PROC. N.S. INST. SCI. (1983)
VOLUME 33, pp. 123-129

INDUCTION AND SUBCULTURE OF CALLUS FROM PETIOLES OF *FRAXINUS AMERICANA* (L.), WHITE ASH.

G. HICKS AND R. BROWNE
Department of Biology
Dalhousie University
Halifax, N.S. B3H 4J1

Terminal buds of *Fraxinus americana* L., white ash, were forced in a 16 hr photoperiod at 28°C. Proximal petiole segments were excised, disinfested, and plated on semi-solid Murashige/Skoog medium, containing 2,4-D and kinetin used singly or in various combinations.

After 3 weeks incubation, the best callus production occurred when 1.0 ppm 2,4-D + 1.0 ppm kinetin were applied to either light- or dark-grown segments. On the same medium, subcultured callus grew better in the light. Some calluses could be subcultured at regular intervals in the dark for as long as 24 months, on Linsmaier/Skoog medium with pyridoxine, 2,4-D and kinetin.

La croissance des bourgeons terminau de *Fraxinus americana* L., le frêne blanc, fut hâtée par une photoperiode de 16 hr. à 28°C. Les segments proximaux des pétioles furent coupés, désinfectés et furent piqués sur un milieu semi-solide de type Murashige/Skoog, content du 2,4-D et de la kinetine séparément on en combinaisons variées. Après trois semaines d'incubations, la meilleure production de cals fut enrégistrée quand les segments croissant en lumière ou à l'obscurité furent traités avec un solution de 1.0 ppm de 2,4-D + 1.0 ppm de kinetine. Pour le même milieu, les sous-cultures de cals croissaient mieux en lumière. Quelque cals ont pa être mis en sous-culture, à intervalle régulier à l'obscurité, pour une période aussi longue que 24 mois en utilisant un milieu de type Linsmaier/Skoog continent de la pyridoxine, de 2,4-D, et de la kinetine.

Introduction

Mott (1981) has reviewed the establishment and uses of callus cultures from trees. He notes the lengthy history of these studies and the fact that over 200 species have been investigated. From this and other reviews (Bonga 1980, 1981; Sommer & Caldas 1981; Karnosky 1981) it seems clear that major breakthroughs in propagative techniques *in vitro* have yet to be realized, especially regarding forest trees. Nonetheless, tree callus has proven to be useful in studies of somatic embryogenesis (Sondahl *et al.* 1981), organogenesis (Venverloo 1973; Srivastava & Steinhauer 1981) and differentiation (Wright & Northcote 1973). In recent decades relatively few studies on the genus *Fraxinus in vitro* have appeared. Wolter and Skoog (1966) started callus on root-sprout segments of *F. pennsylvanica*. Continuous subculture was possible on a modified Reinert and White's medium, with myoinositol, pyridoxine and an auxin. Doley and Leyton (1970) examined growth and differentiation of stem callus from *F. excelsior* L. Both the water potential of the medium and indoleacetic acid were influential.

Recently, we began work on various types of somatic cells of *F. americana* L., white ash, with the long-range goal of vegetative propagation. In this preliminary report we document conditions for callus induction and subculture from petiole sections.

Methods

Most of the experiments used plant material from mature trees on the eastern edge of MacDonald Lake, Dartmouth, Nova Scotia, unless otherwise described. Branches were harvested in January, 1982 and twigs from these were stored in darkness at 4°C. Prior to each experiment, terminal buds were forced under controlled conditions in an attempt to generate "uniform" leaf material for inoculation. Twigs were brought to room temperature, trimmed to a length of

about 25 cm and placed standing in containers of tap water. Batches of such twigs were incubated at 28°C under a 16 h photoperiod. Illumination was provided by 4 fluorescent lamps (General Electric F40 CW) and 3 incandescent ones (General Electric 40 W), set at 40 cm above the containers (55 μ Einsteins sec $^{-1}$ M^{-2}). Terminal buds flushed in about 2 wks usually producing 2 pairs of expanded leaves. Petiole segments proximal to the leaflets were excised and disinfested as follows:

(a) 1 min in 70% ethanol
(b) 3 five-min rinses in sterile distilled water
(c) 10 min in aqueous commercial bleach (Javex: water 1:1)
(d) 3 five-min rinses in sterile distilled water

