# III.4 Micropropagation of *Juglans cinerea* L. (Butternut)

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## 1 Introduction

The genus *Juglans* L. (family Juglandaceae) comprises about 20 species of deciduous trees. These monoecious trees are native to North and South America, and from southeastern Europe to eastern Asia (Bailey and Bailey 1976). They are grown as ornamentals, for the edible nuts, and some species for the fine-grained wood highly valued for furniture, veneer, gunstocks, and cabinet work. The Persian walnut (*J. regia* L.) is the most horticulturally developed and widely cultivated species for nut production (McGranahan and Leslie 1990). Worldwide production of walnuts in 1992 was listed at 918 180 metric tons, with the United States accounting for approximately 20% of world production at 181 400 metric tons (FAO 1992). Black walnut (*J. nigra* L.) is one of the most valuable hardwoods produced in the United States (Williams 1990). Eastern black walnut is also grown for the edible nuts, but it is the species valued economically for its high quality wood, prized for fine furniture, gunstocks, cabinets, and veneer.

Butternut (*J. cinerea* L.) (Fig. 1A), a species native to North America from New Brunswick to Georgia, and west to Minnesota and Arkansas (Rink 1990), is valued economically and ecologically for its wood and edible nuts. The nut is an important wildlife mast and the wood is marketable for many uses including furniture, cabinets, fine woodworking, and paneling. In areas where quality butternut wood is available, it ranks eighth out of the top 28 species for prime veneer and sawlogs (Peterson 1990). Butternut, also known as white walnut, is relatively slow-growing with an average height of 12 – 18 m, but can attain heights of 30 m (Rink 1990). Moist, rich soils of hillsides and stream banks are the preferred growing sites, although butternut can grow quite well on dry, rocky soils.

Male and female flowers (Fig. 1B) are borne separately on the same tree, but mature at different times (McDaniel 1956). Male flowers (catkins) are preformed on the previous year's wood, appearing as small, scaly, cone-like buds (Dirr 1983). Female flowers occur in two- to eight-flowered spikes borne on the current year's shoots. Butternut can be distinguished from black walnut by stems having a chambered, chocolate-brown pith and a large, conspicuous

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leaf scar surmounted by a raised, downy pad. The fruit (Fig. 1C) is a drupe-like, furrowed nut enclosed in a thick, indehiscent husk that develops from a floral involucre (Brinkman 1974). The nut is two-celled, has a hard outer wall (pericarp), and exhibits eight prominent ridges on the shell. Fruits (nuts) are ovoid-oblong, up to 6.4 cm long, sticky-pubescent, occur singly or in clusters of from two to five, and are edible, sweet, and oily. Seeds of butternut, like most *Juglans* spp., have a dormant embryo (Dirr 1983). Dormancy can be broken by fall sowing or by moist, prechilling of seeds at 3 – 5 ºC for 3 – 4 months. Butternut has a ridged and furrowed bark; the ridges are whitish and the furrows are grayish black. The inner bark of butternut has mild cathartic properties, and was used in older times as an orange or yellow dye (Dirr 1983).

The foliage, bark, wood, branches, and fruit of butternut are susceptible to attack by a number of insects and disease organisms. The most serious insect pest is the butternut curculio, *Conotrachelus juglandis* LeConte (Johnson and Lyon 1988). Feeding by the larvae and adults causes severe damage to the nuts, young stems, leaf petioles, and branches of butternut. Butternut canker disease, incited by the fungus *Sirococcus clavigignenti-juglandacearum* N.B. Nair, Kostichka and Kuntz (Nair et al. 1979), has caused widespread mortality (Tisserat and Kuntz 1984) and threatens butternut as a species (Orchard et al. 1982). Perennial cankers can arise on all aboveground parts of the tree, even the buttress roots (Sinclair et al. 1987), and trees of all ages and sizes are susceptible. Eventually, the girdling effect of multiple coalescing cankers kills the tree.

