Androgenesis in *Capsicum annuum* L.—Effects of Carbohydrate and Carbon Dioxide Enrichment

Ramon Dolcet-Sanjuan¹, Elisabet Claveria, and Agustin Huerta

Departament de Genètica Vegetal, Institut de Recerca i Tecnologia Agroalimentàries, Centre de Cabrils, 08348 Cabrils (Barcelona), Spain

ADDITIONAL INDEX WORDS. pepper, dihaploids, anther culture, sugars, CO₂

ABSTRACT. A new and simple protocol for androgenesis in bell pepper is described. The initial medium, a modification of Nitsch and Nitsch's H medium, consisted of a two-phase system of semi-solid and liquid medium and contained maltose as carbon source. The total number of embryos formed was greater with maltose at 40 g·L⁻¹, but embryos developed better at 10 to 20 g·L⁻¹. Depending on the genotype, the number of embryos and plants recovered ranged from 3 to 750 and 0.25 to 8, respectively, per 100 flowers. Further increases in the number of embryos (up to 3561 per 100 flowers) and plants (up to 23 per 100 flowers) could be attained by flushing cultures with air enriched with CO₂ at 900 μL·L⁻¹. The ploidy level and the microspore origin of the recovered plants were determined by flow cytometry and zymograms for isocitrate dehydrogenase. Nearly 65% of the acclimated plants had undergone spontaneous doubling of the chromosome number, as confirmed by flow cytometry of leaf nuclei. Isocitrate dehydrogenase zymograms demonstrated that plants originated from microspores and that the two parental alleles were equally represented among the haploid and dihaploid plants.

Haploid production through androgenesis or parthenogenesis is used frequently in modern breeding programs (Foroughi-Wehr and Wenzel, 1993). Haploids facilitate the recovery of recessive mutations and unique genetic recombinations. Furthermore, chromosome doubling of haploids allows the production of homozygotes, which can be used directly in hybrid production. Dihaploids (DHLs) have been used in pepper breeding for resistance to tobacco mosaic virus (Daubèze et al., 1990; Dumas de Vaulx et al., 1982), potato virus Y (Pochard et al., 1983), *Phytophthora capsici* (Abak et al., 1982; Daubeze et al., 1990), and nematodes (Hendy et al., 1985).

In cultivated pepper and related species, DHLs traditionally were obtained via parthenogenesis in the form of twin seedlings (Pochard and Dumas de Vaulx, 1979). More recently, they have been generated at a higher rate by anther culture (Dumas de Vaulx, 1990; Morrison et al., 1986a; Vagera, 1990). Production of callus, embryoids, and pepper plantlets with haploid chromosome number were first reported by Kuo et al. (1973), George and Narayanaswamy (1973), Novak (1974), and Wang et al. (1973). In these early studies, the medium composition was not well defined, and the numbers of embryoids and recovered plants per cultured anther were low.

Sibi et al. (1979) and Dumas de Vaulx et al. (1981) increased the rate of success by incubating anthers in two successive media at different incubation conditions to induce embryogenesis. This process involved incubating explants in a semi-solid medium containing kinetin at 0.01 mg·L⁻¹ and 2,4-D at 0.01 mg·L⁻¹ for 8 d at 35 °C in darkness and then transferring the explants to light conditions (12-h photoperiod) for 4 d at 25 °C. These explants then

were transferred to a regeneration medium containing kinetin at 0.1 mg·L⁻¹ to promote embryo development and growth of plantlets within 40 to 60 d. Various modified versions of this protocol have been tested since (Mityko et al., 1995; Morrison et al., 1986a, 1986b; Munyon et al., 1989).

Not all *C. annuum* genotypes are responsive to androgenesis and haploid formation from *C. chinense* × *C. annuum* hybrids (Morrison et al., 1986b), chile pepper (Munyon et al., 1989), and other sweet and hot peppers (Mityko et al., 1995; Qin and Rotino, 1993) is sporadic. The frequency of androgenic plants obtained depends highly on the genotype, ranging from 0.5 to 75 embryos per 100 anthers (Dumas de Vaulx et al., 1981; Ltfi and Wenzel, 1994; Mityko et al., 1995; Qin and Rotino, 1993; Sibi et al., 1979). At least 100 DHLs per genotype are desired for breeding purposes (Foroughi-Wehr and Wenzel, 1993); therefore, the low rate of haploids recovered limits the utility of anther culture in pepper breeding.

In this paper, we report a procedure for inducing androgenesis in bell pepper F₁ hybrids using a one-step protocol. The highlights include using a liquid-solid two-phase medium, replacing sucrose with maltose, and enriching the anther culture atmosphere with CO₂.

Materials and Methods

PLANT MATERIAL. Fourteen bell pepper F₁ hybrids (C21, C22, C31, C33, and C40 to C49) were provided by Semillas Fitó S.A., and, since they are proprietary material, the pedigrees of these genotypes can not be provided at this time. Flowers were collected between February and July from plants grown in a screenhouse at >18 °C and a 12-h photoperiod supplemented with high-pressure sodium lights (radiance of 400 W·m⁻²).

STAGE OF MICROSPORES. Anthers containing microspores mainly at the late uninucleate stage (start of first pollen mitosis; Fig. 1A) and ≈10% binucleated pollen were used. At this stage, the corolla is equal to or slightly longer than the calyx and the anthers have a violet tinge at their ends. In addition to morphological indicators, fluorescent microscopy after staining with mithramycin A (Sigma Chemical Co., Alcovendas, Madrid, Spain), a DNA-specific fluorescent dye, was used to confirm the developmental stage of microspores. The methods of Pace et al. (1987) were followed.

