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Micropropagation of juvenile and adult *Quercus suber* L.

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Abstract

This paper describes research on the application of tissue culture techniques to the micropropagation of cork oak (*Quercus suber* L.), a forest species of ecological and industrial importance in the Mediterranean area. Apical buds and nodal stem segments were employed as initial explants. Their origins were young seedlings, stump sprouts and sprouts formed on cuttings collected from old trees.

The action of the mineral medium and growth regulators was studied in the multiplication stage. Media with low concentrations of ions, such as Sommer's or Heller's, are more suitable for growth and proliferation of explants than other media richer in salts. It was also observed that cytokinin (BA) must be present for the culture development. Adding low concentrations of auxin (NAA) to the medium improves the multiplication rate, especially in vegetative material of adult origin.

The auxin type is the most important factor in the promotion of rhizogenesis. The method of application determines the quality of the root system. Treatment with low concentrations of IBA added to the rooting medium gives the best results.

High sucrose concentration also improves rooting. Diluting the mineral rooting medium is slightly favourable, although there is no significant difference between it and the standard mineral concentration.

Abbreviations: D — Durzan's, GD — Gresshoff & Doy's, H — Heller's, L — Lepoivre's, MS — Murashige & Skoog's, SH — Schenk & Hildebrandt's, S — Sommer's medium

Introduction

Cork oak (*Quercus suber* L.) belongs to one of the most important genera in the Mediterranean forest, both in terms of extent and of its ecological role. Moreover, this species is characterized by its cork production, which gives it special silvicultural and economic relevance in the Mediterranean countries.

The genetic improvement of cork oak is confronted by the problem that the species is very heterozygotic. The sexual propagation of selected superior trees is, therefore, not advisable, because there would be a high percentage of unsatisfactory genotypes among progenies. For this species, therefore, vegetative propagation has to be used [20].

To date, conventional methods of vegetative propagation of cork oak have not been satisfactory. Old trees cannot be propagated from cuttings, and the grafting and layering methods have many limitations. This has inspired a search for alternative methods, such as micropropagation.

Jaquiot [7] was the first to observe histogenic

phenomena in cambial tissue cultures of cork oak and two other *Quercus* species, and callus formation after an initial period of phloem formation.

In 1981, Pardos [12] approached the micropropagation of *Quercus suber* using nodal segments taken from one-year-old seedlings, cultivated in MS medium [11], with BA and NAA as growth regulators. He obtained normally formed 15 to 20 mm long sprouts from these buds.

Bellarosa [1] used *Quercus suber* embryos without cotyledons as initial material, and cultured them in medium modified according to Durzan et al. [3]. The mineral solution and, above all, the hormonal balance between BA and NAA, influenced the elongation of the root, the apical meristem or both.

Finally, El Maataoui & Espagnac [9] studied the formation of somatic embryos on cork oak callus, induced in nodal segments of 6 to 8 month old seedlings by the action of IBA and BA in MS medium. Some 3% of the calli showed globular and polar structures that later developed cotyledons. These embryoids, transferred to the same medium, produced new embryoids in their periphery. If they were transferred to medium without growth regulators, the radicle grew in some cases, but no epicotyle growth was observed.

Materials and methods

Apical and nodal segments from 3-month-old seedlings were used as initial explants. The seedlings were obtained from acorns germinated in wet perlite, previously treated with a $1\,g\,l^{-1}$ fernide solution and periodically watered, placed in a chamber with 25°C day temperature, 20°C night temperature and a photoperiod of 14 h.

Two different sources were employed for adult material. The first was 30 to 40 year old stumps of cork oak. The stumps were sprayed with $100\,mg\,l^{-1}$ of BA solution in September. After 3 weeks, the sprouts were 2 to 5 cm long and were collected.

The second source of adult material was cuttings collected in February from branches of 75 to 115 year old trees. The cuttings were stored at 4°C and 7 days later were immersed in $1\,g\,l^{-1}$ fernide solution for 24 h. Then they were also sprayed with $100\,mg\,l^{-1}$ of BA solution. Sprouts some cm long had formed after 6 weeks.