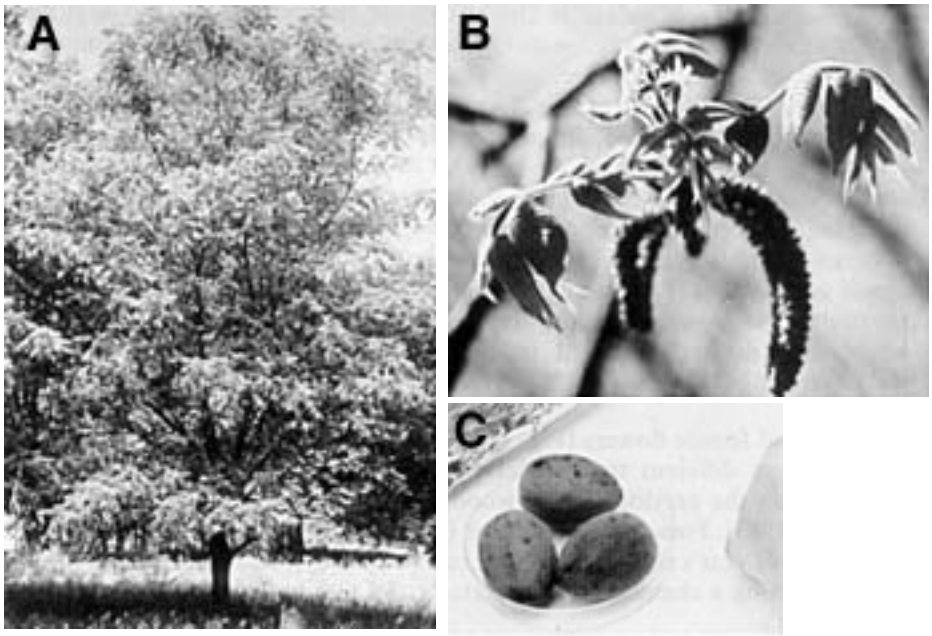


**Fig. 1A – C.** Morphological characteristics of *Juglans cinerea.* **A** Growth habit. **B** Flowering. **C** Fruit. (**A** Courtesy M.E. Ostry)

Propagation of butternut is usually by seed, but evidence exists that the canker disease may have seedborne transmission (Orchard 1984). Vegetative propagation of mature tree specimens, through rooting of cuttings, has been largely unsuccessful. Cultivars of *Juglans* spp. are vegetatively propagated by grafting onto seedling rootstocks. Various degrees of success has been reported for intraspecific as well as interspecific grafting in the genus *Juglans* (Kaeiser and Funk 1971; Xi and Ding 1990). *J. cinerea* can be propagated by grafting onto black walnut rootstock, with limited success.

Maintaining butternut as a viable species, and the potential genetic improvement of existing butternut germplasm, depend on the application and further development of in vitro techniques. In vitro propagation and selection for resistance to biotic and abiotic stresses have the potential to provide valuable germplasm and propagules for a butternut tree improvement program.

## 2 In Vitro Culture and Micropropagation

Research with *Juglans* spp. has focused on several different biotechnological approaches and initial explant material (Table 1) for the purpose of clonal mass propagation and subsequent genetic improvement (see reviews by Preece et al. 1989; Rodriguez et al. 1989). Progress in developing techniques for in vitro culture of about five *Juglans* spp. and hybrids has been very successful over the past 10 years. Plantlets have been obtained via shoot-tip multiplication, cultured nodal segments, and somatic embryogenesis. However, few reports have been published on attempts to micropropagate *J. cinerea* (Pijut 1993a,b, 1994).

### 2.1 Callus Culture

*Juglans cinerea* callus formation has been initiated on stem internodal sections, obtained from 2-3-month-old seedlings, cultured on MS medium supplemented with 0 – 1.2 µM kinetin and 2.3 µM 2,4-D (Pijut, unpubl.). This callus was white, compact, hard, and very slow growing. Attempts to regenerate shoots or somatic embryos from this type of callus have so far been unsuccessful. When BA and 2,4-D were incorporated into the initial culture medium, and leaf pieces were used as explants, explant death ensued.

