Micropropagation of *Ulmus minor* and *U. minor x U. pumila* from 4-year-old ramets

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Abstract

As part of the Spanish breeding programme against Dutch elm disease, investigations were undertaken on reproduction of *Ulmus minor* by means of tissue culture. Microshoots were obtained from nodal segments, collected from terminal twigs of 4-year-old ramets of selected clones of this species and of *U. minor x U. pumila*, and cultured on a modified Murashige & Skoog medium containing 6-Benzylaminopurine (BA). When subcultured, the microshoots or nodal segments excised from these, formed new shoots, their maximum numbers per explant (1.94 to 2) being obtained with the hybrid clone M-TC2 on media containing 3.5 to 8.8 µM BA. The number of microshoots obtained was highest with subcultures of the whole shoot, e.g. 6.9 times superior to control, against 1.5 with nodal segments of V-JR1 in presence of 8.8 µM BA. The addition of 0.5 to 5.3 µM indole-acetic acid of α-naphthaleneacetic acid to the medium induced the highest percentage of root formation from the shoots. Both shoot and root formation varied greatly among clones.

Key words: organogenesis, Dutch elm disease, culture medium.

Resumen

Micropropagación de *Ulmus minor* y *U. minor x U. pumila* a partir de ramets de 4 años de edad

En el presente trabajo se describen los trabajos sobre reproducción de *Ulmus minor* mediante las técnicas de cultivo de tejidos, llevados a cabo en el marco del Programa de Mejora Genética Español de los Olmos Frente a la Grafiosis. Brotes obtenidos a partir de segmentos nodales de ramillos terminales de ramets de 4 años de edad, pertenecientes a clones seleccionados de esta especie y de *U. minor x U. pumila*, fueron cultivados en el medio de Murashige & Skoog modificado conteniendo 6-Bencilaminopurina (BA). Una vez subcultivados los brotes enteros o los segmentos nodales obtenidos a partir del material vegetal *in vitro*, se originaron nuevos brotes, y fue el clon híbrido M-TC2 el que proporcionó un mayor número por explanto (1,94 a 2) sobre medio conteniendo 3,5 a 8,8 µM BA. El número de brotes obtenidos fue mayor cuando todo el brote fue subcultivado, e.g. 6,9 veces superior al control, frente a 1,5 con segmentos nodales de V-JR1 en presencia de 8,8 µM BA. La adición de 0,5 a 5,3 mm de ácido indolacético o α-naftalenacético al medio, provocó el mayor porcentaje de formación de raíz en los brotes. Tanto la formación de brotes como la formación de raíz fue muy variable entre clones.

Palabras clave: organogénesis, grafiosis, medios de cultivo.

Introduction

Dutch elm disease (DED) is a devastating disease responsible for the destruction of the majority of mature elm trees (*Ulmus*) across the world. It was initially caused by the vascular fungi *Ophiostoma ulmi* and later by the highly aggressive *O. novo-ulmi* (Brasier, 1991) and is propagated by bark beetles (*Scolytus*).

Tissue culture methods are particularly useful in the many DED breeding programmes developed over decades in several countries, such as the Netherlands (Heybroek, 1957), USA (Townsend, 1975), Italy (Mittempergher, 1990), Spain (Gil *et al.*, 2003) and France (Dorion *et al.*, 1994). Tissue culture of elms can be used to obtain plant material for *in vitro* studies about the disease and to propagate resistant trees. Cell sus-

pension cultures (Corchete *et al.*, 1993a; Diez and Gil, 1998a; Eshita *et al.*, 2000), callus cultures (Pijut *et al.*, 1990 a, b; Diez, 1996; Gartland *et al.*, 2000) and cell colonies cultured by a plating technique (Biondi *et al.*, 1991; Diez and Gil, 1998b; Diez and Gil, 1999) have been used in order to determine resistance mechanisms to the disease, the efficiency of *in vitro* selection of resistant elms, and to optimise genetic transformation techniques. Extensive research programmes have developed micropropagation procedures for diverse elm species such as *U. campestris* (Biondi *et al.*, 1984), *U. americana* (Bolyard *et al.*, 1991), *U. laevis* (Dorion *et al.*, 1994), *U. parvifolia* (Lange, 1981), *U. procera* (Fenning *et al.*, 1993), *U. pumila* (Corchete *et al.*, 1993b; Kapaun and Cheng, 1997) and *U. minor* (Dorion *et al.*, 1994).