Petiole segments were transferred to a sterile Petri dish, the ends were trimmed off and then 0.5 cm segments were cut for inoculation. The basal culture medium (BM) was that of Murashige and Skoog (Flow Laboratories, Virginia, U.S.A.), with 3% sucrose, 0.8% agar, at pH 5.6. Double distilled water was used throughout. BM was sterilized by autoclaving 15 min. The auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin, kinetin (KIN), were tested for their ability to induce callus formation. Each hormone was tested alone at 0.01, 0.1 and 1.0 ppm, and in all combinations using those 3 concentrations. Stock solutions of hormones were sterilized by passage through a Millipore filter apparatus with 0.22 μ pore size. Requisite volumes of hormone stocks were added to cooling autoclaved solutions of BM. Media were decanted into presterilized plastic Petri dishes (8.5 cm diameter) at 25 ml per dish, and allowed to set. Control plates consisted of BM only. Two petiole segments were inoculated into each dish; dishes were sealed with masking tape. Cultures were incubated for 3 wks in the light under the same conditions as the flushing twigs, or in a dark cabinet in the same room. The dark experiment was attempted three times and the light experiment twice. For most of the 16 treatments, a total of 12-15 petiole segments have been cultured. At termination, a semi-quantitative assessment of callus production was made as follows. The two ends of petioles were examined carefully using a Wild M5 dissecting microscope. Using the 12 X objective and a calibrated ocular micrometer aligned along the long axis of the segment, callus size was measured, from the cut end of the petiole to the outer edge of the callus mass (Fig 1). This procedure afforded a sensitive detection method for even small amounts of petiolar callus. When it became obvious that BM with 1.0 ppm 2,4-D and 1.0 ppm kinetin was the most effective medium, its effects were examined in repeat experiments. In the dark, an additional 34 petiole segments were cultured, and an additional 64 in the light.

To produce callus for subsequent subculture and growth measurements, we first inoculated petioles on BM + 1.0 ppm 2,4-D and 1.0 ppm KIN. After 3 weeks, callus from dark-grown cultures was excised and taken through 2 dark subcultures, each of 3 weeks duration. The medium was of the same composition as before, with 40 ml aliquots dispensed in baby food jars with Parafilm (American Can Co.) closures. This tissue was then cut into cubes measuring 3 mm to the side and inoculated on the usual medium. These cultures were incubated in the dark, or under the same light conditions as previously described. After 3 weeks, the fresh weight of each piece was determined on an analytical balance. This growth experiment was repeated once.

A different collection of plant material was used for long-term experiments. Twigs collected in late June, 1979, in a ravine near Wentworth, Nova Scotia, were stored for 2 weeks in the cold, then flushed as previously described. Disinfested petiole segments were inoculated on Linsmaier/Skoog medium containing kinetin at 0.1 ppm and 2,4-D at 1.0 ppm, in 50 ml Erlenmeyer flasks in the dark. Calluses were subcultured on the same medium in 125 ml Erlenmeyer flasks, with a passage

time of 2-3 weeks for the first four passages, and 4-5 weeks for the remaining passages for a total of 24 months. After the sixth passage, pyridoxine (0.1 mgm L^{-1}) was included in all media.

Results

It soon became evident that the medium BM + 1.0 ppm 2,4-D + 1.0 ppm KIN generated the largest callus masses (Table I). The largest (average) callus was observed on dark-grown petioles on this medium. Individual pieces as large as 4.40 mm were measured. In separate experiments, this result was substantiated. Thus, 34 additional dark-grown segments averaged 2.25 mm callus, whereas 64 segments in the light averaged 1.91 mm callus.

Periodic observations of the segments on this 'optimal' medium revealed that after one week, the cut ends had swollen and callus had just appeared. After two weeks, a compact nodular callus had developed, often wider than the swollen ends (Fig 1). Dark-grown callus was either gray-white or a light tan colour, while in the light, callus was white on its surface, with a light-green cast to the underlying callus tissues. Often there was a substantial difference in the amount of callus produced at the two ends of an individual segment. Some dark-grown segments produced substantial amounts of callus on the petiole surface in contact with the semi-solid medium.

