INFLUENCE OF NUTRIENT MEDIUM UPON SHOOT INITIATION ON VEGETATIVE EXPLANTS EXCISED FROM 15- TO 18-YEAR-OLD PICEA GLAUCA

GINA H. MOHAMMED, DAVID I. DUNSTAN*
AgriForest Technologies Ltd, P.O. Box 178, Kelowna,
British Columbia, Canada V1Y 7N5

and TREVOR A. THORPE
Department of Biology, University of Calgary, Calgary,
Alberta, Canada T2N 1N9

(Received for publication 9 January 1986; revision 4 August 1986)

ABSTRACT

A comparison was made of the growth responses of explants excised from vegetative buds of 15- to 18-year-old **Picea glauca** (Moench) Voss (white spruce) when cultured upon seven nutrient media. One nutrient medium, GMD, was found to be superior in the induction of shoot primordia (average 8.1 per explant) and in its primary multiplication value (average 3.24 shoot primordia per original bud). Five of the other nutrient formulae also gave rise to shoot primordia, though at significantly lower values. Only explants grown on Schenk and Hildebrandt medium failed to give rise to shoot primordia. Medium GMD was devised after a comparison of elemental concentrations within a range of media that have been published for use with various tree tissue cultures.

Keywords: vegetative bud explant; nutrient media; micropropagation; **Picea glauca**.

INTRODUCTION

Picea glauca has a broad distribution in Canada and northern United States (Owens & Molder 1984). In British Columbia it is the most extensively harvested tree species, used primarily for lumber and pulpwood. Natural regeneration and reforestation have often resulted in stands of variable quality. Because elite progeny from seed orchards remain scarce, it is desirable to develop vegetative propagation techniques for the provision of phenotypically superior trees.

Assessment of the superiority of *P. glauca* trees can be made at 12–15 years (K. Illingworth, British Columbia Ministry of Forests, pers. comm.), an age at which vegetative propagation by traditional means is difficult (Bonga 1982; Montain *et al.* 1983). Recently it has become possible to induce shoot primordia *in vitro* upon vegeta-

* To whom requests for reprints should be sent. NRCC No. 25858.
Present address: Plant Biotechnology Institute, National Research Council, 110 Gymnasium Road, Saskatoon, Saskatchewan, Canada S7N OW9.

tive buds excised from older trees of *Abies balsamea* L. Mill. (Bonga 1977), *Picea abies* L. Karst. (Jansson & Bornman 1983; von Arnold 1984), and *Pseudotsuga menziesii* (Mirb.) Franco (Boulay 1979; Thompson & Zaerr 1981; Dunstan *et al.* 1986). The nutrient media that have been used for these species, and for cultures of *Picea glauca in vitro,* are quite varied. They were based on MS (Murashige & Skoog 1962) used for *Pseudotsuga menziesii* (Boulay 1979; Thompson & Zaerr 1981; Dunstan *et al.* 1986); VAE (von Arnold & Eriksson 1977, 1979) used for *Picea abies* (although these authors call their medium LP, it has also become customary to note the medium of Quoirin & Lepoivre (1977) as LP (see Aitken-Christie & Thorpe 1984) and to avoid further confusion, we have adopted the latter usage); SH (Schenk & Hildebrandt 1972) used for *P. abies* (Jansson & Bornman 1983) and for *P. glauca* (Rumary & Thorpe 1984); and CD, used for *P. glauca* (Campbell & Durzan 1975). This paper presents data on *in vitro* growth of explants from vegetative buds of a 15- to 18-year-old *Picea glauca* upon several nutrient media.

MATERIALS AND METHODS

Plant material

Collections were made in the dormant season (between 20 November and 6 March) from a 15- to 18-year-old *Picea glauca* (Tree No. 2) growing in a natural stand in the Penticton Forest District of southern interior British Columbia. Portions of first- and second-order branches bearing dormant vegetative buds were harvested from the lower two-thirds of the tree.

Sterilisation and explant preparation (after Dunstan *et al.* 1986)

Buds, together with 3 mm of subtending stem tissue, were excised from the branches and surface-sterilised for 15 min in a 1.2% chlorine solution (prepared from 5.25% sodium hypochlorite). After sterilisation, buds were rinsed three times with sterile double-distilled water. Bud scales were removed by a circumferential cut at the widest part of the bud. The terminal portion of exposed embryonal shoot axis (comprising the meristem dome and several needle primordia) was excised and discarded. The final explant was the remaining bud base (bud-base explant), which was approximately 50% embryonal shoot axis and 50% subtending crown and stem tissue.