Micropropagation was also achieved with *U. pumila* x *japonica* hybrids «Sapporo gold» (Ben Jouira *et al.*, 1997), and with hybrids of *U. glabra* «Commelin» (Ben Jouira *et al.*, 1998); «Dodoens» (Dorion *et al.*, 1994), «Lobel» (Rodríguez *et al.*, 1997) and «Pioneer» (Fink *et al.*, 1986), but not with *U. minor x U. pumila* hybrids. In many of these studies the cultures have been obtained from seedlings plants, whereas micropropagation from adult trees is preferable (Bonga, 1987) as it is difficult to predict the characteristics of a mature tree from plants still in their juvenile phase. Thus, Solla (2000) found that elms under 4-years old were not appropriate to screen against DED for their juvenile resistance. However, micropropagation from lignified plants is frequently recalcitrant (Corchete *et al.*, 1993b; Quraishi and Mishra, 1998). The purpose of this investigation was to determine the conditions necessary to establish vigorous micropropagated 4-year-old *U. minor* and *U. minor* x *U. pumila* plants to initiate cultures from different Spanish elm clones with ornamental interesting characteristics.

Materials and Methods

Nodal segments collected during the early summer from terminal twigs of 4-year-old ramets cultivated in the elm clonal bank of Puerta de Hierro (Madrid) were used for micropropagation. Five elm genotypes were used (Table 1). Nodal segments were sterilized with ethanol (70%) for 3 min, followed by 10 min in 1.5% sodium hypoclorite containing two drops of Tween 80, and rinsed three times in sterile, distilled water. Following the removal of any excess of tissue, 24 nodal seg-

Table 1. Plant material specifications

Clones	Genotype	Origin	DED response
M-PH1	*U. minor*	Madrid (Spain)	Susceptible
M-PH2	*U. minor*	Madrid (Spain)	Susceptible
M-SF1	*U. minor*	Madrid (Spain)	Unknown
V-JR1	*U. minor*	Valencia (Spain)	Unknown
M-TC2	*U. minor* x *U. pumila*	Madrid (Spain)	Susceptible

ments (0.5 to 1 cm long) from each clone were cultured in glass tubes (Kimax Tubes, 2.5 × 15 mm; covered with Belco Kap-Uts, 2.6 × 3.5 mm) containing 12 mL of sterilised Murashige and Skoog (MS) medium (1962) supplemented with 30 g L^{-1} sucrose and 2.6 µM 6-Benzylaminopurine (BA) and 8 g L^{-1} Difco agar.

After four weeks, the shoots (2 to 3 cm long) protruded from the original explants, or nodal segments excised from microshoots (2 to 3 nodal segments per new shoot), were subcultured separately on MS medium (8 g L^{-1}) with different concentrations of BA (0.4, 0.8, 1.7, 2.2, 3.5, 4.4, 8.8 or 22.1 µM) to determine the effect of BA on the number and/or length of the shoots formed, and/or on the weight of the callus originated at the base of the shoots.

Rooting ability of 2 to 3 cm long shoots derived from both types of explants were assayed in 12 mL of MS medium containing indole-3-acetic acid (IAA) or a-naphthaleneacetic acid (NAA) (0.05, 0.5, 5,3 or 53,7 µM).

The pH of the media was adjusted to 5.7 before the addition of 8 L^{-1} agar and autoclaving at 121ºC for 20 min. Cultures were maintained in the growth chamber at 25ºC with a 16h photoperiod using fluorescent white light (30 mmol m^{-2} s^{-1}).

