In vitro regeneration of the important North American oak species *Quercus alba*, *Quercus bicolor* and *Quercus rubra*

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Abstract North American oak species, with their characteristic strong episodic seasonal shoot growth, are highly problematic for clonal micropropagation, resulting in the inability to achieve a stabilized shoot multiplication stage. The potential for initiating and proliferating shoot cultures derived from *Quercus alba*, *Q. bicolor* and *Q. rubra* explants was investigated, and a micropropagation method for these species was developed. Branch segments from 6 to 7-year-old trees were forced-flushed and the forced shoots were used as source of explants for culture initiation. A consistent shoot multiplication stage was achieved, in 13 of the 15 genotypes established in vitro, although marked differences occurred in explants from different genotypes/species. The control of efficient shoot multiplication involved the culture of decapitated shoots in a stressful horizontal position on cytokinin-containing medium with a sequence of transfers within a 6-week subculture cycle, which was beneficial to overcoming the episodic character of shoot growth. During each subculture cycle, the horizontally placed explants were cultured on media containing 0.2 mg l^{-1} benzyladenine (BA) for 2 weeks with two successive transfers (2 weeks each) to fresh medium with 0.1 mg l^{-1}BA, giving a 6-week subculture cycle. The general appearance and vigour of *Q. alba* and *Q. bicolor* shoot cultures were improved by the inclusion of both 0.1 mg l^{-1} BA and 0.5 mg l^{-1} zeatin in the medium used for the second transfer within the 6-week subculture cycle. Addition of $AgNO_3$ (3 mg l^{-1}) to the shoot proliferation medium of *Q. rubra* had a significant positive effect on shoot development pattern by reducing deleterious symptoms, including shoot tip necrosis and early senescence of leaves. The three species showed acceptable in vitro rooting rates by culturing microcuttings in medium containing 25 mg l^{-1} indolebutyric acid for 48 h with subsequent transfer to auxin-free medium supplemented with 0.4% activated charcoal. Although an initial five-day dark

period generally improved the rooting response, it was detrimental to the quality of regenerated plantlets. However, activated charcoal stimulated not only the rooting frequencies, but it also enhanced plant quality, as evidenced by root, shoot and leaf growth.

Keywords Charcoal · Ethylene inhibitors · Northern red oak · Micropropagation · Swamp white oak · White oak

Abbreviations

BA 6-Benzylaminopurine

GD Gresshoff and Doy (1972) medium

IBA Indole-3- butyric acid

WPM Woody Plant Medium (Lloyd and McCown 1980)

Introduction

The genus *Quercus* contains some of the most commercially important hardwood species in the world. Examples include *Q. robur*, *Q. petraea* and *Q. suber* in Europe (Savill and Kanowski 1993), and *Q. rubra* (northern red oak) and *Q. alba* (white oak) in North America (Schwarz and Schlarbaum 1993). For these and other oak species seed production may be inadequate for operational requirements as good harvests are possible only every 2 to 5 years and seed storage is difficult (Vengadesan and Pijut, 2009). Vegetative propagation is desirable in order to satisfy production demands, as well as being essential for the propagation of genetically superior material obtained by means of genetic improvement programmes (Kleinschmit and Meier-Dinkel 1990). However, *Quercus* species become difficult to propagate vegetatively as consequence of ontogenetic maturation. As regards macropropagation, for example, *Q. robur*, *Q. rubra*, *Q. bicolor* and *Q. macrocarpa* (Chalupa 2000; Fishel et al. 2003; Amissah and Bassuk 2007), have shown little amenability to clonal propagation by rooting of cuttings; while in vitro micropropagation techniques have been of limited scope. The micropropagation systems developed for *Q. petraea* (Chalupa 1993), *Q. robur* (Puddephat et al. 1999), Himalayan oaks (Purohit et al. 2002; Tampta et al. 2008) and endangered oak species such as *Q. euboica* (Kartsonas and Papafotiou 2007) are all based on the proliferation of axillary shoots from juvenile seedling material (Meier-Dinkel et al. 1993).

Micropropagation of adult oak trees has in general likewise relied on obtaining initial explants from material retaining a high degree of juvenility (stump sprouts, or epicormic shoots collected from the basal zone of the trunk), and has been reported for *Q. robur* (Vieitez et al. 1985; Chalupa 1988, 2000; San-José et al. 1988; Juncker and Favre 1989), *Q. petraea* (San-José et al. 1990) and *Q. suber* (Romano et al. 1995). The only *Quercus* species for which the rejuvenation or reinvigoration of harvested mature material has hitherto been reported is *Q. robur*. In this case, the forced flushing of stem sections (Evers et al. 1993) or crown branch segments (Vieitez et al. 1994) of *Q. robur* produces shoots that are sufficiently rejuvenated or reinvigorated as to be usable as a source of initial explants for in vitro micropropagation (Sánchez et al. 1996; Ballester et al. 2009).

As far as we know, all attempts to micropropagate *Q. alba* (Schwarz and Schlarbaum 1993) or the related species *Q. bicolor* (swamp white oak) have been unsuccessful (Gingas 1991). Schwarz and Schlarbaum (1993) reported that even uncontaminated shoot cultures initiated from terminal and lateral buds of young *Q. alba* seedlings died through gradual loss of vigour. These authors concluded that episodic growth in culture was a significant factor in the cultures' demise. In the case of *Q. rubra*, difficulties were encountered even in micropropagation with juvenile seedling material (Rancillac et al. 1991; Vengadesan and Pijut 2007) where shoot tip necrosis, dormancy and decline of shoot growth were common problems described (McCown 2000). Although the micropropagation from epicormic shoots of red oaks was also reported (Vieitez et al. 1993a; Sánchez et al. 1996), sustainable reliable results have been inconsistent for several genotypes indicating that genotypic effects need to be considered in terms of physiological requirements for maximum shoot proliferation. These three species are all typical of woody perennials characterized by strong episodic flushes during the growing season, and the difficulty of their in vitro culture is attributed to the inability to achieve the stabilization stage where uniform and continuous shoot growth is displayed. The highly episodic northern oaks, and specifically *Q. bicolor*, *Q. alba* and *Q. rubra* were pointed out as species that have only rarely been successfully microcultured as shoot cultures (McCown 2000).

