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ORIGIN OF PLANTLETS AND CALLUS OBTAINED FROM CHILE PEPPER ANTHER CULTURES

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SUMMARY

Androgenesis occurred from chile pepper (*Capsicum annuum* L.) anthers incubated in a continuous warm environment (29° C) with continuous light. Forty plantlets and embryoids were retrieved from anther cultures and analyzed for isozyme markers. Of these, 35 exhibited a single allele for markers suggesting microspore origin, while 5 were heterozygous indicating somatic tissue origin. Chromosome numbers were confirmed for 21 plantlets, of which 16 were haploid and 5 were diploid. However, two plants exhibited a single allele for an isozyme marker but possessed the diploid chromosome number, suggesting spontaneous doubling. Anther cultures also produced callus. Nearly 92% of the slow-growing calli sampled were heterozygous for the isozyme marker, suggesting somatic tissue origin. More than 46% of the fast-growing calli exhibited only one allele for the marker, indicating microspore origin. Callus did not regenerate plantlets. The occurrence of both heterozygous and homozygous diploid plantlets from pepper anther cultures has important implications for applied breeding programs.

Key words: chile pepper; *Capsicum*; androgenesis; anther culture; callus; isozyme marker.

INTRODUCTION

The status of tissue, cell, and organ culture technologies in cultivated pepper (*Capsicum annuum* L. and related species) was reviewed recently (3,6). Androgenesis is the most extensively studied of these technologies. Initial anther culture studies in pepper reported the production of both embryoids and callus possessing the haploid chromosome number (5,13). Embryoid frequency was low, occurring from 0.1 to 5% of the cultured anthers. Detailed chromosomal analysis of anther-derived callus revealed the majority was diploid, polyploid, or aneuploid whereas a small proportion was haploid (8).

Temperature shock (4° C) pretreatments provided to anthers containing microspores at the first pollen mitosis enhanced androgenesis, yielding haploid, diploid, or triploid plantlets (10). This technique was optimized by applying heat shock (35° C) during the first 8 d of culture with subsequent transfer to a standard environment (2). From 5 to 50 plantlets per 100 anthers cultured were obtained, but more than 40% of the plantlets did not possess the haploid chromosome number. Pepper anther culture responses were highly dependent on genotype (2,10). Chile pepper genotypes have yet to be evaluated for anther culture response.

It is essential to determine the origin of the diploid and other nonhaploid plantlets produced by pepper anther cultures. Homozygous dihaploid plantlets derived from microspores would be of direct value to breeding programs, whereas heterozygous plantlets from somatic tissue would be of little value. This paper reports the use of enzyme markers available in peppers (11) to identify the origin of anther-derived plantlets and callus from chile peppers.

MATERIALS AND METHODS

Anther donor plant materials. Plants donating anthers for culture were provided by the New Mexico State University chile pepper breeding program, as available. Materials included *C. annuum* long green chile types 'NM 6-4', land races New Mexico Capsicum Accession (NMCA) 81, NMCA 91, Plant Introductions (PIs) 159258, 159264, 164502, 174112; paprika types PIs 127444, 138567, 164464, 174810, NMCA 138; F_1 *C. annuum* 'NM6-4' × *C. chinense* (NMCA 4), BC_1 *C. annuum* × (*C. annuum* × *C. chinense*), and F_1 *C. frutescens* (Mexico INIA BG3324) × *C. chinense* (NMCA 4).

