POLIBOTÁNICA
Núm. 35, pp. 85-97, ISSN 1405-2768; México, 2013

IN VITRO PROPAGATION OF MEXICAN OAKS (*QUERCUS* SPP.)

PROPAGACIÓN *IN VITRO* DE ENCINOS MEXICANOS (*QUERCUS* SPP.)

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ABSTRACT

In order to provide an alternative to conventional propagation, a protocol was developed for the *in vitro* multiplication of four Mexican species of *Quercus*, which are exploited to produce timber and coal and are nowadays threatened. Multiple shoot induction from zygotic embryos of *Quercus castanea*, *Q. eduardii*, *Q. resinosa* and *Q. rugosa* cultured on Murashige and Skoog medium supplemented with 6-benzylaminopurine (BA) or kinetin is described. Results showed that both cytokinins affected shoot regeneration, where BA exhibited highest efficiency. Higher shoot proliferation frequency was observed in *Q. castanea* and *Q. eduardii* (91.7%), giving an average of 6.3 and 6.5 shoots per explant on medium with 3.0 and 4.0 mg l^{-1} BA, respectively. For *Q. resinosa*, the best response was obtained with BA at 3.0 mg l^{-1} producing 3.8 shoots per explant. Finally, *Q. rugosa* yielded 3.6 shoots per explant with 1.0 mg l^{-1} BA. Shoots were excised from the seedlings and transferred to half-strength medium supplemented with 10.0 mg l^{-1} indole-3-butyric acid for two or five days, and then transfer to half-strength medium for rooting. Under these conditions, 100, 100, 66.7 and 83.3% of the shoots of *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa* generated roots, respectively. Plantlets were successfully acclimated to soil in greenhouse conditions with survival frequencies of 80% for *Q. castanea*, 83.3% for *Q. eduardii*, 73.3% for *Q. resinosa*, and 70.0% for *Q. rugosa*. This *in vitro* propagation method can contribute to the production of plants for reforestation thus supporting the conservation and rational use of these species.

Key words: cotyledonary nodes, cytokinins, *in vitro* culture, micropropagation, *Quercus*.

RESUMEN

Con el fin de ofrecer una alternativa a los sistemas convencionales de propagación, se desarrolló un protocolo para la multiplicación *in vitro* de cuatro especies mexicanas de *Quercus*, las cuales son explotadas para la producción de madera y carbón y están actualmente amenazadas. Se describe la inducción de brotes múltiples a partir de embriones cigóticos de *Quercus castanea*, *Q. eduardii*, *Q. resinosa* and *Q. rugosa* cultivados en medio de Murashige y Skoogenriquecido con 6-bencilaminopurina (BA)

o cinetina. Los resultados mostraron que ambas citocininas afectaron la regeneración de brotes, donde la BA mostró la mayor eficiencia. La mayor frecuencia de proliferación de brotes se observó en *Q. castanea* y *Q. eduardii* (91.7%), dando promedios de 6.3 y 6.5 brotes por explante al utilizar 3.0 y 4.0 mg l^{-1} de BA, respectivamente. Para *Q. resinosa*, la mejor respuesta fue obtenida con BA a 3.0 mg l^{-1} produciendo 3.8 brotes por explante. Finalmente, *Q. rugosa* produjo 3.6 brotes por explante con 1.0 mg l^{-1} de BA. Los brotes fueron separados de las plántulas y transferidos a un medio basal a la mitad de su concentración enriquecido con 10.0 mg l^{-1} de ac. indolbutírico por dos a cinco días, y luego transferidos a medio a la mitad de su concentración para su enraizamiento. Bajo estas condiciones, el 100, 100, 66.7 y 83.3% de los brotes de *Q. castanea*, *Q. eduardii*, *Q. resinosa* y *Q. rugosa* generaron raíces, respectivamente. Las plántulas fueron aclimatadas a suelo en condiciones de invernadero de manera exitosa con frecuencias de sobrevivencia de 80% para *Q. castanea*, 83.3% para *Q. eduardii*, 73.3% para *Q. resinosa*, y 70.0% para *Q. rugosa*. Este método de propagación *in vitro* puede contribuir a la producción de plantas con fines de reforestación apoyando así la conservación y uso racional de estas especies.

Palabras clave: citocininas, cultivo *in vitro*, micropropagación, nudo cotiledonar, *Quercus*.

