

## Article

# Hybridization assays in Strawberry tree towards the identification of plants displaying increased drought tolerance

João Martins <sup>1</sup>, Pedro Monteiro <sup>2</sup>, Glória Pinto <sup>2</sup> and Jorge Canhoto <sup>1,\*</sup>

<sup>1</sup> University of Coimbra, Centre for Functional Ecology, Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal; jorgecan@uc.pt (J.C.)

<sup>2</sup> Fungal and Plant Biology Laboratory, Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Portugal; gpinto@ua.pt (G.P.)

\* Correspondence: jorgecan@uc.pt

**Abstract:** *Arbutus unedo* L. is a small Ericaceae tree with a circum-Mediterranean distribution. It has a huge ecological impact on southern Europe forests and a great economic importance, as a source of phytochemicals with bioactive properties and for fruit production. On the foreseen climate change context, breeding towards drought tolerance is necessary in order to ameliorate plant performance. The aim of this work was therefore to study the reproduction mechanisms of strawberry tree, obtain new genetic combinations by hybridization and select genotypes more tolerant to drought stress. A morphological analysis of flowers and pollen was carried out, and controlled pollinations performed both *in vitro* and *ex vitro*. The very first approach on strawberry tree breeding by means of hybridization is also presented. Several physiological parameters were evaluated on 26 genotypes submitted to a water deficit regime. Plant behavior under drought greatly varied among genotypes, which showed a high phenotype plasticity. Three genotypes that were able to cope with water restriction without compromising net CO<sub>2</sub> assimilation were identified as highly tolerant to drought stress. The results obtained elucidate the reproduction mechanisms of strawberry tree and open the way for a long-term breeding program based on the selection of drought tolerant plants.

**Keywords:** *Arbutus unedo* L. Artificial Pollination, Breeding, Drought stress, Microscopy, Pollen, Physiological performance

## 1. Introduction

Strawberry tree (*Arbutus unedo* L.) is an Ericaceae widely distributed mainly around the Mediterranean basin, but also in the Atlantic coasts of Portugal, France and Southern Ireland [1]. Members of this species grow spontaneously on poor rocky and well-drained acidic soils and can stand a wide range of temperatures, easily thriving in marginal soils where other species of trees or bushes can hardly survive. Moreover, its ability to rapidly regenerate after forests fires, prevents the spread of common invasive species in Mediterranean ecosystems such as *Acacia* spp. or *Ailanthus altissima*, avoids soil degradation and helps to retain water, aspects that demonstrate the relevant ecological role of this species. Strawberry tree has several uses, mainly on pharmaceutical, cosmetics and food industries, due to the variability and amount of phytochemicals present in their tissues and organs [2]. However, the edible berries used to produce an alcoholic distillate that reaches high prices in the market still remain as the principal income source for farmers and other stakeholders [3].

Strawberry tree orchards are usually established using seed-derived plantlets on marginal dry areas where water is usually a scarce resource. Considering the expected increase on the frequency and severity of drought events in southern Europe in the near future [4], it is urgent to obtain new genotypes more tolerant to drought stress, in order to ameliorate their performance and increase productivity. Several studies have been carried

out to study the effects of drought stress on water relations, growth rate and photosynthesis in *A. unedo* under field conditions [5]. Although crucial, data focusing on drought performance at early stages of plant development are missing. Thus, early selection decisions are currently only based on productivity/fruit quality traits. Considering the increasing demand of high quality plant stocks of *A. unedo*, *in vitro* propagation protocols were developed to cloning selected genotypes [6–8] and studies to evaluate how these *in vitro* propagation systems change drought tolerance of regenerated plants have been carried out [9].

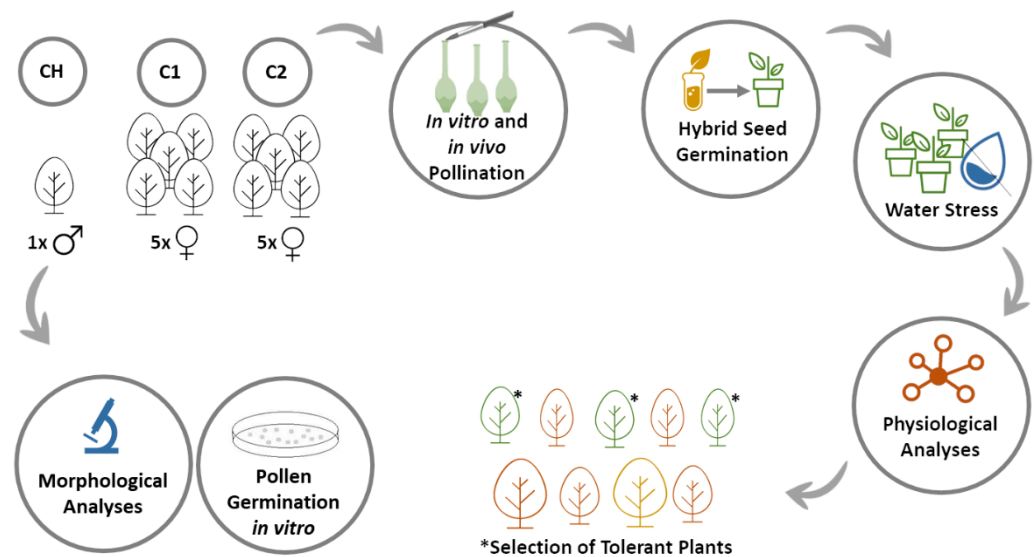
However, as far as is known, no work has been done in order to improve strawberry tree throughout conventional breeding, although some extensive experiments have been carried out on other Ericaceae, such as *Rhododendron* [10,11] and *Vaccinium* [12,13] species. Although conventional breeding is a lengthy process, particularly in tree species with long life-cycles, improved varieties of several tree species such as *Populus* spp., *Platanus* spp. and *Malus x domestica* have been produced through classical breeding [14,15]. The first step to initiate the development of new cultivars based on conventional breeding is a deep knowledge of the mechanisms of sexual plant reproduction, in particular the compatibility between the male and female reproductive structures, as well as the time of their maturation and phenology [16].

In order to set up the basis for a long-term breeding program on strawberry tree, the aim of this work was to study the reproduction system of *A. unedo*, from pollen morphology to pollen-stigma interactions and analyze the tolerance of the F1 plants towards drought. For this purpose, a morphological analysis of flowers and pollen was carried out, and controlled pollinations were made *in vitro* and *in situ* to obtain hybrid plants. Moreover, the plants obtained by the artificial crossings were tentatively selected based on its drought tolerance. For this purpose, they were submitted to a water deficit regime in order to identify individuals able to maintain higher photosynthetic levels under water deficit conditions, that might be used on future micropropagation and/or breeding programs.

## 2. Materials and Methods

### 2.1. Plant material

Flowers from three different populations were used in this study: CH (N 41°42'31.868" W 7°26'32.506", altitude 579m), from Chaves (North Portugal) and populations C1 (N 40°12'17.472" W 8°23'40.929", altitude 103m) and C2 (N 40°11'33.604" W 8°23'37.163", altitude 123m) from Coimbra (Central Portugal). Plants were selected based on its fruit quality and production (data not shown). A tree from population CH was used as a pollen donor for morpho-histological analysis (section 2.2) and germination studies (section 2.3). For pollen release and gathering anthers were removed from the flowers and placed on a Petri dish coated with aluminum foil for 1-2 days at room temperature. *In vitro* and *in vivo* pollinations (section 2.4) were carried out using emasculated flowers from ten trees from C1 and C2 populations (5 from each population) and the collected pollen as described before (Fig. 1).



**Figure 1.** Methodology scheme applied in this work to study the phenology and reproductive anatomy of strawberry tree, as well as selection of drought tolerant plants.

## 2.2. Reproductive phenology and anatomy

Trees from populations C1 and C2 were monthly monitored throughout the year in order to characterize *A. unedo* reproductive phenology. Flowers and fruits were gathered and characterized, including anther position and fruit maturation stages. To analyse its morphology, pollen from CH was treated by the standard method of acetolysis [17]. Briefly, after being washed in water and acetic glacial acid (100%, v/v), pollen grains were treated with the classic acetolysis mixture (9:1, acetic anhydride:sulphuric acid), and heated in a water bath at 70 °C for 5 min. After being treated with acetone (100%, v/v), acetolysed pollen material was mounted in glycerin jelly (Sigma-Aldrich, St. Louis, MO, USA). Measurements (D: diameter of the tetrad; d: single grain diameter; and the ratio D/d) were taken under light microscopy (Nikon EclipseCi) with an ocular micrometer, from 30 randomly chosen pollen tetrads from 3 different slides (10 per slide). Terminology based on that of Punt et al. [18] was used for pollen morphology characterization. For scanning electron microscopy, pollen was placed on stubs and coated with gold on a JEOL JFC 1100 apparatus (JEOL, Musashino, Japan). Pollen observations were performed on a JEOL JSM 5400 microscope. For anther anatomy studies, whole anthers were fixed for 3h at room temperature in glutaraldehyde (1.5%, v/v, Sigma), prepared with phosphate buffer (0.1M) and postfixed in osmium tetroxide (1%, w/v, Sigma) prepared with the same buffer. Samples were further dehydrated with ethanol and embedded with resin [19]. After the polymerization, ultrathin sections (1.5 µm) were obtained on an LKB Ultratome III and the cross sections stained with toluidine blue (1%, w:v) [20] and observed on a light microscope (Nikon EclipseCi) and photographs were collected with a Nikon DS-Fi3 camera and processed with the software NIS-Elements D (version 4.60).