All results are mean values of 18 replicates per genotype and treatment. The data of the assays were analysed using statistical software (Statgraphics Plus 5.0) to determine the significance of differences among treatments by using one-way ANOVA or the Fisher's Exact Test for categorical data. Data processing was undertaken by regression in order to determine the relation between the variables.

Results and Discussion

Shoot bud establishment

The response of the nodal segments to in vitro culture was non uniform. Genotype V-JR1 had the highest
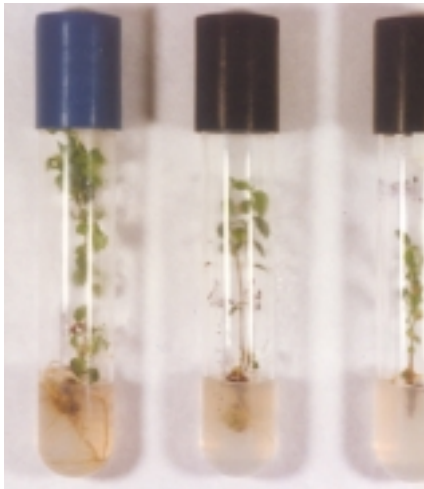
Figure 1. Micropropagated plant obtained from nodal segments of *U. minor x U. pumila* genotype M-TC2.

percentage of proliferated explants (75%) as opposed to M-PH2, M-TC2 and M-PH1, which had the least (41, 42, and 46%, respectively); M-SF1 fell between the two groups (57%). Some of the explants produced shoots, some of them only formed callus. The percentage of initial explants producing but break and shoots was 50% in V-JR1, 40% in M-PH1, 35% in M-SF1, 33% in M-PH2 and 28% in M-TC2. Proliferating cul-

tures in which shoots did not elongate after *in vitro* culture were also found for Dutch elm hybrids (Krajnakova and Greguss, 1992). This variability among clones was previously described for other tree species (Pardos, 1981; Chalupa, 1984; Ahuja, 1986) as well as for elm (Díez, 1996).

Some explants placed in the tubes released excessive amounts of phenolics into the MS media from their cut ends and eventually died. This problem would probably be overcome by suspending the explants in a sterile solution, and by adding PVP 40 to the media (Quraishi and Mishra, 1998), or by weekly transfer of explants to fresh media. The subculture of the microshoots protruding from the original explants or nodal segments, allowed the maintenance of the cultures (Fig. 1).