In view of the limited success of previous approaches to the in vitro culture of these *Quercus* species, and the interest in defining the optimal conditions for clonal

micropropagation, the main objectives of this work are: 1) To study the initiation and stabilization stages of shoot cultures derived from *Q. alba, Q. bicolor* and *Q. rubra* explants; 2) To optimize the shoot proliferation stage by evaluating the effect of different cytokinin treatments (*Q. alba* and *Q. bicolor*), and $AgNO_3$ concentrations (*Q. rubra*); 3) To define the rooting stage of micropropagated shoots for plantlet regeneration of these species.

Materials and Methods

Plant material

Source material consisted of branches collected in February 2006 and February 2007 from trees of *Quercus alba* (8), *Q. bicolor* (4) and *Q. rubra* (3) aged 6-7 years growing in a selected field plant collection at Villanieva de Perales, Spain (see Table 1 for genotype codes). Segments 20-25 cm long were cut from proximal part of branches (1-2 cm thick), set upright in moistened perlite, and forced to flush axillary or epicormic shoots in a growth chamber at 25º C and 80-90% relative humidity under a 16 h photoperiod (90-100 µmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps). After 2-3 weeks, newly sprouted shoots (Fig. 1A) were used as the source of initial explants for in vitro culture establishment. Flushed shoots 3-10 cm long were stripped of leaves and their surfaces were disinfected by immersion for 20 s in 70% ethanol followed by 8 min in a 0.6% solution of free chlorine (Millipore® chlorine tablets) containing 2-3 drops of Tween 80®, after which they were rinsed three times in sterile distilled water. Explants consisting of 5-8 mm shoot tips and nodal segments were cut from the shoots and placed upright in 30 × 150 mm culture tubes containing 16 ml of initial medium. This consisted of GD medium (Gresshoff and Doy1972) supplemented with 0.5 mg l^{-1} benzyladenine (BA), 30 g l^{-1} sucrose and 6.5 g l^{-1} Vitroagar (Hispanlab S.A.), brought to pH 5.6, and autoclaved at 121ºC for 20 min. To avoid contact with excreted phenolics, each explant was moved to the opposite side of its culture tube 1 day after the initiation of culture; thereafter, the explants were transferred to fresh initial medium every 2 weeks. After 4-7 weeks of culture, depending on species, the percentage of explants with sprouting buds (the response rate) and the percentage of explants with shoots ≥ 10 mm in length were determined for each genotype.

All cultures were kept in a growth chamber with a 16 h photoperiod (50-60 µmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps) and temperatures of 25ºC (light) and 20ºC (dark).

Shoot multiplication stage

New shoots longer than 1 cm produced by the initial cultures were excised from the original explants, their leaves and apical 2 mm were removed, and the decapitated shoots were placed horizontally in 500 ml glass jars (with glass lids fixed with plastic film) containing 70 ml of multiplication medium. In view of our previous experience with *Quercus* spp (Vieitez et al. 1985; San-José et al. 1990; Vieitez et al. 1993a), the multiplication media were based on GD medium for *Q. alba* and *Q. bicolor*, and Woody Plant Medium (WPM; Lloyd and McCown 1980) for *Q. rubra*. Both were supplemented with 30 g l^{-1} sucrose, 6.5 g l^{-1} Vitroagar, and BA, and the shoots were transferred to fresh medium every 2 weeks. On the basis of preliminary experiments using 0.5, 0.2 and 0.1 mg l^{-1} BA (results not shown), the BA concentration was 0.2 mg l^{-1} for the first 2 weeks and 0.1 mg l^{-1} for the next 4 weeks, in a six-week multiplication cycle (hereinafter referred to as the standard multiplication cycle). At the end of this period, vigorous shoots longer than 10 mm that had developed from axillary buds were isolated and used for the next subculture. Subculture on the multiplication media was repeated until the number of shoots produced was sufficient to evaluate the modifications in media, conditions and procedure for improving shoot proliferation stage.

Experiments with *Q. alba* and *Q. bicolor*

Influence of mineral composition on Q. bicolor multiplication. The dependence of the multiplication rate of *Q. bicolor* on the mineral composition of the multiplication medium (GD or WPM) was evaluated using clones SWOQ-7, SWOQ-12 and SWOQ-18, which were otherwise subcultured in accordance with the standard multiplication cycle procedure.

Influence of cytokinin regime on the multiplication of Q. alba and Q. bicolor. In a three-cycle experiment, the effect of cytokinin on the shoot multiplication of *Q. alba* and *Q. bicolor* was investigated by subculturing clones WOQ-1, WOQ-4, WOQ-23, SWOQ-7

and SWOQ-18 on GD-based multiplication medium in which the cytokinin treatment was changed from cycle to cycle, as follows:

Cycle 1 - the standard multiplication cycle (0.2 mg l^{-1} BA for the first 2 weeks, with two successive transfers to fresh medium with 0.1 mg l^{-1} BA).

Cycle 2 - in the second and third two-week periods of the cycle, the 0.1 mg l^{-1} BA supplement was replaced with 0.5 mg l^{-1} zeatin.

Cycle 3 - in the third two-week period of the cycle, the medium was supplemented with 0.5 mg l^{-1} zeatin as well as with 0.1 mg l^{-1} BA.

In all these experiments the following variables were determined at the end of the six-week multiplication cycle: the frequency of responsive explants, i.e. the percentage of explants forming shoots; the mean number of shoots 0.5-1.0 cm long among responsive explants; the mean number of shoots longer than 1 cm among responsive explants; the mean length of the longest shoot on responsive explants; and the percentage of shoots with at least two expanded leaves.