INTRODUCTION

Quercus species are widely distributed in the northern hemisphere, mostly in temperate forests and some in tropical and subtropical regions. The genus comprises nearly 500 species (Manos *et al.*, 1999), and its centers of diversity are located at: *a)* Southeast Asia, where about 125 species are present, and; *b)* México, particularly at Central, South and Sierra Madre Oriental regions (Valencia and Flores-Franco, 2006), where the genus reaches its greatest representation with about 160 species (Valencia, 2004). These trees have ecological and economical importance: which are used as food and refuge for many organisms including insects, birds, and mammals (Johnson *et al.*, 2002); they play a vital function for soil and water conservation, and helps to recuperate degraded environments. Oaks also are source of fodder, firewood, charcoal, pulpwood for paper, and its wood is used for furniture manufacture and flooring, sawn wood, crossties, poles, piles, veneer, and plywood elaboration (De la Paz-Pérez *et al.*, 2000). These uses of oak forest are subject to inadequate management that contributes to the decline in oak populations, due to fires, human activities, and the presence of multiple pest and disease problems (Moreno-Rico *et al.*, 2010). Conventional propagation methods of *Quercus* species are not efficient because seeds are recalcitrant and cannot be stored for long periods, and its production is erratic and does not occur every year. Trees require long periods of time to reach reproductive maturity and there are no efficient methods for the vegetative propagation of these species (Díaz-Pontones and Reyes-Jaramillo, 2009). For these reasons, *in vitro* propagation could be an attractive alternative to solve these problems and to allow the rapid multiplication of oaks.

The *in vitro* mass propagation, via axillary shoots proliferation, has been described for *Q. robur* (Favre and Juncker, 1987;San-Jose

et al., 1990), *Q. petraea* (Chalupa, 1993), *Q. suber* (Manzanera and Pardos, 1990), and *Q. alba* and *Q. rubra* (Schwarz and Schlarbaum, 1993). Micropropagation using cotyledonary nodes has been reported for *Q. floribunda* (Purohit *et al.*, 2002a), *Q. leucotrichophora* and *Q.glauca* (Purohit *et al.*, 2002b), and *Q. rubra* (Vengadesan and Pijut, 2009). Furthermore, *in vitro* propagation using petiolar tube, as explants, has been reported for *Q. semecarpifolia* (Tamta *et al.*, 2008). Most of these species are Asian or European. Nevertheless México has the highest number of oak species worldwide, and most of them are endemic, to our knowledge there are no reports of *in vitro* propagation of Mexican *Quercus* species.

Quercus castanea, (red oak, Lobatae) reaches 20 m in height and is widely distributed form México to Guatemala and El Salvador. *Q. eduardii* (red oak, Lobatae) and *Q. resinosa* (white oak, *Quercus*) are endemic species of México. These trees have a trunk heights varying between 5-10, and 6-10 m, respectively. *Q. rugosa* (white oak, *Quercus*), reaches 20 m in height and is widely distributed from west Texas and southern Arizona, U.S.A. to Chiapas, México.The populations of these species are diminishing quickly due to its exploitation for obtaining wood and coal, as well as the opening of new areas for the agriculture and cattle raising. Due to the problems mentioned above, currently there is no production of plantlets for reforestation with these species (De la Paz-Pérez *et al.*, 2000; Valencia, 2004). In this study, we propose a simple procedure for *in vitro* propagation of *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa*, through multiple shoot formation from acorns and its subsequent rooting.

MATERIAL AND METHODS

Plant material

Mature acorns of *Q. castanea* Née, *Q. eduardii* Trel., *Q. resinosa* Liebm. and *Q. rugosa* Née were collected from May to October 2009 from wild trees growing at "Sierra Fría" mountains in Aguascalientes State, in central Mexico at 2,100-2,400 masl (22° 01′ - 22° 15′ N and 102° 44′ - 102° 34′ W). All acorns were collected from the same mother plant according to each species. Only the healthy acorns without apparent damages caused by insects were selected. Acorns were separated from the cupules and rinsed vigorously under running tap water. They were washed three times for 5 min each with liquid soap (Dermoclean 1% v/v), and then rinsed under running tap water. Acorns were treated with 70% (v/v) ethanol for 1 min followed again by rinsing with tap water and surface disinfested with 20% (v/v) commercial bleach solution (5.25% sodium hypochlorite) for 25 min. Finally, they were rinsed three times with sterile distilled water under aseptic conditions.

Shoot induction and multiplication

Once the seed coat was removed from the disinfested acorns, intact embryos (decoated seeds) were treated for 20 min with 0.1% (v/v) Plant Preservative Mixture reagent (PPM, Phytotechnology Lab, Shawnee Mission, KS) and an antioxidant mix (0.01% (w/v) ascorbic and citric acids, filter sterilized). Explants (embryos) were placed in MS basal medium (Murashige and Skoog, 1962), pH 5.7, containing 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Sigma-Aldrich, St. Louis, MO, USA). Media were

supplemented with several concentrations of 6-benzylaminopurine (BA): 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg l^{-1}, or kinetin: 1.0, 2.0, 3.0, and 4.0 mg l^{-1} for multiple shoot induction. MS basal medium without cytokinins was used as control. Twenty four explants were tested per treatment with four explants per culture vessel (Phytotechnology Lab Product C590). All cultures were maintained for two weeks under dark conditions, and then were transferred to continuous light condition for 8 weeks for shoot multiplication (daylight fluorescent lamps, 54 µmol m^{-2} s^{-1}) at 25 ± 2°C. Data on percentage of responding explants, number of shoots per explant and shoot length were recorded after 10 weeks.

