Micropropagation of Selected Trees of *Arbutus unedo* L. through Axillary Shoot Proliferation and Somatic Embryogenesis

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Abstract

***Arbutus unedo* (Strawberry tree) grows spontaneously in several countries of the Mediterranean basin. Fruits can be consumed fresh or can be processed to make a spirit. A project to clone selected trees of this species based on their fruit production was initiated a few years ago. In this work the role of BA on shoot proliferation and the effect of IBA on root formation are evaluated. Preliminary results about somatic embryogenesis induction are also presented. Best shoot proliferation was achieved when 8.9 µM BA were used. In this situation, an average of 1.34 shoots and 1.75 nodes per explant were obtained. Rooting was achieved on a Knop medium containing different IBA concentrations (1 week) followed by subculture (5 weeks) on the same medium without auxin and containing charcoal (1.5%). On these conditions, best rooting frequencies (100%) were obtained with IBA at concentrations of 49.2 µM. For somatic embryogenesis induction, leaves from in vitro propagated material were used. Results showed that a combination of BA (8.8 µM) and NAA (10.7; 26.8 µM) gave the highest frequencies of induction (94.4%). Somatic embryo conversion was achieved when somatic embryos were transferred to a medium without plant growth regulators. Plant acclimatization was successfully accomplished for both types of in vitro propagated material and some of the produced plants are now in the field to be evaluated.**

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. This plant is drought tolerant and has a great potential for regeneration, a characteristic which is particularly important in regions where fires are common, as in southern European countries. The production of a spirit from its fruits represents the main income for farmers growing this crop. More recent uses are related with biomass production and floriculture (Mereti et al., 2002).

Arbutus unedo is common in Portugal and Mediterranean countries. However, in most of the cases, the fruits are picked from wild trees and little work has been done to characterize and propagate the most interesting genotypes. Considering the increasing importance that alternative crops are assuming in the agricultural policy of the European Union, a project to clone selected adult trees based on their fruit production was initiated by our group a few years ago. The main objective is to evaluate the potential of tissue culture techniques to propagate selected trees based on fruit production (Gomes and Canhoto, 2009). Axillary shoot proliferation is the most widely used micropropagation technique for Ericaceae clonal propagation. Members of this family have been successfully micropropagated, such as *Arbutus xalapensis* (Mackay, 1996), *Rhododendron* (Almeida et al., 2005; Anderson, 1984) and *Vaccinium corymbosum* (Isutsa et al., 1994). Previous studies in Strawberry tree have shown that in vitro propagation from juvenile material could be accomplished (Gonçalves and Roseiro, 1994; Mereti et al., 2002, 2003). Micropropagation of adult material of *A. unedo* has been achieved by Mendes (1997) and Gomes and Canhoto (2009). In this work we present

some data related with the propagation of Strawberry tree through axillary shoot proliferation and somatic embryogenesis.

MATERIALS AND METHODS

For culture establishment of adult selected plants, branches (30-40 cm length) were collected in the field. To stimulate the development of epicormic shoots, branches were disinfected with a fungicide, and then maintained in a culture chamber covered with a plastic bag to keep a high humidity environment. Following sterilisation of epicormic shoots, nodal segments were used for culture establishment.

For shoot proliferation four benzyladenine (BA) concentrations (2.2, 4.4, 8.9 and 17.8 μM) were tested and compared with the control (BA 0 μM). The basal medium used in the experiments contained the major salts of De Fossard (De Fossard et al., 1974) medium, MS (Murashige and Skoog, 1962) micro-nutrients, FS (De Fossard et al., 1974) organics and 3% sucrose, as described by Gomes and Canhoto (2009). Shoots 12-18 mm in length, were used in the assays. The multiplication rate was evaluated by the number of shoots formed per culture during the initial culture (4 weeks), and by the number of nodes formed on subsequent subcultures (4 week intervals) on the same proliferation culture medium (4 subcultures).

Shoots (14-20 mm) were transferred to a root induction medium (Knop macronutrients) added of 3-indolebutyric acid (IBA), for a week. Root induction was assayed in the dark. Following this period root induced plants were subcultured (1 month) on the same medium culture without growth regulators and containing charcoal (1.5%) to promote root elongation. During root induction, five IBA treatments (2.5, 4.9, 9.8, 24.6, 49.2 μM for 1 week) were used and compared with the control (IBA 0 μM). Twelve treatments were tested: two earlier reported shoot proliferation media (FS and AND; Anderson, 1984), 5 different IBA concentrations plus the control (IBA 0 μM). Thirty shoots were used per treatment (total of 360 shoots).

