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SOMATIC EMBRYOGENESIS IN ELM

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Elm is a Nothern hemiphere tree that is greatly valued for its landscape, amenity and timber qualities. Elm populations have been devasted by Dutch elm disease (Brasier, 1991) caused, initially, by the vascular wilt fungus *Ophiostoma ulmi* and, later on, by the distinctly different and highly aggressive *O. novo-ulmi* (Brasier, 1994). There have been various attempts to control the disease through international research programs (Burdekin, 1982; Sticklen and Sherald, 1993), none of which have yet succeeded in significantly halting its spread or reducing its impact (Fenning *et al.*, 1996).

Genetic engineering might provide an useful aid in the control of the disease. However, suitable in vitro regeneration systems need to be available for genetic transformation of elms. Micropropagation and regeneration of different elm species through axillary branching has been achieved not only from juvenile material (Fenning *et al.*, 1993) but also from mature trees (Dorion *et al.*, 1987; Corchete *et al.*, 1993). Adventitious buds were achieved from protoplast culture (Dorion *et al.*, 1994), leaf explants (Boylard *et al.*, 1991; George and Tripepi, 1994) and from strips of stem (Ben Jouira *et al.*, 1998). Elm internodes have been demonstrated to be suitable explants for genetic modification via *Agrobacterium tumefaciens* (Boylard *et al.*, 1991; Dorion *et al.*, 1995).

Somatic embryogenesis could represent, not only a way of clonally propagating valuable genotypes, but an important tool for genetic engineering. In spite of the great number of papers reporting plantlet regeneration from axillary branching and/or adventitious shoot induction, there are no reports, to our knowledge, on the capacity of elm tissue to form somatic embryos.
In this report, we describe the induction of somatic embryogenesis from zygotic embryo tissues of *Ulmus minor* and *U. glabra*.

Three 20-year-old *Ulmus minor* Miller trees and two 70-year-old *Ulmus glabra* Hudson trees, growing in the Rosalía de Castro park (Lugo, NW Spain) were used in this study. Samaras were collected between March and April 1997 and 1998 at different intervals. In 1999, samples were collected from March 8 to April 25 at weekly intervals or approximately 0-6 weeks postanthesis (WPA).

Plant material was surface sterlized in 0.5% active chlorine (Millipore® tablets) containing 1% of Tween® 80, for 3 min and then rinsed three times with sterile distilled water. Ovules and/or immature embryos, depending on the sampling date, were excised; when possible, cotyledons were separated from the hypocotyl-radicle axis before culture. Explants were placed on a basal MS medium containing glutamine (1000 mg/l), arginine (50 mg/l), glycine (50 mg/l), sucrose (3%) and bacto Difco agar (0.8%). In the first set of experiment (samples collected in 1997 and 1998), treatments consisted of 4 concentrations of 2,4-D (0.2, 0.5, 1 and 2 mg/l) and one level of BA (0.2 mg/l) arranged

as a factorial in a randomized complete block design. Twenty-four explants were cultured per treatment giving a total of 96 explants per each genotype and explanting date. In the second set of experiments (samples collected in 1999), treatments consisted of MS basal medium additioned with BA 0.2 mg/l alone or in combined with 2,4-D (0.2, 1 mg/l). A MS basal medium control without plant growth regulators was also included. Seventy-two explants were used per treatment for a total of 288 explants per sample date.

In both experiments, the cultures were grown under darkness for a period of 4 weeks, after which, half of the cultures were transferred to the basal medium without growth regulators, and the other half to MS medium containing BA 0.1 mg/l. The cultures remained under darkness for two weeks, and were then grown under light conditions.

In each experiment, the explants were placed (one per tube) in 120x25 mm culture tubes containing 16 ml of induction medium, and after 4 weeks of culture they were transferred to 300-ml jars containing 50 ml of medium and 4-5 explants/jar. The number of responsive explants for callogenesis and somatic embryogenesis induction in relation to each treatment was examined periodically.
For maintenance of the embryogenic competence, embryogenic calli were transferred to petri dishes containing 25 ml of basal medium additioned with BA 0.2 mg/l, and subcultures were performed at four-week intervals.

As it has been stated above, all cultures were incubated for an initial 6-week period of darkness, and were then transferred to light conditions at 24ºC with 30 $\mu molm^{-2}s^{-1}$ of light provided by cool white fluorescent tubes for 16h/day.

The results achieved in the experiments carried out during 1997 and 1998 are shown in Table 1. Survival of initial explants ranged from 13.5% to 100%, and was related to the genotype and, to a lesser extent, to the size (time of explantation) of the zygotic embryos. After 4 weeks of culture in induction medium (in darkness), responsive explants developed a soft, whitish-translucent callus. The amount of callus material produced increased by increasing 2,4-D concentrations. The ability to produce somatic embryos directly from the cotyledons or embryonic axis was not observed. After transference of the cultures to 0.1 mg/l BA medium, free of 2,4-D, the central part of the callus become necrotic but was sorrounded by a fast growing callus which, due to the growth under light conditions, acquired a greeny-yellowish colour. As the culture progressed the callus became nodular with yellowy-whitish nodules which finally produced embryogenic masses. Somatic embryos in different stages were produced at this stage, normally 2-3 months after the initial cultures. Some of the embryos appeared to be individualized, but many of them were fused, mainly through the cotyledons. Within a species, the embryogenic induction capacity was related to the genotype as well as to the presence of 2,4-D. Media lacking this plant regulator did not develop embryogenic callus, regardless of the species/genotype studied. Precocious germination occurred at this early stage: within the embryogenic masses, the embryos developed both epycotil and root, following a similar pattern to the development of the zygotic embryos. After isolation of the germinated somatic embryos, no futher development of the shoot was observed. Isolated

somatic embryos became necrotic and, in some cases, a whitish, friable callus formed on the necrotic epycotil. It was observed that this callus was also embryogenic.

Table 1. Effects of immature zygotic embryo size and 2,4-D concentration on the embryogenic response of 5 elm clones

Clone	WPA[a]	Size(mm)	Survival (%)[b]	Embryogenesis Induction(%) 2,4-D (mg/l)[c]			
				0.2	0.5	1	2
E	4	4.2 ± 0.3	13.5	-	-	-	-
C	4	3.8 ± 0.2	44.3	4.2	-	-	-
PB	2	2.6 ± 0.4	30.2	8.4	-	4.2	4.2
PB	5	5.3 ± 0.4	100	12.6	-	4.2	-
RC	6	6.2 ± 0.3	100	-	-	-	-
AV[d]	6	-	100	12.5	-	-	-

a weeks postanthesis; b after four weeks; c all media are additioned with 0. 2 mg/l BA; d 16 explants per treatment.

There was no evidence of repetitive, direct embryogenesis from a primary somatic embryo, as has been reported in other species such as oak (Cuenca *et al*., 1999). Embryogenic calli and embryo production has been maintained for 26 months by transferring the cultures every 4 weeks. Most of the embryos examined histologically had a vascular system, a well-developed root meristem and a poorly organized shoot meristem. This poor meristem structure could be the cause of the shoot growth failure recorded after isolation of somatic embryos.

The results achieved demonstrated the feasibility of elm immature zygotic embryos for producing somatic embryos. However, much effort is still needed to develop whole plantlets from the somatic embryos induced.

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