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Effect of plant growth regulators and genotype on the micropropagation of adult trees of *Arbutus unedo* L. (strawberry tree)

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Arbutus unedo grows spontaneously around the Mediterranean basin. The species is tolerant to drought and has a strong regeneration capacity following fires making it interesting for Mediterranean forestation programs. Considering the sparse information about the potential of this fruit tree to be propagated *in vitro*, a project to clone selected trees based on their fruit production was initiated a few years ago. The role of several factors on *A. unedo* propagation was evaluated. The results showed that 8.9 μ M kinetin gave the best results although not significantly different from those obtained with benzyladenine or zeatin. The inclusion of thidiazuron or 1-naphthaleneacetic acid promoted callus growth and had deleterious effects on the multiplication rate. The genotype of the donor plants is also a factor interfering with the multiplication. The results also indicated that the conditions used for multiplication influenced the behavior of shoots during the rooting phase.

Introduction

The genus Arbutus (Ericaceae) includes about 20 species from which Arbutus unedo, commonly known as strawberry tree, is the most interesting from an economic point of view. According to Piotto et al. [1] strawberry tree seems to be native to Ireland, southern Europe and the western Mediterranean region growing spontaneously as a bush or small tree in several countries around the Mediterranean basin in rocky and well-drained soils. This species can withstand low temperatures and is drought tolerant [1,2]. Moreover, A unedo easily regenerates after forest fires, a characteristic which is particularly important for forestation programs in southern European countries such as Greece, Italy, Portugal and Spain where fires are common during the dry season. As a species characteristic of Mediterranean ecosystems strawberry tree contributes to maintain the biodiversity and helps to stabilize soils, avoiding erosion [3,4]. From an economic point of view, strawberry tree can be considered a neglected or underutilized crop (NUC) and has been included in the list of NUCs by the Global Facilitation Unit for Underutilized Species [5] because it is used in

small scale in particular areas of Mediterranean countries. In Portugal, it grows in most of the country [2] often associated with other trees such as cork-oaks and maritime-pines but it is in the Algarve region and in the Center that the fruits are most popular to make a spirit called 'medronheira' which is the main income for small farmers. The fruits are usually picked up by local populations from spontaneously growing trees (culture in orchards is unusual) which are then abandoned in the field until the new period of fruit collection (late fall to early winter). The mature red berries can be eaten fresh or used to make jams. Owing to an expanded belief that the fruits are reach in ethanol, they are consumed only in small amounts (unedo means 'eat only one') and therefore are seldom found in supermarkets. The bark has been used in tanning and the plant has been used in folk medicine [6]. The small white flowers take a year to ripen and, during several months, both flowers and fruits are present making the tree an attractive ornamental. Considering the increasing importance that alternative crops are assuming in the agricultural policy of the European Union, a project to select, characterize and clone selected adult trees based on their fruit production and quality was initiated by our group a few years ago.

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The number of papers dealing with the *in vitro* cloning of A. unedo is scarce and the same is true for other members of the Ericaceae family. Members of this family that have been successfully micropropagated include Arbutus xalapensis [7], Kalmia latifolia [8], Oxydendrum arboreum [9], Rhododendron [10,11] and several species of the genus Vaccinium [12-14]. Previous studies on micropropagation of strawberry tree, from juvenile material, have been reported by Gonçalves and Roseiro [15] and Mereti et al. [16,17]. More recently, we have established a protocol for the establishment and micropropagation of adult trees through axillary shoot proliferation [18]. In this work it was reported that shoot apices from epicormic shoots showed higher rates of in vitro establishment when compared with nodal segments. From the three basal medium used in combination with 9.0 µM BA, the FS medium [19] gave the highest rates of multiplication and the inclusion of an auxin significantly increased root formation. Preliminary results about the induction and regeneration of adult plants by somatic embryogenesis in A. unedo have been also published [20]. It is well known that several factors can affect in vitro micropropagation [21]. Among these parameters are the genotype of the donor plants [21,22] and the plant growth regulators (PGRs) included in the culture media [23,24]. According to this we decided to analyze the effect of different cytokinins and one auxin on the propagation of A. unedo to find a suitable combination that can assure high rates of propagation. Several previously selected genotypes were also tested to find those which are more amenable for in vitro cloning. In long-term breeding programs these genotypes can be used to transfer their regeneration potential to more recalcitrant genotypes.

Materials and methods

Establishment and propagation

The conditions for plant establishment have been described before [18]. Briefly, branches (30-40 cm length) of adult selected plants were collected in the field, disinfected with a fungicide (dipped in dichlofluanid, Euparene, 120 mg l^{-1} for 10 min) and kept in a culture chamber covered with plastic bags to maintain a high humidity environment that stimulate epicormic shoot development. Shoot apices and nodal segments from these epicormic shoots were further used to establish in vitro cultures through its culture on a medium (AND) consisting of Anderson major salts [11], Murashige and Skoog (MS) micronutrients [25] and organic compounds of the FS medium [19]. Sucrose 0.087 ${\rm M}$ and 9.0 ${\rm \mu M}$ BA were added. The pH was adjusted to 5.7 before agar addition (7 g l^{-1}) and the media were then autoclaved (121°C, 20 min), being these procedures similar for all prepared culture media. Also, all the genotypes used were established in this way. To propagate the established material, three culture media were tested on shoot multiplication: (1) the Anderson medium above described (AND); (2) the same medium, but with major salts of the MS, reduced at half-strength (1/2 MS) and (3) the same medium with the major salts of the FS medium (FS). After 12 weeks (four subcultures at 3-week intervals) of culture the multiplication rate was evaluated by the number of clusters formed and by the maximum shoot length formed per test tube. For more details see Gomes and Canhoto [18].