## 2.3. Pollen germination

Previously to the pollination assays the viability of the collected pollen was checked. Mature pollen was cultured on Petri dishes containing a basal germination medium [21] composed of  $\text{H}_3\text{BO}_3$  (5 mg  $\text{L}^{-1}$ ),  $\text{CaCl}_2$  (15 mg  $\text{L}^{-1}$ ),  $\text{KNO}_3$  (10 mg  $\text{L}^{-1}$ ), agar (8%, w/v, Duchefa Biochemie B.V, Haarlem, The Netherlands) and different concentrations of sucrose (0, 3, 6, 9, 12, 15 and 18%, w/v, Duchefa), for 6 and 24 hours, at room temperature. Pollen grains were then stained with aceto-carmin and observed under a light microscope. As *A. unedo* pollen grains are dispersed as tetrad units [22], germination rates were determined by scoring 100 pollen tetrads from 5 replicates (a total of 500 tetrads and 2000 pollen grains). A pollen tetrad was considered germinated when the pollen tube length of at least a pollen grain surpassed the diameter of a pollen grain. Different carbon sources as well as the

effect of some plant growth regulators on pollen germination were also tested. For this purpose, pollen was cultivated for 6 hours on the medium described before, with sucrose, glucose and fructose at three different concentrations (3%, 9% and 15%). The effect of NAA (1-naphthaleneacetic acid, Sigma), IBA (indole-3-butyric acid, Sigma) and GA<sub>3</sub> (gibberellic acid, Sigma) was also tested in three concentrations (10, 100 and 500 mg L<sup>-1</sup>), on the same basal germination medium containing 15% sucrose.

#### 2.4. *In vitro* and *in situ* pollination assays

For *in vitro* pollination, flowers immediately before anthesis, from C1 and C2 populations were used. After emasculation, a total of 120 pistils from each population was placed on baby food jars (5 pistils per container) with a jellified medium for support (with water and 8 g L<sup>-1</sup> agar). Pollen from population CH collected as described in section 2.2. was then carefully placed at the stigma using a spatula. Open and closed non-pollinated flowers were used as controls and all treatments were done in triplicate. From each population, a total of 75 pistils were crosspollinated (15 pistils/tree from 5 different trees), 15 autopollinated (from a single tree), and 30 used as negative and positive controls (15 each from a single tree). Following artificial pollination, the pistils were kept in the dark at 25 °C, for 24 h, and the efficiency of the pollination was evaluated. For this purpose, pistils were fixed in FAA (formalin:acetic acid:ethanol, 5:5:90, v/v/v) at room temperature for 24 h, washed in water, softened on a NaOH solution (8N) and mounted with aniline blue (0.1%, w/v, Sigma) as described by Martin [23]. The observations were carried out in a fluorescence microscope (ex: 370 nm, Leica DM4000 B), and pollination was considered efficient when pollen germination was observed on the stigma, and pollen tubes grown along the style and reached the ovaries.

For *in situ* pollination assays pollen with a viability over 80% from a single tree (population CH) was used to hand pollinate flowers from the trees used for *in vitro* pollination. After the emasculation with forceps, the pollen was carefully placed on the stigma and the pollinated flowers covered with polypropylene pollination bags for 7 days, in order to avoid pollen contamination. 10 flowers from three different inflorescences (a total of 30 per tree) were pollinated on each of the 10 trees. All the immature and old flowers from the pollinated inflorescences were removed. During the assays the minimum absolute temperature was 7.1 °C and the maximum absolute temperature was 26.9 °C, while the total precipitation recorded was 145.5 mm, according to the data provided by the meteorological station of Coimbra/Cernache (www.ipma.pt).

#### 2.5. Seed germination and plant development

Mature fruits resulting from hand-pollination were gathered and washed with tap water. Isolated seeds were washed with distilled water for 10 min. Following a 30s surface sterilization with ethanol (70%, v/v, Merck), the seeds were sterilized in a calcium hypochlorite solution (5%, w/v, Sigma) and 2-3 drops of Tween 20 for 10 min, washed 3 times with distilled sterilized water and sowed on sterilized Petri dishes (9 cm) with cotton wool imbibed with sterile distilled water and covered with filter paper. The seeds were kept at 4 °C for 30 days and then transferred to a culture chamber (25 °C) for another 60 days. After this period the germination rate was recorded. Since only viable seeds, selected based on morphological characteristics were used, Relative Germinability (RG) was calculated:  $RG = (\text{number of seeds produced} * 100) / \text{number of viable seeds germinated}$  [24]. Seedlings were then transferred to acclimatization containers with sterilized perlite, and kept in a growth chamber at 25 °C and 70% relative humidity, under a 16 h daily illumination regime of 15–20  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetically active radiation (PAR, cool-white fluorescent lamps). After 15-30 days, the plants were transferred to individual containers (5 dm<sup>3</sup>) with a substrate composed of peat and perlite (3:1, v/v, Siro, Mira, Portugal) for further growth.

#### 2.6. Drought stress assays

Three-year-old plants resulting from cross pollination (a tree from each population) were then submitted to water stress. A total of 26 plants (#1-13 from population C1 and #14-26 from population C2) were watered to full field capacity, and plant performance was evaluated after 24 hours (t0). After that period watering was interrupted and plants submitted to 3 weeks (t3) of water deficit. Leaf gas exchange was evaluated on t0 and t3 while due to the destructive nature of leaf water potential ( $\Psi_w$ ) and leaf relative water content (RWC) measurements, sampling was performed in the end of the experiment (t3). The experiment was conducted during July, and the temperature ranged from 13 °C ( $15.8 \pm 1.4$ ) to 32 °C ( $24.1 \pm 2.7$ ). The average temperature at each sampling point was:  $20.5 \pm 4.5$  °C (t0) and  $17.5 \pm 6.4$  °C (t3).

*In situ* leaf gas exchange measurements (net CO<sub>2</sub> assimilation rate:  $A$ , transpiration rate:  $E$ , stomatal conductance:  $g_s$  and intercellular CO<sub>2</sub> concentration:  $c_i$ ) were measured on a young and fully expanded leaf (normally the fifth leaf from the top) using a portable infrared gas analyzer coupled to a broad leaf chamber (LCpro+, ADC, Hoddesdon, UK), operating in open mode and under the following conditions: photosynthetic photon flux density -  $650 \mu\text{mol m}^{-2} \text{s}^{-1}$  (based on a light curve:  $0\text{--}1750 \mu\text{mol m}^{-2} \text{s}^{-1}$ ); air flux -  $200 \text{mol s}^{-1}$ ; block temperature - 25 °C; and atmospheric CO<sub>2</sub> and H<sub>2</sub>O concentration. Data were recorded when the measured parameters were stable (2–6 min). Water potential was measured with a Scholander-type pressure chamber (PMS Instrument Co., OR, USA). Relative water content (RWC) was calculated as:  $\text{RWC (\%)} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100$ , where FW is the fresh weight of the leaf, TW the turgid weight (after 24 h on distilled water at 4 °C) and DW is the dry weight (after drying at 70 °C for 48 h).

## 2.7. Statistical analysis

Pollen germination and physiological data was analyzed by one-way ANOVA (GraphPad Prism for Windows v. 6.01) followed by a Tukey's multiple comparison test ( $P < 0.05$ ). Data expressed as percentages were first submitted to arcsine transformation. A heatmap with dendrogram, a correlation and a principal component analysis (PCA) were carried out using R software [25] to evaluate the interaction and significance of all the physiological parameters measured on the analyzed trees. A heatmap with physiological data from all the samples was constructed using the Heatmap function and the package ComplexHeatmap [26]. The dendrogram within the heatmap was calculated with Euclidean distance as dissimilarity measure. To evaluate the interaction of the measured variables a correlation was calculated using the ggcor function (Pearson correlation coefficients and pairwise observations) and the packages GGally [27] and ggplot2 [28]. Finally, data was classified with a PCA, using the prcomp function and the package ggbiplot [29].