### 2.2 Axillary Bud Culture

Several reports indicate that *Juglans* species are amenable, to a certain degree, to micropropagation (Somers et al. 1982; Driver and Kuniyuki 1984; Meynier 1985; Heile-Sudholt et al. 1986; Lee et al. 1986; McGranahan et al. 1988; Revilla et al. 1989; Felaliev 1990; Gruselle and Boxus 1990; Leslie and McGranahan 1992).

**Table 1.** Summary of in vitro studies with *Juglans* spp. (see also Preece et al. 1989; Rodriguez et al. 1989; Tulecke et al. 1995)

| Species | Explant | Study/results | Reference |
|---|---|---|---|
| *J. cinerea* | Immature cotyledons | Somatic embryogenesis; callus; roots; germinants | Pijut (1993a,b) |
| *J. nigra* | Immature cotyledons | Somatic embryogenesis; shoot organogenesis | Long et al. (1992) |
| *J. nigra* | Immature to mature cotyledons | Somatic embryogenesis; callus;roots | Neuman et al. (1993) |
| *J. nigra, J. major,* interspecific hybrids | Immature cotyledons | Somatic embryogenesis; germinants | Cornu (1988) |
| *J. nigra* x *J. regia* | Embryonic axes | Shoots | Cornu and Jay-Allemand (1989) |
| *J. nigra* x *J. regia* | Shoot tips | Rooted plantlets | Meynier and Arnould (1989) |
| *J. nigra* x *J. regia* | Cotyledon segments with embryonic axis attached | Roots; Histology of root structures | Jay-Allemand et al. (1991) |
| *J. nigra* x *J. regia* | Immature cotyledons | Maturation, germination of somatic embryos | Deng and Cornu (1992) |
| Interspecific hybrids | Embryonic axes | Improved rooting of microshoots; plants | Jay-Allemand et al. (1992) |
| *J. regia* | Cotyledon | Somatic embryogenesis; plants | Tulecke and McGranahan (1985) |
| *J. regia* | Immature cotyledons | Histology of somatic embryo origin | Polito et al. (1989) |
| *J. regia* | Embryonic axes; nodal segments | Shoots; rooted plants | Revilla et al. (1989) |
| *J. regia* | Apical and lateral buds | Shoot formation | Felaliev (1990) |
| *J. regia* | Axillary buds | Shoot multiplication; rooted plants | Stephens et al. (1990) |
| *J. regia* | Ovules | Somatic embryo origin determined by RFLP and isozyme analysis | Aly et al. (1992) |
| Clone TRS | Microshoots | Improved acclimatization of plantlets | Voyiatzis and McGranahan (1994) |
| *J. regia,* J. hindsii x *J. regia* | Nodal segments | Shoot multiplication; rooted shoots | Gruselle and Boxus (1990) |
| *J. regia* x *J. nigra* | Nodal segments | Pi nutrition in relation to callus and shoot development | Barbas et al. (1993a) |
| *J. regia* x *J. nigra* | Embryonic axes | Gelling agent effects on shoot growth | Barbas et al. (1993b) |

Abbreviations: Pi, orthophosphate; RFLP, restriction fragment length polymorphism.

Micropropagation of butternut by axillary bud culture has been demonstrated by Pijut (unpubl. ). This chapter outlines the successful in vitro propagation of *J. cinerea* from seedling origin, which will provide a basis for the development of techniques to micropropagate selected mature, disease-resistant butternut trees.

Mature, dormant seeds were purchased commercially (F. W. Schumacher Co., Inc., Sandwich, Massachusetts) and stored at 5 ºC before being used. Seeds were then stratified in moist sand at 5 ºC in darkness for 90 – 120 days. After stratification, seeds were germinated (four per pot) in 19-cm plastic pots containing a 1 peat: 1 perlite (v/v) medium. Seedlings were maintained in a growth chamber at 30ºC under clear, krypton, incandescent lamps (54 µmol/ $m^2$/s) with a 12-h photoperiod followed by 20 ºC in darkness for 2 to 3 months.

