agriculture

MDPI

Article

Seed Germination Behavior, Molecular Analysis of Four Populations of *Arbutus andrachne* Species from Greece, and Cultivation Practice for Producing High-Quality Plants

Elias Pipinis [1,*,†], Athanasios Stampoulidis [2,†], Aikaterini Angeliki Kotoula [3,†], Elias Milios [2], Stefanos Kostas [3,*], Stefanos Hatzilazarou [3], Evgenia Papaioannou [4,5], Alexandros Papaeirinaios [2], Kyriaki Kitikidou [2] and Kalliopi Radoglou [2]

1 Laboratory of Silviculture, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
2 School of Forestry and Management of the Environment and Natural Resources, Democritus University of Thrace, Pandazidou 193, 68200 Orestiada, Greece; thanasis.st@gmail.com (A.S.); emilios@fmenr.duth.gr (E.M.); alexpapa131997@hotmail.com (A.P.); kkitikid@fmenr.duth.gr (K.K.); kradoglo@fmenr.duth.gr (K.R.)
3 Laboratory of Floriculture, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; kotoulaa@agro.auth.gr (A.A.K.); hatzilaz@agro.auth.gr (S.H.)
4 Laboratory of Soil Science, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; eapapaioa@agro.auth.gr
5 Laboratory of Forest Soil Science, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
* Correspondence: epipinis@for.auth.gr (E.P.); skostas@agro.auth.gr (S.K.); Tel.: +30-2310992311 (E.P.)
† These authors contributed equally to this work.

check for updates

Citation: Pipinis, E.; Stampoulidis, A.; Kotoula, A.A.; Milios, E.; Kostas, S.; Hatzilazarou, S.; Papaioannou, E.; Papaeirinaios, A.; Kitikidou, K.; Radoglou, K. Seed Germination Behavior, Molecular Analysis of Four Populations of *Arbutus andrachne* Species from Greece, and Cultivation Practice for Producing High-Quality Plants. *Agriculture* **2023**, *13*, 1428. https://doi.org/10.3390/agriculture13071428

Academic Editor: Raúl De la Rosa Navarro

Received: 16 June 2023
Revised: 10 July 2023
Accepted: 17 July 2023
Published: 19 July 2023

Abstract: *Arbutus andrachne*, the Greek strawberry tree, is an evergreen shrub or small tree in the family Ericaceae native to the Mediterranean basin, and is a valuable phytogenetic resource. In the context of sustainable exploitation of *A. andrachne*, four Greek natural populations of species were selected and a detailed germination protocol, molecular analysis, and cultivation practices were reported herein for the first time. The 1 month period of cold stratification or the treatment with gibberellic acid resulted in similar patterns of seed germination over a wide temperature range for all four populations. Differences among the four populations were observed in five of six morphological traits measured in 1-year-old seedlings. The 2-year-old plants grown in plastic bags (2.26 L in volume) showed superior growth rates. Foliage, but not roots, of 2-year-old seedlings supplemented with mineral fertilizers had higher fresh masses than untreated seedlings. The four *A. andrachne* accessions exhibited more genetic variability within than among populations.

Keywords: cold stratification; container size; fertilization; genetic variation; gibberellic acid; plant production

1. Introduction

The genus *Arbutus* L. (Ericaceae) includes about 12 to 20 species (depending on the author) distributed from the West coast of North America through Mexico and Central America, Western Europe, Mediterranean region, Northern Africa, and parts of the Middle East [1,2]. This genus is represented by the species *A. unedo* L., *A. andrachne* L., and their hybrid *A.* × *andrachnoides* Link in the flora of the Mediterranean region [1]. *A. andrachne* (the Greek strawberry tree) is an evergreen species, which usually exists as a shrub or sometimes as a small tree up to 5–8 m in height [3]. It grows mostly along the Mediterranean coasts spontaneously, separately, or in association with the *A. unedo*; however, as it is more tolerant to low winter temperatures than *A. unedo*, *A. andrachne* occurs further inland than *A. unedo* [4].

A. andrachne is a valuable phytogenetic resource due to its medical and floricultural value. Its fruits are a good source of minerals, ascorbic acid, phenols, and antioxidants

and are characterized by the low content in soluble sugars [5–7]. Furthermore, the *A. andrachne* species with the peeling, red-brown bark, the attractive white-cream spring flowers, and the red edible autumn fruits, is drought tolerant, and thus can be a suitable landscaping choice in the arid and semi-arid environments of the Mediterranean basin, even in the urban residential landscapes. *A. andrachne* possesses a combination of food and ornamental qualities that render the species a candidate for genetic improvement and cultivation. Effective propagation protocols for *A. andrachne* will be needed to enable successful cultivation.

In general, the most efficient plant propagation method is by sexual reproduction and true seeds [8]. Sexual reproduction promotes genetic diversity, a consequence that is desirable for sustainability but undesirable in the nursery industry. However, a major constraint to the sexual propagation of many species is the poor germination of their seeds due to low viability or to seed dormancy [9]. Seed dormancy is a physiological state during which a viable seed fails to germinate even when the environment is favorable to germination [8]. In previous studies on germination of *A. andrachne* seeds, conflicting reports have appeared regarding the dormancy. According to Tilki and Guner [10], *A. andrachne* seeds show that physiological dormancy and cold stratification or gibberellic acid are used to overcome it. Furthermore, the effectiveness of these treatments to dormancy breaking and seed germination in *A. andrachne* are inferred by Karam and Al-Salem [11]. In contrast, Bertsouklis and Papafotiou [12] state that seeds of *A. andrachne* do not possess dormancy and they germinate at high percentage at a constant temperature of 15 °C or less. Although, there is enough knowledge about seed germination and dormancy, no attention has been given on the variation in germination behavior among populations of this species. Germination behavior may vary greatly within a single species from one population to another, from year to year, and among individuals [13]. Adaptations to local ecological conditions may lead to differences among populations in terms of seed germination requirements. Variations in seed germination dynamics within species and populations present a challenge to the development of standardized germination protocols.

Despite the promising potential of using *A. andrachne* species in the ornamental and the medicinal sector, a research gap exists regarding the production of seedlings in containers. A main challenge at nurseries is the production of a quality planting stock. In a harsh Mediterranean environment (high temperatures, low water availability, and low soil fertility), the quality of planting stock is very important [14]. According to Radoglou [15], the morphological and physiological attributes of planting stock define the quality of seedlings. Various cultivation practices can affect the morphological and physiological characteristics of plants produced in nurseries, and thus a high-quality planting stock can be produced [16]. The use of containers for seedlings production is the most common technique in nurseries and several studies have related the type of container used (size, spacing, design) with the morphological and physiological characteristics of seedlings and especially their root system [17–19]. Therefore, it is very important to determine which container type has the greatest influence on seedling morphology. Furthermore, the use of fertilizers is one of the most important nursery tools that can affect the quality of producing plants. The majority of fertilization studies have focused on N as an essential nutrient that plants need in higher amount and the changes in its availability induce large variations in seedling performance [20]. Since in the majority of cases the reforestation programs take place on degraded soils, it is important to increase nutrient reserves of plants at the nursery stage [21]. Applications of nitrogen fertilizers in the nursery usually increase the survival and growth of seedlings in the field [20,22].

A research gap also exists considering the variance of *A. andrachne* both on morphological and molecular level. Considering the morphological traits, *A. andrachne* has been studied in Greece [23,24] as well as in the south part of North Macedonia [4]. Most studies focus on the genetic analysis of *A. unedo* using isozyme markers [25], amplified fragment length polymorphism (AFLP) [26,27], simple sequence repeat (SSR) [28,29], and randomly amplified polymorphic DNA (RAPD) [24,28,30,31]. In the present study, inter-simple se-

quence repeat (ISSR) markers have been used for the genetic analysis of *A. andrachne* [31], the only related paper is that of Betsouklis and Papafotiou [24], where the genetic relationship among *A. andrachne*, *A. unedo*, and their natural hybrid *A.* × *andrachnoids* was studied using RAPD markers. This work showed that there is limited biodiversity between the two *A. andrachne* populations, while an analysis with more populations and genotypes would help in understanding whether it is possible to increase the genetic diversity for the prospects of genetic improvement of the species.

In this context, the aim of the present study was the sustainable exploitation of the *A. andrachne* species by (i) investigating the germination behavior of four populations of *A. andrachne*, and more precisely to examine the effect of cold stratification, GA_3, and incubation temperature on seed germination in each population, (ii) evaluating the growth of the 1-year-old seedlings of the four populations, (iii) assessing the effect of container size, substrate, and fertilization on the growth of 2-year-old plants by means of seedling morphological properties, and (iv) providing genetic information about the four populations of *A. andrachne* taxon.

2. Materials and Methods

2.1. Seed Collection

Mature fruits (berries, Figure 1A) of *A. andrachne* were collected in late autumn of 2018 from four natural populations spanning from north to south parts of Greece. In particular, fruits were collected from three populations located in the northeast, northern, and central part of Greece (Rhodope, Chalkidiki, Pieria, correspondingly) and one population located in the southeast part of the country (Rhodes island) (Table 1 and Figure 2). In each population, fruits were collected from 15 healthy individuals that were at least 30 m apart. After collection, the fruits were pulped by hand and the separation of seeds from pulp was achieved using sieves and running water. In addition, floated seeds were removed during cleaning, and subsequently, the clean seeds were spread out on filter papers in laboratory conditions for a week. After drying, the seeds (Figure 1B) were stored in glass containers in the refrigerator (3–5 °C) until they were used in the experiments.

Table 1. Propagation material collection details regarding the four Greek populations of *A. andrachne* studied herein.

Population	Latitude	Longitude	Altitude (m)	Collection Date
Rhodope	41°08′38″ N	25°15′38″ E	250	11 November
Chalkidiki	40°30′20″ N	23°32′44″ E	496	16 November
Pieria	40°11′27″ N	22°19′31″ E	485	25 November
Rhodes	36°19′41″ N	28°03′54″ E	366	23 November

2.2. Seed Treatment and Germination Test

Germination experiments were started the following January and conducted in the Laboratory of Silviculture, Department of Forestry and Management of the Environment and Natural Resources, Democritus University of Thrace.

For each population, an experiment was carried out to determine the effects of gibberellic acid (GA_3), cold stratification (CS), and combination of GA_3 with CS on seed germination. Seeds of each population were soaked in GA_3 solutions of 500, 1000, or 2000 mg·L^{-1} for 24 h. Subsequently, the treated seeds were randomly placed between two moist layers of filter paper in 9 cm plastic Petri dishes, and then stratified at 3–5 °C for 0, 1, or 2 months. In addition, seeds from each population were soaked in distilled water for 24 h (control), and then were placed in plastic Petri dishes and subjected to CS as described above. In total, 12 treatments (combinations between GA_3 concentrations and CS periods) were conducted for each population. In each treatment of each population, there were 8 plastic Petri dishes of 30 seeds. During seeds stratification, the moisture of

filter paper was checked periodically and distilled water was added whenever necessary to keep it moist.