Nutrient media

Growth responses on seven nutrient media were compared. These were SH (Schenk & Hildebrandt 1972), CD (Campbell & Durzan 1975), LP (Quoirin & Lepoivre 1977), VAE (von Arnold & Eriksson 1977), LM (Litvay *et al.* 1981), WPM (Lloyd & McCown 1981), and a medium, GMD, devised during these experiments (Table 1). All media contained benzyladenine (3 mg BA/*l*) for the first 42 days (after which it was removed), sucrose at 30 g/*l,* and agar at 5 g/*l,* and the pH was adjusted to 5.6 prior to addition of agar. Media were dispensed into 150 × 25-mm test-tubes at 20 ml per tube, capped with Kaputs, and autoclaved at 138 kPa for 15 min. After 15 weeks, cultures were grown on 25 ml medium in 125-ml-capacity wide-mouth erlenmeyer flasks, capped with aluminium foil. Explants were transferred to their respective fresh nutrient medium every 21 days. Five explants were inoculated on to each medium and the experiment was repeated five times. Growth conditions were as described by Dunstan *et al.* (1986).

TABLE 1—Compounds present in GMD, a medium devised from a comparison of concentrations present in seven media used for woody plant tissue cultures **in vitro**

Constituent	Quantity (mg/*l*)	Molarity (mM)
$CaCl_2.2H_2O$	183.77	1.25
KNO_3	1819.80	18.00
NH_4NO_3	405.35	5.06
$(NH_4)_2SO_4$	131.18	0.99
$MgSO_4.7H_2O$	394.38	1.60
$NH_4H_2PO_4$	293.35	2.55
$NaH_2PO_4.2H_2O$	7.77	0.05
$MnSO_4.4H_2O$	11.18	0.05
$ZnSO_4.7H_2O$	2.04	0.007
$CuSO_4.5H_2O$	0.025	0.0001
KI	0.88	0.005
$CoCl_2.6H_2O$	0.024	0.0001
H_3BO_3	3.09	0.05
$Na_2MoO_4.2H_2O$	0.024	0.00009
$FeSO_4.7H_2O$	13.90	0.05
Na_2EDTA	18.61	0.05
Myo-Inositol	99.90	0.56
Nicotinic acid	1.97	0.02
Pyridoxine HCl	1.00	0.005
Thiamine HCl	5.06	0.015

RESULTS AND DISCUSSION

Shoot Primordium Induction

Explants increased in size from 2 mm to approximately 4 to 5 mm by the sixth to ninth week, by which time enlargement of the needle primordia was evident (Fig. 1a). Such modified needles were characterised by their paler, more globular appearance compared to other needle primordia. Between the ninth and twelfth weeks, small shoot meristems were observed upon the modified needles (Fig. 1b) and these developed asynchronously into distinct shoot primordia (soft buds) (Fig. 1c) and then 1-cm-long shoots (Fig. 1d) from the fifteenth week. This pattern of adventitious shoot development is similar to that reported for *Picea abies* (von Arnold & Eriksson 1979; Jansson & Bornman 1983) and for some trees of *Pseudotsuga menziesii* (Dunstan *et al.* 1986).

Nutrient Media

Preliminary experiments, which compared the growth of various vegetative bud explants excised from 12- to 15-year-old *Picea glauca* trees, indicated that a limited induction of shoot meristems could occur on explants cultured on MS medium and SH medium containing 1 to 3 mg BA/*l*. This occurrence was, however, very inconsistent and the cultures eventually callused (on MS) or senesced (on SH) (unpubl. data). During such experiments bud-base explants were observed to produce greater quantities of induced meristems than entire bud axes or excised apices (see also Dunstan *et al.* 1986).

