Callus and Somatic Embryogenesis from Cultured Anthers of Service Tree (*Sorbus domestica* L.)

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Abstract. The effects of stage of microspore development, cold pretreatment, and growth regulators on the embryogenic response of anthers from service tree (*Sorbus domestica* L.) are reported. Callus proliferation required the presence of growth regulators in the culture medium, best results being obtained with combinations of auxins and BA. Microspore embryogenesis was only induced when anthers containing tetrads or uninucleate pollen were cultured on BA-supplemented media containing IBA or IAA. A cold pretreatment before anther culture elicited a significant decrease in callus formation and inhibited embryogenesis. Chemical names used: benzyladenine (BA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA).

Although in vitro anther culture of some members of the Rosaceae have been reported (Von Aderkas and Dawkins, 1993), there appears to be no studies with service tree. This hardwood species, well adapted to withstand moisture stress, has good potential for afforestation of degraded Mediterranean areas and is also used as a landscape tree by homeowners. Factors influencing anther culture are extremely diverse and must be determined empirically for each species (Radojevic and Koovor, 1986). This study was designed to assay the influence of pollen development stage, cold pretreatment, and culture medium on morphogenic responses from cultured anthers of service tree.

Floral buds of *Sorbus domestica* (4 to 7 mm long) were collected during March and April from field-grown plants (35 years old and 5 m high) in their natural habitat (Abejuela, Teruel, Spain). For the experiments including a cold pretreatment, flower buds were kept in darkness at 4 to 6°C for 7 days. To determine microspore development, anthers within floral buds from 2 to 7 mm long were stained with mithramycin (M7393; Sigma, St. Louis) as described by Pace et al. (1987). Floral buds were disinfested by successive immersions in 10% Mercryl-Lauril (a medical disinfestant composed of 0.1 g·liter⁻¹ mercurobutol and 40.8 g·liter⁻¹ sodium lauryl sulfate; Labaz S.A., Gerona, Spain) (20 min), 70% ethanol (1 min), 1.5% NaOCl plus 0.1% Tween-20 (20 min), and sterile distilled water. Then, anthers from several buds of various donor plants were dissected out, filaments removed, chosen at

random, and placed directly onto the culture medium (20 anthers per dish). Basal medium contained MS salts (Murashige and Skoog, 1962), vitamins as described by Vieitez et al. (1985), 3% sucrose, and 0.7% Difco Bacto-agar (Difco Laboratories, Detroit); pH of 5.6 to 5.7. Growth regulators were added to the culture medium before autoclaving (120°C for 20 min). Vessels used in the experiments were 15 × 100-mm plastic petri dishes (with 30 ml of medium) or 150 × 25-mm glass tubes (with 20 ml of medium). Unless otherwise stated, cultures were maintained in darkness at 26 ± 2°C.

In a first experiment, anthers isolated from cold-treated flower buds containing microspores in the late uninucleate or binucleate stages were cultured on basal medium supplemented with 0.0, 0.5, 5.2, 26.0, or 52.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), IBA, 1-naphthaleneacetic acid (NAA), or IAA in combination with 0.0, 0.4, or 4.4 BA. Data were recorded after 40 days. The second experiment was designed to examine the effects of microspore development stage and cold treatment. Anthers (cold pretreated or not) containing mainly tetrads, uninucleate microspores, or binucleate pollen were cultured on basal medium containing 5 μM BA combined with 5, 10, or 25 μM 2,4-D, IBA, or IAA. After 40 days, anthers with visible somatic embryos were transferred to tubes with basal medium, and calluses were subcultured on the same medium supplemented with 2.5 μM each BA and IAA. After 60 days at 26 ± 2°C and a 16-h photoperiod of 80 μmol·m⁻²·s⁻¹ provided by cool-white fluorescent lamps (Sylvania GTE Gro-lux, F36W/GRO, Erlangen, Germany), tissues were transferred to basal medium. In both experiments, 100 anthers, distributed in five experimental units (each consisted of 20 anthers), were cultured for each treatment. The percentage of responding anthers and the number of embryos per 100 cultured anthers were recorded. Significance of treatment effects was determined using analysis of variance (ANOVA) (Statgraphics program, version 6; Manugistics, Rockville, Md.). Percent-

age data were subjected to arcsin transformation before analysis. Variation among treatment means was analyzed using Tukey's (1953) procedure.

