concentration in a medium depends on the elements. It is generally accepted that inorganic compounds in gelling agents and the dynamics of the interaction gelling agent-medium-tissue play a major role during tissue growth *in vitro* (Scholten & Pierik, 1998). The chemical analysis of the gelling agents showed that gelrite had a high content of copper, iron, magnesium, zinc and calcium compared to the other five gelling agents, with cornstarch having the lowest content of most of the elements analyzed. However, to be able to explain fully the performances of the gelling agents used in our experiments, further chemical and physical analyses need to be carried out. These would include determining the concentration of other elements including biologically active organic compounds, the electrical conductivity, the water content, the gel strength, the diffusion rates of water and salts and pH, amongst others.

ACKNOWLEDGEMENTS

The authors wish to thank the Faculty of Agriculture and the technical staff at the Faculty of Agriculture, University of Mauritius, for their support and help during this work.

REFERENCES

ADAOHA MBANASO, E.N.A. & ROSCOE, D.H. (1982). Alginate: an alternative to agar in plant protoplast culture. *Plant Science Letters* **25**, 61-66.

ALVARD, D., COTE, F. & TIESSON, C. (1993). Comparison of methods of liquid medium culture for banana micropropagation. *Plant Cell Tissue Organ Culture* **32**, 55-60.

ATTFIELD, E.M. & EVANS, P.K. (1991). Development pattern of root and shoot organogenesis in cultured leaf explants of *Nicotiana tabacum* cv. Xanthi. *Journal of Experimental Botany* **42** (234), 51-57.

BABBAR, S.B. (1998). Isubgol - an inexpensive gelling agent for plant tissue culture. *Agricell Report* **30** (4), 25-26.

BARVE, D.M., IYER, R.S., KENDURKAR, S. & MASCARENHAS, A.F. (1986). An effective method for rapid propagation of some budded rose varieties. *Indian Journal of Horticulture* **41**, 1-7.

BHAGYALAKSHMI, L. & SINGH, N.S. (1995). Role of liquid versus agar-gelled media in mass propagation and *ex-vitro* survival in bananas. *Plant Cell Tissue Organ Culture* **41**, 71-73.

BORNMAN, C.H. & VOGELMANN, T.C. (1984). Effect of rigidity of gel medium on benzyladenine-induced adventitious bud formation and vitrification *in vitro* in *Picea abies*. *Physiologia Plantarum* **61**, 505-512.

BUDIMIR, S. & VUJIEIE, R. (1992). Benzyladenine induction of buds and somatic embryogenesis in *Picea omorika* Purk. *Plant Cell Tissue Organ Culture* **31**, 89-94.

CALLEBERG, E.K., KRISTJANSDOTTIR, I.S. & JOHANSSON, L.B. (1989). Anther cultures of tetraploid solanum genotypes: the influence of gelling agents and correlations between incubation temperature. *Plant Cell Tissue Organ Culture* **19**, 189-197.

CHU, C.Y., KNIGHT, S.L. & SMITH, M.A.L. (1993). Effect of liquid culture on the growth and development of miniature rose (*Rosa chinensis* Jacq. ‘Minima’). *Plant Cell Tissue Organ Culture* **32**, 329-334.

CONNER, A.J. & MEREDITH, C.P. (1984). An improved polyurethane support system for monitoring growth in plant cell cultures. *Plant Cell Tissue Organ Culture* **3**, 59-68.

DEBERGH, P.C. & MAENE, L.J. (1981). A scheme for commercial propagation of ornamental plants by tissue culture. *Scientia Horticulturae* **14**, 335-345.

DEBERGH, P., HARBAOUI, Y. & LEMEUR, R. (1981). Mass propagation of globe artichoke (*Cynara scolymus*) : evaluation of different hypotheses to overcome vitrification with special reference to water potential. *Physiologia. Plantarum* **53**, 181-187.

DEBERGH, P. (1983). Effects of agar brand and concentration on the tissue culture medium. *Physiologia Plantarum* **59**, 270-276.