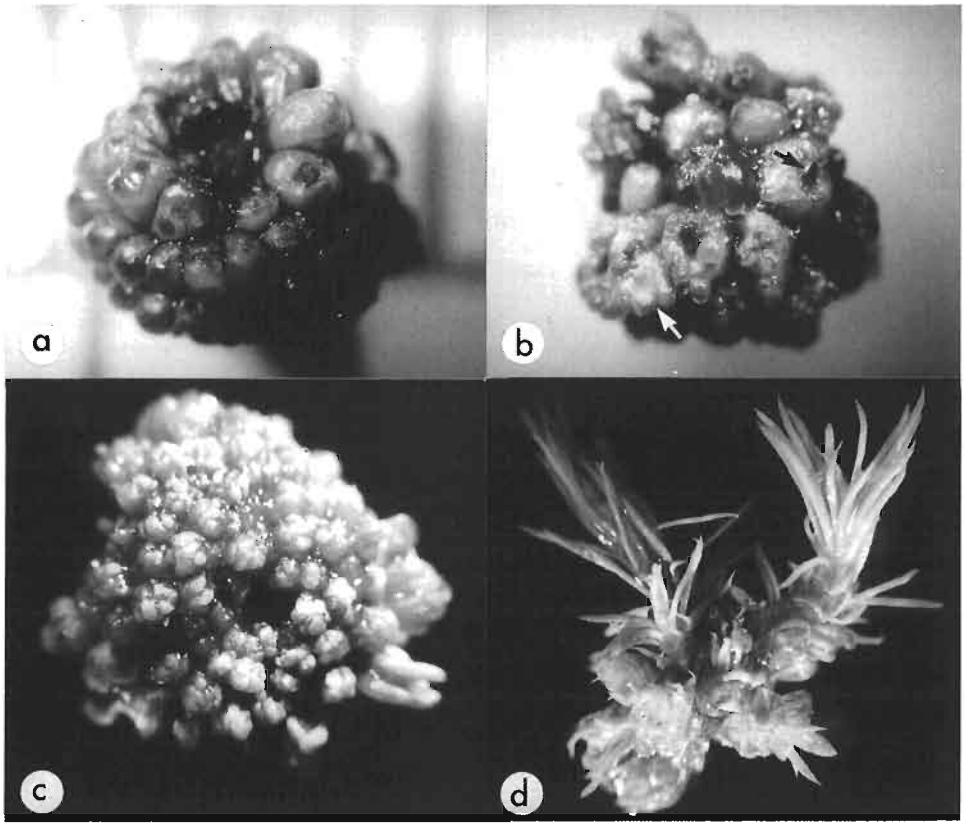

FIG. 1a—Bud-base explant (4.5 mm wide) on medium GMD after 6 weeks, showing modified needles (×11.0).

FIG. 1b—Production of meristems upon modified needles, after 9 weeks on medium GMD. Arrows denote the necrotic needle tip (black arrow) and meristems flanking the tip (white arrow) (×11.0).

FIG. 1c—Development of distinct shoot primordia (soft buds) from meristems such as those in 1b, after 12 to 15 weeks on medium GMD (×4.0).

FIG. 1d—Small shoots that have developed from the bud-base explant after 15 weeks on medium GMD - shoot at right is about 0.75 cm long to its apex (×4.0).

The following research was designed therefore to arrive at a medium that would permit reproducible induction of shoot meristems and development of these into small shoot primordia and eventually into 1-cm shoots. To do this, a comparison was made of the totalled elemental concentrations of all inorganic compounds and of the concentrations of all other constituents present in media used for woody plant tissue cultures (Murashige & Skoog 1962; Schenk & Hildebrandt 1972; Durzan *et al.* 1973; Sommer *et al.* 1975; Horgan & Aitken 1981; von Arnold & Eriksson 1977). The comparison

revealed the extent to which elemental, ionic, and compound concentrations varied amongst the media. This was especially evident in their content of ammonium (NH_4^+) and nitrate (NO_3^-) ions, micro inorganics, and organic compounds (Table 2). The comparison led to the formation of several experimental media possessing various features of the ranges, e.g., with high, low, or average ammonium or nitrate concentrations in combination with high, low, or average concentrations of other constituents. This approach is similar to that used by de Fossard *et al.* (1974) and Dunstan & Short (1977). Medium GMD was observed to be superior amongst these in its ability to support the growth of *P. glauca* bud-base explants. It was therefore subjected to further comparison with nutrient media that had been used in conifer tissue cultures.

