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Shoot proliferation and organogenesis on *Arbutus unedo*: physiological analysis under water stress

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Abstract

Strawberry tree (*Arbutus unedo*) is a small perennial tree that grows spontaneously in the Mediterranean basin, Ireland, and Portugal. In this work, strawberry tree clones were established *in vitro* from epicormic shoots obtained from a young tree, an adult tree, and from a seedling. They were propagated by axillary shoot buds proliferation on solid and in liquid media, and also in a modified De Fossard medium with 9 μM benzylaminopurine. The organogenesis from calli obtained from apical leaves of the *in vitro* grown shoots from the three genotypes was carried out in the same basal liquid medium supplemented with 9 μM thidiazuron. Micropropagation through organogenesis in liquid medium proved to be more efficient than the other tested methods (considering the number of shoots produced), but the shoots were showing hyperhydricity. Shoots were sucessufully rooted on medium with indole-3-butyric acid and acclimatized *ex vitro* with rates higher than 90 %. Six month-old plants from the most proliferative genotype (AU1) and propagated *in vitro* by different methods were submitted to drought stress (no watering for 10 d) and several morphological and physiological parameters were evaluated and compared to a control group (watered to 70 % field capacity). No significant differences were found in plant biomass, root length, and plant height, however, slight differences were observed in water potential, net photosynthetic rate, intercellular CO_2 concentration, and stomatal conductance between the plantlets propagated on solid or liquid medium. In general, the responses to drought stress imposed were was similar in plants micropropagated by different propagation methods.

Additional key words: chlorophyll fluorescence, photosynthesis, stomatal conductance, strawberry tree, transpiration, water potential.

Introduction

Strawberry tree (*Arbutus unedo* L.) is an evergreen shrub or small tree (3 - 8 m) of the *Ericaceae* family native to the Mediterranean countries. It is also found in Portugal, Southern Ireland, and other regions where it has been introduced (Russell *et al*. 2007). *Arbutus unedo* is fairly tolerant to low temperatures (-12 °C) and drought (Piotto *et al*. 2001). These characteristics make *A. unedo* interesting from an ecological point of view since it can be grown in poor marginal lands where other plants hardly thrive. The ability to sprout following fires is also important (Quevedo *et al*. 2013). Strawberry tree fruits are round red berries and have interesting nutritional properties (Özcan and Haciseferoğullai 2007, Ruiz-Rodríguez *et al*. 2011, Miguel *et al*. 2014, Oliveira and Franco 2015). The fruits are mostly used in the production of a distilled beverage (Botelho and Galego 2015). The strawberry tree has been considered as neglected and underutilized crop (NUC) species. In order to meet the high yield expected by producers, selected and improved plant materials need to be available. For this reason, our group has been working on the micropropagation and breeding of *A. unedo* plants (Martins and Canhoto 2014,

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Abbreviations: BA - benzylaminopurine; c_i - intercellular CO_2 concentration; E - transpiration rate; F_v/F_m - variable to maximum fluorescence ratio; g_s - stomatal conductance; IBA - indole-3-butyric acid; P_N - net photosynthetic rate; TDZ - thidiazuron; WS - water stress; WW - well watered; Ψ - water potential; ϕPS II - maximum yield of photosystem II.
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Martins *et al*. 2015, 2016). Several protocols for strawberry tree cloning through axillary shoot proliferation (Mereti *et al*. 2002, Gomes and Canhoto 2009, Gomes *et al*. 2010), and somatic embryogenesis (Martins 2012, Martins *et al*. 2015, 2016) have been developed. In this work, a new protocol for strawberry tree shoot proliferation in liquid medium and organogenesis was developed, and different micropropagation methods were tested, and their potential for mass scale propagation was evaluated. As referred by De Klerk and Ter Brugge (2011), these protocols are only profitable, from the economic point of view, if the performance of *in vitro* derived plants to field conditions is adequate. Water is the most limiting factor for plant acclimatization and growth on field conditions (Guarnaschelli *et al*. 2012) even in species recognized as drought tolerant as *A. unedo* (Munné-Bosch and Peñuelas 2004). Since strawberry tree orchards are usually established on dry and poor soils it is essential to assure drought tolerance of the micropropagated plants (Allen *et al*. 2010). Work done with strawberry tree wild plants on field conditions under water stress has shown that plants follow a conservative water use strategy (Castell and Terradas 1994, Ogaya *et al*. 2003, Munné-Bosch and Peñuelas 2004). However, and as far as we know, the performance of these plants obtained through micropropagation under water deficit conditions has not been tested before.

