that are polymorphic in the walnut parents or in the general pool of walnut pollinators. For those markers that are heterozygous at a single locus, the probability of identifying a nonmaternal genotype is 0.5 (represented by homozygote genotypes) if selfing or crossing to males with the same alleles occurs. Nonmaternal genotypes will also be detected from the presence of nonmaternal alleles, resulting in detection of a nonmaternal genotype frequency from 0.5 to 1.0. The presence of a maternal genotype at the locus does not imply that the embryo is of apomictic origin, since heterozygotes can also occur from fertilization events. Therefore, when discussing probability of apomixis we can only establish a maximum value. The actual frequency of apomixis may be much less. However, given analysis at several loci with an appropriate selection of alleles in potential male parents, it is often possible to eliminate most or all of the embryos as possible apomicts. This was true for the present study. The maximum probability of apomixis is: $P = (0.5)^k$, where P = probability of having the maternal genotype and k = number of heterozygous loci tested. A 95% level of confidence that an offspring is identical to its maternal parent could be established by testing five heterozygous markers ($P = 0.031$). For walnuts, ≈ 20% of our probe/enzyme combinations show heterozygosity. Thus, up to 25 markers could be required to show that apomixis had occurred with >95% confidence. However, as seen in the present study, relatively few markers may eliminate the possibility of apomixis within a given population of embryos. For situations where the possibility of selfing can be dismissed and only nonmaternal alleles are present in the pollinator population, only one marker is needed to test for apomixis. A second marker could be used to confirm the results.

This study illustrates the direct use of molecular genetic markers at an applied level to facilitate plant improvement programs. In addition to evaluation of somatic embryos, these markers may be used to evaluate the products of protoplast fusion experiments or progeny from crosses at the seed or seedling stage. They provide unambiguous results in a very short time.

Literature Cited

Arulsekar, S. and D.E. Parfitt. 1986. Isozyme analysis procedures for stone fruits, almond, grape, walnut, pistachio, and fig. HortScience 21:928-932.

Arulsekar, S., G.H. McGranahan, and D.E. Parfitt. 1986. Inheritance of phosphoglucomutase and esterase isozymes in Persian walnut. J. Hered. 77:220-221.

Castle, A.J., P.A. Horgen, and J.B. Anderson. 1987. Restriction fragment length polymorphisms in the mushrooms *Agaricus brunnescens* and *Agaricus bitorquis.* Applied Environ. Microbiol. 53:816-822.

Dallas, J.F. 1988. Detection of DNA "fingerprints" of cultivated rice by hybridization with a human minisatellite DNA probe, Proc. Natl. Acad. Sci. USA 85:6831-6835.

Dellaporta, S.L., J. Wood, and J.B. Hicks. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rptr. 1:19-21.

McGranahan, G.H., C.A. Leslie, S.L. Uratsu, L.A. Martin, and A.M. Dandekar. 1988. Agrobacterium-mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Biotechnol. 6:800-804.

McGranahan, G.H., C.A. Leslie, S.L. Uratsu, and A.M. Dandekar. 1990. Improved efficiency of the walnut somatic embryo gene transfer system. Plant Cell Rpt. 8:512-516.

Rogstad, S.H., J.C. Patton, and B.A. Schaal. 1988. Ml3 repeat probe detects DNA minisatellite-like sequences in gymnosperms and angiosperms. Proc. Natl. Acad. Sci. USA 85:9176-9178.

Sauer, D.B. and R. Burroughs. 1986. Disinfection of surfaces with sodium hypochlorite. Phy topathology 76:745-749.

Tulecke, W. and G. McGranahan. 1985. Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci. 40:57-63.

Tulecke, W., G. McGranahan, and H. Ahmadi. 1988. Regeneration by somatic embryogenesis of triploid plants from endosperm of walnut, *J. regia* L. cv. Manregian. Plant Cell Rpt. 7:301-304.

Woodworth, R.H. 1930. Meiosis of microsporogenesis in the Juglandaceae. Amer. J. Bot. 17:863-869.

Zamir, D., S.D. Tanksley, and R.A. Jones. 1981. Genetic analysis of the origin of plants regenerated from anther tissues of *Lycopersicon esculentum.* Plant Sci. Let. 21:223-227.

HortScience 27(1):63–65. 1992.