Received for publication 30 Aug. 1996. Accepted for publication 4 Feb. 1997. This work was supported in part by the Spanish seed company Semillas Fitó S.A. Use of trade names does not imply endorsement of the products named or criticism of similar ones not named. We thank Machteld Mok and David Mok, Dept. of Horticulture, Oregon State Univ., for suggestions and review of the manuscript. We also appreciate the help of Jaume Comas, Seveis Científico-Tècnics, Universitat de Barcelona, with the flow cytometry determinations. Finally, we thank Pere Arús, Head of the Dept. of Genetics, IRTA, for his encouraging support and help with isozyme analyses. 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.

¹To whom requests for reprints should be addressed.

THE TWO-PHASE MEDIUM AND CULTURE CONDITIONS. Flower buds were disinfected by spraying with 96% ethanol. Anthers were excised, and, after removal of the filaments, anthers from two buds were placed in each petri dish (6 cm) with 5 mL of the solid phase (SP), after which the 3 mL liquid phase (LP) was added to form a two-phase system. SP and LP were based on the H medium devised

by Nitsch and Nitsch (1969) but had higher FeEDTA (0.1 mM), maltose (20 g·L⁻¹), and a pH of 5.8 before autoclaving. In addition, the SP contained 0.5 % (w/v) activated charcoal and was solidified with 0.3% (w/v) Gelrite.

Cultures were incubated in darkness at 7 °C for 1 week and then at 28 °C for 8 weeks. Embryos formed after this period were

Fig. 1. Pepper androgenesis in the two-phase culture system. Mononucleated microspores labeled with mithramycin in pepper anthers before incubation (A; bar = 20 μ m). Cotyledonary androgenic embryos (B; bar = 1 mm) released from anthers (arrow), after 8 weeks of culture, along with embryos at different stages of development (C; bar = 1 mm). Precociously germinated embryos (D; bar = 5 mm). Developed plantlets in tubes (E) and after acclimation (F).

transferred to petri dishes containing 8 mL of plantlet regeneration 1 medium (PR1). PR1 medium is similar to SP but had Bacto agar (10 g·L⁻¹) in place of Gelrite and no charcoal. After 3 to 4 weeks, green regenerants were transferred to tubes with plantlet regeneration 2 (PR2) medium, which was similar to PR1 but contained sucrose (20 g·L⁻¹) instead of maltose. During plantlet development, cultures were maintained at 28 °C with a 16-h photoperiod (50 μmol·m⁻²·s⁻¹ for PR1 and 70 μmol·m⁻²·s⁻¹ for PR2). Plantlets with four to six normal leaves and a good root system were acclimated in a mixture of 2 peat : 1 perlite (v/v).

COMPARISONS OF ANTHR CULTURE PROTOCOLS. The procedures described by Dumas de Vaulx et al. (1981) and the two-layer system were compared using flowers collected from nine bell pepper genotypes (C41 to C49). Briefly, the protocol of Dumas de Vaulx et al. (1981) involved incubating anthers on a C medium supplemented with 2,4-D at 0.01 mg·L⁻¹, kinetin at 0.01 mg·L⁻¹, vitamin B₁₂ at 0.03 mg·L⁻¹, 0.05 mM FeEDTA, 3% (w/v) sucrose, and 0.8% (w/v) Bacto agar. Cultures were kept at 35 °C in darkness for 8 d and then transferred after 12 d to R1 medium. R1 medium differed from C medium in that it contained kinetin at 0.1 mg·L⁻¹ but no 2,4-D or vitamin B₁₂. Cultures were maintained at 25 °C with a 12-h photoperiod. After 40 to 60 d, plantlets were observed on R1 medium and were transferred to a fresh medium. Embryos with poor development were transferred to R2 medium with kinetin at 2 mg·L⁻¹.

A complete randomized design was used. Each experiment consisted of 25 replicated petri dishes of 10 to 12 anthers (from two flowers) per genotype per protocol. The experiment was repeated five times. The number of responsive flowers per 100 flowers (RF/100 F), of embryos (normal and abnormal) per 100 flowers (E/100 F), and of normal embryos per 100 flowers (NE/100 F) were recorded after 8 weeks. The number of acclimated plants per 100 flowers (AP/100 F) also was scored. It should be noted that results were reported based on the number of flowers rather than anthers because, in the two-phase system, embryos were released into liquid media.

GENOTYPE EFFECTS. Production of androgenic haploids was examined with the two-phase system using 14 genotypes, C21, C22, C31, C33, and C40–C49. Fifty replicated petri dishes of 10 to 12 anthers (from two flowers) were used per genotype, and the experiment was repeated five times. The number of responsive flowers, embryos, and normal embryos were determined after 8 weeks, and the number of acclimated plants was recorded.

THE INFLUENCE OF SUGAR SOURCE AND CONCENTRATION. The effects of maltose; malt extract; and sucrose at 10, 20, 40, 60, and 80 g·L⁻¹ were investigated using the two-phase system. The genotypes C41, C43, C47, and C49 were used. Three petri dishes of 10 to 12 anthers derived from two flowers were used, and the experiment was repeated eight times. The numbers of total and normal embryos were recorded after 8 weeks.