The sprouts were prepared for sterilization by removing the leaves and coating the cut end with paraffin. The material of young origin was sterilized in a solution of 0.1% NaOCl ($174\,g\,l^{-1}$ active chlorine) plus a few drops of Tween 20, agitating for 10 min, followed by three rinses in distilled sterile water, each of 10 min, in a laminar flow cabinet.

The vegetative material of adult origin was sterilized with a solution of 0.2% $HgCl_2$ to which a few drops of Tween 20 had been added, in agitation for 2 min, followed by two rinses, the first with a solution of $2.44\,g\,l^{-1}$ of $CaCl_2$, and the second with $1\,g\,l^{-1}$ ascorbic acid, both for 10 min. Afterwards, the sprouts were separated into apical buds and nodal segments, and were put in test tubes containing 10 ml culture medium.

The following macroelement formulas were tested: Heller [6] modified by multiplying the concentration × 1.25 and adding 1 mM of $(NH_4)_2SO_4$; Schenk & Hildebrandt [15]; Lepoivre [13]; Murashige & Skoog [11]; Greshoff & Doy [4]; Sommer et al. [18]; and Durzan et al. [3] (the latter as modified by Bellarosa [1]).

The microelements used were those of Murashige & Skoog [11], and the following cofactors: $1.76\,mg\,l^{-1}$ of ascorbic acid, $1\,mg\,l^{-1}$ of nicotinic acid, $100\,mg\,l^{-1}$ of m-inositol, $1\,mg\,l^{-1}$ of Ca pantothenate, $1\,mg\,l^{-1}$ of pyridoxine and $1\,mg\,l^{-1}$ of thiamine. Sucrose was used as carbon source ($30\,g\,l^{-1}$), and the medium was solidified with agar ($6\,g\,l^{-1}$). The growth regulators employed were BA, NAA and IBA. The pH was adjusted to 5.5–5.7. The medium was sterilized in autoclave at 0.5 atmospheres (115°C) for 20 min. The cultures were incubated at temperatures of 25°C by day and 20°C by night under a 14 h photoperiod and an irradiance of $3.6\,W\,m^{-2}$.

The different treatments studied in each experiment were compared by analysis of variance. When statistically significant differences were obtained, the Newman-Keuls test was performed [10]. In all calculations the 0.05 significance level was considered satisfactory. The results that were not statistically different were marked with the same letter. All experiments were repeated twice.

Results and discussion

'In vitro' establishment and multiplication

Initial media

Explants belonging to four clones of juvenile origin were cultured in Heller's medium, supplemented with BA at two different concentrations, 0.2 and 1 $mg\,l^{-1}$, plus 0.1 $mg\,l^{-1}$ NAA. The uncontaminated explants were transferred to fresh medium after one week, in order to avoid the inhibitory effect of catabolite exudation.

The apical and axillary buds began to swell and develop six days after being put into the culture tubes. In some cases, the sprouts reached a length of 1 cm in two weeks, without great differences between position (apical or axillar), or BA concentration (0.2 or 1 $mg\,l^{-1}$). The percentage of contaminated explants was very low.

The sterilization with NaOCl was inadequate for material of adult origin. Therefore, $HgCl_2$ (0.2%) was used. The contamination was very rare, but 50% of the explants showed toxicity. The surviving explants began to develop their buds in the same way as the juvenile material.

Seven macronutrient formulas described in 'Material and methods' were compared. The percentage of explants that developed juvenile material and the growth in length are shown in Table 1. There were no statistical differences between the media.

The same experiment was performed with monoclonal material (M13) from an adult source. The percentage of sprouting and the sprout length were measured 60 days later, because growth was slower than in the material of juvenile origin. Table 2 shows the results.

Sommer's formula gave the highest sprouting rate. Other media gave lower percentages, but the sprouts formed had a generally good appearance, except those in MS medium, which was unable to produce any bud break.

Table 1. Effect of media formula on sprouting percentage and sprout length. Explants from juvenile origin.

