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Rapid multiplication of adventitious somatic embryos in peach and nectarine by secondary embryogenesis

R. Raj Bhansali [1], J. A. Driver [2], and D. J. Durzan [3]

[1] Division of Perennial Cropping System, Central Arid Zone Research Institute, Jodhpur-342 003, India
[2] Plant Research Laboratory, Modesto, California, CA 95351, USA
[3] Department of Environmental Horticulture, University of California, Davis, CA 95616-8587, USA

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ABSTRACT

Somatic embryos were multiplied by secondary embryogenesis in cotyledonary cultures of peach and nectarine (*Prunus persica* L.) using a simplified culture medium for immature seeds. A three-stage process with an initial callus phase was established in darkness on a medium containing basal salts (modified MS) supplemented with 2,4-D (5 mg/l), Kn (2 mg/l) and BAP (2 mg/l) and casein hydrolysate (500 mg/l). This was followed by a growth regulator-free medium with activated charcoal for the adventitious and direct multiplication of somatic embryos under continuous light. Somatic embryos (10-15) originated from the epidermal layer of primary somatic embryos of 4-6 mm size. The incidence of morphologically abnormal embryos was reduced by subculturing every 20 days. Calli which were isolated and grown on a 2,4-D medium were more embryogenic than those on NAA. These embryos multiplied continuously for more than 10 months by a repetitive somatic embryogenic process. A third stage medium, supplemented with BAP (2 mg/l), was required for axis elongation, germination and transfer to soil.

INTRODUCTION

Plant regeneration from a tissue culture system is often the most critical step in the success of various biotechnological techniques of any plant improvement program. Rapid regeneration and multiplication rates of propagules are now possible through somatic embryogenesis. This method produces somatic embryos with root and shoot meristems which originate from a single cell with or without a callus stage (McWilliams et al. 1974, Street and Withers 1974, Haccius 1978). The induction of somatic embryogenesis directly on explant tissues or in callus cultures has been reported in many fruit tree species (Raj Bhansali and Arya 1979, Tisserat et al. 1979, Tulecke 1987) including the peach (Hammerschlag 1986).

Offprint requests to: D. J. Durzan

The formation of adventitious secondary embryos in *Prunus persica* using immature seeds and clonal leaf tissues has been already reported (Raj Bhansali et al. 1990). We now describe the factors responsible for the repetitive multiplication of secondary embryos on explanted primary embryos rescued from immature seeds and plant development by repetitive secondary embryogenesis, as distinct from nonadventitious and reconstitutive processes involving polyembryogenesis (Sinnott 1960, Durzan 1988).

MATERIALS AND METHODS

The immature seeds were obtained in 1987 from hybrid crosses performed at Burchell Nursery Inc., Fresno, California, USA. One to four longitudinal slices along with embryonal axes of cotyledons were removed from each immature zygotic seed and placed individually on a modified MS medium (MMS-I) supplemented with sucrose (3% w/v), L-glutamine, *myo*-inositol, and casein hydrolysate (each 500 mg/l). All media were adjusted to pH 5.7 at 23°C before autoclaving for 20 minutes at 121°C. Media were dispensed in approximately 20 ml aliquots in 15 mm petri dishes and solidified with 0.64% w/v Difco agar. Plates were sealed with plastic wrap.

To induce somatic embryos, cotyledonary explants were incubated for 10 days in the dark, followed by continuous light (30 μ $Mm^{-2}S^{-1}$) at 23°C for 20 days on a MMS-I, supplemented with 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/l kinetin (Kn) and 2 mg/l N^6-benzylamino-purine (BAP). The formation of highly acetocarmine-reactive embryogenic cells was ascertained by a double-staining test (Gupta and Durzan 1987) and by light microscopy. Embryogenic cultures were transferred to a second medium (MMS-II) without growth regulators for the development of embryos.

