

Regeneration of peach plants from callus derived from immature embryos

F. A. Hammerschlag¹, G. Bauchan¹ and R. Scorza²

¹ Tissue Culture and Molecular Genetics Laboratory and Field Crops Laboratory, BARC-West, USDA, ARS, Beltsville, MD 20705, USA

² Appalachian Fruit Research Station, USDA, ARS, Kearneysville, WV 25430, USA

Received November 1, 1984; Accepted November 28, 1984

Communicated by Y. Gleba

Summary. Peach plants were repeatedly regenerated from immature embryos but not from callus derived from mature embryos. A white, nodular, highly regenerative callus was obtained when friable, primary callus from immature embryos was transferred from medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid and 0.44 μM benzyladenine (BA) to media containing 0.27 μM α -naphthaleneacetic acid (NAA) and 2.2 μM BA. This callus retained its morphogenetic potential for a minimum of three subcultures. Green nodular callus, that lacked regenerative capacity, was produced from primary callus derived from mature embryos. Maximum regeneration of shoots occurred when highly regenerative callus was transferred to a medium in which the NAA concentration was reduced five times and the BA concentration was increased two times. Regenerated shoots were rooted in the dark on a medium containing 28.5 μM indoleacetic acid. Cytogenetic analysis of regenerated plants indicated that all plants were diploid, $2n=2x=16$. Phenotypic evaluation of regenerated plants, grown under field conditions, is now in progress.

Key words: *Prunus persica* – Peach – Embryo culture – Plant regeneration – Cytogenetic analysis

Introduction

In vitro mutant selection for toxin insensitivity can produce novel disease resistant plants (Behnke 1980; Gengenbach et al. 1977).

This system requires that the toxin or toxic metabolite be involved in disease development and be active at the cellular level. The cultured cells or tissues also must express disease

resistance in vitro and be morphogenetically competent (Hammerschlag 1984b; Yoder 1981; Brettell and Ingram 1979). Hammerschlag (1984a) reported that a toxic metabolite of *Xanthomonas campestris* pv. *pruni* (E.F. Sm.) Dows, causal agent of leaf spot of peach, *Prunus persica* (L.) Batsch, is involved in disease development and is active at the cellular level. In vitro selection to obtain leaf-spot-resistant peach plants can now be realized if it can be demonstrated that peach cells express resistance in culture and if they can be induced to undergo morphogenesis.

To date, there has been only one brief report of plant regeneration from peach endosperm callus (Meng and Zhou 1981). In this paper we describe regeneration of peach plants from callus derived from immature embryos and cytogenetic analysis of the regenerated plants.

Materials and methods

To obtain friable callus, peach fruits were removed from field grown open-pollinated 'Sunhigh' and 'Suncrest' trees when the PF_1 $\left(\frac{\text{embryo length}}{\text{seed length}}\right) \times 100$ values (Kester and Hesse 1955) for the embryos were 3 (approximately 56 days after full bloom), 28 (approximately 70 days after full bloom) and 100 (approximately 125 days after full bloom). The pericarp tissue was removed and seeds were surface-sterilized for 15 min in 0.5% sodium hypochlorite containing 0.01% Tween-20 and then rinsed three times in sterile distilled water. Immature (EI and EII) embryos with PF_1 values of 3 and 28, respectively, and mature (EIII) embryos with PF_1 values of 100 were aseptically removed, wounded slightly and transferred to friable callus-inducing medium (Hammerschlag 1983). This medium (MS) consisted of Murashige and Skoog (1962) salts supplemented with 555.1 μM myoinositol, 4.06 μM nicotinic acid, 2.43 μM pyridoxine HCl, 1.18 μM thiamine HCl, 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.44 μM benzyladenine (BA), 87.6 μM sucrose and 0.6% agar. Cultures were incubated in the dark at 26 °C. Approximately 150 embryos per stage of embryo development per cultivar were excised.

To induce nodular callus, friable callus was removed from embryo explants and transferred to MS medium supplemented with 0.27 μM NAA and 2.2 μM BA. Friable callus from a minimum of 12 embryos per stage of development per seed source were plated. Cultures were incubated at 26 °C with a 16-h photoperiod of approximately 40 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ irradiance provided by cool white fluorescent lamps.

To induce shoot formation, nodular callus was subcultured three times onto the same medium and then transferred to MS medium supplemented with various levels of NAA (0.027–0.27 μM) and BA (2.2–22.0 μM). Culture conditions were the same as those used for nodule induction. Each factorial was replicated a minimum of three times.