Rooting of shoots and transfer of plantlets to soil

For root induction, individual microshoots (2-4 cm) with at least two leaves were transferred to culture vessels containing 50 ml of half-strength MS, pH 5.7, with 3% (w/v) sucrose, 0.25% (w/v) phytagel (Sigma, St. Louis, MO, USA), and four different concentrations of indole-3-butyric acid (IBA): 1.0, 5.0, 10.0, and 20.0 mg l^{-1}, and cultured for 2 or 5 days (root induction medium). Then, shoots were transferred to half-strength MS medium solidified with 0.25% (w/v) phytagel (rooting medium), and free of plant growth regulators. In both steps, cultures were maintained under continuous light condition (daylight fluorescent lamps, 54 µmol m^{-2} s^{-1}) at 25 ± 2°C. Observations on percentage rooting, number of roots formed per shoot, root length, were recorded after 3 weeks of growth on rooting medium. Twenty four shoots were cultured per treatment in culture vessels, containing 50 ml of medium. Four shoots were placed per vessel, six vessels per treatment. Culture vessels were closed with polypropylene caps with membranes that allowed gas exchange (Phytotechnology Lab Product C170). IBA-free half-strength MS served as control in these experiments.

For soil transfer, rooted plants were removed from vessels and roots were gently washed under running tap water to remove residues of culture medium. The plantlets were transplanted to plastic pots containing a mix of sand and peat moss (1:1), and covered with plastic bags to maintain high humidity. The potted plantlets were kept for one month in the bioclimatic chamber under a 16:8 photoperiod (54 µmol m^{-2} s^{-1}) at 25±2°C. Plantlets were gradually acclimatized (over four weeks) to room temperature and humidity conditions by progressively opening the bag until plants were ready to be completely removed from the bags. The plants were then transferred to the greenhouse in polyethylene bags, containing the same potting mixture. Survival was recorded after three months in the greenhouse.

Data analysis

Collected data were analyzed by ANOVA and the differences among means were assessed by the Tukey's test at P = 0.05. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

Multiple shoots of *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa* were induced from intact embryos, placed on culture media supplemented with BA or kinetin. The percentage of response, number of shoots formed, and shoot length varied significant-

ly with concentration and type of cytokinin for the four species (table 1). BA was more effective than kinetin on inducing multiple shoots and promoting shoot growth in the four species tested. The four species grown on MS medium devoid of cytokinins did not show multiple shoot formation. The optimal cytokinin concentration required to induce the best response in percentage of multiple shoot formation, number of shoots per explants, and shoot length differed in the four species. MS containing BA showed the highest percentage of multiple shoot forming explants (91.7%) for *Q. castanea* and *Q. eduardii* with 3.0 and 4.0 mg l^{-1}, respectively. For *Q. rugosa*, the highest percentage of explants responding was 87.5% with BA (1.0 mg l^{-1}). For *Q. resinosa*, the highest percentage of explants responding was 83.3% with at 3.0 mg l^{-1} kinetin (table 1). The present study shows that the incorporation of BA or kinetin to the medium was essential to induce multiple shoot proliferation from a single embryo. The highest shoot number averages were 6.5, 6.3, 3.8, and 3.6 shoots per explant, with BA at 4.0, 3.0, 3.0, and 1.0 mg l^{-1}, for *Q. eduardii*, *Q. castanea*, *Q. resinosa*, and *Q. rugosa*, respectively (table 1, Fig. 1a, and 1b). Similar observations were reported with BA at 1.0 mg l^{-1} for *Q. rubra*, obtaining 6 shoot per explant (Schwarz and Schlarbaum, 1993). In the studied species, some embryos produced shoots in a quantity higher that the average, up to 20-30 new shoots per explant (Fig. 1c). The present study shows that explants exposed to high concentrations of BA did not formed basal callus, which inhibits shoot initiation or restrict their development as reported in *Quercus robur* where concentrations of BA higher than 0.8 mg l^{-1} stimulated callus formation (Puddephat *et al.*,1997). However, we observed that prolonged exposure to BA (over 10 weeks) at high concentrations (5.0, and 6.0 mg l^{-1}), promotes direct somatic embryogenesis at low-frequencies, in the four species tested (results not shown).

