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In Vitro Seed and Clonal Propagation of the Mediterranean Bee Friendly Plant *Anthyllis hermanniae* L.

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Abstract: *Anthyllis hermanniae* L. (Fabaceae) is a perennial Mediterranean shrub with the potential to be used as a bee-friendly ornamental plant in arid and semi-arid regions, valued for its tolerance of barren soils, winds, and strong temperature changes. With the aim of facilitating the introduction of the species into the horticulture industry, its in vitro seed and clonal propagation was investigated for the first time, to our knowledge. Seeds stored in the dark at room temperature for 4, 7, 9, 12, and 18 months germinated at percentages higher than 80% after scarification, when incubated in vitro in solid half-strength Murashige and Skoog (MS) medium at temperatures from 10 to 25 °C, while photoperiod (continuous darkness or 16 h light period/8 h dark) during incubation did not affect germination. Explants excised from in vitro grown seedlings established at higher percentages compared to explants from adult native plants, more efficiently in MS medium with 1.0 mg L^{-1} 6 N benzyladenine (BA). During subcultures in the same medium, juvenile explants formed more and longer shoots than adult ones. Almost all adult explants formed shoots when subcultured in MS medium with 0.0 to 4.0 mg L^{-1} BA, zeatin, kinetin or 6-(γ,γ-dimethylallylamino)purine (2iP). BA at 0.5 to 2.0 mg L^{-1} induced many more shoots (17–21) per explant and much higher multiplication indices compared to all other cytokinins, while longer shoots were produced in a medium without hormones or with 0.5–1.0 mg L^{-1} 2iP. Microshoots cultured in half-strength MS medium with 0.0–4.0 mg L^{-1} indole-3-butyric acid rooted at highest percentage (around 70%) in the medium containing 4.0 mg L^{-1} IBA, while microshoots of juvenile origin developed more and longer roots compared to adult ones. Micropropagated plantlets were successfully acclimatized ex vitro (>97%), regardless of their origin. The efficient micropropagation of *A. hermaniae* will facilitate its sustainable exploitation as a bee-friendly landscape plant, a forage plant for honeybees in Mediterranean areas, and a medicinal plant.

Keywords: acclimatization; auxin; cytokinin; explant origin; germination conditions; micropropagation; native xerophytic ornamental; proliferation; rooting; seed pretreatment

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1. Introduction

Anthyllis hermanniae L. (f. Fabaceae), the lavender-leaved anthyllis, is an evergreen densely branched shrub, typically 45–60 cm tall and wide, with crooked or zigzag branches. The leaves are small (6 × 20 mm), simple or trifoliate, with soft silky hairs mostly at the lower surface, presenting a rather grayish color in winter. Small yellow flowers appear in April–June, along the branches in loose clusters, three to five per node, and the whole plant gets a yellow color. Its flowers attract bees, butterflies, and a host of other pollinators, making it an important honeybee forage plant [1–3]. *A. hermaniae* is found in Anatolia, Greece, the Balkans, and many Mediterranean islands [4,5], in a variety of habitats, from pine forests and maquis to rocky cliffs and sandy beaches [1–3].

Due to the plant's appearance, as well as its tolerance in barren soils, xerothermic conditions, winds, and strong temperature changes, it is recommended to be used as an ornamental plant in sustainable urban and peri-urban green spaces, in rock gardens,

xeriscaping, costal planting and degraded soil areas, as well as in archaeological sites of the Mediterranean region [6]. Its property of attracting pollinators and its limited needs for irrigation serves the current trend for sustainable landscaping. Even urban landscapes present a conservation opportunity to promote pollinators biodiversity through maintaining flowering plants, planting native perennial plant species, and conserving large urban habitats [7]. Apart from this, the species is of nutritional and medicinal interest; it is important as forage for honeybees in Mediterranean coastal areas [8] and has been studied for possible use in the pharmaceutical industry, due to its content of chalcones and isoflavonoids in its aerial parts [9–13].

Species of the Fabaceae family have hard seeds that exhibit physical dormancy [14], as a consequence of impermeability to water and gas due to the thickness and biochemical composition of their testa [15–17].

Usually, physical dormancy is faced by seed scarification before placing in the adequate environmental conditions for germination. Scarification is applied either by using sandpaper or a sharp instrument to chip or pierce the seed coat, or by immersion in sulphuric acid or hot water, and the most appropriate method varies according to the plant species and even the seed lot [18]. Storage period and conditions during storage are of great importance, affecting the germination process as well [19]. Finally, temperature and light conditions are two basic, ecological parameters of great importance for seed germination, affecting both the germination rate and speed [20]. It has been shown that *A. hermaniae* seeds germinated at 5–20 °C after pretreatment with immersion in boiling water [21]. This is the only published work found on propagation of the species.

The investigation of seed germination of *A. hermaniae*, as well as the development of in vitro propagation methods starting from seedlings and adult plants, is necessary to facilitate the commercial exploitation of the species. Propagation by seed favors the higher genetic diversity that is desirable when native plants are reintroduced in the landscape. In addition, the use of seedlings as parent material for micropropagation could lead to high proliferation rates, as shown in *Teucrium capitatum* [22], and help overcome problems of explant sterilization.

In vitro clonal propagation in addition to the advantages of micropropagation, such as the production of certified, disease-free propagation material in large quantities in a short time and limited space without seasonal constraints, in *A. hermanniae* could contribute to the conduct of breeding experiments, as well as its utilization in the pharmaceutical industry. Micropropagation is important to select, multiply, and conserve the critical genotypes of medicinal and aromatic plants [23,24].

Micropropagation protocols have been developed for many native plant species, aiming to facilitate commercial propagation and enable their sustainable use [23–25]. Regarding species of the genus *Anthyllis*, *A. cytisoides* was successfully micropropagated by explants of seedling and adult origin, and higher proliferation rates were obtained when axillary buds from adult plants were used [26], while *A. barba-jovis* was micropropagated by explants of seedling origin [27,28] and showed the highest proliferation when cultures were initiated from of cotyledonary nodes explants [28].

A. cytisoides and *A. barba-jovis* readily rooted in vitro [26–28]; however, other species of the Fabaceae family present difficulties in rooting [29]. In vitro rooting is a result of an interaction between the environment in which rooting takes place, and the explant source [30]. Root formation is affected by, among other parameters, the salt, sugar, and auxin concentration of the rooting medium, the type of auxin and the duration of its application, and often by the type and concentration of cytokinin used in the shoot proliferation medium [29].

Ex vitro acclimatization and establishment of micropropagated plantlets is an important stage of micropropagation protocols. In previous reports, Mediterranean native shrubs of the macchia zone, including *A. cytisoides* and *A. barba-jovis* have been successfully acclimatized to ex vitro conditions [22,26–28,31,32].

Regarding subsequent plant breeding, it is important to determine the appropriate substrate and fertilization levels, especially those of nitrogen (N), which will provide op-

timal and commercially viable plant growth. That is because nitrogen (N) management remains a global challenge for the sustainability of diversified farming systems, as the use of N-fertilizers has contributed to the increase of agricultural yields and food production, but overfertilization may cause major environmental problems, such as soil and water acidification, contamination of surface and groundwater resources, increased ozone depletion and greenhouse gas levels, and loss of biodiversity [33,34]. Among the benefits of native plants is that they have low cultivation requirements, and this has been proved in case of several Mediterranean plants, which grew equally satisfactorily in greenhouse conditions when they were fertilized with half of the recommended concentration of fertilizer [35,36].

In this work, for the first time, to our knowledge, the in vitro seed and clonal propagation of *A. hermanniae* was investigated, with the aim of facilitating the sustainable exploitation of the species, as a bee-friendly landscape plant, forage plant for honeybees in Mediterranean areas, and medicinal plant. In particular, the effects of: (a) pretreatment, temperature, light, and storage period on seed germination, (b) explant origin (juvenile, adult) on in vitro culture establishment and proliferation, (c) cytokinin type and concentration on shoot proliferation, (d) explant origin, auxin concentration, and cytokinin used for proliferation on in vitro rooting, (e) explant origin on ex vitro acclimatization of plantlets, and (f) substrate type and fertilization on ex vitro establishment of plantlets were studied.

