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MICROPROPAGATION

Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants

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Abstract *Arbutus unedo* L. is a species of strawberry tree, widely represented in the Mediterranean climates of southern Europe. Fruits are used to make jellies and a spirit called "medronheira." Shoot apices and nodal segments from epicormic and coppiced shoots of adult plants were used for plant propagation. Shoot apices from epicormic shoots, which were developed in a growth chamber, showed higher rates of *in vitro* establishment. The results also indicated that shoot apices are more effective for plant establishment than nodal segments, with rates of establishment significantly higher after 12 wk of culture. Of the three basal media used in combination with 9.0 μM benzyladenine and 0.087 M sucrose, the FS medium with the micronutrients of the Murashige and Skoog medium gave the highest rates of multiplication, especially when the parameter analyzed was the number of clusters formed. When shoot apices from selected adult plants (AL01–AL06) were tested, the multiplication rate was not significantly different among the plants. However, in the conditions tested, shoots from the clones AL1, AL2, and AL3 showed better development, whereas shoots from AL4, AL5, and AL6 showed an impaired development and could not be rooted. Rooting was achieved in all the conditions tested, even in the absence of auxin. The inclusion of an auxin significantly increased root formation, whereas the addition of charcoal did not improve root formation. Rooted plantlets were acclimatized, and some of them are now in the field for further study.

Keywords Acclimatization · Epicormic shoots · Rooting · Shoot proliferation

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Introduction

The genus *Arbutus* (*Ericaceae*) includes about 20 species from which *Arbutus unedo*, commonly known as strawberry tree, is the most interesting species economically. Strawberry tree seems to be native to Ireland, southern Europe, and the western Mediterranean region where it grows spontaneously in several countries in rocky and well-drained soils (Piotto et al. 2001). The plant is an evergreen shrub or small tree (rarely exceeding 3 m) with a spreading habit, and gray-brown bark (Heywood 1993). The small white blueberry-like flowers are assembled in panicles about 5 cm long. The spherical bright red fruits are warty and about 2 cm in diameter. They take a year to ripen, and during several mo of the year, both flowers and fruits are present in the same tree.

Populations of *A. unedo* can be uniform, but in most cases, this species grows associated with other trees (Neppi 2001), in particular with some species of the family *Fagaceae* (e.g., *Quercus suber*). The fruits are edible and have been traditionally used to make a strong tasting spirit called "medronheira." They can also be used to make preserves or provide pollen for a distinctive honey (Neppi

2001). Fruits are collected from October to December, and each tree produces an average of 7–10 kg. In general, 10 kg are necessary to produce 1 l of the drink (Cardoso 2004). The bark has been used to tan leather, and in folk medicine, the fruits and leaves have been used as an astringent, diuretic, antirheumatic, antidiarrheal, and against urinary infections (Pabuccuoglu et al. 2003; Cardoso 2004). More recent usages are related with biomass for energy production and floriculture, since young branches make attractive floral bouquets (Metaxas et al. 2004).

From an ecological perspective, *A. unedo* is also an interesting plant. As a species characteristic of Mediterranean ecosystems, it contributes to maintain the biodiversity of the fauna, helps to stabilize soils avoiding erosion, has a strong regeneration capacity following fires, and survives quite well in poor soils. In addition, it can withstand low temperatures and is tolerant to drought (Piotto et al. 2001).

The selection of the most interesting trees, based on fruit production/quality or any other characteristic, is relevant for its economic use. Considering this general overview and the growing role of alternative crops in the European Unions's agricultural policy, it is of great importance to start a long-term program for the improvement of *A. unedo* in Portugal based on the genetic diversity occurring among natural populations of different regions (Torres et al. 2002).

The propagation of the selected strawberry trees is particularly important. Seed propagation does not assure genetic stability, and particular characteristics can be lost. Assays of vegetative propagation can be made by conventional vegetative propagation methods such as rooting of cuttings and by micropropagation methods (Hartman et al. 1997). However, the frequencies of rooting are quite low especially when mature cuttings are used (Mereti et al. 2002; Metaxas et al. 2004). Micropropagation may be a valuable alternative when: (a) conventional propagation is difficult to achieve, (b) problems of rejuvenation persist, and (c) pressure to increase multiplication rates occurs (McComb and Bennett 1986; Gomes and Canhoto 2003). Several micropropagation techniques such as somatic embryogenesis (Canhoto et al. 1999), organogenesis (Arezki et al. 2000), and axillary shoot proliferation (several authors in Jain and Häggman 2007) have been applied for *in vitro* propagation of woody plants. Among these methods, axillary shoot proliferation is the most widely used for *Ericaceae* clonal propagation. Members of this family that have been successfully micropropagated include *Arbutus xalapensis* (Mackay 1996), *Kalmia latifolia* (Lloyd and McCown 1980), *Oxydendrum arboreum* (Banko and Stefani 1989), *Rhododendron* (Anderson 1984), and several species of the genus *Vaccinium* (Isutsa et al. 1994; Gajdošová et al. 2007; Ostrolucká et al. 2007). Previous studies in strawberry tree have shown that *in vitro* propagation from juvenile material could be accomplished (Mereti et al. 2002). However, as with seeds, juvenile explants are of unknown genotype, making it difficult for the propagation of selected trees, which can only be achieved through the propagation of adult plants. Previous reports of *A. unedo* micropropagation are scarce. As far as it is known, the only report in which micropropagtion of *A. unedo* has been achieved from adult material was that of Mendes (1997). Therefore, the development of a protocol for an efficient *in vitro* propagation of this species from adult trees is necessary. In this paper, we describe a reliable and reproducible method to propagate adult trees of *A. unedo* through shoot proliferation.