Stems from actively growing seedlings were stripped of leaflets and surface disinfested in 0.8% (v/v) sodium hypochlorite (15% Clorox bleach) for 15 – 20 min, followed by four rinses in sterile, deionized water. Nodal explants (1 to 2 cm long) were excised and placed upright in 25 x 95-mm culture vials containing 12 ml of MS medium (Murashige and Skoog 1962) supplemented with 200 mg/1 casein hydrolysate (CH), 8.9 µM 6-benzyladenine (BA), 3% (w/v) sucrose, and 0.22% (w/v) Phytagel (Sigma Chemical Co., St. Louis, Missouri). The pH of the medium was adjusted to 5.7 before autoclaving at 121 ºC for 20 min. All explants were transferred to fresh medium weekly for 4 weeks prior to rooting studies. Cultures were incubated at 26 ºC with an 18-h photoperiod provided by cool white fluorescent lamps (92 µmol/$m^2$/s).

In preliminary studies we also tested Driver and Kuniyuki (DKW) medium (1984) and woody plant medium (WPM; Lloyd and McCown 1980), various factorial combinations of BA, indole-3-butyric acid (IBA), thidiazuron (NOR-AM Chemical Co., Wilmington, Delaware), α-naphthaleneacetic acid (NAA), and Difco Bacto agar versus Phytagel as gelling agents.

Microshoots (1.5 to 2 cm long) with at least three axillary buds were excised from the original explant and used in rooting experiments. Root initiation treatments consisted of half-strength MS medium containing 0, 2.5, 4.9, 14.8, or 24.6 µM IBA, 100 mg/1 CH, 1.5% (w/v) sucrose, and 0.22% (w/v) Phytagel in Magenta GA7 vessels (five microshoots per vessel containing 50 ml media). Microshoots were pulsed in auxin-containing medium in darkness at 26 ºC for 7, 14, or 21 days for root initiation.

Microshoots were transferred to a root development medium (50 ml in GA7 vessels) of half-strength MS containing 100 mg/1 CH, 2% (w/v) sucrose and 0.22% Phytagel. Cultures were incubated at 26 ºC under an 18-h photo-period provided by cool white fluorescent lamps (92 µmol/$m^2$/s). The number of adventitious roots per shoot, root length (mm), and shoot length (mm) were recorded 3 weeks after transfer to root development medium.

Rooted microshoots were planted in a sterile medium of 1 perlite: 1 vermiculite (v/v) in aluminum deli trays (13 x 10.5 x 4 cm deep) and covered with a clear plastic lid to maintain high relative humidity. Plantlets were watered weekly and maintained under the same environmental conditions as stated above for root development. Plantlets were acclimatized to the ex vitro environment over time (approx. 1 month), by loosening the plastic lid, placing the lid ajar on the tray, followed by complete removal of the plastic lid.

Axillary buds elongated when explants were cultured on a modified MS medium supplemented with 8.9 µM BA for 4 weeks (Fig. 2A). Chalupa (1981) reported successful axillary bud elongation from nodal stem segments of *J. regia* seedlings cultured on MS medium containing 0.4 µM BA and 0.8 µM NAA. Gruselle et al. (1987), using a modified MS medium, found both 4.4 and 8.9 µM BA suitable for culturing nodal explants from young seedlings of *J. regia.* Culturing juvenile *J. cinerea* nodal explants on WPM or DKW media, regardless of growth regulators tested, resulted in the swelling and partial expansion of axillary buds, but with no significant elongation. In contrast, DKW medium has proven to be suitable (and in many cases superior) for the culture of *J. regia* as well as other *Juglans* species (Driver and Kuniyuki 1984; Heile-Sudholt et al. 1986; Lee et al. 1986; McGranahan et al. 1988; Leslie and McGranahan 1992). Weekly transfer of butternut nodal explants to fresh culture medium was necessary to maintain optimum growth and to limit the buildup of phytotoxic exudates in the culture medium. The production of exudates from freshly cultured explants of walnuts has also been a problem, solved by employing explant presoaks and transferring explants frequently to fresh medium (Preece et al. 1989; Leslie and McGranahan 1992).