2. Materials and Methods

2.1. Seed Material and Its Viability

Seeds were collected in August 2013 and July 2014 from the same selected *A. hermanniae* wild plants in the region of "Diomedes Botanic Garden" (38°00′39.24″ B, 23°38′11.32″ E, altitude 157 m), in Haidari, a western suburb of Athens, Greece. Seeds, whose dimensions were about 1.5 mm × 0.8 mm (Figure 1a), were found inside tiny lobs (3–4 mm × 1–1.5 mm) and surrounded by dried floral debris (Figure 1b). The seed material was left to dry spread out in trays in an open-air shaded place for 15 d and then stored in glass vessels at room temperature (about T = 21 °C and darkness). Before each germination experiment, seeds were excised from the lobs by hand, using small forceps or tweezers, while mechanical pressure between two tiles was also tested. The weight of 100 seeds was estimated.

Seed viability was determined once, using seeds collected in August 2013 four months after harvest. Seeds were submitted to 2,3,5-triphenil tetrazolium chloride (TZ) staining (1.0%), at 20 °C, in D, for 24 h. A total of 100 seeds were used (25 seeds/Magenta™ glass vessel, 4 vessels containing 25 mL of TZ-solution each) for each test. The embryo of viable seeds was colored red. Embryos that had less than 1/2 cotyledon colored-red or non-colored hypocotyl were considered non-viable [37]. The coloration of the embryo was observed using a portable QS.20200-P (Euromex Microscopen, Arnhem, The Netherlands) microscope.

2.2. In Vitro Seed Germination

Four months after harvesting (December 2013), seeds were scarified either using sandpaper suitable for metal surfaces (No 100) for 1 min or by dipping in concentrated sulfuric acid (H_2SO_4 > 95%, Fisher Scientific, Leicestershire, UK) for 15 min, or they did not receive any treatment (control), before surface sterilization by 15% *v*/*v* commercial bleach (4.5% *w*/*v* sodium hypochloride) water solution with 1–2 drops of Tween 20 (polyxyethylenesorbitan monolaurate, Merck KGaA, Darmstadt, Germany) for 15 min, followed by three 3-min rinses with sterile distilled water. Then, they were cultured for germination in vitro, in Petri dishes (Figure 1f), with half-strength (1/2) MS medium [38] (Sigma-Aldrich, St. Louis, MO, USA) with 20 g L^{-1} sucrose, at 20 °C and 16 h cool white fluorescent light (37.5 μmol m^{-2} s^{-1})/8 h dark photoperiod.

Nine months after harvest (May 2014), seeds, which either received no treatment or were scarified with sandpaper, were subjected to the same disinfection and placed in Petri dishes with 1/2MS medium at temperatures of 5, 10, 15, 20, 25, or 30 °C, under 16 h light or continuous darkness.

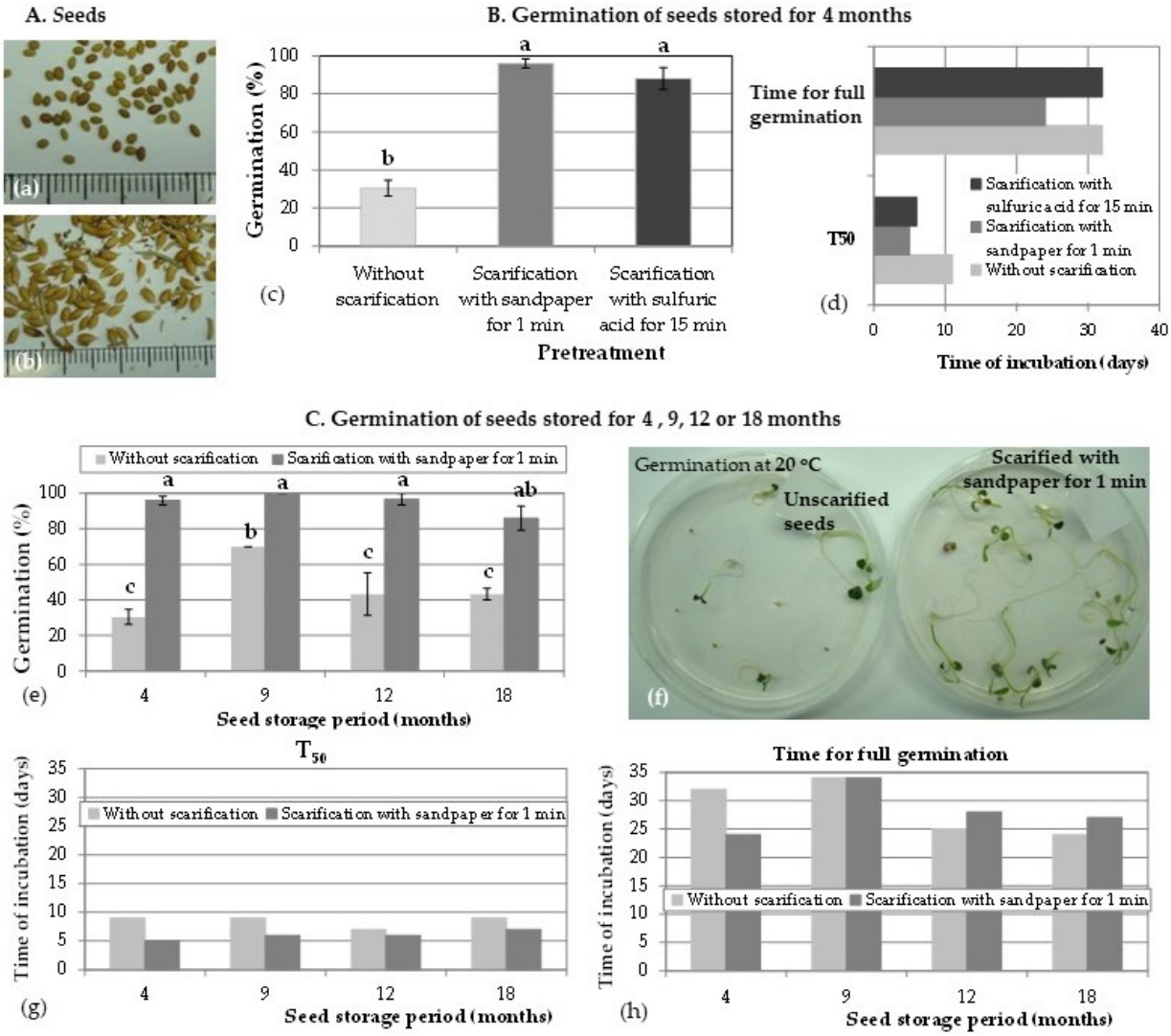

Figure 1. (**A**) Seeds (**a**) and lobs (**b**) of *A. hermanniae*; (**B**) Effect of scarification pretreatment on germination percentage (**c**), as well as time for full germination and T_{50} (**d**) of seeds stored for 4 months; (**C**) Effect of scarification pretreatment and storage period on germination percentage (**e**), T_{50} (**g**) and time for full germination (**h**) of seeds stored for 4–18 months; Typical seed germination at 20 °C of unscarified and scarified seeds (**f**); Germination took place in vitro in Petri dishes with solid half-strength MS medium at 20 °C, under 16 h photoperiod. Mean values (n = 5 repetitions of 10 seeds per dish) in each bar of subfigures (**c**,**e**) followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test.

Aiming to examine the effect of storage period at room conditions on seed germination, in August 2014 and February 2015 (12 and 18 months, respectively, after harvest), seeds, either untreated or scarified with sandpaper, were surface sterilized and placed in Petri dishes with 1/2MS medium at 20 °C, under 16 h of light.

In February 2015, a replicate experiment was carried out using seeds collected in July 2014, 7 months after their harvest, in which seeds were either left untreated or scarified with sandpaper, and placed, after disinfection, on Petri dishes with 1/2MS medium at temperatures 5–30 °C, under 16 h light or continuous darkness.

The observation period was 35–40 days. Five replicates of 20 seeds were used for each treatment and a seed was considered germinated when the radicle was longer than 1 mm. T_{50} was also estimated, which is the time needed for 50% of the final germination value, and it is calculated by linear interpolation from the two germination values closest to median germination.

2.3. In Vitro Culture Establishment Stage

Explants for the establishment of in vitro culture were initially excised from mature native plants (older than 6 years old), grown in the same area where seeds were collected. Single-node explants, approximately 1.0 cm long, from the mid-section of annual shoots (3rd–5th node below the apex, which always ended in a thorn) were collected in June and August 2013 (Figure 2a). Before explant excision, the mother material was washed in water with a light detergent for 10 min under stirring, rinsed under running tap water for 10 s and surface sterilized with 20% v/v commercial bleach water solution with 1–2 drops of Tween 20 for 10 min followed by three 3-min-rinses with sterile distilled water. Following this, explants were cultured in MS medium with 30 g L^{-1} sucrose, either hormone free (hf) or supplemented with 1.0 mg L^{-1} 6-benzyladenine (BA) or zeatin (ZEA) (Sigma-Aldrich, St. Louis, MO, USA).