Effect of plant growth regulators

Shoots (12–18 mm height) of the established explants were used to test the role of PGRs on shoot multiplication. Cultures were placed

in a culture chamber (16 h photoperiod, 40 μ mol m⁻² s⁻¹) and test tubes (Sigma, $25 \text{ mm} \times 150 \text{ mm}$) containing 12 ml of culture medium and covered with plastic caps were used. To test the PGRs the FS medium described in the previous section was used and prepared following the same procedure. The effect of four cytokinins and one auxin (1-naphthalene acetic acid, NAA) on shoot multiplication was evaluated, during four subcultures at 4-week intervals. The experiments were carried out with the genotype AL1 derived from an adult selected tree [18] and the following assays were performed: (1) different BA concentrations were tested (0; 2.2; 4.4; 8.9; 17.8 µM); (2) the cytokinins kinetin (KIN), zeatin (Zt), thidiazuron (TDZ) and N^6 -(2-isopentenyl) adenine (2i-P) were tested and compared with BA at the same concentration (8.9 µM); (3) NAA at different concentrations (0; 0.54; 1.34; 2.69 μM), was tested in combination with BA (8.9 μM). The multiplication rate was evaluated by the following variables assessed per test tube: (1) maximum shoot length (SL), (2) number of shoots formed (SF) and (3) the number of shoots formed per test tube for further multiplication (SNX). Other parameters assessed were callus formation, necrosis and axillary shoot proliferation (referred

Effect of the genotype

To evaluate the role of the genotype on the multiplication rate, ten selected adult genotypes were tested. FS was used as basal medium containing 9.0 μ M of BA and 0.087 M sucrose. All the genotypes were from adult trees selected according to their fruit production and quality. These ten genotypes were obtained from three provenances from center region of Portugal: Oleiros (AL2; AL3; AL4; AL6 and AL7), Alva (IM1; IM2; IM4 and IM6) and Piódão (JF3). The multiplication rate was evaluated by the same variables described before and calculated per test tube.

as callus, necrosis and proliferation in the results).

Rooting and acclimatization

Shoots were rooted on a rooting induction medium containing Knop macronutrients [26], MS micronutrients without potassium iodide and FS organics without riboflavin. The auxin 3-indolebutyric acid (IBA: 14.8; 19.7 or 24.6 µм) was added to promote root differentiation. On a first assay the rooting potential of shoots formed on media containing different concentrations of BA was tested. In this case, IBA at the concentration of 24.6 $\mu \textsc{m}$ was used. On a second set of experiments, shoots formed on media with different cytokinins were rooted in the presence of 14.8 µM IBA. Finally, on a third set of experiments shoots formed on media containing combinations of NAA and BA, or shoots from the different genotypes analyzed were rooted on a medium containing 19.7 µM IBA. In general, root induction (RI) was carried out in dark for seven days. However, other periods (five to ten days) of root induction were also tested (RI-days). Following root induction shoots were transferred to a root development medium (RD) identical to the rooting medium but without IBA and with 1.5% charcoal. Nevertheless, other periods (22-48 days) of root development (RD-days) were also tested. After four to five weeks on this medium, rooted plantlets were transferred to containers (covered) and placed into the greenhouse, as described elsewhere [18]. Rooting ability was evaluated using the following parameters: percentage of rooted shoots, number of roots formed per shoot (NR), length of the longest root (LLR), length of the shortest root

TABLE 1

Effect of different combinations of BA (experiment I), cytokinins (experiment II), combinations of NAA and BA (experiment III) and of the genotype (experiment IV) on the multiplication of A. unedo

Experiment	Variable	Shoot length (SL mm)	SNX
Experiment I BA (µм)	0 µм	$20.11\pm7.93^{\rm a}$	1.43 ± 0.51^{bc}
	2.2 μм	$20.45 \pm 8.28^{\mathrm{a}}$	$1.49\pm0.55^{\rm b}$
	4.4 μм	$16.07\pm 6.39^{ m b}$	1.28 ± 0.47^{c}
	8.9 µм	$19.42\pm7.46^{\rm a}$	$1.75\pm0.75^{\text{a}}$
<i>N</i> = 843	17.8 µм	$14.96\pm7.15^{\rm b}$	$1.33\pm0.69^{\rm c}$
Experiment II cytokinins (8.9 µм)	2-iP	$23.21\pm7.77^{\text{b}}$	$1.20\pm0.41^{\text{b}}$
	BA	$21.44 \pm \mathbf{5.80^{c}}$	$1.29\pm0.45^{\rm a}$
	KIN	$25.38 \pm \mathbf{6.85^a}$	$1.31\pm0.48^{\rm a}$
	TDZ	12.74 ± 4.26^{d}	$1.01\pm0.17^{\rm c}$
<i>N</i> = 945	Zt	24.89 ± 6.98^{a}	$1.28\pm0.46^{\text{ab}}$
Experiment III NAA + BA 8.9 μм	0 µм	$25.31\pm12.0^{\rm a}$	$2.27\pm1.38^{\rm a}$
	0.54 µм	$19.50\pm7.45^{\rm b}$	1.80 ± 1.22^{b}
	1.34 µм	15.91 ± 7.44^{c}	$1.48\pm0.74^{\rm c}$
<i>N</i> = 1283	2.69 μм	$17.34\pm7.51^{\circ}$	1.43 ± 0.85^{c}
Experiment IV genotypes	AL2	16.00 ± 7.21^{a}	$1.71\pm0.90^{\rm ab}$
	AL3	$15.72\pm6.71^{\mathrm{a}}$	$1.48\pm0.67^{\rm b}$
	AL4	$12.64\pm4.52^{\mathrm{b}}$	$1.48\pm0.83^{\text{b}}$
	AL6	11.65 ± 5.93^{bc}	1.57 ± 0.99^{ab}
	AL7	$12.70\pm 6.48^{ ext{b}}$	$1.90\pm0.73^{\text{a}}$
	IM1	8.92 ± 5.65^{c}	0.93 ± 0.51^{d}
	IM2	$12.88\pm4.41^{\rm b}$	1.71 ± 0.98^{ab}
	IM4	11.34 ± 3.82^{bc}	$1.36\pm0.65^{ m bc}$
	IM6	$11.28\pm4.35^{ t bc}$	1.34 ± 0.63^{bc}
<i>N</i> = 560	JF3	$9.85\pm5.78^{ m bc}$	1.00 ± 0.53^{cd}

Each value is the mean \pm SD. Multiplication was evaluated by the maximum shoot length and by the number of shoots formed per test tube for further multiplication (SNX). In each treatment, values followed by the same letter are not significantly different, P > 0.05).