For somatic embryogenesis induction, leaves from in vitro propagated material were used. Entire leaf blades were excised and small cuts were made in the abaxial face. The explants were cultured on AND medium containing different concentrations of benzyladenine (BA) and 1-naphtaleneacetic acid (NAA). Cultures were maintained in the dark, at 25ºC, for 8 weeks. Following this period, somatic embryos were transferred to a medium without growth regulators for maturation. Somatic embryo germination and conversion was achieved when the explants were transferred to a Knop's basal medium without plant growth regulators under a photoperiod of 16h light and 8h dark, at 25ºC for 5 weeks.

For acclimatization plantlets were dipped in a fungicide solution and transferred to containers. These were covered with plastic bags, to maintain a high degree of humidity, and placed in a greenhouse. Perlite (100%, without fertilizer) was used as substrate. After the first month in the greenhouse the plantlets were sprayed with a solution of Knop macronutrients once a week. For plant hardening the levels of humidity were gradually decreased by raising the plastic covers. These were completely removed after 1.5 months of acclimatization. After 2 months, plants were transferred to individual containers (220 ml) with peat, vermiculite, perlite (1:1:1) and at that moment the plant survival rate was registered.

RESULTS AND DISCUSSION

The effect of different BA concentrations on shoot proliferation is shown in Table 1. The results indicate that 8.9 μM BA was the most effective concentration both in terms of the number of shoots and in the total number of nodes produced ($P \leq 0.05$). Shoots formed in the presence of 17.8 μM BA were shorter than those developed on the medium with 8.9 μM BA. Shoot proliferation occurred also in a BA free medium at rates similar to those obtained with 2.2 or 4.4 μM BA.

Rooting assays showed that for both parameters studied (root formation and length of the longest root) significant differences between treatments occurred. Root formation

(Fig. 1A) was affected ($P \leq 0.01$) by IBA concentration and by the type of shoot proliferation medium used as well as by the interaction between these two factors (Table 2). Best results were observed when AND and FS media were used in combination with IBA at concentrations of 49.2, 24.6 and 9.8 μM (Table 2). In general, root formation was higher in higher IBA containing medium and, in several treatments, root formation was induced in 100% of the shoots used. The role of auxins on root formation is well characterized but its interaction with other factors, namely the combination of nutrients used, needs further attention. The results also indicated that the length of the longest root was affected by IBA concentration ($P \leq 0.01$) and by the interaction between the two factors ($P \leq 0.05$; IBA concentration and type of shoot proliferation medium). However, it should be noted that except for the treatments in which no IBA was used (controls), no statistically significantly differences were found among the other treatments. Effective protocols for root induction were also described by other authors working with *A. unedo*, showing that a wide range of conditions can be used for rooting this species (Mendes 1997; Gonçalves and Roseiro, 1994; Mereti et al., 2002; Gomes and Canhoto, 2009). Following acclimatization (Fig. 1B) plant survival was evaluated (Fig. 1C) with the results showing that 98% of the plants reached the appropriate conditions to be transferred to the field. Field tests with the in vitro propagated plants are now being carried out.

Somatic embryos start to appear 1.5-2 months after culture (Fig. 2A), following the browning of the initial explant due to oxidation of phenolic compounds. The role of phenolic compounds on somatic embryo formation was recently evaluated by Reis et al. (2008). Somatic embryos were morphologically identical to their zygotic counterparts. Results have shown that a combination of BA (8.8 μM) and NAA (10.7, 26.8 μM) gave the highest frequencies of induction (94.4%). Somatic embryo conversion (Figs. 2B and C) was obtained after isolation of mature embryos and culture in a medium without plant growth regulators. When the plantlets showed a developed root system and a vigorous shoot (6-8 weeks), they were transferred to pots and acclimatized in a greenhouse (Fig. 2D). Some of the embryos displayed morphological abnormalities thus reducing the frequencies of somatic embryo conversion. A more detailed study concerning the factors affecting somatic embryogenesis induction and conversion are being carried out.

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Tables

Table 1. Effect of the BA on shoot proliferation.