Medium	Sprouting (%)	Elongation (mm)
L	42.80	7.50
SH	41.75	7.83
D	40.80	8.00
S	39.90	7.22
GD	35.75	6.42
MS	35.20	6.15
H	32.25	7.42

Table 2. Effect of media formula on sprouting percentage and sprout length. Explants from adult origin (M13).

Treatment	Number of explants	Sprouting (%)	Elongation (mm)
S	8	50.00	10.7
H	18	27.78	15.0
GD	16	25.00	8.5
SH	16	18.75	12.6
D	22	9.09	12.0
L	17	5.88	14.0
MS	27	0.00	0.0

Effect of mineral formula on the multiplication rate

Six macronutrient media were tested in the multiplication stage (MS, L, D, SH, H and S). The trial was performed on 4 clones of juvenile origin. For greater uniformity, shoot apices 1 cm long were used as explants. In order to avoid the carry-over effect, the explants were subcultured 3 times for a period of 4 weeks. Nevertheless, the responses to the macronutrient formulas were already remarkable at the first subculture. Growth in length at the end of third subculture is shown in Fig. 1, and the number of shoots longer than 0.5 cm per initial explant is shown in Table 3.

Sommer's and Heller's media gave the best results in the four clones as a whole, in elongation as well as in number of shoots formed per explant (Fig. 2). Similar results were obtained by other authors working with *Quercus rubra* [16] and *Q. robur* [21].

Table 3. Effect of media formula on number of shoots per explant. Four clones of juvenile origin (clones 12, 13, 17, 18).

Formula	Mean number of shoots
S	1.44 (a)
H	1.35 (a)
L	1.10 (b)
SH	1.00 (b)
D	1.00 (b)
MS	1.00 (b)

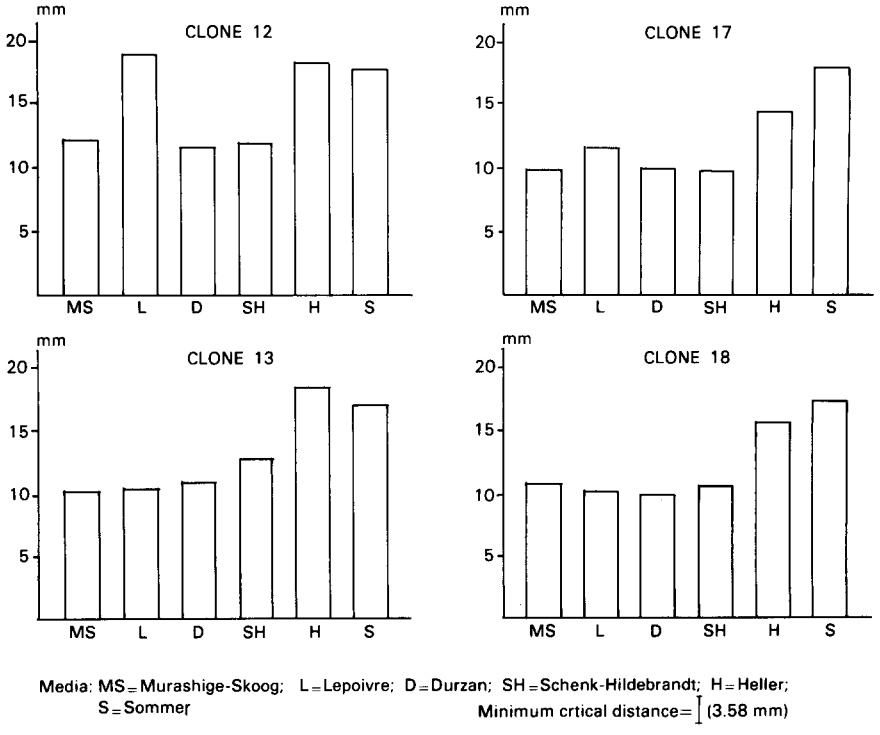

Fig. 1. Mean growth in length of shoots in the multiplication phase. Four clones of juvenile origin (clones 12, 13, 17, 18).

The growth regulators BA and NAA at the multiplication stage

Different levels of BA (0, 0.1, 0.5, 1 and 5 mg l^{-1}) and NAA (0, 0.01, 0.1 and 0.5 mg l^{-1}) were tested in a clone of juvenile origin (12) and another one of mature origin (M13). Elongation and number of shoots formed per explant were measured 4 weeks after beginning the assay.