(LSR) and final shoot length (SL). When apical shoot necrosis or callus formation at the cut end of the shoot was observed they were also registered.

Experimental design and statistics

The multiplication experiments started with 30 shoots per treatment comprising at least three replicas of 10-20 explants. Variables were recorded after each one of the four subcultures at fourweek intervals. For rooting experiments 30 shoots per treatment were also tested. The rooting parameters were recorded after five weeks on medium without auxin and containing charcoal during acclimatization procedures and transference of the in vitro plants to the greenhouse as described by Gomes and Canhoto [18]. For statistics analysis (STATISTICA 6) complementary approaches were tested: an ANOVA was performed, and means were compared using the Duncan test (P < 0.05) followed by a multiple linear regression and a principal components analysis (PCA). The 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 [27].

For the multiple linear regression analysis the effect of the different variables on the multiplication rate (effect of PGRs and genotype) was evaluated considering the number of shoots formed per test tube for further multiplication (SNX) as variable dependent. The results are displayed when multiple R^2 coefficient was higher than 0.67 (R = 0.81). For the PCA all variables were analyzed to achieve a better understanding of the interactions between them and its significance level for the total variance. The results are presented when the percentage of total

variance explained by the two factors of PCA analysis was higher than 49%.

Results

Effect of PGRs

When different BA concentrations were tested best results were achieved with 8.9 μ M (Table 1). When this concentration of BA was used the number of shoots formed per test tube for further multiplication (SNX) was significantly higher than in the other concentrations tested (P < 0.05). The multiple regression analysis (Table 2a) indicated that the dependent variable SNX increased with the concomitant increase of the variables SL, SF and BA (P < 0.01). However, SL and SF showed a higher coefficient (0.658 and 0.451, respectively) than BA (0.098). The multiple regression accounts for 68.9% ($R^2 = 0.689$) of the variable necrosis and shoot proliferation gave non-statistically different (P > 0.05) results. Callus formation was never observed during these assays.

PCA analysis (Fig. 1) shows that the factor 1 accounts for 42% of the total variance showing as significant variables SNX, SL-lenght, SF-formed and shoot proliferation and displaying factor loadings higher than 0.70. This type of analysis also indicated that the variable necrosis affected negatively the culture propagation group (SNX; SL; SF). As it would be expected the variables SF-formed and shoot proliferation are closely related. Factor 2 contributes with 17.5% for the total variance being almost dependent of the variable subculture. Finally, it must be signaled that the multiplication, evaluated by SNX, was not depending on the number of subcultures showing a factor loading of -0.045.

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TABLE 2

Multiple linear regression analysis for shoot multiplication assessed by the dependent variable SNX and the following independent variables: number of subcultures, necrosis, proliferation, number of shoots formed (SF), maximum shoot length (SL) per test tube and callus formation

(a) Concentration of BA (μ M)

Regression	summary	for	dependent	variable:	SNX	(BA	Х	AL1.sta)	R = 0.83002735,	$R^2 = 0.68894540,$	adjusted
$R^2 = 0.68671$	295, F(6,83	36) =	308.61, P < 0).0000, se d	of esti	imate	: 0.	38755			

<i>N</i> = 843	Beta	SE of beta	В	se of B	<i>t</i> (836)	P-level
Intercept			0.535401	0.321380	1.66594	0.096099
BA	0.098694	0.020224	0.049210	0.010084	4.88011	0.000001
Subculture	-0.057959	0.021308	-0.036853	0.013549	-2.71998	0.006664
Necrosis	0.023398	0.023701	0.078847	0.079870	0.98720	0.323832
Proliferation	-0.054316	0.033067	-0.045589	0.027754	-1.64260	0.100842
SF-formed	0.451097	0.032544	0.574731	0.041464	13.86112	0.000000
SL-length	0.658314	0.025438	0.053329	0.002061	25.87933	0.000000

(b) Effect of NAA addition

Regression summary for dependent variable: SNX (NAA X AL1.sta), R = 0.81858498, $R^2 = 0.67008138$, adjusted $R^2 = 0.66827006$, F(7,1275) = 369.94, P < 0.0000, sE of estimate: 0.70917

<i>N</i> = 843	Beta	se of beta	В	se of B	<i>t</i> (1275)	P-level
Intercept			-18.0241	1.833841	-9.82860	0.000000
NAA	0.103040	0.018579	0.1000	0.018037	5.54603	0.000000
Subculture	0.234935	0.020942	0.2263	0.020173	11.21828	0.000000
Necrosis	0.053691	0.016623	0.5771	0.178665	3.22992	0.001270
Proliferation	0.179976	0.017694	0.2408	0.023670	10.17154	0.000000
SF-formed	0.532377	0.018045	0.6825	0.023133	29.50251	0.000000
SL-length	0.468784	0.017620	0.0547	0.002056	26.60552	0.000000
Callus	0.032754	0.021271	0.0845	0.054862	1.53985	0.123846

