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IN VITRO PROPAGATION OF ADULT STRAWBERRY TREE (*ARBUTUS UNEDO* L.) THROUGH ADVENTITIOUS SHOOTS AND SOMATIC EMBRYOGENESIS

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Abstract

A method for induction of adventitious shoots and somatic embryogenesis (SE) of *Arbutus unedo* is presented. Semi-hard woody nodal segments were excised from adult donor tree (12 years old) and cultured for 6 weeks on Murashige and Skoog's (MS) medium without plant growth regulators (PGRs) for the induction of axillary shoots. The induced axillary shoots were then cultured for 8 weeks on MS medium supplemented with TDZ (0-4 mg l^{-1}) for their multiplication. The best axillary shoot multiplication (3.9 shoots/explants) was achieved on MS medium supplemented with 3.0 mg l^{-1} TDZ. Calluses were induced from internodal segments excised from *in vitro* axillary shoots and cultured on MS medium supplemented with various concentrations and combinations of PGRs. Embryogenic calluses developed over a period of 14 days with the highest response (17 embryos / callus) at 5.0 mg l^{-1} BA and 5.0 mg l^{-1} NAA. Following induction, the calluses were proliferated on the same medium and somatic embryos at the globular, heart-shaped, torpedo, and cotyledonary stages developed after 35 days in culture. Plantlets developed at 95% on MS medium without PGRs. Non-embryogenic callusses developed clusters of shoot buds. A combination of 2 mg l^{-1} BA and 0.5 mg l^{-1} NAA promoted the highest number of adventitious shoots (5.3 shoots/callus).

Key words: Arbutoidae, Ericaceae, *in vitro* culture, plant growth regulators, semi-hardwood cuttings

PROOF

INTRODUCTION

Conventional propagation of *A. unedo* is achieved through seeds however, it requires prechilling (4-12 weeks) to accelerate its emergence. Moreover, it requires a long time for seedlings growth (Mereti et al. 2002). The plant is also propagated by cuttings; however it has not been possible to obtain rooting percentages higher than 50% (Crobeddu and Pignatti 2005, Pignatti and Crobeddu 2005). According to Pignatti and Crobeddu (2005), it is necessary to use material obtained from young plants which have been repeatedly pruned to stimulate vigorous sprouting. If this type of material is not used, the rooting probability is nil. Despite the increasing commercial demand of *A. unedo* plants, only a few protocols for tissue culture propagation have been published. Previous reports have shown that *in vitro* shoot proliferation of strawberry tree could be accomplished using shoot explants from either potted greenhouse plantlets (Mereti et al. 2002) or from adult tress (Mendes 1997, Gomes and Canhoto 2009).

The aim of this study was to develop an efficient method for propagation of *A. unedo* through adventitious shoots and somatic embryogenesis (SE) using segments of *in vitro* obtained axillary shoots.

MATERIALS AND METHODS

Plant material and culture establishment

The shoot cuttings, 5-10 cm long, were taken from adult strawberry tree (*Arbutus unedo* L.) at the reproductive stage (12 years old) and than were defoliated, washed under running tap water and dipped in antioxidants solution (150 mg l^{-1} ascorbic acid + 100 mg l^{-1} citric acid + 1 g l^{-1} activated charcoal (AC), Sigma-C6289) for 10 min. Then they were rinsed three times with distilled water and sectioned to 4-5 cm long pieces containing 3-4 nodes and then disinfected for 60 s in 70% ethanol followed by 5 min in a solution of 0.1% (v/v) mercuric chloride containing 2-3 drops of Tween 20 (polyoxyethylene-sorbitan monolaurate). After rinsing three times with sterile distilled water, explants were cultured for 8 weeks on a basal medium consisting of half strength MS nutrients (Murashige and

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Skoog 1962), 3% (w/v) sucrose and solidified with 0.8% (w/v) Sigma agar-agar for induction of axillary shoots. The pH of all variants of the medium was adjusted to 5.7 before autoclaving at 121°C for 20 min. All the cultures were incubated at 25 ± 2°C for 2 weeks under dark conditions (to avoid browning of plant tissue) followed by 4 weeks under a 16 h photoperiod provided by cool-white fluorescent lights at 25 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF).

Axillary shoot multiplication

A. unedo axillary shoots were transferred on MS medium supplemented with different concentrations of thidiazuron (TDZ) at 0, 1, 2, 3 and 4 mg l^{-1}. The shoot growth and shoot multiplication responses were recorded after 8 weeks of culture in terms of number of axillary shoots developed per explant, shoot length as well as fresh and dry weight of the whole explants. Dry weights were determined after drying for 48 h at 70°C. All measurements were obtained from 12 randomly chosen shoots.