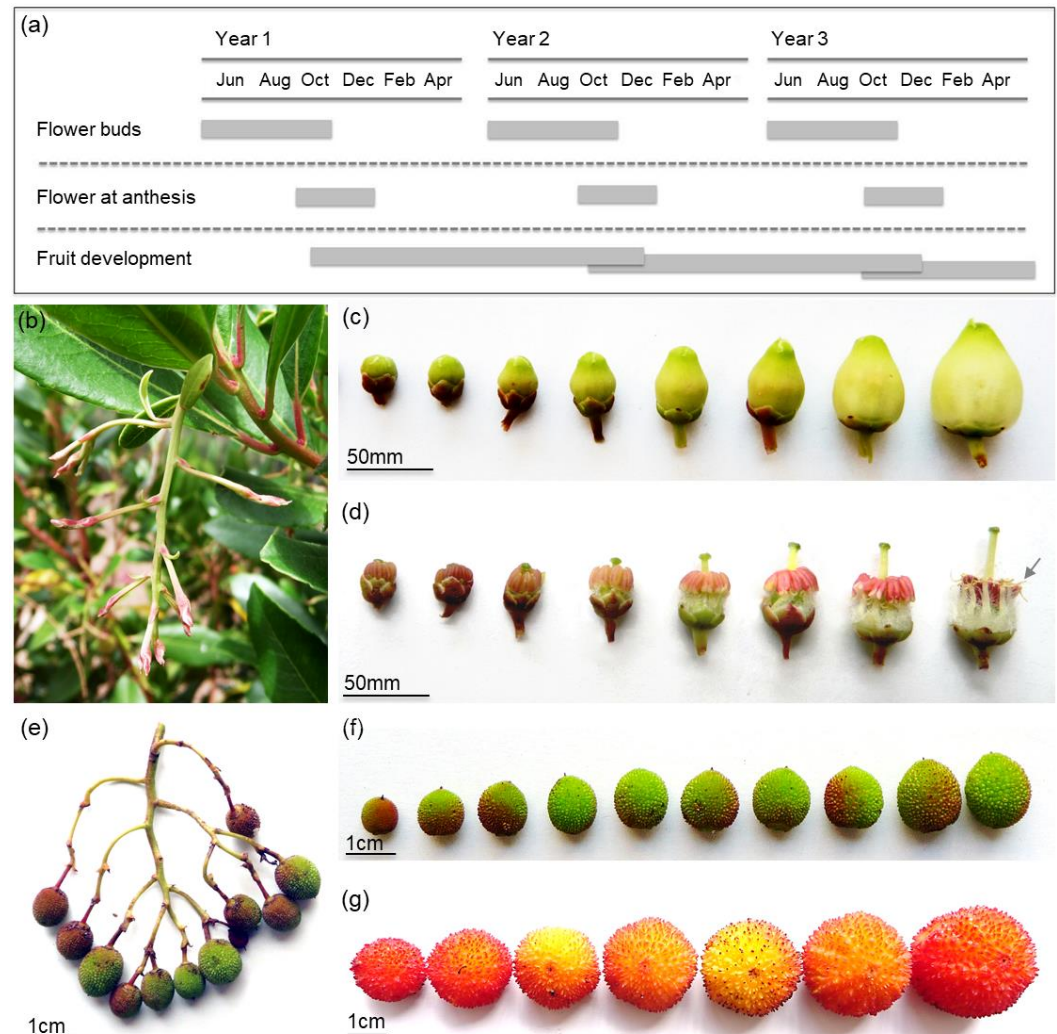
## 3. Results

### 3.1. Reproductive phenology and anatomy

The reproductive cycle of strawberry tree is long, and lasts for almost two years (Fig. 2 A). During this period three distinct stages can be identified: flower buds, flowers at anthesis and fruit development. During June, the inflorescences (panicles) start to appear from terminal meristems of young stems (Fig. 2 B). Flower development proceeds through Summer months and flower anthesis usually begins on October (Fig. 2 C). The flowering period can be long, from early October to late January depending on the trees and location. The flower is complete, bell-shaped, sympetalous and white to slightly pink (Fig. 2 C). Each pistil is formed by a pentalocular ovary, a style and a stigma who becomes receptive to pollen just before flower anthesis. Each stamen possesses a hairy filament and an anther with two pores located at the top. During flower development and just before flower anthesis, anthers suffer an inversion process from an extrorse to an introrse position and develop two appendages on the apical end (Fig. 2 D). After pollination, the slow fruit development process begins. Each infructescence will usually bear between 1 to 20 fruits (Fig. 2 E) that will develop along the year until fully ripe (Fig. 2 F). Consequently, fruit ripening occurs simultaneously with the next flowering period, during autumn (Fig.

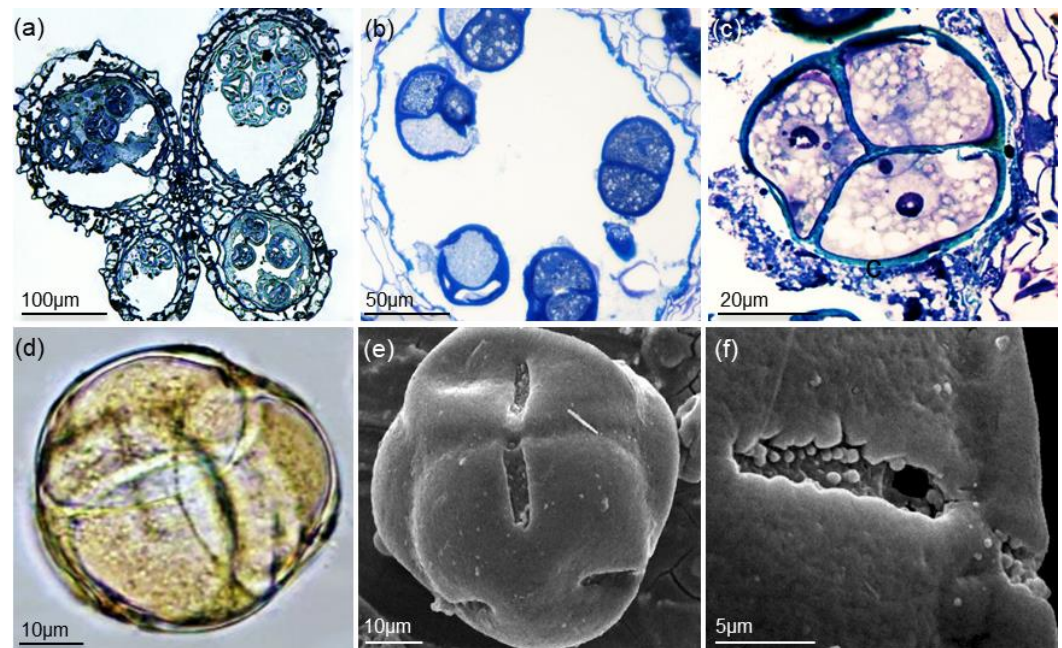


2 A). Fruits at different developmental and ripening stages can be found at the same time on a tree. When fully ripped, fruits present a variable size and shape and a bright red color (Fig. 2 G).



**Figure 2.** Different aspects of strawberry tree phenology and reproductive anatomy: (a) phenological cycle of strawberry tree; (b) terminal hanging panicle at an early development stage during June; (c) general morphology of the bell-shaped flowers from early stages of development to flower anthesis; (d) developmental process of the stamens; (e) infructescence at an early development stage; (f) fruit developmental stages before ripening; (g) mature fruits with different sizes and shapes. Feb - February, Apr - April, Jun - June, Aug - August, Oct - October, Dec - December.

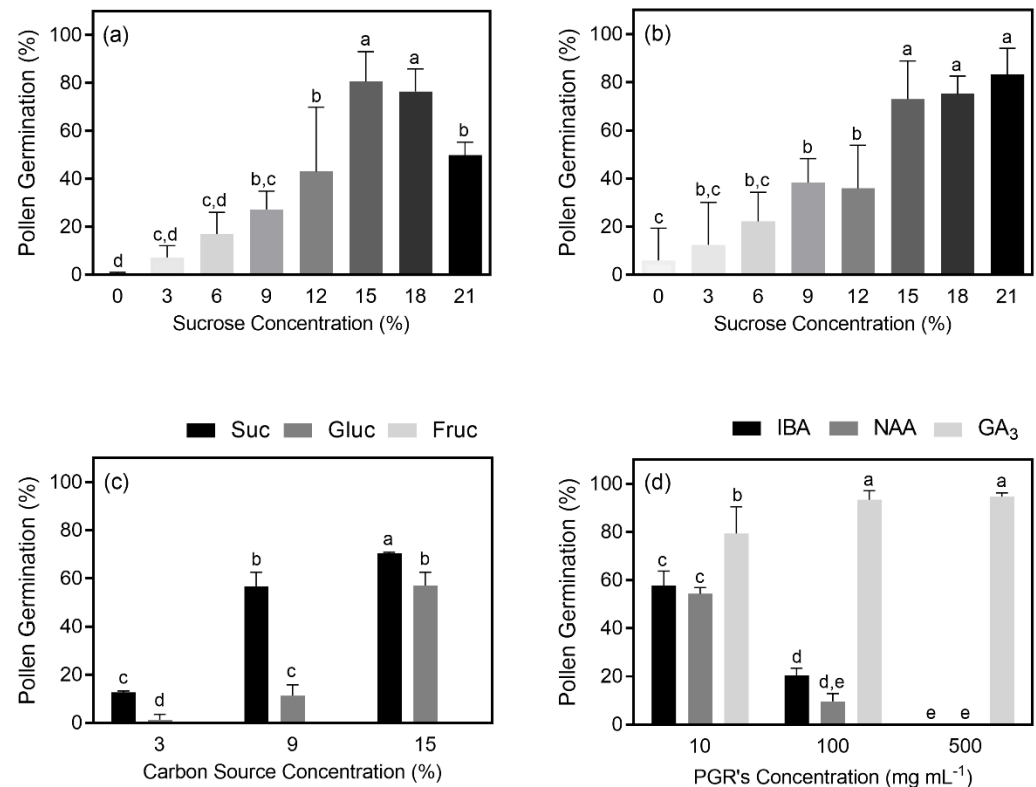
Anthers of *A. unedo* have four microsporangia or pollen sacs arranged in pairs (Fig. 3 A). Pollen is dispersed in groups of four and each anther contains on average 500 pollen units. On the earlier developmental stages some pollen grains were found to be aborted on the pollen tetrad (Fig. 3 B). Pollen tetrads became mature and are released just after flower anthesis. Single pollen grains are 3-zonocolporate and have a circular or slightly elliptic outline on optical slice (Fig. 3 C-D). The ectoapertures are long colpus with a granulate membrane, and the endoapertures are pores with regular outline (Fig. 3 E-F). The exine ( $\approx 1.5 \mu\text{m}$ ) has a psilate surface and is tectate and slightly columellate. The size of the pollen tetrads (D) ranged from  $42 \mu\text{m}$  to  $67 \mu\text{m}$  ( $53.8 \mu\text{m} \pm 3.6$ ) whereas the size of single pollen grains (d) varied from  $22 \mu\text{m}$  to  $36 \mu\text{m}$  ( $29.5 \mu\text{m} \pm 0.8$ ). The relation D/d was between 1.6 and 2.2 ( $1.8 \pm 0.1$ ).



