sustainability

MDPI

Article

Sustainable Exploitation of Greek *Rosmarinus officinalis* L. Populations for Ornamental Use through Propagation by Shoot Cuttings and In Vitro Cultures

Stefanos Kostas [1,†], Aikaterini Kaplani [2,†], Efthymia Koulaouzidou [2], Aikaterini-Angeliki Kotoula [1], Evangelos Gklavakis [3], Parthena Tsoulpha [4], Stefanos Hatzilazarou [1,*], Irini Nianiou-Obeidat [2,*], Angelos K. Kanellis [5] and Athanasios Economou [1]

1 Laboratory of Floriculture, School of Agriculture, Aristotle University, 54124 Thessaloniki, Greece; skostas@agro.auth.gr (S.K.); aikakoto@agro.auth.gr (A.-A.K.); aseconom@agro.auth.gr (A.E.)
2 Laboratory of Genetics and Plant Breeding, School of Agriculture, Aristotle University, 54124 Thessaloniki, Greece; aikaterinikaplani@gmail.com (A.K.); evikoulaouzidou@gmail.com (E.K.)
3 Evangelos Gklavakis Nurseries, 58400 Aridea, Greece; evan.glavakis@gmail.com
4 Laboratory of Forest Genetics and Plant Breeding, School of Forestry and Natural Environment, Aristotle University, 54124 Thessaloniki, Greece; thena@for.auth.gr
5 Laboratory of Pharmacognosy, Group of Biotechnology of Pharmaceutical Plants, School of Pharmacy, Aristotle University, 54124 Thessaloniki, Greece; kanellis@pharm.auth.gr
* Correspondence: hatzilaz@agro.auth.gr (S.H.); nianiou@agro.auth.gr (I.N.-O.)
† These authors contributed equally to this work.

Citation: Kostas, S.; Kaplani, A.; Koulaouzidou, E.; Kotoula, A.-A.; Gklavakis, E.; Tsoulpha, P.; Hatzilazarou, S.; Nianiou-Obeidat, I.; Kanellis, A.K.; Economou, A. Sustainable Exploitation of Greek *Rosmarinus officinalis* L. populations for Ornamental Use through Propagation by Shoot Cuttings and In Vitro Cultures. *Sustainability* **2022**, *14*, 4059. https://doi.org/10.3390/su14074059

Academic Editor: Svein Øivind Solberg

Received: 1 March 2022
Accepted: 25 March 2022
Published: 29 March 2022

Abstract: *Rosmarinus officinalis* L. belongs to the Lamiaceae family and is cultivated worldwide due to its diverse uses as an ornamental evergreen shrub in gardening, as well as a food seasoning and a natural medicine. The present research was conducted to study the morphological traits of seven wild-grown rosemary populations in Greece, as well as the propagation of two selected populations with the most desirable ornamental characteristics, by the use of shoot cuttings and in vitro cultures. From the study of the morphological traits of the seven populations, those with interesting features were grown in the areas of Amaliada and Piperia and, thus, were chosen for studying their propagation. Rooting of the shoot cuttings was carried out during the four seasons of the year, using potassium salt Indole-3-butyric acid (K-IBA) at concentrations of 0.5 and 1 g·L^{-1}, in various substrates under the intermittent mist or fog system. It was found that the shoot cuttings rooted easily in all four seasons, but there were better results for the population of 'Piperia' in autumn, with 1 g·L^{-1} K-IBA (80%), and 'Amaliada' in spring, with 0.5 g·L^{-1} K-IBA (82.5%), while higher rooting percentages were achieved in the fog system, on a substrate consisting of perlite and peat, in a ratio of 2:1 (85%). For the in vitro cultures, shoot tips excised from the two selected populations were successfully disinfested by pre-soaking in an antioxidant solution and then, by sterilizing them in 0.6% (*w*/*v*) NaOCl, followed by transferring them onto a Murashige and Skoog (MS) nutrient medium. 'Amaliada' cultures proved to be the most productive population (2.1 shoots per explant), with the highest shoot formation frequency (91.6%), when cultivated on the MS nutrient medium without plant growth regulators. For 'Piperia' cultures, the highest shoot formation frequency (66.6%) was achieved on the MS nutrient medium supplemented with 0.25 or 0.5 mg·L^{-1} 6-Benzyloaminopurine (BAP) and 0.1 mg·L^{-1} Indole-3-acetic acid (IAA). Spontaneous root formation frequency was noticed on the MS nutrient medium, containing 0.5 mg·L^{-1} BAP and 0.1 mg·L^{-1} IAA, for both 'Amaliada' and 'Piperia' cultures (50% and 41.6%, respectively) in a single stage, with root lengths of 7.1 and 5.3 cm, respectively. Rosemary plantlets, with roots formed in vitro after transplanting them in soilless substrate, were acclimatized adequately in the greenhouse environment (~70%).

Keywords: biodiversity; fog rooting system; intermittent mist; K-IBA; rosemary

1. Introduction

Rosemary is considered one of the most important plants of the Lamiaceae family from an economic perspective. *Rosmarinus officinalis* L., a diploid species (2n = 24), is classified as a member of the class Dicotyledon, order Tubiflorae, and family Lamiaceae. Rosemary is an evergreen, perennial, and tufted shrub, with erect or procumbent growth and a maximum height, usually, up to 1.8 m [1]. It is propagated easily by seeds, cuttings, layering, or root division [2–4]. Although it derives from the Mediterranean basin, it is hardly enough that it can be cultivated in a vast spectrum of environmental conditions. It is also worth mentioning that it is a species which shows significant tolerance in unfavorable conditions, such as drought, frost, or salt stress. When cultivated, it requires, respectively, low quantities of fertilizers. Three kinds of products are obtained from the cultivation of rosemary: essential oil, as well as fresh and dried leaves [5]. Apart from its apparent use as a spice in cooking, rosemary is a rather sought after plant species. It is broadly used in many fields, such as commercial floriculture, landscape architecture, the food industry, cosmetology, and medicine, because of its large number of beneficial characteristics [6–12].

Analysis of morphological traits is a quick and common method to identify and characterize the germplasm through phenotyping. However, phenotyping traits that are influenced by environmental factors may cause elevated diversity in the desirable agronomic characteristics, thus reducing the method's reliability [13]. To avoid the effect of the environment, it is recommended to cultivate plants in the same environment and in a randomized experimental design [14,15].

Seeds of rosemary are rarely used in propagation as they are slow to germinate, taking 3–4 weeks before emergence, with a poor germination rate of 10–20% [3]. Therefore, vegetative propagation is the most common method for propagation. Cutting propagation is, in general, fast, simple, and does not require special techniques and methods, such as grafting and budding, while each cutting can become a plant with desirable genetic properties that are the same as the parent plant [16]. It is also well-known that the plant-produced phytohormones, such as auxins, play a key role in stimulating the adventitious root formation of shoot cuttings, as well as the roots' branching, and increasing the percentage of rooting, root initiation, number, and uniformity of rooting [17]. Previous studies showed that rosemary has a good rooting ability, and the use of the plant growth regulators achieves better results, both in terms of the rooting percentage and the qualitative features of the new root system [18–21]. On the other hand, there are several proposed protocols for achieving the optimal rooting of rosemary cuttings. Thus, at a concentration of 1 $g \cdot L^{-1}$ α-Naphthaleneacetic acid (NAA), 84% rooting of the cuttings was achieved, while at 5 $g \cdot L^{-1}$ Indole-3-butyric acid (IBA), the rooting percentage was 66% [22]. Additionally, Abu-Zahra et al. [19] reported that 3 $g \cdot L^{-1}$ of NAA resulted in the highest rooting percentages of the rosemary cuttings. In another report, IBA (5 $mg \cdot L^{-1}$) was the most effective auxin on the rooting of rosemary cuttings (92%), as compared with NAA or Indole-3-acetic acid (IAA) of the same concentrations [20]. The form of the applied auxin may also be crucial for the rooting success of the rosemary cuttings. As Paradicovic et al. [16] reported, it is highly recommendable to use auxin powder for the propagation of rosemary green cuttings.

The rooting substrate and the collection time of the cuttings may also play an important role in the success of the rooting process of stem cuttings. In general, peat, perlite, or, more often, a combination of both in different proportions, is a key component of most commercial substrates used for cutting propagation [23]. A mixture of peat and perlite (1:1, v/v) was referred to, by Varban et al. [24], as the most effective substrate for the rooting of rosemary stem cuttings. Kiuru et al. [3] also tested four different substrates (vermiculite, topsoil-manure-sand mixture at a ratio of 10:3:1, topsoil, and sand), on the propagation of rosemary by cuttings, and concluded that the sand was the most efficient one. Furthermore, Mendoza-Hernandez et al. [25] tested the replacement of peat, in the rooting substrate, with compost and various vermicomposts of horticultural waste. The various vermicomposts used outperformed compost and peat for the rooting of rosemary cuttings. Regarding the

collection season of cuttings, Silva and Pedras [26] reported that the end of winter stood out as the best season for all analyzed parameters.

A well-established alternative tool for preserving selected wild-grown plant species is micropropagation [27,28]. For rosemary, there is sufficient literature on its propagation in vitro. Chaturvedi et al. [29] described the production of 5000 plants from one nodal segment of *R. officinalis* L. in a single year. Dong et al. [30] and Boix et al. [31] studied the factors affecting callus induction and plant regeneration by using leaf explants of *R. officinalis*. Aman and Afrasiab [32] developed a protocol for somatic embryogenesis from leaf explants of rosemary. Furthermore, Al Masoody and Stanica [33] investigated the effects of different growth regulators on callus formation and the production of somatic embryos from leaf explants of *R. officinalis*. Likewise, the application of different concentrations of growth regulators was tested by El-Zefzafy et al. [34] for callus induction, in vitro, from rosemary shoot tip explants. Irum et al. [35] also used shoot tips and seedling explants, in order to develop a micropropagation protocol, while Darwesh et al. [36] reported the induction of multiple shoots, from sterilized seedlings, obtained from rosemary seeds.

The objective of the present study was to evaluate the morphological traits of seven wild-grown rosemary populations, derived from seven different regions of Greece, and select the most suitable populations for ornamental use. Furthermore, by developing protocols for their macro and micropropagation, it aimed at sustainable exploitation of these native genotypes, for the production of plant material, to meet the demands of the ornamental plant market.

