SOMATIC EMBRYOGENESIS FROM IMMATURE FRUIT OF JUGLANS CINEREA

Paula M. Pijut, USDA Forest Service, North Central Forest Experiment Station, 1992 Folwell Avenue, St. Paul, Minnesota 55108 USA

1. Introduction

1.1 ECONOMIC IMPORTANCE AND GEOGRAPHIC DISTRIBUTION

Butternut (*Juglans cinerea* L.) (Fig. 1a), also known as white walnut or oilnut, is a hardwood species in the family Juglandaceae, section *Trachycaryon* (Manning, 1978), or more appropriately, section *Cardiocaryon* (Fjellstrom & Parfitt, 1994). This hardwood species is valued for its wood and edible nuts. Native to North America from New Brunswick to Georgia, and west to Minnesota and Arkansas (Fig. 1b), butternut is not an abundant species and is seldom found in pure stands, but rather in association with several other tree species such as cherry, basswood, oak, walnut, ash, maple, elm, and hemlock (Rink, 1990). A relatively slow-growing tree, butternut attains an average height of 12-18 m and grows best on moist, rich, deep soils of bottom lands, although it also grows quite well in drier, rocky soils (Dirr 1990). Quality butternut wood commands a high market price for many uses including furniture, veneer, cabinets, paneling, and fine woodworking, and the nut is an important food source for wildlife. Historically, the inner bark of butternut and its nut husks were used to yield an orange or yellow dye for Confederate troop uniforms, and the inner bark has mild cathartic properties (Dirr, 1990; Peattie, 1950). Native Americans extracted oil from crushed butternuts by boiling them in water, and the sap of butternut has been used to make syrup (Goodell, 1984). There has been limited selection of butternuts for superior genotypes, other than for nut quality and production (Goodell, 1984; McDaniel, 1981; Milikan & Stefan, 1989; Milikan et al., 1990).

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Figure 1. a,b. Growth habit (a) and native range (b) of *Juglans cinerea*. (a Courtesy of M.E. Ostry).

1.2 BOTANICAL CHARACTERISTICS

Juglans *cinerea* is considered to be one of the most winter-hardy of the *Juglans* species. In spring, male flowers (catkins) preformed on the previous year's wood appear as small, scaly, cone-like buds, and the female flowers occur in two- to eight-flowered spikes borne on the current year's shoots (Fig. 2a) (Dirr 1990). Male and female flowers mature at different times (McDaniel 1956). The fruit (Fig. 2b) 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. Butternut can be distinguished from black walnut by stems having a chambered, chocolate-brown pith and a large, conspicuous leaf scar surmounted by a raised, downy pad (Dirr 1990). *Juglans cinerea* has a ridged and furrowed bark; the ridges are whitish and the furrows are grayish black, and upon exposure to air the inner bark turns yellow (Dirr 1990). Butternut trees produce seed at about 20 years of age, with good seed crops occurring every 2 to 3 years (Rink 1990). Seeds of butternut, like most *Juglans* spp., have a dormant embryo, but dormancy can be broken by fall sowing or by moist, prechilling of seeds at 3-5°C for 3-4 months (Dirr, 1990).

Figure 2. a,b. Flowering (a) and fruit (b) of *Juglans cinerea.*

1.3 DISEASES AND PESTS

Butternut is susceptible to attack by a number of insects and disease organisms. The most serious insect pest is the butternut curculio, *Conotrachelus juglandis* LeConte (Corneil & Wilson, 1979; Johnson & Lyon, 1988; LeConte, 1876; Wilson et al., 1979). Feeding by the larvae and adults causes severe damage to the nuts, young stems, leaf petioles, and branches of butternut. But, it is butternut canker, caused by the fungus *Sirococcus clavigignenti-juglandacearum* N.B. Nair, Kostichka and Kuntz (Nair et al., 1979), that has caused widespread mortality and threatens butternut as a species (Cummings Carlson, 1993; Orchard et al., 1982; Ostry, 1997; Tisserat & Kuntz, 1984).