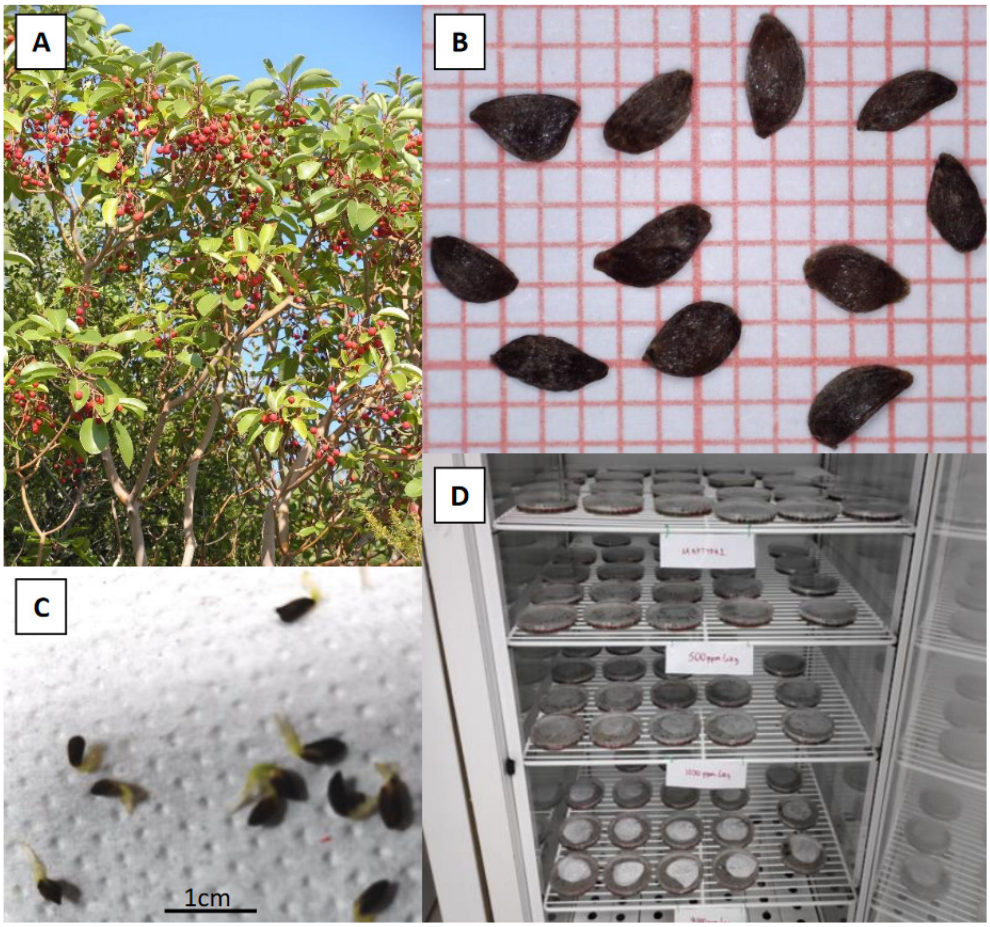

Figure 1. Wild-growing individual of *A. andrachne* with mature fruits (**A**), seeds of *A. andrachne* (**B**), germinated seeds (**C**), and in Petri dishes placed in growth chamber (**D**).

Figure 2. The regions of Greece where the *A. andrachne* fruits were collected. The populations are: 1: Rhodope, 2: Chalkidiki 3: Pieria and 4: Rhodes.

To determine the effect of temperature on seed germination for each population, at the end of each stratification period (0, 1, and 2 months), the 8 Petri dishes that corresponded to each GA_3 concentration (0, 500, 1000, and 2000 mg·L^{-1}) were randomly divided and placed in two temperature controlled growth chambers. Seeds germination response at 2 alternating temperature regimes of 15/10 °C and 25/20 °C were evaluated. The Petri dishes were randomly arranged on the shelves of the growth chambers (Figure 1D), with a 12 h light/12 h dark photoperiod and filter paper was kept moisten, as required along the whole germination test. Germinated seeds were counted and removed every 4 days for a period of 56 days. A seed was considered as germinated (Figure 1C) when at least 2 mm long radicle had emerged through the seed coat.

2.3. The 1-Year-Old Seedling Production

The experiments on growth dynamics of 1-year-old seedlings were conducted from March to November 2019 in the greenhouse of the Laboratory of Silviculture, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, Greece.

In early March, seeds stratified at 3–5 °C for 1 month from each population were sown in 4 QuickPot trays, each with 24 cavities (QuickPot 24 T/16, cavity volume 0.33 L, height 16 cm). This type of QuickPot tray is the most commonly used for forest seedlings production in nurseries of Forest service in Greece. In total, 16 QuickPot trays were filled with a 3:1 (v/v) mixture of enriched peat (Klasmann TS1) and perlite. The sown seeds were carefully covered with sand and the trays were randomly placed on a wire mesh bench to allow for the air pruning of roots at the hole of the base of the cavity, in the greenhouse (Figure 3A). The position of trays was randomized once a week in order to ensure uniform conditions. Watering was applied by the overhead spraying system of the bench. During the phase of seed germination, more than one watering per day was applied to maintain the proper moisture conditions for germination. After the germination phase, the containers were irrigated every 2–3 days depending on the weather conditions.

After the first growing season (middle of November), the morphological variables of all plants from each population were measured. Specifically, the shoot height (SH) and root collar diameter (RCD) of all plants from each population were measured using a metal ruler and a digital calliper, respectively. Furthermore, the sturdiness quotient (ratio of the shoot height (cm) to the root collar diameter (mm) of the seedling) was estimated. Subsequently, 10 plants per population were randomly sampled for the measurement of number of leaves, the root dry biomass (RDB), and above ground part dry biomass (AGDB). Dry weight of plants was determined after oven drying at 74 °C for 48 h.

2.4. Seedling Transplanting

In early March 2020, two experiments were conducted to estimate the effect of pot size, substrate, and fertilization on the growth of *A. andrachne* seedlings. For these experiments, the 1-year-old seedlings from Rhodope and Pieria populations, which exhibited higher growth rates than those of the other two populations (see Section 3), were used.

In one experiment, 1-year-old seedlings of Rhodope population were transplanted into pots of different sizes in volume: 0.65 L in volume (QuickPot 12 T/18, height 18 cm), 1.60 L in volume (QuickPot 6 T/20, height 20 cm), 2.26 L in volume (plastic nursery bags, 12 × 20 cm (D × H)), and 3.4 L in volume (plastic pot, 17 × 14 × 18 cm (upper D × bottom D × H)). In each of the above pots and plastic bags, 16 seedlings grown in QuickPot 24 T/16 trays were randomly selected and transplanted. All the pots and plastic bags were filled with the same mixture enriched peat (Klasmann TS1) and perlite in a ratio of 3:1 (v/v). Furthermore, 16 seedlings were kept for the initial type of tray (QuickPot 24 T/16).

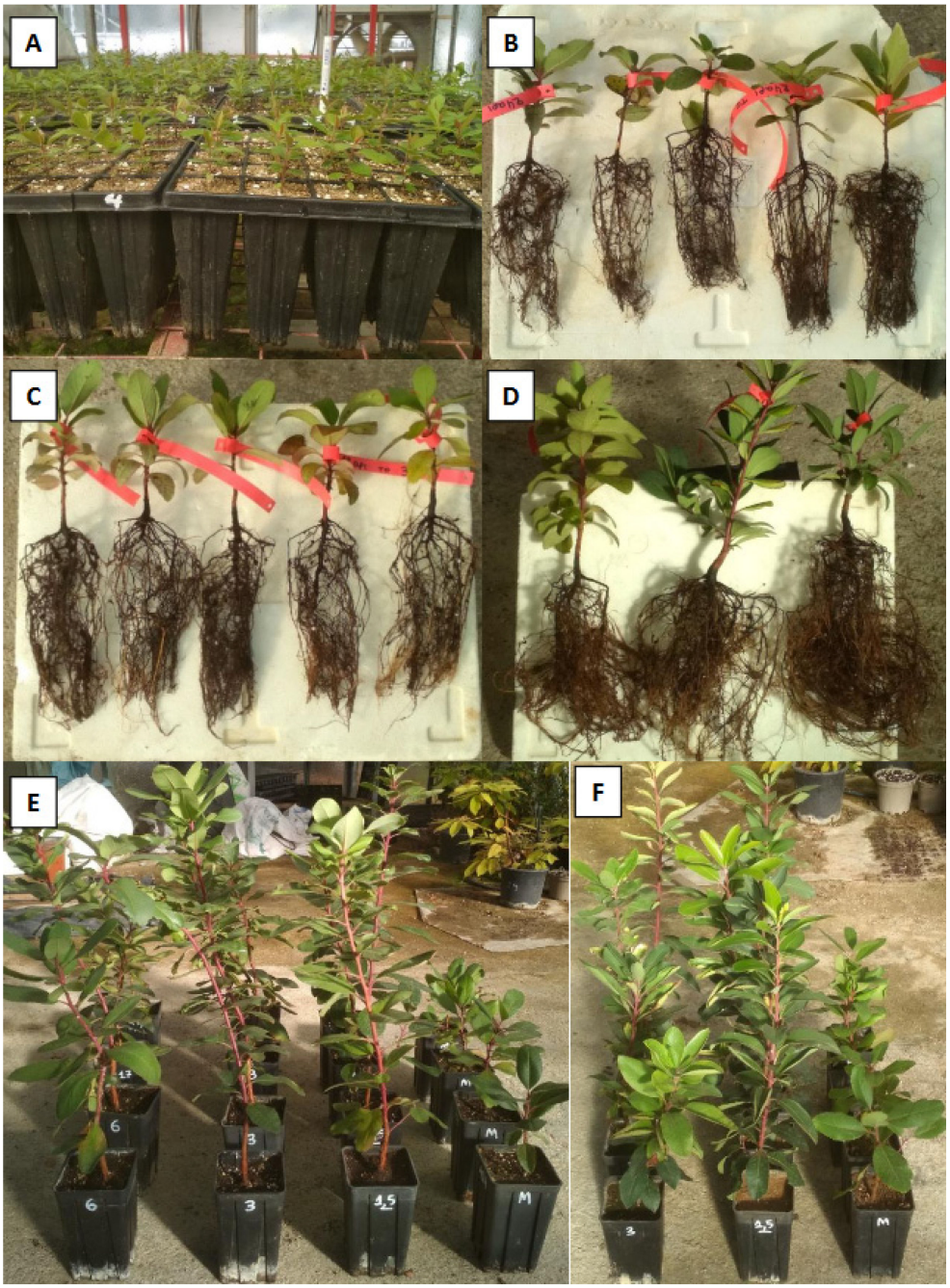

Figure 3. The 1-year-old seedlings of *A. andrachne* growing in QuickPot 24 T/16 trays (**A**), 2-year-old plants grown in: QuickPot 24 T/16 trays (**B**), QuickPot 12 T/18 trays (**C**), and plastic bags (**D**). The 2-year-old plants of Arbutus andrachne grown in plastic square pots filled with: a mixture of peat and perlite in a ratio of 3:1 (**E**), and a mixture of gneiss soil, peat, and perlite in a ratio of 4:3:1 (**F**). In both types of substrates, the plants were fertilized with a commercial inorganic fertilizer (Complesal Suprem 21-5-10 (+3+TE)) at doses of 0, 1.5, 3.0, and 6.0 g per pot.