Highest rooting frequency (75%) occurred on microshoots pulsed on half-strength MS containing 2.5 µM IBA for 7 days in darkness. Adventitious roots began to emerge within 7 days and elongated when microshoots were placed in the light (Fig. 2B). Although roots were also initiated on microshoots cultured on media containing 4.9 or 24.6 µM IBA, only the 2.5 µM IBA-rooted plantlets were successfully acclimatized ex vitro (Fig. 2C). In vitro and ex vitro rooting of micropropagated *Juglans* species has been successful using IBA with or without NAA (Leslie and McGranahan 1992). Much like black walnut and butternut seedlings, in vitro-derived plantlets initially increased root growth and not shoot growth (Fig. 2D).

### 2.3 Somatic Embryogenesis

Somatic embryogenesis has been induced in a number of species of *Juglans* (Table 1) and the subject has been recently reviewed (see Tulecke et al. 1995). In our work (Pijut 1993a), somatic embryogenesis was induced from immature cotyledonary explants of *J. cinerea.* The immature fruits collected at weekly intervals from 4 to 11 weeks postanthesis were surface sterilized in 50% (v/v) ethanol for 20 – 30 s, then 1% (v/v) sodium hypochlorite containing 0.01% Tween 20 for 20 min, followed by three rinses in sterile, deionized water. Embryos were removed aseptically and cotyledon explants (5-mm segments) placed on induction medium for 3 weeks. Explants were then transferred to development medium at 3-week intervals.

Induction media treatments consisted of either a DKW medium (Tulecke and McGranahan 1985) or MS medium supplemented with combinations of 0, 1.1, or 4.4 µM BA, 0 or 9.1 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0 or 9.3 µM kinetin, and 0 or 0.05 µM IBA, with or without 250 mg/1 L-glutamine or 1000 mg/1 CH. All media were supplemented with 3% (w/v) sucrose and