The main objective of this work was to compare different microprogation protocols for the production of strawberry tree and analyse the effect of these protocols on plant performance under drought stress.

Materials and methods

***In vitro* establishment:** Three phenotypes of *Abrutus unedo* L. were selected: AU1 (young tree less than 5-year-old and a shrub-like growing type), AU2 (adult tree more than 10-year-old), and AU3 (seedling). The AU1 and AU2 were propagated *in vitro* using young branches from two different trees that were cut, dipped in 100 mg dm^{-3} fungicide *Aliette* (*Bayer CropScience*, Carnaxide, Portugal) for 10 min, and rinsed with distilled water. After this treatment, branches were kept in containers, covered with a plastic bag, watered with distilled water and placed in a culture chamber at a 16-h photoperiod, an irradiance of 250 µmol m^{-2} s^{-1}, a temperature of 25 ºC, and an air humidity of 75 % for 30 d. The epicormic shoots formed were removed and surface sterilized with 70 % (v/v) ethanol (30 s) and 5 % (m/v) calcium hypochlorite solution (*Sigma-Aldrich*, St. Louis, MO, USA) for 10 min with two drops of *Tween 20*. After 3 washes with sterile distilled water, the epicormic shoots (0.5 - 1 cm) were inoculated in a medium with De Fossard macronutrients and vitamins (De Fossard *et al.* 1974), Murashige and Skoog (1962; MS) microelements, 9 µM benzylaminopurine (BAP; *Sigma-Aldrrich*), 3 % (m/v) sucrose (*Duchefa Biochemie*, Haarlem, The Netherlands) and 0.6 % (m/v) agar (*Duchefa*) and grown in test tubes (25 × 150 mm) with plastic caps (*Duran*, Mainz, Germany). The culture medium was autoclaved at 121 ºC for 20 min and pH was adjusted to 5.7 using KOH or HCl before autoclaving and agar addition. For micro-propagation of AU3, seeds after 1 month of cold stratification (4 ºC) were disinfected following a similar methodology as for shoots and germinated in Petri dishes on filter paper discs moistened with sterile distilled water in a growth chamber set to conditions mentioned above. The obtained seedlings were selected and multiplied by shoot proliferation in the medium described before.

Axillary shoot proliferation: For axillary shoot proliferation in solid medium (SM), AU1, AU2, and AU3 shoots were cultured in test tubes with 10 cm^3 of the medium described before (one explant per tube and 30 tubes per test group). Shoot proliferation in liquid medium (LM) was carried out in Erlenmeyer flasks (100 cm^3) with 25 cm^3 of the same medium without the gelling agent (30 shoots were divided into 5 flasks for each test group). The flasks were placed on an orbital shaker (80 rpm). The cultures were kept in a growth chamber, subculture intervals were 8 weeks. After 6 subcultures, micropropagation rates of the three genotypes were evaluated by the number of shoots longer than 1 cm obtained per initial explant.

Organogenesis: For micropropagation through organogenesis in liquid medium (LMO), calli with organogenic capacity were obtained from apical leaves from shoots obtained by shoot proliferation mentioned before. Leaves were cultured in the liquid medium described before, but supplemented with 9 µM thidiazuron (TDZ; *Sigma-Alrich*), for a month (Martins and Canhoto 2014). Transversal cuts (4 - 6) were made in the central part of the leaves, before they were placed with the abaxial side down on the culture medium. After 2 months, the obtained calli were transferred to liquid medium with the same composition and under the same conditions as for the shoot proliferatrion on liquid media.