Butternut canker was first observed in Wisconsin in 1967. Today, the canker can be found throughout most of the native range (Cummings Carlson, 1993; Davis et al., 1992; Haynes, 1994), and trees of all ages and sizes are susceptible. Perennial cankers can arise on all aboveground parts of the tree, even the buttress roots (Kuntz et al., 1979; Ostry et al., 1996; Sinclair et al., 1987). Removing the bark from around a canker (Fig. 3), illustrates the dark brown to black wood formed in an elliptical pattern, which reduces the quality of the wood and renders many stands unmarketable. Eventually, the girdling effect of multiple coalescing cankers kills the tree. A number of strategies, such as clonal propagation, screening putative resistant germ plasm, and developing silvicultural methods to assure regeneration, in order to maintain and restore butternut have been developed (Ostry et al., 1994).

Figure 3. Bark removed from cankered butternut tree to illustrate defective wood.

1.4 PROPAGATION

Juglans cinerea is usually propagated by seed, but evidence exists that the canker disease may have seedborne transmission (Orchard, 1984). Vegetative propagation of selected mature tree specimens, through rooting of cuttings and by grafting onto (black walnut) seedling rootstock, has proven to be expensive because of the low frequency of success. In vitro techniques offer an alternative for the propagation and preservation of existing butternut germ plasm (Pijut, 1993a,b; Pijut, 1997). The potential genetic improvement of butternut, and selection for resistance to butternut canker disease, exists with the application and further improvement of these techniques. Somatic embryogenesis has been induced and studied for several species of Juglans (Table 1), and in vitro studies with Juglans spp. has been the subject of recent reports (Pijut, 1997; Preece et al., 1989, 1995; Rodriguez et al., 1989; Tulecke et al., 1995). The objective of this communication is to provide an overview of the research on somatic embryogenesis from immature fruit of J. cinerea, in order to maintain butternut as a viable species.

TABLE 1. Summary of somatic embryogenesis and related studies with *Juglans* spp[a]

Species	Explant	Study / Results	References
J. cinerea	Immature cotyledons	Somatic embryogenesis; callus; roots; germinants	Pijut, 1993 a,b
J. cinerea, *J. nigra*, & *J. regia*	Embryo axes	Cryopreservation	Pence, 1990
J. nigra	Immature cotyledons	Somatic embryogenesis; shoot organogenesis	Long et al., 1992, 1995
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 hybrid	Cotyledons	Somatic embryogenesis; physiology & histology	Cornu, 1989
J. nigra hybrid	Somatic embryos	Cryopreservation	de Boucaud & Brison, 1995
J. nigra x *J. regia*	Immature cotyledons	Maturation, germination of somatic embryos	Deng & Cornu, 1992
J. nigra x *J. regia*	Somatic embryos	Cryopreservation	de Boucaud et al., 1994
J. regia	Immature cotyledons	Somatic embryogenesis; germinants	Lee et al., 1988
J. regia	Somatic embryos	Genetic transformation	McGranahan et al., 1988
J. regia	Endosperm tissue	Somatic embryogenesis; plants (triploids)	Tulecke et al., 1988
J. regia	Somatic embryos	Cryopreservation	Lee, 1989
J. regia	Immature cotyledons	Histology of somatic embryo origin	Polito et al., 1989
J. regia	4-11 week embryos	Somatic embryogenesis; plants	Boltivets & Piven, 1990
J. regia	Somatic embryos	Genetic transformation	McGranahan et al., 1990
J. regia	Ovule tissue	Somatic embryo origin, RFLP & isozyme analysis	Aly et al., 1992
J. regia	Leaf petioles	Somatic embryogenesis; histology	Liu et al., 1992
J. regia	Somatic embryos	Genetic transformation	Dandekar et al., 1994
J. regia, *J. hindsii*, & *Pterocarya* sp.	Immature cotyledons	Somatic embryogenesis; plants	Tulecke & McGranahan, 1985
Pterocarya sp. x *J. regia*	Immature cotyledons	Somatic embryogenesis; intergeneric hybrids	McGranahan et al., 1986
Not stated	Somatic embryos	Development & maturation	Huang & Sutter, 1997

[a]See reviews by Preece et al. 1995; Tulecke et al. 1995.

Abbreviations: RFLP, restriction fragment length polymorphism.

2. In Vitro Culture

2.1 EXPLANT

Immature fruit (nuts) were routinely collected 8-9 weeks postanthesis from canker-free J. cinerea specimen trees, located in Knapp, Wisconsin. Fruits were used for culture on the same day or stored at 2-4°C for <1 week. By late-July, early-August, the cotyledons were opaque white and had elongated to fill the locules. At this stage of development the pericarp was hardened and a sterile chisel and hammer were employed to open the fruit.