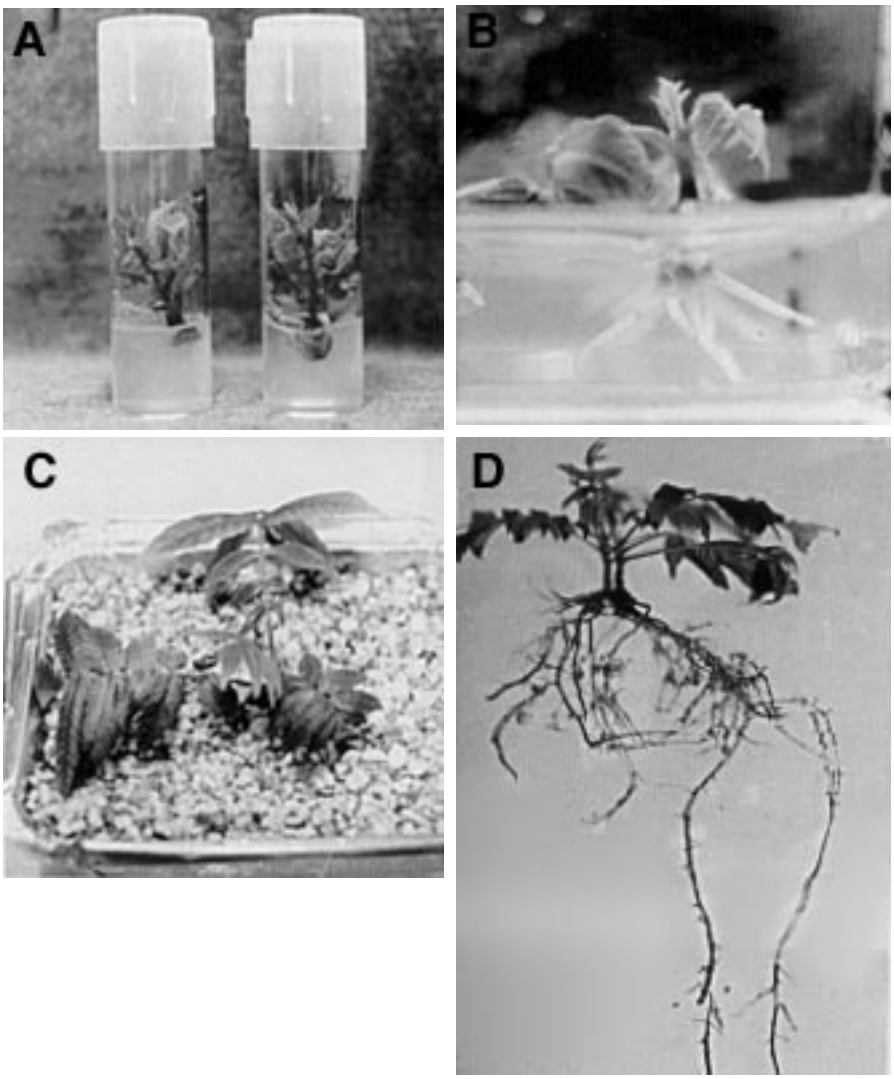


**Fig. 2A – D.** In vitro propagation of juvenile *Juglans cinerea.* **A** Elongation of axillary buds from nodal explants cultured on medium containing 8.9 µM BA. **B** Plantlet rooted on medium containing 2.5 µM IBA. **C** Butternut plantlet established in a perlite: vermiculite medium. **D** Functional and vigorous root system of acclimatized plant

0.24% (w/v) Gelrite (Merck Co.) or 0.7% (w/v) Difco Bacto agar. Development medium was of the same composition as induction media minus plant growth regulators and glutamine, but containing 200 mg/1 CH. Cultures were incubated in darkness at 26 ºC.

Globular to mature somatic embryos formed directly on the surface of cotyledons collected approximately 9 weeks postanthesis (Figs. 3, 4) when cultured on DKW medium supplemented with 250 mg/1 L-glutamine, 0.05 µM IBA, 4.4 µM BA, and 9.3 µM kinetin. Globular somatic embryos could be visualized as early as 8 weeks after culture initiation and appeared to originate adventitiously from the surface of noncallused cotyledon tissue. Explants collected 11 weeks postanthesis failed to initiate somatic embryos on the identical medium, but adventitious root formation occurred.

Embryogenic callus was initiated on cotyledon explants collected 8 – 11 weeks postanthesis when cultured on DKW or MS media containing 1.1 µM BA and 9.1 µM 2,4-D. Embryogenic callus was shiny, cream-colored, and nodular in appearance (Fig. 5). This callus developed directly on explants or on

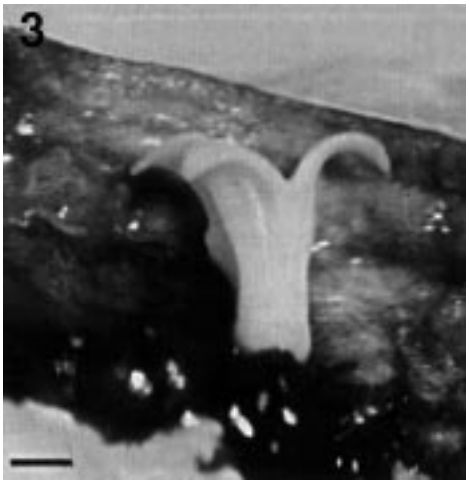


**Fig. 3.** Mature somatic embryo induced directly on immature butternut cotyledon explant collected 9 weeks postanthesis. *Bar* 1 mm. (Pijut 1993a)

