Effect of different nutritional basal media culture on *in vitro* development of *Arbutus unedo* L.

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Abstract

Strawberry tree, *Arbutus unedo* L., is a species widely represented in the Mediterranean climates of southern Europe, being a very economically, medical, and ecological interesting species, been known to withstand some hard climate conditions. The aim of this study was to evaluate the *A. unedo* sensibility according to different nutritional basal media culture in *in vitro* conditions. Based on it, three different basal media were tested: Murashige and Skoog (MS), Woody Plant Medium (WPM) and Driver and Kuniyuki (DKW), with the respective vitamin composition. The culture media were supplemented with 4.44 µM 6-Benzylaminopurine (BAP) and 30gL^{-1} sucrose, with pH adjusted to 5.6 before autoclaving. Biometrics characteristics were evaluated (number of shoots, longest shoot length and multiplication factor) on three *in vitro* cultivation cycles (with 30 days each). None of the variables presented significant differences, regardless the nutritional basal media used. It was also noticed, based on visual analysis of leaves, no indicative of nutritional deficiency (absence) or nutritional toxicity (excess). These results indicate that *A. unedo in vitro* performance is quite independent of the nutritional basal media culture used. These results confirm that this species is very resilient and with a high plasticity in terms of adaptability to different nutritional composition. Therefore, based on these results, is possible to state that *A. unedo* is not responsive to the nutritional basal media used during *in vitro* development and then, the efforts to get development improvement should be focused on finding the more appropriate growth regulators combination. Moreover, finding better adjust of some physical parameters applied on *in vitro* growth condition for this species could improve its performance (light, temperature and photoperiod).

Keywords: strawberry tree, micropropagation, BAP, nutritional media, plasticity.

Resumo

Efeito de diferentes formulações nutricionais de meios de cultura no desenvolvimento *in vitro* de *Arbutus unedo* L.

O medronheiro, *Arbutus unedo* L., é uma espécie amplamente representada nos climas mediterrâneos do sul da Europa, com importante interesse económico, medicinal e ecológico, sendo também conhecida por suportar situações climáticas adversas. O objetivo deste estudo foi a utilização de diferentes formulações nutricionais de meios de cultura *in vitro* aplicados em micropropagação, de forma a avaliar a sensibilidade do *A. unedo* durante o seu crescimento e desenvolvimento *in vitro*. Com base nisso, três conhecidas formulações nutricionais foram testadas: Murashige e Skoog (Meio MS), Woody Plant Medium (meio WPM) e Driver e Kuniyuki (meio DKW) com respetivas composições vitamínicas. Os meios de cultura foram suplementados com 4,44 µM de 6-

Benzilaminopurina (BAP) e 30gL^{-1} de sacarose, com o pH ajustado a 5,6 antes da autoclavagem. Foram realizados três ciclos de cultivo (com 30 dias cada), acompanhados de avaliações biométricas (número de rebentos, comprimento do maior rebento e fator de multiplicação). Os resultados demonstraram que nenhum dos parâmetros avaliados apresentou diferenças significativas, independentemente da formulação nutricional utilizada no meio de cultura basal. Também foi observado, com base na análise visual das folhas, que não ocorreram indicadores de deficiência ou toxicidade nutricional. Os resultados deste estudo indicam que, para os meios testados o desempenho *in vitro* de *A. unedo* é, em algum nível, independente do meio nutricional basal, confirmando que esta é uma espécie muito resiliente e com uma elevada plasticidade em termos de adaptabilidade às diferentes composições nutricionais. Com base nos resultados é possível afirmar que *A. unedo* não é exigente em termos de constituição do meio nutricional para seu desenvolvimento *in vitro* e que os esforços para melhorar a sua performance *in vitro* podem ser focados noutros parâmetros, como por exemplo nos reguladores de crescimento, e/ou nos fatores físicos de controlo de ambiente (luz, temperatura ou fotoperíodo).

Palavras-chave: medronheiro, micropropagação, BAP, meio nutricional, plasticidade.