Materials and Methods

Plant material. Shoots from spontaneous field-growing adult plants were used to obtain the explants used in the experiments. Young shoots (5–10 cm length) were collected in the autumn from 2- to 3-mo-old coppices of a plant (C01) sprouting in a burned area. Woody branches with 30- to 40-cm length and 0.4- to 2.5-cm diameter were collected from another spontaneous adult plant (E05) and from six adult plants selected for its high fruit productivity and growing in an orchard (AL01 to AL06). Woody branches from E05 and AL01 to AL06 plants were stimulated to produce epicormic shoots, which were the source of the explants used for *in vitro* culture establishment.

Surface-sterilization procedures and culture establishment. For explant sterilization, coppiced shoots were defoliated, dipped in a fungicide (dichlofluanid, Euparene, 120 mg l^{-1}) for 2 min, rinsed with running tap water, and placed in a 20% sodium hypochloride solution (5% active chlorine) containing two to three drops of Tween 20, for 18 min. After three washes in sterile distilled water, they were inoculated on the establishment medium. Epicormic shoots were surface-sterilized in a 75% ethanol solution for 2 min dipped in a 10% sodium hypochloride solution (5% active chlorine) for 3 to 6 min and then washed in sterile distilled water three times.

Woody branches were washed under running tap water, dipped in a fungicide (dichlofluanid, Euparene, 120 mg l^{-1}) for 10 min, and rinsed with distilled water. Following this treatment, the branches were transferred to jars containing 1.5 l of sterilized sand and watered with distilled-sterilized water and placed into a greenhouse or in a growth chamber at 25±1°C under a 16-h photoperiod provided by cool-white fluorescent lights (40 μmol m^{-2} s^{-1}) to promote epicormic shoot development. The branches placed in the growth chamber were covered with transparent polythene plastic to keep a high degree of humidity. To promote epicormic shoot development, branches placed in the

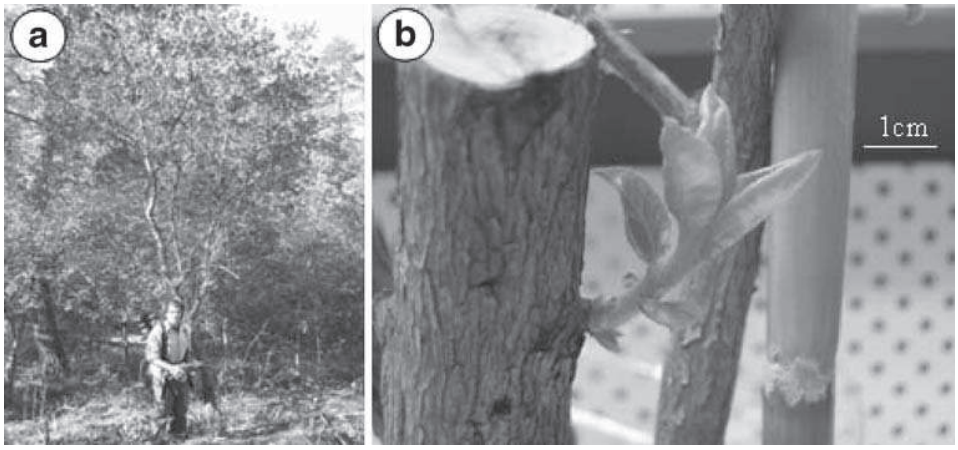

Figure 1. Tree E05 (*a*) and branches (*b*) showing epicormic shoot development after 15 d in the growth chamber.

greenhouse were sprayed with a solution of benzyladenine (BA, 9.0 μM) three times a wk and/or covered with polypropylene plastic bags for 2 mo. Following this period, epicormic shoot development was evaluated by the number of produced shoots and shoot length. The epicormic shoots thus obtained where then used for *in vitro* establishment.

Shoot apices (<2 mm) and nodal segments (10–20 mm) were used as explants to establish the cultures. Explants were incubated individually and subcultured every 3 wk in test tubes (nodal segments) or Petri dishes (shoot apices). To avoid phenol oxidation and tissue necrosis, explants were placed in a growth chamber at 25±1°C, under dark conditions for a wk and then transferred to light (16 h photoperiod, 40 μmol m^{-2} s^{-1}). Test tubes (Sigma, 25× 150 mm, Madrid, Spain) containing 12 ml of culture medium and covered with plastic caps were used. The medium for culture establishment (AND) consisted of Anderson salts (Anderson 1984), Murashige and Skoog (MS) micronutrients (Murashige and Skoog 1962), and organic compounds of the FS medium (De Fossard et al. 1974). We added 0.087 M sucrose and 9.0 μM BA. The medium was solidified with agar (7 g l^{-1}) and the pH adjusted to 5.7 prior to autoclaving (121°C, 20 min.). The best conditions for epicormic shoot development were further applied to obtain epicormic shoots from clones AL01-AL06, which were then used as source of the explants for *in vitro* establishment. For all the assays, the number of established explants and the frequencies of necrosed and contaminated explants were taken after 1 and 12 wk of culture.