In the second experiment, eighty 1-year-old seedlings of Pieria population were randomly selected and transplanted into plastic square pots with a special anti-spiraling veins system on the side and grid on the bottom (10 × 10 × 17 cm dimension, volume 1.4 L, provided by the Bamaplast Company, Pistoia, Italy). Two different substrates were used to fill the pots. The half pots were filled with a mixture of enriched peat (Klasmann TS1) and perlite in a ratio of 3:1 (v/v) (SUB 1), and the other half with a mixture of gneiss soil, enriched peat (Klasmann TS1), and perlite in a ratio of 4:3:1 (v/v) (SUB 2). Forty

pots were filled in each substrate. After the seedlings transplanting, three different doses of a commercial inorganic fertilizer (Complesal Suprem 21-5-10 (+3+TE)) were applied two times during the experimental period: the first in early April and the second in early June. The pots from each substrate were randomly divided into four groups, with ten seedlings/replicates each. Fertilizer dose of 1.5 g was applied in each seedling of the first group, 3.0 g was applied in each seedling of the second group, 6.0 g was applied in each seedling of the third group, and the seedlings of the fourth group were used as control with no application of fertilizer.

Soil from parent material Gneiss has been used as a substrate in pots for plants production [32–34]. The gneiss soil used in the substrate herein was received from a region of North Greece a few days before the substrate preparation. It was collected from the upper 30 cm of soil profile and the 2 cm sieved soil was used in the experiments. Furthermore, before its use, a sample of approximately 1.5 kg was taken and submitted to chemical analyses in order to check its fertility. The results of the chemical analysis, as well as the mechanical analysis, showed that the soil used was characterized by a sandy loam texture, an acidic reaction (pH), and a moderate content of organic matter and nutrients (Table 2).

Table 2. Chemical and physical properties of soil.

pH	Organic Matter (%)	Mechanical Analysis Sand (%)	Silt (%)	Clay (%)
5.48	3.12	59.36	18.21	22.43
Macronutrient concentrations				
N (%)	**P ($mg \cdot kg^{-1}$)**	**K ($cmol_c \cdot kg^{-1}$)**	**Mg ($cmol_c \cdot kg^{-1}$)**	**Ca ($cmol_c \cdot kg^{-1}$)**
0.14	12.53	0.24	2.35	9.17
Micronutrient concentrations ($mg \cdot kg^{-1}$)				
	Fe	**Zn**	**Mn**	**Cu**
	19.67	1.13	16.43	1.23

The plants from both experiments were grown inside the greenhouse of the Laboratory of Silviculture, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, Greece. The pots of both experiments were placed on a wire mesh bench and irrigated every 3 days throughout the experimental period.

In middle of November 2020, the effects of pot size (Figure 3B–D), substrate, and fertilization (Figure 3E,F) on morphological variables of plants were evaluated. In both experiments, the shoot height (SH) and root collar diameter (RCD) of all plants were measured and the sturdiness quotient was calculated. The SH and RCD were measured using a metal ruler and a digital calliper, respectively. In addition, in the first experiment, 5 plants per pot size were randomly sampled, whereas in the second experiment, 4 plants from each combination of substrate and fertilizer level were randomly sampled. In each sampled plant, the number of leaves, the root dry biomass (RDB), and above ground part dry biomass (AGDB) were assessed. Dry weight of plants was determined after oven drying at 74 °C for 48 h.

2.5. Molecular Analysis

In July 2019, eight 1-year-old seedlings were randomly selected from each population, and young leaves from each plant were collected (a total of 32 samples were collected). The leaves were stored at −20 °C until they were ground in liquid nitrogen using pre-cooled mortars and pestles. The cetyltrimethylammonium bromide (CTAB) method [35] was used to extract the total genomic DNA of our samples and to check its quality and quantity by performing gel electrophoresis. The spectrophotometry on a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was performed and the samples were diluted to 20 ng/μL, in order to use them for the polymerase chain reaction (PCR) reactions.

Fourteen ISSR primers were tested at 5 different annealing temperatures and finally 10 of them appeared to be the most polymorphic, and clear and consistent bands were

used. The PCR reactions were performed in SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) in a 15 µL reaction mixture. The mixture consisted of 1.5 µL gDNA (20 ng/µL), 1.5 µL molecular primer, 10.08 µL sterile distilled water, and from the Kapa Biosystems Taq PCR Kit (Kapa Biosystems, Inc. Boston, MA, USA), 0.3 µL KAPA dNTP Mix (10 mM each), 1.5 µL KAPA Taq Buffer A (10×), and 0.12 µL KAPA Taq DNA Polymerase (5 U/µL). The PCR amplification included the initial denaturation for 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at the chosen annealing temperature of each primer, 2 min at 72 °C, followed by a final extension cycle of 2 min at 72 °C. The amplified fragments of DNA were separated by electrophoresis in 1.2% agarose gel together with 100–3000 bp DNA Rainbow Ladder (GeneOn, Ludwigshafen am Rhein, Germany) in 1× TAE buffer, stained with ethidium bromide, and photographed under UV light in a gel Dark HOOD dh-10 (Biostep, Burkhardtsdorf, Germany, Figure 4).

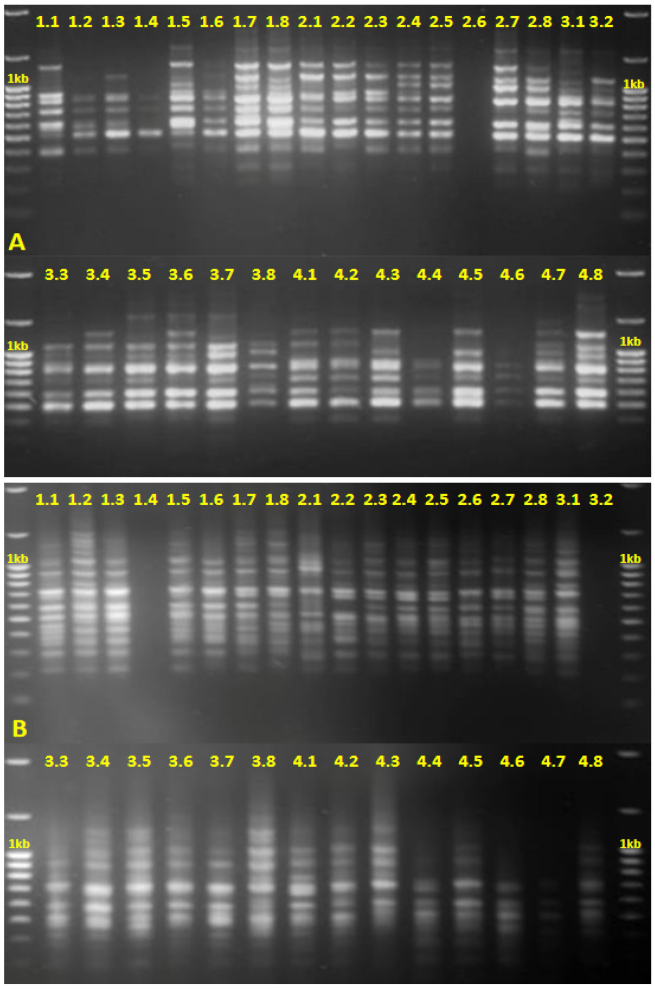

Figure 4. Profiling of *A. andrachne* accessions by ISSR primers UBC-815 (**A**) and UBC-888 (**B**). The samples 1.1–1.8 represent the population from Rhodope, 2.1–2.8 the Chalkidiki population, 3.1–3.8 the Pieria population, and 4.1–4.8 the population from Rhodes.

2.6. Statistical Analysis

For each experiment, a completely randomized design was used. The data were subjected to analysis of variance (ANOVA) and the comparisons of the means were made using Duncan's test at significance level $p \leq 0.05$ [36]. Prior to the ANOVA, only the germination percentage data were transformed to arc-sine square root values [37]. In the experiment, where the effects of substrate and fertilization on plant growth were investigated, the experimental design was a completely randomized design with two factors. The factors were the substrate (two types) and the fertilization (four doses). The

data were analyzed using the ANOVA in the frame of the procedure general linear model (GLM) [38] and the means were compared using Dunnett's T3 test at a significance level $p \leq 0.05$.

To quantify the genetic polymorphism and determine the genetic structure of the four populations studied of *A. andrachne*, the score of ISSR bands was carried in a binary mode, in which 1 indicated the presence and 0 the absence of fragments. For each primer, the number of polymorphic bands (NPB), total amplified bands (TAB), and the percentage of polymorphism bands (PPB) were calculated. The resolving power (RP) [39], the polymorphism information content (PIC) [40], the effective multiplex ratio (EMR) [41], and the marker index (MI) [42] were also calculated. To describe the genetic structure of the populations, the results of the ISSR primers were analyzed through the program GenAIEx 6.501 [43] in a principal coordinate analysis (PCoA) and an analysis of molecular variance (AMOVA) diagram with the calculation of the population subdivision index (PhiPT index) [44]. Furthermore, the data were subjected to the cluster analysis of unweighted pair group method with arithmetic mean (UPGMA) and a dendrogram was constructed through the Mega 4 software [45].

3. Results

3.1. Seed Germination Tests

In all four populations of *A. andrachne* and regardless of GA_3 treatment, at the end of the 2 month period of CS the percentages of germinated seeds ranged from 93.33 to 100%.

In non-stratified seeds of the four populations which were not treated with GA_3 solutions (control seeds), high germination percentages (85.83–98.33%) were observed in seeds incubated at 15/10 °C, whereas seeds incubated at 25/20 °C exhibited very low germination percentages (0.83–12.50%) (Figure 5). At the germination temperature of 15/10 °C, control seeds of Rhodope population exhibited higher germination percentages than those of Chalkidiki and Pieria populations. The treatment with GA_3 increased the germination of seeds incubated at 25/20 °C, whereas it decreased the germination of seeds incubated at 15/10 °C. At 25/20 °C, seeds treated with GA_3 of 500 or 1000 mg·L^{-1} of Rhodope and Rhodes populations exhibited higher germination percentages than those of Chalkidiki and Pieria. In seeds treated with 2000 mg·L^{-1} and then incubated at 25/20 °C, Chalkidiki population exhibited the lowest percentage of germinated seeds. In all four populations, germinated seeds of control treatment were recorded at 12th day and their germination at 15/10 °C was completed after a month from the beginning of the germination test. At 15/10 °C, seeds treated with 500 mg·L^{-1} of Rhodope and Chalkidiki populations exhibited higher germination percentages than those of Pieria and Rhodes. In seeds treated with 1000 mg·L^{-1} and then incubated at 15/10 °C, Rhodope population exhibited the lowest percentage of germinated seeds. In all four populations and regardless of treatment, germinated seeds were recorded at 12th day at 15/10 °C and their germination was completed about a month from the beginning of the germination test (Figure 5). Whereas in seeds treated with GA_3 and incubated at 25/20 °C, the germinated seeds were recorded at 8th day and their germination was completed in less than a month.