Somatic Embryogenesis in Carnation

Les Frey, Yehoshua Saranga, and Jules Janick

Purdue University, 1165 Horticulture Building, West Lafayette, IN 47907-1165

Additional index words. Dianthus caryophyllus, histology

Abstract. **Somatic embryogenesis was induced from internodal callus of 'Scania', 'Improved White Sim', and 'Sandra' carnation** *(Dianthus caryophyllus* **L.). The optimum protocol for the induction of somatic embryogenesis included initiation of callus in liquid basal Murashige and Skoog medium supplemented with 3.0 μM 2,4-D followed by transfer to liquid basal medium lacking 2,4-D for embryo development. Somatic embryos originated from single cells and early embryonic development proceeded conventionally (i.e., via globular, heart-shaped, and torpedo stages), but clearly developed apical or root meristems were not always formed. A few embryos developed into seedlings and were acclimatized to ex vitro conditions. Chemical name used: 2,4-dichlorophenoxyacetic acid (2,4-D).**

Somatic embryogenesis induced from mature somatic tissue is a desirable means of rapid vegetative propagation (Ammirato, 1983; Janick et al., 1989). This study was undertaken to determine the feasibility of inducing somatic embryos from sporophytic tissue of carnation, a species, to our knowledge, for which somatic embryogenesis has not been reported previously,

Stock plants of 'Scania', 'Improved White Sim', and 'Sandra' carnation were maintained in a greenhouse at 16C (days) and 10C (nights) with supplemental light from incandescent bulbs to interrupt winter nights.

In a preliminary study, young internodal tissues from 'Improved White Sim' were disinfested for 30 set in 80% (v/v) ethanol and surface sterilized for 30 min in a 0.5% (v/v) solution of NaOCl [10% laundry bleach (v/v)] containing a few drops of Tween 20. Tissues were then rinsed four to five times with sterile distilled water, cut into explants ≈ 8 mm in length, and cultured on callus induction medium (CIM) consisting of Murashige and Skoog (1962) salts, MS vitamins, 87.6 μM sucrose, and 1.0 g casein hydrolysate/liter. Semisolid media contained 8 g agar/liter (Sigma, St. Louis). Various plant growth regulators (as listed below) were added, pH adjusted to 5.8, and the media were autoclaved for 20 min at 120 kPa. The

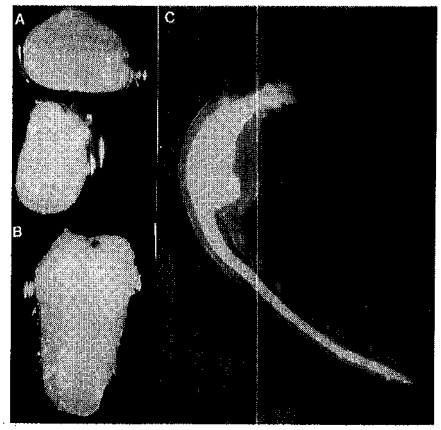

Fig. 1. Somatic embryos of 'Improved White Sim' carnation: (**A**) globular stage; (**B**) heart-shaped stage; (**C**) germinating embryo with poorly differentiated cotyledons.

Received for publication 7 Feb. 1991. Accented for publication 15 July 1991. Journal paper-no. 12,817 of the Purdue Univ. Agriculture Experiment Station. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Table 1. Effects of BA, kinetin, and 2,4-D in callus induction medium on callus fresh weight per petri plate produced from 'Improved White Sim' carnation internodes after 31 days.[z]

2,4-D (μM)	Callus fresh wt (g ± SE)				
	BA			Kinetin	
	0.0 μM[y]	2.0 μM	4.0 μM	2.0 μM	4.0 μM
1.0	0.3 ± 0.1	1.1 ± 0.4	1.9 ± 0.4	0.9 ± 0.3	2.2 ± 0.3
3.0	1.2 ± 0.2	2.0 ± 0.4	2.0 ± 0.6	2.8 ± 0.2	1.1 ± 0.2
5.0	1.3 ± 0.2	2.2 ± 0.5	2.1 ± 0.4	1.9 ± 0.3	2.3 ± 0.2

[z]There were 20 petri dishes per treatment.
[y]Used also as control for kinetin.

following two protocols for embryo initiation and development were used: semisolid CIM followed by transfer to semisolid or liquid embryo production medium (EPM, similar to CIM except casein hydrolysate was not added), or liquid CIM followed by liquid EPM.