Table 1. Effect of BA (9.0 μM) and plastic covers (covered or uncovered) on epicormic shoot development (E05 plant) in branches placed in the greenhouse for 2 mo

Treatment of the branches	Number of shoots per branch	Shoot length (cm)
Covered and BA	4.0±0.58[a]	1.75±0.31[a]
Covered	4.3±2.03[a]	0.81±0.39[ab]
Uncovered	3.0±1.73[a]	0.69±0.49[ab]
Uncovered and BA	1.0±1.0[a]	0.07±0.07[b]

In each *column*, values (mean±SE) followed by different *letters* are significantly different

Shoot multiplication. Established explants were transferred to test tubes containing the same medium and subcultured every 3 wk until enough stock material was available to perform further experiments. Since *in vitro* plant establishment from the C01 plant was easily achieved and a large number of shoots from this material was obtained, this clone was used to test the effect of three culture media on shoot multiplication: (1) the Anderson medium above described (AND), (2) the same medium containing as major salts the MS macronutrients reduced at half-strength (1/2-MS); and (3) the same medium with the major salts of the FS medium (FS). After 12 wk (four subcultures at 3-wk intervals) of culture, the multiplication rate was evaluated by the number of clusters formed and by the maximum shoot length formed per test tube. The best culture medium was then tested on shoot multiplication of the six selected adult clones (AL01–AL06).

Rooting. Micropropagated shoots (14–20 mm) of the C01 clone growing in the AND medium were transferred to the rooting induction medium (RM), which consisted of KNOP macronutrients (Gautheret 1959), MS micronutrients without potassium iodine, and FS organics without riboflavin. Root induction was assayed in darkness, and three treatments with 3-indolebutyric acid (IBA) were tested and compared with the respective control. In one set of experiments, shoots were induced to root on RM medium containing 9.8 or 24.7 μM IBA for 6 d. In another experiment, shoots were dipped in a solution containing 9.8 mM IBA for 15 s. In both cases, auxin treatment was

Table 2. ANOVA

Source	*df*	SS	MSS	*F* ratio	Sig. level
Effect of plastic covers and BA on epicormic shoot length					
A: covers	1	2.4277	2.4277	6.5748	0.0334*
B: BA	1	0.0709	0.0709	0.1906	0.6728
Interaction: A × B	1	1.8323	1.8323	4.9624	0.0565
Error	8	2.9539	0.3692		
Effect of plastic covers and BA pulverization on the number of shoots/branch					
A: covers	1	14.0833	14.0833	2.2237	0.1742
B: BA	1	4.0833	4.0833	0.6447	0.4452
Interaction: A×B	1	2.0833	2.0833	0.3289	0.5820
Error	8	50.6667	6.3333		

*$P \leq 0.05$

followed by culture on the same RM medium without IBA for shoot and root elongation. The role of charcoal (1.5% *w*/*v*) on root elongation was also evaluated. A total of 240 shoots was tested (ten shoots×three replications×four auxin treatments plus the control×two elongation media (with and without charcoal). Root formation was analyzed after 5 wk of culture on the RM medium. The best conditions for root induction and plant survival were then tested in the clones (AL01–AL03).

Acclimatization. After agar removal with tepid tap water, rooted plantlets were dipped in a fungicide solution (benomil, Benlate 0.6 g l^{-1}). Plantlets were then transferred to containers (60×40 cm), containing a mixture of sand and *Siro 30* (1:1; *v*/*v*) previously sterilized and covered with plastic to maintain a high degree of humidity. *Siro 30* is a commercial substrate of composted pinus bark and peat (70:30%; *v*/*v*) supplemented with *Osmocote* slow release fertilizer (0.6 g/plantlet). The containers were placed on an irrigation sheet in a greenhouse, and the levels of humidity were gradually decreased. After 4 mo, the number of surviving plantlets was evaluated. A similar protocol (100% perlite used as substrate) was tested to acclimatize plantlets of the clones AL01, AL02, and AL03. Following this period, plants were planted in an orchard in the county of Oleiros, Castelo Branco (center of Portugal).

Statistical analysis. All quantitative data expressed as percentages were first submitted to arcsine transformation, and the means corrected for bias before a new conversion of the means and standard error (SE) back into percentages (Zar 1996). Statistical analysis was performed by analysis of variance (ANOVA) (Statistica 6), and the significantly different means ($P \leq 0.05$ or $P \leq 0.01$) were identified by using the Duncan's multiple range test (Duncan 1955).

