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Molecular Authentication, Propagation Trials and Field Establishment of Greek Native Genotypes of *Sambucus nigra* L. (Caprifoliaceae): Setting the Basis for Domestication and Sustainable Utilization

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Abstract: In the context of sustainable utilisation of valuable but neglected plant resources, a multifaceted study is presented herein for Greek native genotypes of elderberry (*Sambucus nigra* L., Caprifoliaceae), a species with an appreciated pharmaceutical and nutritional value. At the first phase, fresh plant materials (leaves, softwood cuttings) were collected from nine geographically separated genotypes originating from wild-growing Greek native germplasm of *S. nigra*. The leaf samples were genetically authenticated using DNA barcoding (ITS2). The next phase included the development of an asexual propagation protocol via cuttings which included screening of the collected genotypes in terms of propagation success, and further experimentation over a two-year period on a prioritised genotype. The propagation results highlighted the importance of external application of indole-3-butyric acid (IBA) rooting hormone at levels of 2000–4000 ppm, which consistently presented high rooting rates (100%) of summer softwood, leafy cuttings of apical or sub-apical type. At the same time, rooting quality in terms of root number and length as well as early plant growth after rooting, were improved by external hormone application resulting in high rates of plant survival. This study reports first-time data on multifaceted assessment of Greek native *S. nigra* genotypes on molecular authentication and asexual propagation, thus ultimately setting the basis for domestication and sustainable utilization of this species.

Keywords: elderberry; super fruits; biodiversity; ex situ conservation; domestication; DNA barcoding; asexual propagation; indole-3-butyric acid; phytogenetic resources; medicinal plants

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1. Introduction

A worldwide concern for a healthy diet is becoming increasingly important over the latest years as human health is at the forefront of relevant research and is inevitably linked to diet [1–3]. An issue that has received much attention over the years are free radicals and their effects on human health coupled with the positive impact of natural food products that contain antioxidants and other beneficial compounds, thus considered as superfoods including also wild species [4,5]. A specific category of fruits with ancient and well-known value for human health are small stone fruits and berries of several plant families, including

both commercially cultivated and wild-growing species [6]. Berries, as sources of primary and secondary metabolites, provide excellent antioxidant activity for humans [6]. The latter is particularly important as they provide a number of beneficial functions for human health, such as antioxidant protection and therapeutic benefits, including reduced risk of coronary heart disease, reduced risk of stroke, anticarcinogenic activity, improved visual acuity, and improved cognitive behavior [7,8].

Compared to other antioxidant-rich small fruits, such as strawberry, raspberry and blueberry, *Sambucus nigra* L. or elderberry (Caprifoliaceae) has received to date considerably weaker attention [9,10]. The nutritional value of the fruits of *S. nigra* is very high [9,10] and this is coupled with validated pharmaceutical value, thus allowing for possible commercial exploitation. *S. nigra* has received significant pharmaceutical attention since the 1990s due to the antiviral and antimicrobial capacity of elderberry fruit and flower extracts against human diseases such as influenza, with relevant products such as Sambucol® (Sambucol, Copyright 2011, Pharmacare US Inc., San Diego, CA, USA) being already in the market [11–13]. Recently, the immunomodulatory properties of elderberry fruit phenolic compounds have been proposed for research on COVID–19 treatment [14,15]. Although such research on *S. nigra* is still in its infancy, recent studies to that end are already yielding promising results; for example, evidence has been found during viral entry in vitro on inhibition of binding of the S1 protein receptor of SARS-CoV2 to an enzyme related to the immune system (ACE2) by flower and fruit extracts of both wild-growing and cultivated genotypes of *S. nigra*, with wild germplasm presenting higher inhibitory capacity [16].

Wild plants present sophisticated evolutionary adaptations to the climatic regime in which they thrive naturally [17,18]. This tendency makes the wild-growing plants physiologically balanced in terms of photosynthate production and carbon balance; this, in turn leads to improved function of primary and secondary metabolism, which for fruit species is associated with the production of high-quality fruits [19]. As such, the utilisation of native phytogenetic resources for new crop development that are otherwise neglected or underutilized can bring forth strategic advantages countrywide, especially for Mediterranean countries with a high level of biodiversity, such as Greece [20,21]. Greece is a biodiversity hot spot with more than 6620 taxa, including about 100 species with small fruits and berries [22,23]. However, for sustainable exploitation of such resources, coordinated research efforts and sustainable exploitation strategies are required [20,24,25] as already proposed in other countries of the Mediterranean Basin, including molecular authentication of the plant material collected from the wild as one of the first steps towards its domestication and utilization, and breeding between wild crop relatives (CWRs) and cultivated species to produce new varieties useful for humans [26].

Novel molecular techniques based on the DNA sequence are becoming increasingly common for genetic identification of both model and non-model organisms. A well-established molecular tool that can be used for molecular authentication of plant germplasm across a very wide taxonomical spectrum is the DNA barcoding method coupled with bioinformatic analysis [27]. Molecular analysis is a pillar of phylogenetic studies as well as studies of genetic diversity of species [28]. Recent studies have redefined phylogenetic relationships within two species of *Sambucus* by applying the nrDNA ITS molecular marker designed to nuclear DNA [29], thus discerning one cluster with *S. nigra* populations and a second one of *S. ebulus* populations. However, there is no systematic study to date on the molecular authentication of Greek native populations of *S. nigra* assessing their genetic fingerprinting.

