Pergamon

PII: S0031-9422(97)00286-107

Phytochemistry, Vol. 46, No. 2, pp. 273–278, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

DIGITALIS OBSCURA CARDENOLIDES*. EFFECT OF MACRONUTRIENT CONCENTRATION AND N SOURCE ON GROWTH AND PRODUCTIVITY OF SHOOT-TIP CULTURES

ISABEL GAVIDIA and PEDRO PÉREZ-BERMÚDEZ†

Departamento de Biología Vegetal, Facultad de Farmacia, Universidad de Valencia, Av. V. A. Estellés s/n, 46100 Burjassot (Valencia), Spain

(*Received* 27 *January* 1997)

Key Word Index—*Digitalis obscura*; Escrofulariaceae; biosynthesis; macronutrients; shoot-tip culture; rooting; cardenolides.

Abstract—Changes in nutrient medium formulation in terms of major salt concentration or N source significantly modified cardenolide production by *Digitalis obscura* (genotype T4) shoot-tip cultures. A positive correlation between morphogenetic responses, culture growth, and proliferative capacity on one side and metabolite biosynthesis on the other cannot be established. The final stages of micropropagation, rooting, and acclimatization of *D. obscura* were improved by using paclobutrazol. © 1997 Elsevier Science Ltd

INTRODUCTION

Information concerning the factors regulating secondary metabolism is as important as the selection of high-producing lines in increasing the production of secondary metabolites [1]. In this respect, we have recently determined the influence of different media components on the establishment, maintenance, and cardenolide production by stable *Digitalis obscura* (genotype T4) shoot-tip cultures [2–4].

In this paper, we present additional information for this *in vitro* system with regard to the influence of changes in the major salt strength and the N source of the nutrient media on both morphogenesis and productivity. The beneficial effect of the growth retardant paclobutrazol on rooting of the micropropagated shoots and plantlet acclimatization after transplantation to soil is also described.

RESULTS AND DISCUSSION

Effect of changes in the concentration of macronutrients

The effects of varying the concentrations of MS major salts (BM, 1/2 BM, 1/4 BM) on growth and productivity of *D. obscura* shoot-tip cultures are summarized in Table 1. As macronutrient concentration decreased so did the proliferation rates and the fresh weight and length of the developed shoots. This effect was most marked with 1/4 BM, especially with regard to the final sizes of the shoots. Both 1/2 BM and 1/4 BM significantly inhibited shoot development but only this latter medium provoked significant reductions of fresh weight and proliferation rates in relation to BM. Our results agree with those obtained for hypocotyl cultures of *D. obscura* [5], although these juvenile explants seem to be less sensitive than shoot tips to changes in the major salt formulation.

By contrast, it is known that nutrient media with high salt concentrations are deleterious for some particular genotypes [6] or tissues [7], and other workers have reported best results on different species when

Table 1. Effect of macronutrient concentration on plant regeneration and plantlet growth in shoot-tip cultures of *D. obscura* (T4)

	No. shoots/explant*	Fr. wt (g/explant)*	Shoot length (mm)†
BM	19.8 b	0.88 b	15.4±0.9
1/2 BM	17.5 ab	0.69 ab	13.7±0.4
1/4 BM	13.8 a	0.42 a	12.0±0.4

* For each column, values followed by the same letter are not significantly different according to Tukey's test ($P = 0.05$).

† Values are combined means (±SD) from three independent experiments.

* Part 2 in the series '*Digitalis obscura* cardenolides'. For Part 1 see ref. [4].

† Author to whom correspondence should be addressed.

the strength of the basal medium was reduced or when formulations with low salt concentrations were used [8–10].

With regards to other *Digitalis* species, original and modified MS formulations have been usually employed in different *in vitro* systems and species of this genus [11–17], but it is not possible to make comparisons with out results because such studies do not provide data concerning the possible effect of macronutrient concentration on culture growth and productivity.

In order to study the influence of changes in the concentration of major salts on *D. obscura* cardenolide production, these secondary metabolites were measured in shoots after four months of culture (Table 2). The results showed an increased cardenolide accumulation in the shoots grown in media with reduced levels of macronutrients. This effect was especially significant in 1/2 BM cultures where the shoots had double the cardenolide content of those grown in BM. These differences are due mainly to the higher content in lanatoside A, since the rest of glycosides and genins showed little change (Table 2).