There was a pronounced effect of nutrient medium upon explant growth response, although explant survival over the first 6 weeks was not significantly influenced by medium. By the ninth week statistical differences in growth response became evident with LP and GMD showing the greatest numbers of explants (25 and 20, respectively) possessing modified needles (Table 3, Fig. 1a). Only explants grown on SH failed to produce such needles during these trials. The number of modified needles on each explant was also a function of medium, with medium GMD having the maximum value (average 23.7 modified needles per explant). The number of modified needles on explants growing on LM and LP averaged 13.1 and 9.2, respectively (Table 3).

Similarly, the development of shoot primordia from the modified needles (Fig. 1c) occurred most frequently on explants grown on GMD (average 8.1 per explant). Explants on other media (with the exception of SH) showed similar development of shoot primordia but in lower numbers. A comparison of primary multiplication factors (PMF) accounting for losses due to contamination or senescence, shows the superiority of medium GMD (average 3.24 shoot primordia per original explant; Table 3). The only other media to support PMF values over 1 were LP and LM. For comparison the PMF reported for *P. abies* was on average 3.06 per original explant (see von Arnold 1984), while that reported for *Pseudotsuga menziesii* averaged 8.8 per original explant (Dunstan *et al.* 1986).

The number of shoot primordia that develop into excisable shoots is presently low, occurring only from explants on media GMD (40%) and LP (6%). No other media permitted a similar development of shoot primordia. There have been no previous reports which refer to conditions that affect reproducible shoot elongation from shoot primordia induced on vegetative bud explants from 15- to 18-year-old spruce trees. Keathley (1984) indicated, however, that a low concentration of benzyladenine (10^{-8} M) or a mixture of kinetin (10^{-6} M) with indole acetic acid (10^{-4} M) enhanced shoot growth of whole vegetative bud explants in spring or late summer respectively. Dunstan *et al.* (1986) noted that the elongation of shoots from shoot primordia induced upon bud-base explants of 17- to 20-year-old *Pseudotsuga menziesii* was better from explants exposed to ammonium nitrate during only the initial 42 days of culture (in comparison with 21 or 63 days, or continuous exposures). All the media tested here contained ammonium nitrate either as an ingredient compound or as a result of the dissociation of ammonium (NH_4^+) and nitrate (NO_3^-) ions from other compounds, and no attempt was made to alter their availability. A comparison of inorganic ion concentrations of the media used in these trials was made in order to understand why GMD was the

TABLE 2—Comparative mM concentrations of inorganic elements and organic constituents in media* used for the derivation of GMD (values are corrected to one decimal point or to the nearest whole number)

	MS	SH	DCL	SBK1	SBK2	RW	VAE	GMD
K	20.0	24.7	2.9	13.9	13.0	24.7	21.3	18.0
N (NO_3)	39.4	24.7	30.0	9.9	25.3	24.7	33.8	23.0
N (NH_4)	20.6	2.6	20.0	3.0	12.5	2.6	15.0	9.6
Mg	1.5	1.6	1.5	1.0	0.1	1.6	1.5	1.6
P	1.3	2.6	1.25	0.8	2.2	2.6	2.5	2.6
Ca	3.0	1.4	4.15	1.0	1.5	1.4	1.2	1.3
S	1.6	1.7	1.6	2.6	0.2	1.7	1.7	2.7
Cl	6.0	2.7	0.0002	2.0	0.002	2.7	2.4	2.5
Na (non EDTA)	0.002	0.0008	0.002	0.9	0.003	0.02	0.0002	0.05
I	0.05	0.006	0.005	0.005	0.005	0.006	0.005	0.005
B	0.1	0.08	0.1	0.05	0.05	0.08	0.01	0.05
Mn	0.1	0.06	0.1	0.06	0.06	0.09	0.01	0.05
Zn	0.03	0.003	0.03	0.01	0.01	0.003	0.1	0.007
Mo	0.001	0.0004	0.001	0.001	0.001	0.009	0.0001	0.00009
Cu	0.0001	0.0008	0.0001	0.001	0.001	0.0008	0.00001	0 0001
Co	0.0001	0.0004	0.0001	0.001	0.001	0.0008	0.00001	0.0001
Fe	0.1	0.05	0.1	0.1	0.1	0.05	0.05	0.05
Na_2EDTA	0.1	0.05	0.1	0.1	0.1	0.05	0.05	0.05
L-glutamine	—	—	4.0	—	—	—	—	—
L-glycine	0.03	—	4.0	—	—	—	0.03	—
L-asparagine	—	—	1.5	—	—	—	—	—
L-arginine	—	—	0.3	—	—	—	—	—
L-methionine	—	—	0.2	—	—	—	—	—
Myo Inositol	0.6	5.6	0.6	0.06	0.06	5.6	0.6	0.6
Nicotinic acid	0.004	0.04	—	0.0008	0.0008	0.04	0.02	0.02
Pyridoxine HCl	0.002	0.002	—	0.0005	0.0005	0.003	0.005	0.005
Thiamine HCl	0.0003	0.02	0.001	0.003	0.003	0.03	0.015	0.015