2.2 STERILIZATION

The nuts were surface sterilized in 50% (v/v) ethanol for 20-30 sec, then 1% (v/v) sodium hypochlorite containing 0.01% Tween-20 for 20 min, followed by three rinses in sterile, deionized water.

2.3 INDUCTION OF SOMATIC EMBRYOGENESIS

Embryos (Fig. 4) were removed aseptically and cotyledon explants (5 mm segments) cut from the embryo axes were placed horizontally (adaxial versus abaxial orientation random) in 25 x 95 mm culture vials containing 12 ml of induction media for 3 weeks. All explants were then transferred to development medium at 3-week intervals.

Figure 4. Immature zygotic embryo of *Juglans cinerea* at 8 weeks post-anthesis. Bar 1mm.

Induction media treatments consisted of either a Driver and Kuniyuki (DKW) medium (Driver & Kuniyuki, 1984; see also McGranahan et al. 1987; Tulecke & McGranahan, 1985; Tulecke et al., 1988) or a Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with various combinations of plant growth regulators and organic nitrogen (Table 2). All media were supplemented with 3% (w/v) sucrose and 0.24% (w/v)

Phytagel, and the pH adjusted to 5.7 before autoclaving at 121°C for 20 min. Development medium was of the same composition as induction media minus plant growth regulators, minus L-glutamine (GLUT) for DKW, and reduced casein hydrolysate (CH) [0.2 g/l] for MS. All cultures were incubated in the dark at 26°C.

TABLE 2. Morphogenic response of immature butternut cotyledons to various culture induction media

	Organic nitrogen[b] (mg/l)		Growth regulators[c] (μM)					
Medium[a]	CH	GLUT	BA	KN	IBA	2,4-D	NAA	Morphogenic response[d]
DKW-0	0	250	0	0	0	0	0	Adventitious roots (AR)
DKW-1	0	250	4.4	9.3	0.05	0	0	Nonembryogenic callus (NEC) Direct globular to mature somatic embryos; AR
DKW-2	0	250	1.1	0	0	9.1	0	Embryogenic callus (EC); Globular to mature somatic embryos (SE)
DKW-3	0	250	1.1	0	0	0	10.7	EC; SE
DKW-4	0	250	1.1	0	0	0	26.9	EC; SE
DKW-5	1000	0	1.1	0	0	9.1	0	EC; SE
DKW-6	1000	0	1.1	0	0	0	10.7	NEC; AR
DKW-7	1000	0	1.1	0	0	0	26.9	NEC; AR
MS-1	1000	0	1.1	0	0	9.1	0	EC; SE
MS-2	1000	0	1.1	0	0	0	10.7	EC; SE
MS-3	1000	0	1.1	0	0	0	26.9	EC; SE

[a]DKW, Driver & Kuniyuki medium (Driver & Kuniyuki, 1984; see also McGranahan et al., 1987; Tulecke & McGranahan, 1985; Tulecke et al., 1988); MS, Murashige & Skoog medium (1962).

[b]CH, casein hydrolysate; GLUT, L-glutamine.

[c]BA, 6-benzylaminopurine; KN, kinetin; IBA, indole-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthaleneacetic acid.

[d]AR, adventitious roots; EC, embryogenic callus; NEC, nonembryogenic callus; SE, globular to mature somatic embryos.

2.4 EMBRYO DEVELOPMENT

Globular to mature somatic embryos (SE) formed directly on the surface of cotyledons collected 9 weeks postanthesis (Fig. 5a,b) when cultured on DKW medium supplemented with 4.4 μM 6-benzylaminopurine (BA), 0.05 μM indole-3-butyric acid (IBA), 9.3 μM kinetin (KN), and 0.25 g/l GLUT. 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 (AR) formation occurred (Fig. 6).