EFFECTS OF CO₂ ENRICHMENT. The effect of CO₂ on androgenesis was tested using five genotypes (C41, C43, C47–C49) in a two-phase medium supplemented with maltose at 20 g·L⁻¹. Cultures were incubated for 8 weeks in darkened plexiglass boxes with periodic inflow of air containing CO₂ (in μL·L⁻¹) at 1) 300, 2) 600, or 3) 900. Air was renewed every 48 h. Control samples were held in ambient air (not renewed) that contained CO₂ at ≈340 μL·L⁻¹.

Twenty five petri dishes (10 to 12 anthers from two flowers) were used per treatment, and the experiment was repeated four times. Number of embryos and normal embryos were determined after 8 weeks, and the number of acclimated plants was scored.

STATISTICAL ANALYSES. A preliminary analysis of data revealed a large positive correlation between estimates of the mean and

variance. Therefore, data were transformed before statistical analysis by taking the fourth root (X^{1/4}). Following transformation, analysis of variance, separation of means by the Fisher's protected least significant difference, and planned contrasts (Petersen, 1985) were conducted. Regression analysis were conducted using orthogonal polynomial contrasts. Polynomial coefficients to partition the treatment sum of squares (SST) into components associated with successive terms of a polynomial equation and the regression equation were calculated according to Carmer and Seif (1963).

DETERMINATION OF PLOIDY LEVEL. Flow cytometry was used to determine the DNA content (ploidy level) of acclimated plants, after labeling of isolated leaf nuclei with propidium iodide. Young leaves (200 mg) from actively growing plants were finely chopped and macerated for 5 min into 1 mL LB01 lysis buffer (Dolozel et al., 1989) with 2% (v/v) Triton X-100 to release intact nuclei. The supernatant was filtered through a 56 μm nylon mesh, and 400-μL aliquots were used. The nuclei were labeled with propidium iodide (20 μg·mL⁻¹) and refrigerated at 4 °C in darkness for 30 min. Flow cytometry was performed with an EPICS ELITE (Coulter Electronics, Hialeah, Fla.) instrument with a standard air-cooled argon-ion laser tuned to 488 nm at 15 mW.

Red fluorescence was analyzed using a 675 nm band-pass filter in optical block three. Forward scatter (FSC), side scatter (SSC), red fluorescence, and time as control parameter were collected. Data were analyzed with Elite software, and results were presented in a histogram expressing the distribution of nuclei according to the red fluorescence intensity, which is proportional to the DNA content and combinations of FSC, SSC, and fluorescence parameters to identify the nuclei population.

ISOZYME ANALYSES. The origin of plants obtained from anther culture was determined by isozyme markers (Tanksley, 1984). Preliminary analyses of 16 enzymes (aconitase, alcohol dehydrogenase, catalase, malic enzyme, esterase, acid phosphatase, phosphoglucosomerase, phosphoglucosomutase, 6-phosphoglucosomate dehydrogenase, glutamate dehydrogenase, glutamate-oxaloacetate transaminase, isocitrate dehydrogenase, leucine aminopeptidase, malate dehydrogenase, peroxidase, and shikimate dehydrogenase) indicated that only variant forms of isocitrate dehydrogenase (IDH) occurred in the F₁ hybrids C33, C41, C42, and C44–C48. Therefore, the patterns of IDH-1 (Tanksley, 1984) were determined after isozyme separation by starch gel electrophoresis (Shields et al., 1983; Tanksley, 1984). Young leaves (2 cm²) were macerated in 100 μL extraction buffer (1% w/v glutathione in 0.1 M Tris, pH 8.5). Filter paper wicks (Whatman no. 3; Whatman Scientific, Maidstone, Kent, England) were soaked in the extract and used to load a 12.5% (w/v) starch gel with 5 mM DL-histidine (pH 7.0). The electrode buffer was 0.1 M tris-citrate (pH 7.0). Electrophoresis was performed for 3 h at 200 V. IDH isozymes were stained at 35 °C according to procedures described earlier (Vallejos, 1983). Chi-square analyses (α = 0.05) were used to test the goodness of fit to expected segregations of the *Idh-1* locus.

CHROMOSOME DOUBLING AND SEED PRODUCTION. For chromosome doubling of haploid plants, branches were pruned and axillary buds were removed. Colchicine (0.4% w/w) in lanolin paste then was applied to sites where axillary buds had been removed. Seeds were collected from new shoots originating from secondary meristems as well as from spontaneously doubled plants.

Results and Discussion

EFFICIENCY OF ANTHR CULTURE PROTOCOLS. The two-phase system gave better results than the method devised by Dumas de

Vaulx et al. (1981) (Table 1). The number of RF/100 F was significantly higher ($P < 0.001$) for the two-layer system. Normal and abnormal embryos (Fig. 1 B and C) were floating on the liquid medium or laying on the solid phase after 6 weeks. The developmental stages of the embryos varied, ranging from the globular to the cotyledonary stage. Some embryos had visible radicles, and their cotyledons turned green. On the medium of Dumas de Vaulx et al. (1981), embryos developed directly from anthers but some callus also formed on the anther. The frequency of embryos formed on this medium was significantly low, and many of the embryos were abnormal. For the number of embryos formed, the genotype $\times$ medium interaction was highly significant ($P = 0.001$). Also the number of NE/100 F was significantly higher ($P < 0.001$) in the two-layer culture system. However, the number of acclimated plantlets (Table 1 and Fig. 1E and F) was the same for the two methods.