* MS (Murashige & Skoog 1962); SH (Schenk & Hildebrandt 1972); DCL (Durzan **et al.** 1973); SBK1 and SBK2 (Sommer **et al.** 1975); RW (Horgan & Aitken 1981); VAE (von Arnold & Eriksson 1977).

TABLE 3—Comparison of the growth responses of vegetative bud-base explants grown upon seven nutrient formulations

Parameter	Medium						
	LM	GMD	VAE	SH	LP	WPM	CD
Survival (6 wk)	20/25	25/25	20/25	25/25	25/25	25/25	25/25
Number of explants producing modified needles*	15/20 b	20/25 ab	4/20 c	0	25/25 a	5/25 c	15/25 b
Mean number of modified needles per explant (9 wk)±S.E.†	13.1±0.45 b	23.7±0.67 a	2.75±0.43 d	0	9.2±0.47 c	5.8±0.86 d	4.9±0.46 d
Number of explants producing shoot primordia‡	6/20	10/25	2/20	0	8/25	3/25	5/25
Mean number of shoot primordia per explant (15 wk)±S.E.†	4.8±0.48 b	8.1±0.90 a	1.0 b	0	4.1±0.55 b	2.3±0.33 b	1.4±0.25 b
Primary multiplication factor	1.15	3.24	0.08	0	1.31	0.28	0.28

For each parameter, data followed by the same letter are not significantly different at the 1% level.

* Chi-square test for independence.

† Fisher's least significant difference.

‡ Data are not significantly different at the 1% level, Chi-square test for independence.

superior medium. There were no clear differences which could account for the range of explant responses that have been observed. Nitrogen, in the form of the NH_4^+ ion, was lowest in SH (2.61 mM) compared to GMD (9.59 mM) and its highest value in LM (20.61 mM); zinc was also lowest in SH (3.48 μM) compared to GMD (7.09 μM) and its highest value in LM (149.54 μM). It is probable therefore that the differences in explant response amongst the media were due more to the occurrence of particular inorganic compounds provided in each medium, their relative ease of dissociation into constituent ions, and the subsequent availability of these to the plant tissue. Present work is designed to determine the influence of nitrogen-containing compounds upon shoot initiation and elongation from cultures of *Picea glauca in vitro.*

ACKNOWLEDGMENTS

The authors gratefully acknowledge the funding of this work in part by the British Columbia Ministry of Forests and by the Natural Sciences and Engineering Research Council Canada (Grant GO818 to D.I.D. and T.A.T.).

REFERENCES

AITKEN-CHRISTIE, J.; THORPE, T. A. 1984: Clonal propagation: Gymnosperms. Pp. 82–95 **in** Vasil, I. K. (Ed.) "Cell Culture and Somatic Cell Genetics. Volume 1, Laboratory Procedures and their Applications." Academic Press, Orlando, Florida.

BONGA, J. M. 1977: Organogenesis in **in vitro** cultures of embryonic shoots of **Abies balsamea** (Balsam fir). **In Vitro 13:** 41–8.

——— 1982: Vegetative propagation in relation to juvenility, maturity, and rejuvenation. Pp. 387–412 **in** Bonga, J. M.; Durzan, D. J. (Ed.) "Tissue Culture in Forestry." Nijhoff-Junk, The Hague, Holland.

BOULAY, M. 1979: Propagation **in vitro** du Douglas (**Pseudotsuga menziesii** (Mirb.) Franco) par micropropagation de germination aseptique et culture de bourgeons dormants. "Micropropagation d'Arbres Forestiers". Afocel, Nangis, France. **"Etudes et Recherches" 12:** 67–75.