Callus induction and somatic embryogenesis

Axillary shoots, proliferated from the initial explants, were used for the induction of callus and SEs. The internodes were cut into 2-3 mm segments and cultured on MS medium with 2 g l^{-1} AC and supplemented with different concentrations and combinations of PGRs (Table 2). Medium without PGRs served as the control. To study the effect of light regimes on callus induction and SE, cultures were either incubated under dark condition or incubated under a 16 h photoperiod provided by cool-white flourescent lights at 25 µmol m^{-2} s^{-1} PPF. There were 10 explants per replicate and 3 replicates per treatment. The percentage of callus formation, callus fresh weight, number of embryos formed per callus and characteristics of developed calluses were recorded after 8 weeks of culture.

Somatic embryo formation and conversion to plantlets

Embryogenic calluses obtained on MS medium supplemented with N^6-benzyladenine (BA) 5.0 mg l^{-1} + α-naphthalene acetic acid (NAA) 5.0 mg l^{-1} showing different developmental stages of somatic embryos were transferred onto MS medium without PGRs or MS medium supplemented with different concentrations and combinations of BA and NAA as follows: BA (1.0 mg l^{-1}) + NAA (0.5 mg l^{-1}), BA (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}), BA (2.0 mg l^{-1}) + NAA (1.0 mg l^{-1}) or BA (5.0 mg l^{-1}) alone. Embryogenic calluses were sub-cultured once after 4 weeks of culture. After 8 weeks of culture, the percentage of embryo germination was determined. Embryos with elongated hypocotyls and root growth were counted as germinating.

Adventitious shoot formation from non-embryogenic calluses

All non-embryogenic calluses were transferred onto MS medium with 1 g l^{-1} AC supplemented with different concentrations and combinations of BA and NAA as described above for shoot proliferation or without PGRs. There were 5 explants per Petri dish and 4 replicates per treatment. After 8 weeks of culture, the number of proliferated shoots per callus was recorded. Proliferated shoots obtained through organogenic calluses were individually separated and cultured on MS medium without PGRs for their subsequent growth.

Experimental design and data analysis

Experiments were set up in a completely randomized design and repeated twice. Data were subjected to Duncan's multiple range test using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

RESULTS AND DISCUSSION

Culture establishment and axillary shoot multiplication

In the present study, soaking of explants in antioxidants solution prevented browning, a major problem during culture initiation reported earlier (Gomes and Canhoto 2009). Semi-hard woody cuttings of *A. unedo* developed axillary shoots on MS medium without PGRs. The proliferated shoots were healthy and had no browning. It has been reported earlier that MS nutrients instead of WPM nutrients resulted in extensive oxidative browning of *A. unedo* explants after culture for 3 weeks (Mereti et al. 2002). TDZ at 3.0 mg l^{-1} induced the highest number of axillary shoots (3.9) with maximum fresh (714 mg) and dry weights (76 mg) per explant (Table 1). However, the highest shoot length (52 mm) and the number of leaves (14) were obtained in TDZ-free medium. *A. unedo* shoots, which were cultured on medium with TDZ at 4 mg l^{-1} showed thick broad leaves that were wrinkled, curled and brittle all indicating symptoms of hyperhydricity. The abnormal plant growth associated with high TDZ concentrations already has been demonstrated by Dewir et al. (2006) and Siddique and Anis (2007) in *Spathiphyllum cannifolium* and *Cassia angustifolia*, respectively. TDZ is considered to be one of the most active PGRs for shoot induction and a potent bioregulant of *in vitro* plant morphogenesis (Murthy et al. 1998). TDZ-induced morphogenesis probably depends on the levels of hormones, and also modulates the endogenous auxin levels. TDZ is effective for some species (Heutteman and Preece 1993) but not effective for others (Mitras et al. 2009). The effect depends from the concentration and the duration of its application. TDZ-induced shoot organogenesis from different explants of many recalcitrant species as well as from medicinal plants has been reported earlier (Liu et al. 2003, Thomas 2003).

Table 1. Effects of TDZ concentrations on axillary shoot multiplication and growth of *A. unedo* after 8 weeks of culture incubation.

TDZ concentration (mg l^{-1})	Number of shoots/explant	Number of leaves/shoot	Shoot length (mm)	Shoot fresh weight (mg)	Shoot dry weight (mg)
0.0	1.0 ± 0.00 d	14.0 ± 0.33 a	52 ± 0.20 a	114 ± 3 e	31 ± 0.92 c
1.0	2.9 ± 0.10 c	8.2 ± 0.25 b	21 ± 0.51 c	122 ± 5 d	21 ± 0.72 d
2.0	3.2 ± 0.15 b	8.3 ± 0.19 b	38 ± 0.84 b	332 ± 16 c	62 ± 2.00 b
3.0	3.9 ± 0.12 a	7.8 ± 0.35 b	23 ± 1.02 c	714 ± 21 a	76 ± 3.00 a
4.0	2.9 ± 0.09 c	4.9 ± 0.12 c	16 ± 0.19 d	672 ± 18 b	67 ± 2.00 b
Significance	**	**	**	**	**

Values are means ± standard error. Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test.
** significant at $p \leq 0.01$.