2. Materials and Methods

2.1. Plant Source

A total of 47 rosemary plants were selected from seven populations, wild-grown in seven different natural environments, in Greece, namely: Amaliada, Ioannina, Chios, Variko, Piperia, Archanes, and Kavousi (Figure 1). The morphological characteristics, the durability, the vigor, and the shape of the plants selected were the main criteria for their selection. In addition, the selected populations came from greatly different habitats of Greece, aiming at a sufficient biodiversity of rosemary plants for the needs of this study. The number of collected plants of the aforementioned rosemary populations, and the coordinates of the regions of origin, are presented in Table 1.

Plant material collected from these seven populations was transported to the greenhouse of the Floriculture Laboratory of the Aristotle University in Thessaloniki. In order to obtain sufficient plant material for the various experiments, young plantlets were vegetatively produced, from the 47 genotypes, by the rooting of shoot cuttings, which were then transplanted into 1 L pots containing a substrate of peat TS2 Klasmann (Klasmann-Deilmann, Geeste, Germany) and perlite (Isocon, Athens, Greece) in a ratio of 3:1 (v/v). Six months later, two developed plants from each genotype were transplanted into 2 L pots, filled with the same substrate, and kept in the greenhouse, under natural light conditions and ambient temperatures of 23 $\pm$ 3 °C, for the needs of the in vitro cultures, while three other plants from each genotype were moved and planted in an outdoor experimental field collection, located in the region of Piperia Aridea (latitude 40.964263° N, longitude 22.017363° E). Plant irrigation was done using a drip irrigation system in both the greenhouse and the field collection. After a period of 12 months, on 28 May, the plants of the field collection (47 genotypes $\times$ 3 plants per genotype) were evaluated in order to determine the most appropriate populations for ornamental use.

Figure 1. The seven locations (with red dots) of Greece from which the plants of the seven populations of *R. officinalis* were collected.

Table 1. Origin of the seven populations of *R. officinalis*, number of plants collected, and coordinates of the seven locations of Greece.

Origin—Population	Plants per Population	Latitude N	Longitude E	Elevation (m)
Amaliada (AM)	9	37.769800°	21.303847°	11
Ioannina (I)	3	39.645606°	20.849649°	481
Chios (CH)	3	38.243976°	26.035575°	57
Variko (V)	9	40.192397°	22.550020°	12
Piperia (P)	14	40.964379°	22.017295°	143
Archanes (AR)	6	35.233768°	25.152781°	382
Kavousi (KA)	3	35.118470°	25.856935°	173

2.2. Morphological Traits

The morphological characteristics of ornamental value, measured in situ, were plant height (cm), plant diameter (cm), and number of terminal branches. To avoid errors on the measurements, due to environmental conditions or developmental stages of the plants, all measurements were conducted on the same day of 28 May. For the same reason, three terminal branches from the middle zone of the canopy, of each genotype, were collected on 28 May, transferred to the Lab, and, after being air-dried, were stored in the herbarium, under the code numbers RO101–RO241, for evaluation in due time. On these air-dried terminal branches, the measurements taken were: branch length (cm), number of nodes and leaves per branch, and leaf length (cm) and width (cm). The recorded morphological traits and the number of measurements taken, per plant of each genotype, are shown in Table 2.

The statistical analysis of the data was based on analysis of variance (ANOVA) with the use of the statistical package SPSS 27 (IBM, Armonk, NY, USA). Separation of the means was made with Duncan's multiple range test at $p \leq 0.05$ [5].

Table 2. Morphological traits, recorded on 28 May, for all *R. officinalis* plants planted in the field collection in Piperia Aridea.

	Morphological Traits	Details	Measurements per Plant of each Population
1.	Plant height (cm)	Plant canopy height	1
2.	Plant diameter (cm)	Plant canopy diameter	1
3.	Number of terminal branches	Terminal shoots of new vegetation per plant	1
4.	Length of terminal branches (cm)	Terminal shoots of new vegetation	10
5.	Number of nodes	Nodes per terminal branch	10
6.	Number of leaves	Leaves per terminal branch	10
7.	Leaf length (cm)	Measured from the base to the tip of adult leaf	30
8.	Leaf width (cm)	Measured at the widest part of adult leaf	30

2.3. Propagation by Shoot Cuttings

2.3.1. Plant Material

Shoots of new vegetation of *R. officinalis* were collected from two-year-old mother plants of the populations 'Amaliada' and 'Piperia', which were found to have the most desirable ornamental characteristics after the morphological traits evaluation. Rosemary mother plants were grown in the outdoor experimental field collection, located in Piperia Aridea. From these shoots, the terminal parts were cut and used as cuttings for the rooting experiments.

2.3.2. Effect of K-IBA and Season on Rooting of Cuttings

Terminal shoots, 8–10 cm long, from plants of the two selected *R. officinalis* populations, 'Piperia' and 'Amaliada', were cut during the four seasons of the year (spring: 20 April 2020; summer: 17 July 2020; autumn: 20 October 2020; winter: 19 January 2021) and used for rooting. The leaves on the base of the shoot cuttings were removed, leaving 7–8 leaves on the top part. Then, the basal portions (1.5 cm) of the shoot cuttings were dipped, for 10 s, into aqueous solutions of 0, 0.5, or 1 $g \cdot L^{-1}$ of potassium salt Indole-3-butyric acid (K-IBA) (Sigma-Aldrich, St. Louis, MO, USA) and planted in multi-position plastic discs, of 6 cm depth and 4 cm diameter, with holes at their bases for drainage. The rooting substrate was a mixture of perlite and peat TS2 Klasmann in a ratio of 2:1 (v/v). Afterwards, the plastic disks with the shoot cuttings were placed for rooting in a fog system, with a relative humidity (RH) adjusted to 94–96%. For each population, forty shoot cuttings were used for every treatment of K-IBA and every season. At the end of a 5-week period, rooting (%), number, and length (cm) of roots were measured.

2.3.3. Effect of Substrate and Rooting System on Rooting of Cuttings

In spring, which proved to be the best season for the rooting of *R. officinalis* cuttings, terminal shoots were harvested from the mother plants of the population 'Piperia' (bearing abundant new vegetation) and treated, at their basal parts, with 0.5 g·L^{-1} K-IBA for rooting in the multi-position plastic discs, as described previously. The rooting substrate consisted of different mixtures of perlite and peat TS2 Klasmann (ratios of 1:0, 1:1, or 2:1 v/v, respectively). The shoot cuttings, planted in the multi-position plastic discs, were then placed for rooting on the bench of either the fog or the intermittent mist system. The temperature at the bottom of the benches of both rooting systems, close to the basal ends of the planted cuttings, was set at 21 ± 1 °C using electrical cables. The relative humidity in the fog system was adjusted to 94–96%, while in the intermittent mist system, the water was sprayed every 30 min, from 7:00 to 23:00, lasting 20 s in winter, 30 s in autumn and spring, and 40 s in summer. Forty shoot cuttings were used for every substrate and root system. At the end of a 5-week period, rooting (%), as well as number and length (cm) of roots, were recorded.

2.3.4. Statistical Analysis

The experiments of rooting were conducted in completely randomized designs. Forty cuttings (4 replications of 10 replicates each) per treatment were used. Analysis of variance (ANOVA) was employed for statistical analysis of the data. The rooting percentages were subjected to arcsine transformation, prior to statistical analysis, and were transformed back to percentages for presentation in figures [37]. The transformed data were checked for normality and homogeneity of variances, and then, they were analyzed using one-way ANOVA. The means were compared using the Duncan's multiple range test at $p \leq 0.05$ [38]. The statistical analysis was carried out using the statistical package SPSS 27 (IBM, Armonk, NY, USA).

2.4. Propagation In Vitro

2.4.1. Plant Material

Shoot tips (1–1.5 cm long), used as explants for the present study, were provided from 1-year-old plants of the two selected *R. officinalis* populations of 'Amaliada' and 'Piperia'. These plants, derived from shoot cuttings of the selected populations, were grown in the greenhouse in 2 L pots filled with a substrate of 3:1 (v/v) peat TS2 Klasmann and perlite.

2.4.2. Explant Disinfestation and Establishment of In Vitro Cultures

Twenty different treatments were applied and evaluated in order to choose the best one for explant disinfestation (Table 3). Explant surface disinfestations were conducted as follows: For the treatments 1–14, NaOCl inclusion in the aqueous solution varied in concentration and time duration of the application (0.15–0.60%, 3–30 min, respectively). For the procedures 15–19, 100 mg·L^{-1} of Polyvinyl-pyrrolidone (PVP) was applied, for 30 min, to the explants prior to their immersion into the NaOCl aqueous solution. For procedure 20, the explants were first soaked in a small amount of commercial detergent, with a few drops of Triton-X 100, and then, they were rinsed under running tap water for 45 min. Afterwards, a pre-treatment in a mixture of antioxidants (300 mg·L^{-1} ascorbic acid and 200 mg·L^{-1} citric acid) was applied to explants, for 16 min, prior to their final dipping into 0.6% (v/v) NaOCl for 7 min. All explants, after disinfestation, were rinsed three times by sterilized-distilled water and established, in vitro, on MS nutrient medium [39], supplemented with 2% (w/v) sucrose, 0.25% (w/v) activated charcoal, and 0.8% (w/v) agar (Technobiochem, Athens, Greece), in Magenta vessels (77 × 77 × 77 mm) (Sigma-Aldrich, St. Louis, MO, USA). From preliminary experiments, explants cultured on MS nutrient medium, but lacking activated charcoal, failed to grow, turned brown, and became necrotic; thus, in all MS nutrient media used, activated charcoal was added. The pH of the nutrient media was adjusted to 5.8, with 0.5N NaOH or 0.1N HCl, prior to agar inclusion. All in vitro cultures were maintained in a plant growth chamber with a photoperiod of 16 h,

provided by cool-white fluorescent lamps of a photosynthetic photon flux density (PPFD) of 50 μmol·m^{-2}·s^{-1}, at vessel level. The temperature in the growth chamber was set at 23 ± 0.1 °C. After a 2-week period, the effects of the different disinfestation treatments on explants' survival were assessed. For each disinfestation treatment, three replications, with nine explants each, were used.