Introduction

Commonly known as strawberry tree, *Arbutus unedo* is a species native to Ireland, southern Europe, and the western Mediterranean region where it grows spontaneously in several countries (Piotto et al., 2001). Strawberry tree is a most interesting species economically, with its fruits being edible and traditionally used to make a strong-tasting spirit called "medronheira" (Gomes and Canhoto, 2009). In folk medicine, the fruits and leaves have been used as an astringent, diuretic, antirheumatic and against urinary infections (Cardoso, 2004). From an ecological view, strawberry tree contributed to maintain biodiversity of the fauna, helps to stabilize soils, can be used for land recovery, and it is fire resistant with a strong regeneration capacity (Pedro, 1994; Piotto et al., 2001; Godinho-Ferreira et al., 2005). Moreover, *A. unedo*, can withstand low temperatures and is tolerant to drought (Piotto et al., 2001). In recent years, it has been observed an increasing demand for *A. unedo* plants, which led to a request for a more effective propagation protocols. Micropropagation could present a valuable alternative to the conventional methods, increasing the multiplication rates (McComb and Bennett, 1986; Gonçalves and Roseiro, 1994; Gomes and Canhoto, 2003). Previous studies have shown that *in vitro* shoot proliferation of *A. unedo* could be accomplished using shoot explants from either potted greenhouse plantlets or from adult plants (Mereti et al., 2002; Gomes and Canhoto, 2009). Cytokinins are usually used on the micropropagation media to stimulate shoot proliferation, and promising results were obtained on strawberry tree with the application of 6-Benzylaminopurine (BAP) or thidiazuron (TDZ) to the culture media (Gomes and Canhoto, 2009; El-Mahrouk et al., 2010). The reports for micropropagation on *A. unedo* are yet scarce, and although Gomes and Canhoto (2009) presented a reliable protocol for shoot proliferation on adult trees, there still some variables, such as mineral composition of the culture media, that must be considered for the development of a high efficient micropropagation protocol. The aim of this study was to evaluate the effects of different nutritional basal media on the *in vitro* propagation of *A. unedo*, using explants obtained from an established *in vitro* culture.

Material and methods

Plant material and culture conditions

Strawberry tree (*Arbutus unedo*) explants from the same genotype (clone LV2) previously growing in *in vitro* culture were used for the propagation studies in this work. The explants with two nodal segments were prepared and transferred on three different nutritional basal media: Treatment 1 (T1), Murashige and Skoog (MS) medium, Treatment 2 (T2), Woody Plant Medium (WPM) and Treatment 3 (T3) Driver and Kuniyuki (DKW) medium, with the respective vitamin composition. All three culture media were supplemented with 4.44 μM BAP and 30gL^{-1} sucrose, with pH adjusted to 5.6 before autoclaving. Strawberry tree cultures were maintained at 23 °C ± 2 °C, for 16 h photoperiod with a photosynthetic photon flux density of 50 μmol $m^{-2}s^{-1}$. The media substitution protocol was carried out every 4 weeks for 3 months (3 cycles). The multiplication ability of the culture media was evaluated by the number of shoots per explant, shoot elongation and aspect, and finally multiplication factor (MF = nodal segments number with 1.5 cm obtained from each explant).

Data collection and statistical analysis

Each treatment had 50 replicates, with the previous mentioned parameters being evaluated every month for each treatment. The effect of the three treatments on shoot number, shoot elongation and MF over the 3 culture cycles were evaluated by a one-way analysis of variance (ANOVA) (p<0.05), followed by the Tukey HSD *Post-hoc* test, since data normality was observed and the variances of the groups were homogeneous.

Results and discussion

At the end of three months, after three *in vitro* subculture (30, 60 and 90 days), no morphological differences were found among explants from the different treatments (Figure 1).

The number of new shoots obtained on each *in vitro* cycle, indicates that no significant differences were found among treatments, which shows that, although BAP at 4.44 μM was found for shoot proliferation in this species, the difference of nutritional basal media used on this work are irrelevant for this parameter (Figure 2).

Gomes and Canhoto (2009), indicated that macronutrients composition, particularly levels of nitrogen (NH_4^+ and NO_3^-) could influence the shoot multiplication rates, however on this study, regardless the composition variances (MS medium with 1,650mg.L^{-1}, DKW with 1,416mg.L^{-1} and WPM medium with 400mg.L^{-1} of NH_4NO_3), no differences were found among basal media used. These results require more assays in order to better understand the role of nitrogen and other compounds on *A. unedo in vitro* propagation.

The longest shoot length among treatments was the only variable that appears to be influenced by the basal media used, with treatment with MS nutritional basal medium being statistically different from the other treatments (Figure 3). These results could be once more related to the nitrogen levels on the media, since some studies related the amount and type of nitrogen with *in vitro* morphogenic processes (Young and Cameraon, 1985; Dal Vesco and Guerra, 2001).