Table 3. Establishment of shoot apices from epicormic shoots formed in the growth chamber (GC) or in the greenhouse (GH) after 12 wk of culture on the AND medium

Plant	Epicormic shoot formation	Number of inoculated explants	1 wk of culture			Established explants (12 wk)	
			Survival (%)	Necrosis (%)	Contamination (%)	*N*	Percent
E05	GC	29	79.3	0	20.7	20	68.9[a]
	GH	25	48.0	16.0	36.0	10	40.0[b]
AL 01	GC	101	53.5	11.9	34.6	40	39.6[c]
AL 02	GC	114	64.9	11.4	23.7	74	64.9[a]
AL 03	GC	82	76.8	10.9	12.2	44	53.7[ab]
AL 04	GC	41	68.2	7.3	24.3	21	51.2[bc]
AL 05	GC	16	81.2	6.3	12.5	2	12.5[d]
AL 06	GC	22	36.4	0	63.6	3	13.6[d]
Total AL explants	376		184	48.9			

The percentages of contaminated, dead, and surviving explants after 1 wk of culture were also indicated. In each *column*, values followed by different *letters* are significantly different.

Table 4. ANOVA

Source	*df*	SS	MSS	*F* ratio	Sig. level
Effect of origin of epicormic shoots (GC *vs* GH) on culture establishment					
A: Origin of shoots (GC *vs* GH)	1	837.48	837.48	15.067	0.0037*
Error	9	500.27	55.59		
Effect of explant source on culture establishment					
A: Adult Selected Clone (AL)	5	3434.69	686.94	28.422	0.0001*
Error	31	749.24	24.17		

*$P \leq 0.01$

Results

Culture establishment. Woody branches from the E05 plant (Fig. 1*A*) showed epicormic shoot development after 15 d in the growth chamber (Fig. 1*B*). Epicormic shoot development from branches maintained in the greenhouse developed after 2 mo, much slower than those developed in the culture chamber. The number of shoots per branch was not affected by the treatment applied, since the values obtained for the different situations were not significantly different (Tables 1 and 2). The simultaneous use of BA and uncovered branches gave shorter shoots than covered branches treated with BA (Tables 1 and 2). A higher frequency of necrosis and contamination were noted for epicormic shoots grown in the greenhouse than for epicormic shoots grown in the growth chamber. As a consequence, the number of established explants (12 wk) was significantly higher when explants were formed in the growth chamber (Tables 3 and 4). The analysis of explant establishment for the six adult selected trees (AL01–AL06) showed significant statistical differences; the AL02 and AL03 gave the highest percentage (64.9% and 53.7%, respectively) of established shoots after 12 wk of culture (Tables 3 and 4). When shoot apices (Fig. 2*A* and nodal segments (Fig. 2*B*) were compared for explant establishment, the results showed that the levels of contamination were similar (Tables 5 and 6). However, the levels of necrosis were significantly lower in the shoot apices (Tables 5 and 6). Additionally, the number of explants that survived and were established after 12 wk was significantly higher for shoot apices than for nodal segments (Tables 5 and 6).

Shoot multiplication. The effect of three different culture media on the multiplication of the C01 clone (Fig. 3*A*, Tables 7 and 8) showed that the FS medium gave a significantly higher number of clusters per explant (1.99± 0.11) than the other two media (1/2-MS and AND). No significant difference was observed in shoot length between FS and 1/2-MS media, but shorter shoots were noted on AND medium. The FS medium was further used to test the multiplication potential of the six adult clones (AL01–AL06), and the results showed no significant statistical differences among the clones (Tables 9 and 10). After five subcultures at 3-wk intervals, shoots able to be rooted (Fig. 3*B*) were only obtained from the clones AL01, AL02, and AL03. Clones AL04, AL05, and AL06 were unable to produce shoots elongated enough to be used in the rooting assays (Tables 9 and 10).

Rooting. Clone 01 was used for root induction tests (Fig. 4*A*), which showed that shoots were able to root in

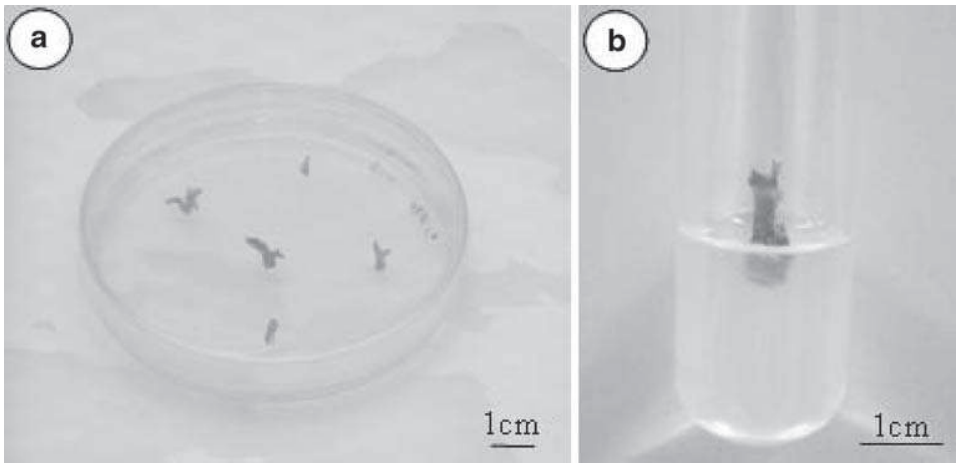

Figure 2. Established shoot apices (*a*) and nodal segments (*b*) of *A. unedo* after 30 and 15 d, respectively, of culture on AND medium.