Semisolid CIM treatments were supplemented with 1, 3, or 5 μM 2,4-D without cytokinin or combined with either 2 or 4 μM *N* -(phenylmethyl)-1 *H* -purin-6-amine (BA) or 2 or 4 μM 6-furfurylaminopurine (kinetin) and 25 ml was dispensed into 100 × 15-mm petri dishes. Cultures were maintained at 25C under cool-white fluorescent lights with a 16-h photoperiod [160 μmol·m^{-2}·s^{-1} photosynthetic photon flux (PPF)]. Thirty-one days after culture initiation, callus from semisolid CIM treatments was harvested, weighed, gently macerated with a spatula, and subcultured either into 60 × 15-mm petri dishes containing semisolid EPM or into 250-ml flasks that contained 50 ml of liquid EPM. The dishes were placed in a growth chamber at 25C with 16-h days (32 μmol·m^{-2}·s^{-1}), and the flasks were maintained at 100 rpm on a gyratory shaker in a growth room (50 μmol·m^{-2}·s^{-1} PPF). After 2 days, liquid EPM cultures were sieved through stainless steel screens (l-mm pore size), washed with EPM, and filtered materials were subcultured into 25 ml of EPM containing either 0 or 2 μM BA or 2 μM kinetin in 125-ml flasks.

The greatest production of callus on semisolid CIM was generally obtained from cytokinin and/or high-auxin treatments (Table 1); however, maximum callus weight was induced with 2 μM kinetin plus 3 μM 2,4-D. No somatic embryos were induced after transfer of this callus to semisolid EPM.

In liquid CIM treatments, internodes were cultured in 25 ml of liquid medium containing either 3 μM 2,4-D alone or combined with 2 or 4 μM kinetin in 125-ml flasks; these were maintained on a gyratory shaker at 100 rpm with a 16-h photoperiod (50 μmol·m^{-2}·s^{-1}). Callus was produced in liquid media with all plant growth regulator combinations, but yields were not determined. This callus was sieved on day 31, subcultured into 250-ml flasks of EPM, washed with EPM after 2 days, subcultured into 125-ml flasks of the same three EPM treatments listed above, and cultured as before, except that light intensity was 25 μmol·m^{-2}·s^{-1}.

Embryo production from either semisolid CIM to liquid EPM or liquid CIM to liquid EPM was visually evaluated 30 days after embryo initiation. Putative embryonic structures (Fig. lA-C) were observed on all three EPM treatments (Table 2). There was no evidence that cytokinin in either liquid or semisolid CIM improved embryo formation. Embryo ratings were highest from liquid CIM without kinetin. Only the 3 μM 2,4-D treatments were tested in liquid CIM. On semisolid CIM, embryo ratings increased with either 3 or 5 μM 2,4-D on all EPM treatments. Callus initially grown on semisolid CIM with 2 μM kinetin plus 1 μM 2,4-D, then transferred to liquid EPM plus BA, produced structures that were interpreted as a proembryonic callus mass. In this treatment, leafy tissues also were observed (Fig. 2) and were considered to be cotyledonary structures, as observed in cucumber by Chee (1990). Leafy tissues were subcultured into 60 × 15-mm petri dishes containing EPM with 7 g agar/liter and placed in a growth chamber at 25C with a 16-h photoperiod (32 μmol·m^{-2}·s^{-1}) 39 days after embryo initiation. These tissues proliferated, and after 21 days, 55 explants were either placed in 70 × 50-mm glass (baby food) jars half-filled

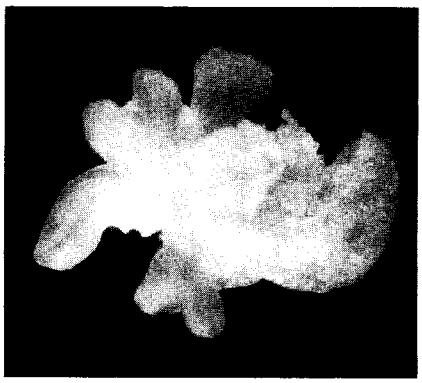

Fig. 2. Proembryonic callus mass with leafy, cotyledonary-like structures.

Fig. 3. Plant developing from proembryonic callus mass.