**Figure 3.** Anther and pollen morphology of strawberry tree: (a) anther cross section stained with toluidine blue; (b) pollen sac with aborted pollen grains; (c) pollen tetrad section stained with toluidine blue showing a bi-nucleated pollen grain; (d) non acetolysed pollen tetrad; (e) pollen tetrad on SEM; (f) aperture and ornamentation detail of the pollen tetrad on SEM.

### 3.2. Pollen germination

Pollen germination was higher on media with higher sucrose concentrations for both periods analyzed (6 and 24 h). After 24 h, best germination rates were obtained on 15% and 18% sucrose. However, a decrease on germination was observed on the medium with 18% sucrose after 6 h (Fig. 4 A-B). The highest germination rates were obtained on a medium with 18% sucrose, after 24 hours ( $83.29\% \pm 10.85$ ), and 15% sucrose after 6 hours ( $80.52\% \pm 12.55$ ; Fig. 4 A-B). In most of the pollen tetrads scored as germinated, only one of the pollen grains developed a pollen tube ( $70.98\% \pm 1.81$ ), whereas the germination of more than two pollen grains was only observed occasionally (Fig. 5 A). When different carbon sources were tested, sucrose gave the best results on the three concentrations tested, with the maximum germination rate obtained with 15% sucrose ( $70.33\% \pm 1.89$ ). Although the glucose was not as efficient as sucrose, a germination rate of  $57.00\% \pm 4.55$  was obtained with the maximum concentration tested (Fig. 4 C). No pollen germination was observed when fructose was used as carbon source. Likewise, NAA and IBA had an inhibitory effect on pollen germination, even on the lowest concentration tested ( $10 \text{ mg L}^{-1}$ ), when compared to the control group. When these two auxins were applied at higher concentrations, pollen germination was completely inhibited (Fig. 4 D). On the other hand,  $\text{GA}_3$  highly promoted pollen germination. When a concentration of  $10 \text{ mg L}^{-1}$  was applied, no statistic significant differences were observed when compared to the control. However, a germination rate of  $93.33 \pm 3.09$  was obtained with  $100 \text{ mg L}^{-1} \text{ GA}_3$  and  $94.67 \pm 1.25$  with  $500 \text{ mg L}^{-1}$  (Fig. 4 D). When the concentration of  $\text{CaCl}_2$  was highly incremented on the germination medium (10 x increase), similar results were obtained (data not show).

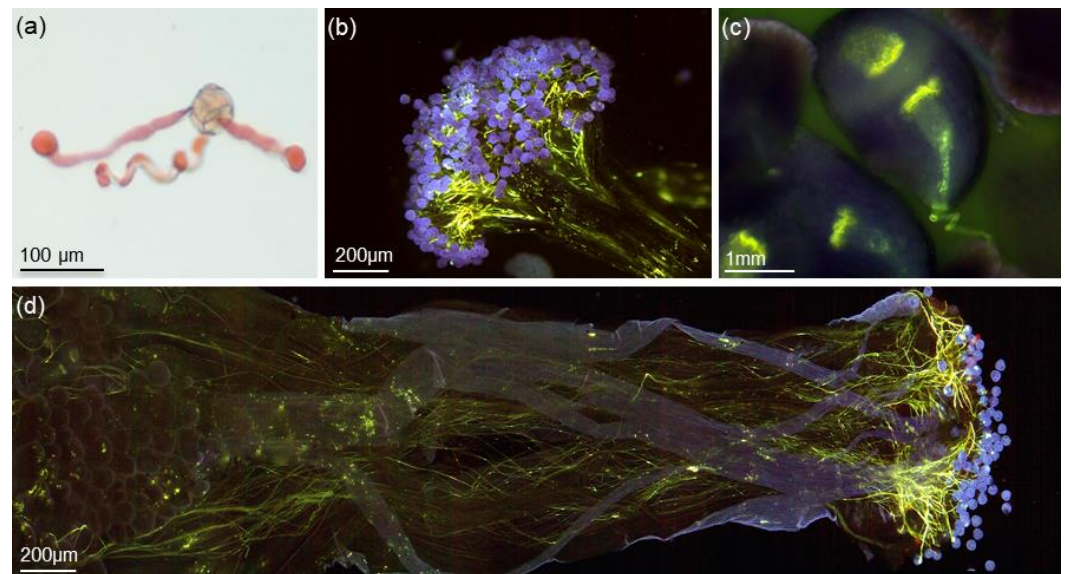


**Figure 4.** Pollen germination rates (%): (a-b) on a medium with different concentrations of sucrose (0, 3%, 6%, 9%, 12%, 15% and 18%) after 6 and 24 hours; (c) with different carbon sources (sucrose, glucose and fructose); (d) germination rates with different plant growth regulators (IBA, NAA and GA<sub>3</sub>) (D). Means  $\pm$  SDs,  $n = 5$ , different letters indicate significant differences between treatments at  $P \leq 0.05$ .

### 3.3. *In vitro* and *in situ* pollination assays

From the 12 combinations of crosses carried out *in vitro*, including 2 auto-pollinations, the average success rate obtained was  $78.9\% \pm 22.7$ . Pollen germination was observed on stigma 1-2 hours after the pollination (Fig. 5 B). The pollen tubes grow along the style, reaching the ovary in 24 h (Fig. 5 D), and the tips of pollen tubes enter the micropyle (Fig. 5 C). The effectiveness of the cross-pollinations was  $82.51\% \pm 19.81$ , while the effectiveness of the self-pollinations was  $71.65 \pm 29.50$ . It was observed the accumulation of callose along the pollen tubes as well as on the tips. In some cases, pollen showed no signs of germination, both on self- and cross-pollinations. The growing pattern of pollen tubes seemed to be very similar on all the crosses made. In most of the flowers from the positive control (open flowers) pollen germination and pollen tube growth were observed, while all the flowers from the negative control (closed flowers) showed no signs of pollen in the stigma. Most of the pollinated flowers *in situ* were lost along the fruit developmental process. From the total of 300 pollinated flowers, after one year under development, only 3 fruits reached the mature stage which represents a very low success rate of only 1%.

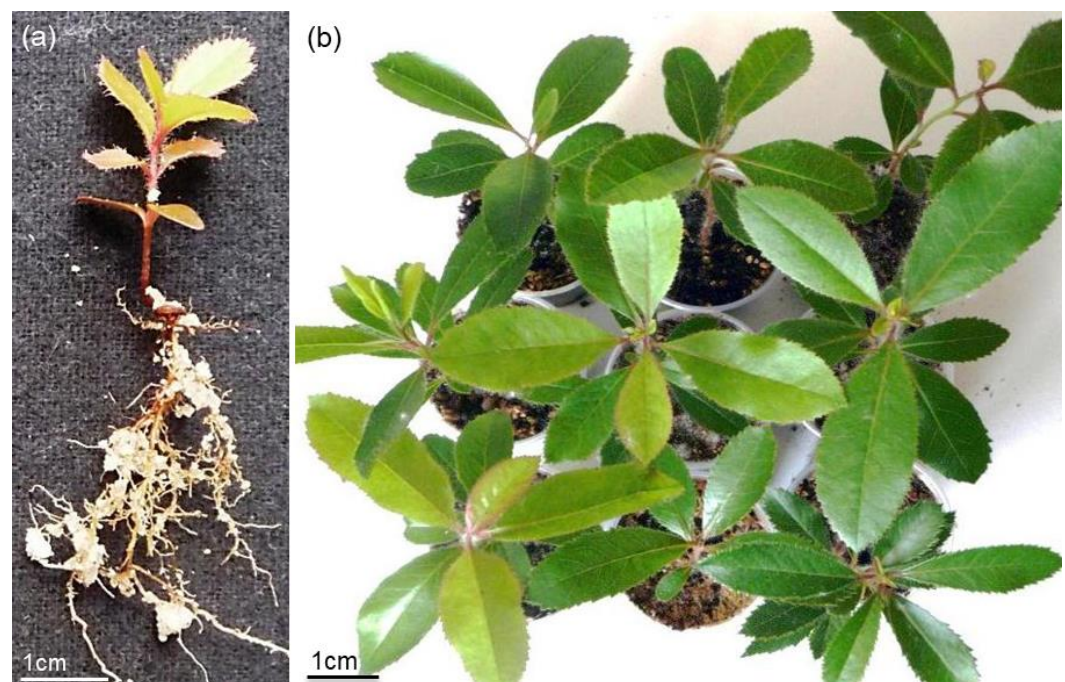




**Figure 5.** Strawberry tree pollen germination: (a) germinated pollen tetrad stained with aceto-carmin; (b) germination of pollen grains on stigma; (c) pollen tube entering the ovule micropyle; (d) pollen germination on the stigma and pollen tube growth along the pistil.