(c) Effect of the genotype

Regression summary for dependent variable: SNX (genotype X.sta), R = 0.81891367, $R^2 = 0.67061961$, adjusted $R^2 = 0.66644268$, F(7,552) = 160.55, P < 0.0000, sE of estimate: 0.45108

<i>N</i> = 843	Beta	se of beta	В	se of B	<i>t</i> (552)	P-level
Intercept			1.151140	0.836645	1.37590	0.169411
Genotype	-0.097547	0.029674	-0.026269	0.007991	-3.28732	0.001076
Subculture	0.086869	0.030444	0.081721	0.028640	2.85338	0.004488
Necrosis	0.166336	0.030580	0.696983	0.128138	5.43931	0.000000
Proliferation	0.371883	0.027196	0.468467	0.034260	13.67405	0.000000
SF-formed	0.323954	0.029216	0.662036	0.059706	11.08832	0.000000
SL-length	0.601141	0.028436	0.072102	0.003411	21.13996	0.000000
Callus	-0.017154	0.033793	-0.031296	0.061650	-0.50763	0.611912

When cytokinins were tested and compared with BA at the same concentration, KIN was shown to be most effective (Table 1, experiment II). In the presence of KIN shoot length reached an average of 25.38 ± 6.85 mm. However, this value was not significantly different from the results obtained with Zt (average shoot length of 24.89 ± 6.98 mm). Furthermore, the multiplication rate evaluated by the number of shoots formed per test tube for further multiplication (SNX) was not significantly different on the media containing KIN, BA or Zt (Table 1, experiment II). Both Zt and KIN promoted shoot elongation, but BA also induced shoot proliferation (Fig. 2a-c). When TDZ was used, callus formation was often observed (Fig. 2d and e) whereas shoot growth was inhibited $(12.74 \pm 4.26 \text{ mm})$. Moreover, in the presence of TDZ, callus formation increased with the number of subcultures while and shoot growth was consistently impaired. For this set of experiments multiple regression analysis is not shown because a low coefficient $(0.46 - \text{the multiple } R^2)$ was obtained. PCA analysis (Fig. 3) shows that in this case the multiplication (SNX; factor 1) is more correlated with shoot elongation (SL) than with shoot formed (SF). Axilary shoot proliferation and subsquently shoot development was only observed when BA was tested (Fig. 2c). This feature is confirmed by the low factor loading associated with the variable SF (0.31; Fig. 3). The data also indicate that multiplication (SNX) is also positively affected by cytotokinins whereas callus formation, induced by TDZ, has a negative effect. Then, factor 1 of the PCA analysis shows that cytokinins promoted (factor loadings 0.60 and 0.72) multiplication (SNX) while inhibiting callus formation (-0.66).

When the auxin NAA was included on the culture media, the results showed that NAA was unable to improve the multiplication rate (Table 1, experiment III). Best results (P < 0.05) were achieved on media without NAA (25.32 ± 12.44 mm SL; 2.27 ± 1.38 SNX). Moreover, NAA induced callus formation, and unorganized growth increased in the follow-up subcultures. When NAA was tested at concentrations higher than $1.34 \,\mu$ M, callus formation completely inhibited shoot growth and multiplication.



FIGURE 1

Evaluation of the effect of different BA concentrations on shoot multiplication by PCA analysis considering the following variables: BA concentration, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL, and SNX. (The variables are associated with each factor 1 and 2, according to the coefficients 'factor loadings', presented on the left table. The highest factor loadings are marked whether they are correlated or are relevant to explain the total variance expressed by the factors 1 and 2).

The multiple regression analysis performed for this assay (Table 2b) contributes to 67% ($R^2 = 0.67$) of the variance of the dependent variable (SNX). The values obtained for the variable callus showed to be not significant (P > 0.05) while SL-length or SF-formed exhibited the highest coefficients (0.46 and 0.53, respectively). PCA analysis (Fig. 4) points out to a positive interaction between the variables multiplication (SNX), SL-length, SF-formed and proliferation (with high factor loadings, Fig. 4, factor 1). It also confirms that callus formation increases with the increasing number of subcultures (Fig. 4, factor 2).

Effect of the genotype

When selected adult clones were tested (Table 1, experiment IV), ANOVA analysis showed that the genotype significantly affects the

multiplication rate (P < 0.05) both in terms of shoot length (SL) and in terms of number of shoots (SNX) with some genotypes sharply giving better results than others. The multiple regression analysis of SNX (Table 2c, $R^2 = 0.67$) showed significant differences (P < 0.01) for all variables analyzed with the only exception being callus formation. PCA analysis for multiplication assessment showed a positive interaction between the variables multiplication (SNX), SL-length, SF-formed and proliferation (Fig. 5, factor 1). The results also demonstrated that the genotype has an important factor loadings in terms of both multiplication (variables, which are pointed out by factor 1 SNX, SL, SF and proliferation) and callus formation (factor 2). Once again, PCA analysis indicates that when genotypes display callus formation they have a tendency to increase this feature in the next subcultures (Fig. 5).



FIGURE 2

Micropropagation of A. unedo on different culture conditions. (a) Kinetin, (b) zeatin, (c) BA and (d) and (e) TDZ. When TDZ was used callus was often formed (d) and shoot growth inhibited (e). Marks on tubes indicate the initial shoot length.



FIGURE 3

Evaluation of the effect of different cytokinins (8.9 µM) on shoot multiplication by PCA analysis considering the following variables: type cytokinin, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL and SNX. For more details see Fig. 1.