Regarding the multiplication factor (MF), the most important variable evaluated for strawberry tree *in vitro* propagation, no significant differences were found between

treatments (Figure 4). Each basal medium used presented similar number of new propagation explants, indicating that regardless the nutritional basal media used for *in vitro* propagation, this species presented related multiplications rates. Although shoot elongation on MS medium showed up to be higher, that do not correspond to a significant higher MF, which could be related that a higher shoot length do not directly correspond to a higher number of axillary shoots, which leads to a similar number of ideal propagation explants for every nutritional basal medium used. These results contradicted those found by Mereti et al. (2002), which showed that woody plant medium (WPM) combined with BAP gave the best rates of shoot proliferation for *A. unedo* when compared with MS nutrients. Nonetheless, the genotype used can also influence *in vitro* response and thus its effect should not be excluded (Gomes and Canhoto, 2009).

Although it was not evaluated, no differences were found among treatments regarding the number of abnormal shoots, or hyperhydricity, with only four explants found with these characteristics between the different nutritional basal media used.

Conclusions

Our results led to the confirmation that *A. unedo* is, in fact, a species with a high plasticity, which promotes a good adaptability to different composition, thus nutritional basal media does not seemed to influence significantly the *in vitro* multiplication and development of *A. unedo*. Therefore, and although BAP was found to promoting the best multiplication rates for *A. unedo*, the use of other cytokinins and auxins may result in a more effective multiplication rates, and further studies should be focused on this. Other conditions such as genotype, carbon source and physical parameters should also be considered.

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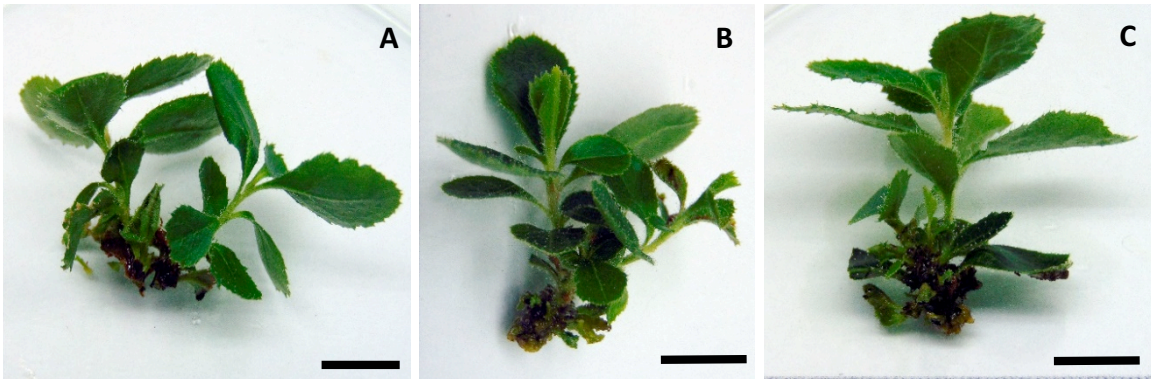

Figure 1 - Morphological aspect among explants from different treatments: **A** (MS nutritional basal medium); **B** (WPM nutritional basal medium) and; **C (**DKW nutritional basal medium). Bars = 1 cm.