### 3.4. Seed germination and plant development

From the three fruits retrieved from the field, a relative germination rate of 85.0% was obtained for group C1 and 86.7% for C2. After *in vitro* germination, seedling development proceeded rapidly, and after the acclimatization period the root system was well developed (Fig. 6 A). The hybrid plants were morphological diverse (Fig. 6 B) in terms of height and leaf morphology (data not shown). A total of 35 plants were obtained, 17 from group C1 and 13 from C2. After 3 years under development, 13 plants from each group were submitted to drought stress.



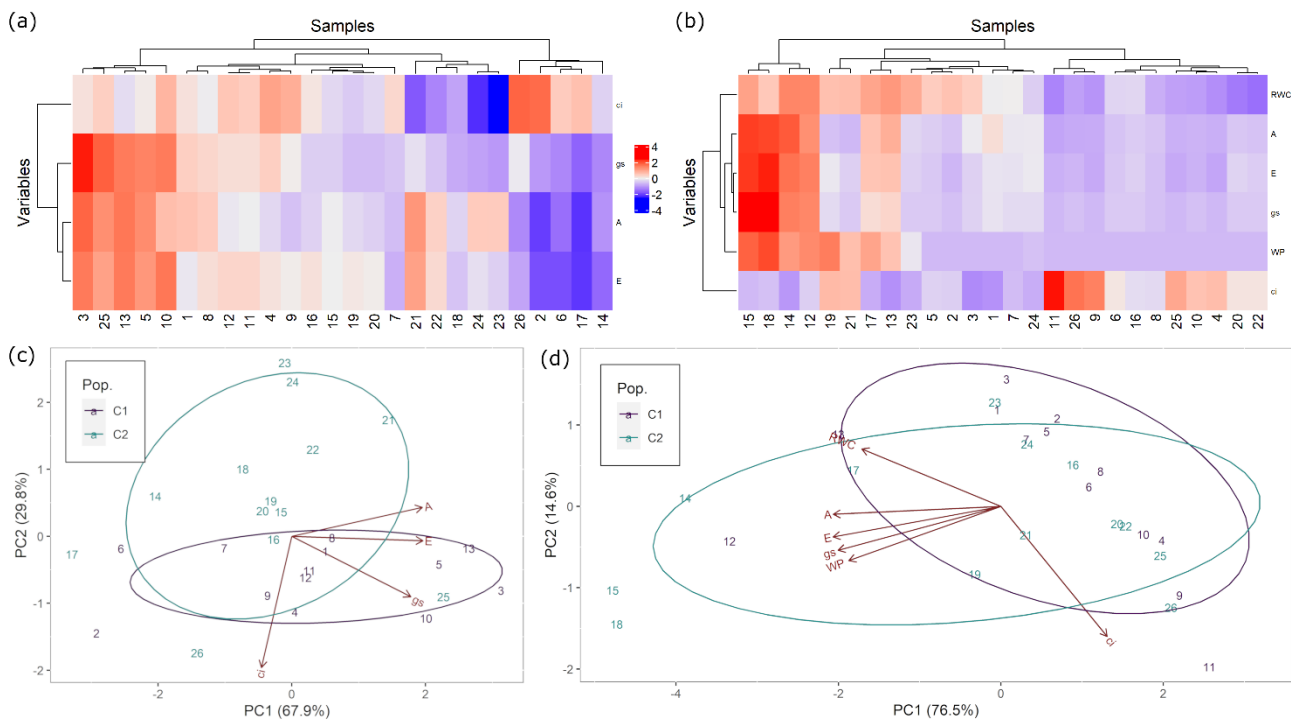
**Figure 6.** Strawberry tree hybrid plants development: (a) two months-old hybrid seedlings; (b) six months-old hybrid plants with distinct morphological characteristics.

### 3.5. Plant water status and gas exchange

Before the imposed water stress deficit regime ( $t_0$ ) a considerable variance was found among genotypes on all the physiological parameters measured. While some of the tested genotypes presented higher net CO<sub>2</sub> assimilation rates (*e.g.*, 3, 5, 13, 21 and 25), others had considerably lower values (*e.g.*, 2 and 17) (Table 1, Fig. 7 A). After 3 weeks under water deficit, stomatal conductance, net CO<sub>2</sub> assimilation and transpiration rates decreased (Table 1, Fig. 7 B). On the other hand, intercellular CO<sub>2</sub> concentration increased on most of the plants throughout the imposed water stress. Although a great reduction on stomatal conductance, transpiration and net CO<sub>2</sub> assimilation rates was observed on genotype 13 after drought stress, these parameters are still considerably higher than on most of the evaluated genotypes. A similar behavior was observed on genotypes 12 and 17, but with a less marked decreased on net CO<sub>2</sub> assimilation rate. Meanwhile, these parameters remained unchanged or slightly increased on genotypes 14, 15 and 18. Relative water content and water potential were in general higher on plants with a higher net CO<sub>2</sub> assimilation rate.

**Table 1.** Gas exchange related parameters water status of 26 hybrid plants on  $t_0$  (control) and  $t_3$  (3 weeks under water deficit). ci - intercellular CO<sub>2</sub> concentration, E - transpiration rate, gs - stomatal conductance, A - net CO<sub>2</sub> assimilation rate, RWC - relative water content, WP - water potential. + plants with an intermediate performance. ++ plants identified with the best performance. \* equipment detection limit. Tables should be placed in the main text near to the first time they are cited.

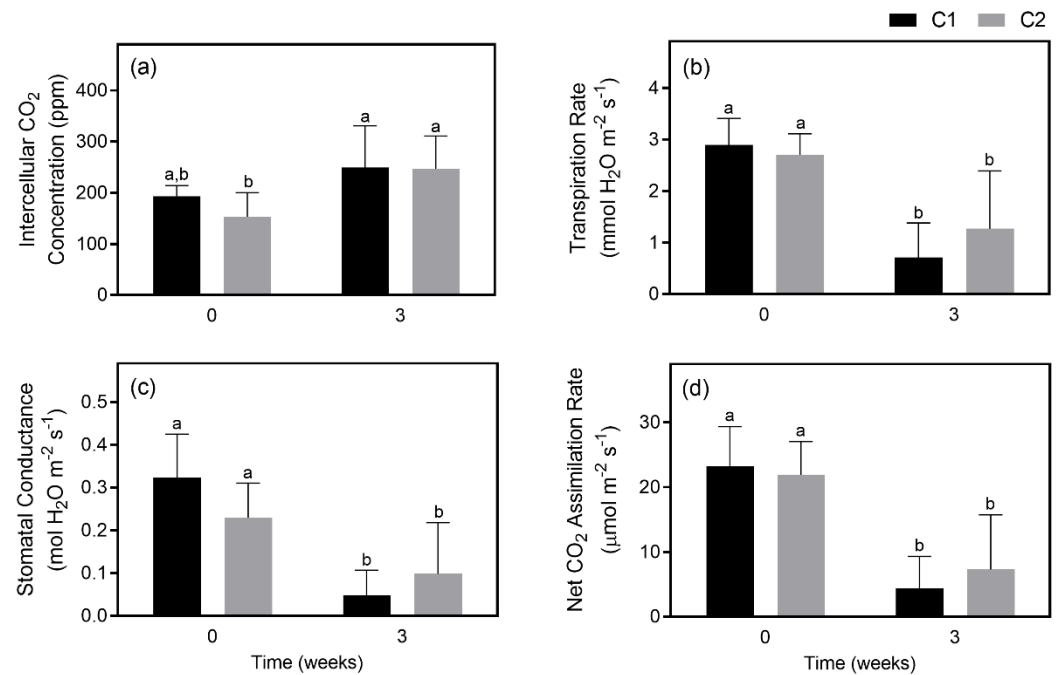
Genotype	ci		E		gs		A		RWC	WP
	$t_0$	$t_3$	$t_0$	$t_3$	$t_0$	$t_3$	$t_0$	$t_3$		
1	181	166	2.76	0.86	0.32	0.06	25.87	7.67	63.4	-50*
2	243	220	1.98	0.34	0.18	0.02	11.81	2.31	72.8	-50*
3	181	159	3.5	0.64	0.51	0.04	32.36	5.49	70.5	-50*
4	215	302	2.88	0.14	0.32	0.01	21.2	0.45	47.5	-50*
5	176	223	3.34	0.55	0.42	0.03	29.84	3.07	70.9	-50*
6	193	258	1.98	0.53	0.16	0.03	14.7	2.21	59.4	-50*
7	186	204	2.48	0.84	0.23	0.05	19.59	5.18	63.9	-50*
8	172	230	2.91	0.45	0.31	0.02	25.23	2.32	53.0	-50*
9	209	364	2.82	0.19	0.28	0.01	19.85	0.15	48.6	-50*
10	205	296	3.52	0.29	0.43	0.02	26.22	0.82	50.0	-50*
11	192	435	2.96	0.27	0.3	0.01	22.51	-0.46	45.7	-50*
12 <sup>+</sup>	196	220	2.96	2.52	0.3	0.22	22.1	15.61	84.6	-21
13 <sup>+</sup>	165	165	3.5	1.63	0.43	0.11	31.25	12.51	81.2	-32
14 <sup>++</sup>	160	171	2.11	2.7	0.16	0.23	16.92	20.27	84.7	-24
15 <sup>++</sup>	163	201	2.8	3.27	0.24	0.33	22.5	22.19	79.4	-14
16	179	232	2.89	0.63	0.24	0.03	20.86	2.89	59.6	-50*
17 <sup>+</sup>	199	190	1.87	1.59	0.12	0.1	11.24	10.47	78.8	-26
18 <sup>++</sup>	139	208	2.58	3.41	0.2	0.34	20.96	21.7	71.7	-10.5
19	157	305	2.85	0.91	0.22	0.05	21.26	2.01	73.3	-18.5
20	163	260	2.82	0.68	0.22	0.03	20.68	2.45	44.0	-50*
21	105	302	3.28	0.63	0.26	0.03	29.13	1.24	74.0	-31.25
22	125	260	2.93	0.6	0.23	0.03	24.77	2.16	42.3	-50*
23	76	188	2.64	0.64	0.18	0.03	25.25	3.73	77.6	-42.5
24	87	191	2.72	0.92	0.19	0.05	25.27	5.3	58.2	-48.5
25	191	325	3.28	0.19	0.46	0.01	29.66	0.55	50.6	-50*
26	246	377	2.42	0.33	0.27	0.02	16.36	0.02	50.5	-50*