Influence of $AgNO_3$ on the multiplication of *Q. rubra*

To optimize shoot production and quality of *Q. rubra* cultures, the effect of adding $AgNO_3$ to the multiplication medium was studied by culturing clones ROQ-8 and ROQ-10 on WPM-based multiplication medium supplemented with 0, 1, 3 or 6 mg l^{-1} $AgNO_3$ in accordance with the standard multiplication cycle procedure. The $AgNO_3$ concentration was maintained throughout the six-week multiplication cycle. The following variables were determined: the mean number of shoots 0.5-1.0 cm long; the mean number of shoots longer than 1 cm; the mean length of the longest shoot; the percentage of shoots exhibiting shoot tip necrosis; the percentage of shoots exhibiting leaf senescence and/or leaf abscission; and the percentage of shoots exhibiting anomalous development pattern (recumbent shoots with folding leaves appearance).

In all shoot multiplication experiments carried out in the three species, there were six replicate jars per treatment and clone, with six shoot explants per jar. Each experiment was repeated at least twice.

184 Rooting experiments

185 In a preliminary experiment, shoots 15-20 mm long were isolated from multiplication
186 cultures of the *Q. alba* clone WOQ-1 and the *Q. bicolor* clone SWOQ-12, and were
187 placed for 24 h in root induction medium consisting of GD basal medium with half-
188 strength macronutrients, 30 gl^{-1}sucrose, 6.5 gl^{-1} Vitroagar and supplemented with
189 25 $mg\,l^{-1}$ indole-3-butyric acid (IBA), after which they were transferred to rooting
190 medium of the same composition except for the absence of IBA (root expression
191 medium) for the remainder of the 1-month rooting period.

192 In the main experiment, in which the duration of the initial 25 $mg\,l^{-1}$ IBA treatment
193 was 48 h, 15-20 mm shoots from cultures of WOQ-1, SWOQ-12 and the *Q. rubra*
194 clones ROQ-8 and ROQ-10 (for which GD with half-strength macronutrients was
195 replaced by WPM with half-strength macronutrients) were rooted in accordance with a
196 2×2 factorial design to evaluate the effects of an initial 5-day dark period (which
197 included the 2-day root induction period) and the inclusion of activated charcoal at a
198 concentration of 0.4% in the IBA-free root expression medium.

199 In both sets of experiments, each treatment and clone was evaluated using four
200 replicate 100 ml glass jars, each containing 30 ml of rooting medium and 5 or 6 shoots
201 (20-24 shoots per treatment). All experiments were repeated three or four times, and the
202 variables determined at the end of the 1 month rooting period were the percentage of
203 shoots that had rooted, the mean number of roots per rooted shoot, and the production of
204 surviving shoots as the percentage of rooted shoots with no signs of apical necrosis and
205 no leaf drop.

206 Statistical analysis

207 The influence of the main experimental factors was evaluated statistically by two-way
208 analysis of variance (factorial design) in the following experiments: i) Cytokinin
209 regimes in various genotypes of *Q. alba* and *Q. bicolor* (cycle treatment x genotype)
210 within each of the two species studied (Table 2); ii) Effect of $AgNO_3$ concentration in
211 two genotypes of *Q. rubra* ($AgNO_3$ x genotype; Table 3); iii) Rooting experiment
212 (illumination x activated charcoal; Table 4).

The arcsine square root transformation was applied to proportional data prior to analysis; the data presented in the figures and tables are untransformed.

Results

Culture initiation

After 2-3 weeks in the growth cabinet, flushing occurred in branch segments of all genotypes providing shoots 3-10 cm long (Fig. 1A) that had developed from lateral buds or from accessory buds associated with branch scars. That the contamination rates of the initial explants cut from these shoots were in general low (Table 1) is attributed to the shoots having been grown in a growth cabinet. Also, transfer of the explants to another area of the culture medium appears to have succeeded in limiting the negative effects of phenolics and other exudates. Explants with sprouting buds were obtained in all the genotypes tested, though with marked differences in their in vitro performance. Bud sprouting and shoot growth was slower in *Q. alba* (7 weeks after culture initiation) followed by *Q. rubra* (6 weeks) and *Q. bicolor* (4-5 weeks). The response rates of all *Q. bicolor* and *Q. rubra* genotypes were quite high (79-99%), but those of *Q. alba* genotypes ranged from 29% to 93% (Table 1). Similarly, whereas for all *Q. bicolor* genotypes and two of the three *Q. rubra* genotypes more than 50% of explants were at least 1 cm in length, only one of the eight *Q. alba* genotypes, WOQ-1, had more than 50% of explants that long, the rates of the others ranging from 11% to 40% (Table 1).

Shoot proliferation

Shoots excised from initial explants were subcultured following the standard multiplication cycle of 6 weeks. Only new developed shoots exhibiting vigorous growth were used in successive subcultures. Following this procedure all *Q. bicolor* and *Q. rubra* genotypes, and all except two *Q. alba* genotypes (WOQ-2 and WOQ-6, lost after 5 months) became stabilized, though the time required ranged from 4 months for three of the four *Q. bicolor* genotypes to 9-12 months for all established *Q. alba* genotypes except WOQ-1 (Table 1). In all cases it was possible to re-culture subcultured tissue repeatedly, on fresh medium, after successive harvests of its most vigorous shoots. However, *Q. alba* and *Q. bicolor* genotypes tended to form elongated

shoots with reduced leaf development giving rise to small or scale-like leaves, especially in the case of SWOQ-18, and *Q. rubra* cultures tended to suffer from shoot tip necrosis and leaf anomalies (folding, chlorosis or abscission). To address these problems, the experiments reported on in the next two subsections were performed.