Rooting and acclimatization: For root induction, 3 cm long shoots were cultured in test tubes, in a medium with KNOP salts (Gautheret 1959), De Fossard vitamins without riboflavin, MS microelements without KI, and 24.7 µM indole-3-butyric acid (IBA; *Sigma-Aldrich*) at 25 ºC under the dark for 10 d. Shoots were then transferred to a medium with the same composition but without IBA,

1 % (m/v) charcoal (*Sigma-Aldrich*) was added and the shoots were grown in a growth chamber at a 16-h photoperiod, an irradiance of 15 - 20 µmol m^{-2} s^{-1} (cool-white fluorescent lamps), and a temperature of 25 ºC for 3 weeks. Rooting rates were calculated and the number of roots per shoot was counted (only roots longer than 1 mm), before plant acclimatization in covered containers with *Perlite* (*Siro*, Mira, Portugal) moistened with water in a culture chamber (*FitoClima 10000 HP*, Aralab, Portugal) under a 16-h photoperiod, an irradiance of 250 µmol m^{-2} s^{-1}, a temperature of 25 ºC, and 70 % relative humidity. The container cover was gradually removed and after a month, acclimatization rates were calculated and plants transferred to individual 200 cm^3 containers with peat and sand (1:1, v/v).

Drought stress assays: From the acclimatized six-month-old plants, obtained by the three methods of *in vitro* propagation, with at least 10 well developed leaves, 6 plants were randomly chosen and placed under two water regimes: WW - well watered (watered to 70 % field capacity) or WS - water stressed (without watering). After 10 d under these conditions plant performance was evaluated based on growth and physiological parameters. Only genotype AU1 was used in this assay, since it presented the highest multiplication rates at all propagation methods tested.

Root length and plant height were measured at the end of the WS period. Total plant dry mass was determined after drying the samples at 70 °C until constant mass. Leaf area was determined using *ImageJ* (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016). Water potential (Ψ) was measured with a Scholander-type pressure chamber (*PMS Instrument Co*., Albany, OR, USA). Plant water status was further assessed by the determination of the relative water content (RWC) calculated as [(fresh mass - dry mass)/(water saturated mass - dry mass)] × 100. Water saturated mass was determined after 24 h on distilled water at 4 °C, and dry mass after drying at 70 °C for 48 h.

In situ leaf gas exchange measurements (net photosynthetic rate, P_N, transpiration rate, *E*, intercellular CO_2 concentration, c_i, and stomatal conductance, g_s) were performed on apical fully expanded leaves using a portable infrared gas analyser (*LCpro+*, *ADC*, Hoddesdon, UK) operating in open mode under the following conditions: irradiance of 350 µmol m^{-2} s^{-1}, air flow of 200 mol s^{-1}; block temperature of 25 ºC, and atmospheric CO_2 and H_2O concentrations. Data were recorded when the measured parameters were stable (after 2 - 6 min).

A Chl *a* fluorescence was determined *in situ* in fully expanded leaves, with a portable fluorimeter *Mini-PAM* (*Walz*, Effeltrich, Germany) as described by Jesus *et al*. (2015). Values of F_v/F_m.= $(F_m - F_0)/F_m$ and $\Phi_{PSII} = (F'_m - F'_0)/F'_m$ were determined as described in this paper.

Statistical analysis: Values were given as means ± standard deviantions of 30 replicates for micropropagation assays, and 6 replicates for drought stress. Micro-propagation data were analyzed by one way *ANOVA* (*GraphPad Prism* for *Windows v. 6.01*), followed by a Tukey´s multiple comparison test ($P < 0.05$). Comparisons of eco-physiological parameters of the plants submitted to two water regimes was done by a Student *t*-test.