**Figure 7.** Physiological parameters measured on 26 hybrid plants from populations C1 and C2 on t0 and t3 (3 weeks under water deficit): (a-b) heatmap with dendrogram on t0 and t3; (c-d) principal component analysis (PCA) on t0 and t3. ci - intercellular CO<sub>2</sub> concentration, E - transpiration rate, gs - stomatal conductance, A - net CO<sub>2</sub> assimilation rate, RWC - relative water content, WP - water potential.

This result is confirmed by the dendrogram within the heatmap (Fig. 7 A-B) as well as the PCA biplot (Fig. 7 C-D), that revealed a high positive correlation between E, gs and A with relative water content and water potential (Fig. 7 B). Moreover, a negative correlation was found between these parameters and ci. On most of the plants, water potential was below the detection limit (-50 MPa). The PCA analysis have also revealed a very diverse behavior of plants, regardless of their provenience either on t0 or t3 (Fig. 7 C-D). Thus, plants with best and worst performance under water stress are from both proveniences. On t0, principal component 1 (PC1) contributes with 67.8% to the total variance and A, gs and E are the parameters with a higher weight on this component, whereas PC2 contributes with 29.8% to the total variance and ci is the variable that most contributes to this variance. The genotypes identified due to a better performance (3, 5, 13, 21 and 25) are grouped (Fig. 7 C). On t3, principal component 1 (PC1) contributes with 76.5% to the total variance. A, gs, E, relative water content and water potential are the parameters with a higher weight on this component. PC2 contributes with 14.6% to the total variance. As mentioned before, some of the tested plants showed a better overall performance in terms of net CO<sub>2</sub> assimilation under drought. Thus, genotypes 12, 13, 14, 15, 17 and 18 are grouped together, by the influence of some gas exchange parameters (gs, E and A), relative water content and water potential. Genotype 19 is also on this cluster as it was able to maintain relatively high values of relative water content and water content, in spite of its low performance in terms of net CO<sub>2</sub> assimilation. Plants with a worst overall performance are grouped together by the influence of ci (Fig. 7 D).

When comparing the two groups (C1 and C2), although no statistical differences were found between groups (Fig. 8 A), transpiration rates greatly decreased after 3 weeks on both groups (Fig. 8 B), as well as stomatal conductance (Fig. 8 C) and net CO<sub>2</sub> assimilation rate (Fig. 8 D). No statistical difference was found for relative water content as well, with values of  $62.4 \pm 13.0\%$  on C1 and  $65.0 \pm 14.7\%$  on group C2.



**Figure 8.** Gas exchange related parameters of hybrid plants from populations C1 and C2 on t0 and t3 (3 weeks under water deficit): (a) intercellular CO<sub>2</sub> concentration; (b) transpiration rate; (c) stomatal conductance; (d) net CO<sub>2</sub> assimilation rate (D). Means ± SDs, n = 13, different letters indicate significant differences between treatments at  $P \leq 0.05$ .

#### 4. Discussion

The long phenological cycle observed on the trees analyzed on this work, is similar to the one that has been reported by Villa [30]. The inversion process of the anthers observed, as well as the development of the two appendages, has been described as a characteristic feature of the Ericaceae family [31]. The pollen morphology observed is similar to data reported by Villa [30], but slightly differs from that described by Mateus [22]. According to this author, the endoapertures of strawberry tree pollen are endocolpus. In contrast, on this study the endoapertures observed were endopores. The size of the pollen tetrads is slightly different as well: the diameter of the tetrads (D) determined on this work range from 42 μm to 67 μm, compared to 49-66 μm obtained by Mateus [22], while the size of single pollen grains (d) range from 22 μm to 36 μm compared to 33-41 μm.

The pollen germination rates obtained on the germination medium with 12% sucrose (89.40%) are similar to the ones obtained on other species, such as *Prunus domestica* [32] and *Pistacia* spp. [33], and much higher than the germination rates obtained on *Annona charimola* [34] and *Olea europaea* [35]. Therefore, the germination medium used is adequate for strawberry tree and should be used for pollen viability tests. Although it has been reported by Cane [36] that 90% of the tetrads generated 3-4 pollen tubes on other member of the Ericaceae (*Vaccinium macrocarpon*), we found that most of the strawberry tree pollen tetrads analyzed had only one pollen grain germinated. This can be due to the fact that some of the grains on the pollen tetrads were found aborted, even in the initial developmental stages. The inhibition of pollen germination when fructose is included on the medium has been reported on the literature by Okusaka and Hiratsuka [37]. According to these authors, fructose completely inhibited pear pollen germination, but without pollen viability loss, since it was able to germinate when transferred to a different germination medium without fructose. Thus, fructose is not an adequate sugar for strawberry tree pollen germination, and other carbon sources should be used instead, preferably sucrose. Plant growth regulators (PGRs) can be an extremely useful tool for plant breeders, either as gametocides or on the contrary by promoting pollen germination and eventually increasing fruit seed-set. Different PGRs have been tested on diverse crop species including rye [38], barley [39], onion, tomato, eggplant, pepper, watermelon [40] and wheat [41].



Our results showed an inhibitory effect of IBA and NAA on strawberry tree pollen germination. The inhibitory effect of IBA and NAA on pollen germination and in similar concentrations to those tested on our work has been reported. on the literature: the application of NAA (50 mg L<sup>-1</sup>) on eggplant and IBA or NAA (10-100 mg L<sup>-1</sup>) on onion, proved to have an efficient gametocide effect [40]. On the other hand, GA<sub>3</sub> greatly promoted pollen germination when compared to the control group. This effect has also been observed on other species namely strawberry [42] and blueberry where the application of GA<sub>3</sub> on flowers lead to an increased fruit set [43]. For this reason, the effect of GA<sub>3</sub> on strawberry tree pollen germination and fruit set should be further tested due to its potential as a breeding aiding tool.

Although a previous study had suggested that pollen tubes growth speed is slower on self-pollination due to higher rates of attrition [44], this was not the case in strawberry tree and similar pollen tube growth patterns on self- and cross-pollinations was observed. Moreover, no difference was found between the effectiveness of self- and cross-pollinations. The observed accumulation of callose on pollen tubes of strawberry tree has also been observed in *Chaenomeles japonica* [45]. Only a small portion of the hand-pollinated flowers on the field were able to complete the long development process and bear fruits. Due to the long development process that takes a year to be completed, strawberry tree pollinated flowers and fruits under development are subjected to a wide range of environmental conditions and interferences and only a small portion of fruits is able to complete its development. This might help to explain the low success rate of hand-pollinations along with the high manipulation required to carry the pollination procedure. In fact, it has been reported that fruit production on *Vaccinium* spp. is lower when plants were cross- or self-pollinated by hand than when natural pollination occur [13]. Thus, the improvement of pollinations conditions is something to be pursued in the near future in order to increase success rates of hand-pollinations. The increase of the amount of pollen placed on the stigma and/or the use of PGRs (e.g., GA<sub>3</sub>) should be considered, as well as the implementation of open pollinated seed orchards. Size and seediness of fruits may also be affected when hand-pollination is carried out, as referred by Usui et al. (2005). On this work, the average of viable seeds obtained on hand-pollinated fruits was 50%, much lower than the 77% obtained on open pollinated trees (data not shown). Nonetheless, due to the low amount of hybrid fruits obtained, these results are not significative and further analyses should be carried out in the future. The high germination rates obtained are similar to other works [46–48] indicating that germination ability of the hybrid seeds is not compromised. However, such rates were obtained after cold stratification, a procedure that should be followed in order to break seed dormancy.

When the hybrid plants were submitted to drought stress (t3), a decrease on transpiration rates, stomatal conductance as well as net CO<sub>2</sub> assimilation rate was observed, has a consequence of the efficient conservative water use strategy adopted by strawberry tree [49]. As mentioned before, strawberry tree is an isohydric species, with a tight stomatal control. When under water deficit conditions, plant will adopt a conservative water use strategy by closing stomata, thus keeping a low gas exchange rate [50,51]. The increase of intercellular CO<sub>2</sub> concentration throughout the imposed water stress is caused by a reduction of the photosynthetic machinery due to stomatal limitations and probably oxidative stress [5]. The high positive correlation found between water availability (relative water content and water potential) and gas exchange parameters evaluated is not surprising, as water is one of the most limiting factors on the entire photosynthetic process. Thus, plants with a higher ability to maintain their water status have as expected a better overall performance.