DEBERGH, P., AITKEN-CHRISTIE, J., COHEN, D., ARNOLD, V., ZIMMERMAN, R. & ZIV, M. (1992). Reconsideration of the term "vitrification" as used in micropropagation. *Plant Cell Tissue Organ Culture* **30**, 135-140.

DOUGLAS, G.C. (1984). Propagation of eight cultivars of Rhododendron *in vitro* using agar-solidified and liquid media and direct rooting of shoots *in vitro*. *Scientia Horticulturae **24**, 337-347.*

GEORGE, E.F. & SHERRINGTON, P.D. (1984). In *Plant Propagation by Tissue Culture*, pp 236-237. Eastern Press, Reading, Berks, UK.

GORINOVA, N., ATANASOV, A., ALEXANDROVA, K., VELKOVA, R. & KASACHKA, A. (1993). Comparison of agar and microcrystal cellulose as gelling agent for *in vitro* culture of *Nicotiana tabacum* stem explants. *Biologia Plantarum* **35** (2), 217-221.

HENDERSON, W.E. & KINNERSLEY, A.M. (1988). Corn starch as an alternative gelling agent for plant tissue culture. *Plant Cell Tissue Organ Culture* **15**, 17-22.

KURATA, K. & MITA, H. (1996). Qualitative analysis of liquid movement within shake culture flasks using a flow visualisation technique. *Acta Horticulturae* **440**, 545-548.

LESHEM, B., SHALEV, D.P. & IZHAR, S. (1988). Cytokinin as an inducer of vitrification in melon. *Annals of Botany* **61**, 255-260.

MACLEOD, K. & NOWAK, J. (1990). Glass beads as a solid matrix in *in vitro* study of the role of polyamines in cold hardiness of white clover. *Plant Cell Tissue Organ Culture* **22**, 113-117.

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

NAIRN, B.J., FURNEAUX, R.H. & STEVENSON, T.T. (1995). Identification of an agar constituent responsible for hydric control in micropropagation of radiata pine. *Plant Cell Tissue Organ Culture* **43**, 1-11.

PAQUES, M. & BOXUS, P. (1987). Vitrification: review of literature. *Acta Horticulturae* **212**, 155-166.

ROBERTS, A.V. & SMITH, E.F. (1990). The preparation *in vitro* of chrysanthemum for transplantation to soil - protection of roots by cellulose plugs. *Plant Cell Tissue Organ Culture* **21**, 129-132.

ROCHE, T.D. (1996). Gaseous and media related factors influencing *in vitro* morphogenesis of Jerusalem artichoke (*Helianthus tuberous*) 'nahodka' node cultures. *Acta Horticulturae* **440**, 588-592.

ROMBERGER, J.A. & TABOR, C.A. (1971). The *Picea abies* shoot meristem in culture. I: agar and autoclaving effects. *American Journal of Botany* **58**, 131-140.

SCHOLTEN, H.J. & PIERIK, R.L.M. (1998). Agar as a gelling agent: chemical and physical analysis. *Plant Cell Reports* **17**, 230-235.

SKIDMORE, D.I., SIMONS, A.J. & BEDI, S. (1988). *In vitro* culture of *Pinus caribaena* on a liquid medium. *Plant Cell Tissue Organ Culture* **14**, 129-136.

STOLZ, L.P. (1971). Agar restriction of the growth of excised mature Iris embryos. *Journal of the American Society of Horticultural Science* **96**, 618-684.

SULLUMAN, H. & MAZIERE, M. (1992). Influence of growth room and vessel humidity on the *in vitro* development of rose plants. *Plant Cell Tissue Organ Culture* **30**, 121-125.

VON ARNOLD, S.V. & ERIKSSON, T. (1984). Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies*. *Plant Cell Tissue Organ Culture* **3**, 257-264.

ZIV, M., MEIR, G. & HALEVY, A. (1983). Factors influencing the production of hardened carnation plantlets *in vitro*. *Plant Cell Tissue Organ Culture* **2**, 55-60.

ZIV, M., SCHWARTS, A. & FLEMINGER, D. (1987). Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implications for hardening. *Plant Science* **52**, 127-134.