Experiments with *Q. alba* and *Q. bicolor*

Both the number and the length of shoots produced by SWOQ-12 and SWOQ-18 cultures were independent of whether the multiplication medium was GD-based or WPM-based, but SWOQ-7 proliferated better on the former (Fig. 2). Moreover, although SWOQ-18 shoots with more than two expanding leaves were twice as frequent on WPM as on GD, these leaves tended to be thinner and drier than those produced on GD medium, which would be of inconvenience for acclimatization purposes.

In the experiments comparing different cytokinin regimes (Table 2), most of the evaluated genotypes produced between 90-100% of responsive explants. Although genotype differences were evident within each species (Table 2), acceptable multiplication rates were obtained following subculturing in cycle 1. With this treatment, however, a considerable proportion of shoots exhibited poor leaf development; hence, the frequency of shoots with expanded leaves was lower than those achieved with the other subculture cycles in all genotypes of the two species studied. With regard to this variable, a significant interaction between genotype and cycle treatment was also found for *Q. alba* ($P \leq 0.05$) and *Q. bicolor* ($P \leq 0.01$). While in cycle 2 subculturing gave rise to the highest percentages of cultures with normal appearance for determined genotypes, it was considered inadequate for shoot proliferation as cycle 2 also afforded a reduction in shoot number ($P \leq 0.0001$) for both species, along with a reduction of shoot length in *Q. alba* genotypes ($P \leq 0.0001$) and the SWOQ-7 genotype (significant interaction for *Q. bicolor*). Thus cycle 3, in which both 0.5 mg l^{-1} zeatin and 0.1 mg l^{-1} BA were included in the medium in the final 2 weeks of the cycle, was the most efficient in terms of shoot number and shoot length, also giving rise to relatively high frequencies of vigorous and normal shoots (Fig. 1 B, C). Similar behaviour was shown by genotypes WOQ-3, WOQ-5 and SWOQ 12 (results not shown).

Experiments with *Q. rubra*

Analysis of variance showed that, regardless of the genotype, addition of $AgNO_3$ to the multiplication medium had a significant positive effect ($P \leq 0.0001$) on the appearance of ROQ-8 and ROQ-10 cultures, to the extent that with 3 or 6 mg l^{-1} $AgNO_3$ cultures developed normally, producing straight, vigorous shoots with dark green leaves (Fig. 1D, E) and, at most, a low incidence of shoot tip necrosis (Table 3). $AgNO_3$ also tended to produce more shoots, though somewhat shorter ($P \leq 0.01$). Analysis of variance also indicated that the genotype had a significant effect on shoot number and shoot length, as well as on the frequency of anomalous shoot development (Table 3), whereas in genotype ROQ-8, 3 mg l^{-1} $AgNO_3$ treatment significantly increased the production of shoots longer than 1 cm (significant interaction at $P \leq 0.05$). On balance, a concentration of 3 mg l^{-1} was deemed the most appropriate for the shoot proliferation stage of red oak.

Rooting experiments

Treatment of cultures with IBA for 24 h achieved rooting rates of only 24% for WOQ-1 and 8.3% for SWOQ-12. With 48 hours' treatment, these clones achieved rates of at least 40% regardless of whether initial darkness or activated charcoal was used (Fig. 3A). Rooting started around day 10 or 11 for shoots of WOQ-1 and SWOQ-12, and between days 13 and 16 for the *Q. rubra* clones; in all cases root emergence was completed between days 21 and 24. In all genotypes, initial darkness accelerated root emergence by 1-3 days.

For all clones, the effect of initial darkness on rooting rate (Fig. 3A) depended on whether activated charcoal was present or absent, and the effect of activated charcoal on whether initial darkness was applied (significant interaction of the two factors, Table 4). In the absence of activated charcoal, rooting rates were always higher with initial darkness (≈ 45-90%) than without (≈ 30-55%), whereas in its presence darkness was associated with slightly lower rooting rates for all clones except ROQ-8 (Fig. 3A). Activated charcoal always greatly increased rooting rates to values of 80-90% when an initial dark period was not applied, and when it was, slightly increased the rates of all clones except WOQ-1, affording values of 70-90%.

Neither activated charcoal nor initial darkness had any significant influence on the number of roots produced by SWOQ-12 shoots (Table 4). WOQ-1 shoots produced

most roots if dark was applied in the absence of charcoal, or charcoal without initial darkness (Fig. 3B). Both factors had statistically significant additive positive influences on the number of roots on ROQ-8 shoots, but in clone ROQ-10 the use of both charcoal and initial darkness had a less positive influence than the use of either factor by itself (Fig. 3B). A further difference we observed was that roots developed in the charcoal supplemented medium produced more secondary roots than those in medium lacking charcoal, although this variable was not measured.

In all clones, WOQ-1 and SWOQ-12 especially, a number of rooted shoots exhibited browning and senescence of the apical zone, and initial darkness favoured the appearance of these deleterious symptoms (Fig. 3C, Table 4). In all except ROQ-8, the survival rate was greater in the presence of activated charcoal than in its absence.

Overall, the best rooting treatment was deemed to consist of an initial 48 h treatment with 25 mg l^{-1} IBA, followed by transfer to auxin-free medium containing activated charcoal at a concentration of 0.4%, with no initial dark period (Fig. 1F). Application of these conditions to clones WOQ-4 and SWOQ-7 afforded rooting rates of 83% and 71%, respectively, but that of SWOQ-18 was only 19%. However, the rooting rate of SWOQ-18 increased to 89% when the initial 48 h IBA treatment was prolonged for a further 24 h.