Regenerated shoots were rooted and acclimatized as described previously (Hammerschlag 1982). Cytogenetic analysis consisted of determining the chromosome number from root tip cells. Root tips were collected from five regenerated plants per seed source, placed in distilled water and incubated for 24 h at 4 °C. Root tips were fixed in Carnoy's solution (ethanol, chloroform, acetic acid, 6:3:1) for 24 h, hydrolyzed in 1 N HCl at 60 °C for 15 min and stained in Feulgen's reagent for 4 to 24 h. Root tips were squashed in 1% acetocarmine. A minimum of 50 cells per plant were analyzed.

Results

Callus initiation

'Sunhigh' and 'Suncrest' embryos and calli derived from embryos responded similarly in that both friable and

nodular callus formation was positively related to stage of embryo development (Tables 1 and 2). Maximum friable callus formation occurred from mature embryos. Either rough-green (Fig. 1a) or smooth-white (Fig. 1b) nodular callus formed from friable callus after 28 days of culture. The percentage friable callus that produced nodular callus is seen in Table 2. Maximum production of smooth-white nodular callus occurred from friable primary callus derived from EI embryos. Predominantly rough-green nodular calli were produced from friable callus derived from EIII embryos.

Induction of smooth-white nodular callus was effected by the length of time on friable callus medium. This was determined by subculturing friable primary calli from five embryos per stage of development per seed source every 3–4 weeks for 3 months and then subsequently transferring the calli to nodule-inducing medium. This experiment was repeated three times. Most of the calli died after being transferred to nodule-induced medium. Only one genotype at one stage of embryo development (EI) repeatedly produced callus that could be subcultured and then induced to produce white nodular callus.

Regeneration

Plants could only be regenerated from smooth-white nodular callus derived from EI and EII primary callus. This nodular callus retained its morphogenetic potential for a minimum of three subcultures. Smooth-white nodular callus originating from EIII primary callus and from EI callus maintained on friable callus medium for 4 months, produced only large swollen abnormal nodules and abnormal leafy structures when transferred to regeneration medium.

Highly regenerative, smooth-white nodular callus produced embryoid-like structures (Fig. 1c) and shoots

Table 1. Percentage of peach embryos at different stages of development that produced friable callus. Stage of development is indicated by PF₁ value which equals $\frac{\text{embryo length}}{\text{seed length}} \times 100$. Approximately 100 to 150 embryos per stage of development per seed source were cultured

Seed source	PF ₁ value		
	3	28	100
'Sunhigh'	13	41	100
'Suncrest'	18	45	100

Table 2. Percentage of friable callus, derived from peach embryos at different stages of development, that produced nodular callus. Stage of development is indicated by PF₁ value which equals $\frac{\text{embryo length}}{\text{seed length}} \times 100$. Friable callus from a minimum of 12 embryos per stage of development per seed source were plated

Seed source	PF ₁ value					
	3		28		100	
	White nodules	Green nodules	White nodules	Green nodules	White nodules	Green nodules
'Sunhigh' (primary callus)	45	55	10	90	0	100
'Suncrest' (primary callus)	33	67	10	90	10	90

