Genotype, Temperature, and Sampling Date Affect Embryogenesis in Asparagus Anther Culture

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Abstract. *Asparagus (Asparagus officinalis L.)* anthers from flowers of field-grown plants were cultured for five genotypes, four incubation temperatures, and three sampling dates. Treatments were evaluated for total and embryogenic callus production. Incubating anthers at 35C was optimal for initiating embryogenic callus for three genotypes. Another line performed best and equally well at 29 and 32C, while one was recalcitrant to embryogenic callus formation at the temperatures evaluated. For all genotypes, almost half of the anthers produced callus for at least one temperature treatment, but the percentage of these calli that was embryogenic ranged from 0% to 50%. Sampling date affected response only for specific genotype-temperature combinations. Embryo recovery ranged from six to 14 per callus. For the four responsive genotypes, 77% to 100% of plantlets was haploid. Culturing anthers at several temperatures ranging from 29 to 35C, with repeated samplings of flowers from the field, likely will allow recovery of haploid embryos from many selections. This result will expand the germplasm base to develop all-male asparagus hybrids.

Asparagus is a dioecious perennial in which gender is controlled by a single gene, *M*; males are *Mm* and females are *mm* (Rick and Hanna, 1943; Snee, 1953). Breeding efforts focus on developing all-male hybrids produced by crossing a female and a supermale (*MM*). Although supermales can be obtained from the progeny of occasional hermaphroditic flowers on male plants, producing perfect flowers is genotype- and environment-dependent. Recovering doubled haploid supermales through anther culture may enhance breeding efforts and expand the germplasm base to develop all-male hybrids.

Anther culture has been used to obtain doubled haploid asparagus (Doré, 1974; Falavigna et al., 1985; Hondelman and Wilberg, 1973; Hsu et al., 1988; Inagaki et al., 1983; Pelletier et al., 1972; Rotondo, 1984; Tsay and Hsu, 1986; Tsay et al., 1982; Yakuwa et al., 1972). Efficiency was often low; fewer than 10 plants were recovered per 100 anthers cultured (Falavigna et al., 1990; Tsay and Hsu, 1986) and $\leq 10\%$ of the plants was haploid (Falavigna et al., 1985; Hsu et al., 1988; Torrey and Peirce, 1984; Tsay and Hsu, 1986). Response also depended on genotype; 0% to 93% of anthers produced calli and 0% to 31% of calli produced shoots (Qiao and Falavigna, 1990; Tsay and Hsu, 1986; Tsay et al., 1982).

Significant genotypic and environmental effects for anther culture response have been

identified in various species. Embryo recovery per anther ranged from four to 196 for rapeseed (*Brassica napus* L.) genotypes surveyed (Chuong et al., 1988). In barley (*Hordeum vulgare* L.) anther culture, 73% and 11% of the total variation observed were explained by genotypic and environmental effects, respectively (Knudsen et al., 1989).

Recently, we reported that 15% of anthers for the G203 genotype incubated at 32C produced embryogenic calli (Feng and Wolyn, 1991). Each callus produced 10 to 15 embryos, of which 50% germinated and 76% was haploid. The objective of the study reported here was to determine the effects of genotype, incubation temperature, and sampling date on producing haploid embryos in asparagus anther culture.

Field-grown flowers with microspores at the late uninucleate developmental stage were collected on 10 and 24 July and 10 Aug. 1991 for genotypes G127, G133, G171, G193, and G203 and stored at 5C for 24 h. Anther culture was conducted as described by Feng and Wolyn (1991), except initial incubation temperatures varied. Anthers were placed on a solid MS (Murashige and Skoog, 1962) medium containing (mg·liter⁻¹) 500 casein hydrolysate, 800 glutamine, 2 α -naphthalenacetic acid (NAA), 1 N⁶-benzyladenine (BA), and 5% sucrose and incubated at 26, 29, 32, or 35C for 4 weeks in darkness. All treatments had four replications, each consisting of a petri plate with 18 anthers. Calli were grown at 25C for 4 weeks with a 16-h photoperiod. Subsequently, developing embryos and calli were transferred to embryo maturation medium [MS containing (mg·liter⁻¹) 0.1 NAA, 0.1 kinetin, 0.65 α -cyclopropyl- α -(4-methoxyphenyl)-5-pyrimidinemethanol (ancymidol), and 6% sucrose] for 4 weeks.

Embryos were germinated on MS containing 1 mg gibberellic acid (GA₃)/liter.

Calli were classified as embryogenic or nonembryogenic. The number of embryos per embryogenic callus was determined. Chromosomes from randomly selected plants for each genotype were counted according to Grant (1982) with carbol fuchsin stain (Zhu, 1982).

Data for the number of embryogenic calli-transformed and nontransformed values were not distributed normally; many replications had zero values. Consequently, means and standard errors are presented.

Genotype, incubation temperature, and sampling date affected the total and embryogenic number of calli in asparagus anther culture (Table 1). For the most responsive genotypes at their optimum temperature-G127 and G203 at 35C-half of the anthers produced calli and $\approx 50\%$ of the calli was embryogenic. The response at this temperature was consistent for the three sampling dates. G171 anthers rarely produced embryogenic calli, although 50% produced green or brown calli at 29 and 32C. About 10% of G133 and G193 anthers produced embryogenic calli; optimum response was observed at 35C for the former and at 29 and 32C for the latter.