After a 1 month period of CS, in seeds of all four populations which were treated or not with GA_3 solutions and incubated at 15/10 °C, very high germination percentages were observed (>96.67%) (Figure 6). At 15/10 °C, in control seeds as well as in seeds treated with GA_3, no significant differences were observed among the germination percentages of the four populations (Figure 6). At 25/20 °C, significant differences among the germination percentages of the four populations were observed only in treatments with 500 and 2000 mg·L^{-1} GA_3. Specifically, in seeds treated with 500 mg·L^{-1} GA_3, Pieria and Rhodes populations exhibited the lowest germination percentages, whereas in seeds treated with GA_3 of 2000 mg·L^{-1}, the Pieria population exhibited the lowest germination percentage. In seeds stratified for a month, their germination was completed until the 12th day from the beginning of the germination test. Furthermore, in seeds of Rhodope

and Chalkidiki populations which were treated with GA_3 (500 and 1000 mg·L^{-1}) or not (control), germinated seeds were recorded at the end of the 1 month period of CS (Figure 6).

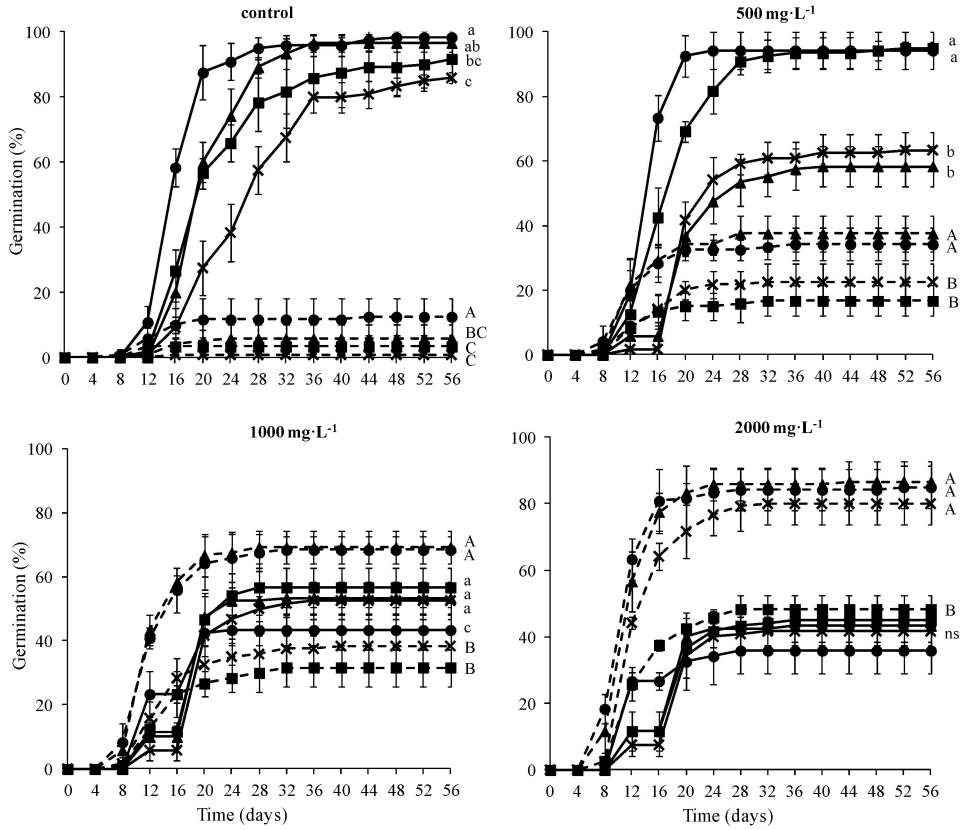

Figure 5. Cumulative germination percentage diagrams of *A. andrachne* seeds treated only with GA_3 of the four populations (● Rhodope, ■ Chalkidiki, × Pieria, and ▲ Rhodes) incubated at 10–15 °C (—) or 20–25 °C (— — —). At the germination temperature 10–15 °C, the means of the four populations are statistically different at $p < 0.05$, when they do not share a common small letter. At the germination temperature 20–25 °C, the means of the four populations are statistically different at $p < 0.05$, when they do not share a common capital letter. ns: non-significant differences. The comparisons were made using Duncan's test.

3.2. Seedlings Growth

Significant differences were observed on morphological traits among the 1-year-old seedlings of the four populations (Table 3). More precisely, the seedlings of Rhodope and Pieria populations exhibited the highest shoot height, whereas the seedlings of the other two populations (Chalkidiki and Rhodes) had the lowest root collar diameter. The sturdiness quotient of seedlings of Chalkidiki and Pieria populations was significantly lower than that of seedlings of Rhodope. Root dry biomass was significantly higher for seedlings of Rhodope compared to seedlings of Chalkidiki and Rhodes populations. Seedlings of Chalkidiki population exhibited the lowest value in above ground part dry biomass.

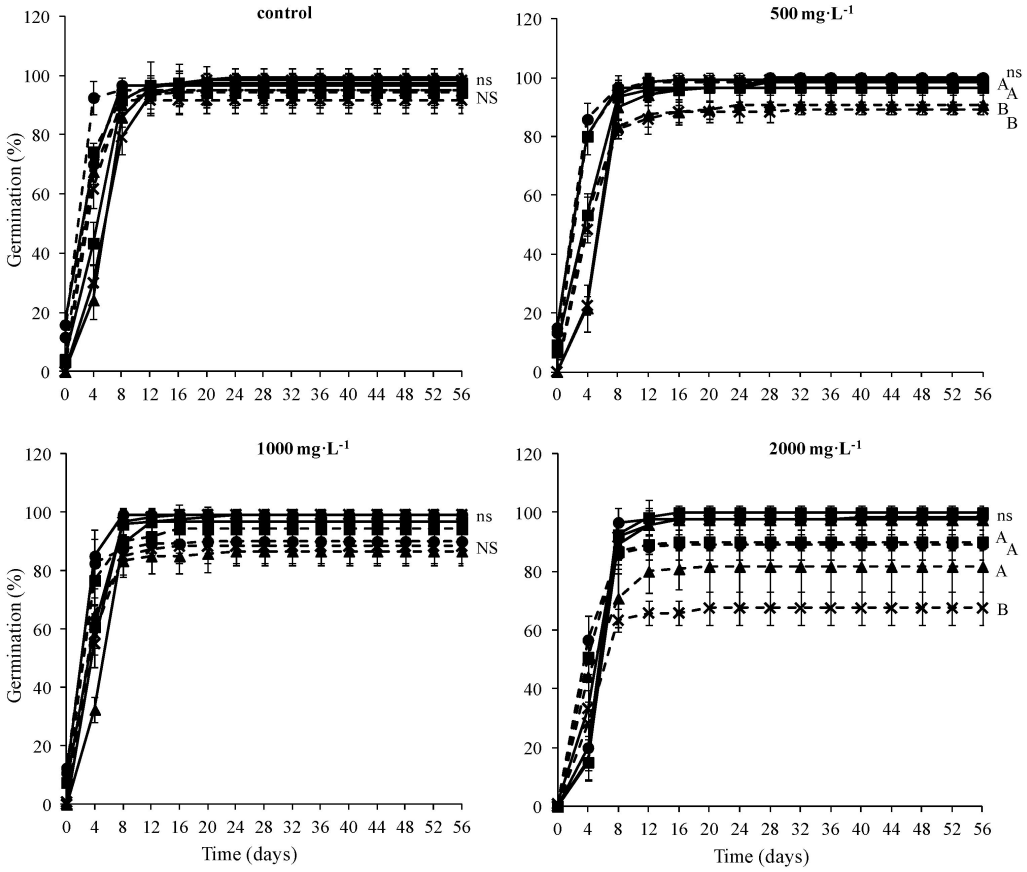

Figure 6. Cumulative germination percentage diagrams of *A. andrachne* seeds treated with GA_3 and stratified for 1 month of the four populations (● Rhodope, ■ Chalkidiki, × Pieria, and ▲ Rhodes) incubated at 10–15 °C (—) or 20–25 °C (– – –). At the germination temperature 10–15 °C, the means of the four populations are statistically different at $p < 0.05$, when they do not share a common small letter. At the germination temperature 20–25 °C, the means of the four populations are statistically different at $p < 0.05$, when they do not share a common capital letter. ns: non-significant differences. The comparisons were made using Duncan's test.

Table 3. Effect of population on morphological characteristics of *A. andrachne* 1-year-old seedlings. Means and (±) standard deviation values are given.

	Rhodope	Chalkidiki	Pieria	Rhodes
Shoot height (cm)	6.47 ± 1.37 a [1]	5.50 ± 1.27 b	6.57 ± 1.26 a	5.81 ± 1.22 b
Root collar diameter (mm)	2.69 ± 0.44 b	2.45 ± 0.38 c	2.92 ± 0.48 a	2.50 ± 0.42 c
Sturdiness quotient (cm·mm^{-1})	2.42 ± 0.44 a	2.24 ± 0.39 b	2.26 ± 0.37 b	2.34 ± 0.37 ab
Number of leaves	9.90 ± 1.79 a	9.50 ± 1.65 a	10.40 ± 2.01 a	11.20 ± 2.04 a
Root dry biomass (g)	1.05 ± 0.18 a	0.70 ± 0.14 c	0.93 ± 0.17 ab	0.81 ± 0.18 bc
Above ground part dry biomass (g)	0.94 ± 0.21 a	0.74 ± 0.15 b	0.96 ± 0.20 a	1.00 ± 0.22 a

[1] Values, in the same row, followed by the same letter are not significantly different ($p > 0.05$) according to Duncan's test.

In the 2-year-old plants of Rhodope population, the different container sizes resulted in significant effects on all morphological parameters (Table 4). Plants that were transplanted to QuickPot 12 T/18 exhibited lower values for all morphological parameters at the end of growing season compared to plants that were grown in the other containers (QuickPot 6 T/20, plastic bags, and plastic pots). Plants that were grown in plastic bags had the highest shoot height and sturdiness quotient value. Root collar diameter was significantly higher for seedlings grown in plastic bags and plastic pots.

Table 4. Effect of container size on morphological characteristics of *A. andrachne* 2-year-old plants. Means and (±) standard deviation values are given.

	QuickPot 24 T/16	QuickPot 12 T/18	QuickPot 6 T/20	Plastic Bags 2.26 L	Plastic Pot 3.4 L
Shoot height (cm)	7.34 ± 1.24 d [1]	8.87 ± 1.42 d	17.29 ± 3.12 c	25.53 ± 4.54 a	21.24 ± 3.94 b
Root collar diameter (mm)	3.54 ± 0.37 d	4.51 ± 0.51 c	6.60 ± 0.86 b	7.70 ± 1.13 a	7.47 ± 1.19 a
Sturdiness quotient ($cm \cdot mm^{-1}$)	2.08 ± 0.34 c	1.97 ± 0.29 c	2.62 ± 0.35 b	3.35 ± 0.60 a	2.88 ± 0.51 b
Number of leaves	11.40 ± 1.52 c	13.40 ± 2.07 c	22.60 ± 3.05 b	27.80 ± 5.36 ab	29.40 ± 5.90 a
Root dry biomass (g)	1.88 ± 0.29 c	2.41 ± 0.19 c	7.86 ± 1.44 b	11.03 ± 2.18 a	9.59 ± 1.79 ab
Above ground part dry biomass (g)	1.94 ± 0.39 b	2.34 ± 0.05 b	7.62 ± 1.29 a	7.89 ± 2.03 a	8.42 ± 1.64 a

[1] Values, in the same row, followed by the same letter are not significantly different ($p > 0.05$) according to Duncan's test.