Table 3. Disinfestation treatments applied to the explants of the two selected *R. officinalis* populations, 'Amaliada' and 'Piperia', and percentages of contamination-free explants.

Code Number	PVP *	Ascorbic and Citric Acid **	NaOCl (%)	Disinfestation Duration (min)	Contamination-Free Explants (%)
1	−	−	0.5	7	88.9 ab ***
2	−	−	0.625	8	88.9 ab
3	−	−	0.5	5	88.9 ab
4	−	−	0.625	5	100.0 a
5	−	−	0.3	7	100.0 a
6	−	−	0.3	5	0.0 f
7	−	−	0.3	3	0.0 f
8	−	−	0.4	4	0.0 f
9	−	−	0.4	3	22.2 e
10	−	−	0.4	4	0.0 f
11	−	−	0.3	5	0.0 f
12	−	−	0.3	6	44.4 cde
13	−	−	0.4	3	0.0 f
14	−	−	0.3	4	22.2 e
15	+	−	0.5	5	100.0 a
16	+	−	0.15	30	66.7 bc
17	+	−	0.15	20	55.6 cd
18	+	−	0.15	15	33.3 de
19	+	−	0.15	10	33.3 de
20	−	+	0.6	7	100.0 a

* PVP (Polyvinyl-pyrrolidone, 100 mg·L^{-1}). ** Ascorbic acid (300 mg·L^{-1}) and citric acid (200 mg·L^{-1}). *** Different letters in the column indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

2.4.3. Effect of Growth Regulators on Shoot Multiplication and Rooting

Uncontaminated explants of 'Amaliada' and 'Piperia' populations, from the previous stage, were subcultured on fresh MS nutrient medium supplemented with 2% (*w*/*v*) sucrose, 0.8% (*w*/*v*) agar, and 0.25% activated charcoal. The in vitro cultures were maintained in a plant growth chamber with the same conditions as described previously. Five different combinations of the plant growth regulators, BAP (Sigma-Aldrich, St. Louis, MI, USA) and IAA (Sigma-Aldrich, St. Louis, MI, USA), were tested in the nutrient media (MS1–MS5) for their effect on shoot multiplication, elongation, and rooting (Table 4). Multiplication of shoots was based on axillary branching, while spontaneous rooting occurred on the same nutrient media in almost all combinations of the plant growth regulators tested. After a 5-week period, shoot formation frequency (%), number and length (cm) of shoots formed, as well as rooting response (%) and length (cm) of roots were measured.

Table 4. Growth regulator combinations, in the MS nutrient media, for the in vitro cultures of *R. officinalis*.

Nutrient Medium	IAA (mg·L^{-1})	BAP (mg·L^{-1})
MS1	0	0
MS2	0.1	0
MS3	0.1	0.25
MS4	0.1	0.5
MS5	0.1	1

2.4.4. Plantlet Acclimatization

The in vitro rooted plantlets of the 'Amaliada' and 'Piperia' populations were taken out of the agar-solidified MS nutrient media, and, after thorough rinsing in water for the removal of any medium residues, they were transplanted in nursery trays filled with a 2:1 (*v*/*v*) mixture of peat and perlite. The trays with the plantlets were placed in the greenhouse under controlled environmental conditions. For acclimatization, the plantlets were initially kept under 95% relative humidity, which was progressively lowered to 75% after a 2-week period. The light irradiance gradually increased, approaching the conditions of the natural environment, while the ambient temperature was stable at 23 ± 2 °C. After four weeks, plantlet survival was evaluated for both the 'Amaliada' and 'Piperia' populations.

2.5. Statistical Analysis

The experiments were conducted using completely randomized designs. For each disinfestation treatment, three replications of nine explants were used. For both shoot multiplication and rooting in vitro, three replications of 12 explants per treatment were employed for each population. In the case of acclimatization, two replications of 20 plantlets, for each population, were assessed. The statistical analysis of the data was based on analysis of variance (ANOVA). Prior to statistical analysis, data expressed as percentages were subjected to arcsine transformation for proportions, and they were then transformed back to percentages in order to be presented in tables. All statistical analyses were conducted using the SPSS 23 software (SPSS Inc. Statistical Package for the Social Sciences, Chicago, IL, USA). The mean separations were carried out with Duncan's multiple range test at $p \leq 0.05$ [38].

3. Results and Discussion

3.1. Morphological Analysis of the Traits

From a macroscopic observation, on the plant branches of the seven populations, it is clear that there are differences in their morphological features (Figure 2). It is known that the traits "plant height" and "plant diameter" contribute to the shape of the shrub's canopy, and thus, they provide important information for the growth rate of the plants. For both traits, the population 'Piperia' had the highest values, and the population 'Chios' had the lowest (Figure 3A,B), while the same picture appeared for the "number of terminal branches per plant" (Figure 3C). Furthermore, the populations 'Piperia' and 'Amaliada' had the longest terminal branches, in contrast to the population of 'Ioannina', which ranked last on the "terminal branches length" trait (Figure 3D). For the "number of nodes per terminal branch" and "number of leaves per terminal branch", the 'Amaliada' population achieved the highest values (Figure 3E,F). In relation to "leaf length" and "leaf width", it appeared that the populations 'Piperia', 'Amaliada', and 'Variko' had the largest leaves, whereas the other four populations were found to bear the smallest leaves. According to the morphological analysis of the traits, it seems that there is a significant phenotypic variability among the selected rosemary populations of Greece. Based on the results of the phenotypic evaluation, of the populations of *R. officinalis*, the populations with the overall better morphological traits seemed to be 'Amaliada' and 'Piperia', and thus, they were chosen for the propagation experiments via shoot cuttings and tissue culture.

Figure 2. Terminal branches taken from the previous year vegetation, of the seven wild-grown *R. officinalis* populations, from the regions of Variko (V), Piperia (P), Amaliada (AM), Ioannina (I), Chios (CH), Archanes (AR), and Kavousi (KA).

R. officinalis is one of the most important species of Lamiaceae family, which appears to have interesting properties as an aromatic-medicinal plant, as well as a plant for ornamental use [40]. Carrubba et al. [41] has noticed that, contrary to other medicinal and aromatic plants, there is not an official descriptor list for the morphological traits of *R. officinalis* among the different regions in which it grows. The variability and the lack of characterization, of the different phenotypes of *R. officinalis*, have been acknowledged in previous studies in different regions of the Mediterranean basin [20,41–43]. Carrubba et al. [41] reported that the cultivators select the species phenotypes with the largest leaves, without including in their preferences, and prioritize the factor of production of secondary products by the rosemary plants, such as the aromatic oils. However, other researchers examined the connection between morphological traits of the plant and the products of their secondary metabolism [44–46].

Furthermore, the name of the *Rosmarinus* clade, which is derived from the words "ros" and "mare", meaning, in Latin, "drew" and "sea", indicates its capability of surviving near the sea [47]. This is confirmed from several studies, which examine the tolerance of *R. officinalis* plants to drought stress [48,49] and salt stress [50–52]. All of the above suggest that rosemary is a suitable landscaping choice for environments with high salinity and temperatures.

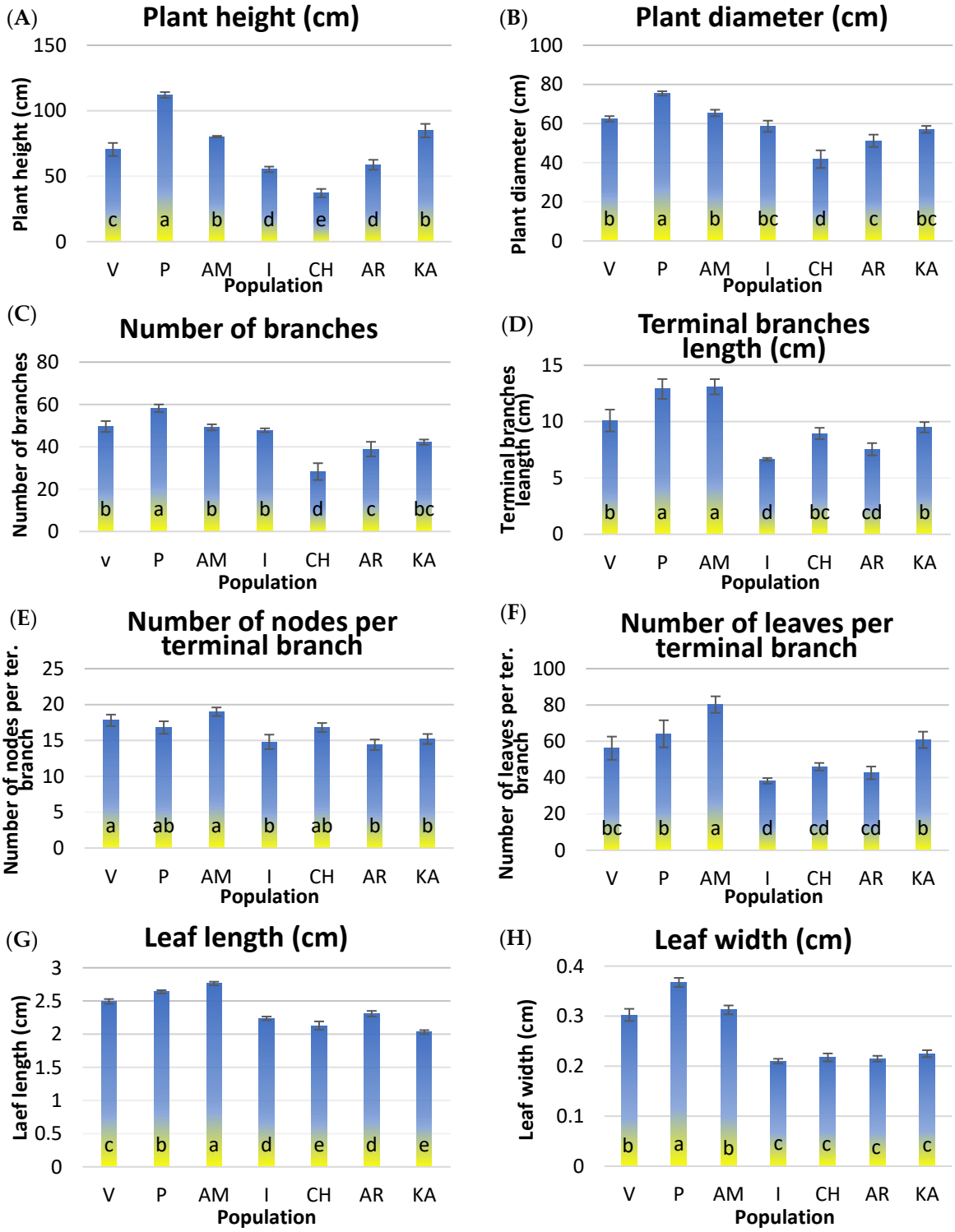

Figure 3. Mean values for eight morphological traits of ornamental use [(**A**) plant height (cm), (**B**) plant diameter (cm), (**C**) number of terminal branches per plant, (**D**) terminal branches length (cm), (**E**) number of nodes per branch, (**F**) number of leaves per terminal branch, (**G**) leaf length (cm), (**H**) leaf width (cm)] measured on the plants of the seven populations of *Rosmarinus officinalis*: 'Variko' (V), 'Piperia' (P), 'Amaliada' (AM), 'Ioannina' (I), 'Chios' (CH), 'Archanes' (AR), and 'Kavousi' (KA). Different letters, in columns for each of the traits, indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