Propagule multiplication

The number of shoots formed at the base of nodal segments used was affected by the amount of BA ($p < 0.01$, Fig. 2). The optimum BA concentration for the formation of microshoots in the hybrid M-TC2 genotype ranged from 3.5 to 8.8 μM. The number of shoots formed in the *U. minor* clone V-JR1 was slightly less in all treatments except in 22.1 μM BA. Leaching
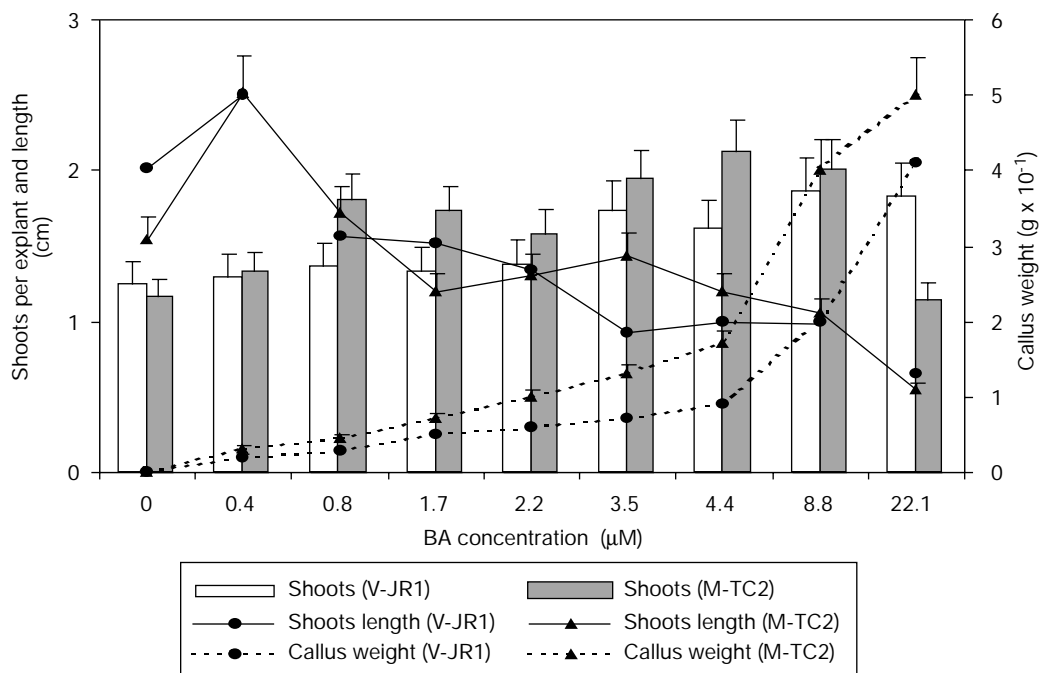

Figure 2. Influence of BA concentration on shoot proliferation, shoot length and callus weight from nodal segments of V-JR1 *(Ulmus minor)* and M-TC2 *(U. minor x U. pumila)* genotypes one month after subculture. Vertical bars indicate standard errors.
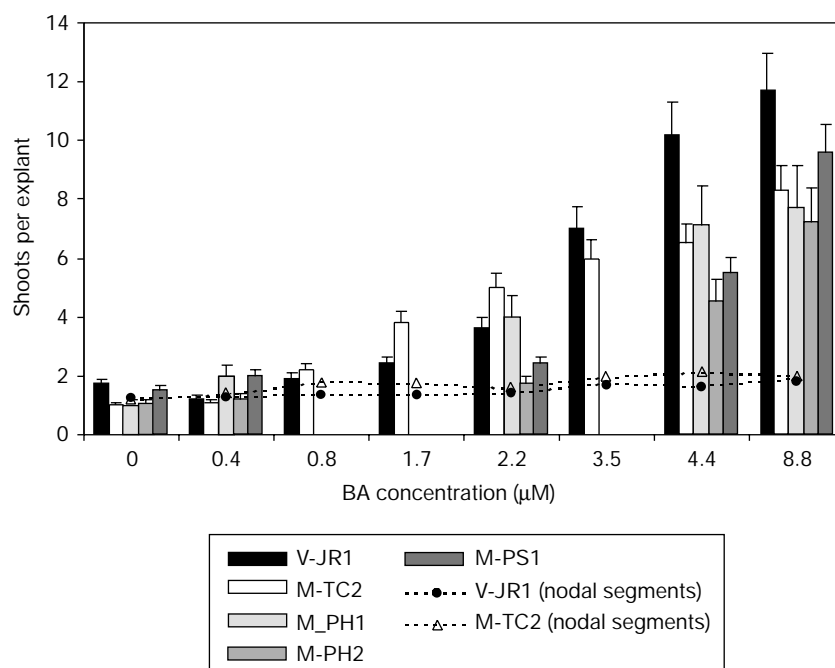
Figure 3. Influence of BA concentration on shoot proliferation from entire elm shoots of M-PH1, M-PH2, M-PS1, V-JR1 and M-TC2, or nodal segments of V-JR1 and M-TC2 genotypes (broken lines) one month after subculture. Vertical bars indicate standard errors.

of phenolics was absent at this stage. Growth regulator concentration influenced elm shoot multiplication, as previously described for cultures of *U. americana* (Fink *et al.*, 1986) and *U. pumila* (Corchete *et al.*, 1993b).