Discussion

The achievement of uniform, continuous in vitro shoot growth is highly problematic for woody species with a strongly episodic growth habit (McCown 2000); such is the case of *Q. alba*, *Q. bicolor* and *Q. rubra*. When these shoots were cultured upright for 4-6 weeks with or without transfer to fresh medium (preliminary experiments), the episodic character of the shoots was observed, with arrested shoot growth generally followed by explant death. The successful proliferation of shoot cultures in this study may have been helped by the culture of decapitated shoots in a stressful horizontal position which promoted vigorous shoot development, as has previously been reported in *Q. robur* (Vieitez et al. 1994). In addition, the fortnightly transfer of cultures to fresh cytokinin-containing medium in a 6-week subculture cycle was beneficial in overcoming the episodic character. Changes of medium within subculture cycles have also proved

334 necessary for the stabilization of other *Fagaceae*, including *Fagus* sp. (Vieitez et al.
335 1993b) and *Q. robur* (Vieitez et al. 1994).

336 When the only cytokinin in the multiplication medium was BA, both *Q. alba* and *Q.*
337 *bicolor* tended to form elongated shoots with small or scale-like leaves (Fig. 1 B).
338 Scale-like leaves have also been observed on shoots developed from germinating
339 *Q. suber* somatic embryos, the frequency of scales increasing with the BA concentration
340 applied to the latter (González-Benito et al. 2002). In previous work we found that
341 although *Q. robur* (Vieitez et al. 1985) and *Q. rubra* (Vieitez et al. 1993a) required only
342 BA treatment for axillary shoot culture systems, the in vitro growth of shoots and leaves
343 of beech species was favoured by combining BA with zeatin (Vieitez et al. 2003). The
344 present study shows that the same is true of *Q. alba* and *Q. bicolor*. Zeatin is one of the
345 natural cytokinins found in higher plants, and our results suggest the occurrence of a
346 possible imbalance in levels of endogenous zeatin or its different derivatives in shoots
347 of these species cultured in presence of BA-supplemented medium. This finding is in
348 keeping with those of Werner et al. (2001), who concluded that the growth of leaves in
349 cytokinin-defficient tobacco plants not only required cytokinins, but also the fine
350 adjustment of natural cytokinin levels. Similarly, Perrin et al. (1997) have also reported
351 that the recovery of capacity for axillary shoot organogenesis by in vitro rubber tree
352 shoots is related to an increase in endogenous zeatin levels.

353 Although the horizontal position of subcultured shoot explants successfully
354 promoted the initially vigorous development of shoots, horizontal culture is a form of
355 mechanical stress, and as such may have favoured the synthesis of ethylene and its
356 accumulation in the atmosphere of the culture vessel (Anten et al. 2006). Ethylene-
357 induced shoot growth anomalies similar to those observed in non-Ag^+-treated *Q. rubra*
358 cultures in this study - shoot tip necrosis and leaf abscission – have been observed in
359 shoot cultures of *Annona squamosa* (Armstrong et al. 1997), *Holostemma ada-Kodien*
360 (Martín 2002) and habanero pepper (Santana-Buzzy et al. 2005). $AgNO_3$ and silver
361 thiosulphate have previously been reported to have beneficial effects on shoot growth
362 and organogenesis in a variety of culture systems (Faria and Segura 1997; Reis et al.
363 2003; Burgos and Alburquerque 2003; Alaska-Kennedy et al. 2005; Qin et al. 2005),
364 but as far as we know there have hitherto been no studies of the effects of ethylene
365 inhibitors on the growth and development of oak shoots in vitro. It may be noted that in

366 the concentration range used in this study, $AgNO_3$ appears not to have any of its known
367 undesirable side effects (Kumar et al. 1998).

368 For all three species studied, acceptable rooting rates were achieved by optimizing
369 the initial IBA treatment (48 or, exceptionally, 72 h) and including activated charcoal in
370 the root expression medium. Moreover, activated charcoal not only stimulated rooting,
371 but also benefited root, shoot and leaf growth. These effects of charcoal are attributable
372 to its adsorbing excess of plant growth regulators or detrimental substances that may be
373 released by the plant tissue or by the medium, and to its partial darkening of the
374 environment (Pan and van Staden 1998). The positive effect of darkness on rooting
375 frequency may be due to a dark-induced decrease in peroxidase activity, which may
376 delay the degradation of plant growth regulators. Also, Ahn et al. (2007) have suggested
377 that the number of cells that are competent to initiate adventitious root development
378 may be higher in dark-treated tissues since etiolation of stems has been reported to
379 result in a high level of undifferentiated cells, and cell dedifferentiation is required for
380 acquiring organogenic competence (Sugiyama 1999). In this study, in the absence of
381 activated charcoal, rooting rates were always increased by an initial dark period of
382 5 days, the time taken by *Q. robur* microcuttings to differentiate root initial cells and
383 meristemoids (Vidal et al. 2003). However, darkness treatment during this period
384 favoured shoot senescence and necrosis, as has also been observed in the case of *Q.*
385 *robur* (Sánchez et al. 1996).

386 Finally, that the plant material employed in the present study derived from
387 relatively juvenile source plants (6-7 years old) may seem to throw doubt on whether
388 the results of the study are of use for genetic improvement purposes. However,
389 correlations between the values of growth parameters such as height or diameter at
390 different ages indicate that, at least in the case of *Q. alba*, selection of the fastest
391 growing families can be identified at a relatively early (6-9 years) age (Schlarbaum
392 1993).

393 In conclusion, the above results show the possibility of successful in vitro micro-
394 propagation of species representative of the *Quercus* subgenera *Lepidobalanus* (*Q. alba*,
395 *Q. bicolor*) and *Erythrobalanus* (*Q. rubra*) - all of them woody plants of episodic
396 growth habit and consequently difficult establishment in vitro - starting from crown

material obtained from trees 6-7 years old. Given the analogous results reported for *Q. robur* (Sánchez et al. 1996; Ballester et al. 2009) and *Q. suber* (Romano et al. 1995; Romano and Martins-Louçao 2003), this means that in spite of their reputation for recalcitrance to microculture, all the most important *Quercus* species could now be micropropagated via axillary shoot cultures.