All donor lines used, except 'NM6-4', were heterozygous for isozyme-coding locus 2 of phosphoglucomutase (*Pgm-2*). The interspecific F_1 and BC_1 between *C.*

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TABLE 1

CALLUS FORMATION AND DIRECT ANDROGENESIS FROM CULTURED CHILE PEPPER ANTHERS IN THE HEAT SHOCK INCUBATION SCHEME[a]

Genotype	Number of Anthers Cultured	Percent Cultured Anthers with	
		Callus	Androgenetic Plants
PIs 127444, 164464, 174810, F_1 (*C. annuum* × *C. chinense*)	1570	1.4	0
PIs 159258, 159264, 164502	955	0	0
PI 174112	510	3.5	0.6[b]
Total	3035	1.3%	0.1%

[a]Following procedures of Dumas de Vaulx et al. (2).
[b]Each androgenetic response consisted of a single plant per anther.

annuum and *C. chinense* were heterozygous for both *Pgm-2* and locus 1 of phosphoglucoisomerase (*Pgi-1*). The interspecific F_1 between *C. frutescens* and *C. chinense* was heterozygous for *Pgm-2*, loci 1 and 4 of malate dehydrogenase (*Mdh-1*, *Mdh-4*), and locus 1 of shikimate dehydrogenase (*Skdh-1*).

Donor plants were grown in the greenhouse or in a growth chamber at 25° C/12-h d and 18° C/12-h night. Floral buds were collected when the corolla was even with or just longer than the calyx, generally corresponding to the uninucleate microspore stage. Buds were surface sterilized by passage through 70% ethanol for 2.5 min, 2% sodium hypochlorite (40% commercial bleach) for 5 min, and rinsed in sterile deionized water for at least 5 min.

Anther culture conditions. Three incubation environments were used in different experiments. a) Anthers excised from buds exposed to 4° C for daily increments up to 9 d, to provide cold shock pretreatments, and controls (no cold shock) were cultured at 25 ± 1° C with a 16-h photoperiod (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$). b) Anthers were cultured for 1 wk at 35 ± 1° C in the dark (heat shock), then transferred to 25 ± 1° C with a 12-h photoperiod (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$). c) Anthers were cultured at 29 ± 1° C (continuous warm temperature) with continuous light (15 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

The hormone compositions of the media reported by Dumas de Vaulx et al. (2) were used as controls in all anther culture experiments, using the Murashige and Skoog (7) basal nutrient medium (MS) with vitamins according to the L2 recipe (9). Induction media included 0.01 mg·liter^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (picloram) or indole-3-acetic acid (IAA) in combinations with 0.01 mg·liter^{-1} kinetin or 6-benzylaminopurine (BA). Embryoids were grown out by transfer to MS medium including 0.1 or 2.0 mg·liter^{-1} kinetin, or 0.05 mg·liter^{-1} each of IAA and BA. Media were utilized in 60 × 15-mm petri dishes and sealed with Parafilm for incubation. Subcultures to fresh media were performed at 2- to 3-wk intervals following Dumas de Vaulx et al. (2).

Anther responses were tallied after 4 mo. of culture. Plantlets obtained from cultured anthers were then transferred to 0.1-strength B5 (4) basal nutrient medium or to the Singapore formula (1) medium, both without hormones, to encourage further development. After 1 mo. the plantlets were transplanted into peat pellets under high relative humidity for 2 wk, and plants were then established in the greenhouse.

Several confirmed haploid plants were treated for doubling of the chromosome number. A mixture of 1% colchicine in water-soluble lanoline was applied to nearly mature, decapitated plants at the subtending axillary bud regions. Emerging shoots were observed for fertility.

Cytogenetic and enzyme electrophoretic analyses. Plants obtained from anther cultures were evaluated for chromosome number. Root tips were fixed for 5 min in three parts 95% ethanol: one part acetic acid, hydrolyzed in 1 *N* HCl for 10 min, rinsed in 70% ethanol, macerated, and stained in 1% acetocarmine overnight. At least 10 intact cells showing good chromosome spreads were counted for each plantlet to determine ploidy.

All available plants and embryoids and random samples of callus obtained from anther cultures were evaluated electrophoretically. Starch gel electrophoresis followed the procedures of Tanksley (11) and Vallejos (12) for enzyme activities corresponding to the markers identified above, using samples of plant leaf tissues or 5-mm cubes of callus.