Details of statistical analysis of the second experiment, including the significance of main effects (substrate type, fertilization) and its interaction are provided in Table 5 and Figure 6. The fertilization significantly affected all the morphological variables measured in A. andrachne 2-year-old plants of Pieria population (Table 5). However, plants mortality occurred mainly after the second fertilizer addition and appeared to increase with dosage in both substrates. More precisely, in substrate type SUB 1, the fertilizer doses of 1.5, 3, and 6 g caused the mortality in 2, 4, and 6 plants out of 10 plants of each treatment, respectively. In SUB 2, the doses of 1.5 and 3 g caused the mortality in 1 and 4 plants out of 10 plants of each treatment, respectively; whereas all the plants which were fertilized with 6 g were dead at the end of the second growing period.

Table 5. Main effects of substrate and fertilization addition and their interaction on morphological characteristics of *A. andrachne* 2-year-old plants. Means and (±) standard deviation values are given.

	SH (cm)	RCD (mm)	Sturdiness Quotient ($cm \cdot mm^{-1}$)	Number of Leaves	RDB (g)	AGDB (g)
Substrate (SUB)	ns	ns	ns	ns	ns	ns
SUB 1	32.53 ± 15.73	6.76 ± 1.00	4.77 ± 2.19	35.06 ± 15.12	3.44 ± 0.76	14.34 ± 7.18
SUB 2	35.04 ± 14.16	7.06 ± 1.09	4.92 ± 1.74	37.42 ± 14.89	3.75 ± 1.51	13.83 ± 6.21
Fertilization (F)	*	*	*	*	*	*
control	17.12 ± 5.16	6.59 ± 0.81	2.58 ± 0.65	15.75 ± 4.27	4.24 ± 1.02	5.75 ± 1.90
1.5 g	47.14 ± 6.52	7.79 ± 0.91	6.08 ± 0.76	45.50 ± 6.28	4.00 ± 0.85	20.81 ± 2.09
3.0 g	41.72 ± 8.48	6.43 ± 0.88	6.49 ± 1.02	46.50 ± 8.02	2.84 ± 1.07	16.33 ± 4.69
6.0 g	39.25 ± 5.84	6.36 ± 1.14	6.25 ± 0.83	37.00 ± 7.33	2.84 ± 0.97	13.06 ± 3.01
SUB × F	*	ns	*	ns	*	*

ns: not significant ($p > 0.05$). *: significant effect ($p < 0.05$).

Furthermore, the interaction of the main factors (substrate and fertilization) had a significant effect on shoot height, sturdiness quotient, root dry biomass, and above ground part dry biomass (Table 5). In the presence of a significant interaction, it was considered that the interpretation of main effects was of less importance. In substrate type SUB 1, the fertilized plants (regardless of the dose of fertilizer) exhibited higher values of shoot height, sturdiness quotient, and above ground part dry biomass than the control plants (Figure 7). However, only in above ground part dry biomass, a significant difference among the doses of fertilizer was observed. More precisely, the dose of 1.5 g was more effective in increasing above ground part dry biomass than the dose of 6 g. In substrate type SUB 2, the dose of 1.5 g of fertilizer produced the tallest plants, and the plants fertilized with 3 g exhibited the lowest root dry biomass. Furthermore, the fertilized plants (regardless of the dose of fertilizer) exhibited higher value of sturdiness quotient than the control plants.

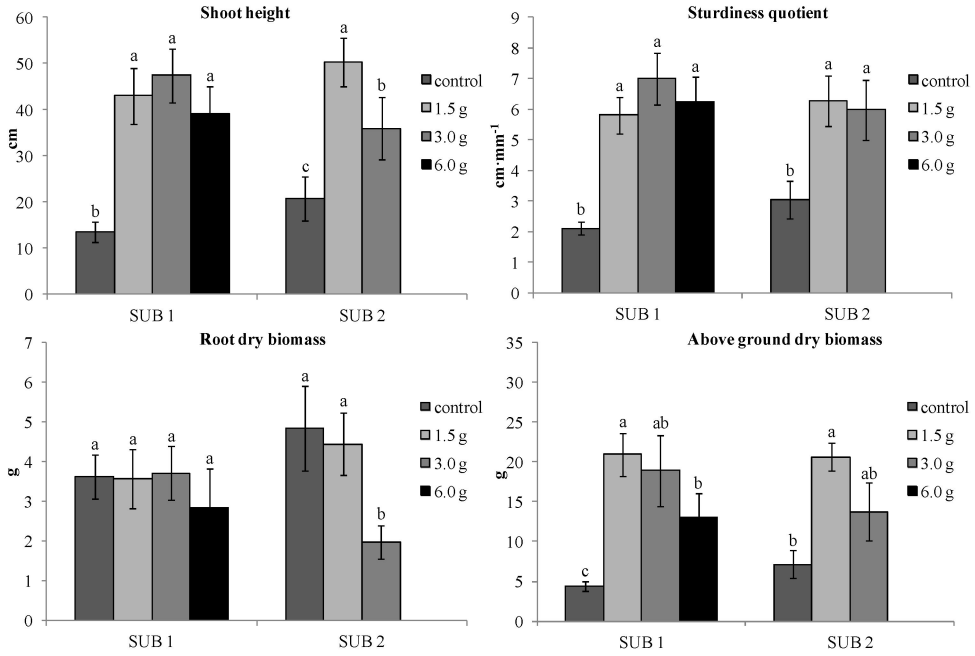

Figure 7. Effect of fertilization addition on shoot height, sturdiness quotient, root dry biomass, and above ground part dry biomass of *A. andrachne* 2-year-old plants in each substrate (SUB 1: mixture of enriched peat and perlite in a ratio of 3:1, SUB 2: mixture of gneiss soil, enriched peat, and perlite in a ratio of 4:3:1). In each substrate, columns accompanied with the same letter do not differ significantly ($p > 0.05$) according to Dunnett's T3 test.

3.3. Genetic Analysis of Arbutus Andrachne Taxon

3.3.1. ISSR Polymorphism

The 10 ISSR primers used in this study generated 122 well-defined and reproducible DNA fragments, out of which 96 (77.39%) were polymorphic. Table 6 gave a description of the criteria calculated for the selected ISSR molecular markers. The total number of bands per primer varied between 7 (UBC-808) and 18 (UBC-821) with an average of 12.2. To determine the PIC values of each ISSR marker, the mean of PIC value was analyzed for all amplified fragments. The PIC values were between 0.19 (UBC-845) and 0.36 (UBC-834) with an average of 0.28. The marker index (MI) was also calculated to examine the usefulness of the markers and is equivalent to PIC. Therefore, the highest value of MI was 3.66 (UBC-845) and the lowest 0.78 (UBC-834).

3.3.2. Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA)

Figure 8 shows the principal coordinate analysis (PCoA) based on the ISSR primers. The first three principal coordinate components accounted for 16.97, 9.68, and 7.21% variation, respectively. At first glance, it appears that the populations stand out from each other. The two populations of Rhodope (red elipse) and Rhodes (yellow cycle) are grouped by themselves in the right and left parts of the PcoA, respectively. This shows that these two populations are genetically isolated and different from the other two studied populations. Moreover, the genotypes of Chalkidiki (green elipse), apart from genotype 2.1 which is genetically close to the population of Rhodope, are also grouped together in the upper left part of the PCoA figure. The individuals of this population are also genetically close to each other. Finally, the genotypes from the population of Pieria (blue elipse) seem to be more distant from each other. They are located in the upper left and right parts of the PCoA with greater distances between them, compared to the rest of the populations, which indicates that they are not as homogeneous as the other three populations. Moreover, some genotypes (3.1 and 3.2) are genetically close to the Chalkidiki genotypes.

Table 6. ISSR primers used in the genetic analysis of *A. andrachne* and their parameters.

Primer	Sequence (5 → 3)	Ta (°C)	NPB	TAB	PPB (%)	PIC	MI
UBC-808	AGA GAG AGA GAG AGA GC	56	5	7	71.43	0.33	1.06
UBC-810	GAG AGA GAG AGA GAG AT	50	8	9	88.89	0.29	1.78
UBC-811	GAG AGA GAG AGA GAG AC	58	7	11	63.64	0.27	1.06
UBC-814	CTC TCT CTC TCT CTC TCTA	50	6	10	60.00	0.25	1.08
UBC-815	CTC TCT CTC TCT CTC TG	50	9	11	81.82	0.25	1.41
UBC-818	CAC ACA CAC ACA CAC AG	50	14	17	82.35	0.25	2.46
UBC-821	CTC TCT CTC TCT CTC TT	58	16	18	88.89	0.36	3.66
UBC-834	AGA GAG AGA GAG AGA GYT	56	7	11	63.64	0.19	0.78
UBC-845	CTC TCT CTC TCT CTC TRG	52	10	11	90.91	0.29	1.41
UBC-888	CAC ACA CAC ACA CA BDB	56	14	17	82.35	0.28	2.61
Total			96	122			
Average			9.6	12.2	77.39	0.28	1.73

Ta (°C): annealing temperature; NPB: number of polymorphic bands; TAB: total amplified bands; PPB (%): percentage of polymorphic bands (%); PIC: percentage of polymorphism information content; MI: marker index of primer.

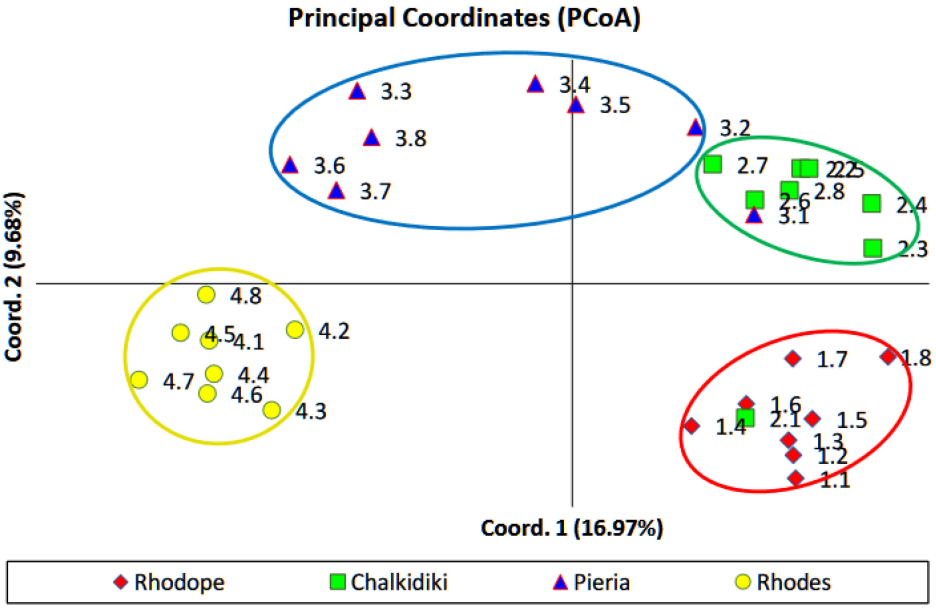

Figure 8. PCoA based on ISSR markers data from 32 genotypes of the four populations of *A. andrachne*.