Table 5. Effect of the type of the explant on survival, contamination, and necrosis

Type of explant	1 wk of culture			Estab. exp. (%) 12 wk
	Survival explants (%)	Necrosed explants (%)	Contaminated explants (%)	
Shoot apices	38.65±9.78[a]	8.44±4.46[a]	52.91±9.89[a]	26.13±8.20[a]
Nodal seg.	4.49±2.63[b]	43.04±11.05[b]	52.47±11.21[a]	2.55±1.78[b]

The results were evaluated after 1 wk (dark conditions) and 12 wk of culture. Explants from the C01 plant and the AND medium were used. In each *column* values (mean±SE) followed by different *letters* are significantly different.

all conditions tested, even when no auxin was used (Tables 11 and 12). Rooted shoots showed neither callus formation at the shoot base nor apical necrosis (Fig. 4*A*). High frequencies of root induction (between 76.7% and 93.3%) were achieved either after a treatment with 24.7 µM IBA for 6 d or by an auxinic pulse (15 s dipping in 9.8 mM IBA) followed by root development on a medium containing (1.5%) charcoal or in a charcoal-free medium (Tables 11 and 12). The ANOVA analysis showed that the only factor affecting root formation was the IBA treatment and that there is no interaction between IBA and charcoal on root formation. In a second set of experiments, shoots (a total of 317) from the clones AL01, AL02, and AL03 were tested for rooting in a medium containing 24.7 µM IBA for 6 d followed by culture in a charcoal containing medium (1.5% *w*/*v*). Shoot rooting varied between 78.1±6.7% for the clone AL02 and 66.9±3.8% for the clone AL01 with the clone AL03 showing intermediate values (72.3±0.7%). However, no statistically significant differences were found between these three AL clones and between them and the C01 clone rooted in the same conditions (Tables 11 and 12).

Acclimatization. Acclimatization of plantlets of the clone C01 (Fig. 4*B*) showed an average of success of 84.7±4.6% after a period of 4 mo in the greenhouse (Fig. 4*C*), and the results were not conditioned by the type of auxin treatment used for root induction. Rooted plantlets never showed signals of callus formation or apical necrosis. Plantlets from clones AL01, AL02, and AL 03 showed the following rates of plant survival: AL01 (98.2% of plants acclimatized), AL02 (85% acclimatization), and AL03 (94.4% acclimatization). Following acclimatization, 229 plantlets were placed in a nursery. Some are now in the field (Fig. 4*D*) to undergo testing in growth, fruit production, and fruit quality.

Discussion

Juvenile explants are often used for micropropagation of woody species due to the difficulties encountered when explants from adult plants are used for cloning (Rathore et al. 2004). However, juvenile plants are usually of unknown genotype, making it difficult to clone adult selected trees (Hackett 1985). This is also the case in *A. unedo* in which previous assays of micropropagation used potted plants growing in the greenhouse (Mereti et al. 2002). Partially successful attempts to propagate *A. unedo* from adult plants were obtained by Mendes (1997). However, the results showed that the multiplication rate was greatly impaired by

Table 6. ANOVA

Source	*df*	SS	MSS	*F* ratio	Sig. level
Type of explant *versus* percentage of surviving explants (1 wk)					
Type of the explant	1	6,317.09	6,317.09	11.6432	0.00228**
Error	24	13,021.34	542.56		
Type of explant versus percentage of necrosis (1 wk)					
Type of the explant	1	5,563.48	5,563.48	6.9495	0.01446*
Error	24	19,213.33	800.56		
Type of explant versus contamination rate (1 wk)					
Type of the explant	1	34.02	34.02	0.02988	0.864206
Error	24	27,321.03	1,138.38		
Type of explant versus percentage of established cultures (12 wk)					
Type of the explant	1	3,757.75	3,757.75	7.7109	0.01047*
Error	24	11,073.39	461.391		