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Table 1 Results on the *in vitro* establishment of shoot cultures of several genotypes of *Quercus alba*, *Q. bicolor* and *Q. rubra*, evaluated when initial explants had been cultured for the times shown in parentheses in column 1. The time required for stabilization of shoot proliferation cultures is also shown.

Species	Genotype	Number of initial explants	Contamination rate (%)	Response rate (%)*	Explants with shoots ≥ 1 cm (%)	Period needed for stabilization (months)
Q. alba						
(7 wks)	WOQ-1	32	6.3	93.3	60.0	6
	WOQ-2	117	5.1	48.6	16.2	lost
	WOQ-3	54	1.8	71.7	17.0	9-10
	WOQ-4	56	0	69.6	37.5	9-10
	WOQ-5	51	1.9	84.0	40.0	10
	WOQ-6	138	9.4	38.7	20.2	lost
	WOQ-14	58	1.7	29.4	10.5	12
	WOQ-23	71	1.4	77.1	35.7	10
Q. bicolor						
(4-5 wks)	SWOQ-7	140	6.4	78.9	54.1	4
	SWOQ-12	96	2.1	92.6	76.2	4
	SWOQ-13	111	25.2	90.4	83.1	10
	SWOQ-18	103	4.9	98.9	93.8	4
Q. rubra						
(6 wks)	ROQ-8	48	0	89.4	53.2	7-8
	ROQ-10	48	45.8	81.8	54.5	8
	ROQ-11	47	0	82.9	38.3	6

*Explants with sprouting buds.

546 **Table 2** Effect of genotype and sequence of cytokinin application (cycles 1, 2 and 3) on the in
547 vitro multiplication of *Q. alba* and *Q. bicolor*. Statistical significance (P-values) of the terms of
548 a two-way ANOVA with genotype and cycle treatment as the two main factors.

549

Genotype	Cycle	Responsive explants (%)	Number of shoots ≥1 cm	Number of shoots 0.5-1.0 cm	Total number of shoots	Longest shoot length (mm)	Shoots with expanded leaves (%)
Quercus alba							
WOQ-1	1	100±0.0	4.9±0.5	1.9±0.4	6.9±0.8	19.6±1.1	47.7±3.8
	2	97.1±2.6	3.7±0.5	1.3±0.3	4.9±0.7	17.2±1.3	62.9±3.1
	3	97.5±2.3	3.9±0.6	2.3±0.2	6.2±0.8	19.0±1.1	80.5±3.0
WOQ-4	1	100±0.0	2.4±0.2	2.1±0.2	4.4±0.3	16.8±1.0	75.0±3.7
	2	94.3±3.1	1.0±0.1	1.2±0.2	2.1±0.2	10.1±0.5	89.9±3.1
	3	97.1±2.6	3.2±0.2	2.8±0.6	5.8±0.4	16.5±1.2	78.5±2.2
WOQ-23	1	97.1±2.6	1.8±0.1	2.1±0.4	3.8±0.5	14.8±0.9	55.8±6.7
	2	87.4±3.0	0.8±0.1	1.1±0.2	1.9±0.2	10.2±0.8	82.5±8.2
	3	100±0.0	2.7±0.3	1.4±0.2	4.1±0.3	15.7±0.5	88.0±3.2
F-test							
Cycle treatment (A)			P ≤0.0001	P ≤ 0.0001	P ≤ 0.0001	P ≤ 0.0001	P ≤0.0001
Genotype (B)			P ≤ 0.0001	ns	P ≤ 0.0001	P ≤ 0.0001	P ≤ 0,001
A x B			P ≤ 0.05	ns	ns	ns	P ≤ 0,05
Quercus bicolor							
SWOQ-7	1	94.3±2.9	2.5±0.2	2.3±0.3	4.8±0.3	17.0±0.8	66.6±4.3
	2	80.0±6.1	0.8±0.2	1.3±0.1	2.1±0.2	10.8±0.9	89.6±3.8
	3	100±0.0	3.9±0.3	2.1±0.1	6.0±0.4	19.3±1.5	74.7±2.7
SWOQ-18	1	100±0.0	3.7±0.3	2.5±0.2	6.2±0.4	21.6±1.3	20.6±2.9
	2	94.9±6.3	1.6±0.2	0.9±0.2	2.5±0.3	25.4±2.2	74.2±4.7
	3	97.1±5.7	3.3±0.2	1.6±0.2	4.8±0.3	20.4±0.8	70.7±1.8
F-test							
Cycle treatment (A)			P ≤ 0.0001	P ≤ 0.0001	P ≤ 0.0001	ns	P ≤ 0.0001
Genotype (B)			P ≤ 0.05	ns	ns	P ≤ 0.0001	P ≤ 0.0001
A x B			P ≤ 0.05	ns	P ≤ 0.01	P ≤ 0.0001	P ≤ 0.01

Data represent means ± SE of six replicate jars with six shoot explants per jar.