Results

Strawberry tree was successfully micropropagated on solid and in liquid medium (Fig. 1*A,B*) and statistically significant differences were found in the number of shoots obtained in both treatments in the three genotypes tested (AU1, AU2, and AU3). However, the number of shoots obtained using organogenesis was substantially higher when compared to shoot proliferation. Although this difference was observed in all three tested genotypes, it was higher for genotype AU2, especially when compared to the propagation on solid medium, as the number of shoots obtained by organogenesis was almost 8 times higher (15.57 ± 2.21) compared to axillary shoot proliferation (2.20 ± 0.40; Fig. 2*A*). Despite the highest multiplication rates achieved, hyperhydricity was observed on some of the shoots obtained on liquid medium, either by shoot proliferation or organogenesis, causing phenotypic differences, such as abnormal leaf shape and lower chlorophyll content (data not show).

The rooting rates obtained were mostly higher than 80 % in most of the groups tested and no statistically significant differences were observed among propagation methods. Considering plants produced on solid medium the rooting rate was of 100 % for genotype AU2. Moreover, the number of roots was similar on the plants produced on liquid and in solid medium, except in genotype AU2, where an average of 13.87 ± 5.8 roots was observed in shoots obtained on solid medium, compared to 5.2 ± 0.8 in liquid medium (Fig. 2*C*). Throughout the rooting process, most of the plants showing hyperhydricity and corresponding modifications recovered their normal phenotype.

In all groups, most of the plants survived after the acclimatization (Fig. 1*C*), and no differences were found among propagation methods, with rates higher than 80 %, except for plants produced through organogesis from genotype AU3, established *in vitro* from a seedling (Fig. 2*D*). Three months after acclimatization, the plant height and leaf area of those obtained by shoot proliferation, both in solid and on liquid medium, was very similar. However, the plants obtained through organogenesis showed lower height (Fig. 3*A*) and smaller leaf area (for genotypes AU2 and AU3, Fig. 3*B*).

Mostly no significant differences were found in total plant biomass, height and root lenght under WW and WS regimes (Fig. 4*D-F*) in plants obtained by different micropogation methods. Similar results were also obtained regarding leaf area, although sligtly less values were found for plants propagated on solid medium (Fig. 4*C*).

Fig. 1. Micropropagation of strawberry tree by axillary shoot proliferation on solid medium (*A*) or by organogenesis in liquid medium (*B*), and *ex vitro* acclimatized plants after three months (*C*) (*bar* = 1 cm).