*$P \leq 0.05$, **$P \leq 0.01$

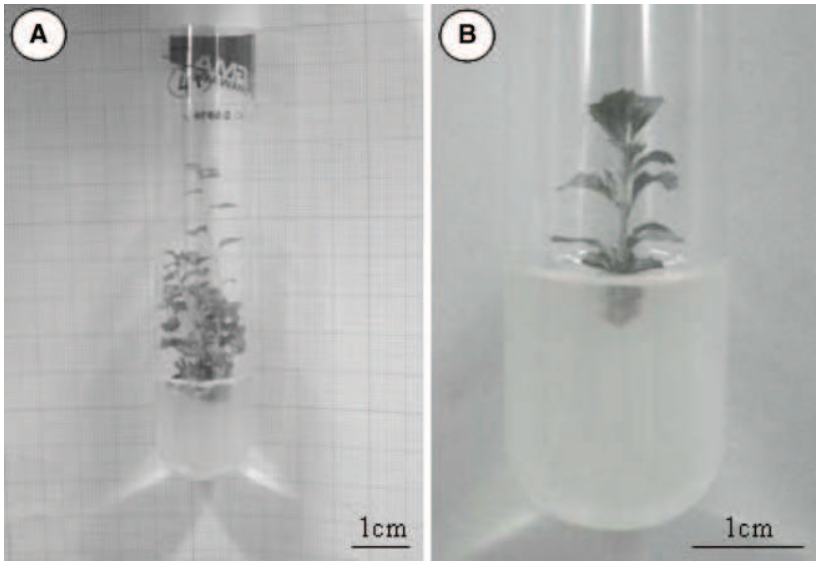

Figure 3. *A. unedo* propagation from shoot apices *A*) Cluster of shoots after 21 d on the FS medium containing 9.0 µM BA *B*) Shoot just before transference to the rooting medium.

the high number of contaminated cultures and the short plant establishment period. Our results showed that epicormic shoots from branches of adult trees are a good source of explants (shoot apices and nodal segments) for the propagation of mature trees (E05 and AL01 to AL06). This is particularly true when the branches were maintained in the growth chamber, probably because the controlled light and temperature were more adequate for shoot growth.

The early work of Murashige (1974) indicated that plant regeneration through axillary shoot proliferation involves several steps and that the success of each step is conditioned by the rate of success in the previous phase. The results obtained with *A. unedo* showed that the levels of explant establishment could reach values over 60%, in particular, when explants from the plants E05 and AL02 were used. However, explants from other plants (AL05 and AL06) displayed considerably lower rates of establishment, and in some cases, the percentages of explant establishment after 12 wk of culture were lower than 15%. Contamination of the explants contributed to the reduced number of established plants from AL06 explants, and senescence in culture contributed to the reduced number of established plants from AL05 explants. It does not seem plausible that the differences between explant establishment in *A. unedo* could be attributed to the genotype. Instead, it seems more reasonable to improve the conditions of disinfection and culture of the explants in the cases in which the rate of success is too low.

The data obtained indicated that shoot apices are more effective for explant establishment than nodal segments. Similar results have been found in other woody species studied in our lab, such as carob (Panteleitchouk 2002), *Eucalyptus nitens* (Gomes and Canhoto 2003), and *Leucadendron laureolum*×*Leucadendron salignum* cv. Safari Sunset (Ferreira et al. 2003). Since the levels of contamination were similar in the two explant types, the better performances of shoot apices are due to the lower rates of tissue senescence. The reason for this behavior is not clear; it is possible that nodal segments exude a higher number of phenolic compounds, which inhibited plant growth upon oxidation. At the time of culture, the shoot apical meristem is not a dormant structure and is surrounded by several leaf primordial. The leaf primordia produce plant growth regulators (PGRs), which promote cell division and

Table 7. Effect of culture media on the multiplication rate and shoot length (cm) after four subcultures, at 3-wk intervals, in the same culture medium

Culture Media	No of clusters	Maximum shoot length (cm)
FS	1.99±0.11[a]	3.30±0.11[a]
1/2-MS	1.47±0.10[b]	3.00±0.11[a]
AND	1.34±0.07[b]	2.45±0.10[b]

Explants (shoot apices) of the clone C01 were used. In each *column*, values (mean±SE) followed by different *letters* are significantly different.

Table 8. ANOVA

Source	*df*	SS	MSS	*F* ratio	Sig. level
Effect of culture media in the number of clusters formed					
Culture media	1	21.9865	10.9933	10.0015	0.000067*
Error	247	271.4932	1.0992		
Effect of culture media on shoot length					
Culture media	1	31.628	15.814	14.523	0.00001**
Error	247	268.964	1.089		

*$P \leq 0.01$

Table 9. Multiplication rates of six adult selected clones (AL01–AL06) during the first five subcultures, at 3-wk intervals

Subculture	Clone											
	AL 01		AL 02		AL 03		AL 04		AL 05		AL 06	
	Number of clusters	Mult. rate	Number of clusters	Mult. rate	Number of clusters	Mult. rate	Number of clusters	Mult. rate	Number of clusters	Mult. rate	Number of clusters	Mult. rate
1st	40		74		44		21		2		3	
		1.0		1.1		1.1		1.2		1.0		1.0
2nd	40		79		50		26		2		3	
		1.3		1.3		1.4		1.0		0.5		1.0
3rd	50		102		69		27		1		3	
		1.2		1.3		2.2		1.5		1.0		1.0
4th	59		128		152		40		1		3	
		2.4		1.2		1.1		0.9		2.0		1.0
5th	140		153		174		35		2		3	
Mult rate	1.45±0.31[a]		1.20±0.05 [a]		1.47±0.25 [a]		1.16±0.13 [a]		1.13±0.31 [a]		1.0±0.0 [a]	
Shoots[z]	40		38		11		0		0		0	

Values (mean±SE) followed by the same *letter* are not significantly different. FS medium was used both for shoot multiplication and shoot elongation. The number of clusters was evaluated at the end of each subculture

[z] Total number of shoots able to be rooted after the fifth subculture

elongation and retard senescence. These characteristics may explain the better performance of this type of explant. However, other factors may also be involved, since in some species, nodal segments are very effective for plant micropropagation (Gonzalez et al. 2000; Ibañez et al. 2005). This study suggests that further experiments on *A. unedo* micropropagation should preferentially use shoot apices as explants.