The value of PhiPT genetic distances detected by the AMOVA (Table 7) for the genetic analysis of *A. andrachne* was 0.243. The AMOVA showed that the majority of variation were detected within populations (76%), with only 24% of variation being due to differences among populations, which supports the high genetic distances between the samples of the populations Pieria, Chalkidiki, and Rhodope in the PCoA diagram (Figure 8).

Table 7. AMOVA summary for A. andrachne with ISSR markers.

Source of Variation	Degree of Freedom	Sum of Squares	Mean Squares	Variance Component	% of Total Variance	Probability of Significances
Among populations	3	150.87	50.29	4.525	24%	<0.001
Within Populations	28	394.50	14.09	14.09	76%	<0.001
						PhiPT = 0.243

3.3.3. Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

To examine the genetic relationships among the 32 samples of *A. andrachne*, cluster analysis was performed using the UPGMA method. As it appeared on the PCoA diagrams, the samples from the populations Rhodope (1.1–1.8), Chalkidiki (2.1–2.8), and three out of eight from Pieria (3.1, 3.2 and 3.5) were included in group A (Figure 9). The other five samples of Pieria were grouped to cluster B.I and the population of Rhodes was grouped by itself in cluster B.II. Therefore, the Rhodes population was distinguished, and as in the PCoA diagram, the rest of the populations also create distinct subgroups with some genetic admixture from the transferred genetic material.

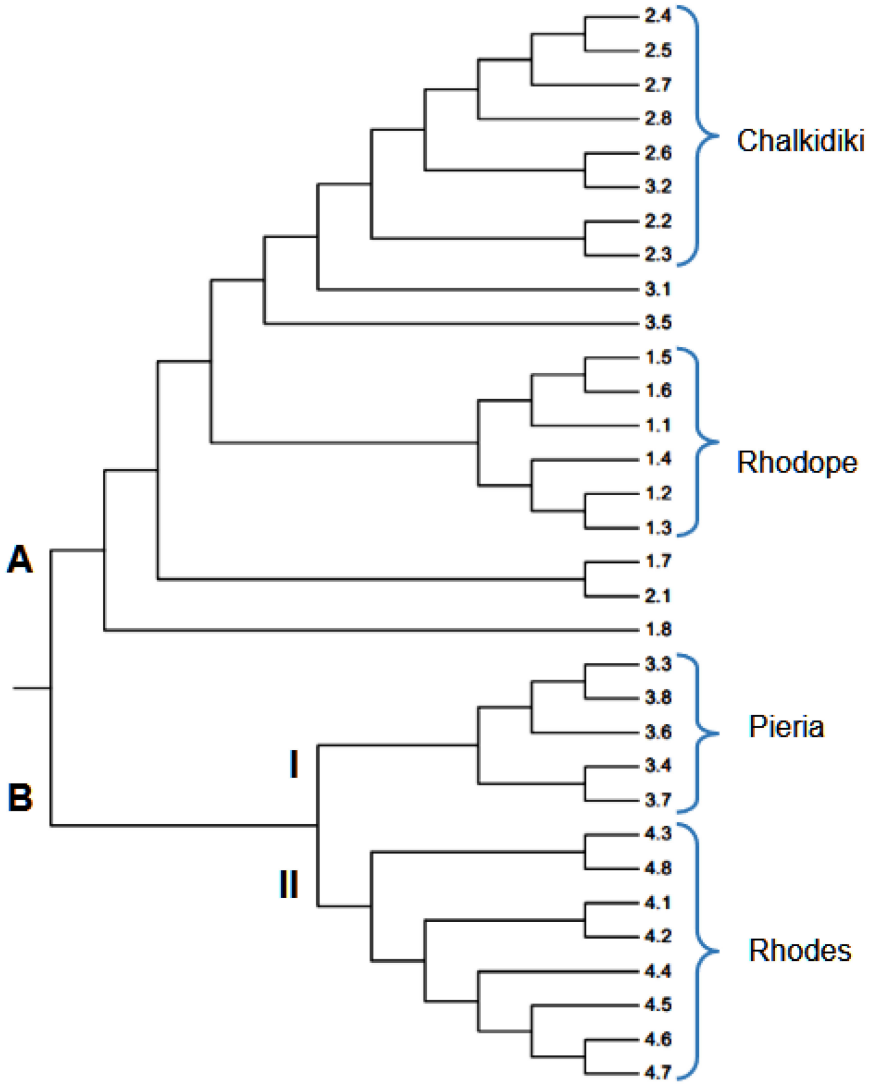

Figure 9. Dendrogram of *A. andrachne* based on genetic distance according to ISSR markers, using the UPMGA method. 1.1–1.8: Rhodope, 2.1–2.8: Chalkidiki, 3.1–3.8: Pieria, 4.1–4.8: Rhodes.

The above grouping was mainly influenced by the geographical factor. This is especially clear in the case of Rhodes, which is an island and geographically isolated from the rest of the populations, where individuals clustered separately in both PCoA and dendrogram. Moreover, it is observed that the Rhodope population, which is geographically distant from the rest, has a separate population group in the PCoA and in the dendrogram, confirming the theory of the correlation between geographic and genetic grouping. Furthermore, it seems logical that some individuals of Chalkidiki are grouped together with genotypes of Pieria, as the two areas are relatively close geographically.

4. Discussion

Generally, similar patterns of germination behavior were observed in all four *A. andrachne* populations. Only non-stratified seeds (control) of all four *A. andrachne* populations germinated at low temperatures (15/10 °C) and exhibited very high germination percentages (85.83–98.33%), whereas they exhibited very low germination percentages (0.83–12.50%) at high temperatures (25/20 °C). *A. andrachne* is native to the Mediterranean region and it is well documented that seed germination of species from the Mediterranean region is observed at relatively low temperatures and this behavior is considered an adaption of plants to Mediterranean climate [46–48]. However, the observed differences in germination characteristics (rate and percentage of germination) among populations could be attributed to different degrees of dormancy. Since seeds of a species collected in various locations can vary in degree of dormancy, as reflected by germination percentages of fresh seeds [13]. It has been reported that *A. andrachne* seeds germinate at high percentages when they incubated at 10 or 15 °C, whereas they germinate at low percentage or fail to germinate when they incubated at 20 °C or 25 °C, respectively [12]. Furthermore, in previous studies, very low germination percentages of *A. andrachne* seeds have been referred at temperatures higher than 20 °C [10,11].

Over all four populations, the CS of control seeds resulted in the loss of effect of temperature on germination and seeds germinated over a wide range of temperatures. After a 1 month period of CS, control seeds of all four populations, regardless of incubation temperature, germinated at very high percentages (>91%) and in a very short period of time (12 days). This provides evidence that *A. andrachne* seeds of the population from the northern latitudes of Greece (Rhodope, Chalkidiki) do not differ fundamentally from seeds of the population from the southern latitudes of Greece (Rhodes) in their response to cold stratification. Few germinated seeds were found at the end of 1 month of CS, only in the Rhodope and Chalkidiki populations. Whereas in all four populations of *A. andrachne* and regardless of GA_3 treatment, at the end of the 2 month period of CS high percentages of germinated seeds were observed. A similar effect of CS on germination of *A. andrachne* seeds has been reported [10–12]. Vis-à-vis the dormancy issue, the response to temperature and cold stratification confirms the existence of some degree of dormancy in *A. andrachne* seeds. According to Baskin and Baskin [13], if cold stratification reduces the temperature requirement for germination or increases the speed of germination, the species is listed as having physiological dormancy rather than a species having non-dormant seeds. Specifically, this germination behavior indicates that seeds of *A. andrachne* exhibit the Type 1 non-deep physiological dormancy and that germination is controlled by conditional dormancy.

Treatment with GA_3 had a positive effect on germination of non-stratified seeds which were incubated at 25/20 °C. Over all four populations, the increase in GA_3 concentration resulted in an increase in germination percentages at 25/20 °C. However, the GA_3 did not have the same effect on germination of non-stratified seeds in the four populations at 25/20 °C (see Figure 5). Specifically, non-stratified seeds of Chalkidiki population which were treated with 2000 $mg \cdot L^{-1}$ exhibited significantly lower germination percentage (48.33%) than the other three populations (80.00–86.67%). On the other hand, GA_3 treatment negatively affected the germination of non-stratified seeds which were incubated at 15/10 °C. Regardless of the population, the treatment of seeds with 2000 $mg \cdot L^{-1}$ resulted in lower germination percentages (35.83–45.00%) than control seeds (85.83–98.33%) (see Figure 5).

Concerning the growth of the 1-year-old seedlings, differences among populations of *A. andrachne* were observed in five of the six measured morphological traits. Variation in seedling morphological traits during the nursery phase is usually considered genetic in origin [49]. Differences in growth of seedlings from the four populations of *A. andrachne* may be due to the ecological adaption and geographic range of distribution of this species. The seedlings from Rhodope and Pieria populations exhibited superior growth rates than those of the other two populations. Specifically, the seedlings from Rhodope differed

significantly from the seedlings from Chalkidiki for five traits and from seedlings from Rhodes for three traits; the seedlings from Pieria differed significantly from seedlings from Chalkidiki for four traits and from seedlings from Rhodes for two traits. Taking into account the above, these two populations (Rhodope and Pieria) were selected to evaluate the effect of container size, substrate, and fertilization on seedling growth. Many studies have been conducted related to the effect of containers size on the quality of planting stocks [18,50,51]. Although there are differences in species response [52], in general, larger container volumes result in better seedling growth. However, there is no information on the cultivation of *A. andrachne* in containers that improve the quality of plants produced in a nursery.

In the present study, the volume of container had a significant influence on morphological traits. The 1-year-old *A. andrachne* seedlings from the population of Rhodope which were transplanted to plastic bags or plastic pots exhibited superior growth rates at the end of the second growth period. Growing in plastic bags produced plants with the greatest height and the highest value of sturdiness quotient. However, in all types of containers, the plants exhibited a sturdiness quotient value of below 6, which is recommended for container plants [53]. On the other hand, low value of the shoot to root dry mass ratio (ratio not shown in the results) was observed for plants growing in plastic bags. The shoot to root dry mass ratio relates the evaporating surface to the water-absorbing surface. Therefore, these plants may have better survival in field after out-planting, since their root system is adequate to absorb the necessary water and nutrients.