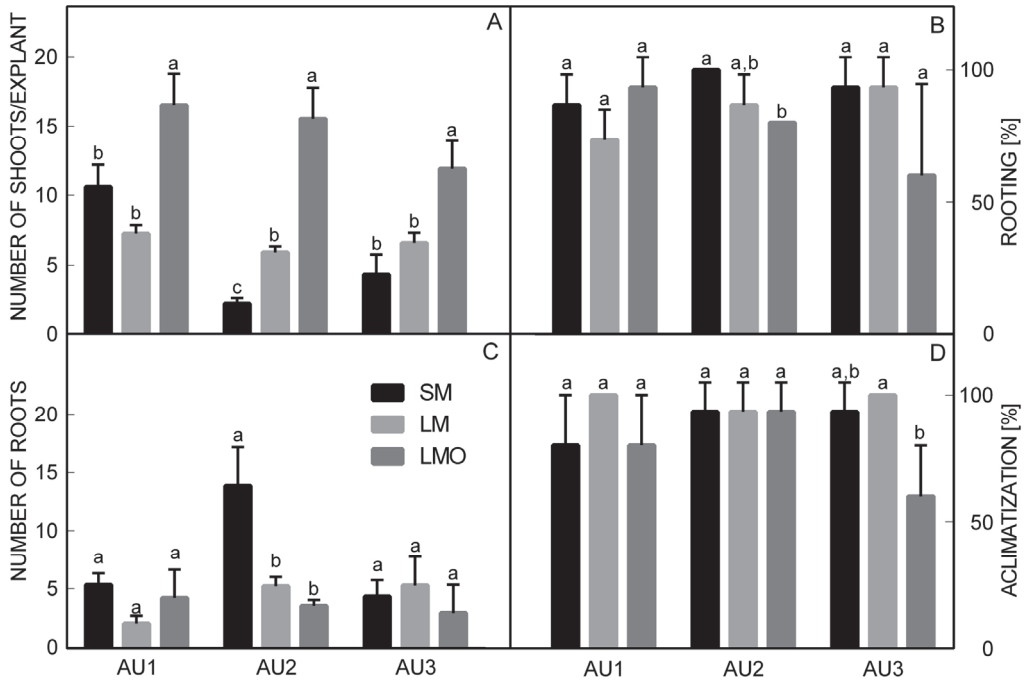

Fig. 2. Number of shoots per explants (*A*), rooting rate (*B*), number of roots per plantlet (C), and acclimatization percentage (*D*) in genotypes AU1, AU2, and AU3 by using three micropropagation methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium). Means ± SDs, $n = 3$, on each genotype different letters indicate significant differences between treatments at $P \leq 0.05$.

Water potential was lower on plants under drought stress conditions, when compared to well-watered plants. However, it was statistically different only for plants produced in liquid medium by axillary shoot proliferation (Fig. 4*A*) **check values!**.Very slight differences were found in RWC and only in plants propagated on solid medium (Fig. 4*B*).

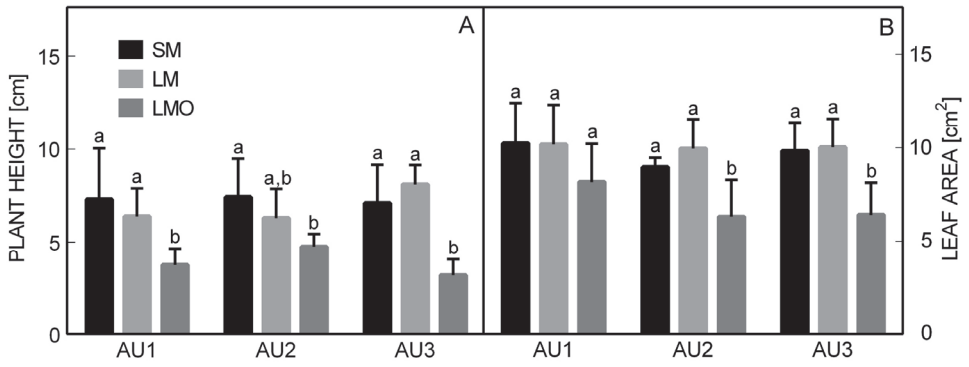

Fig. 3. Effect of three micropropagation methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium) on height (*A*) and leaf area (*B*) of plants of different genotypes (AU1, AU2, and AU3) measured three months after acclimatization. Means ± SDs, $n = 3$, different letters indicate significant differences between treatments at $P \leq 0.05$.