Results and Discussion

Temperature shock incubation schemes. Initial experiments evaluating chile peppers for anther culture response involved cold shock (10) or heat shock (2)

TABLE 2

CALLUS FORMATION AND DIRECT ANDROGENESIS FROM CULTURED CHILE PEPPER ANTHERS IN THE CONTINUOUS WARM INCUBATION ENVIRONMENT

Genotype	Number of Anthers Cultured	Anthers with Callus, %	Proportion of Callus Fast-growing, %	Anthers with Androgenetic Embryos, %	Complete Plants per 100 Anthers
NM 6-4	389	20.6	5.0	0.3	0
PI 138567	337	11.6	5.1	1.5	0.9
PI 164502	295	10.8	21.9	1.7	1.0
PI 174810	1229	27.6	24.8	5.9	3.3
F_1 (*C. annuum* × *C. chinense*)	337	26.7	12.2	2.1	0.3
F_1 (*C. frutescens* × *C. chinense*)	81	9.9	12.5	7.4	6.2
Total	2668	22.0	18.5	3.6	2.0

incubation schemes following previous reports on pepper androgenesis. In one experiment, cold shock pretreatments were provided to floral buds in daily increments from 0 (control) to 9 d before culture of the anthers. A total of 1365 anthers were cultured from the lines NMCA 81, NMCA 91, NMCA 138, PI 174112, and the BC_1 between *C. annuum* and *C. chinense*. No androgenetic plantlets were obtained. Callus was produced from 22% of the cultured anthers and did not seem to be influenced by the length of the cold shock pretreatment or by the medium used. Callus classified as fast-growing was obtained from only one line, NMCA 91, representing 11% of the callus obtained from that line. No plants were regenerated from callus.

Another experiment closely duplicated the heat shock incubation scheme described by Dumas de Vaulx et al. (2). PI 174112 was the only genotype available for use in this experiment that was also used in the cold shock experiment (*see* above). The only genotype responding with androgenetic plantlets in the heat shock experiment was PI 174112 (Table 1). The frequency of androgenesis in this genotype was low (0.6% of the anthers cultured), and only a single plant was obtained from each responsive anther. Callus was obtained from only certain genotypes, and at lower frequency in this experiment (1.3% of the cultured anthers) compared to the cold shock experiment (22%). Fast-growing callus lines were obtained from PIs 174112, 174810, and the interspecific F_1, representing 10% of all callus obtained in the experiment. No plants were regenerated from callus.

Continuous warm temperature incubation. This experiment evaluated a constant anther culture environment (29° C, continuous light), as opposed to the discontinuous temperature shock incubation schemes evaluated in the previous experiments. Genotypes available for this experiment that had also been used in the heat shock experiment (*see* above) included PIs 164502, 174810, and the F_1 (*C. annuum* × *C. chinense*). All of the chile pepper genotypes evaluated in this experiment produced androgenetic embryos or plantlets at an average frequency of 3.6% of the cultured anthers (Table 2). Several of these androgenetic responses consisted of multiple embryos or plantlets from the same anther, but not all embryos developed into complete plants. The use of 2,4-D in the induction medium was superior to the use of picloram or IAA (data not shown), accounting for 85% of all recovered plants. All genotypes produced callus in this experiment, at an average frequency of 22% of the cultured anthers, and more than 18% of the callus was fast-growing (Table 2). No plants were regenerated from callus.

The results of the experiments presented here confirm the importance of genotype in pepper androgenetic response as observed previously (2,10) and represent the first report of successful anther culture of chile pepper genotypes. Previous researchers have postulated that temperature shock is critical to achieve androgenesis in pepper anther cultures. However, the results of the present investigation suggest that extreme temperature shocks per se are not required for induction of androgenesis in chile pepper anther cultures, but rather that androgenesis is dependent on specific environmental conditions during the incubation period. The development of procedures for large-scale haploid breeding or genetics programs via androgenesis, therefore, will involve the identification of specific genotype-by-incubation environment interactions. Such interactions are evident in callus formation from cultured anthers as well as androgenetic plant formation in the present investigation.