The analysis of variance of the results showed that BA was the only factor affecting elongation in the clone of juvenile origin (Fig. 3), whereas BA, NAA and the interaction affected the number of shoots per explant (Fig. 4). In the mature clone (M13), both BA and NAA influenced the elongation of shoots (Fig. 5), and BA, NAA and their interaction affected the number of shoots formed per explant (Fig. 6).

The optimal BA level was 0.1 mg l^{-1}, regardless of whether the explants were of young or adult origin.

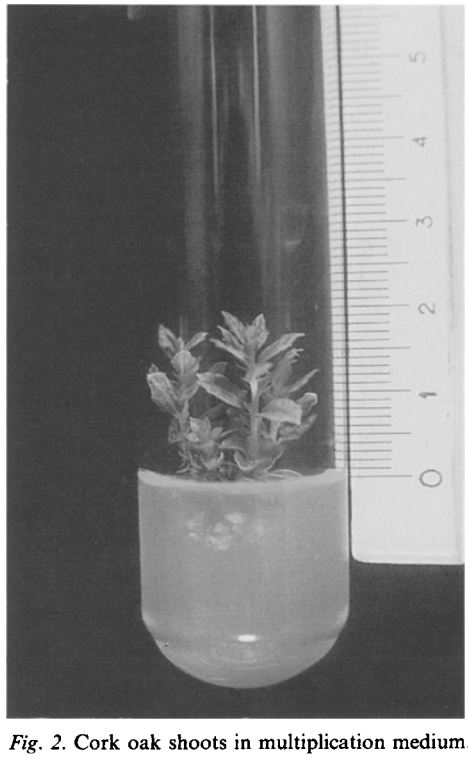

Fig. 2. Cork oak shoots in multiplication medium.

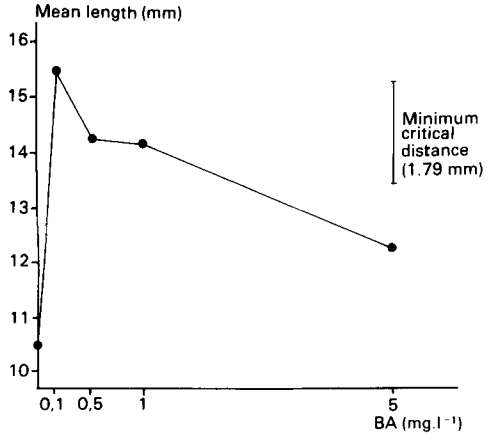

Fig. 3. Growth in length of apical buds of clone 12 (juvenile).

Rooting induction

Type of auxin and application method

Two auxins, IBA and NAA, and two different application methods were tested. One method was dipping the base of the shoots in a solution of 1 g l^{-1} of auxin for 30 s and then transferring them to medium without growth regulators. The second method consisted of cultivating the shoots for 7 days in medium supplemented with 1 mg l^{-1} of auxin, and then transferring them to medium without growth regulators (Fig. 7).

The auxin was very significant in influencing rooting percentage (Fig. 8). There was no difference among clones; this allows us to generalize the results.

Rooting of shoots of adult origin

Three clones of adult origin and four concentrations of IBA were tested: 0, 0.1, 1 and 5 mg l^{-1}. The explants were cultivated for 1, 7 or 14 days, and then transferred to medium lacking auxin.