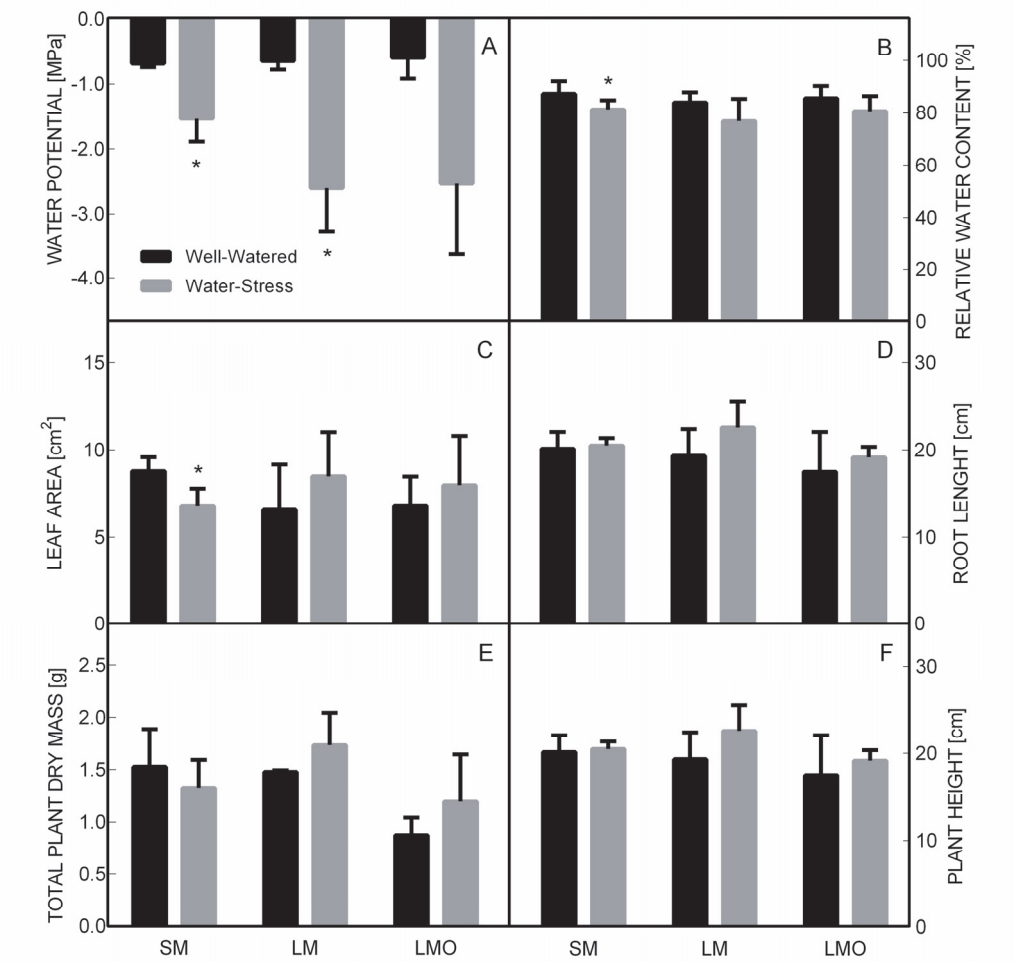

Fig. 4. Effects of water stress (WS) imposed to AU1 plants propagated by three methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium) and acclimatized for 6 month on water potential (*A*), relative water content (*B*), leaf area (*C*), root length (*D*), total plant dry mass (*E*), and plant height (*F*) in comparison with well watered plants (WW). Means ± SDs, $n = 5$,* indicate significant differences between treatments at $P \leq 0.05$.

As concern gas exchange parameters, stomatal conductance, intercellular CO_2 concentration, and net photosynthetic rate (Fig. 5*ACD*) were decreased under water deficit, especially when the plants were obtained by axillary shoot proliferation on solid and liquid medium. No marked differences were found in transpiration rates (Fig. 5*B*). The chlorophyll fluorescence parameters, the yield of photosystem II under steady-state conditions and maximum efficiency of photosystem II were very similar in all treatments (Fig. 5*E*,*F*). Only a slight increase in ϕPS II was observed for plants from solid medium when submitted to water stress.