Table 1. Androgenic response of bell pepper F_1 hybrids to the two-layer or the Dumas de Vaulx et al. (1981) culture system.

F_1 hybrids	Response to culture systems ^{z,y}							
	RF/100 F ^x		E/100 F ^x		NE/100 F ^x		AP/100 F ^x	
	1	2	1	2	1	2	1	2
C41	66.0	7.5	228.0	12.5	12.6	1.5	2.0	0.6
C42	13.1	17.5	22.1	35.8	3.8	2.5	0.8	0.0
C43	64.2	3.8	426.3	5.1	24.4	0.4	0.0	0.4
C44	20.5	2.6	20.8	3.2	9.1	0.5	0.0	0.5
C45	19.3	9.1	21.4	12.3	3.3	4.0	1.6	4.0
C46	4.4	1.0	3.3	1.0	0.0	0.0	1.1	0.0
C47	67.7	15.1	227.2	30.6	19.3	4.3	2.0	1.4
C48	33.9	3.2	80.9	3.9	7.6	0.8	0.8	0.0
C49	59.8	1.8	119.1	3.2	6.0	0.0	5.5	0.0
Mean of nine genotypes	37.6 a	6.8 b	111.9 a	11.9 b	9.2 a	1.6 b	1.3 a	0.8 a

^zCulture systems 1 and 2 correspond to the two-layer and Dumas de Vaulx et al. (1981) media, respectively.

^yTreatment means with the same letter are not significantly different in the Fisher's protected least significant difference ($P = 0.05$).

^xF = number of flowers, RF = number of responsive flowers, E = number of embryos at all developmental stages, NE = number of normal embryos with cotyledons and radicles, and AP = number of acclimated plants.

Table 2. Differences on the androgenic response of bell pepper F_1 hybrids to the two-phase culture system.

F_1 hybrids	Androgenic response (mean $\pm$ SE) ^{z,y}			
	RF/100 F	E/100 F	NE/100 F	AP/100 F
C31	75.2 $\pm$ 5.5 a	600.2 $\pm$ 167.6 a	39.29 $\pm$ 13.3 a	8.32 $\pm$ 3.42 a
C21	48.8 $\pm$ 6.0 ab	207.5 $\pm$ 61.3 bc	25.58 $\pm$ 10.3 a-c	7.93 $\pm$ 3.69 ab
C33	43.6 $\pm$ 9.9 a-c	81.9 $\pm$ 27.4 c-e	18.94 $\pm$ 9.0 a-c	2.95 $\pm$ 2.32 a-c
C49	48.7 $\pm$ 7.0 bc	203.4 $\pm$ 91.5 cd	54.64 $\pm$ 33.8 b-d	4.15 $\pm$ 2.45 b-d
C47	66.1 $\pm$ 3.5 a	284.7 $\pm$ 60.7 ab	22.15 $\pm$ 3.9 a	1.34 $\pm$ 0.53 b-d
C41	50.7 $\pm$ 7.1 a-c	307.1 $\pm$ 90.7 bc	15.86 $\pm$ 6.3 b-d	0.67 $\pm$ 0.38 b-e
C43	56.0 $\pm$ 7.6 ab	750.7 $\pm$ 289.6 ab	25.31 $\pm$ 8.6 ab	0.81 $\pm$ 0.44 c-c
C45	12.2 $\pm$ 4.3 ef	12.9 $\pm$ 6.1 fg	1.66 $\pm$ 0.7 d-f	0.59 $\pm$ 0.41 c-e
C22	45.4 $\pm$ 9.4 a-c	95.4 $\pm$ 13.4 cd	9.29 $\pm$ 4.3 c-f	0.57 $\pm$ 0.57 c-e
C48	35.3 $\pm$ 6.1 cd	97.3 $\pm$ 28.3 de	6.53 $\pm$ 1.3 b-e	0.75 $\pm$ 0.46 c-e
C46	4.9 $\pm$ 2.9 f	2.9 $\pm$ 1.6 g	0.71 $\pm$ 0.7 f	0.33 $\pm$ 0.33 c-e
C42	9.9 $\pm$ 3.0 ef	10.7 $\pm$ 5.6 fg	1.34 $\pm$ 0.9 ef	0.25 $\pm$ 0.25 c-e
C44	6.6 $\pm$ 3.2 f	6.2 $\pm$ 3.7 fg	2.64 $\pm$ 2.3 ef	0.00 $\pm$ 0.00 de
C40	21.3 $\pm$ 5.0 de	22.6 $\pm$ 7.4 ef	2.48 $\pm$ 1.5 f	0.00 $\pm$ 0.00 c

^zTreatment means with the same letter are not significantly different in the Fisher's protected least significant difference ($P = 0.05$).

^yF = number of flowers; RF = number of responsive flowers, E = number of embryos at all developmental stages, NE = number of normal embryos with cotyledons and radicle, and AP = number of acclimated plants.

Cold (at 7 °C) or heat (at 35 °C) preconditioning preceded the cultures with two-phase medium and Dumas de Vaulx media, respectively. Omission of cold treatment resulted in lower rates of embryogenesis (data not shown). Although the mechanism of temperature shock in inducing higher androgenesis is not known, one of the possibilities suggested is the reduction of ABA levels in the cultured anthers (Johansson et al., 1982).