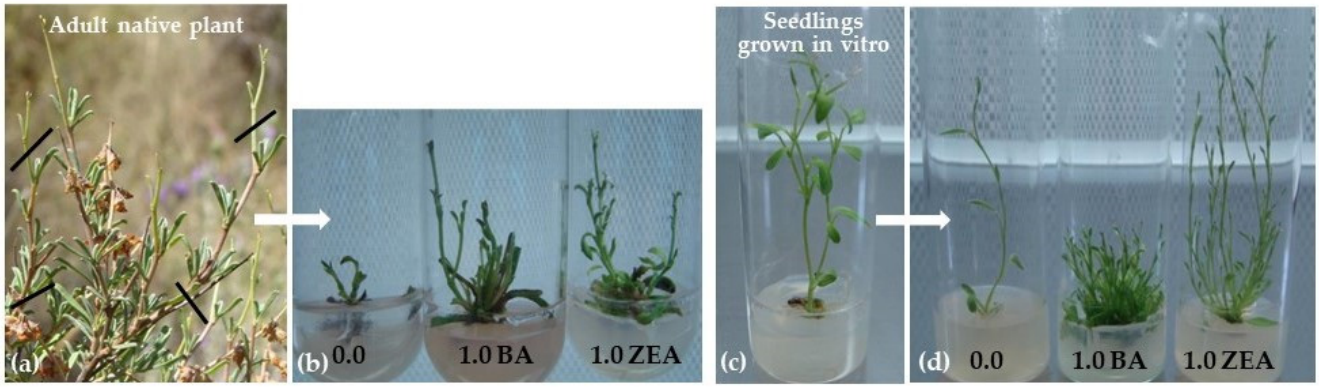

Figure 2. Characteristic morphology of *A. hermanniae* adult mother stems from which adult explants were excised (**a**) and typical in vitro response of adult explants (**b**); seedling morphology from which juvenile explants were excised (**c**) and typical in vitro response of juvenile explants (**d**). In vitro establishment took place in MS medium with marked cytokinin type and concentration (mg L^{-1}).

In vitro culture was also established from seedlings grown in vitro. After completion of germination, the most vigorous seedlings were transferred to grow for six weeks in test tubes containing MS medium with 30 g L^{-1} sucrose. Shoot tip and single-node explants, approximately 0.6 cm long, were obtained from the in vitro grown seedlings (Figure 2c) and cultured on the same substrates as explants from adult plants.

Twenty-four explants were used for each treatment and data were collected after 40 days of culture.

2.4. Shoot Multiplication Stage

Cultures established (in June and August) from native adult plants (adult cultures), as well as the culture established from in vitro seedlings (juvenile culture) were maintained by continuous subcultures every six weeks in MS medium with 1.0 mg L^{-1} BA. Moreover, the cytokinins BA, ZEA, kinetin (KIN), or 6-(γ,γ-dimethylallyamino) purine (2iP) at concentrations of 0.0 (control), 0.5, 1.0, 2.0, or 4.0 mg L^{-1} were tested for their efficacy on shoot proliferation using explants from the adult culture established in June. All cytokinins used were purchased from the company Sigma-Aldrich, St. Louis, MO, USA.

Thirty explants were used for each treatment. Data were collected after 40 days of culture. The "multiplication index" of each culture was calculated by multiplying the percentage of explants that produced shoots by the mean number of shoots per responding explant, and by the mean length of produced shoots, divided by 0.6, which was the length of explants used for subculture. The "multiplication index" gave the proliferation potential of each culture by showing the number of explants that the culture could provide for a subsequent subculture.

2.5. In Vitro Rooting

Microshoots, 1.0–2.0 cm long, produced in MS medium containing 1.0 mg L^{-1} BA, were excised from both the adult and juvenile cultures and placed for rooting in half-strength MS medium with 20 g L^{-1} sucrose supplemented with indole-3-butyric acid (IBA) (Sigma-Aldrich, St. Louis, MO, USA) at 0.0, 0.5, 1.0, 2.0, or 4.0 mg L^{-1}, either for six weeks or for one week followed by transfer to hf-1/2MS medium for five more weeks.

Apart from the effect of the physiological state (adult or juvenile) of microshoots on rooting, the effect of the cytokinin type used in the shoot proliferation stage on the subsequent rooting of the produced microshoots was also investigated. For this purpose, microshoots obtained from adult and juvenile culture and grown in a medium containing either BA or ZEA (1.0 mg L^{-1}) were cultured for rooting on the above IBA media for six weeks.

Thirty, forty-five, or sixty microshoots were used for each treatment, depending on the experiment (relevant information is given in data tables), and data were recorded after 42 days of culture.

2.6. Ex Vitro Acclimatization and Plant Growth

For acclimatization, plantlets of adult or juvenile origin were transferred ex vitro into trays (eight plantlets per 500 mL volume tray) with a mixture of peat: perlite 1:1 (v/v). The trays were covered with plastic wrap (SANITAS, Sarantis S.A., Athens, Greece) and placed in a growth chamber (20 °C and 16 h cool white fluorescent light 37.5 μmol m^{-2} s^{-1}) for one week, then the cover was removed, and the trays were transferred to a heated greenhouse, on a semi-shaded bench, for a further 7 weeks. Four replicates of 8 rooted microshoots were used and two acclimatization experiments were carried out during autumn 2014 and late winter 2015. Plantlets survival was estimated 7 weeks after transfer to the greenhouse.

Regarding subsequent growth, plantlets were transplanted individually in plastic pots (1.3 L) either on a mixture of peat: perlite 2:1 (v/v) and fertilized monthly with 2 or 4 g L^{-1} soluble fertilizer (Nutrileaf 60, 20-20-20, Miller Chemical and Fertilizer Corp., Hanover, PA, USA), 100 mL of solution per pot, or on a mixture of enriched peat: perlite 2:1 (v/v) and received no extra fertilization. Thirteen plants per treatment were used and this experiment lasted from April to July 2014. Data were recorded monthly during this period. Average monthly temperatures in the greenhouse ranged between 17.3 and 29.3 °C; the average minimum temperature, between 24.8 and 31.5 °C; the average maximum temperature and between 21.5 and 30.2 °C; the average day temperature, starting with lower temperatures in April and ending with the highest temperature in July 2014.

2.7. In Vitro Culture Conditions

All culture media were solidified with 8 g L^{-1} agar (M. Roumboulakis S.A., Athens, Greece) and their pH was adjusted to 5.7 before agar addition and autoclaving (at 121 °C for 20 min). Initial cultures took place in test tubes (25 × 100 mm) with 10 mL medium, while subcultures and rooting took place in 75 mL glass vessels with 20 mL medium (three explants and five microshoots per vessel, respectively), all of them covered with plastic wrap (SANITAS, Sarantis S.A., Greece). All in vitro cultures were maintained at 25 °C with a 16 h photoperiod at 37.5 μmol m^{-2} s^{-1} fluorescent light, provided by cool-white fluorescent lamps.

2.8. Statistical Analysis

A completely randomized design was used in all experiments. The significance of the results was tested either by one- or two- or three-way analysis of variance (ANOVA) and the means of the treatments were compared by the Student's t test at $p \leq 0.05$ (JMP 13.0 software, SAS Institute Inc., Cary, NC, USA, 2013).

3. Results

3.1. Seed Material and Its Viability

A. hermanniae seed material, collected from wild plants during two successive years, 2013 and 2014, consisted of tiny lobs, each one containing one seed, and dried floral debris. The floral debris was easily removed by rubbing the pods between each other by hand, but then each pod had to be carefully broken with small forceps or, better, eyebrow tweezers to receive the clean seed. Cleaning the seed by rubbing the gross seed between two tiles was also tried, but this resulted in several seeds breaking as well. It was also observed that only 20% of the lobs that were collected in August 2013 contained seeds, whereas the following year, when seeds were collected earlier, in July 2014, about 80% of the lobs contained seeds.

In the 2013 seed collection, the weight of 100 seeds was 0.137 g, while tetrazolium test showed that 99.0% of seeds were viable four months after harvest.