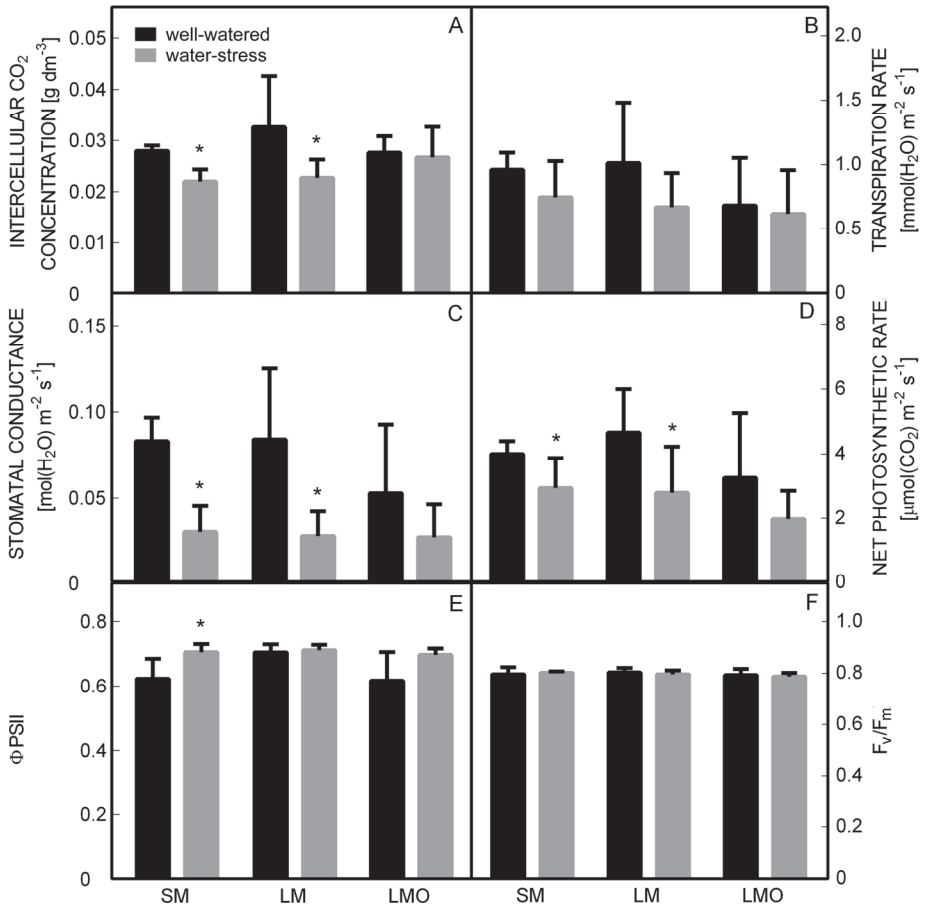

Fig. 5. Effects of water stress (WS) imposed to AU1 plants propagated by three methods (SM - axillary shoot proliferation on solid medium, LM - axillary shoot proliferation on liquid medium, LMO - organogenesis on liquid medium) and acclimatized for 6 month on intercellular CO_2 concentration (*A*), transpiration rate (*B*), stomatal conductance (*C*), net photosynthetic rate (*D*), and chlorophyll fluorescence parameters quantum yield of photosystem II (*E*) and photochemical efficincy of PS II (*F*) in comparison with well watered plants (WW). Means ± SDs, $n = 5$,* indicate significant differences between treatments at $P \leq 0.05$.

Discussion

The protocol developed for the micropropagation of strawberry tree in liquid medium was proved to be very efficient. Similar results have been reported for some other species, such as *Camellia sinensis* (Sandal *et al.* 2001), *Dioscorea japonica* (Kadota and Niimi 2004), and *Catharanthus roseus* (Pati *et al.* 2011). These kinds of protocols tend to be more effective when compared to the ones on solid medium in terms of proliferation rates, shoot size, and biomass. This might be related to a more efficient uptake of nutrients due to the large area of contact with the medium, since nutrients can be uptaken also by leaves *via* stomata and aqueous pores (De Klerk and Ter Brugge 2011) and even through the cuticule, which in leaves of *in vitro* growing shoots or plantlets is thin and may allow the change between the culture medium and plant tissues (Dias Ferreira *et al*. 2003). However, some of the drawback observed on strawberry tree, particularly the hyperhydricity of shoots, were also mentioned previously (Sandal *et al.* 2001, Kadota and Niimi 2004, Pati *et al.* 2011). Although some phenotypic differences were observed in strawberry tree plants produced in liquid medium either by shoot proliferation or organogenesis

(Martins and Canhoto 2014), they were only a transitory and according to the parameters evaluated six-month after *ex vitro* transfer, they did not affect overall plant performance when submitted to drought stress.