Callus induction and somatic embryogenesis

Internodal segments developed callus at the cut surfaces within 15 days of culture. The callus, subsequently, covered the entire surface of a segment. Callus formation could not be achieved in the absence of PGRs. Callus formation was dependent on the combination of PGRs and light conditions (Table 2). Under dark conditions, all explants formed callus irrespective of the PGR used, whereas light was inhibitory to callus formation. The highest percentage of callus formation (100%) was observed on variants of the medium containing NAA, 2,4-D or combination with Kin or BA. However, the highest callus fresh weight (60 mg) was observed in the medium supplemented with 2.0 mg l^{-1} TDZ. The callus growth rate increased from the second week of culture initiation till eighth week, after which it declined. Calluses formed on variants of the medium containing either auxins alone or cytokinins alone did not form somatic embryos. A combination of auxins and cytokinins was a prerequisite for SE. The embryogenic calluses developed on the surfaces of the explants 5-6 weeks after culture initiation and were pale yellow or white and had a friable texture. Numerous small clumps of compact cells ranging in diameter from 3 to 4 mm were developed into a mass of somatic embryos. The highest number of somatic embryos per callus (17.0) was observed in medium containing BA (5.0 mg l^{-1}) and NAA (5.0 mg l^{-1}). Also, variants of the medium supplemented with 1.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} Kin + 1.7 mg l^{-1} IAA or 1.0 mg l^{-1} 2,4-D + 5.0 mg l^{-1} BA + 5.0 mg l^{-1} NAA produced somatic embryos (10.7, 10.3) and (8.3, 7.7) under dark and light conditions, respectively.

Light can be an important determinant of somatic embryo induction. In eggplant, somatic embryo induction was reported to have an absolute requirement for light (Gleddie et al. 1983). Similarly, SE for garden leek *(Allium porrum* L.) also required light (Hong and Debergh 1995). In contrast, somatic embryo induction of *Camellia* (San-Jose and Vieitez 1993) and olive (Rugini and Caricato 1995) had an absolute requirement for darkness. In Norway spruce, SE was reported to be possible only in complete darkness when ammonium was present in the medium. The removal of ammonium was necessary for SE to take place in the light (Verhagen and Wann 1989). In the present study, light inhibited callus formation and SE in *A. unedo.* It has been reported that a yellowish non-compact callus of *A. unedo* was induced on half strength MS medium supplemented with IAA and Zeatin but no signs of SE and/or shoot organogenesis were observed (Giordani et al. 2005). The emergence of somatic embryos occurred after 14 days of culture on medium containing 5.0 mg l^{-1} BA + 5.0 mg l^{-1} NAA. Once embryogenic cells have been formed, they continued to proliferate on the same medium. The embryos progressed through the globular, heart, torpedo and cotyledonary stages. Cotyledonary-stage somatic embryos were formed after 35 days of culture of the primary calluses.

Somatic embryos cultured on MS medium without PGRs showed the highest germination percentage in which 96% of somatic embryos converted into plantlets with well developed roots (Fig. 1). Somatic embryos could not germinate under dark conditions. The embryos became watery, succulent and exhibited abnormal growth (data not shown).

Adventitious shoot formation

The non-embryogenic calluses were pale yellow greenish in color and compact. Non-embryogenic calluses induced on medium containing 3 mg l^{-1} 2,4-D and incubated either under light (callus A) or under dark (callus B) were able to form shoots. A combination of 2 mg l^{-1} BA and 0.5 mg l^{-1} NAA promoted the highest number of shoots (5.3 and 4.3) for callus A and callus B, respectively. In general, callus A regenerated higher number of shoots than callus B (Fig. 2) which indicated that light had a stimulatory effect on shoot regeneration of *A. unedo*. Many reports have provided evidence

Table 2. Effects of light regime and plant growth regulators on callus induction and somatic embryogenesis of *A. unedo* after 8 weeks of culture incubation.