3.2. In Vitro Seed Germination

Regarding the 2013 seed collection, four months after harvest and storage in the dark, at room conditions, seeds that had been scarified by either sandpaper or H_2SO_4 germinated at higher percentages at 20 °C (Figure 1c) and reached T_{50} faster than those without any pretreatment, while the shortest time to complete germination was observed in sandpaper-treated seeds (Figure 1d).

With regard to the effect of storage period on seed germination, 18 months after harvest, a slight decrease (not statistically significant) of approximately 10% was observed in the germination rate of the scarified seeds (Figure 1e). Although seeds stored for 18 months did not show a significant reduction in germination when scarified, seedlings showed reduced growth compared to those produced from recently harvested seeds. Storage period did not affect T_{50} and time for full germination (Figure 1g,h). Non-pretreated seeds generally germinated at lower rates, by about 50%, compared to the scarified ones, regardless of storage period; however, an increase in their germination percentage was observed when stored for nine months, which was reduced again after further storing (Figure 1e).

The effect of temperature and photoperiod on germination of scarified and unscarified seeds was examined with two replicate experiments, using seeds collected in 2013 and 2014, nine and seven months after harvest, respectively. Both experiments resulted in similar germination parameters. Three-way ANOVA of the 2013 seeds (nine months storage) experiment showed significant interaction of the three experimental, while in the experiment with 2014 seeds (seven months storage) there was an interaction between pretreatment and temperature, while photoperiod did not affect germination percentage (Table 1). Unscarified seeds germinated at higher percentages when they were incubated at 20 °C, while scarified seeds showed expanded temperature ranges for maximum germination, i.e., 10–25 °C (Table 1). At 30 °C, the germination percentage was greatly reduced and seedlings were dehydrated, while at 5 °C, only the seeds of 2014 germinated at low percentages, starting their germination after 27 days, much more slowly than seeds at higher temperatures, and showing greatly increased T_{50} compared to germination at all other temperatures (Table 1). The application of scarification increased seed germination percentages, while the incubation under 16-h light or under continuous darkness had no significant effect (Table 1 and Figure 1d). Scarified seeds reached T_{50} faster, while the time for full germination was not significantly affected by the treatment seeds had received (Table 1).

Taking into account the high germination percentage and the fast germination rate, as determined by T_{50} and the time for full germination, the best temperatures for germination were 15–20 °C (Table 1).

3.3. In Vitro Culture Establishment Stage

Explants obtained from in vitro grown seedlings were established at higher percentages, formed more shoots per explant, and showed a higher multiplication index compared to explants from adult native plants, which responded at slightly higher percentages in the beginning than at the end of summer (Figures 2b,d and 3a,b,d). Regarding the con-

tamination of explants from native plants, this occurred at a higher percentage in June (28%) than in August (7%). In both adult and juvenile explants, the highest values of shooting percentage and mean shoot number per explant were recorded in the substrate with 1.0 mg L^{-1} BA and the lowest in the control (Figure 3a,b). The medium with BA induced the most shoots, while that with ZEA the longest ones (Figure 3b,c).

Table 1. The effect of mechanical scarification pretreatment, incubation temperature, and photoperiod on in vitro germination of *A. hermanniae* seeds, collected in August 2013 (A13) and July 2014 (J14), 9 and 7 months after harvesting and storage at room temperature, respectively.

Pretreatment	Photoperiod	Germination Temperature (°C)	Germination (%)	T_{50} (Days)	Time for Full Germination (Days)
	Seed collecting time:		A13/J14	A13/J14	A13/J14
No scarification	16 h light/ 8 h dark	5	0.0 k [Z]/6.0 h	-/30	-/33
		10	50.0 h/42.0 cde	10/10	19/27
		15	35.0 i/40.0 de	15/7	19/24
		20	70.0 de/58.0 c	9/10	37/27
		25	50.0 h/35.0 def	16/12	34/24
		30	20.0 j/18.0 fgh	9/9	10/21
	Continuous darkness	5	0.0 k/12.0 gh	-/30	-/33
		10	40.0 i/28.0 efg	8/16	22/27
		15	55.0 gh/44.0 cde	8/7	28/27
		20	50.0 h/46.0 cd	8/5	19/27
		25	25.0 j/40.0 de	7/15	37/27
		30	25.0 j/36.0 de	4/12	31/27
Mechanical scarification with sandpaper for 1 min	16 h light/ 8 h dark	5	0.0 k/40.0 de	-/28	-/33
		10	75.0 d/84.0 ab	8/9	28/27
		15	100.0 a/86.0 ab	6/7	28/27
		20	100.0 a/94.0 a	6/7	37/15
		25	85.0 c/76.0 b	6/10	37/21
		30	65.0 ef/46.0 cd	6/9	19/30
	Continuous darkness	5	0.0 k/30.0 def	-/28	-/33
		10	95.0 ab/90.0 ab	8/9	16/24
		15	85.0 c/98.0 a	6/5	22/15
		20	90.0 bc/98.0 a	4/5	16/12
		25	90.0 bc/86.0 ab	6/12	40/30
		30	60.0 fg/44.0 cde	6/10	40/30
	Significance [§]				
	$F_{pretreatment}$		-/-		
	$F_{photoperiod}$		-/NS		
	$F_{temperature}$		-/-		
	$F_{pretreatment \times photoperiod}$		-/NS		
	$F_{pretreatment \times temperature}$		-/**		
	$F_{photoperiod \times temperature}$		-/NS		
	$F_{pretreatment \times photoperiod \times temperature}$		**/NS		
	$F_{one\text{-}way\ ANOVA}$		**/**		

[Z] Mean values (n = 5 repetitions of 20 seeds) in each column followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. [§] NS or **: non-significant at $p \leq 0.05$ or significant at $p \leq 0.01$, respectively.

Especially in the medium with 1.0 mg L^{-1} BA, which was the best treatment for both origins of explants, it was found that explants of juvenile origin were established at double percentage (Figure 3a), formed three times the number of shoots (Figure 3b), and showed a five-fold multiplication index (Figure 3d) compared to explants of adult origin, while no differences were observed in terms of the length of the produced shoots (Figure 3c).