The shoot length and the weight of the basal callus were also affected by the concentration of BA in the media (p < 0.01, Fig. 2). Concentrations of 8.8 and 22.1 µM BA increased callus formation at the base of the stem, thus decreasing the culture performance and shoot length in both genotypes. The weight of the basal callus increased with the concentration of BA as a square root model ($R^2 = 0.93$, p < 0.01), but not however the length of the shoots, which decreased progres-

sively as a reciprocal model ($R^2 = 0.92$, p < 0.01). A reciprocal relationship between both variables was found ($R^2 = 0.85$, p < 0.01).

When the whole shoots were subcultured, new shoots were derived from anywhere along the shoot and callus explant. The number of microshoots formed per explant when using the entire shoot as an explant increased with higher concentrations of BA (Fig. 3). A square root relationship between both variables was found ($R^2 = 0.90$, p < 0.01). The five genotypes tested reached a maximum at 8.8 µM BA (11.7 shoots per explant) for V-JR1. The number of shoots formed in the *U. minor x U. pumila* hybrid M-TC2 was similar to the

Table 2. Effect of indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) on rooting percentage of elm microshoots in vitro one month after subculture. Numbers followed by the same letter are not significantly different (Fisher's Exact Test, p < 0.05)

Genotype	Control	IAA (µM)			NAA (µM)			
		0.05	**0.5**	**5.3**	**0.05**	**0.5**	**5.3**	**53.7**
M-PH1	0 a	11 ab	28 b	17 ab	0 a	17 ab	39 bc	22 ab
M-PH2	—	22 ab	17 ab	0 a	—	—	—	—
M-SF1	0 a	0 a	0 a	11 ab	11 ab	0 a	33 bc	0 a
V-JR1	11 ab	11 ab	78 c	67 c	28 b	78 c	44 bc	—
M-TC2	0 a	22 ab	22 ab	11 ab	55 bc	78 c	22 ab	17 ab

number of shoots formed in the *U. minor* genotypes.

The number of new shoots per explant was higher when the whole shoots were subcultured than when nodal segments were used (6.9 times greater than control, compared to 1.5 using nodal segments for clone V-JR1 at 8.8 μM BA concentration, p < 0.001) (Fig. 3). This effect may be caused by a higher metabolic activity existing in this material as a result of the presence of functional leaves, and by the role of reserves. The profitability and simplicity of this method makes it particularly suitable for the *in vitro* propagation of elms in spite of the number of explants lost initially (microshoots contain 2 or 3 nodal segments).

Rooting

IAA and NAA added to the MS medium stimulated root formation in the shoots of all clones tested (Table 2), as was observed for *U. pumila* (Corchete *et al.*, 1993b). However, as previously described by Dorion *et al.* (1987) and Krajnakova and Greguss (1992), differences among genotypes in the *in vitro* rooting ability were found.

Microshoot cultures of V-JR1 and M-TC2 reached a high rooting percentage (78%) at 0.5 μM IAA and NAA, and in 0.5% NAA, respectively. Root formation was failed in some treatments with M-SF1 and M-PH1. V-JR1 was the only clone that formed roots in control treatments. For M-PH1 and M-TC2 at 53.7 μM NAA, large callus was formed at the base of the shoots, impeding shoot elongation, even after root formation. The micropropagated plants acclimatized to *ex vitro* conditions in the greenhouse and transplanted to the field have shown to date a normal growth (data not shown).

In this work it has been demonstrated that *in vitro* propagation techniques may allow the cloning of *U. minor* or *U. minor x U. pumila* ramets at the age of four years, the appropriate for testing DED resistance. Recalcitrant clones interesting for their ornamental value, or DED resistance already obtained within the Spanish breeding programme (Solla, 2000) may be successfully propagated by this technique.