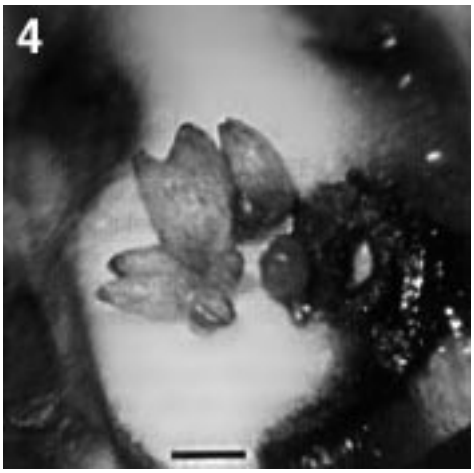


**Fig. 4.** Direct somatic embryos at various stages of development on the surface of original cotyledon explant. *Bar* 1 mm. (Pi-jut 1993a)

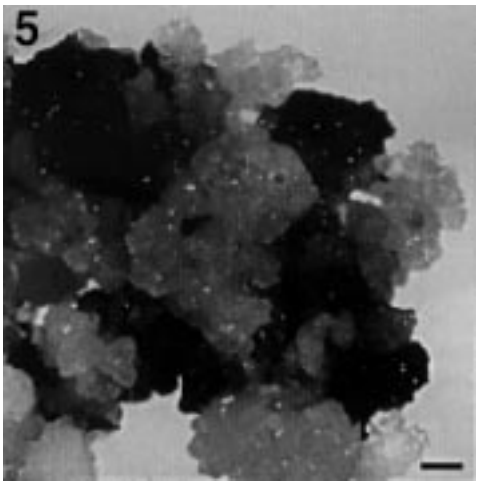


**Fig. 5.** Embryogenic callus produced from cotyledonary explants initiated on MS medium supplemented with BA and 2,4-D. *Bar* 1 mm. (Pijut l993a)

explants that had formed callus, but the callus had turned brown. Globular to mature somatic embryos were observed to dedifferentiate from this callus (Figs. 6, 7). Proliferation of somatic embryos was maintained by subculturing embryogenic callus every 3 weeks on development medium. Somatic embryos with developed cotyledons were subjected to a cold treatment (8 weeks at 5 ºC) in an attempt to break dormancy. This treatment resulted in swelling and greening of tissue after embryos were incubated in the light, but somatic embryos failed to germinate.

In another investigation, butternut somatic embryos were induced to break dormancy and germinate at a low frequency (1.7%) into visually normal plantlets (Pijut 1993b). Mature somatic embryos that were opaque with starch were stored at 5 ºC for 16 weeks in darkness, on half-strength MS or DKW

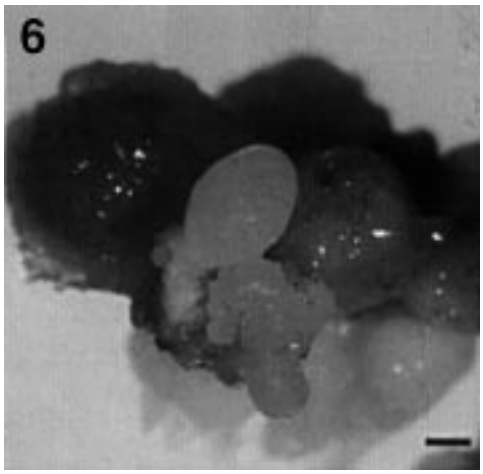


**Fig. 6.** Globular somatic embryo arising from nodular embryogenic callus produced on cotyledonary explants collected 9 weeks postanthesis. *Bar* 1 mm. (Pijut 1993a)