Individual or groups of two to four embryos were recovered from the initially explanted tissues. These somatic embryos were subjected to a two-stage culture procedure, used for cotyledonary tissues, or were inoculated on different media containing various levels of growth regulators, i.e., 2,4-D, NAA, Kn and BAP (1 to 4 mg/l) for the assessment of multiplication of somatic embryos. All somatic embryos were incubated under complete darkness or in continuous light under the conditions described earlier.

Multiplication rates were determined by counting the number of secondary adventitious embryos induced per primary somatic embryo. The development of adventitious embryos was followed through distinct stages and referenced to zygotic embryo morphology and development, e.g., globular/pea seed shape, heart shape, torpedo/elongated late-heart stage, and emerging cotyledons. Somatic embryos were classified as normal or aberrant (e.g. lacking distinct stages or morphologically abnormal in shape and size).

Elongated mature embryos recovered from the primary somatic embryos were cultured on a third stage medium (MMS-III, 2.0 mg/l BAP with 500 mg/l casein hydrolysate) for further development of the shoot/root axis. Leafy plantlets developed on a medium containing 1.0 mg/l BAP, 0.1 mg/l NAA and 0.03% (w/v) activated charcoal. Germinated embryos were transferred to half-strength basal medium in vermiculite and incubated under continuous bright light.

In all studies, a minimum of 20 embryos was cultured per treatment, and generally 50 embryos were used per treatment.

RESULTS AND DISCUSSION

Adventitious somatic embryos attached to the rescued parental cotyledons did not undergo further development, even though they often possessed distinct root and shoot poles (Fig. 1). Therefore, for embryo multiplication, embryos were separated and isolated either singly or in small groups of 2 to 4 embryos (when the embryos were not easily separable).

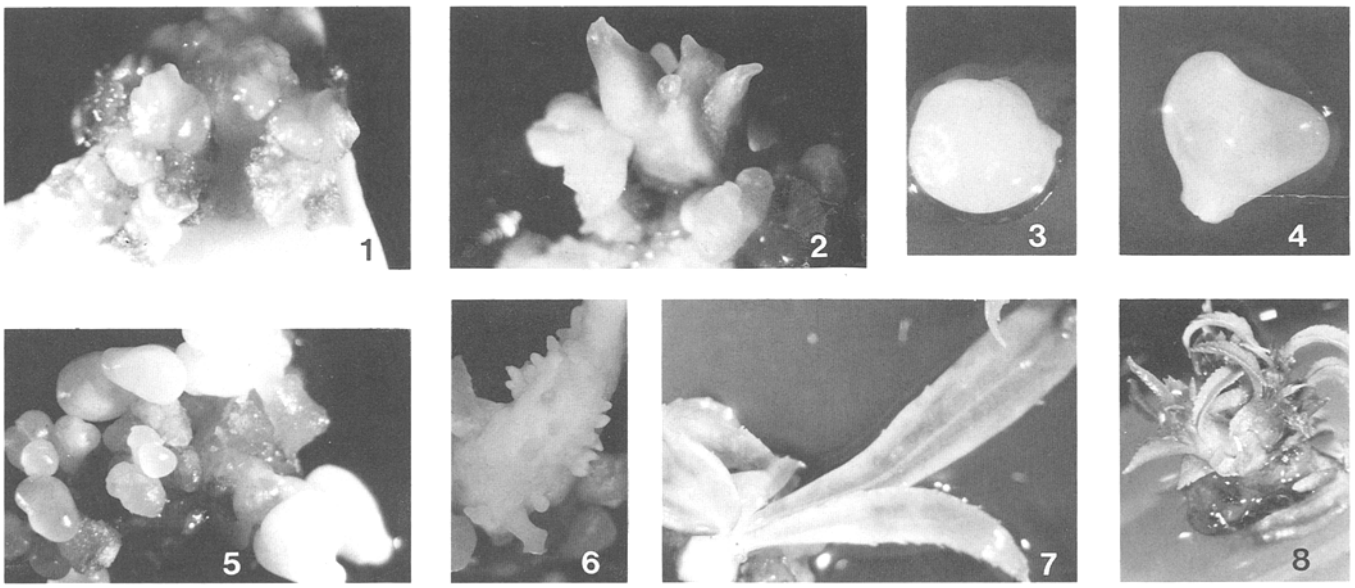

Figure 1. Somatic embryos induced on the embryonal axis region at base of immature peach cotyledons. (20×)