In other oak species, low concentrations of BA (0.2; 0.6; 1.0 mg l^{-1}) favored higher shoot multiplication as seen in *Q. robur* and *Q. petraea*, where three shoots were produced per explant (San-Jose *et al.*, 1990; Chalupa, 1988; Chalupa,1993; Puddephat *et al.*,1997). Kartsonas and Papafotiou (2007), and Vieitez *et al.* (1993), reported that the number of shoots formed per explant was lower (1.6 and 2.3 shoots per explant) for *Q. euboica*, and *Q. rubra*, respectively.

Shoots of *Q. robur* generated with BA concentrations higher than 1.0 mg l^{-1} showed an abnormal development exhibiting thickened and reduced internodes, twisted leaves, rosette development, vitrification, and callogenesis (Favre and Juncker, 1987; Puddephat *et al.*, 1997). In this study, shoot number per explant was significantly ($P < 0.001$) increased by high concentrations (4.0 mg l^{-1}) of BA in *Q. castanea* and *Q. eduardii*. Even for *Q. rugosa* at concentrations higher than 2.0 mg l^{-1} there was no vitrification nor callogenesis, which may inhibit shoot elongation and survival. Similar to our findings, a high concentration (5.0 mg l^{-1}) of BA when used alone, it was found to be effective for *Q. floribunda*, where the average number of shoots per cotyledonary node increased to 11.0 shoots per explant (Purohit *et al.*, 2002a). In *Q. semecarpifolia* (Tamta *et al.*, 2008), with 4.5 mg l^{-1} BA 5 shoots were obtained. In *Q. leucotrichophora* and *Q. glauca* (Purohit *et al.*, 2002b), similar results were obtained with high concentrations of BA. However their

Table 1. Effect of BA and Kinetin on shoot formation and shoot length in *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa*.

Species	Cytokinin	(mg l^{-1})	Explants responded (%)	Shoots per explant	Shoot length (cm)
Q. castanea	None	0.0	66.7 ± 25.8abc	0.9 ± 0.3f	3.4 ± 0.2cd
	BA	1.0	83.3 ± 12.9a	2.0 ± 0.5cde	4.3 ± 0.1a
		2.0	83.3 ± 20.4a	2.9 ± 0.5bc	3.5 ± 0.3bcd
		3.0	91.7 ± 12.9a	6.3 ± 0.8a	3.5 ± 0.3bc
		4.0	79.2 ± 10.2ab	3.7 ± 0.6b	3.6 ± 0.2bc
		5.0	62.5 ± 13.7abc	2.5 ± 0.5cd	3.2 ± 0.2cd
		6.0	45.8 ± 18.8c	1.9 ± 1.0de	3.0 ± 0.1d
	Kinetin	1.0	83.3 ± 12.9a	2.4 ± 0.5cde	4.0 ± 0.4ab
		2.0	75.0 ± 0.0abc	1.6 ± 0.1def	3.4 ± 0.4cd
		3.0	66.7 ± 12.9abc	1.5 ± 0.2ef	3.4 ± 0.1cd
		4.0	50.0 ± 15.8bc	0.6 ± 0.1f	3.5 ± 0.2cd
Q. eduardii	None	0.0	75.0 ± 22.4abcd	0.9 ± 0.3e	3.8 ± 0.4bc
	BA	1.0	79.2 ± 18.8abcd	2.1 ± 0.5d	4.2 ± 0.2ab
		2.0	83.3 ± 12.9abc	2.9 ± 0.3c	4.4 ± 0.4a
		3.0	87.5 ± 13.7ab	3.7 ± 0.4b	3.5 ± 0.3bcd
		4.0	91.7 ± 12.9a	6.5 ± 0.4a	2.8 ± 0.2e
		5.0	62.5 ± 13.7bcde	3.0 ± 0.4c	2.6 ± 0.4ef
		6.0	58.3 ± 12.9cde	2.9 ± 0.2c	2.1 ± 0.1f
	Kinetin	1.0	66.7 ± 12.9abcde	1.7 ± 0.3d	3.4 ± 0.4cd
		2.0	87.5 ± 13.7ab	2.0 ± 0.2d	3.7 ± 0.4bcd
		3.0	54.2 ± 10.2de	1.8 ± 0.2d	3.1 ± 0.4cde
		4.0	45.8 ± 10.2e	0.8 ± 0.2e	3.1 ± 0.5de
Q. resinosa	None	0.0	54.2 ± 18.8bc	0.6 ± 0.3e	5.4 ± 1.0abcd
	BA	1.0	62.5 ± 20.9abc	1.2 ± 0.3de	6.5 ± 0.3a
		2.0	66.7 ± 12.9abc	1.3 ± 0.6cde	5.3 ± 1.1abcd
		3.0	75.0 ± 15.8ab	3.8 ± 0.4a	4.0 ± 1.1de
		4.0	62.5 ± 13.7abc	2.3 ± 0.3b	3.9 ± 0.6de
		5.0	45.8 ± 10.2c	1.7 ± 0.6bcd	3.4 ± 0.5ef
		6.0	45.8 ± 18.8c	1.3 ± 0.4cde	2.1 ± 0.3f
	Kinetin	1.0	66.7 ± 12.9abc	1.9 ± 0.3bc	4.3 ± 0.6cde
		2.0	66.7 ± 12.9abc	1.4 ± 0.2cd	4.6 ± 1.1bcde
		3.0	83.3 ± 12.9a	2.0 ± 0.4bc	6.1 ± 0.9ab
		4.0	79.7 ± 10.2ab	1.6 ± 0.3bcd	5.9 ± 1.5abc

Table 1. Conclusion.