3.2. Propagation by Shoot Cuttings

3.2.1. Effect of K-IBA and Season on Rooting of Cuttings

The rooting of *R. officinalis* cuttings was influenced by both K-IBA application and the season of the cuttings' collection (Figure 4). Thus, when K-IBA was applied, in most treatments, the rooting rate significantly increased, or even over doubled. Regardless, its concentration, as compared with the control, succeeded figures up to 82.5%. The concentration of 1 g·L^{-1} K-IBA was more effective on rooting than 0.5 g·L^{-1} in the winter collection of cuttings for both 'Piperia' and 'Amaliada' (Figure 4D), whereas for 'Amaliada', cuttings were less effective in spring (Figure 4A). In all other cases, both concentrations of K-IBA achieved similar percentages of rooting (Figure 4A–C). Furthermore, in spring, higher rooting percentage was noticed at 0.5 g·L^{-1} K-IBA, as compared with 1 g·L^{-1} (82.5% and 60%, respectively) in the 'Amaliada' population, while in the 'Piperia' population, no statistically significant difference was noticed (Figure 4A). Additionally, in autumn and summer, the rooting of cuttings was almost at the same range for both auxin concentrations tested (Figure 4B,C). Cuttings of 'Amaliada' rooted better in spring, with 0.5 g·L^{-1} K-IBA (82.5%), than in the other seasons, while cuttings of 'Piperia' achieved the highest rooting percentages in autumn, with either K-IBA concentration tested (80–82.5%). In all cases, the number of roots was significantly higher with the use of K-IBA than the control, except for 'Amaliada' in autumn, without differences between the two concentrations (Table 5). However, the root length was not increased with the use of K-IBA in spring, autumn, and winter, while in summer, the control cuttings produced longer roots in comparison to the 1 g·L^{-1} K-IBA treatment. No differences in root length were found between the concentrations of 0.5 and 1 g·L^{-1} K-IBA (Table 5).

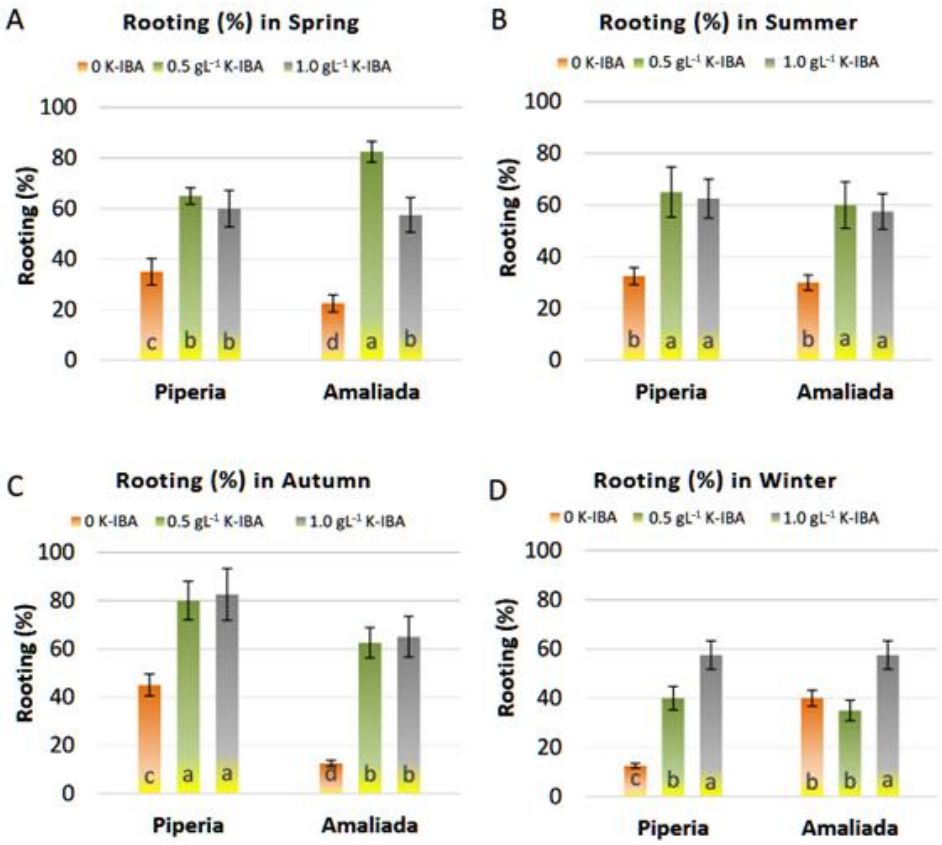

Figure 4. Effect of K-IBA and season [(**A**) spring, (**B**) summer, (**C**) autumn, and (**D**) winter] on the rooting (%) of *R. officinalis* 'Piperia' and 'Amaliada' shoot cuttings. Different letters, in columns for both populations in each season, indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

Table 5. Effect of K-IBA and season (spring, summer, autumn, and winter) on number and length (cm) of roots of *R. officinalis* 'Piperia' and 'Amaliada' shoot cuttings.

	Population	K-IBA g·L^{-1}	Spring	Summer	Autumn	Winter
Number of roots	'Piperia'	0	3.11 ± 0.51 * b **	3.35 ± 0.42 b	3.34 ± 0.59 b	2.77 ± 0.18 b
		0.5	5.73 ± 0.29 a	5.72 ± 0.38 a	5.94 ± 0.51 a	4.33 ± 0.19 a
		1	6.10 ± 1.10 a	6.28 ± 0.52 a	6.41 ± 0.31 a	4.75 ± 0.42 a
	'Amaliada'	0	2.79 ± 0.22 b	3.11 ± 0.25 b	5.18 ± 0.70 a	2.68 ± 0.37 b
		0.5	5.86 ± 0.47 a	5.95 ± 0.36 a	5.70 ± 0.55 a	4.54 ± 0.50 a
		1	6.07 ± 0.85 a	6.16 ± 0.68 a	6.69 ± 0.46 a	4.71 ± 0.46 a
Length of roots (cm)	'Piperia'	0	7.70 ± 0.61 a	8.19 ± 0.91 a	8.58 ± 0.68 a	5.91 ± 0.49 a
		0.5	7.60 ± 0.57 a	7.58 ± 0.58 ab	8.93 ± 0.75 a	5.57 ± 0.52 a
		1	7.17 ± 1.02 a	7.31 ± 0.64 b	7.77 ± 0.34 a	6.36 ± 0.47 a
	'Amaliada'	0	7.56 ± 0.93 a	7.95 ± 0.61 a	7.34 ± 0.55 a	6.18 ± 0.38 a
		0.5	7.44 ± 0.74 a	7.63 ± 0.69 ab	7.88 ± 0.28 a	5.42 ± 0.40 a
		1	8.21 ± 0.39 a	6.27 ± 0.45 b	8.51 ± 0.47 a	5.34 ± 0.51 a

* Standard Deviation. ** Means in columns, for number and length of roots, with different letters indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

Previous studies reported that rosemary shoot cuttings have a good rooting ability, and the application of plant growth regulators induces even greater rooting percentages and qualitative features of the new root system [18–21]. Shahhoseini et al. [22] reported that, at a concentration of 1 g·L^{-1} NAA, the rooting of rosemary cuttings reached 84%, while at 5 g·L^{-1} IBA, the rooting percentage was 66%. In the same trend, Abu-Zahra et al. [19] found that 3 g·L^{-1} of NAA triggered the highest rooting percentages on rosemary cuttings. In another report, IBA at only 5 mg·L^{-1} stimulated the rooting of rosemary cuttings up to 92% [20]. The degree of lignification, and the physiological status of shoot cuttings, vary among the four seasons, and these factors influence their rooting response differently. In this study, spring and autumn appeared to be more efficient for rooting, whereas Silva and Petras [26] reported that the end of winter was the best season for the rooting of rosemary cuttings.

3.2.2. Effect of Substrate and Rooting System on Rooting of Cuttings

Significant differences were found regarding the influence, of the type of substrate and the kind of rooting system, on the rooting percentage of shoot cuttings collected from 'Piperia' plants (Figure 5). Rooting in the fog system was higher than in the intermittent mist system when the substrate contained peat. Similar rooting percentages were achieved in fog and intermittent mist system when the substrate consisted of only perlite. The highest rooting of cuttings was observed on substrates of perlite and peat, in proportions of 2:1 and 1:1, in the fog system (85% and 75%, respectively) (Figure 5). There were no statistically significant differences in the number and length of the roots formed on the cuttings, among the three substrate formulations, for both fog and intermittent mist (Table 6). From the above, it appeared that shoot cuttings of *R. officinalis* rooted adequately on the substrate of perlite and peat (2:1, *v*/*v*) in the fog rooting system (Figure 6).

The substrate plays an important role in the rooting process because, apart from anchoring, it permits the aeration (i.e., oxygen) and provides the needed moisture at the bases of the cuttings, stimulating root formation in this way. Peat and perlite alone, or a combination of both in various proportions, are the main components of most commercial substrates used for the successful rooting of cuttings [23]. Varban et al. [24] found the mixture of perlite and peat, in a ratio of 1:1 (*v*/*v*), as the most effective substrate for the rooting of rosemary shoot cuttings.