Overall, the measured values of physiological parameters are in accordance with those obtained on a previous study [9]. However, net CO<sub>2</sub> assimilation rates measured were higher than those obtained on previous reports [9,52]. This difference can be related to the older age of the plants evaluated on this study, that hypothetically might have a higher photosynthetic ability. Considerably lower values of water potential were also measured on this work, which is probably related with the period under water stress as

well as the age of the plants that might have more lignified tissues and a higher resistance to cavitation and low water potential. Still, this hypothesis should be further tested and confirmed on future analysis.

Although most of the tested plants showed a poor performance under drought stress, we successfully identified two groups of plants that followed a different strategy to cope with water deficit and were able to maintain a high stomatal conductance and consequently higher net CO<sub>2</sub> assimilation rates. Genotypes 14, 15 and 18 were able to maintain their basal levels of photosynthesis, which was accomplished by maintaining stomata open as these plants were able to maintain relatively high levels of water (relative water content and water potential). Genotypes 12, 13 and 17 showed to have an intermediate performance under drought stress. Although these plants were able to maintain the photosynthetic mechanisms active after 3 weeks under water deficit conditions, they were already probably close to their resistance limits, and a significant drop on net CO<sub>2</sub> assimilation rates was expected after a few more days under stress. Finally, some genotypes (*e.g.*, 19) were able to maintain relatively high levels of water (relative water content and water potential), but were unable to maintain satisfactory levels of net CO<sub>2</sub> assimilation which might be due to biochemical limitation of photosynthesis rather than stomatal constraints. From the genotypes identified on t<sub>0</sub> has to have higher net CO<sub>2</sub> assimilation rates, only genotype 13 was able to maintain a similar performance under stress. On the other hand, genotypes with lower net CO<sub>2</sub> assimilation rates on t<sub>0</sub> (*e.g.*, 17) were able to cope with drought stress and maintain the levels measured at t<sub>0</sub>. The genotypes identified on t<sub>0</sub> for its high net CO<sub>2</sub> assimilation rates (3, 5, 13, 21 and 25) might have high potential and their productivity should be evaluated. Nonetheless, on the water restriction scenario we hypothesize on this work, with the exception of genotype 13, they generally fail to cope with water stress.

Besides revealing the importance of genotype on strawberry tree physiological performance and response to drought, these results show that strawberry tree plants have a high phenotypic plasticity and are able to adjust differential strategies to cope with stress. The role of the genotype on plant response under drought have already been stressed by other authors [51,53], and is crucial for future breeding strategies that considers climate adaptation. Therefore, we are currently investigating the effect of genotype on plant response to drought stress in order to provide further insights on the mechanisms underlying phenotype plasticity.

Taking the isohydric behavior of strawberry tree into account and the obtained results, the parameters used as a selection criterion proved to be adequate to identify genotypes more tolerant to drought. However, due to the plasticity observed the physiological performance of plants without water restrictions can't be used to infer its behavior under water deficit. Therefore, in order to select water stress tolerant genotypes, we consider that only the physiological response under specific water stress conditions should be considered, which compromise early plant selection. In order to facilitate this process and considerably reduce the necessary required time for selection, the identification of other adequate selection parameters should be pursued. In particular, metabolites like phenols, proline, chlorophyll, anthocyanins and several hormones (*e.g.*, abscisic acid, jasmonic acid and salicylic acid), that are known to be essential on plant response mechanisms to drought stress, might be used as markers to identify plants with a better aptitude to undergo extreme drought events.

Although a great variance was observed between individuals from the same population, no differences were observed between populations on all the tested parameters. These results suggest that intra-population variation should be take into account and prioritized over inter-population on future selection endeavors, and a large number of individuals from within a population must be sampled. In contrast, results obtained by Vasques et al. [52] showed that seedlings provenience might influence the tolerance of plants under water stress, thus suggesting local adaptations of plant populations, which reinforces the importance of inter-population variance on plant behavior. Due to its implication on plant selection, this hypothesis should be further investigated. Overall, the

obtained results will have important repercussions on strawberry tree phenotyping and early plant selection as well as breeding towards the obtention of drought stress resistant genotypes.

## 5. Conclusions

As a basis for any breeding program that includes plant hybridization, a deep knowledge of the plant reproduction system is necessary. This work provides the first insights of strawberry tree reproduction system which will be crucial on future breeding attempts. A detailed morphological description of the flower and pollen is provided. No incompatibility barriers have been found on pollinations and although strawberry tree has a longer phenological cycle than most tree species, this is not an impediment for the obtention of hybrid plants. Nonetheless, the success of *in situ* pollinations needs to be considerably improved. As a tool for plant selection, the physiological parameters used on this study proved to be adequate. However, the analysis of biochemical parameters could not only elucidate the tolerance mechanism of *A. unedo* but also identify key metabolites (e.g., phenols, hormones and pigments) that could be used as markers for early plant selection. Three genotypes (14, 15 and 18) showed a particular aptitude to cope with water stress and may be the basis for a future breeding program. However, due to the influence of genotype on plant response to water stress and the observed phenotypic plasticity, the analysis of a large number of individuals should be carried out in order to develop a long-term breeding program. The selection and breeding of strawberry tree genotypes more tolerant to drought stress is essential in order to maintain species sustainability and our promising results are a step forward in order to ameliorate strawberry tree adaptation while preserving productivity on drought prone areas.

**Author Contributions:** Conceptualization, J.M. and J.C.; formal analysis, J.M. and P.M.; data analysis, J.M.; writing—original draft preparation, J.M.; writing—review and editing, G.P. and J.C.; supervision, G.P. and J.C.; funding acquisition, G.P. and J.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** Please add: “This research received no external funding” or “This research was funded by Foundation for Science and Technology (Portugal), who supported J. Martins PhD fellowship (SFRH/BD/122478/2016). The authors also acknowledge CESAM within PT2020 Partnership Agreement and Compete 2020 (UID/AMB/50017/2013-POCI-01-0145-FEDER-007638) and ReNATURE project (CENTRO-01-0145-FEDER-000007). This work was carried out at the R&D Unit Center for Functional Ecology - Science for People and the Planet (CFE), with reference UIDB/04004/2020, financed by FCT/MCTES through national funds (PIDDAC).

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

1. Torres, J.A.; Valle, F.; Pinto, C.; García-Fuentes, A.; Salazar, C.; Cano, E. *Arbutus unedo* L. communities in southern Iberian Peninsula mountains. *Plant Ecol.* **2002**, *160*, 207–223, doi:10.1023/A:1015864821706.
2. Morgado, S.; Morgado, M.; Plácido, A.I.; Roque, F.; Duarte, A.P. *Arbutus unedo* L.: From traditional medicine to potential uses in modern pharmacotherapy. *J. Ethnopharmacol.* **2018**, *225*, 90–102, doi:10.1016/j.jep.2018.07.004.
3. Alarcão-e-Silva, M.L.C.M.M.; Leitão, A.E.B.; Azinheira, H.G.; Leitão, M.C.A. The *Arbutus* Berry: Studies on its color and chemical characteristics at two mature stages. *J. Food Compos. Anal.* **2001**, *14*, 27–35, doi:10.1006/jfca.2000.0962.
4. Bussotti, F.; Ferrini, F.; Pollastrini, M.; Fini, A. The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. *Environ. Exp. Bot.* **2014**, *103*, 80–98, doi:10.1016/j.envexpbot.2013.09.013.
5. Munné-Bosch, S.; Peñuelas, J. Drought-induced oxidative stress in strawberry tree (*Arbutus unedo* L.) growing in Mediterranean field conditions. *Plant Sci.* **2004**, *166*, 1105–1110, doi:10.1016/j.plantsci.2003.12.034.
6. Martins, J.F.; Correia, S.I.; Canhoto, J.M. Somatic Embryogenesis Induction and Plant Regeneration in Strawberry Tree (*Arbutus unedo* L.). In *Methods in molecular biology*, vol. 1359; Germana, M., Lambardi, M., Eds.; Humana Press: New York, 2016; Vol. 1359, pp. 329–339.
7. Martins, J.F.; Santos, T.; Correia, S.I.; Canhoto, J.M. Somatic embryogenesis in *Arbutus unedo* L. and other Ericaceae. In *Vegetative Propagation of Forest Trees*; Park, Y.S., J., B., Moon, H.K., Eds.; National Institute of Forest Science: Seoul, 2016; pp. 565–590.