Analysis of plantlet and callus origins. All available anther-derived plants and embryoids were analyzed for isozyme markers and chromosome numbers (Table 3). Of the 40 plants and embryoids analyzed by electrophoresis, 5 plants showed isozyme heterozygosity, suggesting somatic tissue origin. The remaining 35 plants showed only one segregating allele at marker loci, suggesting microspore origin. Where multiple isozyme markers were available, alleles segregated independently in the hap-

TABLE 3

CLASSIFICATION OF CHILE PEPPER PLANTS AND EMBRYOIDS RECOVERED FROM ANTHER CULTURES BASED ON ISOZYME MARKER OR CHROMOSOME NUMBER ANALYSES, OR BOTH

Genotype	Number of Plants			
	Analyzed	Haploid	Homozygous Diploid	Heterozygous Diploid
Complete plants from anther cultures				
PI 138567	2	2	0	0
PI 164502	3	1	1	1
PI 174810	18	15	1	2
F_1 (*C. annuum* × *C. chinense*)	1	1	0	0
F_1 (*C. frutescens* × *C. chinense*)	1	1	0	0
Incompletely developed plantlets and embryoids[a]				
PI 174810	8	7	—	1
F_1 (*C. annuum* × *C. chinense*)	2	1	—	1
F_1 (*C. frutescens* × *C. chinense*)	5	5	—	0

[a]Chromosome number could not be confirmed; classification is haploid for isozyme homozygotes or diploid for isozyme heterozygotes.

TABLE 4

ANALYSIS OF PLOIDY STATUS AMONG CHILE PEPPER ANTHER-DERIVED CALLI USING DONOR PLANTS HETEROZYGOUS FOR *PGM-2* OR *PGI-1*[a]

Callus Growth Rate	Number of Calli Tested	Isozyme Assay		Proportion of Calli of Putative Haploid Origin, %
		One Allele	Two Alleles	
Slow-growing	63	5	58	7.9
Fast-growing	89	41	48	46.1

[a]All genotypes were tested for *Pgm-2* isozyme activity, except F_1 (*C. annuum* × *C. chinense*) which was tested for *Pgi-1*.

loids (data not shown). Five of the 21 plants examined cytologically were diploid (2N=24), and the remaining 16 plants were haploid (N = 12). Two plants possessed the diploid chromosome number but showed only a single allele at the isozyme marker locus (Table 3), suggesting microspore origin and spontaneous doubling of chromosomes.

All diploid plants raised to maturity were fertile and set seed. Ten haploid plants were treated with colchicine for chromosome doubling, but only two plants survived (both from PI 174810). One of these plants produced fertile fruits on all emerging branches, whereas only one emerging branch of the other plant proved fertile.

Anther-derived calli were sampled for isozyme marker analysis (Table 4). Less than 8% of the sampled slow-growing calli exhibited a single allele at the marker locus, suggesting the majority of these calli were from somatic tissue origin. In contrast, more than 46% of the sampled fast-growing calli showed a single allele, suggesting microspore origin.

The results of this study demonstrate that pepper anther cultures give rise to plants both from microspore origin and from somatic tissue origin. Only those derived from microspores will be of potential use in breeding programs. It is interesting to note that two plants seemed to be spontaneously doubled haploids, and therefore have direct value in a breeding program. However, diploid plants obtained from anther cultures must be screened to eliminate heterozygotes, because heterozygotes are duplicates of the donor lines and add no value to a breeding program. Callus obtained from anther cultures also seemed to have formed from both microspores and somatic tissues. Fast-growing callus seemed to consist of a higher proportion of microspore-derived colonies. The development of appropriate procedures to regenerate pepper plants from callus, combined with screening to identify callus of microspore origin, would greatly enhance the yield of haploid plants for breeding purposes.

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