Activated charcoal was beneficial in anther culture of a number of plant species because it may adsorb inhibitory substances from agar or the air or those produced by the anthers (Horner et al., 1977; Johansson, 1983; Kohlenbach and Wernicke, 1978; Mensuali-Sodi et al., 1993). However, activated charcoal also can adsorb nutrients, organic substances (FeEDTA, pyridoxin, nicotinic acid, thiamine, biotin, and folic acid), or growth-promoting hormones released by anther tissues (Johansson et al., 1990, 1983; Vagera, 1990; Vagera and Havranek, 1985; Weatherhead et al., 1979)

resulting in the small abnormal embryos found with pepper (Table 1 and Fig. 1C).

The plants were recovered from the anther culture systems (Fig. 1 E and F). Acclimation of plants in a mixture of peat and perlite was effective. Overall, the double-phase medium is most effective in producing embryos. Further tests were focused on improving the number of normal embryos and conversion to plants.

GENOTYPE EFFECTS. The genotype of the donor plant had a significant effect ($P < 0.001$) on androgenesis as well as development of plantlets (Table 2). This result was expected since genotypic differences have been reported for many other species (Bajaj, 1983, 1990; Maheshwari et al., 1980) as well as pepper (Dumas de Vaulx et al., 1981; Kristiansen and Andersen, 1993; Ltifi and Wenzel, 1994; Mityko et al., 1995; Morrison et al., 1986b; Munyon et al., 1989; Qin and Rotino, 1993; Sibi et al., 1979). Optimizing growth conditions of the donor plants did not eliminate such genotypic effects (Kristiansen and Andersen, 1993). The differential responses of genotypes to the two media (Table 1) will make it possible to choose the right medium for each genotype. For example, the Spanish cultivar Piquillo was unresponsive to the medium of Dumas de Vaulx (Mityko et al., 1995); however, with the double-phase system, we were able to regenerate plants from this cultivar at a rate similar to that for bell peppers (12 AP/100 F).

THE INFLUENCE OF SUGAR SOURCE AND CONCENTRATION. The type and concentration of sugar had highly significant effects ($P < 0.001$) on E and NE/100 F (Fig. 2). Maltose was most beneficial. At 40 g·L⁻¹, the total number of embryos obtained was the highest; however, the highest frequency of normal embryos was obtained with 20 g·L⁻¹. The beneficial effect of maltose in inducing haploid formation has been reported for barley (Finnie et al., 1989) and wheat (Karsai et al., 1994), but, to our knowledge, this study is the first to confirm it for pepper. The basis of the stimulatory effects of maltose is not known, but it was suggested that microspores are sensitive to glucose and fructose (Finnie et al., 1989). Maltose, however, may be converted to glucose more slowly, thus avoiding the inhibitory effects of other sugars.

EFFECTS OF CO₂ ENRICHMENT. Anthers from five bell pepper F₁ hybrids were used to test the effect of CO₂-enriched atmosphere.

Embryogenesis, measured by the number of E/100 F (Fig. 3A), was significantly enhanced ($P < 0.001$) in atmosphere enriched with CO₂ at either 600 or 900 $\mu\text{L}\cdot\text{L}^{-1}$. The frequency of embryos developing into plants also was higher under CO₂ at 900 $\mu\text{L}\cdot\text{L}^{-1}$ (Fig. 3C). However, the number of normal embryos was not different from the lower (300 $\mu\text{L}\cdot\text{L}^{-1}$) CO₂ enrichment (Fig. 3B). Periodically refreshing the culture atmosphere by allowing air flow (every 48 h), regardless of the CO₂ concentration, appeared to have a positive effect on normal embryo formation, as the number of normal embryos was significantly higher for CO₂ at the 300 $\mu\text{L}\cdot\text{L}^{-1}$ treatment than the control ($P = 0.024$; Fig. 3B). The beneficial effects of air movement may be related to the removal of accumulated ethylene. These stimulatory effects of CO₂ on androgenesis have been reported for several other species as well (Johansson et al., 1982, 1990; Johansson and Ericksson, 1984).

Fig. 2. Effects of different concentrations of maltose (■), malt extract (□), and sucrose (○) on the number of total embryos (A) and normal embryos (B) per 100 flowers after 8 weeks in the two-phase culture system. Vertical bars = SE. For number of total embryos, the polynomial regression was quadratic for maltose and linear for malt extract and sucrose. For number of normal embryos, the regression was cubic for maltose and malt extract and quadratic for sucrose.

Fig. 3. Effect of the concentration of CO₂ on total embryos (A), normal embryos (B), and acclimated plants (C) per 100 flowers after 8 weeks in the two-phase culture system. Treatment means with the same letter are not significantly different in the Fisher's protected least significant difference ($P = 0.05$). Vertical bars = SE.

ORIGIN OF ANDROGENIC PLANTS. The ploidy level and the origin of androgenetic plants were determined using flow cytometry (Fig. 4) and isozyme patterns (Fig. 5), respectively. Haploid pepper plants could be morphologically differentiated by their narrower leaves, shorter internodes, and reduced vigor compared to diploid plants. However, when using these criteria, some mistakes were corroborated by flow cytometry. Of all the androgenetic plants obtained, 34% were haploids, 65% were spontaneously doubled diploids, and the remainder (0.8%) were triploids (Table 3). The frequency of spontaneous diploids was higher in this study than the other reports for pepper (Dumas de Vaulx et al., 1981; Mityko et al., 1995; Morrison et al., 1986b; Munyon et al., 1989; Qin and Rotino, 1993; Sibiel et al., 1979; Vagera, 1990; Vagera and Havranek, 1985). Such diploids will not require colchicine treatment to restore fertility.