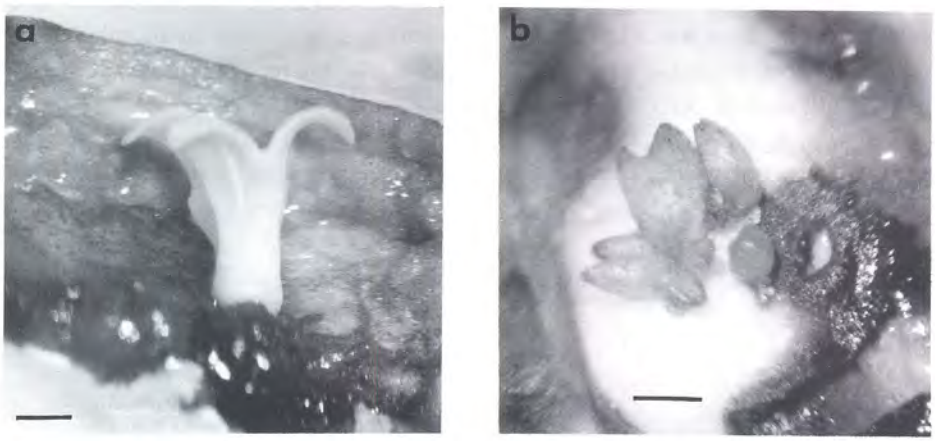

Figure 5. a Mature somatic embryo induced directly on immature butternut cotyledon explant collected 9 weeks postanthesis. b Direct somatic embryos at various stages of development on the surface of original cotyledon explant. (Pijut, 1993a) Bars 1mm.

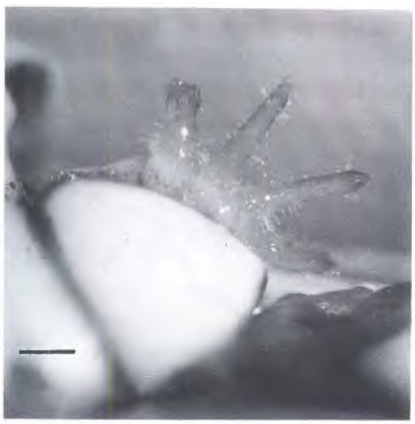

Figure 6. Adventitious roots observed on cotyledon explants collected 11 weeks postanthesis. (Pijut 1993a) Bar 1mm.

Embryogenic callus (EC) was initiated on cotyledon explants collected 8 and 9 weeks postanthesis on all induction media treatments tested (Table 2), except DKW-0, DKW-1, DKW-6, and DKW-7. Embryogenic callus (Fig. 7a) was shiny, cream-colored, and nodular in appearance. This callus developed directly on explants or on explants that had formed callus, but the callus had turned brown. Nonembryogenic callus (NEC) was light to dark brown in color, granular in appearance, but never produced EC or somatic embryos (Fig. 7b). Globular to mature somatic embryos dedifferentiated from embryogenic callus cultures (Figs. 8 a,b) for all genotypes tested. Genotypic differences were observed for

somatic embryo maturation and germination based on induction medium (Table 3). Although somatic embryos were initiated on either DKW or MS medium, somatic embryo germination only occurred on DKW medium. Tree #25 produced the most germinated embryos. It may, therefore be a good tree to use for optimization of somatic embryo germination and plantlet establishment. Proliferation of somatic embryos was maintained by subculturing embryogenic callus every 3 weeks on development medium. Although secondary somatic embryos arose from other somatic embryos (repetitive embryogenesis), many abnormalities were observed to occur in butternut cultures, especially in cotyledon development (Figs. 9 a,b,c). These cultures multiplied over time, but it was difficult or impossible to stimulate them to grow into well formed somatic embryos.

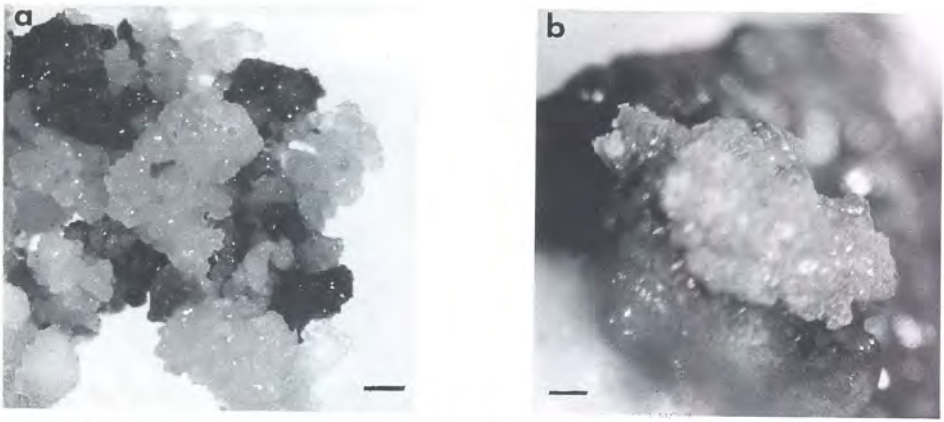

Figure 7. a Embryogenic callus produced from cotyledonary explants initiated on DKW or MS medium. (Pijut 1993a), b Nonembryogenic callus formed on immature butternut cotyledonary explants. Bars 1mm.