Acknowledgements

References

AHUJA M.R., 1986. Mikrovegetativevvermehrung bei Forstbäumen. Allgemeine Forstzeitschrift 41, 1303-1306.

BEN JOUIRA H., BIGOT C., DORION N., 1997. Plant regeneration from cambium explants of the hybrid elm «Sapporo Gold 2» (*Ulmus pumila x Ulmus japonica*). Acta Hort (ISHS) 447, 125-126.

BEN JOUIRA H., BIGOT C., DORION N., 1998. Adventitious shoot production from strips of stem in the Duth elm hybrid «Commelin»: plantlet regeneration and neomycin sensitivity. Plant Cell, Tiss Org Cult 53, 153-160.

BIONDI S., CANCIANI L., BAGNI N., 1984. Uptake and translocation of benzyladenine by elm shoots cultured *in vitro*. Can J Bot 62, 2385-2390.

BIONDI S., MIRZA J., MITTEMPERGHER L., BAGNI N., 1991. Selection of elm cell culture variants resistant to *Ophiostoma ulmi* culture filtrate. J Plant Physiol 3, 631-634.

BOLYARD M.G., SRINIVASAN C., CHENG J.P., STICKLEN M., 1991. Shoot regeneration from leaf explants of American and Chinese elm. HortScience 26, 1554-1555.

BONGA J.M., 1987. Clonal propagation of mature trees: problems and possible solutions. In: Cell and Tissue Culture in Forestry, Vol.3. Bonga J.M. and Durzan D.J. eds. Martinus Nijhoff, Dordrecht, pp. 249-271.

BRASIER C.M., 1991. *Ophiostoma novo-ulmi* sp. nov. causative agent of current Dutch Elm Disease pandemics. Mycopathologia 115, 151-161.

CHALUPA V., 1984. Autovegetativní rozmnozôvání vybraných drêvin tkáñvými kulturami, etapa 02 Rýchlé vegetatívní mnození topolu, osiky, jilmu a jerábu in vitro. Jíloviste-Strnady, pp. 27.

CORCHETE P., DIEZ J.J., VALLE T., 1993a. Phenylalanine ammonia-liase activity in suspension cultures of *Ulmus pumila* and *U. campestris* treated with spores of *Ceratocystis ulmi*. Plant Cell Rep13, 111-114.

CORCHETE P., DIEZ J.J., VALLE T., 1993b. Micropropagation of *Ulmus pumila* L. from mature trees. Plant Cell Rep 12, 534-536.

DIEZ J.J., 1996. Desarrollo y evaluación de técnicas de selección «in vitro» de olmos resistentes a la grafiosis. PhD Thesis. Universidad Politécnica de Madrid, Spain. 154 pp.

DIEZ J.J., GIL L.A., 1998a. Variability in phenylalanine ammonia-lyase activity in elm among cell cultures of elm clones inoculated with *Ophiostoma novo-ulmi* spores. Plant Pathol 47, 687-692.

DIEZ J.J., GIL L.A., 1998b. Effects of *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* culture filtrates on elm cultures from genotypes with different susceptibility to Dutch Elm Disease. Eur J For Pathol 28, 399-407.

DIEZ J.J., GIL L.A, 1999. Culturing of elm cell tissues within the Spanish breeding programme against Dutch Elm Disease. In: Espinel S, Ritter E eds. Proceedings of International congress «Applications of biotechnology to forest genetics» Biofor 99. Vitoria-Gasteiz, pp. 307-311.

DORION N., BIGOT C., NEUMANN P., 1994. Evaluation of Dutch Elm Disease susceptibility and patogenicity of *Ophiostoma ulmi* using micropropagated elm shoots. Eur. J For Pathol 24,112-122.