The best results for shoot multiplication were obtained with FS medium, especially when the number of clusters formed by the clone C01 was evaluated. The three basal media used in our experiments differed only in the composition of the macronutrients, and the results obtained must necessarily reflect these differences. The AND medium has reduced levels of macronutrients that might explain the reduced rates of shoot multiplication. FS and MS media have similar compositions with FS medium possessing reduced levels of nitrogen both in the form of NH_4^+ and NO_3^-. The amount and type of nitrogen can influence *in vitro* morphogenic processes (Young and Cameraon 1985; Dal Vesco and Guerra 2001), but in the case of *A. unedo*, more assays are necessary to evaluate the role of nitrogen on *in vitro* propagation. Another possibility is that the higher levels of sodium present in the FS medium can have a role on shoot proliferation. Sodium is a trace element important for some plants (Salisbury and Ross 1992), but its role on the micropropagation process has not been evaluated. Finally, we can speculate that these variations in the mineral composition can interfere with membrane transport and affect the pH of plant cells, thus influencing the *in vitro* response (Niedz 1994). However, it should be noted that experiments performed with *A. unedo* and other *Ericaceae* indicate that different culture media, other cytokinins, and sucrose were also able to promote shoot proliferation. Thus, Mereti et al. (2002) showed that woody plant medium (Lloyd and McCown 1980) combined with BA gave the best rates of shoot proliferation, whereas Mendes (1997) pointed out that AND and the cytokinin 2-iP (6-(-dimethylallylamino) purine) were particularly effective to propagate adult material. In *Vaccinium corymbosum*, a member of the strawberry tree family, high sucrose levels in the propagation medium increased shoot proliferation (Cao et al. 2003). Some of these factors such as sucrose and other cytokinins are now being tested with *A. unedo* in order to increase the success of the micropropagation process.

Our data suggests that shoot multiplication might be influenced by the genotype of the explants. Although we could not find statistical differences in the multiplication rate of the clones, the data showed that some (AL04, AL05, and AL06) were difficult to propagate, since they formed shoots that were unable to elongate. On the other hand, shoots from clones AL01, AL02, and AL03 displayed a normal growth, which makes them more interesting both for shoot multiplication and rooting. The role of the

Table 10. ANOVA: Effect of the type of plant on multiplication rate

Source	*df*	SS	MSS	*F* ratio	Sig. level
Clone AL	5	0.6967	0.1393	0.7484	0.5978
Error	18	3.3512	0.1862		