The genotype plays and important role on micropropagation, as it has already been reported for strawberry tree (Gomes *et al*. 2010) as well as for other species such as *Paeonia suffruticosa* (Beruto *et al*. 2004) and *Hagenia abyssinica* (Feyissa *et al*. 2005). Our results show that organogenesis was the most effective method for the propagation of all strawberry tree genotypes. However, the results also show that different genotypes behaved differently accordingly to micropropagation method tested. For example, all the genotypes seemed to display no differences when the organogenesis was used whereas more clear differences were found when shoot proliferation was used. This might be due to inadequate culture conditions for specific genotypes, such as medium pH or nutrient composition as well as added plant growth regulators (Gomes *et al*. 2010). The age of the mother plant, as well as its physiological condition, may also be important factors for micropropagation efficiency (Chowdhury *et al.* 2004, Shukla *et al.* 2013). In fact, genotype AU1 (established *in vitro* from a young tree), has higher micropropagation rate by axillary shoot proliferation on solid medium when compared to genotype AU2, whose mother plant is an older tree.

In the present study, no significant morphological differences were observed among the plants acclimatized *ex vitro* for 6 months, which were originally obtained by the different micropropagation methods and also when they were submitted to different water regimes. A reduction in growth was reported in previous studies when strawberry tree plants and seedlings were submitted to a water deficit (Ogaya *et al.* 2003, Ogaya and Peñuelas 2004, Vasques *et al.* 2013). This is a common response to dry environments, as plants tend to increase belowground biomass allocation in order to improve water uptake. However, no differences were observed in this assay in terms of biomass allocation, probably due to the short duration of the experiment because some time is required to activate the complex mechanisms induced by drought stress (Chaves 2002, Chambel *et al.* 2005). Despite that, a slight reduction in leaf area was observed in plants produced by shoot proliferation on solid medium when submitted to water limitation. The reduced investment in foliar area is a relevant strategy to cope with water deficit conditions (Lopez *et al*. 1997).

In general, the physiological parameters evaluated in plants produced by different propagation methods were not significantly different after imposition of mild water stress, and were in accordance with the values previously reported (Castell and Tarradas 1994, Vasques *et al.* 2013). Many of them were not significantly different between two water regimes. However, the water potential was significantly reduced in plants produced by shoot proliferation in the solid medium. Plants obtained by the other two methods were probably more dynamic in maintaining the physiological traits, which are particularly important for breeding drought-tolerant plants.

The stomatal conductance was lower in plants submitted to water stress, with a statistically significant difference for plants produced by shoot proliferation in the solid and liquid medium, which may indicate a better performance of plants produced by this technique, to be tested on future research. In fact, plants have evolved several mechanisms in order to adapt to unfavourable environmental conditions by reducing resources consumption and adjusting their growth (Osakabe *et al*. 2014). In water deficit conditions, changes in ion- and water-transport systems across membranes in guard cells stimulates stomatal closure, which directly affects photosynthetic rates and plant productivity due to lower levels of CO_2 available (Osakabe *et al.* 2014). According to previous reports (Castell and Terradas 1994, Gratani and Varone 2004, Navarro-García *et al.* 2011), the reduction of stomatal conductance usually occurs under drought stress as part of the efficient water use strategy of strawberry tree (Castell and Terradas 1994, Gratani and Varone 2004, Navarro-García *et al.* 2011), a typical drought resistant sclerophyllous Mediterranean species. Activation of anti-oxidative protection mechanisms has also been reported as a mechanism that might be used by this species to cope with water stress (Munné-Bosch and Peñuelas 2004).

The micropropagation in liquid medium was found very efficient for strawberry tree, especially by organogenesis, in the three genotypes tested. Nevertheless, strawberry trees micropropagated by different methods showed a very similar morphological and physiological performance and were not strongly affected under the imposed drought stress. However, plants produced by shoot proliferation on solid medium showed a slightly superior performance in some of the evaluated parameters, indicating different adaptation strategies to the imposed water stress, that should be taken into account when selecting a propagation technique. Overall, we can conclude that the tested propagation methods were efficient and did not considerably affect plant performance and productivity and can be used for strawberry tree micropropagation.

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