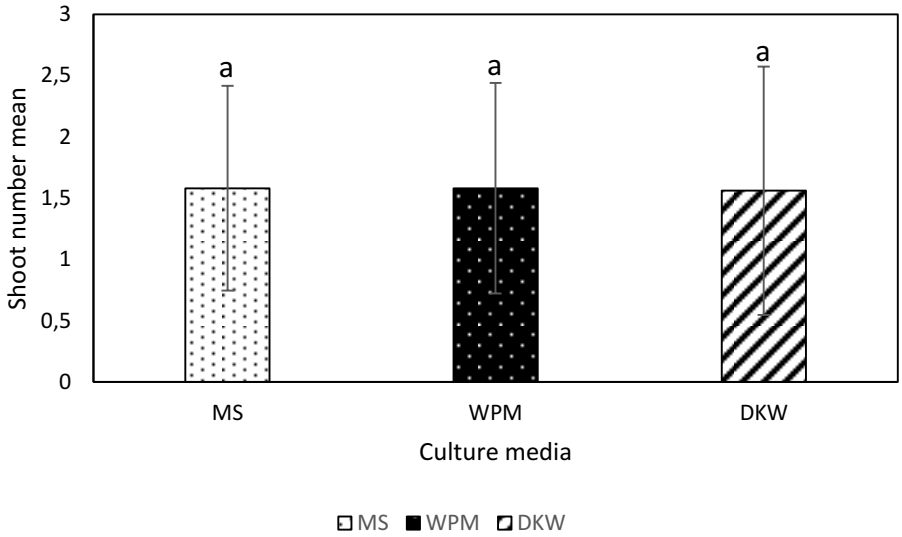

Figure 2 – Average number of new emitted shoot number mean among treatments (MS, WPM and DKW nutritional basal media) at the end of three months, after three *in vitro* subculture (30, 60 and 90 days). Different letter represents statistical different ($p<0.05$).

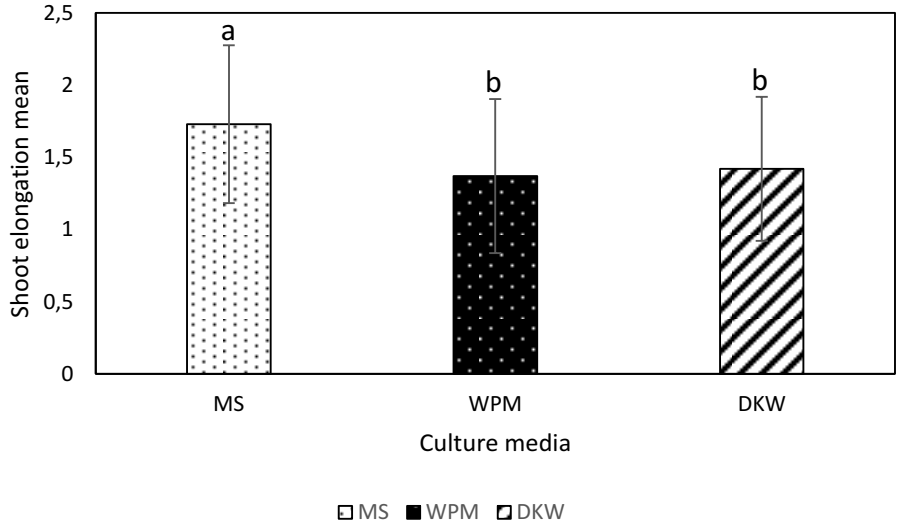

Figure 3 - Shoot elongation mean among treatments (MS, WPM and DKW nutritional basal media) at the end of three months, after three *in vitro* subculture (30, 60 and 90 days). Different letter represents statistical different ($p<0.05$).

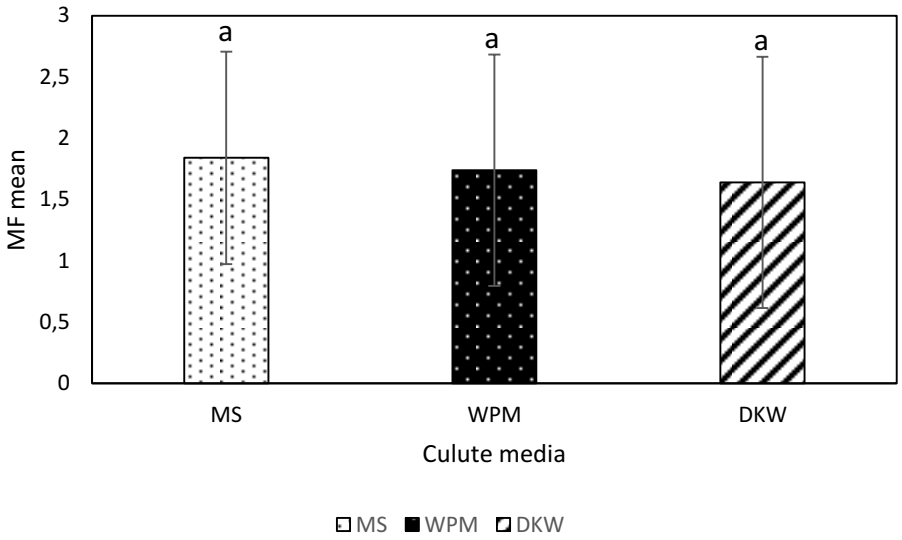

Figure 4 - MF mean among treatments (MS, WPM and DKW nutritional basal media) at the end of three months, after three *in vitro* subculture (30, 60 and 90 days). Different letter represents statistical different ($p<0.05$).