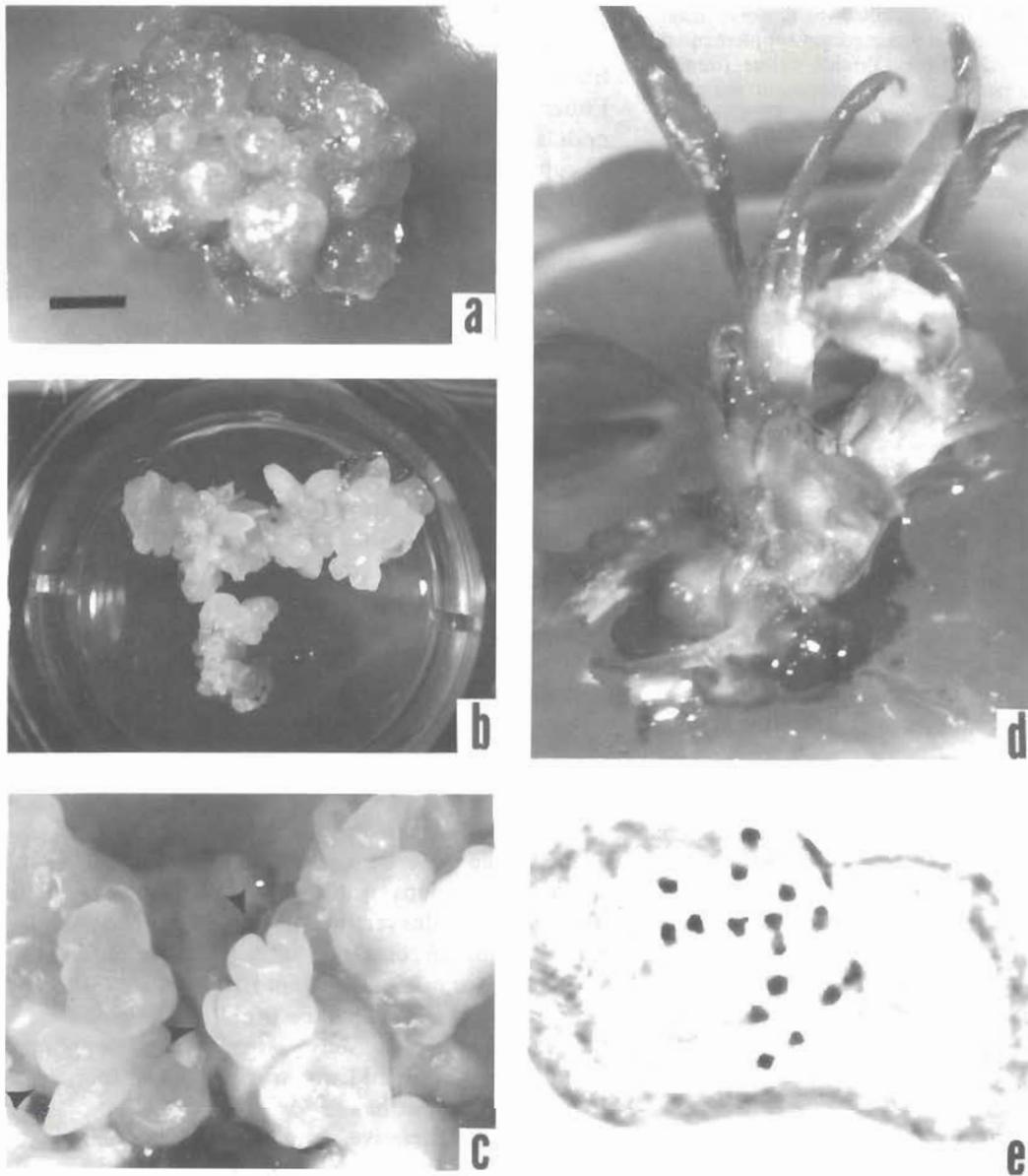


Fig. 1. **a** Green nodular callus formed from friable callus derived from mature peach embryos; **b** white highly regenerative nodular callus formed from friable callus derived from immature peach embryos; **c** embryoid-like structures (*arrows*) produced from highly regenerative callus; **d** shoots regenerated from highly regenerative callus; **e** Feulgen stained root tip chromosomes in *Prunus persica* ($2n=2x=16$) regenerated from highly regenerative callus. Scale bar: **a** = 1 mm; **b** = 4.5 mm; **c** = 1 mm; **d** = 1.5mm; **e** = 5 μ m

(Fig. 1d). The number of plantlets produced from calli and the changes in growth-regulator level required to induce shoot formation are seen in Table 3. Maximum shoot production occurred when the BA level was increased either two or five times, regardless of the NAA level. No plants were produced when the BA level remained the same. Increasing the BA level 10 times also proved to be inhibitory to shoot formation.

Regenerated peach plants were rooted and acclimatized and planted in the field. Chromosome

counts of root-tip cells indicated that all plants were diploid $2n=2x=16$ (Fig. 1e).

Discussion

To date, there has been only one report of peach plant regeneration from callus (Meng and Zhou 1981). In that study, peaches were regenerated from endosperm callus. The dearth of information on regeneration of

Table 3. The number of plantlets regenerated from white nodular calli derived from immature peach embryos. The data are based on three replicate experiments each containing four genotypes per seed source per EI ($PF_1 = 3$) embryos and two genotypes per seed source per EII ($PF_1 = 28$) embryos. The PF_1 value equals $\frac{\text{embryo length}}{\text{seed length}} \times 100$. Growth regulator level is in relation to that present in nodule-inducing media (0.27 μM NAA and 2.2 μM BA)

BA level	NAA level	No. of regenerated plantlets
same	same	0
same	-5 \times	0
same	-10 \times	0
+2 \times	same	14
+2 \times	-5 \times	29
+2 \times	-10 \times	13
+5 \times	same	17
+5 \times	-5 \times	17
+5 \times	-10 \times	11
+10 \times	same	5
+10 \times	-5 \times	9
+10 \times	-10 \times	0

peach plants from callus cultures impedes the use of tissue culture and genetic engineering technology for improvement of this species. The data presented in this paper provide evidence for the totipotency of callus tissue derived from immature embryos and open up the possibility of using highly regenerative peach callus, derived from immature peach embryos, for in vitro mutant selection.