Mithramycin staining allowed us to correlate bud length with pollen development. The three bud size categories, 2 to 3, 4 to 5, and 6 to 7 mm contained mainly tetrads, uninucleate, or binucleate pollen, respectively. In the first experiment, cultured anthers only produced callus. This response required the presence of growth regulators in the culture medium. When auxins alone were tested, only >0.5 μM 2,4-D promoted consistent cell proliferation. The addition of BA to auxin-supplemented media enhanced callus formation, with best results being obtained in the presence of IAA, IBA, or 2,4-D (data not presented). The ANOVA of the second experiment data (Table 1) showed that all main effects that were tested significantly affected the percentage of anthers with callus ($P \leq 0.001$). Further, significant interactions between pollen development stage and cold pretreatment or auxin type, as well as between auxin type and concentration, also were evident. Thus, cold pretreatment drastically reduced callus proliferation, although microspore stage significantly influenced this response in nontreated anthers, with tetrads producing the highest frequency of calluses ($P \leq 0.0001$). Treatments including 2,4-D were the most effective in promoting callus formation from anthers containing uninucleate or binucleate pollen, but the three auxins produced similar frequencies of callus formation in anthers with tetrads ($P \leq 0.0001$). Irrespective of the anther stage, increasing levels of IBA appeared to favor callus, whereas there were no significant differences among the levels of 2,4-D or IAA tested ($P = 0.0004$).

Callus proliferation usually preceded embryo differentiation, but sometimes direct embryogenesis was recorded. The embryogenic response was also related to cold pretreatment, anther stage, and the type and concentration of auxin (Table 1). Cold inhibited embryo formation and this response was only observed when anthers containing tetrads or uninucleate pollen were cultured in the presence of IBA : BA or IAA : BA ratios of 25 or 10 to 5. Although the beneficial effect of cold treatment is well established for many species, in others, a negative effect has been reported (Wenzel and Foroughi-Wehr, 1994). The developmental stage of the microspores at the time of culture has also been cited as one of the most important factors for successful anther culture (Chen, 1987). The close relationship between anther stage and somatic embryo formation in our study emphasizes the importance of this factor for service tree anther culture. The subculture of the anther-derived callus to basal medium containing IAA and BA favored callus growth. This treatment promoted embryo formation in only 8% of calluses formed from tetrads. Although several stages of somatic embryo development were observed, none of these embryos developed into plants when transferred to basal medium.

Our results demonstrate that somatic em-

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Table 1. Effects of cold pretreatment, growth regulators and pollen development stage on callus induction (percentage of anthers with callus) from cultured anthers of *Sorbus domestica*. Values in parentheses are the percentage of anthers forming somatic embryos.

Growth regulators (μM)	Tetrad		Uninucleate		Binucleate	
	Cold	No cold	Cold	No cold	Cold	No cold
IAA/BA						
5/5	0	75	0	6	0	4
10/5	0	81 (2)	1	2	0	0
25/5	0	74 (3)	0	48	0	0
IBA/BA						
5/5	9	75	1	3	1	2
10/5	4	62 (7)	12	30 (4)	2	26
25/5	10	61 (8)	27	92 (5)	9	64
2,4-D/BA						
5/5	3	70	11	95	35	72
10/5	47	77	25	79	28	71
25/5	36	66	0	67	41	74

Mean separations for significant interactions^a

<i>Cold pretreatment $\times$ pollen development</i>			
	<u>Tetrad</u>	<u>Uninucleate</u>	<u>Binucleate</u>
Cold	12.1 a	8.6 a	12.9 a
No cold	71.2 d	46.9 c	34.8 b
<i>Auxin type $\times$ auxin concentration</i>			
	<u>5 μM</u>	<u>10 μM</u>	<u>25 μM</u>
IAA	14.2 a	14.0 a	20.3 a
IBA	15.2 a	22.7 a	43.8 b
2,4-D	47.7 b	54.5 b	47.3 b
<i>Auxin type $\times$ pollen development stage</i>			
	<u>Tetrad</u>	<u>Uninucleate</u>	<u>Binucleate</u>
IAA	38.3 d-f	9.5 ab	0.7 a
IBA	36.8 de	27.5 cd	17.3 bc
2,4-D	49.8 ef	46.2 ef	53.5 f

^aFor each interaction, mean separation by Tukey's test ($P \leq 0.05$). Culture time was 40 days. Values are means of five replications consisting of 20 anthers each.

bryogenesis can be induced from anthers of service tree. Although under the culture conditions tested it was not possible to promote the conversion of the somatic embryos into plants, our results indicate that the potential exists to obtain haploid plants from *S. domestica* for use in genetic improvement of the species.

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