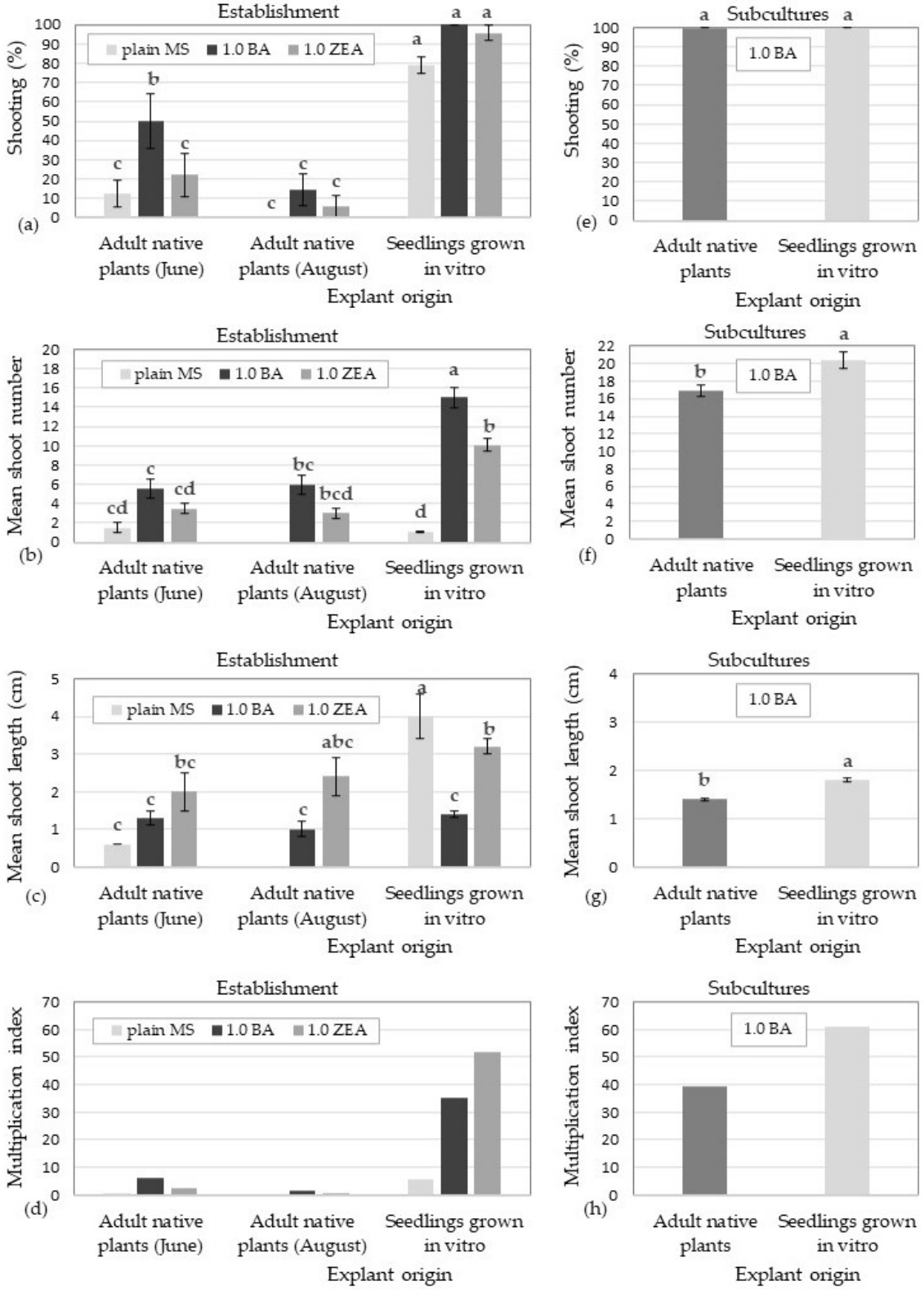

Figure 3. Effect of explant adult or juvenile origin and cytokinin type on shoot multiplication of *A. hermanniae* explants, during in vitro establishment (**a**–**d**, $n = 24$) and subcultures (**e**–**h**, $n = 90$). Mean values in each figure followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. Multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6. In each subfigure, mean values in each bar followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test.

3.4. Shoot Multiplication Stage

During subcultures in MS medium with 1.0 mg L^{-1} BA, all explants of both origins responded producing shoots (Figure 3e), although explants of juvenile origin formed more (Figure 3f) and longer shoots (Figure 3g) and showed a higher multiplication index (Figure 3h) than that of adult explants.

Adult explants, which were also subcultured in MS medium with various concentrations and types of cytokinin, all formed shoots, except those cultured in a medium containing 0.5–2.0 mg L^{-1} KIN (Table 2). The number of shoots formed was greater in explants cultured in a medium containing 0.5–4.0 mg L^{-1} BA than in those containing ZEA, KIN or 2iP or the hf-medium (Table 2 and Figure 4). The longest shoots were formed in the hormone-free medium or that with 0.5–1.0 mg L^{-1} 2iP (Table 2 and Figure 4). The highest values of multiplication index were recorded in media with 0.5–2.0 mg L^{-1} BA (Table 2).

Table 2. Effect of cytokinin type and concentration (mg L^{-1}) on shoot multiplication of *A. hermanniae* explants, excised from cultures established from adult native plants in June.

Cytokinin Type	Cytokinin Concentration	Shooting (%)	Mean Shoot Number	Mean Shoot Length (cm)	Multiplication Index
Control [#]	0.0	100.0 a [Z]	1.5 h	2.0 a	4.9
BA	0.5	100.0 a	17.3 b	1.4 cdef	40.9
	1.0	100.0 a	17.8 b	1.4 cdef	40.9
	2.0	100.0 a	20.8 a	1.3 f	44.4
	4.0	100.0 a	17.3 b	0.9 g	27.1
ZEA	0.5	100.0 a	7.0 e	1.4 cdef	16.3
	1.0	100.0 a	8.6 de	1.5 cdef	20.8
	2.0	100.0 a	10.2 d	1.5 cde	26.2
	4.0	100.0 a	14.9 c	1.4 def	33.8
KIN	0.5	93.3 b	1.3 h	1.3 ef	2.7
	1.0	90.0 b	1.6 gh	1.6 bcd	3.8
	2.0	93.3 b	1.6 gh	1.6 bc	4.0
	4.0	100.0 a	2.5 gh	1.4 cdef	6.8
2iP	0.5	100.0 a	1.3 h	1.8 ab	3.9
	1.0	100.0 a	2.2 gh	1.8 ab	6.7
	2.0	100.0 a	3.1 g	1.6 bc	9.2
	4.0	100.0 a	5.0 f	1.4 cdef	11.9
Significance [§]					
$F_{\text{cytokinin type}}$		**	-	-	
$F_{\text{cytokinin concentration}}$		NS	-	-	
$F_{\text{cytokinin type} \times \text{concetration}}$		NS	**	*	
$F_{\text{one-way ANOVA}}$		*	**	**	

BA = 6-benzyladenine; 2iP = 6-g-g-(dimethylallylamino)-purine. [Z] Mean values (n = 30) in each column followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. [§] NS or * or **: non-significant at $p \leq 0.05$ or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively. [#]: The control was excluded from two-way ANOVA. Multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6.

3.5. In Vitro Rooting

Both adult and juvenile microshoots, cultured in half-strength MS medium with 0.0–4.0 mg L^{-1} IBA for six weeks, rooted at the highest percentage (67 and 71%, respectively) in the medium containing 4.0 mg L^{-1} IBA (Table 3). More roots were formed by microshoots of juvenile origin grown in 1/2MS medium containing 4.0 mg L^{-1} IBA compared to lower IBA concentrations or microshoots of adult origin. Regarding the length of the produced roots, this was greatest in the medium with 4.0 mg L^{-1} IBA, regardless of adult or juvenile origin, while at lower IBA concentrations, microshoots of juvenile origin produced significantly longer roots than those of adult origin (Table 3).

When microshoots were cultured for a single week in an IBA-medium, followed by transfer to hf-1/2 MS, juvenile microshoots rooted at a higher percentage, and formed more and longer roots than microshoots of adult origin, at all IBA concentrations tested (Table 3,

two-way ANOVA). Only juvenile microshoots were favored by the one-week cultivation in a medium with 2.0 or 4.0 mg L^{-1} IBA, presenting a corresponding percentage of rooting to microshoots cultivated continuously for six weeks in a medium with 4.0 mg L^{-1} IBA, as well as longer roots (Table 3). In contrast, microshoots of adult origin showed poorer rooting when cultured in IBA-media for one week compared to continuous culture in them (Table 3).

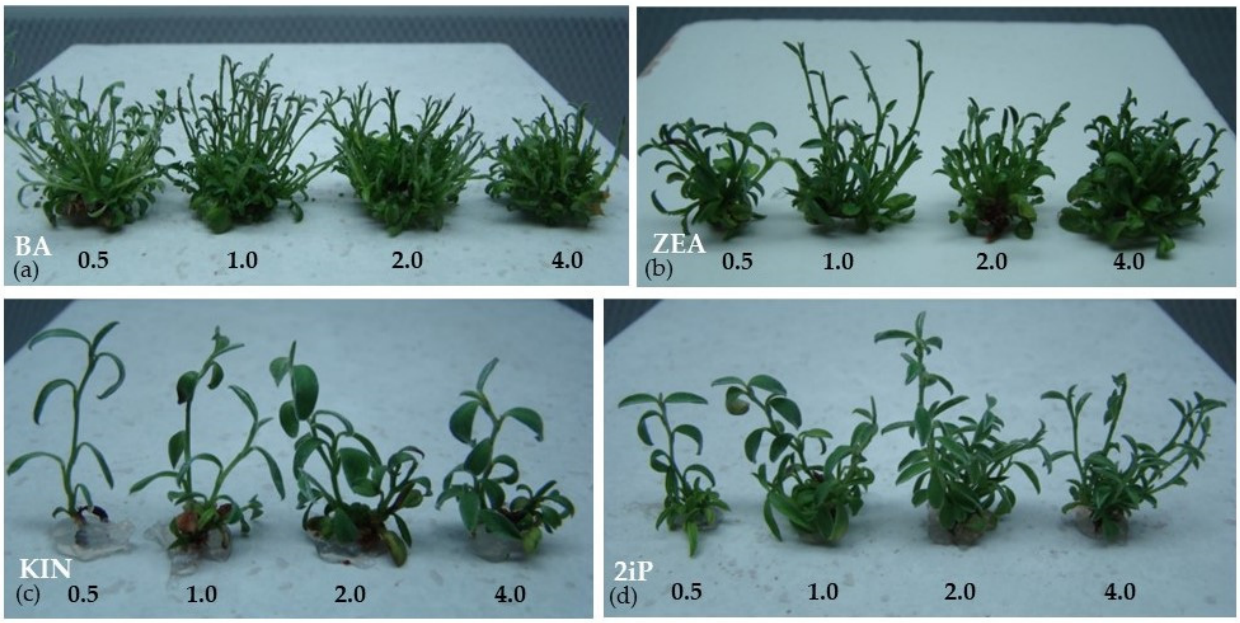

Figure 4. Typical shoot multiplication response of *A. hermanniae* explants from adult native plants during subculture in MS medium with the cytokinins BA (**a**), ZEA (**b**), KIN (**c**), and 2iP (**d**), at marked concentrations (mg L^{-1}).