These data suggest that the productivity of *D. obscura* cultures could be increased by reducing mineral salt strength in the nutrient medium, which is in agreement with similar results reported on different *in vitro* systems where secondary metabolite accumulation improved upon transference of the regenerants to media with lower salt concentrations [18, 19].

It is known that in some species (e.g. *Catharanthus roseus*, *Lithospermum erythrorhizon*, *Coleus blumei* and *Thuja occidentalis*) it has not been possible to define an optimum nutrient medium for both growth and productivity, and this necessitates changing the medium formulation at the end of the growth phase [18, 20]. According to our results, this approach could also be applied to *D. obscura* shoot-tip cultures since BM, which is the optimum medium for growth and shoot multiplication, limited cardenolide production which was, however, significantly improved by 1/2 BM.

Effect of changes in the source of nitrogen

In a previous study [21], we investigated the influence of NH_4^+ and NO_3^- on shoot-tip cultures established from different *D. obscura* genotypes. The results did not show genotype-dependent responses and demonstrated that when NO_3^- was used as the sole nitrogen source it did not support satisfactory growth and a small amount of NH_4^+ was essential for good proliferation and development.

In the present experiment, BM composition was modified using different nitrogen salts (reduced or oxidized forms) to produce varying NO_3^-/NH_4^+ ratios (Table 3).

The use of BM-1, with a NO_3^-/NH_4^+ balance (2/1) similar to BM, led to a drastic reduction of growth and proliferation rates. However, these parameters were not significantly affected by BM-2, although this medium, with increased NO_3^- concentration (4/1), significantly favoured shoot development (Table 4). Very poor responses were obtained on the rest of media tested, BM-3 and BM-4, which had raised NH_4^+ concentrations, negatively affected culture growth and development (Table 4).

Although in some species ammonium [22, 23] or nitrate [24. 25] have been successfully employed as the sole nitrogen source, most cultures require the simultaneous presence of both inorganic nitrogen forms [18, 26].

In addition to the influence of the absolute amount of nitrogen, it has also been reported that the NO_3^-/NH_4^+ ratio may play a determinant morphogenetic role, and its optimum value has to be adjusted depending on the species, the type of explant or the stage of culture [8, 27–29].

According to our data, BM, which contained the original MS formulation, was the best medium overall, but it is not possible to confirm as optimal the NO_3^-/NH_4^+ ratio (2/1) for this medium. In this respect, we point out the low response observed in cultures grown on BM-1, which is similar to BM in the total nitrogen level and NO_3^-/NH_4^+ ratio. Since the major differences

Table 2. Influence of macronutrient concentration on cardenolide content ($\mu g\ g^{-1}$ dry wt) in shoot-tip cultures of *D. obscura* (T4)

Cardenolide	BM	1/2 BM	1/4 BM
Digitoxigenin	10±2	5±2*	3±1*
Purpureaglycoside A	59±11	70±19	35±6*
Evatromonoside	119±37	112±38	24±7*
Lanatoside A	537±105	1354±486*	906±203*
Digitoxigenin bis-digitoxoside	85±47	133±27	33±6
Digitoxin	40±13	20±3*	46±8
Gitoxigenin	13±3	34±7*	25±9
Total	863±156	1728±567*	1072±228

Values are combined means (±SD) from three extracts with 2 analyses each.
* Significantly different from BM.

Table 3. Nitrogen source, Cl^- and Na^+ concentrations (mM) in different nutrient media tested

	KNO_3	$NaNO_3$	NH_4NO_3	NH_4Cl	NO_3^-/NH_4^+	Cl^-	Na^+
BM	18.8		20.6		2/1	6.0	0.2
BM-1	18.8	20.6		20.6	2/1	26.6	20.8
BM-2	18.8	29.2		12.0	4/1	18.0	29.4
BM-3	18.8		10.3	19.7	1/1	25.7	0.2
BM-4	18.8			41.2	1/2	47.2	0.2

Table 4. Effect of different nutrient media on plant regeneration and plantlet growth in shoot-tip cultures of *D. obscura* (T4)

	No. shoots/ explant*	Fr. wt (g/explant)*	Shoot length (mm)†
BM	19.8 c	0.88 c	15.4±0.9
BM-1	8.9 a	0.55 b	14.3±0.7
BM-2	15.6 bc	0.90 c	17.1±0.6
BM-3	9.6 ab	0.33 ab	14.7±0.6
BM-4	5.9 a	0.15 a	8.5±0.4

* For each column, values followed by the same letter are not significantly different according to Tukey's test ($P = 0.05$).