Of 16 isozymes examined, isocitrate dehydrogenase was the only one found to be useful since three NADP-dependent bands of activity were observed in eight of the 14 F_1 hybrids. As the enzyme is encoded by a single locus (*Idh-1*), the isozyme pattern suggests a dimeric enzyme (Tanksley, 1984). Only one band occurred in each of the anther-derived haploids and diploids (Fig. 5), except for one diploid plant, which had multiple bands of the enzyme (Table 3), suggesting the plant may have derived from $2n$ pollen or somatic tissues. With molecular markers at more loci, we could ascertain whether the spontaneous diploids had a sporophytic origin or had originated from an unreduced microspore (Cloutier and Landry, 1994). A much higher proportion (five of 40) of pepper plants originated from somatic tissue in the studies of Munyon et al. (1989), probably due to using the medium of Dumas de Vaulx et al. (1981), which often induces callus.

According to Vagera (1990), more dihaploids are formed spontaneously in pepper than in other species of the Solanaceae

family. Our findings agree with this result since we obtained high proportions of dihaploid plants (Table 3) but much lower proportions (20% to 40%) of dihaploids in *Solanum melongena* (unpublished results). The high rate of diploidization in pepper facilitates recovery of inbred lines. However, in some plants diploidization occurred late in plant development. For production of large numbers of inbred lines, we found it preferable to evaluate the ploidy of plants early, when the second side shoot was formed, and to treat those that had remained haploid with colchicine.

To fully exploit the genetic variation in donor plants, it is important that the population of plants derived from anther culture represents a broad sample of gametes. Segregation analyses of several species, such as broccoli (Orton and Browers, 1985), rye grass (Hayward et al., 1990), barley (Thompson et al., 1991), rice (Guiderdoni et al., 1991), and rapeseed (Foisset et al., 1993) showed that populations derived from anther culture exhibit distorted segregation ratio. Also for pepper, marker segregation in three dihaploid populations were skewed toward one or the other parent (Lefebvre et al., 1992). In our work with the IDH-1 isozyme, no distortion from the expected 1:1 segregation occurred (Table 3). However, we focused on only one isozyme marker; thus, additional differences might be found with more extensive analyses or using restricted fragment length polymorphism analysis markers instead of isozymes.

Conclusion

We have shown that success in culturing pepper anthers can be enhanced significantly by using the two-phase culture system, with activated charcoal in the solid phase and maltose as the sugar source, and by flushing the culture environment with CO_2 . The limiting factor does not reside in the number of embryoids formed but in the further development of embryos and conversion to plants. Too many of the embryos were abnormal in one aspect or another and could not be stimulated to develop into normal plants. Future efforts should focus on further refinements of the medium and culture conditions to normalize embryo development. Despite the problems, this technique is routinely used in pepper breeding programs to achieve complete homozygosity in one generation and DHLs are used as parental lines for the production of commercial bell pepper hybrids. Moreover, pepper has not benefited yet from isolated microspore culture, which might circumvent problems inherent to anther culture and may be useful in the direct production of transgenic plants.

Literature Cited

- Abak, K., E. Pochard, and R. Dumas de Vaulx. 1982. Transmission of resistance to *Phytophthora capsici* on roots and stems of pepper plants: Study of doubled haploid lines issued from the cross 'PM 217' x 'Yolo Wonder' through anther culture. *Capsicum Nwsl.* 1:62-63.
- Bajaj, Y.P.S. 1983. In vitro production of haploids, p. 228-287. In: D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. Yamada (eds.). *Handbook of plant cell culture*, vol. 1. Macmillan Publishing Co., New York.
- Bajaj, Y.P.S. 1990. In vitro production of haploids and their use in cell genetics and plant breeding, p. 3-44. In: Y.P.S. Bajaj (ed.). *Biotechnology in agriculture and forestry*, vol. 12. Haploids in crop improvement I. Springer-Verlag, Berlin, Germany.
- Carmer, S.G. and R.D. Seif. 1963. Calculation of orthogonal coefficients when treatments are unequally replicated and/or unequally spaced. *Agron. J.* 55:387-389.
- Cloutier, S. and B. Landry. 1994. Molecular markers applied to plant tissue culture. *In Vitro Cell. Dev. Biol.* 30P:32-39.
- Daubèze, A.M., A. Palloix, and E. Pochard. 1990. Resistance of androgenetic autodiploid lines of pepper to *Phytophthora capsici* and tobacco

Fig. 4. Histograms of flow cytometric analysis of DNA content of nuclei released from leaves of a diploid ($2n$) and haploid (n) pepper plant.

Fig. 5. Isocitrate dehydrogenase profiles from C47 and C33 F₁ hybrids (bands with arrows) and corresponding androgenic plants (remaining bands).

Table 3. Ploidy distribution and segregation of the enzyme locus *Idh-1* in adnrogenic plants produced from bell pepper F₁ hybrids.