Rooting and acclimatization

When roots formed on media containing different concentrations of BA were induced to root on a IBA-containing medium for seven days followed by transference to an auxin-free medium, it was found that best frequencies of root formation were obtained with shoots formed on a medium containing 8.9 μ M but the results were not significantly different from those obtained with shoots grown in other BA concentrations (Table 3). At this concentration (8.9 μ M) shoot necrosis was never detected. When the parameter evaluated was the number of roots per shoot the results showed significant differences among the treatments with shoots formed on a medium containing BA 17.8 μ M giving the best results (Table 3). However, as indicated before, this medium gave poor results in terms of shoots for further multiplication (SNX) than the medium with 8.9 μ M BA (see Table 1). The results of the PCA analysis (Fig. 6) could explain 65.75% of total variance observed and indicated that the number of roots (NR) is related to the LLR, displaying a factor loading higher than 0.70. The data also clearly show that shoot length is strongly affected by BA concentration even after rooting had occurred. In fact, when BA was used at the



FIGURE 4

Evaluation of the effect of different NAA concentration on shoot multiplication by PCA analysis considering the following variables: NAA concentration, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL and SNX. For more details see Fig. 1.

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FIGURE 5

Analysis of the effect of different genotype on shoot multiplication by PCA analysis considering the following variables: genotypes, number of subcultures, necrosis, proliferation, callus formation, number of shoots formed/SF, maximum shoot length per test tube/SL and SNX. For more details see Fig. 1.

highest concentration, shoot length attained its lowest value after acclimatization (Table 3). On these conditions, apical shoot necrosis was often observed (8.7%; P > 0.05) as well as callus formation at the basis of the shoots. Considering these results it was decided to reduce the level of IBA in the next rooting assays. Thus, when shoots originated from media containing different cytokinins were rooted the concentration of IBA used was reduced to 14.8 μ M instead of the 24.6 μ M previously tested. This IBA drastic

reduction (maintaining the same induction period of seven days) is probably responsible for the low root rate observed (average of $2.00 \pm 4.14\%$). Therefore, on the following assays IBA 19.7 µM was used. Rooting of shoots formed on a medium containing NAA (plus BA 8.9 µM) showed no significant differences on the frequencies of rooting and on the number of roots per shoot (Table 3) with averages of 94.61% and 7.31, respectively. However, shoot length, measured after root induction and development (five weeks), was

TABLE 3

Rooting of shoots formed in different culture conditions								
Conditions of shoot form	nation	Rooting (%)	Number of roots	Shoot length (mm)				
ВА (μм)	ВА 0 μм	$56.7\pm40.4^{\rm a}$	$6.6\pm4.0^{\mathrm{ab}}$	$51.65\pm14.42^{\rm a}$				
	ВА 2.2 μм	$53.3\pm23.1^{\rm a}$	6.9 ± 2.5^{ab}	$47.07\pm12.10^{\rm ab}$				
	ВА 4.4 μм	$53.3\pm37.9^{\rm a}$	$5.0\pm2.5^{ ext{b}}$	43.34 ± 9.43^{ab}				
	ВА 8.9 μм	$60.0\pm10.0^{\rm a}$	$5.7\pm3.1^{ ext{b}}$	40.64 ± 13.56^{b}				
<i>N</i> = 150	ВА 17.8 μм	$50.0\pm26.5^{\text{a}}$	$8.7\pm3.5^{\text{a}}$	$36.77 \pm \mathbf{18.94^{b}}$				
NAA + BA 8.9 μм	0 µм	$95.38\pm11.63^{\rm a}$	$7.53\pm3.55^{\rm a}$	$56.80\pm12.14^{\rm a}$				
	0.54 µм	$94.59\pm16.77^{\rm a}$	7.09 ± 2.80^{a}	$46.10 \pm 14.21^{ m b}$				
	1.34 µм	$80.00\pm33.47^{\rm a}$	7.08 ± 2.52^{a}	45.81 ± 16.83^{b}				
<i>N</i> = 120	2.69 μм	$100.00\pm0.00^{\text{a}}$	$7.07\pm3.09^{\rm a}$	$44.24\pm10.50^{\rm b}$				
Days on RI	5 days	$94.55\pm9.34^{\rm a}$	$7.60\pm3.36^{\rm b}$	52.89 ± 10.10^{ab}				
	6 days	$94.87\pm15.02^{\rm a}$	$6.99\pm3.04^{\rm b}$	$54.93\pm13.20^{\rm a}$				
	7 days	$93.33\pm18.26^{\rm a}$	$6.46\pm2.50^{\rm b}$	$45.24\pm17.80^{\rm c}$				
	8 days	$96.36\pm12.06^{\rm a}$	$\textbf{7.27}\pm\textbf{3.02}^{b}$	$48.93\pm13.74^{\rm abc}$				
	9 days	86.67 ± 32.66^{a}	$\textbf{7.04} \pm \textbf{2.50}^{b}$	$47.96 \pm 14.00^{\texttt{bc}}$				
N = 494	10 days	$97.50\pm6.83^{\rm a}$	9.04 ± 3.95^{a}	$51.68\pm10.78^{\rm ab}$				
Genotype	AL1	$88.12\pm15.36^{\rm a}$	$8.25\pm2.89^{\rm b}$	$38.23\pm9.55^{\mathrm{a}}$				
	AL2	$89.00\pm12.72^{\rm a}$	$11.67\pm5.16^{\rm ab}$	$41.69\pm5.88^{\rm a}$				
	AL3	$89.17\pm15.81^{\rm a}$	$15.00\pm3.16^{\rm a}$	38.84 ± 6.58^{a}				
<i>N</i> = 120	AL4	$88.17 \pm \mathbf{16.74^a}$	12.50 ± 3.54^{ab}	39.52 ± 0.69^{a}				
Days on RD	22 days	$89.17\pm14.21^{\rm a}$	$8.13\pm2.65^{\rm b}$	37.15 ± 8.86^{a}				
	27 days	$87.61 \pm \mathbf{12.16^{a}}$	7.00 ± 2.74^{b}	$41.84\pm9.74^{\rm a}$				
N = 90	48 days	$88.49 \pm \mathbf{15.34^a}$	$12.14\pm4.35^{\rm a}$	$41.12\pm8.62^{\rm a}$				

The following parameters were analyzed: percentage of shoot forming roots, number of roots per shoot and final shoot elongation. Values are the mean \pm sp. For each treatment, values followed by the same letter are not significantly different. P > 0.05.