† Values are combined means (±SD) from three independent experiments.

between these media were higher Cl^- and Na^+ concentrations in BM-1 (Table 3), it appeared that one or both of these ions limited culture development.

Based on our previous work [21] and on the present data (Tables 3 and 4), we suggest that sodium concentration did not play a decisive role in limiting the response of *D. obscura* cultures, and that Cl^- was responsible for BM-1 toxicity. Moreover, the poor results obtained with BM-3 and BM-4 could be due not only to their low NO_3^-/NH_4^+ ratios but also to the high Cl^- level; in both media the Na^+ concentration was very low (0.2 mM) and similar to that of BM (Table 3).

NO_3^-/NH_4^+ ratios greater than 2/1 were found to increase multiplication rates in seedling explants of *D. obscura* [5]. The expected similar results with BM-2 (NO_3^-/NH_4^+ 4/1) have been probably counteracted by the toxic Cl^- level in this nutrient medium (Table 3).

These conclusions are supported by the results obtained in numerous studies focused on the response of different species to Cl^- and Na^+ salinity. Thus, with few exceptions there is a relatively higher uptake of Cl^- than Na^+, Na^+ salinity is less extended than Cl^- salinity and this ion can be responsible for growth inhibition in many species including *Digitalis* spp. [30, 31]. Except for halophytes, chloride levels higher than 7 mM are potentially toxic. This is not a static threshold and can increase with high concentrations of nitrate [32] which, reciprocally, shows a limited uptake in the presence of high chloride concentrations [33].

Chloride toxicity has also been observed in various *in vitro* systems, especially in woody plants, and nutrient media with minimized Cl^- levels have been formulated [34]. In this respect, *in vitro* morphogenic responses of *Digitalis purpurea* improved when the Cl^- concentration was reduced in the culture medium [17].

With regard to the cardenolide content of shoots grown on media with varying NO_3^-/NH_4^+ ratios, we observed a close relationship between their productivity and the source of nitrogen employed in the nutrient media (Table 5). None of the modifications tested improved on the levels obtained with the original formulation. Thus only those shoots grown with high levels of oxidized nitrogen (BM-1 and BM-2) were found to contain cardenolide in amounts similar to control cultures. The deleterious effects of the rest of modifications on culture development made it impossible to measure the cardenolide content (BM-4) or provoked a highly significant reduction in productivity (BM-3).

These results are in agreement with those previously reported on different *Digitalis* cultures where high NO_3^-/NH_4^+ ratios favoured cardenolide accumulation. This ratio was 2/1 for *D. purpurea* cell cultures [35] while in embryogenic systems of *D. lanata* the optimum was 10/1 [13] or ranged between 2/1 and 30/1 depending on the maltose concentration used [36].

This behaviour is not exclusive to *Digitalis* species since, with few exceptions [37], the best yields of numerous important secondary metabolites have been obtained in cultures which had high NO_3^-/NH_4^+ ratios [38–40].

Our results clearly show that relative concentrations of NO_3^- or NH_4^+ not only affect culture growth and proliferative capacity but also metabolite production. Nevertheless, as in BM-1 cultures, these effects may not be correlated. This has also been reported on different species such as *Lithospermum erythrorhizon* [41], *Catharanthus roseus* [42], and *Nicotiana tabacum* and *Phytolacca americana* [18].

Rooting and acclimatization

Shoots were excised from proliferating cultures and treated with IAA. After seven days, the explants were transferred to either agar-solidified medium or to a peat-moss–perlite mixture which included reduced

Table 5. Cardenolide content ($\mu g\ g^{-1}$ dry wt) in shoot-tip cultures of *D. obscura* (T4) grown on different nutrient media

	BM	BM-1	BM-2	BM-3
Digitoxigenin	10±2	—	3±1*	—
Purpureaglycoside A	59±11	62±6	25±19*	14±2*
Evatromonoside	119±37	82±18	82±19	35±7*
Lanatoside A	537±105	626±50	520±133	66±6*
Digitoxigenin bis-digitoxoside	85±47	48±10	50±36	13±1*
Digitoxin	40±13	62±6*	25±19	14±2*
Gitoxigenin	13±3	10±2	10±3	15±9
Total	863±156	859±53	739±195	152±7*

Values are combined means (±SD) from three extracts with 2 analyses each.
* Significantly different from BM.

nutrients and different dosages of the growth retardant paclobutrazol (PBZ).