As in other regeneration studies (Heyser and Nabors 1982; Heyser et al. 1983), a specific type of callus (white, compact, nodular) could be selected and induced to produce embryoid-like structures and subsequently shoots. The development of smooth-white nodular callus which lacked morphogenetic potential, from primary EIII callus and from friable EI callus that was maintained on callus medium for 4 months, suggests that visual selection of callus for morphogenetic potential may not be sufficient. These data also demonstrate that physiological and chronological age of the explant as well as the period of time in vitro can influence organ formation. Other studies have cited the importance of age of explant (Raju and Mann 1970; Green and Phillips 1975) as well as duration in vitro (Fridborg and Eriksson 1975; Heyser and Nabors 1982) on morphogenetic potential of tissue cultures. The changes occurring during embryo development and in vitro culture that result in the loss of morphogenetic potential of callus derived from embryos require investigation. Since regenerative capacity can be restored to some calli (Fridborg and Eriksson 1975) changes in gene expression and not permanent changes in the genome may be involved.

Peaches that have been regenerated are now being evaluated in the field for somaclonal variation. Studies are also under way to utilize highly regenerative peach callus in in vitro selection for insensitivity to the toxic metabolite produced by *X.c. pv. pruni*.

Acknowledgement. We thank Mrs. Karen Harris for her excellent technical assistance.

References

- Behnke M (1980) General resistance to late blight of *Solanum tuberosum* plants regenerated from callus resistant to culture filtrates of *Phytophthora infestans*. *Theor Appl Genet* 56: 151–152
- Brettell RI, Ingram DS (1979) Tissue culture in the production of novel disease resistant crop plants. *Biol Rev* 54: 329–345
- Fridborg G, Eriksson T (1975) Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiol Plant* 34: 306–308
- Gengenbach BG, Green CE, Donovan CM (1977) Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. *Proc Natl Acad Sci USA* 74: 5113–5117
- Green CE, Phillips RI (1975) Plant regeneration from tissue cultures of maize. *Crop Sci* 15: 417–421
- Hammerschlag FA (1982) Factors influencing in vitro multiplication and rooting of the plum rootstock Myrobalan (*Prunus cerasifera* Ehrh.). *J Am Soc Hortic Sci* 107: 44–47
- Hammerschlag FA (1983) Factors influencing callus formation among cultured peach anthers. *HortSci* 18: 210–211
- Hammerschlag FA (1984a) Optical evidence for an effect of culture filtrates of *Xanthomonas campestris* pv. *pruni* on peach mesophyll cells. *Plant Sci Lett* 34: 295–304
- Hammerschlag FA (1984b) In vitro approaches to disease resistance. In: Collins GB, Petolino JF (eds) Applications of genetic engineering to crop improvement. Martinus Nijhoff/W. Junk, The Hague
- Heyser JW, Nabors MW (1982) Long term plant regeneration, somatic embryogenesis and green spot formation in secondary oat (*Avena sativa*) callus. *Z Pflanzenphysiol* 107: 153–160
- Heyser JW, Dykes KJ, De Mott KJ, Nabors MW (1983) High frequency, long term regeneration of rice from callus cultures. *Plant Sci Lett* 29: 175–182
- Kester DE, Hesse CO (1955) Embryo culture of peach varieties in relation to season of ripening. *Proc Am Soc Hortic Sci* 65: 265–273
- Meng X, Zhou W (1981) Induction of embryoid and production of plantlets in vitro from endosperm of peach. *Acta Agric Univ Peking* 7: 95–98
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Raju MVS, Mann HE (1970) Regenerative studies on detached leaves of *Echeveria elegans*. Anatomy and regeneration of leaves in sterile culture. *Can J Bot* 48: 1887–1891
- Yoder OC (1981) Assay. In: Durbin RD (ed) Toxins in plant disease. Academic Press, New York, pp 47–48