8. Gomes, F.; Canhoto, J.M. Micropropagation of strawberry tree (*Arbutus unedo* L.) from adult plants. *Vitr. Cell. Dev. Biol. - Plant* **2009**, *45*, 72–82, doi:10.1007/s11627-008-9164-8
9. Martins, J.F.; Correia, S.; Correia, B.; Pinto, G.; Canhoto, J.M. Shoot proliferation and organogenesis on *Arbutus unedo*: Physiological analysis under water stress. *Biol. Plant.* **2019**, *63*, 278–286, doi:10.32615/bp.2019.032.
10. Doorenbos, J. Shortening the breeding cycle of rhododendron. *Euphytica* **1955**, *4*, 141–146, doi:10.1007/BF00039639.
11. Escaravage, N.; Pornon, A.; Doche, B.; Till-Bottraud, I. Breeding system in an alpine species: *Rhododendron ferrugineum* L. (Ericaceae) in the French northern Alps. *Can. J. Bot.* **1997**, *75*, 736–743, doi:10.1139/b97-084.
12. Lyrene, P.M. Value of various taxa in breeding tetraploid blueberries in Florida. *Euphytica* **1997**, *94*, 15–22, doi:10.1023/A:1002903609446.
13. Usui, M.; Kevan, P.G.; Obbard, M. Pollination and breeding system of lowbush blueberries, *Vaccinium angustifolium* Ait. and *V. myrtilloides* Michx. (Ericaceae), in the boreal forest. *Can. Field-Naturalist* **2005**, *119*, 48–57, doi:10.22621/cfn.v119i1.80
14. Igarashi, M.; Hatsuyama, Y.; Harada, T.; Fukasawa-Akada, T. Biotechnology and apple breeding in Japan. *Breed. Sci.* **2016**, *66*, 18–33.
15. Aravanopoulos, F.A. Breeding of fast growing forest tree species for biomass production in Greece. *Biomass and Bioenergy* **2010**, *34*, 1531–1537.
16. Fryxell, P. Mode of reproduction of higher plants. *Bot. Rev.* **1957**, *23*, 135–233, doi:10.2307/4353564.
17. Erdtman, G. The acetolysis method in a revised description. *Sven. Bot. Tidskr. Lund* **1960**, *54*, 561–564.
18. Punt, W.; Hoen, P.P.; Blackmore, S.; Nilsson, S.; Le Thomas, A. Glossary of pollen and spore terminology. *Rev. Palaeobot. Palynol.* **2007**, *143*, 1–81, doi:10.1016/j.revpalbo.2006.06.008.
19. Spurr, A.R. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **1969**, *26*, 31–43, doi:10.1016/S0022-5320(69)90033-1.
20. Roland, J.C. General preparation and staining of thin sections. In *Electron microscopy and cytochemistry of plant cells*; Hall, J.L., Ed.; Elsevier/North-Holland Biomedical Press: Amsterdam, 1978; pp. 1–62.
21. Jahier, J.; Chevre, A.M.; Eber, F.; Delourme, R.; Tanguy, A.M. *Techniques de cytogénétique végétale*; INRA: Paris, 1992; ISBN 9782738003966.
22. Mateus, J. Pollen morphology of Portuguese Ericales. *Rev. Biol.* **1989**, *14*, 135–208.
23. Martin, F.W. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technol.* **1959**, *34*, 125–128, doi:10.3109/10520295909114663.
24. Yang, J.; Lovett-Doust, J.; Lovett-Doust, L. Seed germination patterns in green dragon (*Arisaema dracontium*, Araceae). *Am. J. Bot.* **1999**, *86*, 1160–1167, doi:10.2307/2656980.
25. R Core Team R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/> 2017.
26. Gu, Z.; Eils, R.; Schlesner, M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **2016**, *32*, 2847–2849, doi:10.1093/bioinformatics/btw313.
27. Schloerke, B.; Crowley, J.; Cook, D.; Briatte, F.; Marbach, M.; Thoen, E.; Elberg, A.; Larmarange, J. GGally: Extension to “ggplot2”. R package version 1.4.0. <https://CRAN.R-project.org/package=GGally> 2018.
28. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4. <https://ggplot2.tidyverse.org> 2016.
29. Vu, V.Q. ggbiplot: A ggplot2 based biplot. R package version 0.55. <http://github.com/vqv/ggbiplot> 2011.
30. Villa, R.S. Ricerche sulla biologia di *Arbutus unedo* L. (Ericaceae): ciclo di sviluppo. *Boll. della Soc. sarda di Sci. Nat.* **1982**, *21*, 309–317.
31. Hermann, P.M.; Palser, B.F. Stamen development in the Ericaceae. I. Anther wall, microsporogenesis, inversion, and appendages. *Am. J. Bot.* **2000**, *87*, 934–957, doi:10.2307/2656993.
32. Čalić, D.; Devrnja, N.; Kostić, I.; Kostić, M. Pollen morphology, viability, and germination of *Prunus domestica* cv. Požegača. *Sci. Hortic. (Amsterdam)*. **2013**, *155*, 118–122, doi:10.1016/j.scienta.2013.03.017.
33. Acar, I.; Gopal, V. The effects of temperature on *in vitro* pollen germination and pollen tube growth of *Pistacia* spp. *Sci. Hortic. (Amsterdam)*. **2010**, *125*, 569–572, doi:10.1016/j.scienta.2010.04.040.
34. Rosell, P.; Herrero, M.; Galán Saúco, V. Pollen germination of cherimoya (*Annona cherimola* Mill.). *Sci. Hortic. (Amsterdam)*. **1999**, *81*, 251–265, doi:10.1016/S0304-4238(99)00012-6.
35. Vuletin Selak, G.; Perica, S.; Goreta Ban, S.; Poljak, M. The effect of temperature and genotype on pollen performance in olive (*Olea europaea* L.). *Sci. Hortic. (Amsterdam)*. **2013**, *156*, 38–46, doi:10.1016/j.scienta.2013.03.029.
36. Cane, J.H. Pollen viability and pollen tube attrition in cranberry (*Vaccinium macrocarpon* Aiton). *Acta Hortic.* **2009**, *810*, 563–566, doi:10.17660/ActaHortic.2009.810.74.
37. Okusaka, K.; Hiratsuka, S. Fructose inhibits pear pollen germination on agar medium without loss of viability. *Sci. Hortic. (Amsterdam)*. **2009**, *122*, 51–55.
38. Nátróvá, Z.; Hlaváč, M. The effect of gametocides on microsporogenesis of winter rye. *Biol. Plant.* **1975**, *17*, 256–262, doi:10.1007/BF02921216.
39. Verma, M.M.; Kumar, J. Ethrel-a male gametocide that can replace the male sterility genes in barley. *Euphytica* **1978**, *27*, 865–868, doi:10.1007/BF00023727.
40. Saimbhi, M.S.; Brar, J.S. A review of the practical use of gametocides on vegetable crops. *Sci. Hortic. (Amsterdam)*. **1978**, *8*, 11–17, doi:10.1016/0304-4238(78)90064-X.



41. Chakraborty, K.; Devakumar, C. Ethyloxanilates as specific male gametocides for wheat (*Triticum aestivum* L.). *Plant Breed.* **2006**, *125*, 441–447, doi:10.1111/j.1439-0523.2006.01254.x .
42. Voyiatzis, D.G.; Paraskevopoulou-Paroussi, G. Factors affecting the quality and *in vitro* germination capacity of strawberry pollen. *J. Hortic. Sci. Biotechnol.* **2002**, *77*, 200–203, doi:10.1080/14620316.2002.11511479.
43. Cano-Medrano, R.; Darnell, R.L. Effect of GA<sub>3</sub> and pollination on fruit set and development in rabbiteye blueberry. *Hortic. Sci.* **1998**, *33*, 632–635.
44. Horsley, T.N.; Johnson, S.D. Is *Eucalyptus* cryptically self-incompatible? *Ann. Bot.* **2007**, *100*, 1373–1378, doi:10.1093/aob/mcm223.
45. Kaufmane, E.; Rumpunen, K. Pollination, pollen tube growth and fertilization in *Chaenomeles japonica* (Japanese quince). *Sci. Hortic. (Amsterdam)*. **2002**, *94*, 257–271, doi:10.1016/S0304-4238(01)00371-5.
46. Demirsoy, L.; Demirsoy, H.; Celikel, G.; Macit, I.; Ersoy, B. Seed treatment with GA<sub>3</sub> or stratification enhances emergence of some strawberry tree genotypes – Short communication. *Hortic. Sci.* **2010**, *37*, 34–37.
47. Ertekin, M.; Kirdar, E. Breaking seed dormancy of strawberry tree (*Arbutus unedo*). *Int. J. Agric. Biol.* **2010**, *12*, 57–60.
48. Tilki, F. Improvement in seed germination of *Arbutus unedo* L. *Pakistan J. Biol. Sci.* **2004**, *7*, 1640–1642.
49. Castell, C.; Terradas, J. Effects of water and nutrient availability on water relations, gas exchange and growth rate of mature plants and resprouts of *Arbutus unedo* L. *Ann. Bot.* **1994**, *73*, 595–602, doi:10.1006/anbo.1994.1074.
50. Raimondo, F.; Trifilò, P.; Lo Gullo, M.A.; Andri, S.; Savi, T.; Nardini, A. Plant performance on Mediterranean green roofs: Interaction of species-specific hydraulic strategies and substrate water relations. *AoB Plants* **2015**, *7*, plv007, doi:10.1093/aobpla/plv007.
51. Forner, A.; Valladares, F.; Aranda, I. Mediterranean trees coping with severe drought: Avoidance might not be safe. *Environ. Exp. Bot.* **2018**, *155*, 529–540, doi:10.1016/j.envexpbot.2018.08.006.
52. Vasques, A.; Chirino, E.; Vilagrosa, A.; Vallejo, V.R.; Keizer, J.J. The role of seed provenance in the early development of *Arbutus unedo* seedlings under contrasting watering conditions. *Environ. Exp. Bot.* **2013**, *96*, 11–19, doi:10.1016/j.envexpbot.2013.08.004.
53. Correia, B.; Pintó-Marijuan, M.; Neves, L.; Brossa, R.; Dias, M.C.; Costa, A.; Castro, B.B.; Araújo, C.; Santos, C.; Chaves, M.M.; Pinto, G. Water stress and recovery in the performance of two *Eucalyptus globulus* clones: physiological and biochemical profiles. *Physiol. Plant.* **2014**, *150*, 580–592, doi:10.1111/ppl.12110.