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552 **Table 3** Effect of genotype and addition of $AgNO_3$ to the shoot multiplication medium on the
553 development of shoots by *Q. rubra*. SN: number of shoots of the stated size per explant.
554 Anomalous shoot development: presence of recumbent shoots with folded leaves. Statistical
555 significance (P-values) of the terms of a two-way ANOVA with genotype and $AgNO_3$ treatment
556 as the two main factors.
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Genotype	Treatment $AgNO_3$ (mg l^{-1})	SN ≥ 1cm	SN (0.5–1cm)	Longest shoot length (mm)	Shoot tip necrosis (%)	Shoots with leaf senescence and/or abscision (%)	Anomalous shoot development (%)
ROQ-8	0	1.9±0.1	1.2±0.2	16.8±1.7	22.1±2.8	37.1±4.1	53.0 ±4.7
	1	2.9±0.4	1.8±0.3	15.7±0.9	15.8± 3.2	31.9±6.3	24.8± 3.1
	3	3.3±0.3	1.7±0.2	16.6±0.8	4.9±2.1	2.0 ±0.8	0
	6	2.0±0.1	1.5±0.3	13.2±0.6	6.8 ±2.5	2.1± 1.3	0
ROQ-10	0	3.9±0.3	1.4±0.2	22.8±1 .7	44.1±2.6	37.0±3.7	66.1±5.6
	1	3.3±0.4	2.9±0.3	18.1±0.8	17.3± 2.8	28.0±2.9	37.4±1.2
	3	4.3±0.6	1.9±0.3	17.8±1.3	3.2 ±1.0	3.7±0.6	7.2 ±2.9
	6	4.5±0.5	2.9±0.4	17.5±1.0	3.3± 0.7	3.9±1.4	2.2± 0.8
F-test							
Treatment (A)		ns	P ≤ 0.01	P ≤ 0.01	P ≤ 0.0001	P≤ 0.0001	P ≤ 0.0001
Genotype (B)		P ≤ 0.0001	P ≤ 0.0001	P ≤ 0.0001	ns	ns	P ≤ 0.0001
A x B		P ≤ 0.05	ns	ns	ns	ns	ns

Data represent means ± SE of six replicate jars with six shoot explants per jar.

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Table 4 Statistical significance (*P*-values) of the terms of a two-way ANOVA model for the data shown in Figure 3.

Genotype	Rooting (%)	Number of roots	Survival (% of rooted shoots)
WOQ-1			
Illumination (A)	P≤ 0.05	ns	P≤0.001
Activated charcoal (B)	ns	ns	P≤0.01
A x B	P≤0.001	P≤0.01	ns
SWOQ-12			
Illumination (A)	ns	ns	P≤0.001
Activated charcoal (B)	P≤0.001	ns	ns
A x B	P≤0.01	ns	ns
ROQ-8			
Illumination (A)	P≤0.001	P≤0.05	P≤0.05
Activated charcoal (B)	P≤0.001	P≤0.001	ns
A x B	P≤0.01	ns	P≤0.05
ROQ-10			
Illumination (A)	ns	P≤0.001	P≤0.01
Activated charcoal (B)	P≤0.001	P≤0.05	P≤0.05
A x B	P≤0.01	P≤0.001	ns

583 **Legend for the figures**

584 **Figure 1 a** Forced flushing of branch segments in the growth chamber. **b-c** Shoot
585 multiplication in *Q. bicolor* (clone SWOQ-18) following different cytokinin regimens:
586 shoot cultures in cycle 1 (**b**) and cycle 3 (**c**). Scale bars in mm. **d-e** Shoot appearance of
587 *Q. rubra* (clone ROQ-8) after culture in multiplication medium devoid of (**d**) or
588 supplemented with (**e**) 3 mg l^{-1} $AgNO_3$. **f** Root development on *Q. alba* shoots (clone
589 WOQ-1) treated with 25 mg l^{-1} IBA for 48 h with subsequent transfer to auxin-free
590 medium containing 0.4% activated charcoal. Scale bars in mm (**b-d**) and cm (**e**).

591 **Figure 2** Effects of the mineral composition of the multiplication medium (GD or
592 WPM) on shoot development in the *Q. bicolor* clones SWOQ-7, SWOQ-12 and
593 SWOQ-18. Values represent means ± SE.

594 **Figure 3** Effects of an initial 5-day darkness period, and of the presence of activated
595 charcoal (AC) in the rooting medium, on rooting rate (**a**), mean number of roots (**b**) and
596 rooted shoot survival rate (**c**) of *Q. alba* (clone WOQ-1), *Q. bicolor* (clone SWOQ-12)
597 and *Q. rubra* (clones ROQ-8 and ROQ-10) microcuttings. Values represent means ± SE.

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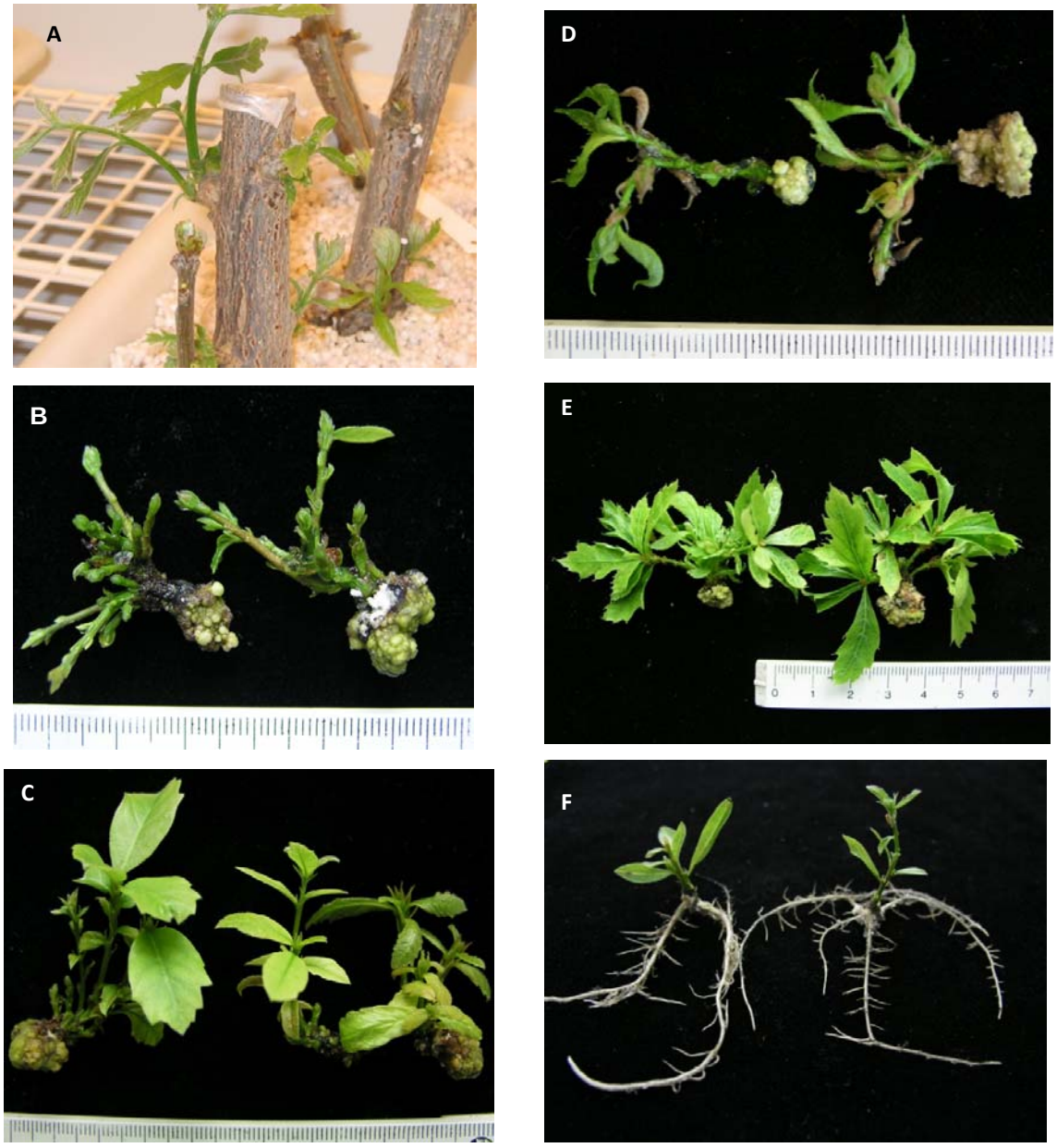
610 Figure 1