BAP (μM)	Number of shoots	Number of nodes
0	1.11 ± 0.36 ^{c}	1.43 ± 0.51 ^{bc}
2.2	1.10 ± 0.32 ^{c}	1.49 ± 0.55 ^{b}
4.4	1.07 ± 0.32 ^{c}	1.28 ± 0.47 ^{c}
8.9	1.34 ± 0.67 ^{a}	1.75 ± 0.75 ^{a}
17.8	1.22 ± 0.61 ^{b}	1.33 ± 0.69 ^{c}

In each column values (mean ± SD) followed by different letters are significantly different ($P \leq 0.05$), identified by using the Duncan's test.

Table 2. Effect of different treatments on root formation and elongation.

Treatments Proliferation media x IBA (μM)	Root formation (%)	Longest root (mm)
AND x 0	5.6 ± 4.81 [d]	17.5 ± 3.54 [b]
FS x 0	9.1 ± 0.83 [d]	16.7 ± 12.50 [b]
FS x 4.9	43.9 ± 6.94 [c]	34.9 ± 12.46 [a]
FS x 2.5	57.6 ± 23.92 [c]	35.7 ± 15.32 [a]
AND x 4.9	83.3 ± 5.77 [b]	42.0 ± 9.74 [a]
AND x 2.5	85.6 ± 5.30 [b]	41.9 ± 12.82 [a]
FS x 9.8	93.3 ± 5.77 [ab]	40.8 ± 9.82 [a]
FS x 24.6	96.7 ± 5.77 [a]	35.8 ± 9.78 [a]
AND x 9.8	100.0 ± 0.00 [a]	37.2 ± 7.57 [a]
AND x 24.6	100.0 ± 0.00 [a]	41.3 ± 6.47 [a]
AND x 49.2	100.0 ± 0.00 [a]	39.4 ± 6.94 [a]
FS x 49.2	100.0 ± 0.00 [a]	37.1 ± 7.49 [a]

In each column values (mean ± SD) followed by different letters are significantly different ($P \leq 0.05$), identified by using the Duncan's test.

Figures

Fig. 1. Root formation and acclimatization. A - Rooted plantlet of *A. unedo* after 4 weeks on the root elongation medium. B - Several plants during the acclimatization phase. C - Plant of *A. unedo*, after 2 months of the acclimatization period, at that moment plants are transferred to individual containers with peat, vermiculite, perlite (1:1:1).

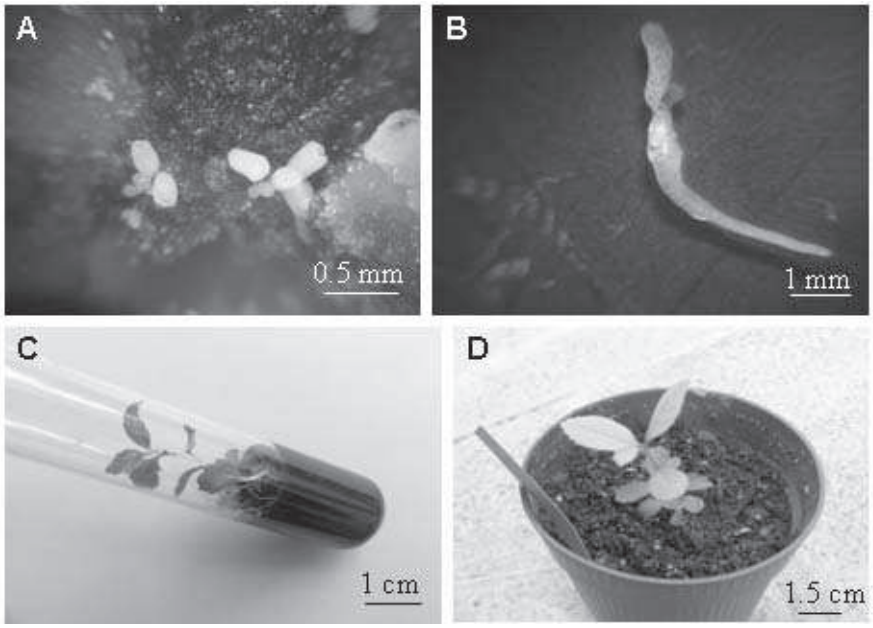

Fig. 2. Somatic embryo formation and plant regeneration. A. Several somatic embryos. B. Somatic embryo germination. C. Plantlets originated by somatic embryogenesis. D. Potted somatic embryo-derived plants.