PGRs concentration (mg l^{-1})		Callus formation (%)	Callus fresh weight (mg)	No. of somatic embryos per callus
		Dark		
Control		0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
NAA	1.0	100 ± 0.00 a	29 ± 0.19 e	0.0 ± 0.00 d
2,4-D	1.0	92.3 ± 0.84 b	26 ± 1.00 e	0.0 ± 0.00 d
2,4-D	3.0	100 ± 0.00 a	37 ± 0.50 d	0.0 ± 0.00 d
TDZ	2.0	53.5 ± 0.96 e	60 ± 0.19 a	0.0± 0.00 d
TDZ	4.0	74.5 ± 0.38 d	35 ± 0.33 d	0.0 ± 0.00 d
2,4-D 2.0 + NAA 0.5		100 ± 0.00 a	33 ± 0.17 d	0.0 ± 0.00 d
BA 5.0 + NAA 5.0		94.5 ± 0.51 b	36 ± 1.52 d	17.0 ± 0.88 a
2,4-D 1.0 + Kin 2.0 + IAA 1.7		100 ± 0.00 a	44 ± 1.33 c	10.7 ± 0.38 b
2,4-D 1.0 + BA 5.0 + NAA 5.0		88.3 ± 0.96 c	43 ± 1.00 c	10.3 ± 0.19 b
		Light		
Control		0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
NAA	1.0	0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
2,4-D	1.0	0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
2,4-D	3.0	95.0 ± 1.60 b	52 ± 1.33 b	0.0 ± 0.00 d
TDZ	2.0	0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
TDZ	4.0	0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
2,4-D 2.0 + NAA 0.5		0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
BA 5.0 + NAA 5.0		0.0 ± 0.00 f	0.0 ± 0.00 f	0.0 ± 0.00 d
2,4-D 1.0 + Kin 2.0 + IAA 1.7		100.0 ± 0.00 a	43 ± 1.00 c	8.3 ± 0.50 c
2,4-D 1.0 + BA 5.0 + NAA 5.0		100.0 ± 0.00 a	53 ± 1.56 b	7.7 ± 0.19 c
Significance				
Light regime (LR)		***	***	***
PGRs		***	***	***
PGRs × LR		***	***	***

Values are means ± standard error. Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test.
*** significant at $p \leq 0.001$.

that photoperiod significantly impacted regeneration. In most cases, culture of the explants in darkness for a certain period seemed to be beneficial for shoot regeneration (Gu and Zhang 2005, Espinosa et al. 2006). Exposure to a dark period may possibly modify the ratio of hormones like cytokinins and auxins, which interact with PGRs in the culture medium (Gentile et al. 2002). Photoperiod might affect the regeneration frequency or regeneration pattern (direct or indirect). For example, indirect shoot regeneration of *Citrus sinensis* from callus was detected under darkness, while direct induction of shoot buds took place only under 16 h photoperiod

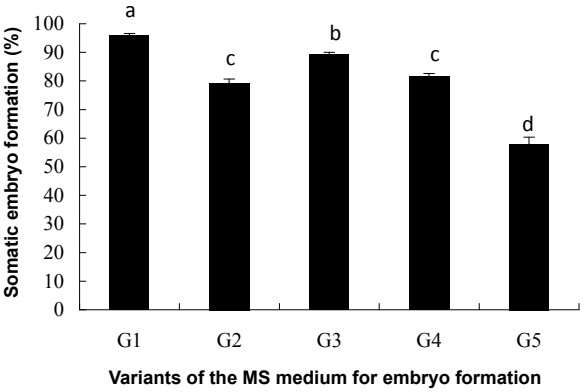

Fig. 1. Effect of BA and NAA concentrations on somatic embryo formation (G1: PGR- free medium, G2: BA (1.0 mg l^{-1}) + NAA (0.5 mg l^{-1}), G3: BA (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}), G4: BA (2.0 mg l^{-1}) + NAA (1.0 mg l^{-1}), G5: BA (5.0 mg l^{-1}). Values are means ± standard error. Different letters above bars indicate significant differences ($p \leq 0.05$) among means across all treatments.

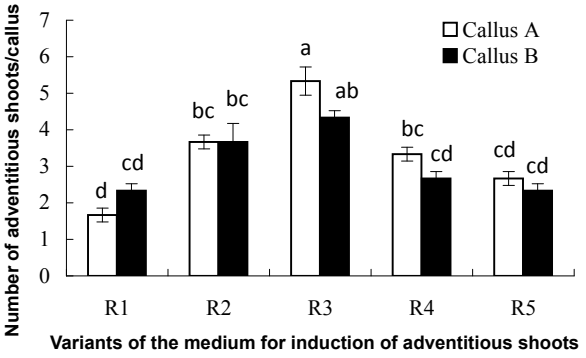

Fig. 2. Effect of BA and NAA concentrations on the adventitious shoot formation (R1: PGR-free medium, R2: BA (1.0 mg l^{-1}) + NAA (0.5 mg l^{-1}), R3: BA (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}), R4: BA (2.0 mg l^{-1}) + NAA (1.0 mg l^{-1}), R5: BA (5.0 mg l^{-1}). Values are means ± standard error. Different letters above bars indicate significant differences ($p \leq 0.05$) among means across all treatments.

(Almeida et al. 2003). Nodular calluses of *A. unedo* were observed after 4 week of culture and small green meristems were visible on the surface of calluses after 4 week of culture. These meristems developed into shoot buds which developed into leafy shoots by the end of 8 weeks on the same medium.

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