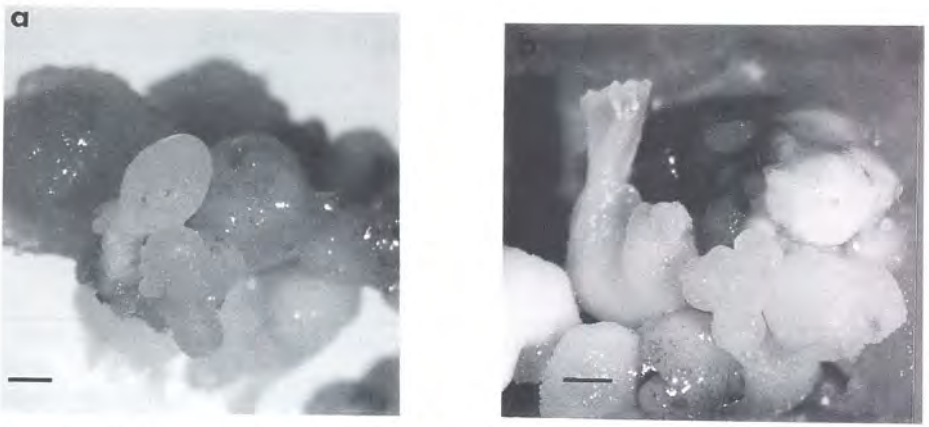

Figure 8. a Globular somatic embryo arising from nodular embryogenic callus produced on cotyledonary explants collected 9 weeks postanthesis, b Embryogenic callus with globular and torpedo-shaped somatic embryos. (Pijut 1993a) Bars 1mm .

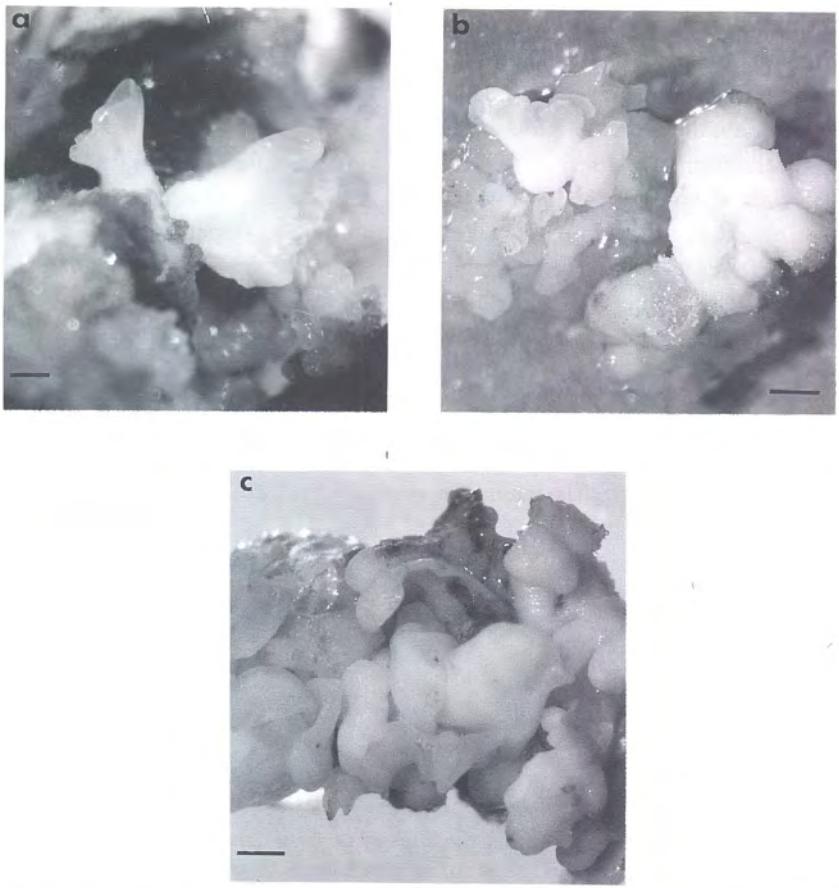

Figure 9. Somatic embryos with fused cotyledons (a,b) and abnormal growth that seldom produced somatic embryos capable of germination (c).