Table 3. Effect of adult and juvenile origin of A. hermanniae microshoots, produced in the medium that contained 1.0 mg L^{-1} BA, and of IBA concentration (mg L^{-1}) on their in vitro rooting, after culture in half-strength MS medium with marked IBA concentrations either for 6 weeks (6 w) or for 1 week followed by transfer to plain 1/2 MS for 5 more weeks (1 w).

Microshoot Origin	IBA (mg L^{-1})	Callus (%)	Rooting (%)	Mean Root Number	Mean Root Length (cm)
		6 w/1 w	6 w/1 w	6 w/1 w	6 w/1 w
Adult native plants	0.0	4.8 b [Z]/0.0 c	48.8 abc/15.0 b	1.9 c/3.7 ab	3.4 bc/4.8 bcd
	0.5	6.7 b/0.0 c	39.1 c/25.0 b	2.7 bc/1.6 bc	3.4 bc/2.0 d
	1.0	6.7 b/5.0 bc	42.3 c/20.0 b	2.6 bc/1.3 c	4.2 abc/3.0 cd
	2.0	16.3 ab/10.0 ab	45.9 bc/30.0 b	3.0 bc/1.2 c	3.3 c/5.9 bc
	4.0	16.5 ab/15.0 a	67.0 ab/35.0 b	2.6 bc/1.9 bc	5.3 a/6.1 bc
In vitro grown seedlings	0.0	0.0 b/0.0 c	33.0 c/33.3 b	2.5 bc/3.2 ab	5.1 ab/9.9 a
	0.5	21.8 ab/0.0 c	35.1 c/50.0 ab	2.7 bc/3.1 ab	4.9 abc/7.6 ab
	1.0	15.2 ab/0.0 c	39.3 c/42.9 ab	3.1 bc/3.5 a	4.9 abc/7.1 b
	2.0	30.6 ab/0.0 c	46.0 bc/68.6 a	3.4 b/3.7 a	4.4 abc/6.8 b
	4.0	41.1 a/0.0 c	71.8 a/70.0 a	4.8 a/3.7 a	5.4 a/7.1 b
Significance [§]					
$F_{\text{microshoot origin}}$		NS/-	NS/**	*/**	**/**
$F_{\text{IBA concentration}}$		NS/-	**/*	**/NS	*/NS
$F_{\text{microshoot origin} \times \text{IBA concentration}}$		NS/*	NS/NS	NS/NS	NS/NS
$F_{\text{one-way ANOVA}}$		*/**	*/**	**/**	*/**

[Z] Mean values (6 w: origin adult $n = 60$ and juvenile $n = 45$, 1 w: $n = 30$) in each column followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. [§] NS or * or **: non-significant at $p \leq 0.05$ or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively.

Comparing the response of adult and juvenile microshoots in terms of their reaction during rooting in vitro on the substrate containing 4.0 mg L^{-1} IBA (the best treatment for both types of explants), no significant differences were found as to their rooting percentage and root length, but only in the number of roots produced, which was greater in microshoots of juvenile origin compared to adult ones (Table 3).

When studying the effect of cytokinin type used for shoot proliferation on the subsequent rooting of juvenile microshoots, it was found that microshoots grown in ZEA-medium rooted at higher percentages than those grown in BA-medium, being cultivated for rooting in a medium with the corresponding concentrations of IBA. An exception was microshoots cultured in a medium with 4.0 mg L^{-1} IBA, which rooted at an equally high percentage regardless of the cytokinin from which they were derived (Table 4). No significant differences were observed in the number and length of roots produced, although both were slightly greater in ZEA-grown microshoots (Table 4 and Figure 5). When repeating this experiment with adult microshoots, higher rooting percentages were also observed from microshoots grown in ZEA-medium compared to those grown in BA-medium and cultured for rooting in a medium with the corresponding concentrations of IBA. However, in this case, the number and length of the produced roots were slightly greater in the BA-grown microshoots (Table 4 and Figure 5).

Table 4. Effect of cytokinin type in shoot multiplication medium and of IBA concentration (mg L^{-1}) in rooting medium on in vitro rooting of *A. hermanniae* microshoots, excised from cultures established from adult native plants (A) or seedlings grown in vitro (J).

Cytokinin Type (mg L^{-1})	IBA (mg L^{-1})	Callus (%)	Rooting (%)	Mean Root Number	Mean Root Length (cm)
		A/J	A/J	A/J	A/J
1.0 BA	0.0	0.0 a [Z]/0.0 a	46.7 abc/33.0 d	2.0 cd/2.5 d	3.8 a/5.1 a
	0.5	0.0 a/19.6 a	33.3 c/35.1 d	3.4 ab/2.7 d	4.0 a/4.9 a
	1.0	0.0 a/15.2 a	26.7 c/39.3 cd	4.0 ab/3.1 cd	3.2 abc/4.9 a
	2.0	0.0 a/30.6 a	46.7 abc/46.0 bcd	3.7 ab/3.4 cd	3.4 ab/4.4 a
	4.0	0.0 a/41.1 a	40.0 bc/71.8 ab	4.2 a/4.8 ab	3.5 ab/5.4 a
1.0 ZEA	0.0	0.0 a/2.1 a	71.7 a/44.3 bcd	3.1 ab/2.7 d	3.0 abc/6.2 a
	0.5	0.0 a/15.2 a	71.7 a/66.1 abc	3.8 a/3.7 bcd	2.6 bcd/5.3 a
	1.0	0.0 a/21.7 a	66.7 ab/57.5 abcd	1.5 d/4.5 abc	1.9 d/6.6 a
	2.0	0.0 a/26.3 a	50.0 abc/74.0 a	2.9 bc/4.7 abc	2.4 bcd/6.0 a
	4.0	0.0 a/26.3 a	71.7 a/72.2 ab	3.3 ab/5.3 a	2.3 cd/5.2 a
Significance [§]					
$F_{\text{cytokinin in shoot multipl. medium}}$		NS	**/**	-/*	**/*
$F_{\text{IBA concentration in rooting medium}}$		NS	NS/*	-/**	NS/NS
$F_{\text{cytokinin multipl.} \times \text{IBA concentration}}$		NS	NS/NS	**/NS	NS/NS
$F_{\text{one-way ANOVA}}$		NS	**/*	**/**	**/NS

[Z] Mean values (origin adult $n = 30$ and juvenile $n = 45$) in each column followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. [§] NS or * or **: non-significant at $p \leq 0.05$ or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively.

3.6. Ex Vitro Acclimatization and Plant Growth

Plantlets transferred ex vitro on a substrate of peat-perlite 1:1 (v/v) were readily acclimatized to the highest percentage, regardless of their adult or juvenile origin (Figure 6b,c).

During the subsequent three-month growth period, all plants survived presenting greater and faster growth, regarding main shoot length and axillary shoot number per main shoot and per plant, when they were transplanted in a substrate of peat-perlite 2:1 (v/v) and were fertilized monthly with 4 g L^{-1} fertilizer 20-20-20, compared to fertilization with 2 g L^{-1} or transplantation in a substrate of enriched peat-perlite 2:1 (v/v) without the application of extra fertilization (Figure 7).

Figure 5. Characteristic rooting of *A. hermanniae* microshoots excised from culture established from (**A**) adult native plants and (**B**) seedlings grown in vitro and produced in medium with BA ((**a**,**c**)) or ZEA ((**b**,**d**)), respectively, in 1/2MS medium with marked IBA concentration (mg L^{-1}).