Species	Cytokinin	(mg l^{-1})	Explants responded (%)	Shoots per explant	Shoot length (cm)
Q. rugosa	None	0.0	54.2 ± 10.2c	0.6 ± 0.1f	3.9 ± 0.8a
	BA	1.0	87.5 ± 13.7a	3.6 ± 0.1a	3.7 ± 0.3ab
		2.0	75.0 ± 15.8abc	2.1 ± 0.6b	3.2 ± 0.6abcd
		3.0	66.7 ± 12.9abc	1.9 ± 0.3bc	3.0 ± 0.3bcd
		4.0	62.5 ± 13.7bc	1.8 ± 0.3bcd	2.8 ± 0.5cd
		5.0	54.2 ± 10.2c	1.5 ± 0.2cde	2.7 ± 0.1cd
		6.0	54.2 ± 10.2c	1.1 ± 0.3ef	2.7 ± 0.2d
	Kinetin	1.0	83.3 ± 12.9ab	1.5 ± 0.4cde	3.3 ± 0.2abcd
		2.0	79.2 ± 10.2ab	1.4 ± 0.3cde	3.6 ± 0.1abc
		3.0	70.8 ± 10.2abc	1.3 ± 0.4cde	3.5 ± 0.7abcd
		4.0	66.7 ± 12.9abc	1.3 ± 0.2de	3.5 ± 0.3abcd

Means (± s.d.) with different letters within a species are significantly different (ANOVA and Tukey's multiple comparison test; $P \leq 0.05$).

results were better when BA was combined with gibberellic acid (GA3). In *Q. rubra* (Vengadesan and Pijut, 2009), only this combination was successful, obtaining up to 16 shoots per explant, though BA alone did not favor higher shoot proliferation. For this reason, some species need the combination of growth regulators, which had a positive effect on number of shoot per explant, as in *Q. suber* (Romano *et al.*, 1992).

The highest average of shoot length (4.3,4.4, 6.5, and 3.9 cm) were observed with 1.0, 2.0, 1.0 mg l^{-1} BA, and without growth regulator for *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa*, respectively, after 10 weeks of explant incubation (table 1). On the other hand, shoot length was decreased significantly with an increase in BA concentration, same finding was observed with kinetin, except for *Q. resinosa*, where shoot length increased by increasing the concentration. Those results are similar to previous reports: such as in *Q. euboica* (Kartsonas and Papafotiou, 2007), *Q. leucotrichophora*, *Q. glauca* (Purohit *et al.*, 2002b), *Q. semecarpifolia* (Tamta *et al.*, 2008), and *Q. rubra* (Vengadesan and Pijut, 2009). However, in these species it was found that shoot elongation was favored with a combination of BA and GA3.

Rooting of shoots and establishment of plants in soil

Preliminary rooting experiments carried with microshoots maintained continually in root induction medium (several concentrations of IBA) produced shoot senescence and necrosis. Therefore, the rooting efficiency was low (less than 23% on average). This also has been reported for *Q. robur*

Fig. 1. *In vitro* propagation of *Q. castanea*, *Q.eduardii*, *Q. resinosa* and *Q. rugosa*: **a** Multiple shoot induction from intact embryos of *Q. resinosa* on MS with 3 mg l^{-1} BA after 10 weeks of culture initiation; **b** Multiple shoots induction from intact embryos of *Q. eduardii* on MS with 4 mg l^{-1} BA after 10 weeks of culture initiation; **c** Multiple shoots induction from intact embryos of *Q. rugosa* on MS with 1 mg l^{-1} BA after 10 weeks of culture initiation; **d** Shoots of *Q. eduardii* rooted with 10 mg l^{-1} BA; **e** Plantlet of *Q. castanea* with well-developed roots; **f** Plantlet of *Q. rugosa* with well-developed roots; **g** *In vitro* generated plant of *Q. castanea* growing on pot; **h** Acclimatized plantlet of *Q. resinosa* one month after *ex vitro* transfer. Bar 1 cm.