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FIGURE 6

Evaluation of the effect of BA concentration on rooting by PCA analysis considering the following variables: BA concentration, number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR) and shoot length (SL). The variables are associated with each factor 1 and 2, according to the coefficients, factor loadings, presented on the left table. For more details see Fig. 1.

negatively influenced (P < 0.05) by the presence of NAA in the multiplication medium (Table 3). The period of root induction (five to ten days) had no impact on the percentage of rooted shoots (Table 3); however, the number of roots per explant (9.04 ± 3.95) was significantly higher when a ten-day period of exposition to auxin was used.

Shoot genotype was another parameter tested for shoot rooting. The data obtained indicated that the percentage of rooting as well as shoot length was not influenced by the genotype (Table 3). However, the number of roots per explant is genotype dependent with the genotypes AL3, AL4 and AL2 giving the best results (15.0, 12.5 and 11.7, respectively).

When different periods of root development (RD-days: 22–48) were tested before acclimatization, the data obtained indicated that the same parameters were not influenced by the root development period (Table 3). However, on RD-48, the plantlets displayed a number of roots (NR) significantly higher (12.14 \pm 4.35) than on RD-22 or RD-27 (8.12 \pm 2.65 and 7.00 \pm 2.74, respectively). In these assays, PCA analysis (Fig. 7) explained 57.2% of total variance, showing that the number of roots (NR) is related to the LLR, root development period (RD) and genotype (factor loadings \geq 0.70). By contrast, more extensive callus formation is generally accompanied by a reduced root growth (LSR) and, in some cases, a complete inhibition of the root development.

Discussion and conclusions

The results so far obtained indicate that the multiplication rate evaluated by the number of shoots obtained per test tube that can be used for further multiplication (SNX) is dependent on the genotype and PGRs tested. When different BA concentrations were used best results were achieved with 8.9 μ M. Cytokinins are usually used on the micropropagation media to stimulate axillary shoot proliferation [22,28–30]. However, the ideal concentrations differ

from species to species and need to be established accurately to achieve the effective rates of multiplication. Moreover, some problems related with the use of cytokinins have been pointed out such as callus growth, poor shoot growth and vitrification [22,30]. In other members of the Ericaceae family, such as Rhododendron ponticum [10] and Vaccinium macrocarpon [31] cytokinins have been also commonly included in the culture media to promote shoot proliferation. Our data indicated that there are differences in the effect of the different cytokinins analyzed. Thus, it was found that KIN was most effective in promoting shoot growth whereas TDZ, a urea-derived cytokinin, induced callus proliferation in the explants. This situation must be avoided because these calli can display organogenic potential leading to the production of adventitious shoots that can exhibit some kind of variability. Experiments performed with A. unedo and other members of the Ericaceae family indicate that beyond cytokinins other factors such as culture media composition and sucrose can also influence shoot proliferation. Gonçalves and Roseiro [15] and Mereti et al. [16] showed that WPM (woody plant medium) combined with BA gave the best rates of shoot proliferation. According to Mendes (M.L.A. Mendes, Multiplicação vegetativa in vitro de medronheiro, Master Thesis, ISA-UTL, 1997) the Anderson medium and the cytokinin 2-iP were particularly effective to propagate adult material. In V. macrocarpon [31] the highest rates of shoot production were obtained when nodal segments were cultured on a medium supplemented with 12.3–24.6 µM of 2-iP without auxin. Also, in Vaccinium myrtillus and Vaccinium vitis-idaea [32] higher rates of micropropagation were obtained when the cytokinin 2-iP was used at concentrations of 49.2 µM and 24.6 µM, respectively.

Our results showed that the auxin NAA was unable to increase the multiplication rate. Moreover, inclusion of the NAA in the multiplication phase induced callus formation and this feature increased with the number of subcultures. Some species may



FIGURE 7

Evaluation of the effect of different genotypes on rooting by PCA analysis considering the following variables: genotype, root development period, number of roots (NR), length of the longest root (LLR), length of the shortest root (LSR), shoot length (SL) and basal callus formation (callus). For more details see Fig. 1.

require a low concentration of auxin in combination with high levels of cytokinin to increase shoot proliferation [30]. Nevertheless, this does not seem to be the case in A. unedo or in related species as our results and those obtained in A. xalapensis [7] might suggest. The observation that both the auxin NAA and the cytokinin TDZ are able to induce callus formation in A. unedo seems to indicate, as pointed out by other authors, that TDZ may act through the modification of the endogenous levels of auxins increasing the amounts of indol-3-acetic acid or other endogenous auxin-like compounds that promote cell proliferation [33-35]. Callus formation may be interesting if they have the ability to undergo shoot formation without loss of the genetic uniformity of the regenerated plants. This possible alternative for plant regeneration in strawberry tree needs to be analyzed in more detail. For now it was only observed that on the conditions tested shoot formation was never recorded on these calli. In some sporadic situations, morphologically abnormal underdeveloped leaves were seen arising from the calli but further shoot growth was impaired. However, this kind of observation indicates that the calli thus obtained are able to organize meristematic regions showing its potential for future research.