IBA concentration significantly affected rooting

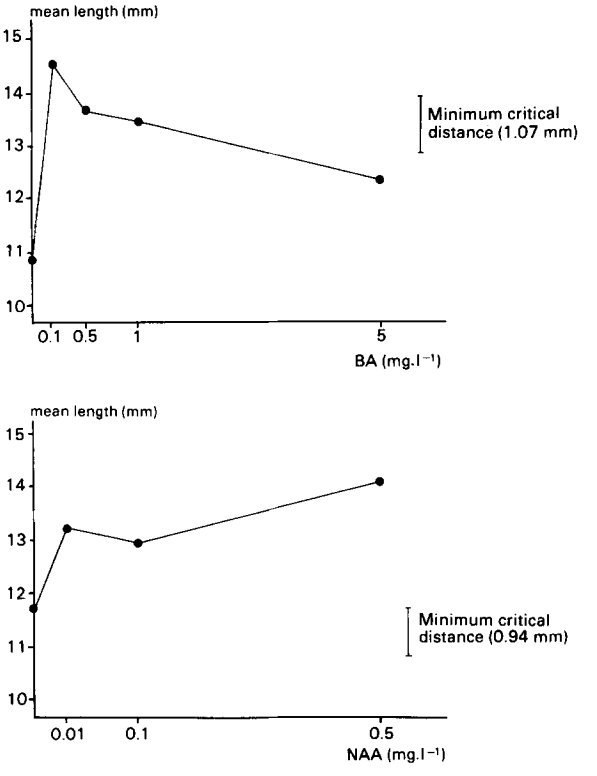

Fig. 5. Growth in length of apical buds of clone M13 (mature).

percentage, mean number and length of roots. Time of exposure was only significant for rooting percentage (Table 4). For all clones, rooting was best at 5 mg l^{-1}. There was no statistical difference between 7 or 14 days of exposure to IBA, and rooting was only significantly lower for 1 day.

Concentration and type of rooting substrate

Three concentrations of the mineral medium were compared in this experiment: Sommer's formula, the same formula at half strength, and at 1/10 strength. Two types of substrate were also tested:

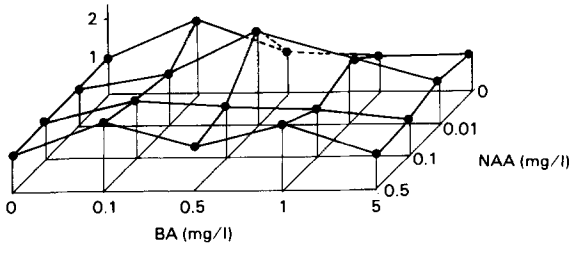

Fig. 4. Number of shoots per explant. Clone 12.

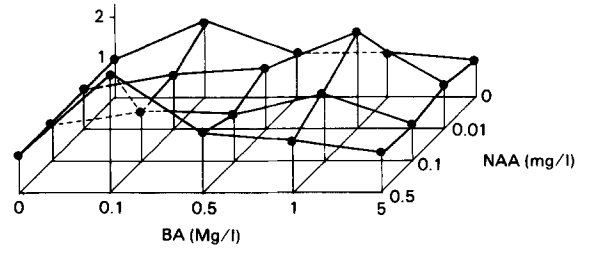

Fig. 6. Number of shoots per explant. Clone M13.

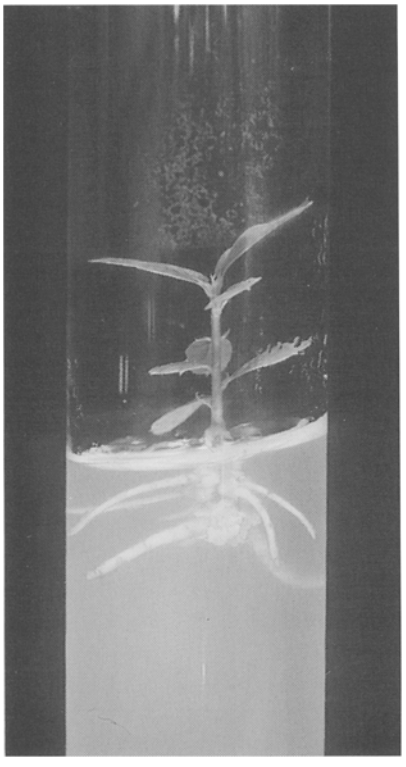

Fig. 7. Root development 20 days after IBA treatment.

agar, as used before, and perlite in liquid medium. The results after using IBA treatment are shown in Fig. 9. There were only significant differences in root length for the substrate: roots were longer in agar than in perlite.

Table 4. Rooting percentage of clones A5, A6 and M13 as affected by cultivation time and IBA concentration.