F ₁ hybrids	AP ^z	Ploidy				<i>Idh-1</i> genotypes			χ^2	1:1
		n	2n	3n	%2n	aa	bb	ab		
C21	40	12	27	1	68	---	---	---	---	
C22	10	3	7	0	70	---	---	---	---	
C31	52	28	24	0	46	---	---	---	---	
C33	10	5	5	0	50	2	8	0	3.600	NS
C40	9	2	7	0	78	---	---	---	---	
C41	14	6	8	0	57	8	6	0	0.286	NS
C42	1	1	0	0	0	1	0	0	0.000	NS
C43	29	7	31	1	72	---	---	---	---	
C44	2	0	2	0	100	1	1	0	0.000	NS
C45	13	7	6	0	46	6	7	0	0.077	NS
C46	1	0	1	0	100	0	1	0	0.000	NS
C47	113	35	77	1	68	65	51	1	1.690	NS
C48	40	10	30	0	75	22	20	0	0.095	NS
C49	55	17	38	0	69	---	---	---	---	
Total	389	133	253	3	65	105	94	1	0.608	NS

^zAP = number of acclimated plants.

^{NS}Nonsignificant deviation at $P = 0.05$.

mosaic virus under high temperature. *Capsicum* Nwsl. 8-9:47-48.

Dolozel, J., P. Binarova, and S. Lucretti. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol. Plant.* 31:113-120.

Dumas de Vaulx, R. 1990. Haploidy and pepper breeding: A review. *Capsicum* Nwsl. 8-9:13-17.

Dumas de Vaulx, R., D. Chambonnet, and E. Pochard. 1981. Culture in vitro d'anthères de piment (*Capsicum annuum* L.): Amélioration des taux d'obtention de plantes chez différents génotypes par des traitements à +35 C. *Agronomie* 1:859-864.

Dumas de Vaulx, R., E. Pochard, and D. Chambonnet. 1982. Distribution of TMV—Susceptible and resistant doubled haploid lines from anther culture of heterozygous L⁺/L⁻ hybrids. *Capsicum* Nwsl., 1:52-53.

Finnie, S.J., W. Powell, and A.F. Dyer. 1989. The effect of carbohydrate composition and concentration on anther culture response in barley (*Hordeum vulgare* L.). *Plant Breeding* 103:110-118.

Foisset, N., R. Delourme, M.O. Lucas, and M. Renard. 1993. Segregation analysis of isozyme markers on isolated microspore-derived embryos in *Brassica napus* L. *Plant Breeding* 110:315-322.

Foroughi-Wehr, B. and G. Wenzel. 1993. Andro- and parthenogenesis, p. 261-277. In: M.D. Hayward, N.O. Bosemark, and I. Romagosa (eds.). *Plant breeding: Principles and prospects*. Chapman and Hall, London.

George, L. and S. Narayanaswamy. 1973. Haploid *Capsicum* through experimental androgenesis. *Protoplasma* 78:467-470.

Guiderdoni, E., J.C. Glazmann, and B. Courtois. 1991. Gametic selection in anther culture of rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 81:406-412.

Hayward, M.D., A. Olesen, I.K. Due, R. Jenkins, and P. Morris. 1990. Segregation of isozyme marker loci amongst androgenetic plants of *Lolium perenne* L. *Plant Breeding* 104:68-71.

Hendy, H., E. Pochard, and A. Dalmasso. 1985. Transmission de la résistance aux nématodes *Meloidogyne chitwood* (Tylenchida) portée par 2 lignées de *Capsicum annuum* L.: Étude de descendances homozygotes issues d'androgénèse. *Agronomie* 5:93-100.

Horner, M., J.A. McComb, A.J. McComb, and H.E. Street. 1977. Ethylene production and plantlet formation by *Nicotiana* anthers cultured in the presence and absence of charcoal. *J. Expt. Bot.* 28:1365-1372.

Johansson, L. 1983. Effects of activated charcoal in anther cultures. *Physiol. Plant.* 59:397-403.

Johansson, L., B. Andersson, and T. Eriksson. 1982. Improvement of anther culture technique: activated charcoal bound in agar medium in combination with liquid medium and elevated CO₂ concentration. *Physiol. Plant.* 54:24-30.

Johansson, L. and T. Eriksson. 1984. Effects of carbon dioxide in anther cultures. *Physiol. Plant.* 60:26-30.

Johansson, L.B., E. Calleberg, and A. Gedin. 1990. Correlations between activated charcoal, Fe-EDTA and other organic media ingredients in cultured anthers of *Anemone canadensis*. *Physiol. Plant.* 80:243-249.

Karsai, I., Z. Bedö, and P.M. Hayes. 1994. Effect of induction medium pH and maltose concentration on in vitro androgenesis of hexaploid winter triticale and wheat. *Plant Cell Tissue and Org. Cult.* 39:49-53.

Kohlenbach, H.W. and W. Wernicke. 1978. Investigations on the inhibitory effect of agar and the function of active carbon in anther culture. *Z. Pflanzenphysiol.* 86:463-472.

Kristiansen, K. and S.B. Andersen. 1993. Effects of donor plant temperature, photoperiod, and age on anther culture response of *Capsicum annuum* L. *Euphytica* 67:105-109.

Kuo, J.S., Y.Y. Wang, N.F. Chien, S.J. Ku, M.L. Kung, and H.C. Hsu. 1973. Investigations on the anther culture in vitro of *Nicotiana tabacum* L. and *Capsicum annuum* L. *Acta Bot. Sinica* 15:47-52.

Lefebvre, V., T. Prevost, and A. Palloix. 1992. Segregation of molecular markers in doubled haploid progenies of pepper, p. 232-237. In: VIIIth Meeting Genetics and Breeding on *Capsicum* and Eggplant, EUCARPIA, Rome, Italy, 7-10 Sept.