Table I Callus production from ash petioles on various media

Hormone supplement (ppm)		Average callus size (mm) Dark-grown	Light-grown
None	(BM)	0.04	0.14
2,4-D	(0.01)	0.10	0.17
	(0.1)	0.26	0.18
	(1.0)	0.57	0.50
KIN	(0.01)	0.07	0.06
	(0.1)	0.14	0.14
	(1.0)	0.16	0.09
2,4-D	(0.01)		
+KIN	(0.01)	0.14	0.13
	(0.1)	0.11	0.18
	(1.0)	0.16	0.19
2,4-D	(0.1)		
+KIN	(0.01)	0.24	0.22
	(0.1)	0.16	0.18
	(1.0)	0.42	0.37
2,4-D	(1.0)		
+KIN	(0.01)	0.38	0.30
	(0.1)	0.46	0.44
	(1.0)	1.80	1.21

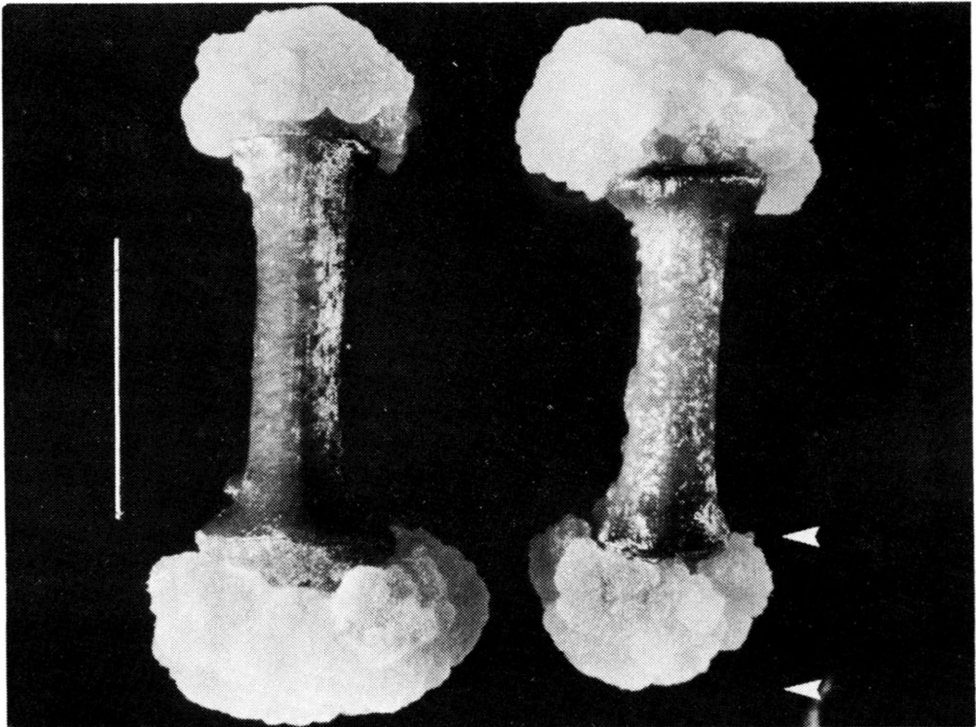

Fig 1 Petiole segments cultured for 21 days in the light. Arrows indicate the measurement taken as an index of callus size. Bar is equal to 0.5 mm.

The majority of the other hormone supplements produced only modest amounts (less than .50 mm) of callus (Table I), except for 2,4-D at 1.0 ppm. Within any one of the low-yielding treatments a variable number of cut ends had no measurable callus. In some treatments, the proportion of such segments was over 50% of the sample. Contrastingly, BM + 1.0 ppm 2,4-D + 1.0 ppm KIN invariably yielded some callus on all cut ends.

The average starting weight of callus cubes used in the growth determinations was 30 mg (Table II). Over the two experiments, the light-grown pieces showed a higher average growth index (6.5) than did their dark-grown counterparts (5.4, Table II). Obviously there was considerable variation in growth, as indicated by the final weight ranges.

Calli from the Wentworth material had a requirement for pyridoxine. Unless the latter was included in media after the 6th passage, calli turned brown and stopped proliferating. With pyridoxine, calli continued as white proliferating masses in the dark. After 23 passages, a growth index of 2.2 was measured for these cultures.