Figure 2. Morphologically abnormal and vitrified peach embryos in 60-day-old cultures. (20×)

Figure 3. Globular-shaped normal peach embryo. (40×)

Figure 4. Heart-stage development of globular peach embryo. (40×)

Figure 5. Repetitive somatic embryogenesis in peach resulting from regular subculturing at 20 day intervals. (20×)

Figure 6. Induction of profuse secondary embryos from epidermal cells of a primary peach embryo. (40×)

Figure 7. Germination of somatic peach embryo by producing leafy shoot on stage III medium. (5×)

Figure 8. Multiple shoot formation from germinated nectarine embryo. (2.5×)

Table I
Production of secondary embryos and plant development from 1-3; 4-6 mm size somatic embryos of peach and nectarine after 40 days under standard three stage culture system. n > 10

Plant	Somatic embryo size (mm)	No. of embryos/somatic embryo		No. of shoots/embryo	
		Stage II	Stage III	Stage II	Stage III
Peach	1-3	7 ± 0.6	2 ± 1.9	-	3/10
	4-6	15 ± 1.7	5 ± 2.2	1/10	5/10
Nectarine	1-3	5 ± 1.3	2 ± 1.6	1/10	2/10
	4-6	12 ± 2.0	3 ± 1.3	2/10	6/10

Embryo Multiplication

Forty to sixty days after subculture, the regenerated adventitious primary embryos were sorted into two sizes, *viz.* 1-3 and 4-6 mm long. These embryos were inoculated onto 2nd and 3rd stage media for repetitive secondary somatic embryogenesis (Table I). Secondary embryos formed more quickly and in greater number and were sized from 4-6 mm, compared to 1-3 mm for primary embryos (Table I). For both *Prunus* genotypes, the response was similar for secondary embryo formation and shoot development. Moreover, morphogenesis by direct secondary embryogenesis was very similar to somatic embryogenesis from zygotic embryo explants (Raj Bhansali et al. 1990).

Direct secondary somatic embryogenesis offers a rapid multiplication method. Embryos cloned in this way could be used for encapsulation as artificial seeds (Redenbaugh et al. 1984). Clonal populations of embryos are needed for the screening against pathotoxins to screen for resistant plants (Sacristan 1986). Clonal embryos provide replicated germplasm for the study of gene frequencies in quantitative genetics and for genetic improvement using recombinant DNA methods (Parzkowski and Saul 1985, Ow et al. 1986).

Subculture Frequency Rates

In both genotypes, secondary embryo development improved with increased subculture frequency (Table II). However, lengths of subculture of 20 days or more drastically affected embryo morphology. This resulted in abnormal embryos (Fig. 2). The rate of embryo multiplication decreased on quick subculturings (10 days) on MMS-I medium. Embryos were now very poorly developed, and further development was arrested. Most embryos were small, globular and heart-shaped (Figs. 3 and 4). Small protuberances on explanted tissues were evident. These increased slightly in size, but rarely initiated shoots.

Table II
Effect of subculture frequency on secondary embryogenesis of peach and nectarine embryos (3-6 mm size) at 2nd stage culture procedure. n > 10

Plant	Subculture frequency (days)	No. of embryos/embryo	
		Normal	Abnormal
Peach	10	5 ± 1.6	3 ± 1.6
	20	10 ± 5.1	8 ± 5.2
	30	6 ± 2.9	14 ± 4.0
	No subculture up to 60	5 ± 3.2	15 ± 6.5
Nectarine	10	3 ± 1.9	1 ± 1.0
	20	7 ± 2.8	5 ± 1.6
	30	8 ± 3.7	14 ± 6.4
	No subculture up to 60	8 ± 6.3	16 ± 3.8

Table III
Effect of plant growth regulators on induction of normal or abnormal secondary adventitious embryos and callus in peach and nectarine.