CAMPBELL, R. A.; DURZAN, D. J. 1975: Induction of multiple buds and needles in tissue cultures of **Picea glauca. Canadian Journal of Botany 53:** 1652–7.

de FOSSARD, R. A.; MYINT, A.; LEE, E. C. M. 1974: A broad spectrum tissue culture experiment with tobacco (**Nicotiana tabacum**) pith tissue callus. **Physiologia Plantarum 30:** 125–30.

DUNSTAN, D. I.; SHORT, K. C. 1977: Improved growth of tissue cultures of the onion, **Allium cepa. Physiologia Plantarum 41:** 70–2.

DUNSTAN, D. I.; MOHAMMED, G. H.; THORPE, T. A. 1986: Shoot production and elongation on explants from vegetative buds excised from 17- to 20-year-old **Pseudotsuga menziesii. New Zealand Journal of Forestry Science 16:** 269–82.

DURZAN, D. J.; CHAFE, S. C.; LOPUSHANSKI, S. M. 1973: Effects of environmental changes on sugars, tannins, and organized growth in cell suspension cultures of white spruce. **Planta (Berl.) 113:** 241–9.

HORGAN, K.; AITKEN, J. 1981: Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. **Physiologia Plantarum 53:** 170–5.

JANSSON, E.; BORNMAN, C. H. 1983: Morphogenesis in dormant embryonic shoots of **Picea abies**: influence of crown and cold treatment. **Physiologia Plantarum 59:** 1–8.

KEATHLEY, D. E. 1984: Micropropagation of mature spruce. Pp. 58–63 **in** "Proceedings of International Symposium on Recent Advances in Forest Biotechnology". Michigan Biotechnology Institute, Traverse City, Michigan, 10–14 June.

LITVAY, J. D.; JOHNSON, M. A.; VERMA, D.; EINSPAHR, D.; WEYRAUCH, K. 1981: Conifer suspension culture medium development using analytical data from developing seeds. **Institute of Paper Chemistry, IPC Technical Paper 115.**

LLOYD, G.; McGOWN, B. H. 1981: Commercially feasible micropropagation of mountain laurel (**Kalmia latifolia**) by use of shoot tip culture. **Proceedings of the International Plant Propagators' Society 30:** 421–97.

MONTAIN, C. R.; HAISSIG, B. E.; CURTIS, J. D. 1983: Differentiation of adventitious root primordia in callus of **Pinus banksiana** seedling cuttings. **Canadian Journal of Forest Research 13:** 195–200.

MURASHIGE, T.; SKOOG, F. 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum 15:** 473–97.

OWENS, J. N.; MOLDER, M. 1984: "The Reproductive Cycle of Interior Spruce". Information Services, British Columbia Ministry of Forests, Victoria.

QUOIRIN, M.; LEPOIVRE, P. 1977: Etude de millieux adaptés aux cultures **in vitro** de **Prunus**. **Acta Horticulturae 78:** 437–42.

RUMARY, C.; THORPE, T. A. 1984: Plantlet formation in black and white spruce. I. **In vitro** techniques. **Canadian Journal of Forest Research 14:** 10–6.

SCHENK, R. U.; HILDEBRANDT, A. C. 1972: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. **Canadian Journal of Botany 50:** 199–204.

SOMMER, H .E.; BROWN, C. L.; KORMANIK, P. P. 1975: Differentiation of plantlets in long leaf pine (**Pinus palustris** Mill.) tissue cultured **in vitro**. **Botanical Gazette 136:** 196–200.

THOMPSON, D. G.; ZAERR, J. B. 1981: Induction of adventitious buds on cultured shoot tips of Douglas fir (**Pseudotsuga menziesii** (Mirb.) Franco). Pp. 167–74 **in** "Colloque International sur la Culture **In Vitro** des Essences Forestières". IUFRO, Fontainebleau, France, 31 August to 4 September.

von ARNOLD, S. 1984: Importance of genotype on the potential for **in vitro** adventitious bud production of **Picea abies**. **Forest Science 30:** 314–8.

von ARNOLD, S.; ERIKSSON, T. 1977: A revised medium for growth of pea mesophyll protoplasts. **Physiologia Plantarum 39:** 251–60.

——— 1979: Induction of adventitious buds on buds of Norway spruce (**Picea abies**) grown **in vitro**. **Physiologia Plantarum 45:** 29–34.