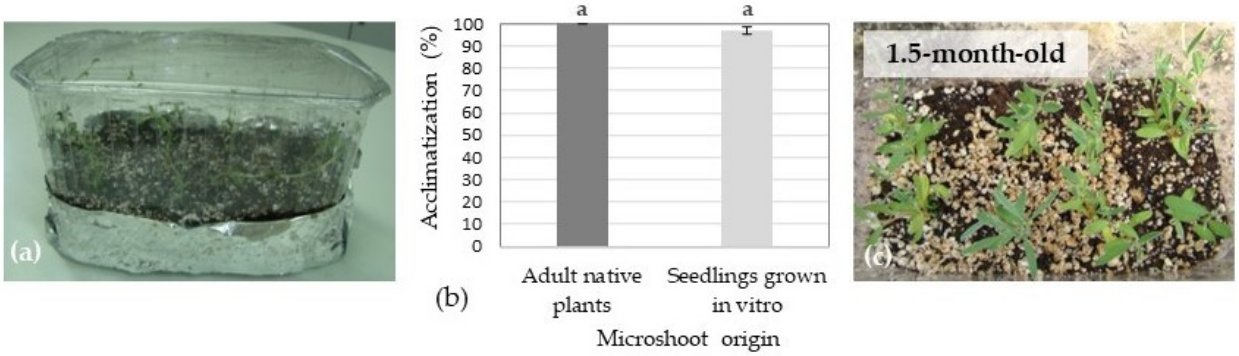

Figure 6. Microplants placed for ex vitro acclimatization in trays covered with plastic wrap (**a**), Effect of explant adult or juvenile origin on ex vitro acclimatization of *A. hermanniae* rooted microshoots, $n = 64$ (**b**); Acclimatized plantlets, 45 days old, on a substrate of peat-perlite 1:1 (v/v) (**c**). Mean values in each bar of subfigure (**b**) followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test.

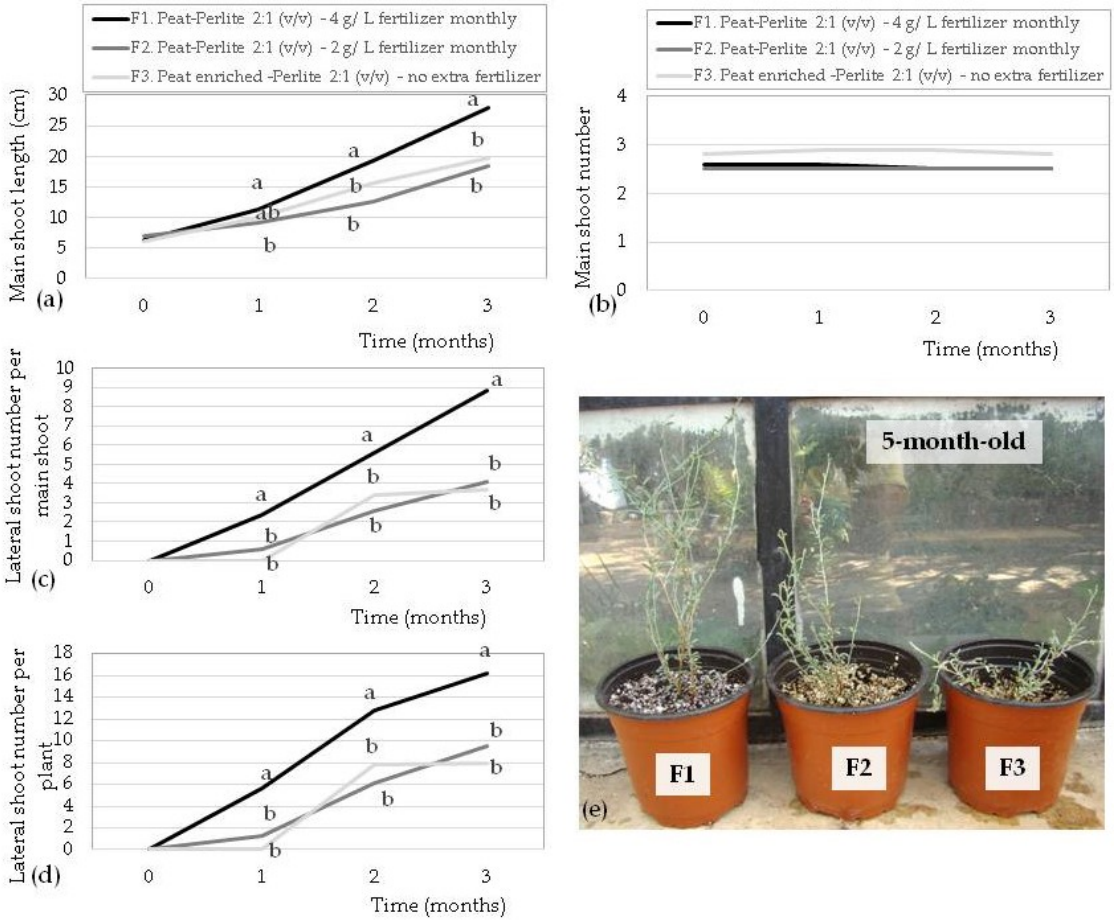

Figure 7. Effect of substrate type and fertilization on mean shoot length (**a**), mean shoot number (**b**), lateral shoot number per main shoot (**c**), lateral shoot number per plant (**d**), and canopy appearance (**e**) of *A. hermanniae* plantlets, during the period from April to July 2014 (n = 13), after transplantation on a peat-perlite substrate 2:1 (v/v) and application of monthly fertilization with 4 (F1) or 2 (F2) g L^{-1} fertilizer 20-20-20 or on enriched peat-perlite substrate 2:1 (v/v) without any extra fertilization (F3). In each subfigure, mean values in each month followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test.

4. Discussion

4.1. Collection of Seed and Tissue Culture Material

The collection of seeds and explants of *A. hermanniae* for the establishment of in vitro culture should follow precise instructions to be successful, which are determined by the characteristics of growth and seed production of the mother plants. Ripe lobs should be collected early after their formation (before the end of July in the climatic conditions of Attika) because they soon fall from the plant compared to seedless. Differences in the amount of seed production from year to year were also observed, as seed production has been reported to be strongly responsive to environmental variation [39] and an environmental stress, such as low rainfall, may have an overriding impact on it [40].

When annual shoots from adult plants are used as parent material for the establishment of in vitro cultures, only node explants can be excised, from the third node and below, because the shoots terminate in a thorn, whereas in case of in vitro grown seedlings both shoot-tip and single-node explants are available.

4.2. In Vitro Seed Germination

Based on all experiments conducted on seed germination, it can be concluded that seed pretreatment with scarification and incubation temperature affected germination,

while photoperiod had no effect. Photoperiod had no significant effect on germination of *A. barba-jovis* either [41], as well as other Mediterranean xerophytes such as *Teucrium capitatum* [22] and *Coridothymus capitatus* [42]. An intermediate response toward light was found for some Lamiaceae of Crete, such as *Satureja thymbra* [42], *Origanum dictamnus*, *Sideritis syriaca* ssp. *syriaca*, *Salvia pomifera* ssp. *pomifera*, and *Salvia fruticosa* [43], in which seed germination was partially manifested in darkness but it was significantly enhanced by light. On the other hand, an absolute light requirement was found for *Origanum vulgare* subsp. *hirtum* [42].

Both scarification techniques, either sandpaper for 1 min or concentrated H_2SO_4 for 15 min, greatly promoted seed germination by facilitating water entry and gas exchange, in agreement with several hard-seeded species such as *A. barba-jovis* [41,44], *Anthyllis cytisoides* [45], *Teucrium capitatum* [22], *Astragalus* sp. [46], and Mediterranean *Salvia* sp. [47]. However, mechanical scarification was preferred against chemical one, because it is more environmentally friendly and less risky for the user and seed integrity [46]. The positive effect of scarification on germination indicates coat-imposed dormancy. Due to impermeable coat, seeds ensure their longevity and obtain ecological adaptation to rapidly recolonize burnt area after fire and to withstand ingestion by animals and birds [48].

Although seeds generally germinated at a range of temperature from 10 to 25 °C, the optimum incubation temperature was affected by scarification, being 20 °C in unscarified seeds and 15 to 25 °C in scarified ones. In this way, scarification also made seeds less sensitive to temperature giving them the ability to germinate at temperature 20 ± 5 °C. In *A. barba-jovis* too, equal temperatures (15–25 °C) were optimal for germination of seeds stored up to 12 months, whereas older seeds (24 or 36 months after harvest) germinated at higher percentages at 20 °C [41]. Other researchers [44,45] found that 20 °C was most favorable for seed germination of other *Anthyllis* spp., while the optimal temperatures for germination of other Mediterranean species overall are in the range of 15–25 °C [22,42,49,50].