The results from the present study indicated that morphological characteristics (shoot height, root collar diameter, sturdiness quotient, leave number, root dry biomass, and above ground part dry biomass) of the plants were not significantly affected by the type of substrate (see Table 5). Therefore, a soil with similar properties as the one used in the present study could replace a part of the peat in the substrate reducing the cost of plant production. The application of fertilizers produced morphologically different *A. andrachne* plants from Pieria population. The availability of nitrogen results in an increase in the photosynthetic rate in plant leaves, and consequently in the enhancement of plant growth [54]. The changes in most morphological traits showed the same trend with the increase in fertilization. In both substrate types, the fertilization resulted in the production of larger plants than the control, and consequently at higher mass only of above ground dry biomass. This may be ascribed to the higher availability of nitrogen availability which influenced plant growth. It has been well documented in previous studies that plants with high nitrogen fertilization are larger and have higher shoot to root mass ratio than low fertilized or unfertilized plants [20,55,56]. Furthermore, studies on Mediterranean species have demonstrated that fertilization of plants at the nursery stage enhances the success of their establishment in field conditions [20,22,56]. It has been observed that fertilized plants with high rates of N-P-K in the nursery stage exhibited increased root growth after transplanting [56,57]. This fact emphasizes the importance of fertilization for nutrient-loaded plants in the nursery stage to ensure increased root growth after transplanting, which can promote the survival of plants. Therefore, regardless of the two types of substrates studied herein, *A. andrachne* plants that were fertilized with 1.5 g of the inorganic fertilizer 21-5-10 (Complesal Suprem) two times during the second growth period may have better survival in field after out-planting. However, fertilization of plants tended to negatively affect the root growth only in the substrate consisting of a mixture of gneiss soil, enriched peat, and perlite in a ratio of 4:3:1 (SUB 2), and specifically in plants fertilized with 3.0 g, the root dry biomass significantly declined possibly due to toxicity. In this type of substrate, the fertilizer dose of 6 g caused the mortality of all plants of the treatment. Plant mortality was presumably a consequence of the osmotic effect of high salt concentrations in the substrate due to soluble fertilizer. Due to the lower water holding capacity of gneiss soil than peat, the concentration of salts was higher in substrate SUB 2 (gneiss soil, enriched peat, and perlite in a ratio of 4:3:1) than SUB 1 (enriched peat and perlite in a ratio of 3:1) resulting in higher plant mortality, and specifically the plants fertilized with 6 g.

In this research, the genetic analysis of *A. andrachne* of 32 samples, eight from four different regions in Greece: Rhodope, Chalkidiki, Pieria, and Rhodes were studied. The parameter PIC of a primer estimates the degree of genomic polymorphism that varies from 0 to 0.5 [58]. ISSR primers produced the highest level of polymorphisms (0.28), and thus were the most useful for measuring genetic variability in *A. andrachne*. Marker index (MI) is a method to evaluate the ability of primers to distinguish between genotypes [37,59]. The combination of the high values of the statistical analysis of the primers indicates that ISSR markers are capable of studying genetic variation. The same conclusion has been drawn from the related species, *A. unedo* [31]. Therefore, the ISSR markers are a useful molecular tool for future genetic analyses. The AMOVA of *A. andrachne* (Table 7) has shown high genetic variance within the populations (76%) in contrast to the variance among the populations (24%), similar to the other study of *A. unedo* species with ISSR and RAPD markers analysis [31]. Furthermore, the populations of Rhodes and Rhodope on the PCoA diagram stood out (Figure 8); meanwhile, the samples from the other two populations are grouped geographically, except for the presence of one genotype, 2.1 from Chalkidiki, in the group of plants from Rhodope. The UPMGA dendrogram (Figure 9) clustered the samples similarly into two main groups, the first one including the populations of Rhodope and Chalkidiki and part of the samples of Pieria, whilst the second cluster includes the samples from Rhodes with the rest of the samples originating from Pieria. Since it is the first time that remote populations of *A. andrachne* have been genetically studied using molecular markers, no comparison can be made with other works. In another paper by Bertsouklis and Papafotiou [24], two populations of *A. andrachne* were studied, but they grow naturally close among them (Kalamos and Varympompi, distance about 12 km). In this paper, the relationship among species *A. andrachne*, *A. unedo*, and their natural hybrid *A.* × *andrachnoides* were studied with RAPD markers. It was observed that higher genetic similarity between samples of *A. andrachne* than the other two species and that the plants with intermediate characteristics, which were considered to be *A.* × *andrachnoides*, were genetically more similar to *A. andrachne* than to *A. unedo*. Between the two populations of *A. andrachne*, from Kalamos and Varympompi, no groupings were observed as in the present work, probably due to the small distance in which the plants were located [24]. In a study carried out on the related species *A. unedo* originating from nine countries using nine AFLP markers, two populations originating from Greece (specifically from Athens and Sithonia), it was found that the populations have a genetic uniformity among them, and they could be distinguished from the Atlantic and Mediterranean groups [26]. In addition, studying the genetic distribution of *A. unedo* with isozyme markers in Tunisia, it was concluded that the genetic structure of the species is mainly related to ecological factors rather than geographical [25].

5. Conclusions

In this study, a detailed sexual propagation protocol as well as a plant production protocol for sustainable exploitation of *A. andrachne* taxon is reported. The seeds of four Greek natural populations of *A. andrachne* studied herein exhibit the same germination behavior. The results of the study indicated a range-specific temperature dependence in germination of *A. andrachne* seeds. However, the cold stratification or the treatment of seeds with GA_3 results in the loss of effect of temperature on germination and seeds can germinate over a wide range of temperatures. Taking into account the aforementioned results and the first-time detected effect of container size and fertilization on the growth of *A. andrachne* plants, this can help in paving the way for plant production at the nurseries of this species with promising potential in different economic sectors (medicinal and horticulture). Genetic analysis on produced seedlings of the four populations of *A. andrachne* showed high genetic differentiation within populations in contrast to the variance among the populations. In addition, the four populations were genetically distinct according to the degree of geographic isolation, and thus the populations of Rhodope and Rhodes, which are further away from the populations of Pieria and Chalkidiki, are more isolated than the rest

of the populations. This observed genetic variation, in addition to ensuring the survival of a population in the case of biotic and abiotic stress, can form the basis for the development of new cultivars with desirable characteristics, for example, breeding programs could focus on populations whose plants exhibit superior growth or superior horticultural traits.

Author Contributions: Conceptualization, E.P. (Elias Pipinis), E.M., S.K., K.K. and K.R.; methodology, E.P. (Elias Pipinis), A.S., A.A.K., E.M., S.K., S.H., E.P. (Evgenia Papioannou) and K.K.; software, E.P. (Elias Pipinis), E.M., K.K., S.K. and A.A.K.; validation, E.P. (Elias Pipinis), A.S., A.A.K., E.M., S.K., S.H., E.P. (Evgenia Papioannou), A.P., K.K. and K.R.; formal analysis, E.P. (Elias Pipinis), A.A.K., E.M., S.K. and K.K.; investigation, E.P. (Elias Pipinis), A.S., A.A.K., E.P. (Evgenia Papioannou) and A.P.; resources, E.P. (Elias Pipinis), E.M., S.K., S.H., E.P. (Evgenia Papioannou) and K.R.; data curation, E.P. (Elias Pipinis), A.S., A.A.K., E.M., S.K., S.H., E.P. (Evgenia Papioannou) and K.K.; writing—original draft preparation, E.P. (Elias Pipinis), A.S., A.A.K., E.M. and S.K.; writing—review and editing, all authors; visualization, E.P. (Elias Pipinis), E.M. and S.K.; supervision, E.P. (Elias Pipinis), E.M., S.K. and S.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: The authors would like to thank the anonymous reviewers for their valuable comments and suggestions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hileman, L.C.; Vasey, M.C.; Parker, V.T. Phylogeny and Biogeography of the Arbutoideae (Ericaceae): Implications for the Madrean-Tethyan Hypothesis. *Syst. Bot.* **2001**, *26*, 131–143.
2. Torres, J.A.; Valle, F.; Pinto, C.; Garcia-Fuentes, A.; Salazar, C.; Cano, E. *Arbutus unedo* communities in southern Iberian Peninsula mountains. *Plant Ecol.* **2002**, *160*, 207–223. [CrossRef]
3. Boratynski, A.; Browicz, K.; Zielinski, J. *Chorology of Trees and Shrubs in Greece*; Institute of Dendrology, Kornik Polish Academy of Sciences: Kórnik, Poland, 1992; p. 286.
4. Markovski, A. Morphological characteristics of greek strawberry tree (*Arbutus andrachne* L.) genotypes. *Acta Agric. Serb.* **2017**, *22*, 193–206. [CrossRef]
5. Seker, M.; Toplou, C. Determination and comparison of chemical characteristics of *Arbutus andrachne* L. (family Ericaceae) fruits. *J. Med. Food* **2010**, *13*, 1013–1018. [CrossRef]
6. Serce, S.; Ozgen, M.; Torun, A.A.; Ercisli, S. Chemical composition, antioxidant activities and total phenolic content of *Arbutus andrachne* L. (family Ericaceae) (the Greek strawberry tree) fruits from Turkey. *J. Food Compos. Anal.* **2010**, *23*, 619–623. [CrossRef]
7. Saral, O.; Ersen Bak, F.; Olmez, Z. Determining total phenolic content and antioxidant activity in fruits and flowers of naturally grown *Arbutus andrachne* L. in Artvin. *Artvin Coruh Univ. J. For. Fac.* **2017**, *18*, 51–54.
8. Macdonald, B. *Practical Woody Plant Propagation for Nursery Growers*; Timber Press, Inc.: Portland, OR, USA, 2006; p. 669.
9. Mackay, A.C.; Mcgill, C.R.; Fountain, D.W.; Southward, R.C. Seed dormancy and germination of a panel of New Zealand plants suitable for re-vegetation. *N. Z. J. Bot.* **2002**, *40*, 373–382. [CrossRef]
10. Tilki, F.; Guner, S. Seed germination of three provenances of *Arbutus andrachne* L. in response to different pretreatments, temperature and light. *Propag. Ornam. Plants* **2007**, *7*, 175–179.
11. Karam, N.S.; Al-Salem, M.M. Breaking dormancy in *Arbutus andrachne* L. seeds by stratification and gibberellic acid. *Seed Sci. Technol.* **2001**, *29*, 51–56.
12. Bertsouklis, K.F.; Papafotiou, M. Seed germination of *Arbutus unedo*, *A. andrachne* and their natural hybrid *A. andrachnoides* in relation to temperature and period of storage. *HortScience* **2013**, *48*, 347–351. [CrossRef]
13. Baskin, C.C.; Baskin, J.M. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*, 2nd ed.; Academic Press: San Diego, CA, USA, 2014.
14. Kostopoulou, P.; Radoglou, K.; Dini-Papanastasi, O.; Spyroglou, G. Enhancing planting stock quality of Italian cypress (*Cupressus sempervirens* L.) by pre-cultivation in mini-plugs. *Ecol. Eng.* **2010**, *36*, 912–919. [CrossRef]
15. Radoglou, K.M. Effect of environmental stress on planting stock and field performance. In Proceedings of the National Forestry Conference: Planting Stock of Woody Species, Thessaloniki, Greece, 28–29 January 1999; pp. 15–26. (In Greek).
16. O'Reill, C.; Arnott, J.T.; Owens, J.N. Effects of photoperiod and moisture availability on shoot growth, seedling morphology, and cuticle and epicuticular wax features of container-grown western hemlock seedlings. *Can. J. For. Res.* **1989**, *19*, 122–131. [CrossRef]