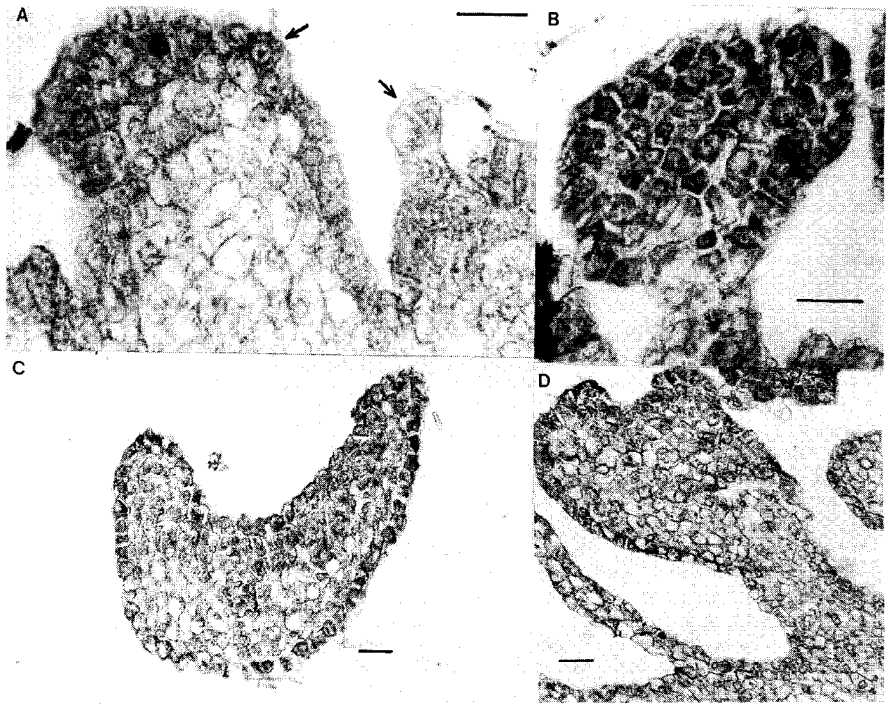

Fig. 4. Histology of somatic embryogenesis: (**A**) (left arrow) unicellular stage with prominent nucleus and nucleolus, (right arrow) three-celled stage; (**B**) globular stage showing suspensor; (**C, D**) heart-shaped stages. Bar = 200 μm.

Table 2. Effect of callus induction medium (CIM) and embryo production medium (EPM) on 'Improved White Sim' carnation somatic embryogenesis evaluated after 61 days (31 days in CIM and 30 days in EPM).[z]

Medium	2,4-D treatment in CIM (μM)	Mean rating for embryo development[y] ± SE							
		Semisolid CIM					Liquid CIM		
		BA			Kinetin		Kinetin		
		0 μM	2 μM	4 μM	2 μM	4 μM	0 μM	2 μM	4 μM
EPM	1	0.7 ± 0.3	0.5 ± 0.5	0.5 ± 0.5	1.3 ± 0.3	1.3 ± 0.3			
	3	1.3 ± 0.6	1.3 ± 0.7	0.3 ± 0.3	2.0 ± 0.0	1.7 ± 0.3	3.7 ± 0.3	2.3 ± 0.3	1.7 ± 0.7
	5	2.0 ± 0.0	0.3 ± 0.3	1.0 ± 0.0	2.5 ± 0.5	2.0 ± 0.0			
EPM + BA (2μM)	1	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.9[x]	1.0 ± 0.0			
	3	2.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	1.7 ± 0.3	4.0 ± 0.0	1.3 ± 0.3	0.7 ± 0.7
	5	1.0 ± 0.0	1.0 ± 0.0	0.5 ± 0.5	2.0 ± 0.0	1.5 ± 0.5			
EPM + kinetin (2μM)									
	1	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.7	1.3 ± 0.3			
	3	2.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	1.5 ± 0.5	4.0 ± 0.0	2.3 ± 0.3	0.3 ± 0.3
	5	1.0 ± 0.0	1.0 ± 0.0	0.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0			

[z]There were 20 petri dishes or flasks/treatment.
[y]0 (no formation) to 4 (extensive formation).
[x]Leafy tissue and proembryonic callus masses formed in this treatment.

Table 3. Effects of cultivar and 2,4-D concentration in callus induction medium on the mean number of carnation embryos produced after 103 days (41 days in CIM and 62 days in hormone-free EPM).[z]

2,4-D[y] (μM)	Mean no. of embryos (±SE)/treatment			
	Scania	Improved White Sim	Sandra	Mean
1.0	9.2 ± 0.9	8.9 ± 0.9	5.2 ± 0.7	7.5 ± 0.5
3.0	25.0 ± 3.2	45.6 ± 4.6	38.1 ± 2.5	35.4 ± 2.2
5.0	17.1 ± 2.4	29.1 ± 3.7	34.6 ± 2.8	27.2 ± 2.1
Mean	16.8 ± 1.6	26.5 ± 3.0	23.9 ± 2.4	

[z]There were 20 flasks/treatment.
[y]2,4-D applied in CIM.