Discussion

In our view, we have established a reliable method for consistently inducing ash callus which can then be subcultured. For good callus proliferation on the primary inocula, both 2,4-D and kinetin were essential. Somewhat similar results have been found with leaf explants from other woody species. For example, leaf *blade* explants responded by callusing well on media supplemented with naphthalene acetic acid (NAA) and kinetin, in the case of *Citrus grandis* (Chaturvedi and Mitra 1974), *Malus pumila* Mill cv. Golden Delicious (Mehra and Sachdeva 1979) and

Table II Fresh weight increases of subcultured ash callus[a]

Experiment	Average callus weight (mg) Initial	Final	Final Weight Range (lowest-highest)	Growth Index[b]	Average Growth Index
Light-grown					
1.	29.6	213.1	(131.2-	7.2	
	(30)[c]	(20)[d]	294.4)		6.5
2.	27.5	156.6	(82.0-	5.7	
	(30)	(24)	318.4)		
Dark-grown					
1.	29.6	165.3	(70.6-	5.6	
	(30)[c]	(14)[d]	373.0)		5.4
2.	27.5	140.6	(70.4-	5.1	
	(30)	(20)	225.0)		

[a]Growth period = 3 weeks; [b]Average final weight/average initial weight; [c]For each experiment, 30 representative cubes were weighed and discarded; [d]Number of replicate pieces of callus.

Pyrus communis var. communis (Mehra and Jaidka 1979). In contrast, leaf blade tissue of *Prunus amygdalus* seedlings required only an auxin for callus formation (Mehra and Mehra 1974). In an extensive study using 7 mm^2 leaf squares of *Coffea arabica* cv. "Bourbon", kinetin and 2,4-D in a 2:1 ratio were found to be superior to a wide range of other auxin/cytokinin combinations (Sondahl *et al.* 1981). Leaf *petiole* explants of *Coffea arabica* cv. "Mundo novo" callused on L/S medium with 2,4-D and kinetin (Sharp *et al.* 1973), as in the present study. Petioles of *Tilia americana* L. callused on White's medium with 2,4-D and 20% coconut milk (Barker 1969). An auxin and a cytokinin have been used for callus induction in other tissues from woody species. For example, stem segments of 11 species of Rosaceae callused in response to both hormones (Coffin *et al.* 1976) as in several cultivars of *Malus pumila* (Chong and Taper 1974). On the other hand, root sprouts of *Populus tremuloides* callused on Wolter's medium with 2,4-D only (Winton 1968a, 1968b), and hypocotyl segments of *Acer pseudoplatanus* needed only NAA for callus induction (Wright and Northcote 1973). Stump sprouts of *Fraxinus pennsylvanica* callused on Reinert and White medium with kinetin at 1 mg L^{-1} (Wolter and Skoog 1966). It thus appears that the hormonal requirements for callus induction in woody species are quite different between species. Precise comparisons are obviously difficult, due to differences in basal media, plant age and culture conditions.

We have also demonstrated that ash petiole callus can be readily subcultured in the light or dark, and for prolonged periods. Growth indices of subcultured callus of our *F. americana* may be compared with indices in other studies. Growth indices similar to white ash have been reported for *Prunus persica, Prunus nigra* (Coffin et *al.* 1976) and for *Malus pumila* cv. Cortland (Chong and Taper 1972). Considerably higher growth indices were documented for callus of *Prunus cerasus, Prunus avium, Pyrus communis, Amelanchier arborea, Crataegus* x *mordensis, Malus*

pumila var. *niedzwetzkyana* and *Amelanchier alnifolia* (Coffin *et al.* 1976) and for *Acer pennsylvanicum* (Mathes 1967). Somewhat lower growth indices than those in the present work have been noted for *Malus robusta* No. 5 (Chong and Taper 1972), *Acer pennsylvanicum* and *A. pseudoplatanus* (Mathes *et al.* 1971). Again, the interpretation of these differences is rendered difficult by major differences between studies, such as in the growth period, basal media, etc. Two notable differences in the behaviour of subcultured callus of *F. americana* and *F. pennsylvanica* (Wolter and Skoog 1966) should be mentioned. The latter callus had much higher growth indices and secondly, that callus had an absolute requirement for pyridoxine in the culture medium as did the Wentworth calli. These differences may relate to the different origins of callus of each species, and to differing culture conditions.

Acknowledgements

It is a pleasure to acknowledge the technical help of Pamela Craigie and Yvon Rochon.

We are grateful for the financial support of the Natural Sciences and Engineering Research Council of Canada.

References

Barker, W.G. 1969. Behaviour in vitro of plant cells from various sources within the same organism. *Can. J. Bot. 47:* 1334-1336.