Cultivation time (day)	Clone	IBA ($mg\,l^{-1}$)			
		0	0.1	1	5
1	A5	20	10	30	30
	A6	30	20	40	80
	M13	10	20	0	50
7	A5	10	40	70	70
	A6	40	40	70	60
	M13	10	10	70	70
14	A5	40	20	50	70
	A6	20	40	70	70
	M13	20	20	60	100

Rooting was slightly better in the more diluted media. Other authors have also reported that reducing the concentration of salts in the rooting medium has beneficial effects [2, 8 and 17].

Sucrose

Two clones of adult origin, M13 and A5, and six sucrose levels were tested. The results are given in Table 5. The lowest sucrose concentration ($10\,g\,l^{-1}$) was very negative for the rooting of both clones; the rooting percentage and number of roots per explant rose with the sucrose level. The optimum depends on the clone.

Haissig [5] and Thorpe [19] have stated that rooting initiation is a process demanding much energy, which requires sucrose as a carbon source. Our results in *Quercus suber* corroborate this.

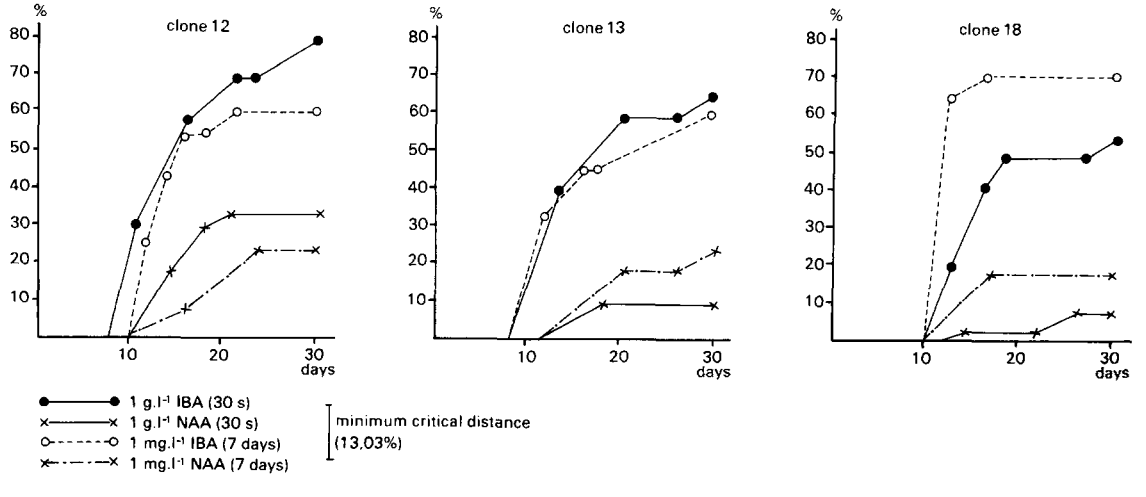

Fig. 8. Effect of type of auxin and application method on rooting percentage. Clones 12, 13, 18 (juvenile).