After one month in culture, the results suggested that the type of physical support affected both the survival and the rooting of the explants (Table 6). Higher rates being obtained with agar (82 and 64%, respectively) than with peat-moss–perlite (67 and 25%). The effect of PBZ also depended on the rooting substrate used. Thus, PBZ increased the survival of the explants grown on agar (92–100%), but did not improve the rooting percentage. By contrast, all dosages of PBZ favoured root formation in peat-moss–perlite cultured shoots (58–83%), and all of the explants survived when the highest concentration (3 mg l^{-1}) was employed (Table 6). The total number and length of roots varied with the treatment, but neither the type of physical support nor the PBZ significantly affected these parameters (Table 6).

After rooting, plantlets were transplanted to pots and maintained in a greenhouse. Although the survival rates substantially decreased during the acclimatization process, 67% of the shoots initially cultured on peat-moss–perlite with 3 mg l^{-1} PBZ developed into plants which exhibited normal growth *ex vitro* (Table 6).

Our findings suggest that PBZ favoured rooting, hardening, and acclimatization of *in vitro D. obscura* regenerants. The best results were obtained when, in addition to PBZ supplementation, mixtures of peat-moss–perlite were used as physical support throughout those processes.

In most species, root formation depends on auxin treatment which is favoured by the use of media with low levels of salts [43]; *Digitalis* is not an exception [14, 44, 45]. However, the success of micropropagation is frequently limited by transplantation shocks, mainly desiccation and low photosynthetic efficiency, since tissue culture plants are often structurally and physiologically abnormal [46]. In this respect, the use of paclobutrazol during rooting and/or hardening has been reported to improve acclimatization of plantlets grown *in vitro*: the spectrum of PBZ effects include smaller leaves, increased epicuticular wax, shorter and thicker roots and shoots, darker green leaves, and slower rates of water loss associated with smaller stomatal apertures [47–50].

EXPERIMENTAL

Plant material. Vegetative shoots were harvested from a selected *Digitalis obscura* L. (genotype T4) and the axillary buds used for *in vitro* culture establishment. Leaves from *in vitro* regenerants (shoots or plantlets) were employed for cardenolide extraction.

In vitro *culture.* Axillary-bud cultures from genotype T4 were established according to Gavidia *et al.*

Table 6. Effect of paclobutrazol and physical support on rooting of shoots grown from *D. obscura* (T4) shoot-tip cultures and survival of acclimatized plants

Physical support	PBZ (mg l^{-1})	% Survival (1 month)	% Rooting (1 month)	Root length (cm)	No. roots/ explant	% Survival (*ex vitro*)
Agar	0	82	64	2.2±1.6	6±3	27
Agar	1	100	27	1.3±0.2	11±5	25
Agar	2	92	67	1.6±0.5	4±1	50
Agar	3	92	58	1.6±0.4	8±2	33
Peat-moss–perlite	0	67	25	1.3±0.5	7±2	15
Peat-moss–perlite	1	50	67	0.9±0.4	6±2	33
Peat-moss–perlite	2	67	58	1.9±0.9	9±4	33
Peat-moss–perlite	3	100	83	1.0±0.7	11±3	67

Values are combined means from two independent experiments of 12 replications each.

[2, 3]. Shoot-tips (1 cm) isolated from proliferating cultures and devoid of developed leaves were employed for large scale multiplication by alternative subculture on basal medium (BM) and on BM with 1 μM benzyladenine (BA) for 30 days each. BM contained MS nutrients [51], 3% sucrose and 0.8% Difco-Bacto agar (pH 5.7). Shoot tips from this multiplication chain were cultured on modified BM with either MS macronutrients reduced to one-half or one-fourth strength (1/2 BM, 1/4 BM), or with different NO_3^-/NH_4^+ ratios maintaining the original nitrogen concn (Table 3). Media were initially supplemented with 5 μM BA (30 days), and regenerated shoots subcultured on their respective modified media according to the above multiplication chain (4 months). A total of 24 explants in three separate experiments were cultured for each nutrient medium. In all cases, cultures were maintained in a growth chamber at $26 \pm 2°$ and a 16 hr photoperiod with light supplied by Sylvania (GTE Gro-lux, F36W/GRO) fluorescent tubes (80 μmol m^{-2} per sec irradiance at culture level).