Table 2. Effect of IBA concentration and application period (days), on rooting percentage, root number and root length in shoots of *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa.*

Species	IBA (mg l^{-1})	Time (d)	Rooting (%)	Roots per shoot	Root length (cm)
Q. castanea	0.0	2	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0e
		5	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0e
	1.0	2	33.3 ± 14.4cd	0.3 ± 0.1bc	1.2 ± 0.2d
		5	41.7 ± 14.4c	0.6 ± 0.3bc	1.7 ± 0.4cd
	5.0	2	58.3 ± 14.4bc	1.1 ± 0.4bc	1.8 ± 0.1c
		5	66.7 ± 14.4abc	1.4 ± 0.5bc	2.4 ± 0.3b
	10.0	2	91.7 ± 14.4ab	4.7 ± 0.5a	3.0 ± 0.3a
		5	100.0 ± 0.0a	6.3 ± 1.2a	2.4 ± 0.1b
	20.0	2	66.7 ± 14.4abc	2.3 ± 0.3b	2.2 ± 0.2bc
		5	91.7 ± 14.4ab	5.1 ± 1.7a	1.9 ± 0.2bc
Q. eduardii	0.0	2	0.0d	0.0c	0.0d
		5	0.0d	0.0c	0.0d
	1.0	2	25 ± 0.0 cd	0.4 ± 0.1c	0.9 ± 0.1cd
		5	33.3 ± 14.4bcd	0.8 ± 0.8c	1.7 ± 0.8bc
	5.0	2	41.7 ± 14.4bcd	1.0 ± 0.3bc	1.8 ± 0.2bc
		5	75.0 ± 25.0ab[c]	3.3 ± 2.4abc	2.4 ± 0.0ab
	10.0	2	91.7 ± 14.4a	5.1 ± 1.5ab	3.0 ± 0.6a
		5	100.0 ± 0.0a[c]	5.6 ± 2.1a	1.6 ± 0.2bc
	20.0	2	58.3 ± 14.4abc[c]	1.6 ± 0.4abc	2.0 ± 0.1abc
		5	83.3 ± 28.9ab[c]	5.3 ± 2.8a	1.8 ± 0.6bc
Q. resinosa	0.0	2	0.0b	0.0d	0.0d
		5	0.0b	0.0d	0.0d
	1.0	2	33.3 ± 14.4ab	0.8 ± 0.3cd	1.1 ± 0.3c
		5	41.7 ± 14.4ab	0.7 ± 0.4cd	2.0 ± 0.4abc
	5.0	2	33.3 ± 14.4ab	0.8 ± 0.4bcd	1.4 ± 0.2bc
		5	41.7 ± 14.4ab	1.3 ± 0.5abcd	2.0 ± 0.3ab
	10.0	2	41.7 ± 14.4ab	1.8 ± 0.6abc	2.0 ± 0.4abc
		5	66.7 ± 14.4a	2.6 ± 0.6a	2.3 ± 0.1ab
	20.0	2	50.0 ± 25.0a[c]	1.7 ± 1.1abc	2.2 ± 0.6bc
		5	66.7 ± 14.4a[c]	2.3 ± 0.6ab	2.4 ± 0.1a

Table 2. Conclusion.

Species	IBA (mg l^{-1})	Time (d)	Rooting (%)	Roots per shoot	Root length (cm)
	0.0	2	0.0d	0.0f	0.0d
Q. rugosa		5	0.0d	0.0f	0.0d
	1.0	2	25.0 ± 0.0cd	0.3 ± 0.1ef	1.1 ± 0.2c
		5	33.3 ± 14.4c	0.6 ± 0.1ef	1.1 ± 0.1c
	5.0	2	33.3 ± 14.4c	0.7 ± 0.1def	1.0 ± 0.1c
		5	41.7 ± 14.4bc	0.9 ± 0.4cde	1.9 ± 0.3a
	10.0	2	41.7 ± 14.4bc	1.4 ± 0.4bcd	1.3 ± 0.2bc
		5	83.3 ± 14.4a	2.8 ± 0.4a	2.1 ± 0.3a
	20.0	2	50.0 ± 0.0bc[c]	1.5 ± 0.0bc	1.4 ± 0.2bc
		5	66.7 ± 14.4ab[c]	1.8 ± 0.5b	1.8 ± 0.1ab

Means (± s.d.) with different letters within a species are significantly different (ANOVA and Tukey's multiple comparison test; $P \leq 0.05$). [c] basal callus formation.