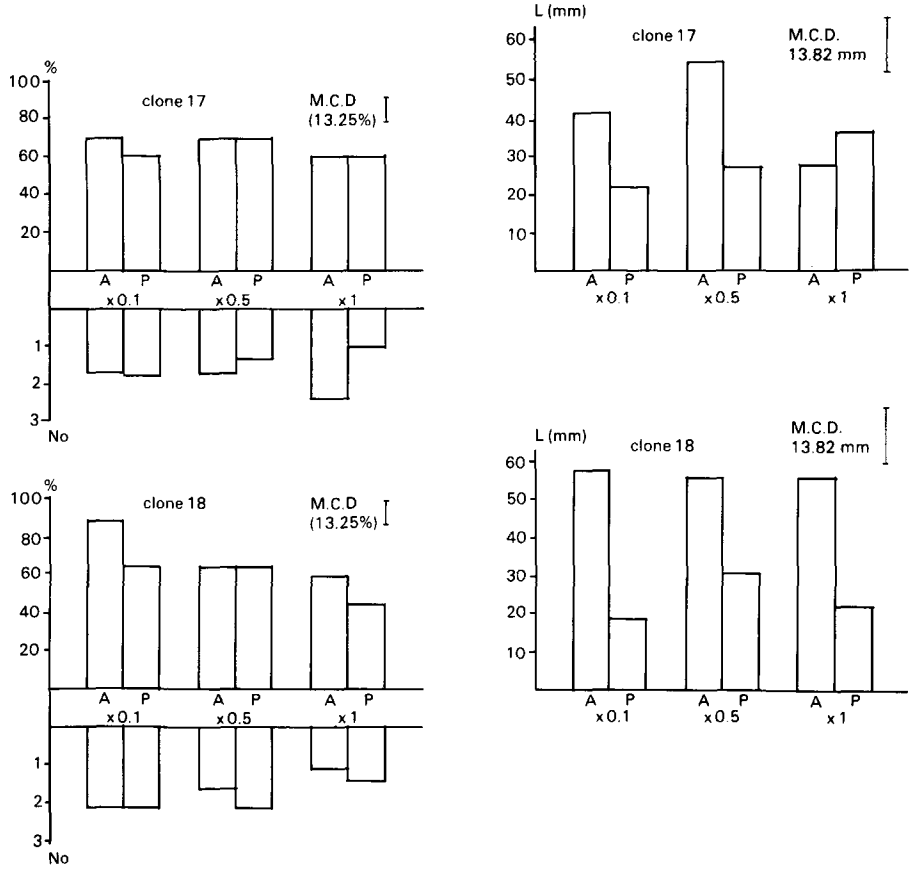

Fig. 9. Effect of medium concentration (× 0.1, × 0.5, × 1) and type of substrate (A = agar, P = perlite) on rooting (%), mean number of roots (No), and mean length of the longest root per explant (L). M.C.D. = minimum critical distance.

Transfer to soil

After 30 days of the rooting treatment, the plantlets have developed their adventitious rooting system sufficiently to be transferred to 'in vivo' conditions. This phase is critical, because the plantlets undergo a great change in the form of nutrition and in environmental conditions.

The cork oak plantlets were placed in plastic vessels with turf substrate in the glasshouse (Fig. 10). The plantlets were protected from direct

Table 5. Rooting percentage and mean number of roots per explant for clones A5 and M13, as affected by sucrose concentration.

Sucrose conc. (gl^{-1})	Rooting (%)		Mean number of roots per explant	
	A5	M13	A5	M13
10	0.0b	0.0b	0.0b	0.0b
20	15.0ab	20.0ab	1.0ab	1.5ab
30	25.0ab	68.75a	1.6ab	2.7ab
40	60.0a	40.0ab	2.9a	2.0ab
60	50.0a	0.0b	1.8ab	0.0b
80	35.0ab	70.0a	1.8ab	3.0a

Fig. 10. Plantlets transferred to the glasshouse.

sunlight with a net and periodically fertilized with a liquid solution of Sommer's macroelements. After 50 days, the percentage of surviving plantlets in active growth was 11.53%.

Conclusions

Our results imply that mature trees of *Quercus suber* can be micropropagated using rejuvenation techniques on stump sprouts, or by spraying cuttings with BA.

Whereas in the case of vegetative material of juvenile origin mineral composition of the medium was not important during the establishment of cultures, in the case of the material of adult origin, Sommer's medium was the best.

IBA was better than NAA for the rooting percentage and mean number of roots per explant. The best application method for explants of juvenile origin was to culture them for 7 days in medium with $1\,mg\,l^{-1}$ of IBA. The treatment by basal dipping in a liquid solution of $1\,g\,l^{-1}$ gave similar rates, but the quality of the root system was worse. For shoots of adult origin, the best concentration was $5\,mg\,l^{-1}$ of IBA added to the rooting medium, and treatment periods of 7 to 14 days.

Reducing the concentration of the rooting medium to 1/10 strength of Sommer's standard formula improved rooting. Agar permitted more elongation of the roots than liquid medium in perlite substrate. High levels of sucrose gave better results. 'In vitro' regeneration of *Quercus suber* plantlets was achieved.

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