17. Landis, T.D.; Tinus, R.W.; McDonald, S.E.; Barnett, J.P. Containers and growing media. In *The Container Tree Nursery Manual: Agriculture Handbook 674, Volume 2*; U.S. Department of Agriculture, Forest Service: Washington, DC, USA, 1990; p. 88.
18. Dominguez-Lerena, S.; Herrero Sierra, N.; Carrasco Manzano, I.; Ocana Bueno, L.; Penuelas Rubira, J.L.; Mexal, J.G. Container characteristics influence *Pinus pinea* seedling development in the nursery and field. *For. Ecol. Manag.* **2006**, *221*, 63–71. [CrossRef]
19. Chirino, E.; Vilagrosa, A.; Hernandez, E.L.; Matos, A.; Vallejo, V.R. Effects of a deep container on morpho-functional characteristics and root colonization in *Quercus suber* L. Seedlings for reforestation in Mediterranean climate. *For. Ecol. Manag.* **2008**, *256*, 779–785. [CrossRef]
20. Vilar-Salvador, P.; Puertolas, J.; Penuelas, J.L.; Planeles, R. Effect of nitrogen fertilization in the nursery on the drought and frost resistance of Mediterranean forest species. *For. Syst.* **2005**, *14*, 408–418. [CrossRef]
21. Buendia Velazquez, M.V.; Lopez Lopez, M.A.; Cetina Alcala, V.M.; Diakite, L. Substrates and nutrient addition rates affect morphology and physiology of *Pinus leiophylla* seedlings in the nursery stage. *IForest* **2016**, *10*, 115–120. [CrossRef]
22. Oliet, J.; Planelles, R.; Artero, F.; Valverde, R.; Jacobs, D.F.; Segura, M. Field performance of *Pinus halepensis* planted in Mediterranean arid conditions: Relative influence of seedling morphology and mineral nutrition. *New For.* **2009**, *37*, 313–331. [CrossRef]
23. Koukos, D.; Meletiou-Christou, M.S.; Rhizopoulou, S. Leaf surface wettability and fatty acid composition of *Arbutus unedo* and *Arbutus andrachne* grown under ambient conditions in a natural macchia. *Acta Bot. Gall.* **2015**, *162*, 225–232. [CrossRef]
24. Bertouklis, K.F.; Papafotiou, M. Morphometric and molecular analysis of the three *Arbutus* species of Greece. *Not. Bot. Horti Agrobot.* **2016**, *44*, 423–430. [CrossRef]
25. Takrouni, M.M.; Ali, I.B.E.H.; Messaoud, C.; Boussaid, M. Genetic variability of Tunisian wild strawberry tree (*Arbutus unedo* L.) populations interfered from isozyme markers. *Sci. Hort.* **2012**, *146*, 92–98. [CrossRef]
26. Santiso, X.; Lopez, L.; Gilbert, K.J.; Barreiro, R.; Whitlock, M.C.; Retuerto, R. Patterns of genetic variation within and among populations in *Arbutus unedo* and its relation with selection and evolvability. *Perspect. Plant Ecol. Evol. Syst.* **2015**, *17*, 185–192. [CrossRef]
27. Santiso, X.; Lopez, L.; Retuerto, R.; Barreiro, R. Population structure of a widespread species under balancing selection: The case of *Arbutus unedo* L. *Front. Plant Sci.* **2016**, *6*, 1264. [CrossRef] [PubMed]
28. Gomes, F.; Costa, R.; Ribeiro, M.M.; Figueiredo, E.; Canhoto, J.M. Analysis of genetic relationship among *Arbutus unedo* L. genotypes using RAPD and SSR markers. *J. For. Res.* **2013**, *24*, 227–236. [CrossRef]
29. Ribeiro, M.M.; Piotti, A.; Ricardo, A.; Gaspar, D.; Costa, R.; Parducci, L.; Vendramin, G.G. Genetic diversity and divergence at the *Arbutus unedo* L. (Ericaceae) westernmost distribution limit. *PLoS ONE* **2017**, *12*, e0175239. [CrossRef] [PubMed]
30. Takrouni, M.M.; Boussaid, M. Genetic diversity and population's structure in Tunisian strawberry tree (*Arbutus unedo* L.). *Sci. Hort.* **2010**, *126*, 330–337. [CrossRef]
31. Lopes, L.; Sa, O.; Pereira, J.A.; Baptista, P. Genetic diversity of Portuguese *Arbutus unedo* L. populations using leaf traits and molecular markets: An approach for conservation purpose. *Sci. Hort.* **2012**, *142*, 57–67. [CrossRef]
32. Chatzistathis, T.; Papaioannou, E.; Giannakoula, A.; Papadakis, I.E. Zeolite and Vermiculite as Inorganic Soil Amendments Modify Shoot-Root Allocation, Mineral Nutrition, Photosystem II Activity and Gas Exchange Parameters of Chestnut (*Castanea sativa* Mill) Plants. *Agronomy* **2021**, *11*, 109. [CrossRef]
33. Tampakis, A.; Papaioannou, E.; Chatzistathis, T.; Karanikola, P. Comparing Soil Substrates of Low Cost for the Production of Calabrian Pine (*Pinus brutia* Ten) Seedlings Resilient to Unfavorable Conditions having in Mind the Climatic Change Phenomenon. *WSEAS Trans. Environ. Dev.* **2022**, *18*, 653–661. [CrossRef]
34. Papaioannou, E.; Gasparatos, D.; Stefanou, S.; Chatzistathis, T.; Karamanoli, K.; Matziris, E. Effect of Soil Mixtures Based on a Gneiss-Derived Soil and Two Forest Floor Types on Growth and Nutritional Status of *Castanea sativa* Mill. Seedlings. *J. Soil. Sci. Plant Nut* **2023**, *23*, 1339–1350. [CrossRef]
35. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
36. Klockars, A.; Sax, G. *Multiple Comparisons*; Sage Publications: Newbury Park, CA, USA, 1986; p. 87.
37. Snedecor, G.W.; Cochran, W.C. *Statistical Methods*, 7th ed.; The Iowa State University Press: Ames, IA, USA, 1980; p. 507.
38. Gomez, K.A.; Gomez, A.A. *Statistical Procedures for Agricultural Research*, 2nd ed.; John Wiley and Sons Publications: Hoboken, NJ, USA, 1984.
39. Prevost, A.; Wilkinson, M.J. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet.* **1999**, *98*, 107–112. [CrossRef]
40. De Riek, J.; Calsyn, E.; Everaert, I.; van Bockstaele, E.; De Loose, M. AFLP based alternatives for the assessment of Distinctness, Uniformity and Stability of sugar beet varieties. *Theor. Appl. Genet.* **2001**, *103*, 1254–1265. [CrossRef]
41. Milbourne, D.; Meyer, R.; Bradshaw, J.E.; Baird, E.; Bonar, N.; Provan, J.; Powell, W.; Waugh, R. Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. *Mol. Breed* **1997**, *3*, 127–136. [CrossRef]
42. Varshney, R.K.; Chabane, K.; Hendre, P.S.; Aggarwal, R.K.; Graner, A. Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys. *Plant Sci.* **2007**, *173*, 638–649. [CrossRef]
43. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef]
44. Nei, M.; Li, W.H. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 5269–5273. [CrossRef] [PubMed]

45. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599. [CrossRef]
46. Perez-Garcıa, F.; Iriondo, J.M.; Gonzalez-Benito, M.E.; Carnes, L.F.; Tapia, J.; Prieto, C.; Plaza, R.; Perez, C. Germination studies in endemic plant species of the Iberian Peninsula. *Isr. J. Plant Sci.* **1995**, *43*, 239–247. [CrossRef]
47. Krichen, K.; Mariem, H.B.; Chaieb, M. Ecophysiological requirements on seed germination of a Mediterranean perennial grass (*Stipa tenacissima* L.) under controlled temperatures and water stress. *S. Afr. J. Bot.* **2014**, *94*, 210–217. [CrossRef]
48. Pipinis, E.; Hatzilazarou, S.; Kostas, S.; Bourgou, S.; Megdiche-Ksouri, W.; Ghrabi-Gammar, Z.; Libiad, M.; Khabbach, A.; El Haissoufi, M.; Lamchouri, F.; et al. Facilitating conservation and bridging gaps for the sustainable exploitation of the Tunisian local endemic plant *Marrubium aschersonii* (Lamiaceae). *Sustainability* **2022**, *14*, 1637. [CrossRef]
49. Ngulube, M.R.; Hall, J.B.; Maghembe, J.A. Fruit, seed, and seedling variation in *Uapaka kirkiana* from natural populations in Malawi. *Forest Ecol. Manag.* **1997**, *98*, 209–219. [CrossRef]
50. Ward, T.M.; Donnelly, J.R.; Carl, C.H. The Effects of Containers and Media on Sugar Maple Seedling Growth. *Tree Plant. Notes* **1981**, *32*, 15–17.
51. Keever, G.J.; Cobb, G.S.; Reed, R.B. Effects of container dimension and volume on growth of three woody ornamentals. *HortScience* **1985**, *20*, 276–278. [CrossRef]
52. McConnughay, K.D.M.; Bazzar, F.A. Is physical space a soil resource? *Ecology* **1991**, *72*, 94–103. [CrossRef]
53. Roller, K.J. *Suggested Minimum Standards for Containerized Seedlings in Nova Scotia*; Information Report M-X-69; Canadian Forestry Service Department of Fisheries and the Environment: Fredericton, NB, Canada, 1977; p. 18.
54. Zhang, H.; Zhao, Q.; Wang, Z.; Wang, L.; Li, X.; Fan, Z.; Zhang, Y.; Li, J.; Gao, X.; Shi, J.; et al. Effects of Nitrogen Fertilizer on Photosynthetic Characteristics, Biomass, and Yield of Wheat under Different Shading Conditions. *Agronomy* **2021**, *11*, 1989. [CrossRef]
55. Graff, J.E.; Hermann, R.K.; Zaerr, J.B. Dry matter and nitrogen allocation in western red cedar, western hemlock, and Douglas fir seedlings grown in low-and high-N soils. *Ann. For. Sci.* **1999**, *56*, 529–538. [CrossRef]
56. Vilar-Salvador, P.; Planeles, R.; Enriquez, E.; Penuelas-Rubina, J.L. Nursery cultivation regimes, plant functional attributes, and field performance relationship in the Mediterranean oak *Quercus ilex* L. *For. Ecol. Manag.* **2004**, *196*, 257–266. [CrossRef]
57. van de Driessche, R. Change in drought resistance and root growth capacity of container seedlings in response to nursery drought, nitrogen, and potassium. *Can. J. For. Res.* **1992**, *22*, 740–749. [CrossRef]
58. Roldan-Ruiz, I.; Dendauw, J.; Van Bockstaele, E.; Depicker, A.; De Loose, M. AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Mol. Breed* **2000**, *6*, 125–134. [CrossRef]
59. Powell, W.W.; Koput, K.W.; Smith-Doerr, L. Interorganizational collaboration and the locus of innovation: Networks of learning in biotechnology. *Adm. Sci. Q.* **1996**, *41*, 116–145. [CrossRef]