TABLE 3. *Juglans cinerea* somatic embryogenesis induction, maturation, and regeneration of plants based on tree selection and induction medium for 1995 collections

Genotype (tree number)	Induction medium	Number of mature somatic embryos[a]	Number of germinated somatic embryos[b]	Number of plants
2	DKW-2	7	0	0
	DKW-3	2	0	0
	MS-2	3	0	0
	MS-3	2	0	0
4	DKW-3	6	0	0
	MS-2	5	0	0
	MS-3	6	0	0
5	DKW-2	11	0	0
	DKW-3	3	0	0
	DKW-4	17	2	2
	MS-2	4	0	0
	MS-3	2	0	0
11	DKW-2	11	3	3
	DKW-3	1	0	0
	DKW-4	1	0	0
	MS-1	1	0	0
	MS-2	1	0	0
	MS-3	2	0	0
14	DKW-3	7	0	0
	MS-1	14	0	0
	MS-2	24	0	0
	MS-3	17	0	0
20	DKW-2	2	0	0
	DKW-5	10	0	0
21	DKW-2	5	0	0
	MS-1	9	0	0
22	DKW-2	8	0	0
	DKW-3	28	1	1
	MS-1	7	0	0
25	DKW-2	14	8	0
	DKW-3	2	2	0
	DKW-5	11	1	0

[a]Embryos transferred to half-strength medium with 2% (w/v) sucrose and stored at 5°C in darkness for 16 weeks.

[b]Embryos transferred to half-strength medium with 1.5% (w/v) sucrose and incubated in the light (16-h photoperiod) at 26°C until germinated.

2.5 EMBRYO MATURATION AND GERMINATION

Mature somatic embryos with well developed cotyledons and opaque with starch (similar to Fig. 5a) were stored at 5°C for 16 weeks in darkness, on half-strength MS or DKW media containing 2% (w/v) sucrose. Somatic embryos germinated (Fig. 10) when placed in the light (16-h photoperiod, 26°C) on half-strength basal 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 3-4 months.

Figure 10. Butternut plantlet regenerated from a somatic embryo subjected to cold treatment to induce germination (Pijut 1993b)

3. Discussion

A somatic embryogenesis protocol was developed as a means for propagation and for the potential genetic improvement of *Juglans cinerea* L. Somatic embryos were differentiated directly on immature cotyledonary explants and indirectly from embryogenic callus initiated from cotyledon cultures. Somatic embryogenesis was genotype dependent. Overall, best results were obtained when cotyledon explants collected 8-9 weeks postanthesis were induced to form embryogenic callus in darkness on a DKW medium supplemented with 1.1 µM BA, 9.1 µM 2,4-D, and 0.25 g/l GLUT, or on a MS medium containing 1.1 µM BA, 9.1 µM 2,4-D, and 1 g/l CH. Somatic embryo development occurred after removal of plant growth regulators, GLUT (for DKW), and reduced (0.2 g/l) CH (for MS). Globular to mature somatic embryos were differentiated, and conversion of somatic embryos into whole plants was complete, although at a very low frequency. Plantlets survived for approximately 3-4 months after acclimatization from the in vitro environment.

4. Conclusions

Immature cotyledonary tissue of *J. cinerea* is amenable to somatic embryogenesis. A developmental window exists between anthesis and fruit maturation for the expression of somatic embryogenesis in butternut cultures (Pijut, 1993a,b). Although days postanthesis

were routinely used as a measure of time for collecting immature fruit of butternut, genotypes and environmental factors influence the development of butternut fruit and thus, the subsequent success of induction of somatic embryogenesis may vary. Germination of somatic embryos and plantlet survival for 3-4 months were achieved, although at a low frequency. Refinement of the regeneration protocol to increase the frequency of "normal" somatic embryos able to germinate into whole plants and survive acclimatization to the greenhouse must be achieved in order to attain the goal of increasing the butternut population. If maturation and germination techniques can be improved upon, and the number of plantlets increased, the potential exists for genetic improvement and multiplication of butternut via somatic embryogenesis.

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