Following the establishment of a distinct genetic identity of the collected material, the next logical step in the domestication process propagation-wise is the development of a solid asexual propagation protocol. This is due to the fact that sexual propagation by seeds does not secure the steady transfer of desirable agronomic and fruit characteristics to the offspring due to the rearrangement of the genetic make-up that takes place during the fertilization process [30]. In addition, an efficient asexual propagation protocol is able to allow the production of large amounts of plant material to be transferred to commer-

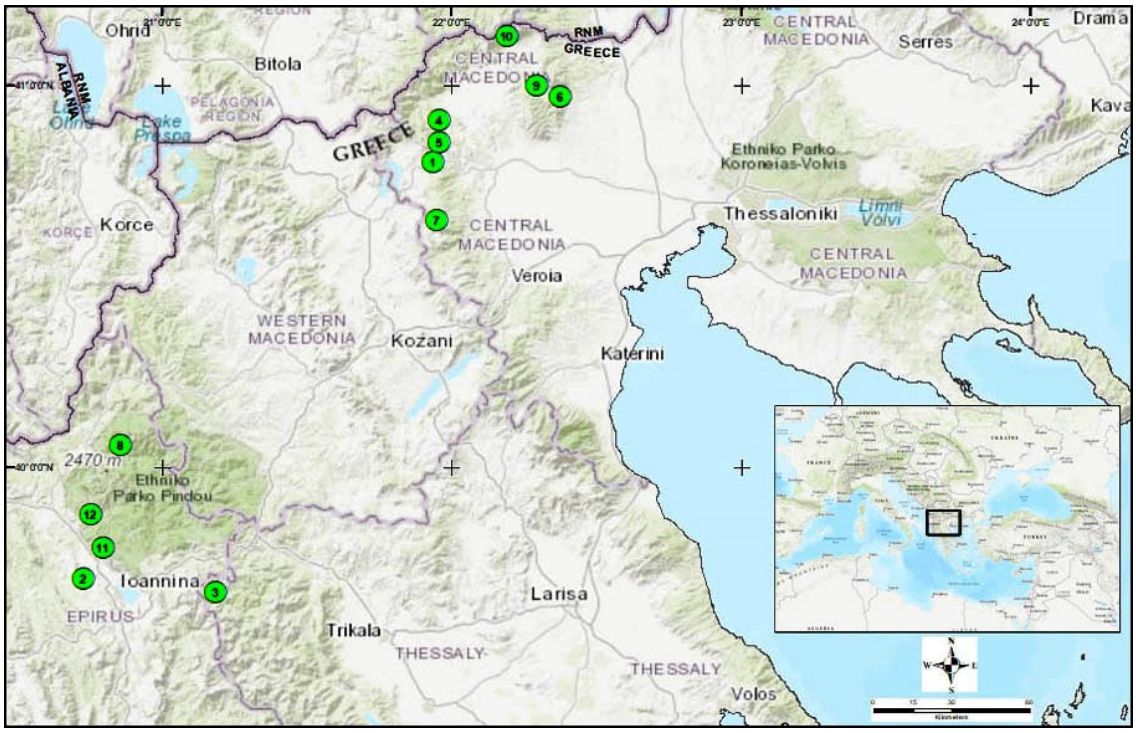
cial production in a sustainable and economically viable manner. *S. nigra* cuttings from populations originating from different regions have been shown to root with the use of commercial auxin compounds such as indole-3-butyric acid (IBA) [31–33]; however, similar work has not yet been conducted on Greek populations of *S. nigra*. Furthermore, close monitoring and recording of early plant establishment after rooting of cuttings is crucial for assessing the protocol's efficiency, since new plants need to survive in order to produce new mother plants for further propagation cycles [34–36]. Moreover, the newly established plants need to be further assessed under field conditions to enable the establishment of efficient and fruitful pilot commercial production systems which will eventually secure the sustainable utilization of the native genetic resources of *S. nigra*.

Considering the above framework, the aims of the current study were basically two-fold, as follows: (i) the collection of originally documented plant genotypes from the wild of Greece (fresh softwood plant parts, and leaves) and the molecular authentication of the collected material (DNA fingerprinting of different genotypes stemming from wild-growing populations); (ii) the development of asexual propagation protocols via cuttings on the successfully characterized genotypes. The latter was conducted in the following three phases: the first phase aimed at screening of different genotypes in terms of propagation success through rooting of cuttings; secondly, propagation experiments were conducted on a prioritized genotype over a two-year period to study the rooting capacity and quality in detail; lastly, the results of the second phase were complemented with early plant growth and development data aiming at shedding light on the physiological basis of plant survival after rooting and assessing the protocol's efficiency. A third aim of this study was the establishment and evaluation of a pilot cultivation system for Greek native *S. nigra* characterized genotypes sourced from the gene pool of this wild plant species. The latter aimed to screen the different genotypes in terms of plant growth rates under field conditions with productivity and fruit quality assessments (ongoing project). The authenticated identity of genotypes and the development of a consolidated, easy to implement and economically viable propagation protocol which is supported by establishment of a successful cultivation system are proposed herein as a baseline for the sustainable utilisation of valuable Greek native germplasm resources of *S. nigra*.