Rooting and acclimatization. Rooting experiments were carried out with shoots (3 cm long) grown from control cultures of T4 shoot-tips. Shoots were cultured in tubes containing 20 ml BM and 3.0 μM indoleacetic acid (IAA). After 7 days, shoots were transferred to tubes containing 20 ml: (a) modified BM containing half-strength salts and vitamins, reduced sucrose (1%), 0.8% agar, and 0, 1, 2 or 3 mg l^{-1} of filter-sterilized paclobutrazol (PBZ) and (b) peat-moss–perlite (1:1) and 10 ml of liquid modified BM including the above dosages of PBZ. Rooted shoots were then transferred to Phytacon vessels (Sigma) containing peat-moss–perlite (2:1). During the rooting and hardening procedures (2 months) the cultures were maintained in growth chambers (60% relative humidity, $24 \pm 1°$, 16 hr photoperiod). Finally, surviving plantlets were transplanted individually to 100 ml pots containing a mixt. of peat-moss–perlite (3:1) and covered with plastic bags. The plants were placed in a greenhouse and acclimatized increasing aeration progressively.

Cardenolide determinations. Experimental conditions employed were those described by Gavidia and Pérez-Bermúdez [4].

REFERENCES

1. Dörnenburg, H. and Knorr, D., *Enzyme and Microbial Technology*, 1995, **17**, 674.
2. Gavidia, I., Segura, J. and Pérez-Bermúdez, P., *Journal of Plant Physiology*, 1993, **142**, 373.
3. Gavidia, I., Del Castillo Agudo, L. and Pérez-Bermúdez, P., *Plant Science*, 1996, **121**, 197.
4. Gavidia, I. and Pérez-Bermúdez, P., *Phytochemistry*, 1997, **45**, 81.
5. Lapeña, L., Pérez-Bermúdez, P. and Segua, J., *In Vitro Cellular Developmental Biology*, 1992, **28P**, 121.
6. Turk, B. A., Swartz, H. J. and Zimmerman, R. H., *Plant Cell Tissue and Organ Culture*, 1994, **38**, 11.
7. Pérez-Bermúdez, P., Cornejo, M. J. and Segura, J., *Plant Cell Tissue and Organ Culture*, 1985, **5**, 63.
8. Flinn, B. S., Webb, D. T. and Georgis, W., *Canadian Journal of Botany*, 1986, **64**, 1948.
9. Declerck, V. and Korban, S. S., *Plant Cell Tissue and Organ Culture*, 1994, **38**, 57.
10. Hilton, M. G. and Rhodes, M. J. C., *Plant Cell Tissue and Organ Culture*, 1994, **38**, 45.
11. Garve, R., Luckner, M., Vogel, E., Tewes, A. and Nover, L. *Planta Medica*, 1980, **40**, 92.
12. Rücker, W., in *Biotechnology in Agriculture and Forestry* 4, ed. Y. P. S. Bajaj. Springer, Berlin, 1988, p. 388.
13. Kuberski, C. H., Scheibner, H., Steup, C., Dietrich, B. and Luckner, M., *Phytochemistry*, 1984, **23**, 1407.
14. Schöner, S. and Reinhard, E., *Planta Medica*, 1986, **52**, 478.
15. Luckner, M. and Diettrich, B., in *Biotechnology in Agriculture and Forestry* 19, ed. Y. P. S. Bajaj. Springer, Berlin, 1992, p. 400.
16. Stuhlemmer, U., Kreis, W., Eisenbeiss, M. and Reinhard, E., *Planta Medica*, 1993, **59**, 539.
17. Chaturvedi, H. C. and Jain, M., *Plant Cell Tissue and Organ Culture*, 1994, **38**, 73.
18. Endress, R., *Plant Cell Biotechnology*. Springer, Berlin, 1994.
19. Carman, J. G., in *Current Issues in Plant Molecular and Cellular Biology*, ed. M. Terzi, R. Cella and A. Falavigna. Kluwer Academic Publishers, Dordrecht, 1995, p. 393.
20. Constabel, F. and Tyler, R. T., in *Plant Cell and Tissue Culture*, ed. I. K. Vasil and T. A. Thorpe. Kluwer Academic Publishers, Dordrecht, 1994, p. 271.
21. Gavidia, I., Pérez-Bermúdez, P., Falcó, J. M. and Segura, J., *Planta Medica*, 1991, **57**, 28.
22. Gamborg, O. L. and Shyluk, J. P., *Plant Physiology*, 1970, **45**, 598.
23. Chaleff, R. S., *Plant Cell Tissue and Organ Culture*, 1983, **2**, 29.
24. Wetherell, D. F. and Dougall, D. K., *Physiologia Plantarum*, 1976, **37**, 97.
25. Pérez-Bermúdez, P. and Sommer, H. E., *Plant Cell Tissue and Organ Culture*, 1987, **11**, 25.
26. Kirby, E. G., Leustek, T. and Lee, M. S., in *Cell and Tissue Culture in Forestry* 1, ed. J. M. Bonga and D. J. Durzan. Martinus Nijhoff, The Hague, 1987, p. 67.
27. David, A., David, H. and Mateille, T., *Physiologia Plantarum*, 1982, **56**, 102.
28. Grimes, H. D. and Hodges, T. K., *Journal of Plant Physiology*, 1990, **136**, 362.
29. Niedz, R. P., *Plant Cell Tissue and Organ Culture*, 1994, **39**, 1.
30. Bernal, C. T., Binham, F. T. and Oerfili, J., *Soil Science, American Proceedings*, 1975, **38**, 777.