In *A. hermanniae*, storage period of seeds started affecting germination of scarified seeds after 18 months of storage reducing slightly their germination percentage, while unscarified seeds showed stable, low germinability throughout the storage period. In *A. barba-jovis,* storage for a longer period (more than 24 months) reduced seed germination, mostly of unscarified seeds [41].

4.3. In Vitro Clonal Propagation

At the in vitro establishment stage, *A. hermanniae* explants from seedlings (juvenile explants) responded at higher percentages to produce shoots than explants from adult wild plants (adult explants). In MS medium with 1.0 mg L^{-1} BA, explants from seedlings produced significantly more shoots (14) compared to explants from adult native plants (6), verifying work on *A. cytisoides* [26]. In that work, the response of seedling-origin explants was also superior, although the number of shoots produced was low (5) in MS medium with 0.5 mg L^{-1} BA, and even fewer were the number of shoots (2) produced from seedling-origin explants of *A. barba-jovis* in the same medium as that of the present study [27,28]. Furthermore, explant establishment was favored by a medium containing BA, although it was also satisfactory in ZEA-medium. For in vitro culture establishment of other *Anthyllis* spp., cytokinin was also shown to be required for the development of lateral shoots and the formation of multiple shoots, especially when explants of adult origin were used [26–28].

During the multiplication stage, juvenile explants showed higher proliferation potential compared to adult ones, in accordance with what has been reported for other, mainly tree, species [51–56]. This happens because in vitro cloning of adult woody plants is adversely affected by characteristics accompanying maturation such as reduced growth rate [57]. However, response of adult explants of *A. hermanniae* was rather satisfactory.

Regarding tested cytokinin types and concentrations, BA at concentration 0.5–2.0 mg L^{-1} was the most effective for shoot multiplication, followed by ZEA, whereas explants response with KIN and 2iP was as poor as in the hf-medium. In *A. barba-jovis* though, BA and ZEA at 1.0 mg L^{-1} were equally effective in shoot multiplication [27]. Regarding shoot number

during subcultures, juvenile and adult explants of *A. hermanniae* formed 20 and 16 shoots, respectively, which were more than double the number of shoots produced in *A. barba-jovis*, which reached a maximum of eight shoots per juvenile explant [27,28]. BA has been proved superior to ZEA, KIN, or 2iP in several works on Mediterranean plants [22,32,58]

In vitro cultures of *A. hermanniae* presented no hyperhydricity, as opposed to other *Antyllis* species in which this was a serious problem [26–28,59]. In our experiments, plastic wrap was used to cover the culture vessels, versus a propylene closure or Magenta B-caps used in the other works, [26,28], respectively, and this may have prevented the hyperhydricity allowing better gas exchange, as the growth conditions mostly associated with hyperhydricity include limited aeration and ethylene accumulation in the aerial part of the culture vessel [60]. In our laboratory, plastic wrap has been used successfully as a cover for culture vessels on both shrub and tree species micropropagation, such as *T. capitatum* [22], *Lithodora zahnii* [61], *Quercus euboica* [62], and ×*Malosorbus florentina* [56]. In *Lithodora zahnii*, not only was hyperhydration eliminated when culture vessels were covered with plastic wrap instead of a transparent semi-hard plastic film, but also the rate of shoot multiplication was increased [61]. In *Quercus euboica*, the semi hard transparent plastic film, as well as the metallic and plastic caps caused high relative humidity and different gas concentrations compared to plastic wrap and magenta caps, which affected the morphological and anatomical characteristics of the leaves of the microshoots [62].

During in vitro rooting phase, the physiological state of the microshoots may not have affected their rooting percentage, since microshoots of both origins rooted at a higher percentage (about 70%) in half-strength MS medium with 4.0 mg L^{-1} IBA, but it affected the characteristics of the formed roots, as the juvenile-origin microshoots produced more and longer roots than the microshoots of adult-origin. To the contrary, *A. cytisoides* microshoots of adult and juvenile origin had similar rooting behavior, reaching 90–100%, regardless of auxin treatment [26]. Juvenile-origin microshoots of *A. barba-jovis* also rooted more efficiently (90–100%) than *A. hermanniae* microshoots in half-strength MS medium either without plant growth regulators or with 0.5 or 2.0 mg L^{-1} IBA [28], while in full-strength MS with the same IBA concentrations rooting percentages were reduced by half (38–55%) [27]. Reduced or total lack of rooting ability is another characteristic accompanying maturation, which adversely affects in vivo and in vitro cloning of adult woody plants [57] and it has been observed in micropropagation of many tree species [51–56]. However, in *Anthyllis* sp., the efficiency of in vitro rooting was not affected significantly by the physiological state of microshoots.

Regarding the effect of a reduced culture period in auxin medium, followed by transfer to an auxin-free medium, only the microshoots of juvenile origin were favored, whereas adult-origin microshoots rooted poorly compared to six-week culture at corresponding IBA concentrations. Probably, juvenile-origin microshoots had a higher concentration of endogenous auxin compared to adult-origin ones. Nevertheless, this technique has been used in rooting microshoots of apple [63,64], *T. capitatum* [22], or those of the endangered difficult-to-root ×*Malosorbus florentina* [56], improving rooting and preventing callus formation. An auxin concentration that is optimal for one phase of in vitro rooting may be supraoptimal for the next, as the three phases of adventitious rooting (dedifferentiation, induction, and outgrowth) may have differences in auxin requirements [65].

As regards the effect of the type of cytokinin used to produce shoots during the shoot proliferation stage on subsequent rooting, microshoots of both origins produced in ZEA-medium rooted at higher percentages than those produced in BA-medium. However, in juvenile microshoots, the number and length of produced roots were greater in ZEA-medium, while in adult microshoots, it was greater in BA-medium. Cytokinin content of regeneration medium has been reported to affect rooting ability of apple [66], which could be attributed to the carrying over effect from cytokinins in the shoot proliferation medium [67].

Finally, the ex vitro acclimatization of plantlets was highly successful irrespectively of their adult or juvenile origin, as it has been reported for *A. cytisoides* [26] and *A. barba-jovis* [27,28], as well as other xerophytic shrubs [22,32]. As the in vitro culture conditions

result in plantlets with changed morphology, anatomy, and physiology, the ultimate success of a micropropagation protocol depends on the ability to transfer plantlets ex vitro with high survival rate [68]. After acclimatization, *A. hermanniae* plantlets were successfully established under greenhouse conditions in a 2:1 (*v*/*v*) peat-perlite substrate and their growth during the following three months was favored by fertilization with the recommended concentration (4 g L^{-1}) of soluble 20-20-20 fertilizer compared to halved concentration (2 g L^{-1}), while in other Mediterranean plants, such as *Limoniastrum monopetalum* [35] and *T. capitatum* [42], satisfactory growth of rooted cuttings was induced by both concentrations of this fertilizer.

5. Conclusions

In the present study, *A. hermanniae* was successfully propagated in vitro both by seed and clonal propagation for the first time, to our knowledge. High seed germination percentages, higher than 80%, were achieved in seeds stored from 4 to 18 months at room temperatures in the dark, after scarification with sandpaper for 1 min, in a temperature range of 10 to 25 °C, regardless of photoperiod. Efficient micropropagation protocols were developed by initiating in vitro cultures from explants excised from either seedlings (juvenile cultures) or adult native plants (adult cultures). It is recommended to culture explants in MS medium containing 0.5–2.0 mg L^{-1} BA for shoot multiplication and microshoots in half-strength MS medium with 4.0 mg L^{-1} IBA for rooting. Ex vitro acclimatization of microplants was achieved at percentages higher than 95% in a substrate of peat and perlite (1:1, *v*/*v*). The subsequent growth of plantlets was best on a substrate of peat and perlite (2:1, *v*/*v*) with the application of monthly fertilization with 4 g L^{-1} of water-soluble 20-20-20 fertilizer. The juvenile culture was more efficient than the adult one in various stages of micropropagation, excepting ex vitro acclimatization. Nevertheless, the productivity of the adult culture was also considered highly satisfactory. Micropropagation by either seed or adult origin parental material was particularly effective in all its stages and could facilitate sustainable exploitation of *A. hermanniae* in commercial floriculture as a bee-friendly landscape plant, in the apiculture and pharmaceutical industry, as well as in breeding programs.

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