The proportion of total calli that was embryogenic differed among temperature treatments for some genotypes. Similar numbers of embryogenic calli were produced at 26, 29, and 32C for G193, although the number of total calli varied twofold. Total callus recovery for G203 anthers incubated at 32 and 35C was similar, while the number of embryogenic calli ranged from 1.0 to 5.4.

Sampling date affected total and embryogenic callus formation for specific genotype-temperature combinations. The response of G127, G133, and G171 anthers collected on 10 July was lower than that for other sampling dates for certain temperature treatments. The G203 genotype was not affected substantially by sampling date.

The number of embryos produced per embryogenic callus, pooled for temperature treatments and sampling dates, was 9.7 ± 0.5 for G127, 10.3 ± 0.6 for G133, 6.0 ± 1.2 for G171, 13.9 ± 1.3 for G193, and 13.4 ± 0.6 for G203. Since interactions were observed among genotypes, incubation temperatures, and sampling dates for callus formation, these data are presented only to indicate the potential embryo yield. For the five genotypes, germination ranged from 45% to 58%. To maximize the recovery of genetically distinct haploids for a breeding program, one would use a single plant from a callus; multiple embryos on a callus could have arisen from the same microspore.

Most (77% to 100%) plants sampled randomly for four of the five genotypes were haploid (Table 2). Few embryos were produced from G171; however, many diploid plantlets were obtained through organogenesis from green, nonembryogenic calli.

When data from these experiments were compared with those from initial studies (Feng and Wolyn, 1991), the response of G203 at 32C varied between years. For experiments

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Table 1. Effects of genotype, incubation temperature, and sampling date on total and embryogenic callus formation in asparagus anther culture.

Genotype	Sampling date	Callus type ^a	Incubation temp (°C)			
			26	29	32	35
			<i>No. calli^b</i>			
G127	10 July	Tot	0.3 ± 0.2 ^a	0.5 ± 0.2	0.9 ± 0.3	8.9 ± 0.5
		Emb	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	5.6 ± 0.8
	24 July	Tot	2.3 ± 0.8	3.1 ± 0.7	4.7 ± 0.6	9.0 ± 0.3
		Emb	0.0 ± 0.0	0.1 ± 0.1	1.2 ± 0.3	5.3 ± 0.5
G133	20 Aug.	Tot	---	---	3.5 ± 0.5	11.0 ± 0.4
		Emb	---	---	0.5 ± 0.5	4.0 ± 0.9
	10 July	Tot	0.4 ± 0.2	2.8 ± 0.4	3.4 ± 1.0	6.9 ± 0.3
		Emb	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.3
G171	24 July	Tot	5.4 ± 1.3	9.1 ± 1.4	9.2 ± 0.8	8.5 ± 0.6
		Emb	0.0 ± 0.0	0.6 ± 0.3	0.2 ± 0.2	2.2 ± 0.4
	10 July	Tot	3.9 ± 0.6	8.4 ± 0.6	9.4 ± 0.6	2.8 ± 0.4
		Emb	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
G193	24 July	Tot	11.3 ± 0.6	15.5 ± 0.4	10.6 ± 0.5	9.4 ± 1.1
		Emb	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.1
	20 Aug.	Tot	12.0 ± 1.1	14.8 ± 1.7	12.3 ± 0.6	10.0 ± 1.2
		Emb	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
G203	20 Aug.	Tot	9.6 ± 0.4	10.1 ± 0.5	4.9 ± 0.4	1.9 ± 0.5
		Emb	1.4 ± 0.4	2.0 ± 0.5	2.2 ± 0.2	0.2 ± 0.2
G203	10 July	Tot	1.6 ± 0.4	6.4 ± 0.8	7.3 ± 0.7	8.0 ± 0.5
		Emb	0.1 ± 0.1	0.8 ± 0.4	1.0 ± 0.2	4.2 ± 0.9
	24 July	Tot	2.8 ± 0.4	5.0 ± 0.6	8.6 ± 0.5	10.8 ± 0.7
		Emb	0.2 ± 0.1	1.0 ± 0.5	1.1 ± 0.3	6.6 ± 0.9

^aTot = total calli; Emb = embryogenic calli.

^bEighteen anthers per replication.

^cMean ± SE.

Table 2. Ploidy of plants recovered in asparagus anther culture from embryogenic callus.

Genotype	No. plants	Haploid (%)	Diploid (%)	Tetraploid (%)
G203	10	90	10	0
G193	13	77	23	0
G171 ^a	12	0	92	8
G133	6	100	0	0
G127	21	78	22	0

^aPlants recovered only from green, nonembryogenic callus.

conducted in 1990 at 32C with three sampling dates, ~45% of anthers produced calli, of which 30% was embryogenic. When the same treatment was repeated for two sampling dates in 1991, 41% to 48% of anthers produced calli, but only 13% to 14% was embryogenic. Since the effects were consistent for sampling dates in a given year, the physiological status of the plant may have differed between seasons to produce these results.

Anthers from new selections or from specific genotypes in different years should be cultured at several incubation temperatures ranging from 29 to 35C. Since a genotype was recalcitrant for the procedure outlined here, additional modifications may be necessary to recover doubled haploids from all selections. Despite the effects of sampling date for callus production from anthers of field-grown flowers, we recommend them for anther culture; more anthers from field-grown flowers produced embryogenic callus than those from the greenhouse (Feng and Wolyn, 1991). Although

interactions between genotype, temperature, sampling date, and years were observed for response in anther culture, haploid embryos were produced for several breeding selections; thus, the supermale germplasm available for developing all-male hybrid asparagus cultivars was expanded.

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