31. Morales, C., Cusido, R. M., Palazón, J. and Bonfill, M., *Journal of Plant Nutrition*, 1993, **16**, 327.
32. Teasdale, R. D., in *Cell and Tissue Culture in Forestry* 1, ed. J. M. Bonga and D. J. Durzan. Martinus Nijhoff, The Hague, 1987, p. 17.
33. Marschner, H., *Mineral Nutrition of Higher Plants*. Academic Press, London, 1995.
34. McCown, B. H. and Sellmer, J. C., in *Cell and Tissue Culture in Forestry* 1, ed. J. M. Bonga and D. J. Durzan. Martinus Nijhoff, The Hague, 1987, p. 4.
35. Hagimori, M., Matsumoto, T. and Obi, Y., *Plant Cell Physiology*, 1982, **23**, 1205.
36. Tuominen, U., Toivonen, L., Kauppinen, V., Markkanen, P. and Björk, L., *Biotechnology and Bioengineering*, 1989, **33**, 558.
37. Ikeda, T., Matsumoto, T. and Noguchi, M., *Agriculture and Biological Chemistry*, 1977, **41**, 1197.
38. Nigra, H. M., Alvarez, M. A. and Giuletti, A. M., *Plant Cell Tissue and Organ Culture*, 1990, **21**, 55.
39. Wysokinska, H. and Swiatek, L., *Plant Science*, 1991, **76**, 249.
40. Oksman-Caldentey, K. M., Sevon, N., Vanhala, L. and Hiltunen, R., *Plant Cell Tissue and Organ Culture*, 1994, **38**, 263.
41. Tabata, M. and Fujita, Y., in *Biotechnology in Plant Science: Relevance to Agriculture in the Eighties*, ed. P. Day, M. Zaitlen and A. Hollaender. Academic Press, Orlando, 1985, p. 207.
42. Vazquez-Flota, F., Moreno-Valenzuela, O., Miranda-Ham, M. L. and Coello-Coello, J., *Plant Cell Tissue and Organ Culture*, 1994, **38**, 273.
43. Preece, J. E., *Plant Tissue Culture and Biotechnology*, 1995, **1**, 26.
44. Rücker, W., Jentzsch, K. and Wichtl, M., *Zeitschrift für Pflanzenphysiologie*, 1976, **80**, 323.
45. Herrera, M. T., Cacho, M., Corchete, M. P. and Fernández-Tárrago, J., *Plant Cell Reports*, 1990, **22**, 179.
46. Bhojwani, S. S. and Dhawan, V., in *Applications of Biotechnology in Forestry and Horticulture*, ed. V. Dhawan. Plenum Press, New York, 1989, p. 249.
47. Marino, G., *Plant Growth Regulation*, 1988, **7**, 237.
48. Smith, E. F., Roberts, A. V. and Mottley, J., *Plant Cell Tissue and Organ Culture*, 1990, **21**, 133.
49. Eliasson, M. K., Beyl, C. A. and Barker, P. A., *Journal of Plant Growth Regulation*, 1994, **13**, 137.
50. Roberts, A. V. and Matthews, D., *Plant Cell Tissue and Organ Culture*, 1995, **40**, 191.
51. Murashige, T. and Skoog, F., *Physiology of Plants*, 1962, **15**, 473.