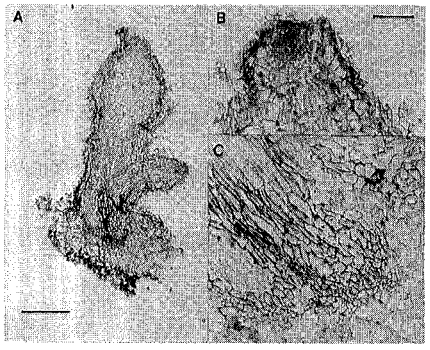

Fig. 5. Histological sections through one somatic embryo showing: (**A**) axillary budding; (**B**) shoot meristem; and (**C**) root meristem. Bar = 200 μm in **A** and 300 μm in **B, C.**

with sterilized peatlite and irrigated with liquid EPM, or in 15 × 25-mm Bellco culture tubes (Vineland, N.J.) containing half-strength MS basal medium (1/2MS) without hormones and solidified with 7 g agar/liter. After 22 days, 80% of the leafy tissue explants on 1/2MS and 50% on peatlite had formed roots, whereas 30% of all tissues had developed shoots. Numerous shoots, ≈ 1 cm tall, were present on 1/2MS and peatlite (Fig. 3). The shoots on peatlite were normal, but those on 1/2MS were slightly vitrified,

The putative embryonic structures produced in various treatments were fixed in formalin acetic acid (FAA) on days 61 or 69, dehydrated in an ethanol-tertiary butanol series, embedded in Paraplast (Monoject Scientific, St, Louis), cut into 7-μm-thick longitudinal sections, and then double-stained with safranin-fast green (Sass, 1951). Somatic embryo development began at the periphery of proembryonic callus masses, where the unicellular stage with prominent nuclei and nucleoli (Fig. 4A, left) and two- and three-celled stages (Fig. 4A, right) were observed. Embryos were attached to the callus mass by suspensors (Fig. 4 B and D). Multicellular globular (Fig. 4B) and heart-shaped stages (Fig. 4 C and D) were found. Somatic embryo verification was based on the presence of root and shoot meristems plus vascular development (Fig. 5). All embryonic structures were not strictly bipolar, however, because the majority of apical meristems were not clearly differentiated. This aberrant morphogenesis represents a breakdown of shoot apical formation and is similar to somatic embryogenesis reported in many species, including borage *(Borago officinalis* L.) (Quinn et al., 1989).

As there was no evidence that BA or kinetin (in all liquid cultures) improved induction of somatic embryos in 'Improved White Sim' compared to EPM alone, no cytokinins were used in a second experiment with all three cultivars (Table 3). Internodal explants were cultured for 41 days in 25 ml of liquid CIM amended with 1, 3, or 5 μM 2,4-D in 125-ml flasks. Cells were sieved as previously described, then held in EPM for 2 days, and recultured in EPM for an additional 60 days. Cultures were evaluated for embryo production 62 days after embryo initiation. Embryonic structures were observed in all treatments for all three cultivars, with the most globular and torpedo-shaped embryos observed from CIM supplemented with 3 μM 2,4-D (Table 3).

This study, the first report, to our knowledge, of somatic embryogenesis in carnation, demonstrated that somatic embryos may be induced from internodal callus. Many somatic embryos, however, were abnormal and lacked either apical or root meristems, but in a few cases, embryos eventually developed ex vitro into viable plants. Optimum embryo initiation was obtained on liquid CIM supplemented with 3 μM 2,4-D for 4 to 6 weeks, with embryo development occurring on 2,4-D-free medium after an additional month.

Further protocol refinement is needed to increase the number of somatic embryos that convert to acclimatized plants. Internodal tissue was investigated because it provided abundant callus, but other explant sources need to be examined. Since somatic embryos apparently were derived from single cells, somatic embryogenesis could be used as a technique for gene transfer.

Literature Cited

Ammirato, P.V. 1983. Embryogenesis, p. 82-123. In: D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. Yamada (eds.). Handbook of plant cell culture, vol. 1: Techniques for propagation and breeding. Macmillan, New York.

Chee, P.P. 1990. High frequency of somatic embryogenesis and recovery of fertile cucumber plants. HortScience 25:792-793.

Janick, J., S.L. Kitto, and Y.-H. Rim. 1989. Production of synthetic seed by desiccation and encapsulation. In Vitro Cell. Dev. Biol. 25:1167-1172.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assay with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Quinn, J., J.E. Simon, and J. Janick. 1989. Histology of zygotic and somatic embryogenesis in borage. J. Amer. Soc. Hort. Sci. 114:516-520.

Sass, J.E. 1951. Botanical microtechnique. Iowa State College Press, Ames. p. 5-77.