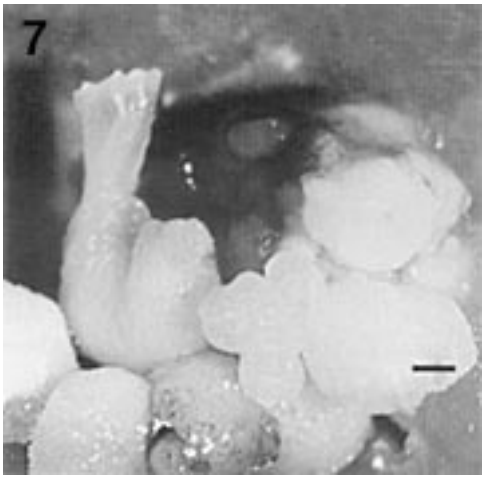


**Fig. 7.** Embryogenic callus with globular and torpedo-shaped somatic embryos. *Bar* I mm. (Pijut 1993a)

media containing 2% (w/v) sucrose. Somatic embryos germinated (Fig. 8) when placed in the light (16-h photoperiod, 26 ºC) on half-strength basal DKW medium containing 1.5% (w/v) sucrose. Plantlets were transferred to a vermiculite-perlite medium and survived acclimatization from the in vitro environment for approximately 3 months.

## 3 Summary and Conclusions

The clonal propagation of *J. cinerea* through axillary bud culture holds great promise for maintaining butternut as a viable species, and for the production

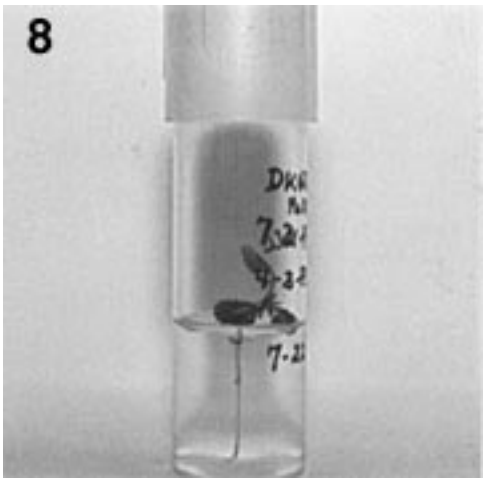


**Fig. 8.** Butternut plantlet regenerated from a somatic embryo subjected to cold treatment to induce germination. (Pijut 1993b)

of clones with unique genotypes, especially resistance to butternut canker disease. Optimum multiplication and plantlet survival rates of microshoots still need to be determined.

Immature cotyledonary tissue of *J. cinerea* is amenable to somatic embryogenesis. The ability to produce embryogenic cultures was dependent upon the developmental stage of the cotyledon explants. This developmental "window,, was 8 – 11 weeks postanthesis. Inclusion of 2,4-D in the induction medium was necessary for differentiation of embryogenic callus cultures. Germination of somatic embryos and plantlet survival were achieved, although at a low frequency. Refinement of the regeneration protocol to increase the frequency of somatic embryos able to germinate into whole plants will be useful in a research program aimed at increasing the butternut population. If maturation and germination techniques can be improved, and the number of plantlets increased, the potential exists for genetic improvement and multiplication of butternut.

## 4 Protocol

For micropropagation, culture nodal segments from seedlings for 4 weeks (18-h photoperiod) on MS medium supplemented with 200 mg/1 CH, 3% sucrose (w/v), 8.9µM BA, and 0.22% Phytagel. Initiate roots on excised microshoots by pulsing for 7 days in darkness on half-strength MS medium containing 100 mg/1 CH, 1.5% sucrose (w/v), and 2.5 µM IBA. For adventitious root elongation, transfer microshoots to half-strength MS with 2% sucrose (w/v), no growth regulators, and place cultures in the light. Rooted plantlets can then be successfully acclimatized ex vitro.

For somatic embryogenesis, excise immature zygotic embryos 8 – 11 weeks postanthesis and culture cotyledon segments on induction media for 3 weeks, then transfer to plant growth regulator-free development medium. Direct somatic embryos will form on explants cultured on DKW supplemented with 250 mg/1 L-glutamine, 0.05 µM IBA, 4.4 µM BA, and 9.3 µM kinetin. Embryogenic callus will form on explants cultured on DKW or MS media supplemented with 1.1 µM BA and 9.1 µM 2,4-D. Incubate all cultures in darkness at 26 ºC. Somatic embryos continue to develop when cultures are routinely transferred (every 3 weeks) to fresh development medium.

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