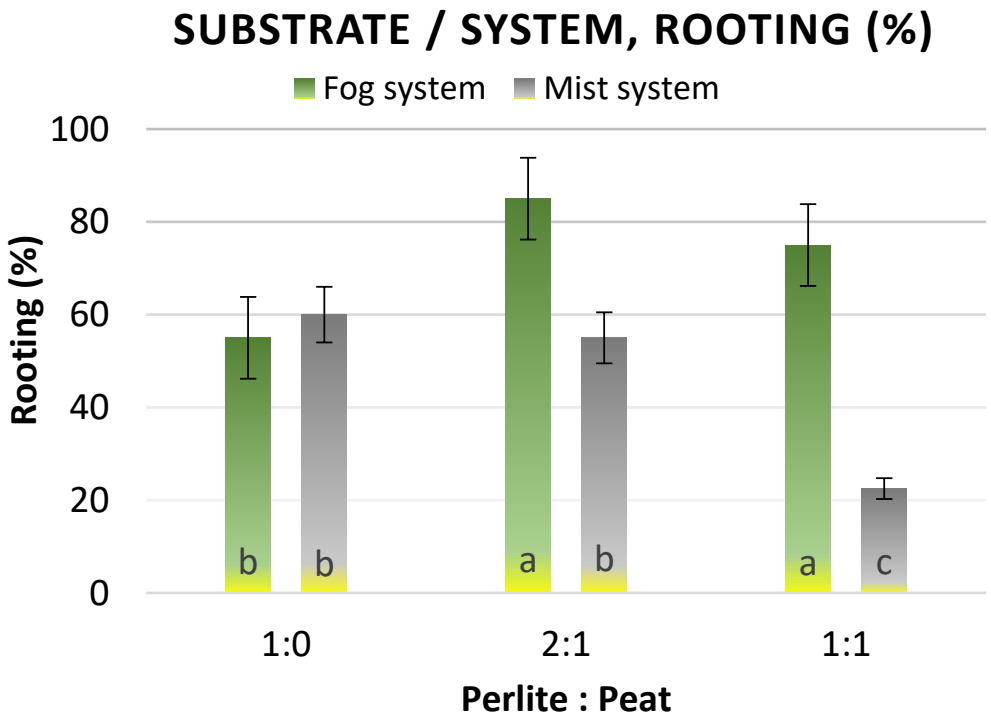

Figure 5. Effect of the rooting system and the substrate composition on the rooting (%) of *R. officinalis* 'Piperia' shoot cuttings. Different letters, in the columns of all substrates, indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

Table 6. Effect of the rooting system and the substrate composition on the number and length (cm) of roots of *R. officinalis* 'Piperia' shoot cuttings.

	Number of Roots		**Length of Roots (cm)**	
Perlite: Peat	**Fog**	**Mist**	**Fog**	**Mist**
1:0	5.74 a *	5.86 a	3.71 a	3.57 a
2:1	5.59 a	6.20 a	4.14 a	3.86 a
1:1	5.47 a	6.01 a	3.92 a	3.78 a

* Means in columns with different letters indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

3.3. Propagation In Vitro

3.3.1. Explant Disinfestation and Establishment of In Vitro Cultures

Disinfestation treatments with code numbers 4, 5, 15, and 20 were effective for removing surface contaminants (100%) from the explants of the two selected populations (Table 3). However, despite the effectiveness of disinfestation treatments No. 4, 5, and 15, some explants exhibited tissue browning, probably due to the high endogenous phenolic content of rosemary. Disinfestation treatment No. 20 was the most beneficial as, besides the optimal results in terms of explant survival (100% healthy), it also eliminated browning due to pretreatment with the aqueous solution of antioxidants (ascorbic and citric acid) (Table 3).

Figure 6. Planted cuttings of *R. officinalis* during rooting in the fog system: 'Piperia' cutting(s) (**A**) in substrate of perlite and peat (1:1, *v*/*v*) in plastic multi-position disks, (**B**) rooted in substrate of perlite and peat (1:1, *v*/*v*), (**C**) rooted in substrate of perlite and peat (2:1, *v*/*v*), and (**D**) 'Amaliada' cuttings in substrate of perlite and peat (2:1, *v*/*v*) in plastic multi-position disks.

The explants are often major sources of contaminants covering their surface or even appearing within the vascular system. Explant surfaces, covered with thick wax and epidermal trichomes, can host numerous microorganisms. Besides contamination, which often occurs in the tissue culture by various pathogens, another problem is browning by phenolic compounds of the culture, causing severe damage to the explants [53]. These substances tend to inhibit explant establishment and further development. The use of antioxidants (ascorbic and citric acid) in this study proved to be crucial in solving the browning problem of rosemary explants. By the same means, other researchers have successfully eliminated browning in their cultures during surface sterilization of the explants, such as: El-Zefzafy et al. [34] for *R. officinalis* and Tsoulpha et al. [54] for *Pyrus spinosa*. Sakr et al. [55], by using antioxidants in the form of lemon juice, in combination with H_2O_2, obtained high disinfestation rates (90–100%) for three rosemary cultivars.

3.3.2. Effect of Plant Growth Regulators on Shoot Multiplication and Rooting

The culture medium and its content in plant growth regulators was a determining factor for the process of the in vitro cultures in this study (Figure 7). In particular, the frequency and the rate of regenerated shoots in vitro varied between the two rosemary populations and the five different combinations of BAP and IAA used. In the cultures of 'Amaliada', shoot formation frequency ranged from 50.0–91.6%, and the number of shoots formed was 0.92–2.0, while for 'Piperia', shoot formation frequency was 8.33-66.6%, and the number of shoots formed was 0.08–0.67 per explant for all the media (MS1-MS5) tested (Table 7). The cultures of the 'Amaliada' population exhibited the highest shoot formation frequency (91.6%) and the greatest number of new shoots per explant (2.0) on

the MS medium without plant growth regulators (control). Regarding the length of new shoots formed, it ranged from 0.68 to 0.94 cm for 'Amaliada', and for 'Piperia', it ranged from 0.35 to 1.26 cm. Cultures of the 'Piperia' population were not as prolific as 'Amaliada' population, since shoot formation frequency reached up to 66.6%, and the number of new shoots was only 0.67 on MS3 and MS4 media. In general, the length of the new shoots produced was less than 1 cm, although on the MS4 medium, the shoot length was 0.94 cm for 'Amaliada' and 1.26 cm for the 'Piperia' population (Table 7).

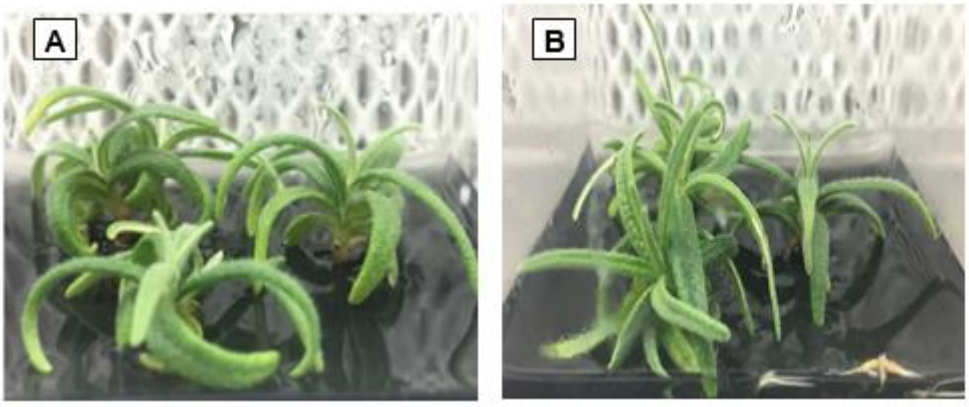

Figure 7. In vitro cultures of *R. officinalis*, after 5 weeks from establishment, for (**A**) 'Amaliada' on MS1 and (**B**) 'Piperia' on MS4.

Table 7. Effect of plant growth regulators on shoot and root formation in in vitro cultures of 'Amaliada' and 'Piperia' populations, after five weeks of culture.

Media	Population	Shoot Formation Frequency (%)	Number of Shoots	Length of Shoots (cm)	Root Formation Frequency (%)	Length of Roots (cm)
MS1	'Amaliada'	91.6 ± 14.4 * a **	2.00 ± 0.21 a	0.68 ± 0.04 e	16.6 ± 14.4 b	6.25 ± 0.75 ab
	'Piperia'	33.3 ± 14.4 d	0.33 ± 0.14 e	1.08 ± 0.19 ab	25.0 ± 25.0 ab	2.50 ± 0.29 f
MS2	'Amaliada'	66.6 ± 14.4 bc	1.50 ± 0.42 ab	0.91 ± 0.08 bc	41.6 ± 14.4 ab	2.80 ± 0.21 ef
	'Piperia'	8.3 ± 14.4 e	0.08 ± 0.08 f	0.35 ± 0.15 f	0 c	0 g
MS3	'Amaliada'	75.0 ± 25.0 ab	1.25 ± 0.28 bc	0.79 ± 0.07 de	33.3 ± 14.4 ab	3.85 ± 0.72 d
	'Piperia'	66.6 ± 14.4 bc	0.67 ± 0.14 cd	0.93 ± 0.16 bc	8.3 ± 14.4 bc	3.75 ± 0.75 de
MS4	'Amaliada'	58.3 ± 14.4 bc	1.08 ± 0.31 bc	0.94 ± 0.05 bc	50.0 ± 25.0 a	7.08 ± 0.71 a
	'Piperia'	66.6 ± 14.4 bc	0.67 ± 0.14 cd	1.26 ± 0.18 a	41.6 ± 14.4 ab	5.30 ± 0.54 bc
MS5	'Amaliada'	50.0 ± 25.0 cd	0.92 ± 0.36 bcd	0.85 ± 0.08 cd	25.0 ± 25.0 ab	4.00 ± 0.58 d
	'Piperia'	58.3 ± 14.4 bc	0.58 ± 0.15 de	0.83 ± 0.05 cd	16.6 ± 14.4 b	4.25 ± 0.75 cd

* Standard Deviation. ** Means in columns, for the same measurement, with different letters indicate statistically significant differences, according to Duncan's multiple range test at $p \leq 0.05$.