and *Q. rubra* (Sánchez *et al.*, 1996). For this reason, in the subsequent experiments shoots were treated only with auxin pulses of two or five days to induce rooting. The rooting percentage of microshoots was increased significantly ($P < 0.05$) both by IBA concentration and application period (table 2). For all four species studied, acceptable rooting rates were achieved by optimizing the length of the IBA pulse treatment (two or, exceptionally, five days). After the pulse, rooting of *in vitro* regenerated shoots (2 to 4 cm long) was achieved in half-strength MS basal medium without IBA (table 2). Root initiation on shoots occurred after two weeks on this medium. No root formation was observed when shoots were cultured initially in the medium devoid of auxin (without pulse), even after 30 days of culture. In the present study, it was observed that the microshoots treated with the pulse of five days to 10.0 mg l^{-1} IBA and then transferred to plant growth regulators-free MS medium showed highest frequency of rhizogenesis (100, 100, 66.7, and 83.3%), with maximum root number (6.3, 5.6, 2.6, and 2.8 roots per shoot) and length (3.0, 3.0, 2.3, and 2.1 cm) averages for *Q. castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa*, respectively. Adding 20.0 mg l^{-1} IBA was found to increase the number of roots formed and reduce the time of their emergence. Of the four species tested, *Q. castanea* and *Q. eduardii* showed better results compared to *Q. resinosa* and *Q. rugosa* (table 2, Fig. 1d-f). Concentrations of IBA bellow 5.0 mg l^{-1} showed a reduction in rooting response. Although all IBA treatments resulted in root induction, treatment with 20.0 mg l^{-1} IBA for two or five days, as well as the treatment with 10.0 mg l^{-1} for five days, gave a high percentage of rooting. However, the shoots produced roots with basal callus formation, which is

not desirable because affects the survival of plantlets in soil (table 2). These results are consistent with previous studies of rooting of oak shoots, where high concentration or prolonged exposure periods of IBA affected rooting in *Q. robur* (Puddephat *et al.*, 1999), *Q. floribunda* (Purohit *et al.*, 2002a), *Q. leucotrichophora*, *Q. glauca* (Purohit *et al.*, 2002b), *Q. semecarpifolia* (Tamta *et al.*, 2008), and *Q. rubra* (Vengadesan and Pijut, 2009). The results contrast with the findings for *Q. euboica* (Kartsonas and Papafotiou, 2007), where the continuous presence of auxin in the rooting medium did not affect root number nor root length. The most effective treatment for root induction without basal callus formation was the use of half-strength MS supplemented with 10.0 mg l^{-1} IBA for a period of two days for *Q. eduardii* and five days for *Q. castanea*, *Q. resinosa*, and *Q. rugosa*, followed by transfer to half-strength basal medium. These treatments were sufficient to allow root formation and reducing the risk of damage during subsequent transfer to soil. In subsequent rooting experiments, additional use of activated charcoal (0.3% w/v) in the rooting medium did not improve the percentage of shoots forming roots, but significantly increased the number of roots per shoot and root length (data not shown). Besides, there were vigorous shoots with more leaves and more secondary roots. On the other hand, the use of gas permeable caps in the culture vessels in this stage is an important factor to avoid falling of leaves (in the previous experiments in which gas permeable caps were not used, a poor development and fall of the leaves was observed).

Rooted Plantlets of *Q.castanea*, *Q. eduardii*, *Q. resinosa*, and *Q. rugosa* were successfully acclimatized, obtaining 80.0, 83.3, 73.3, and 70.0% of survival, respectively, after three months in the greenhouse.

CONCLUSIONS

The present study is the first report of *in vitro* propagation of any species of Mexican oaks. The results show the possibility of successful *in vitro* propagation via multiple shoot formation and subsequent rooting of the four representative species, two of *Quercus* (*Q. resinosa*, Q. *rugosa*) and two of Lobatae (*Q. castanea*, *Q. eduardii*) sections. Therefore, zygotic embryos proved to be excellent explants for micropropagation and this methodology would allow generating multiple plants from each seed. The procedures reported here may facilitate improvement, conservation, and mass propagation of these important Mexican oaks.

ACKNOWLEDGEMENTS

We express our sincere thanks to Roberto Rico Martínez for his helpful and valuable suggestions on this manuscript, to IMAE (Instituto del Medio Ambiente del Estado de Aguascalientes) and Carlos Dávila Figueroa for his help in field works.Thanks to FONDO MIXTO CONACYT-GOBIERNO DEL ESTADO DE AGUASCALIENTES for financial support (AGS-2006-C01-61890), and CONACYT for the fellowship to JSDDL.

LITERATURE CITED

Chalupa, V., 1988."Large scale micropropagation of *Quercus robur* L. using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation". *Biol. Plant*, **30**: 414-421.

Chalupa, V., 1993. "Vegetative propagation of oak (*Quercus robur* and *Q. petraea*) by cutting and tissue culture". *Ann. Sci. For.*, **501**: 295-307.

De la Paz-Pérez, O.C., S.R. Dávalos y C.E. Guerrero, 2000."Aprovechamiento de la madera de encino en México". *Mad. y Bosq.*, **6**: 3-13.

Díaz-Pontones, D. y I. Reyes-Jaramillo, 2009. "Producción y almacenamiento de bellotas de *Quercus hintonii* Warburg (Fagaceae) de la Depresión del Balsas,México". *Polibotánica*, **27**: 131-143.