Ltifi, A. and G. Wenzel. 1994. Anther culture of hot and sweet pepper (*Capsicum annuum* L.): Influence of genotype and plant growth temperature. *Capsicum and Eggplant Nwsl.* 13:74-77.

Maheshwari, S.C., A.K. Tyagi, K. Malhotra, and S.K. Sopory. 1980. Induction of haploidy from pollen grains in Angiosperms—The current status. *Theor. Appl. Genet.* 58:193-206.

Mensuali-Sodi, A., M. Panizza, G. Serra, and F. Tognoni. 1993. Involvement of activated charcoal in the modulation of abiotic and biotic ethylene levels in tissue cultures. *Scientia Hort.* 54:49-57.

Mityko, J., A. Andrasfalvy, G. Csillery, and M. Fari. 1995. Anther-culture

- response in different genotypes and F_1 hybrids of pepper (*Capsicum annuum* L.). *Plant Breeding* 114:78–80.
- Morrison, R.A., R.E. Koning, and D.A. Evans. 1986 a. Pepper, p. 552–573. In: D.A. Evans, W.R. Sharp, and P.V. Ammirato (eds.). *Handbook of plant cell culture*, vol. 4, Techniques and applications. Macmillan Publ. Co., New York.
- Morrison, R.A., R.E. Koning, and D.A. Evans. 1986 b. Anther culture of an interspecific hybrid of *Capsicum*. *J. Plant Physiol.* 126:1–9.
- Munyon, I.P., J.F. Hubstenberger, and G.C. Phillips. 1989. Origin of plantlets and callus obtained from chile pepper anther cultures. *In Vitro Cell. Dev. Biol.* 25P:293–296.
- Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. *Science* 163:85–87.
- Novak, F.J. 1974. Induction of a haploid callus in anther cultures of *Capsicum* sp. *Z. Pflanzenzüchtg* 72:46–54.
- Orton, T.J. and M.A. Browers. 1985. Segregation of genetic markers among plants regenerated from cultured anthers of broccoli (*Brassica oleracea* var. *italica*). *Theor. Appl. Genet.* 69:637–643.
- Pace, G.M., J.N. Reed, L.C. Ho, and J.W. Fahey. 1987. Anther culture of maize and the visualization of embryogenic microspores by fluorescent microscopy. *Theor. Appl. Genet.* 73:863–869.
- Petersen, R.G. 1985. *Design and analysis of experiments*. 1st ed. Marcel Dekker, New York.
- Pochard, E. and R. Dumas de Vaulx. 1979. Haploid parthenogenesis in *Capsicum annuum* L., p. 455–472. In: J.G. Hawkes (ed.). *The biology and taxonomy of the Solanaceae*. Acad. Press, London.
- Pochard, E., K.G. Sclassi, and G. Marchoux. 1983. Oligogenic resistance to potato virus Y pathotype 1-2 in the line 'Perennial'. *Capsicum Nwsl.* 2:137–138.
- Qin, X. and G.L. Rotino. 1993. Anther culture of several sweet and hot pepper genotypes. *Capsicum and Eggplant Nwsl.* 12:59–62.
- Shields, C.R., T.J. Orton, and C.W. Stuber. 1983. An outline of general resource needs and procedures for the electrophoretic separation of active enzymes from plant tissue, p. 443–468. In: S.D. Tanksley and T.J. Orton (eds.). *Isozymes in plant genetics and breeding*, part A. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
- Sibi, M., R. Dumas de Vaulx, and D. Chambonnet. 1979. Obtention de plantes haploïdes par androgénèse *in vitro* chez le piment (*Capsicum annuum* L.). *Ann. Amélior. Plantes* 29:583–606.
- Tanksley, S.D. 1984. Linkage relationships and chromosomal locations of enzyme-coding genes in pepper, *Capsicum annuum*. *Chromosoma* 89:352–360.
- Thompson, D.M., K. Chalmers, R. Waugh, B.P. Forster, W.T.B. Thomas, P.D.S. Caligri, and W. Powell. 1991. The inheritance of genetic markers in microspore-derived plants of barley *Hordeum bulgare* L. *Theor. Appl. Genet.* 81:487–492.
- Vagera, J. 1990. Pepper (*Capsicum* spp.): In vitro induction of haploids, p. 374–392. In: Y.P.S. Bajaj (eds.). *Biotechnology in agriculture and forestry*, vol. 12, haploids in crop improvement I. Springer-Verlag, New York.
- Vagera, J. and P. Havranek. 1985. In vitro induction of androgenesis in *Capsicum annuum* L. and its genetic aspects. *Biol. Plant. (Praha)* 27:10–21.
- Vallejos, C.E. 1983. Enzyme activity staining, p. 469–516. In: S.D. Tanksley and T.J. Orton (eds.). *Isozymes in plant genetics and breeding*, part A. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
- Wang, Y.Y., C.S. Sun, C.C. Wang, and N.F. Chien. 1973. The induction of the pollen plantlets of *Triticale* and *Capsicum annuum* from anther culture. *Scientia Sinica* 16:147–151.
- Weatherhead, M.A., J. Burdon, and G.G. Henshaw. 1979. Effects of activated charcoal as an additive plant tissue culture media: Part 2. *Z. Pflanzenphysiol* 94:399–405.