DORION N., DANTHU P., BIGOT C., 1987. Multiplication vegetative «in vitro» de quelques espèces d´ormes. Ann Sci Forest 44, 103-118

ESHITA S.M., KAMALAY J.C., GINGAS V.M., YAUSSY D.A. 2000. Establishment and characterization of American elm cell suspension cultures. Plant Cell Tissue Organ Cult 61, 245-249

FENNING T.M., GARTLAND A., BRASIER M., 1993. Micropropagation and regeneration of English elm *Ulmus procera* Salisbury. J Exp Bot 44, 1211-1217.

FINK C., STICKLEN M., LINEBERGER D., DOMIR C., 1986. «In vitro» organogenesis from shoot tip, internode, and leaf explants of *Ulmus x «Pioneer»*. Plant Cell Tissue Organ Cult 7, 237-245.

GARTLAND J.S., MCHUGH A.T., BRASIER C.M., IRVINE R.J., FENNING T.M., GARTLAND K.M.A. 2000. Regeneration of phenotypically normal English elm (*Ulmus procera*) plantlets following transformation with an *Agrobacterium tumefaciens* binary vector. Tree Physiol 20, 901-907.

GIL L.A., SOLLA, A., IGLESIAS, S. 2003. Los olmos ibéricos. Conservación y mejora frente a la grafiosis. Ministerio de Medio Ambiente(MMA). Serie Técnica, Madrid, 432 pp.

HEYBROEK H.M., 1957. Elm breeding in the Netherlands. Silvae Genetica.6, 112-117.

KAPAUN J.A., CHENG Z.M., 1997. Plant regeneration from leaf tissues of Siberian elm. Hortscience 32, 301-303.

KRAJNAKOVA J., GREGUSS L., 1992. Micropropagation of resistant elm cultivars using the organ cultures. Acta Instituti Forestalis Zvolenensis 8, 9-20.

LANGE D.D., 1981. Techniques for isolating, purifying, and culturing *Ulmus* protoplast. In: Biotechnology in agriculture and forestry. Vol 1. Trees 1. Y. P. S. Bajaj ed. Springer Verlag, Heidelberg New York, pp. 326-340.

MITTEMPERGHER L., 1990. La mejora genética del olmo en el proyecto de investigación de la CEE sobre la grafiosis. In: Los olmos y la Grafiosis en España. Gil L. ed. Ministerio de Agricultura Pesca y Alimentación (MAPA), Madrid, pp. 29-68.

MURASHIGE T., SKOOG F., 1962. Revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plantarum 15, 473-497

PARDOS J.A., 1981. *In vitro* plant formation from stem pieces of *Quercus suber* L. In: AFOCEL ed. Conférence sur la Culture *in vitro* des Essences Forestières. IUFRO Fontainebleau, pp. 186-190.

PIJUT P.M., DOMIR S., LINEBERGER D., SCHREIBER L., 1990a. Use of culture filtrates of *Ceratocystis ulmi* as a bioassay to screen for disease tolerant *Ulmus americana*. Plant Sci 70, 191-196.

PIJUT P.M., LINEBERGER D., DOMIR S., ICHIDA J.M., KRAUSE C.R., 1990b. Ultrastructure of cells of *Ulmus americana* cultured in vitro and exposed to the culture filtrate of *Ceratocystis ulmi*. Phytopathology 80, 764-767.

QURAISHI A., MISHRA S.K., 1998. Micropropagation of nodal explants from adult trees of *Cleistanthus collinus*. Plant Cell Rep 17, 430-433.

RODRÍGUEZ N., TORRES M., PÉREZ C., 1997. Micropropagación del Clon «Lobel» de olmo resistente a grafiosis. In: II Reunión de la Sociedad Española de Cultivo de Tejidos y Células Vegetales (SECIVTV). Barcelona, pp. 37-38

SOLLA A. 2000. Mejora genética de *Ulmus minor* Miller: Selección de ejemplares resistentes a la grafiosis. PhD Thesis. Universidad Politécnica de Madrid, Spain, 136 pp.

TOWNSEND A.M., 1975. Crossability patterns and morphological variation among elm species and hybrids. Silvae Genetica 24, 18-23.