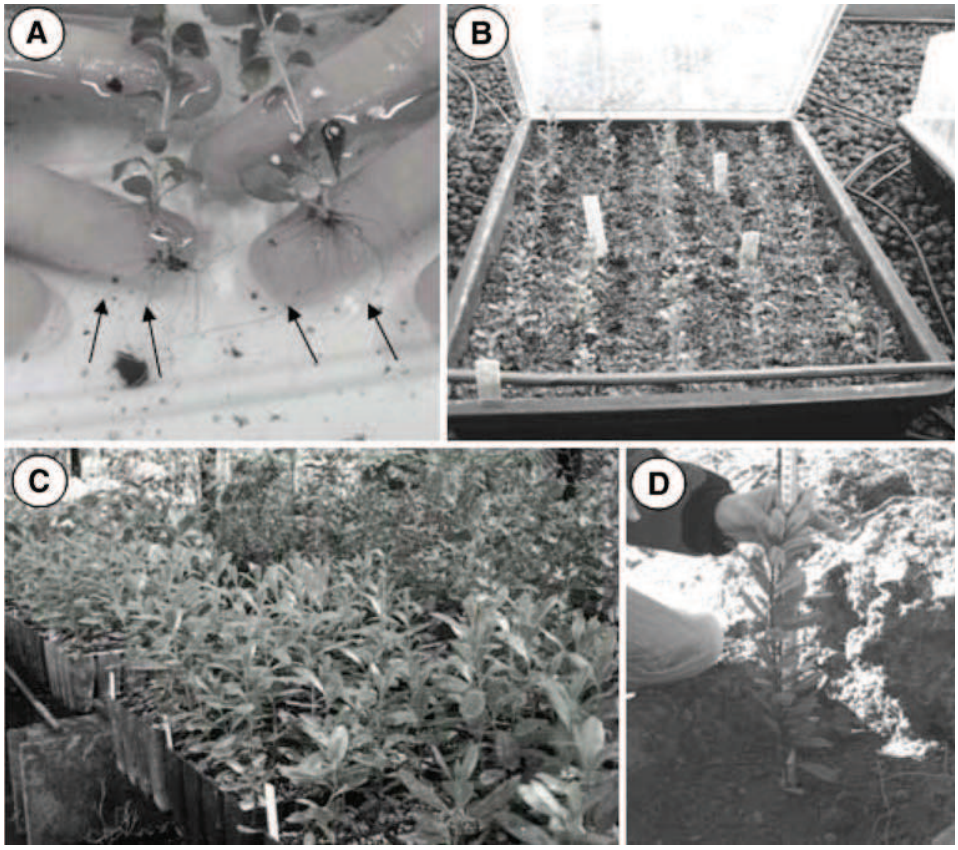

Figure 4. Rooting, acclimatization and field-growth of *A. unedo* micropropagated plants. *A*) Two rooted plants showing a well-developed root system (*arrows*). *B*) Rooted plants in the early stages of acclimatization. *C*) 4-mo old plants propagated *in vitro*. *D*) Plant growing in an orchard.

genotype in several morphogenic processes occurring *in vitro* is well established (Gajdošova et al. 2007) and may be related with the levels of endogenous growth regulators in the explants of different genotypes. In our case, it is possible that the slow-growing shoots possess unbalanced levels of endogenous cytokinins and/or auxins, which, in the conditions tested, were inhibitory for shoot growth. If this is the case, the manipulation of auxin and cytokinin concentration or the inclusion of other growth regulators, such as gibberellins, may result in a more effective growth of these shoots, thus promoting the rates of multiplication (Hansen et al. 1999).

Our data showed that shoots of *A. unedo* have a high rooting ability, since shoots rooted even in media without auxins. However, an auxin treatment for 6 d with IBA or a pulse of 15 s at high IBA concentrations considerably increased the rates of root formation over the control. In all plantlets obtained, callus formation at the shoot base or apical necrosis was never observed. Similar frequencies of induction were obtained by Mereti et al. (2002). The same authors also showed that IBA is more effective than indole-3-acetic acid on root formation. An auxinic shock proved to

Table 11. Effect of several treatments on root formation after 5 wk in the elongation medium

IBA (μM)	Auxin treatment	Elongation medium	Rooting (%)
–	–	Charcoal	40.00 ± 20.0^{bc}
–	–	–	53.33 ± 3.3^{abc}
9.8	6 d	Charcoal	30.00 ± 15.3^{c}
9.8	6 d	–	43.33 ± 28.5^{abc}
24.7	6 d	Charcoal	93.33 ± 6.7^{a}
24.7	6 d	–	83.33 ± 16.7^{ab}
9.8×103	Pulse (15 s)	Charcoal	93.33 ± 3.3^{ab}
9.8×103	Pulse (15 s)	–	76.67 ± 3.3^{abc}

Shoots of the clone C01 and FS medium were used. In each *column* values (mean±SE) followed by different *letters* are significantly different.

Table 12. ANOVA: effect of auxin treatment (IBA) and charcoal on shoot rooting

Source	*df*	SS	MSS	*F* ratio	Sig. level
A: auxin (IBA)	3	6,852.1	2,284.05	5.5027	0.00861*
B: Charcoal (elongation medium)	1	0.83	0.83	0.0020	0.96482
Interaction: A× B	3	817.86	272.62	0.6568	0.59040
Error	16	6,641.21	415.08		

*$P \leq 0.01$

be effective in the assays carried out by Mackay (1996) in *A. xalapensis* and by Mendes (1997) in *A. unedo*. In the case of *A. xalapensis*, charcoal inhibited root formation probably because this compound is also able to adsorb PGRs such as auxins (Kohlenbach and Wernicke 1978), thus reducing the optimal levels of this PGR necessary for root induction. We were also unable to find a positive role for charcoal on root formation. Root induction and elongation are complex processes that are influenced by a large number of factors, such as genotype, type and concentration of PGRs, and culture conditions (Bennett et al. 1994; Mylona and Dolan 2002). In this way, it is not surprising that the conditions to achieve root formation are widely variable between different species and in the same species or cultivar.

The success of any process of plant cloning can be evaluated by the number of regenerated plants that can survive in field conditions, following acclimatization and hardening (Ziv 1986). Our results with *A. unedo* showed that plant survival rates after 4 mo in the greenhouse ranged from 84% to 98% according to the different clones. Assays of acclimatization carried out by other authors working with *A. unedo* showed also that plantlets obtained *in vitro* are easily acclimatized (Mereti et al. 2002), and the same is true for other species of the same family such as *V. corymbosum* and hybrids of *Vaccinium angustifolium* × *V. corymbosum* (Isutsa et al. 1994).

Taken together, our results show that the procedures adopted in this work are the basis of a reliable and reproducible protocol to the cloning of selected adult trees of *A. unedo*. However, the method's success is lessened by the high number of contaminations in some clones and the slow growth of shoots during the multiplication phase. Therefore, attempts to reduce these factors through the refinement of the technique are being carried out. Alternative ways of micropropagation through the formation and conversion of somatic embryos and organogenesis are also being carried out with very promising results (Canhoto et al. 2007).

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