It is worth it to emphasize the incorporation of activated charcoal into all tested nutrient media and its essential role for obtaining healthy rosemary explants and phenolics-free cultures. This, according to the literature, is mainly related to activated charcoal's ability to absorb phenolic compounds, released by the explants, to the nutrient medium and, thus, reducing their oxidation and browning, leading, finally, to greater culture survival rates [56]. The beneficial addition of activated charcoal, in various concentrations (0.5–4.0 $g \cdot L^{-1}$), in the nutrient medium of other Lamiaceae family plants has been reported by many researchers [57–60]. Moreover, Pan and Staden [61], as well as Buckseth [62], emphasize, in their reviews, the important role of activated charcoal for cell growth and development in all aspects of in vitro plant cultures.

The studied populations of *R. officinalis* showed differences in shoot multiplication and development on the tested growth regulator combinations and control treatments. In partic-

ular, increased new shoot formation and elongation was promoted in the absence of growth regulators for the 'Amaliada' population. Mascarello et al. [63] have also reported that, even without plant growth regulators, seedlings of *R. officinalis* showed a decent multiplication capacity and shoot growth in vitro. On the other hand, the combination of 0.5 mg·L^{-1} BAP and 0.1 mg·L^{-1} IAA (MS4) was the best for 'Piperia' cultures. Likewise, for rosemary cultures, Husain and Jawad [64] found high production of new shoots with the same combination of growth regulators in vitro. The effect of genotype of the two populations is obviously responsible for the different responses of their cultures in vitro.

Similarly to our work, with regard to the micropropagation of other Lamiaceae plants, Singh and Sehga [65] proposed the addition of IAA and BAP in the culture medium of holy basil (*Ocimum sanctum*) in order to achieve a significant increase in the production of new shoots. Grzegorczyk et al. [66], investigating the in vitro culture of *Salvia officinalis*, also reported the formation of an average of three new shoots per each initial explant, on the MS nutrient medium with 0.1 mg·L^{-1} IAA and 0.45 mg·L^{-1} BAP, within 5 weeks. Combinations of BAP, with NAA or IBA, were effective for shoot multiplication of *Ocimum basilicum* and *Perovskia abrotanoides*, which both belong to the Lamiaceae family as well [67,68].

Spontaneous formation of roots occurred on the rosemary shoot cultures, in vitro, in almost all the tested nutrient media. In particular, for cultures of 'Amaliada' root, frequency ranged between 16.6 and 50%, and for 'Piperia' it ranged between 8.3 and 41.6% on all nutrient media except that of MS2 (0%) (Table 7). The combination of 0.5 mg·L^{-1} BAP and 0.1 mg·L^{-1} IAA (MS4) led to the highest root formation frequency and the longest roots for both populations (50%, 7.08 cm for 'Amaliada' and 41.6%, 5.30 cm for 'Piperia') (Table 7, Figure 8).

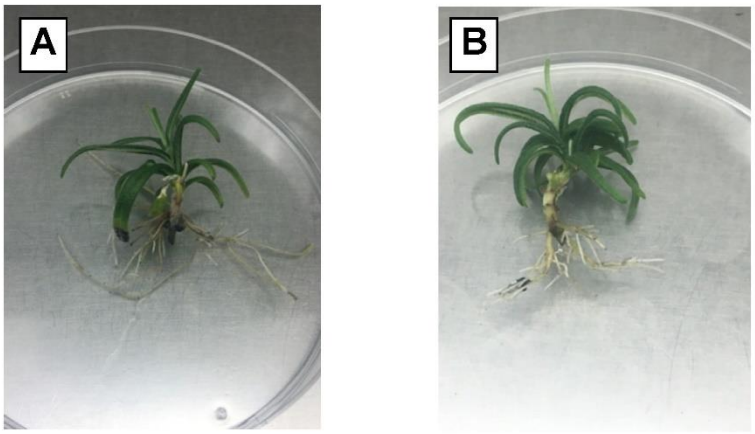

Figure 8. 'Amaliada' (**A**) and 'Piperia' (**B**) plantlets with developed roots, after 5 weeks in MS nutrient medium, supplemented with 0.5 mg·L^{-1} BAP and 0.1 mg·L^{-1} IAA.

Cultures of both populations formed roots promptly in almost all media, including control, exhibiting adequate rooting ability and a well-formed rooting system. This surprising phenomenon, besides the labor and cost saving procedure, provided us with the alternative of developing a simple and functional micropropagation protocol, for rosemary, consisting of only two in vitro steps. Rosemary is a relatively easy species to root in vitro. According to the research of Sakr et al. [55], two rosemary cultivars readily rooted in vitro by simply transferring the explants from a cytokinin-containing nutrient medium to a cytokinin-free MS nutrient medium. Similarly, Mascarello et al. [63], using in vitro germinated rosemary seeds as starting material, reported 50% rooting on nutrient medium without plant growth regulators, while, with the addition of 0.5 or 1 mg·L^{-1} IAA in the nutrient medium, rooting reached 75 and 78.6%, respectively. However, Dong et al. [30], starting from rosemary leaves, found that MS nutrient medium containing 0.1 mg·L^{-1} NAA promoted rooting at 65%.

In this study, the addition of activated charcoal was also an essential factor for the rooting of cultures of both rosemary populations in vitro. The positive effect of activated charcoal on rooting was also demonstrated for conifers, which are generally considered as species that are difficult to root, such as *Pinus pinaster* [69]. Tien Vinh et al. [57] concluded that 1 $g \cdot L^{-1}$ of activated charcoal is the most effective concentration to achieve the maximum number and length of shoots, as well as roots, in vitro in lavender cultures.

3.3.3. Plantlet Acclimatization

At the end of the 4-week period, the acclimatization of the in vitro rooted plantlets, to ex vitro conditions, succeeded a 70% survival rate (data not shown), and the young plantlets of both 'Amaliada' and 'Piperia' populations presented healthy appearance and normal characteristics (Figure 9). Afterwards, the young rosemary plantlets continued their growth and development in the greenhouse under natural environmental conditions (Figure 10). Chaturvedi et al. [29] reported 60% survival rate of in vitro rooted rosemary shoots, which were transplanted directly to sterilized soil in pots, while Husain and Jawad [64] obtained 90% survival rate for acclimatized rosemary plantlets.

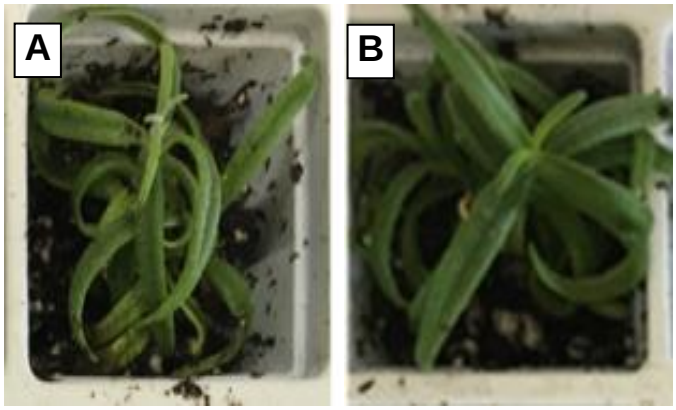

Figure 9. Acclimatized plantlets of 'Amaliada' (**A**) and 'Piperia' (**B**) at the end of the 4-week acclimatization period.

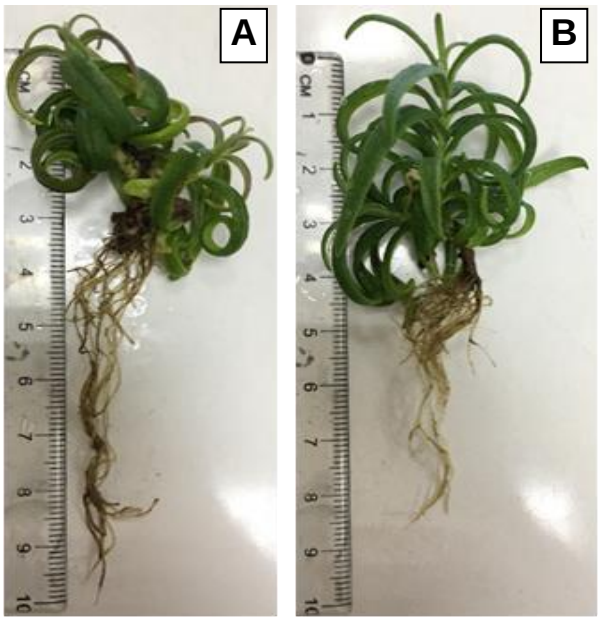

Figure 10. Bare-root plantlets of 'Amaliada' (**A**) and 'Piperia' (**B**), one week after the acclimatization period.

Acclimatization of in vitro-produced plantlets is a stressful process of micropropagation, and it requires gradual adaptation to the external environment and transition of the root system from a non-functional structure to a functional one [70]. Gradual reduction in the ambient relative humidity and, at the same time, gradual increase in the light irradiance greatly contributes to plantlet hardening towards a successful acclimatization. Implementation of this procedure, to the young rosemary plantlets of both populations, resulted in liming the losses.

4. Conclusions

The findings of this study could be useful in an effort of using, in a sustainable way, native rosemary populations with desirable traits, as the primary plant material for the local market, through efficient propagation methods. Plants from the selected populations, 'Amaliada' and 'Piperia', can be produced easily from the shoot cuttings rooted, with the application of 0.5–1 g·L^{-1} K-IBA, on a substrate of perlite and peat (2:1, *v*/*v*) in the fog system in spring or autumn. In addition to cutting propagation, the micropropagation protocol developed in this study can provide abundant propagation material to growers. In the present micropropagation protocol, the use of antioxidants and activated charcoal play key roles in avoiding explant browning, due to oxidation and the inclusion of 0.25 or 0.5 mg·L^{-1} BAP and 0.1 mg·L^{-1} IAA on the MS nutrient medium, for shoot formation. Furthermore, spontaneous rooting occurs on this nutrient medium, and the young plantlets, after being transplanted to soilless substrate, can be adequately acclimatized in the greenhouse. Hence, this two stage micropropagation protocol is simple, economic, and functional in producing great numbers of rosemary plantlets.