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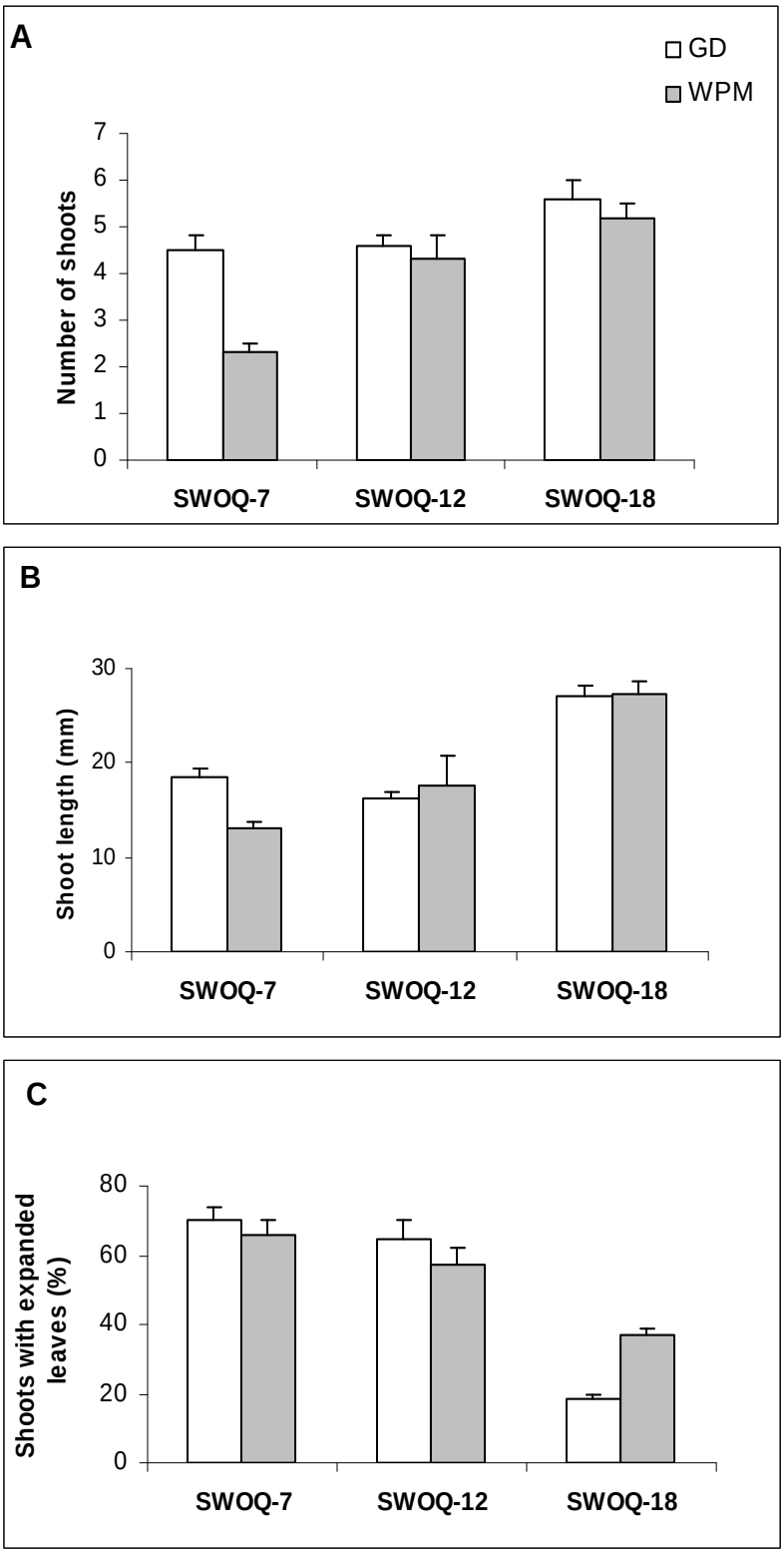
618 Figure 2



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620 Figure 3

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