Our results also showed that the genotype is another important factor involved on shoot proliferation of *A. unedo*. It is well known that *in vitro* culture is highly dependent on the genotype of donor material. In fact different types of morphogenic responses *in vitro* such as somatic embryogenesis, organogenesis, shoot proliferation, rooting and microspore embryogenesis are strongly determined by the genotype of the explants [12,36,37]. This seems to indicate that specific genetic combinations found in some genotypes are more prone to undergo a particular type of morphogenesis than others. In our experiments we found that shoot multiplication and callus formation were highly genotype-dependent. Because these factors are also highly dependent on the PGRs present in the culture media it is plausible to assume that different

genotypes possess different levels of endogenous auxins and/or cytokinins that influence their behavior *in vitro*. Experiments of somatic embryogenesis induction in this species carried out at our lab also showed that somatic embryo formation is dependent on the genotype (data not published). Although these results need to be supported by a more detailed analysis they point out to a strong variability in *A. unedo* genotypes in what concerns *in vitro* culture. However, it should be referred that many of the published genotype effects may in fact result from less understood interactions between the culture environment and the genetic background of the explant. A genotype displaying a low multiplication rate can be only the result of deficient culture conditions that can be improved to achieve a better response. Further research on *A. unedo* micropropagation is necessary to better understand the role of the genotype on *in vitro* morphogenesis.

Rooting is a crucial step to the success of micropropagation. Without an effective root system plant acclimatization will be difficult and the rate of plant propagation may be severely affected [38]. In a previous paper we established the conditions for *in vitro* rooting in A. unedo [18]. In that paper the role of the multiplication conditions on rooting was evaluated. According to the conditions now tested it can be concluded that a ten-day period of root induction is more suitable for A. unedo whereas plant acclimatization should not be carried out before 35-40 days on the root development medium. The statistic analysis performed showed that the frequency of root formation is not affected by previous multiplication conditions. However, when the number of roots formed per explant was the factor considered the results showed a strong influence of factors such as the concentration of BA, genotype, and the periods of root induction and development. Metaxa et al. [4] have also found that the genotype and growth regulators are the main factors involved on root formation in cuttings of A. unedo. The eventual role of the genotype was already discussed and what was stated then can also apply to the results obtained during the rooting phase. A remarkable result obtained in A. unedo was the observation that shoots produced on higher cytokinin containing medium are more amenable to root induction than shoots obtained with the lowest concentrations of BA. A review of the literature clearly points out to a negative effect of cytokinins on shoot rooting [30] although a positive role has been occasionally referred [39,40]. Once again these results may be explained by the complex interactions between endogenous and exogenous growth regulators (mainly auxins and cytokinins) occurring during in vitro cultures. Only a time-course evaluation of the growth regulators present in the explants during root morphogenesis can bring some information about these interactions. However, other compounds such as ethylene and phenolics may also be involved [24]. Finally it should be noted that the tendency of some genotypes to produce callus at the basis of the shoots is a major drawback for further rooting because it can avoid the establishment of normal connections between the vascular system of the forming roots and the shoot.

Assays of acclimatization carried out by authors working with *A. unedo* showed that plantlets obtained *in vitro* are easily acclimatized [15,16,18] and the same is true for other members of the Ericaceae family, such as several *Vaccinium* species [41]. The

References

- 1 Piotto, B. et al. (2001) La ripresa della vegetazione dopo gli incendi nella regione mediterrânea. In Propagazione per seme di alberi e arbusti della flora mediterranea, (Piotto, B. and Noi, A., eds), pp. 32–38, Dip. Prev. Risa. Ambientali, ANPA
- 2 Ferreira, P.G. et al. (2005) Carta da Tipologia Florestal de Portugal Continental. Silva Lusitana 13, 1–34
- 3 Neppi, M. (2001) Alberi ed arbusti della flora mellifera della regione mediterrânea. In *Propagazione per seme di alberi e arbusti della flora mediterranea*, (Piotto, B. and Noi, A., eds), pp. 44–49, Dip. Prev. Risa. Ambientali, ANPA
- 4 Metaxas, D.J. *et al.* (2004) Peroxidases during adventitious rooting in cuttings of *Arbutus unedo* and *Taxus baccata* as affected by plant genotype and growth regulator treatment. *Plant Growth Regul.* 44, 257–266
- 5 Web site of the Global Facilitation Unit for Underutilized Species. (2008) (www.underutilized-species.org/default.asp)
- 6 Pabuccuoglu, A. et al. (2003) Antioxidant activity of Arbutus unedo leaves. Fitoterapia 74, 597–599
- 7 Mackay, W.A. (1996) Micropropagation of Texas madrone, Arbutus xalapensis HBK. HortScience 31, 1028–1029
- 8 Lloyd, G.B. and McCown, B.H. (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Proc. Int. Plant Prop. Soc.* 30, 421–427
- 9 Banko, T.J. and Stefani, M.A. (1989) *In vitro* propagation of *Oxydendrum arboreum* from mature trees. *HortScience* 24, 683–685
- 10 Almeida, R. et al. (2005) In vitro propagation of endangered Rhododendron ponticum L. subsp. baeticum (Boissier & Reuter) Handel-Mazzetti. Biodivers. Conserv. 14, 1059– 1069
- 11 Anderson, W.C. (1984) A revised tissue culture medium for shoot multiplication of *Rhododendron. J. Am. Soc. Hort. Sci.* 109, 343–347
- 12 Gajdošová, A. (2007) Protocol for micropropagation of Vaccinium vitis-idaea L. In Protocols for Micropropagation of Woody Trees and Fruits (Jain, S.M. and Häggman, H., eds), pp. 457–464, Springer
- 13 Ostrolucká, M.G. (2007) Protocol for micropropagation of selected Vaccinium. In Protocols for Micropropagation of Woody Trees and Fruits (Jain, S.M. and Häggman, H., eds), pp. 445–455, Springer
- 14 Ratnaparkhe, M.B. (2007) Blueberry. In Genome Mapping and Molecular Breeding in Plants, vol. 4, Fruits and Nuts (Kole, C., ed.), pp. 217–227, Springer-Verlag
- 15 Gonçalves, J.C. and Roseiro, R.J. (1994) Establishment and in vitro multiplication of Arbutus unedo L. seedlings. VIII International Congress of Plant Tissue and Cell Culture. Abst 20, Firenze, Italy
- 16 Mereti, M. et al. (2002) Micropropagation of the strawberry tree, Arbutus unedo L. Sci. Hort. 93, 143–148

conditions used for acclimatization in our experiments seem also to work well allowing the regeneration of many individuals from different genotypes which are now growing in the field.