Author Contributions: Conceptualization, S.K., S.H., A.E., I.N.-O. and A.K.K.; investigation, S.K., A.K., E.K., A.-A.K., E.G., S.H. and I.N.-O.; resources, S.K., A.K., E.K., A.-A.K., S.H. and A.K.K.; methodology, S.K., A.K., E.K., A.-A.K., E.G., S.H., A.E., P.T. and I.N.-O.; visualization, S.K., I.N.-O., S.H., A.E. and A.K.K.; data curation, A.K., E.K., A.-A.K. and E.G.; writing-original draft preparation, S.K., A.K., P.T., S.H. and I.N.-O.; writing-review and editing, A.E., S.K., P.T., I.N.-O. and S.H.; funding acquisition, I.N.-O.; project administration, S.K. and A.K.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Programme Competitiveness, Entrepreneurship and Innovation, under the call "RESEARCH-CREATE-INNOVATE" (Project code: T1EDK-03919).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets in this paper are available from the corresponding authors on reasonable request.

Acknowledgments: Thanks to the anonymous reviewers for their helpful comments and suggestions.

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

References

1. González-Minero, F.J.; Bravo-Díaz, L.; Ayala-Gómez, A. *Rosmarinus officinalis* L. (Rosemary): An ancient plant with uses in personal healthcare and cosmetics. *Cosmetics* **2020**, *7*, 77. [CrossRef]
2. Hammer, M.; Junghanns, W. Rosmarinus officinalis L.: Rosemary. In *Medicinal, Aromatic and Stimulant Plants*, 1st ed.; Novak, J., Blüthner, W.-D., Eds.; Springer: Cham, Switzerland, 2020; Volume 12, pp. 501–521. [CrossRef]
3. Kiuru, P.; Muriuki, S.J.N.; Wepukhulu, S.B.; Muriuki, S.J.M. Influence of growth media and regulators on vegetative propagation of rosemary (*Rosmarinus officinalis* L.). *East Afr. Agric. For. J.* **2015**, *81*, 105–111. [CrossRef]
4. Mehrabani, V.L.; Kamran, R.V.; Hassanpouraghdam, M.B.; Kavousi, E.; Aazami, M.A. Auxin concentration and sampling time affect rooting of *Chrysanthemum morifolium* L. and *Rosmarinus officinalis* L. *Azar. J. Agric.* **2016**, *3*, 11–16. Available online: http://azarianjournals.ir/wp-content/uploads/aja15121402.pdf (accessed on 2 February 2022).

5. German, T.; Mekonnen, M.; Mengesha, M.; Philiphos, M. *Rosemary Production and Utilization*, 1st ed.; Ethiopian Institute of Agricultural Research: Addis Ababa, Ethiopia, 2003; p. 27. Available online: https://www.researchgate.net/publication/313030290_Cultivation_Processing_and_Utilization_of_Rosemary_Rosemarinus_officinalis_L (accessed on 15 February 2022).
6. Andrade, J.M.; Faustino, C.; Garcia, C.; Ladeiras, D.; Reis, C.P.; Rijo, P. *Rosmarinus officinalis* L.: An update review of its phytochemistry and biological activity. *Future Sci. OA* **2018**, *4*, FSO283. [CrossRef] [PubMed]
7. Carrubba, A.; La Torre, R.; Piccaglia, R.; Grandi, S. Chemical and botanical characterization of a *Rosmarinus officinalis* biotype from Sicily. *Acta Hortic.* **2006**, *723*, 197–202. [CrossRef]
8. De Baets, S.; Poesen, J.; Knapen, A.; Barberá, G.G.; Navarro, J.A. Root characteristics of representative Mediterranean plant species and their erosion-reducing potential during concentrated runoff. *Plant Soil* **2007**, *294*, 169–183. [CrossRef]
9. De Mastro, G.; Ruta, C.; Mincione, A.; Poiana, M. Bio-morphological and chemical characterization of rosemary (*Rosmarinus officinalis* L.) biotypes. *Acta Hortic.* **2004**, *629*, 471–482. [CrossRef]
10. Lagouri, V.; Alexandri, G. Antioxidant properties of Greek *O. dictamnus* and *R. officinalis* methanol—HPLC determination of phenolic acids. *Int. J. Food Prop.* **2013**, *16*, 549–562. [CrossRef]
11. Malvezzi, L.; Mendes, E.; Militao, L.; Tundisi, L.L.; Ataide, J.A.; Souto, E.B.; Mazzola, P.G. Rosemary (*Rosmarinus officinalis* L., syn *Salvia rosmarinus* Spenn.) and its topical applications: A review. *Plants* **2020**, *9*, 651. [CrossRef]
12. Nunziata, A.; Cervelli, C.; De Benedetti, L. Genotype confidence percentage of SSR HRM profiles as a measure of genetic similarity in *Rosmarinus officinalis*. *Plant Gene* **2018**, *14*, 64–68. [CrossRef]
13. Ganopoulos, I.V.; Kazantzis, K.; Chatzicharisis, I.; Karayiannis, I.; Tsaftaris, A.S. Genetic diversity, structure and fruit trait associations in Greek sweet cherry cultivars using microsatellite based (SSR/ISSR) and morpho-physiological markers. *Euphytica* **2011**, *181*, 237–251. [CrossRef]
14. Sarrou, E.; Martens, S.; Chatzopoulou, P. Metabolite profiling and antioxidative activity of Sage (Salvia fruticosa Mill.) under the influence of genotype and harvesting period. *Ind. Crops Prod.* **2016**, *94*, 240–250. [CrossRef]
15. Motta, E.V.; Sampaio, B.L.; Costa, J.C.; Teixeira, S.P.; Bastos, J.K. Quantitative analysis of phenolic metabolites in *Copaifera langsdorffii* leaves from plants of different geographic origins cultivated under the same environmental conditions. *Phytochem. Anal.* **2019**, *30*, 364–372. [CrossRef] [PubMed]
16. Paradiković, N.; Zeljković, S.; Tkalec, M.; Vinković, T.; Dervić, I.; Marić, M. Influence of rooting powder on propagation of sage (*Salvia officinalis*) and rosemary (*Rormarinus officinalis*) with green cuttings. *Poljoprivreda* **2013**, *19*, 10–15.
17. Elhaak, M.A.; Matter, M.Z.; Zayed, M.A.; Gad, D.A. Propagation principles in using Indole-3-butyric acid for rooting rosemary stem cuttings. *J. Hortic.* **2015**, *2*, 121. [CrossRef]
18. Talia, M.A.C.; Viola, F.; Forleo, L.R. Vegetative propagation of two species of the Mediterranean maquis (*Rosmarinus officinalis* L., *Viburnum tinus* L.) for applications in naturalistic engineering. *Italus Hortus* **2004**, *11*, 89–92.
19. Abu-Zahra, T.R.; Al-Shadaideh, A.N.; Abubaker, S.; Qrunfleh, I. Influence of auxin concentrations on different ornamental plants. *Int. J. Bot.* **2013**, *9*, 96–99. [CrossRef]
20. Koleva-Gudeva, L.; Trajkova, F.; Mihajlov, L.; Troiciki, J. Influence of different auxins on rooting of rosemary, sage and elderberry. *Annu. Res. Rev. Biol.* **2017**, *12*, 1–8. [CrossRef]
21. Lasheen, F.F.; Hewidy, M.; Emam, O.N.K. Impact of rooting promoters (PHOS ROOT-X) with or without using IBA on rooting of terminal and middle cuttings of rosemary (*Rosmarinus officinalis* L.). *Scient. J. Flowers Ornam. Plants* **2021**, *8*, 153–163. [CrossRef]
22. Shahhoseini, R.; Moghaddam, M.; Kiani, D.; Mansori, R. Effect of different concentrations of IBA and NAA on rooting of semi-hardwood cuttings of rosemary (*Rosmarinus officinalis* L.). *Iran. J. Med. Arom. Plants* **2015**, *31*, 574–585.
23. Davies, F.; Geneve, R.; Wilson, S.; Hartmann, H.; Kester, D. *Hartmann and Kester's Plant Propagation: Principles and Practices*, 9th ed.; Pearson Prentice Hall: Upper Saddle River, NJ, USA, 2017; p. 915.
24. Varban, D.; Varban, R.; Ghete, A.; Odagiu, A.; Czako, R. The influence of some products on the rooting of *Rosmarinus officinalis* L. cuttings. *Hop. Med. Plants* **2018**, *26*, 94–100.
25. Mendoza-Hernandez, D.; Fornes, F.; Belda, R. Compost and vermicompost of horticultural waste as substrates for cutting rooting and growth of rosemary. *Sci. Hortic.* **2014**, *178*, 192–202. [CrossRef]
26. Silva, C.; Pedras, J. Early rooting in rosemary's (*Rosmarinus officinalis* L.) cuttings under the influence of chemical treatments and collecting time. *Acta Hortic.* **1999**, *502*, 34. [CrossRef]
27. Souza, D.M.S.C.; Fernandes, S.B.; Molinari, L.V.; Avelar, M.L.M.; Brondani, G.E. Activated charcoal application for the micropropagation of *Cattleya crispata* (Thunb.) Van den Berg. *Nativa* **2021**, *9*, 352–358. [CrossRef]
28. Grigoriadou, K.; Krigas, N.; Sarropoulou, V.; Papanastasi, K.; Tsoktouridis, G.; Maloupa, E. *In vitro* propagation of medicinal and aromatic plants: The case of selected Greek species with conservation priority. *Vitr. Cell. Dev. Biol. Plant* **2019**, *55*, 635–646. [CrossRef]
29. Chaturvedi, H.C.; Misra, P.; Sharma, M. *In vitro* multiplication of *Rosmarinus officinalis* L. *Zeitschr. Pflanzenphys.* **1984**, *113*, 301–304. [CrossRef]
30. Dong, Y.; Wang, R.; Li, Z.; Qi, C.; Liu, B.; Duan, R.; Liu, Y. Callus induction and plant regeneration from rosemary leaves. *Biosci. Methods* **2012**, *3*, 21–26. [CrossRef]
31. Boix, Y.F.; Arruda, R.C.O.; Defaveri, A.C.A.; Sato, A.; Lage, C.L.S.; Victorio, C.P. Callus in *Rosmarinus officinalis* L. (Lamiaceae): A morphoanatomical, histochemical and volatile analysis. *Plant Biosyst.* **2013**, *147*, 751–757. [CrossRef]