Bonga, J.M. 1980. Plant propagation through tissue culture, emphasizing woody species. In: *Plant cell cultures: results and perspectives.* (ed F. Sala, B. Parisi, R. Cella and O. Ciferri). Elsevier North-Holland Biomedical Press. pp. 253-263.

Bonga, J.M. 1981. Vegetative propagation of mature trees by tissue culture. In: *Proceedings of the committee on science and technology in developing countries. Symposium on tissue culture of economically important plants.* (ed A.N. Rao). Singapore. pp. 191-196.

Chaturvedi, H.C. and **Mitra, G.C.** 1974. Clonal propagation of *Citrus* from somatic callus cultures. *Hortscience* 9: 118-120.

Chong, C. and **Taper, C.D.** 1972. *Malus* tissue cultures. I. Sorbitol (D-glucitol) as a carbon source for callus initiation and growth. *Can. J. Bot. 50:* 1399-1404.

Chong, C. and **Taper, C.D.** 1974. Influence of light intensity on sorbitol metabolism, growth and chlorophyll content of *Malus* tissue cultures. *Ann. Bot. 38:* 359-362.

Coffin, R., Taper, C.D. and **Chong, C.** 1976. Sorbitol and sucrose as carbon sources for callus culture of some species of the *Rosaceae. Can. J. Bot. 54:* 547-551.

Doley, D. and **Leyton, L.** 1970. Effects of growth regulating substances and water potential on the development of wound callus in *Fraxinus. New Phytol. 69:* 87-102.

Karnosky, D.F. 1981. Potential for forest tree improvement via tissue culture. *Bioscience 31:* 114-120.

Mathes, M. 1967. The in vitro growth of *Acer saccharum* and *Acer pennsylvanicum callus tissue. Can. J. Bot. 45:* 2195-2200.

Mathes, M., Morselli, M. and **Marvin, J.W.** 1971. The in vitro growth of *Acer saccharum* tissue. *Can. J. Bot. 49:* 495-500.

Mehra, P.N. and **Jaidka, K.** 1979. In vitro morphogenetic studies in pear. *Phytomorphol. 29:* 286-297.

Mehra, A. and **Mehra, P.N.** 1974. Organogenesis and plantlet formation in almond. *Bot. Gaz. 135:* 61-73.
Mehra, P.N. and **Sachdeva, S.** 1979. Callus cultures and organogenesis in apple. *Phytomorphol. 29:* 310-323.
Mott, R.L. 1981. Trees. In: *Cloning agricultural plants via in vitro cloning techniques.* (ed. B.V. Conger). CRC Press, Inc. Boca Raton, Florida. pp. 217-254.
Sharp, W.R., Caldas, L.S., Crocomo, O.J., Monaco, L.C. and **Carvalho, A.** 1973. Production of *Coffea arabica* callus of three ploidy levels and subsequent morphogenesis. *Phyton 31: 67-74.*
Sommer, H.E. and **Caldas, L.S.** 1981. In vitro methods applied to forest trees. In: *Plant tissue culture. Methods and applications in agriculture.* (ed. T. Thorpe). New York. pp. 349-358.
Sondahl, M.R., Monaco, L.C. and **Sharp, W.R.** 1981. In vitro methods applied to coffee. In: *Plant tissue culture. Methods and applications in agriculture.* (ed. T.A. Thorpe). Academic Press, New York. pp. 325-347.
Srivastava, P.S. and **Steinhauer, A.** 1981. Isozymes in differentiating shoot cultures of *Betula pendula* Roth. *Z. pflanzenphysiol. 103:* 341-346.
Venverloo, C.J. 1973. The formation of adventitious organs. I. Cytokinin-induced formation of leaves and shoots in callus cultures of *Populus nigra* L. "Italica". *Acta Bot. Neerl. 22:* 390-398.
Winton, L.L. 1968a. The initiation of friable aspen callus. *Phyton 25:* 15-21.
Winton, L.L. 1968b. Initiation of friable aspen callus under different light environments. *Phyton 25:* 23-28.
Wolter, K.E. and **Skoog, F.** 1966. Nutritional requirements of *Fraxinus* callus cultures. *Am. J. Bot. 53:* 263-269.
Wright, K. and **Northcote, D.H.** 1973. Differences in ploidy and degree of intercellular contact in differentiating and non-differentiating sycamore calluses. *J. Cell Sci. 12:* 37-53.