Favre, J.M. and B. Juncker, 1987. "*In vitro* growth of buds taken from seedlings and adult plant material in *Quercus robur* L.". *Plant Cell Tiss. Org. Cult.*, **8**: 49-60.

Johnson, P.S., S.R. Shifley and R. Rogers, 2002. The ecology and silviculture of oaks. CABI. New York. USA, 503 pp.

Kartsonas, E. and M. Papafotiou, 2007. "Mother plant age and seasonal influence on *in vitro* propagation of *Quercus euboica* Pap., an endemic, rare and endangered oak species of Greece". *Plant Cell Tiss. Org. Cult.*, **90**: 111-116.

Manos, P.S., J.J. Doyle and K.C. Nixon, 1999. "Phylogeny, biogeography, and processes of molecular differentiation in *Quercus* subgenus *Quercus* (Fagaceae)". *Mol. Phylogenet. Evol.*, **12**: 333-349.

Manzanera, J.A. and J.A.Pardos, 1990."Micropropagation of juvenile and adult *Quercus suber* L.". *Plant Cell Tiss. Org. Cult.*, **21**: 1-8.

Moreno-Rico, O., R.Velásquez-Valle, G. Sánchez-Martínez, M.E. Siqueiros-Delgado, M. de La Cerda-Lemus y R. Díaz-Moreno, 2010. "Diagnóstico fitopatológico de las principales enfermedades en diversas especies de encinos y su distribución en la Sierra Fría de Aguascalientes, México". *Polibotánica*, **29**: 165-189.

Murashige, T. and F. Skoog, 1962. "A revised medium for rapid growth and bio assays with tobacco tissue cultures". *Physiol. Plant.*, **15**: 473-497.

Puddephat, I.J., P.G. Alderson and N.A. Wright, 1997. "Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quercus robur* L. *in vitro*". *J. Exp. Bot.*, **48**: 951-962.

_____ , 1999. "*In vitro* root induction in axillary microshoots of *Quercusrobur* L.". *Ann. Appl. Biol.*, **134**: 233-239.

Purohit, V.K., L.M.S. Palni, S.K. Nandi and H.C. Rikhari, 2002a. "*In vitro* regeneration of *Quercus floribunda*Lindl. through cotyledonary nodes: an important tree of Central Himalaya". *Current Science*, 833: 312-316.

Purohit, V.K., S. Tamta, S. Chandra, P. Vyas, L.M.S. Palni and S.K.Nandi, 2002b. "*In vitro* multiplication of *Quercus*

leucotrichophora and *Q. glauca*: Important Himalayan oaks". *Plant Cell Tiss. Org. Cult.*, **69**: 121-133.

Romano, A., C. Noronha and M.A. Martins-Loucao, 1992. "Influence of growth regulators on shoot proliferation in *Quercus suber* L.". *Ann. Bot.*, **70**: 531-536.

Sánchez, M.C., M.C. San-Jose, A. Ballester and A.M.Vieitez, 1996."Requirements for *in vitro* rooting of *Quercus robur* and *Q. rubra* shoots derived from mature trees". *Tree Physiol.*, **16**: 673-680.

San-Jose, M.C., A.M. Vieitez and A.Ballester, 1990."Clonal propagation of juvenile and adult trees of sessile oak by tissue culture techniques". *Silvae. Genet.*, **392**: 50-55.

Schwarz, O.J. and S.E. Schlarbaum, 1993. "Axillary bud proliferation of 2 North American oak species: *Quercus alba* and *Quercus rubra*". *Ann. Sci. For.*, **50**: 340-343.

Tamta, S., L.M.S. Palni,V.K. Purohit and S.K. Nandi, 2008."*In vitro* propagation of brown oak (*Quercus semecarpifolia* Sm.) from seedling explants". *In Vitro Cell. Dev. Biol-Plant*, **44**: 136-141.

Valencia, A. S., 2004. "Diversidad del género *Quercus* (Fagaceae) en México". *Bol. Soc. Bot. Mex.*, **75**: 33-53.

Valencia, A.S. and G. Flores-Franco, 2006. "Catálogo de Autoridad Taxonómica del género *Quercus*, Fagaceae en México". Herbario FCME, Facultad de Ciencias, UNAM. Base de Datos SNIB-CONABIO proyecto CS008. México, DF.

Vengadesan, G. and P.M. Pijut, 2009."*In vitro* propagation of northern red oak (*Quercus rubra* L.)". *In Vitro Cell. Dev. Biol-Plant*, **45**: 474-482.

Vieitez, A.M., F. Pintos, M.C. San-Jose and A.Ballester, 1993. "*In vitro* shoot proliferation determined by explant orientation of juvenile and mature *Quercus rubra* L.". *Tree Physiol.*, **12**: 107-117.

Recibido: 19 octubre 2011. Aceptado: 17 diciembre 2012.