32. Aman, N.; Afrasiab, H. Primary and secondary somatic embryogenesis from leaf explants of rosemary (*Rosmarinus officinalis* L.-Lamiaceae). *Pakist. J. Bot.* **2014**, *46*, 903–909.
33. Al Masoody, M.M.M.; Stanica, F. Effect of growth regulators on in vitro callus formation of rosemary plant (*Rosmarinus officinalis* L.). *Bull. UASVM Hortic.* **2015**, *72*, 131–137. [CrossRef]
34. El-Zefzafy, M.; Dawoud, G.; Shahhat, I. Physiological and phytochemical responses of rosemary (*Rosmarinus officinalis* L.) plant on in vitro callus formation. *Eur. J. Med. Plants* **2016**, *17*, 1–16. [CrossRef]
35. Irum, S.; Khan, F.A.; Erum, S.; Nouroz, F.; Muhammad, A.; Kanwal, S. *In vitro* propagation of selected medicinal plants species. *Asian J. Agric. Rural Dev.* **2017**, *7*, 40–47. [CrossRef]
36. Darwesh, H.Y.; Alayafi, A.A. *In virto* propagation response of *Rosmarinus officinalis* L. to biotic and abiotic elicitors on phenolic content and photosynthetic pigments. *J. Agric. Sci.* **2018**, *10*, 301. [CrossRef]
37. Snedecor, G.W.; Cochran, W.G. *Statistical Methods*, 7th ed.; The Iowa State University Press: Ames, IA, USA, 1980; p. 507.
38. Klockars, A.J.; Sax, G. Post hoc comparisons: The Scheffe test. In *Multiple Comparisons*; Klockars, A.J., Sax, G., Eds.; Sage Publications: Beverly Hills, CA, USA, 1986; Volume 61, pp. 58–61.
39. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
40. Zhao, F.; Chen, Y.P.; Salmaki, Y.; Drew, B.T.; Wilson, T.C.; Scheen, A.C.; Celep, F.; Bräuchler, C.; Bendiksby, M.; Wang, Q.; et al. An updated tribal classification of Lamiaceae based on plastomephylogenomics. *BMC Biol.* **2021**, *19*, 1–27. [CrossRef]
41. Carrubba, A.; Abbate, L.; Sarno, M.; Sunseri, F.; Mauceri, A.; Lupini, A.; Mercati, F. Characterization of Sicilian rosemary (*Rosmarinus officinalis* L.) germplasm through a multidisciplinary approach. *Planta* **2020**, *251*, 37. [CrossRef]
42. Herrera, J. Flower size variation in *Rosmarinus officinalis*: Individuals, populations and habitats. *Ann. Bot.* **2005**, *95*, 431–437. [CrossRef]
43. Li, G.; Cervelli, C.; Ruffoni, B.; Shachter, A.; Dudai, N. Volatile diversity in wild populations of rosemary (*Rosmarinus officinalis* L.) from the Tyrrhenian Sea vicinity cultivated under homogeneous environmental conditions. *Ind. Crops Prod.* **2016**, *84*, 381–390. [CrossRef]
44. Del Baño, M.J.; Lorente, J.; Castillo, J.; Benavente-García, O.; Del Río, J.A.; Ortuño, A.; Quirin, K.W.; Gerard, D. Phenolic diterpenes, flavones, and rosmarinic acid distribution during the development of leaves, flowers, stems, and roots of *Rosmarinus officinalis*. Antioxidant activity. *J. Agric. Food Chem.* **2003**, *51*, 4247–4253. [CrossRef]
45. Martín, J.P.; Bermejo, J.E.H. Genetic variation in the endemic and endangered *Rosmarinus tomentosus* Huber-Morath & amp; Maire (Labiatae) using RAPD markers. *Heredity* **2000**, *85*, 434–443. [CrossRef]
46. Zaouali, Y.; Bouzaine, T.; Boussaid, M. Essential oils composition in two *Rosmarinus officinalis* L. varieties and incidence for antimicrobial and antioxidant activities. *Food Chem. Toxicol.* **2010**, *48*, 3144–3152. [CrossRef] [PubMed]
47. Room, A. *A Dictionary of True Etymologies*, 1st ed.; Routledge (Taylor & Francis Group): New York, NY, USA, 1988; p. 198.
48. Munné-Bosch, S.; Lopez-Carbonell, M.; Alegre, L.; Van Onckelen, H.A. Effect of drought and high solar radiation on 1-aminocyclopropane-1-carboxylic acid and abscisic acid concentrations in *Rosmarinus officinalis* plants. *Physiol. Plant.* **2002**, *114*, 380–386. [CrossRef] [PubMed]
49. Olmos, E.; Sánchez-Blanco, M.J.; Ferréndez, T.; Alarcón, J.J. Subcellular effects of drought stress in *Rosmarinus officinalis*. *Plant Biol.* **2007**, *9*, 77–84. [CrossRef] [PubMed]
50. Alarcon, J.; Morales, M.; Ferrandez, T.; Sanchez-Blanco, M. Effects of water and salt stresses on growth, water relations and gas exchange in *Rosmarinus officinalis*. *J. Hortic. Sci. Biotechnol.* **2006**, *81*, 845–853. [CrossRef]
51. Nogués, I.; Muzzini, V.; Loreto, F.; Bustamante, M. Drought and soil amendment effects on monoterpene emission in rosemary plants. *Sci. Total Environ.* **2015**, *538*, 768–778. [CrossRef]
52. Toscano, S.; Ferrante, A.; Romano, D. Response of Mediterranean ornamental plants to drought stress. *Horticulturae* **2019**, *5*, 6. [CrossRef]
53. Cassells, A.C. Problems in tissue culture: Culture contamination. In *Micropropagation*; Springer: Dordrecht, The Netherlands, 1991; pp. 31–44. [CrossRef]
54. Tsoulpha, P.; Alexandri, S.; Tsaktsira, M. Critical factors affecting an efficient micropropagation protocol for *Pyrus spinosa* Forskk. *J. Appl. Hortic.* **2018**, *20*, 190–195. [CrossRef]
55. Sakr, S.S.; Amin, A.Y.; El-Mewafy, E.A.; Eid, N.M. *In vitro* comparative study on *Rosmarinus officinalis* L. cultivars. *Middle East J. Agric. Res.* **2018**, *7*, 703–715.
56. Thomas, T.D. The role of activated charcoal in plant tissue culture. *Biotechnol. Adv.* **2008**, *26*, 618–631. [CrossRef]
57. Tien Vinh, D.; Hoa, M.T.; Khai, P.C.; Van Minh, T. Micropropagation of lavender (*Lavandula angustifolia*). *J. Innov. Pharm. Biol. Sci.* **2017**, *4*, 7–11.
58. Otroshy, M.; Moradi, K. Micropropagation of medicinal plant *Dracocephalum kotschyi* Boiss. via nodal cutting technique. *J. Med. Plant Res.* **2011**, *5*, 5967–5972. [CrossRef]
59. Van Tan, P. Micropropagation of *Curcuma* sp., a threatened medicinal plant. *Adv. Biosci. Biotechnol.* **2016**, *7*, 418. [CrossRef]
60. Kereša, S.; Andrijanić, Z.; Kremer, D.; HabušJerčić, I.; Barić, M.; BateljaLodeta, K.; Bolarić, S.; Bošnjak-Mihovilović, A. Efficient micropropagation and rooting of *Micromeria croatica* (Pers.) Schott (Lamiaceae). *Poljoprivreda* **2018**, *24*, 27–33. [CrossRef]
61. Pan, M.J.; Staden, J.V. The use of charcoal in *in vitro* culture—A review. *Plant Growth Regul.* **1998**, *26*, 155–163. [CrossRef]
62. Buckseth, T.; Singh, R.K.; Sharma, A.K.; Sharma, S.; Moudgil, V.; Saraswati, A. Optimization of activated charcoal on *in vitro* growth and development of potato (*Solanum tuberosum* L.). *Int. J. Curr. Microbiol. Appl. Sci.* **2018**, *7*, 3543–3548. [CrossRef]

63. Mascarello, C.; Sacco, E.; Pamato, M.; Di Silvestro, D.; Bassolino, L.; Cervelli, C.; Ruffoni, B. *Rosmarinus officinalis* L.: Micropropagation and callus induction for cell biomass development. *Acta Hortic.* **2017**, *1155*, 631–636. [CrossRef]
64. Husain, Z.M.A.; Jawad, L.K. Effect of some growth regulators on the multiplication and stimulating the production of the volatile oil of *Rosemary officinalis* in vitro. *Plant Arch.* **2019**, *19*, 1773–1782.
65. Singh, N.K.; Sehgal, C.B. Micropropagation of 'Holy Basil' (*Ocimum sanctum* Linn.) from young inflorescences of mature plants. *Plant Growth Regul.* **1999**, *29*, 161–166. [CrossRef]
66. Grzegorczyk, I.; Bilichowski, I.; Mikiciuk-Olasik, E.; Wysokińska, H. In vitro cultures of *Salvia officinalis* L. as a source of antioxidant compounds. *Acta Soc. Bot. Pol.* **2005**, *74*, 17–21. [CrossRef]
67. Dode, L.B.; Bobrowski, V.L.; Braga, E.J.B.; Seixas, F.K.; Schuch, M.W. In vitro propagation of *Ocimum basilicum* L. (Lamiaceae). *Acta Sci. Biol. Sci.* **2003**, *25*, 435–437. [CrossRef]
68. Ghaderi, S.; Ebrahimi, S.N.; Ahadi, H.; Moghadam, S.E.; Mirjalili, M.H. In vitro propagation and phytochemical assessment of *Perovskia abrotanoides* Karel. *(Lamiaceae)—A medicinally important source of phenolic compounds. Biocatal. Agric. Biotechnol.* **2019**, *19*, 101–113.
69. Dumas, E.; Monteuuis, O. *In vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants: Influence of activated charcoal. *Plant Cell Tissue Organ Cult.* **1995**, *40*, 231–235. [CrossRef]
70. Economou, A.S. From macrocutting rooting to microplant establishment: Key points to consider for maximum success in woody plants. *Acta Hortic.* **2013**, *988*, 43–46. [CrossRef]