Plant hormone (mg/l)		Per cent distribution					
		Peach			Nectarine		
		Normal	Abnormal	Callus	Normal	Abnormal	Callus
Basal medium + 0.2 Kn + 0.2 BAP							
2,4-D	4.0	--	--	60	--	--	65
	2.0	--	--	78	--	--	62
	1.0	65	85	72	59	91	65
Basal medium + 0.2 Kn + 0.2 BAP							
NAA	4.0	--	--	40	--	--	55
	2.0	38	42	40	52	48	50
	1.0	55	45	38	60	40	50
Basal medium + 0.4 2,4-D + 0.2 Kn							
BAP	4.0	--	--	--	--	--	--
	2.0	20	80	76	25	75	60
	1.0	35	65	76	38	62	65
Basal medium + 0.4 2,4-D + 0.2 BAP							
Kn	4.0	--	--	36	--	--	48
	2.0	--	--	48	--	--	50
	1.0	28	72	40	30	70	55

A minimum of 50 explants was used for each treatment

Secondary embryos developed directly from the epidermal layer of explant tissues within 20 days (Fig. 6). By 40 days on the same medium, most embryos became abnormal due to fused cotyledonary lobes and a tendency for increasingly unorganized growth. Also, abnormal embryos were repetitively formed (Fig. 5). Developmental abnormalities were minimized in both genotypes by subculturing at a frequency of 20 days.

Direct somatic adventitious and repetitive embryogenesis has recently been observed in several woody species (Tulecke and McGranahan 1985, Merkle et al. 1987, Muralidharan and Mascarenhas 1987, Krull 1987). Our process adds to this list, extends the earlier work on peach (Hammerschlag 1986), and provides highly useful germplasm for tree improvement.

Effect of Hormones

With the second stage medium, NAA and 2,4-D were tested for their ability to control embryo multiplication (Table III). 2,4-D at 1 to 4 mg/l was more effective than NAA in both genotypes. Both auxins showed a distinct effect on the morphology of cellular embryos. NAA produced more normal morphology, but the frequency of embryogenesis was very low (Fig. 1). Very little callusing was observed on the NAA-containing medium compared with 2,4-D. However, the callus developed with 2,4-D was highly embryogenic. A creamy yellow and granular texture was characteristic of embryonic potential. Large numbers of embryos emerged from calli grown on 2,4-D (1.0 mg/l) medium. The comparison among concentrations showed differences in callus initiation in both genotypes. Auxin type and concentration had a specific effect on the production of embryogenic calli, secondary embryogenesis, and on the morphology of embryos. A similar effect of NAA and 2,4-D on somatic embryogenesis was noticed in soybean culture (Lazzeri et al. 1987).

The addition of BAP and Kn (each at 4.0 mg/l) to 2,4-D had a strong inhibitory effect on callus production (Table III). However, at the lower levels of BAP and Kn, secondary

embryos were visible, with very little callus production. Most of these embryos developed an abnormal morphology (Fig. 2). In these cases, the quality of direct somatic embryo production was not greater than without plant growth regulators. An inhibitory effect of cytokinins in embryogenic media containing 2,4-D has already been reported (Lippmann and Lippmann 1984).

Plant Recovery

Somatic embryos on 3rd stage medium with BAP and casein hydrolysate initiated shoot growth and elongation under continuous light (Fig. 7). Roots emerged from the hypocotyl region of germinated embryos. Leafy plantlets were developed on the medium containing BAP, NAA and activated charcoal. Nectarine showed faster growth of the main shoot and root than peach (Fig. 8). Axillary buds sprouted very quickly on the charcoal-containing medium. The multiplication of somatic embryos and recovery of plants in peach and nectarine occurred within 4 to 5 months. This appears to be an efficient method for rapid clonal propagation of these genotypes.

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