The results so far obtained on the micropropagation of *A. unedo* have established the conditions to clone selected trees and to propagate them in large scale to be evaluated in the field for fruit quality and productivity. Following that analysis the more indicated genotypes will be produced in large scale to be distributed to the farmers interested in this crop. This is part of our strategy for breeding strawberry tree making it a competitive species for fruit production. These selected clones are now being characterized by molecular markers (microsatellites) and conserved *in vitro* and in the field in a germplasm bank. These genotypes are also being evaluated for its potential to undergo somatic embryogenesis and the first preliminary results about somatic embryo formation in strawberry were recently published [20].

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- 17 Mereti, M. et al. (2003) In vitro rooting of strawberry tree (Arbutus unedo L.) in medium solidified by peat-perlite mixture in combination with agar. Proc First Int Symp on Acclimat. and Estab of Microp. Plants pp. 207–210
- 18 Gomes, F. and Canhoto, J. (2009) Micropropagation of strawberry tree (Arbutus unedo L.) from adult plants. In Vitro Cell. Dev. Biol. 45, 72–82
- 19 De Fossard, R.A. et al. (1974) Tissue and organ culture of Eucalyptus. New Zeal. J. For. Sci. 4, 267–278
- 20 Gomes, F. et al. (2009) Micropropagation of selected trees of Arbutus unedo L. trough axillary shoot proliferation and somatic embryogenesis. Acta Hort. 839, 111–116
- 21 George, E.F. and Debergh, P.C. (2008) Micropropagation: uses and methods. In *Plant Propagation by Tissue Culture* (edn 3) (George, E.F. *et al.* eds), pp. 29–64, Springer
- 22 Chawla, H.S. (2009) Introduction to Plant Biotechnology. Springer
- 23 Gomes, F. and Canhoto, J.M. (2003) Micropropagation of *Eucalyptus nitens* Maiden (Shining gum). In Vitro Cell. Dev. Biol. 39, 316–321
- 24 Machakova, I. *et al.* (2008) Plant growth regulators I: introduction; auxins, their analogues and inhibitors. In *Plant Propagation by Tissue Culture* (edn 3) (George, E.F. *et al.* eds), pp. 175–204, Springer
- 25 Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant.* 15, 473–497
- 26 Gautheret, R.J. (1959) La culture des tissues végétaux, techniques et réalisations. Masson
- 27 Zar, J.H. (1996) Biostatistical Analysis (edn 3), Prentice-Hall
- 28 Tornero, et al. (2009) Citrus limon micropropagation: effect of different phytohormones on multiplication and rooting. Acta Hort. 839, 57–62
- 29 El-Agamy, S.Z. (2009) *In vitro* propagation of some grape root stocks. *Acta Hort*. 839, 125–132
- 30 Van Staden, *et al.* (2008) Plant growth regulators II: cytokinins, their analogues and inhibitors. In *Plant Propagation by Tissue Culture* (edn 3) (George, E.F. *et al.* eds), pp. 205–226, Springer
- 31 Debnath, S. and McRae, K. (2001) An efficient *in vitro* shoot propagation of cranberry (*Vaccinium macrocarpon* Ait.) by axillary bud proliferation. *In Vitro Cell. Dev. Biol.* 37, 243–249
- 32 Jaakola, L. (2001) Effect of N⁶-isopentenyladenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. *Plant Cell Tiss. Org. Cult.* 66, 73–77
- 33 Visser, C. et al. (1992) Substitution of the auxin–cytokinin requirement for the induction of somatic embryogenesis by TDZ suggests the possibility of a novel mode of its action. Plant Physiol. 99, 1704–1707
- 34 Maxwell, P.A.J. (2007) The mode of action of thidiazuron: auxins, indoleamines and ion channels in the regeneration of *Echinacea purpurea* L. *Plant Cell Rep.* 26, 1481–1490

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- 35 Sedlak, J. and Paprstein, F. (2009) In vitro proliferation of newly bred Czech pear cultivars. Acta Hort. 839, 87–92
- 36 Gahan, P.B. and George, E.F. (2008) Adventitious regeneration. In Plant
- Propagation by Tissue Culture (edn 3) (George, E.F. et al. eds), pp. 335–401, Springer
 Bhau, B.S. and Wakhlu, A.K. (2001) Effect of genotype, explant type and growth regulators on organogenesis in Morus alba. Plant Cell Tiss. Org. Cult. 66, 25–29
- 38 Gonçalves, J.C. et al. (1998) In vitro propagation of chestnut (Castanea sativa × Castanea crenata): effects of rooting treatments on plant survival,

peroxidise activity and anatomical changes during adventitious root formation. *Sci. Hort.* 72, 265–275

- 39 Nemeth, G. (1979) Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured in vitro. Z. Pflanzenphysiol. 95, 389–396
- 40 Bennett, I.J. et al. (1994) Alternating cytokinins in multiplication media stimulates in vitro shoot growth and rooting of Eucalyptus globulus Labill. Ann. Bot. 74, 53–58
- 41 Isutsa, D.K. et al. (1994) Rapid propagation of blueberry plants using ex-vitro rooting and controlled acclimatization of micropropagules. HortScience 29, 1124–1126