2. Materials and Methods

2.1. Collection of Plant Material

Under the auspices of the research project "Highlighting local traditional varieties and wild native forest fruit trees and shrubs" (acronym: EcoVariety, T1EΔK-05434), several authorised botanical expeditions were performed in 2019 to explore different regions of northern and north-central Greece (Macedonia, Epirus and Thessaly) for *S. nigra* native germplasm. The collections were conducted using a special permit to the Institute of Plant Breeding and Phytogenetic Resources, Hellenic Agricultural Organization–Demeter (Permit 82336/879 of 18 May 2019 & 26895/1527 of 21 April 2021) issued by the Greek Ministry of Environment and Energy. During these expeditions, the wild habitats of *S. nigra* were located and the initial material for the study was collected. Nine geographically separated populations were sampled in total (Figure 1), which were taxonomically identified based on established diagnostic features [37] for members of *Sambucus*. From each of the nine selected population of Greek native germplasm (Table 1), we sampled the following: (a) leaves from 20 individuals destined for DNA analysis, and (b) sets of fresh soft-wood, leafy stem cuttings as starting propagation material. Consecutively, each genotype was allocated a unique IPEN (International Plant Exchange Network) accession number given by the Balkan Botanic Garden of Kroussia (BBGK), Institute of Plant Breeding and Genetic Resources (IPB & GR), Hellenic Agricultural Organization–Demeter (ELGO–Demeter).



(**A**)

(**B**)

(**C**)

(**D**)

(**E**)

Figure 1. Map outlining the collection sites (numbers as in Table 1) of the *Sambucus nigra* Greek native germplasm sampled (**A**), typical appearance of plant individual (**B**), collected softwood cuttings (**C**), inflorescences and leaves (**D**), and flowers (**E**) of *S. nigra* GR-1-BBGK-19,479 (IPEN accession numbers as in Table 1) used for taxonomic identification, DNA barcoding and propagation trials.

Table 1. Genotypes of Greek native *Sambucus nigra* that were sampled from wild-growing populations in various habitats of northern and north-central Greece assigned with different IPEN (International Plant Exchange Network) accession numbers.

No.	IPEN Accession Number	Greek Prefecture	Area	Coordinates (HGRS87/EGSA87)	Altitude (m)	Sampling
1	GR-1-BBGK-19,73	Central Macedonia	Mt Voras	40.801549999999999, 21.935230000000001	462	SWSC, LS
2	GR-1-BBGK-19,192	Epirus	Ioannina	39.704830000000001, 20.727409999999999	490	SWSC, LS
3	GR-1-BBGK-19,425	Thessaly	Trikala	39.667850000000001, 21.184380000000001	1117	SWSC, LS
4	GR-1-BBGK-19,478	Central Macedonia	Mt Voras	40.909920000000000, 21.955359999999999	566	LS
5	GR-1-BBGK-19,479	Central Macedonia	Mt Voras	40.878860000000003, 21.959170000000000	749	SWSC, LS
6	GR-1-BBGK-19,503	Central Macedonia	Kilkis	40.970910000000003, 22.376750000000001	790	LS
7	GR-1-BBGK-19,562	Central Macedonia	Mt Vermio	40.649999999999999, 21.949999999999999	1615	SWSC, LS
8	GR-1-BBGK-19,574	Epirus	Ioannina	40.056390000000000, 20.856770000000001	930	SWSC, LS
9	GR-1-BBGK-19,584	Central Macedonia	Kilkis	41.002249999999997, 22.294530000000002	1224	LS
10	GR-1-BBGK-19,596	Central Macedonia	Mt Tzena	41.128920000000001, 22.190809999999999	1263	SWSC
11	GR-1-BBGK-19,629	Epirus	Ioannina	39.787129999999998, 20.797750000000001	990	SWSC
12	GR-1-BBGK-19,637	Epirus	Ioannina	39.873710000000003, 20.752459999999999	955	SWSC

SWSC: Soft-wood stem cuttings for propagation; LS: Leaf samples for DNA analysis.

2.2. DNA Isolation

Approximately 30 mg of dried leaf sample was completely grounded in liquid nitrogen. Total DNA was isolated from leaf samples of *S. nigra* using a Nucleospin Plant II (Macherey-Nagel) kit following the manufacturer's instructions.

2.3. Polymerase Chain Reaction (PCR) Amplification

One primer set of the nuclear ITS2 barcode region suggested by Chen et al. [38] was used for amplification and sequencing. The PCR amplification was performed according to Madesis et al. [28].

2.4. Sequence Analysis

PCR products were directly sequenced in two directions of each fragment with a Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an automated ABI 3730 sequencer (PE Applied Biosystems). The sequences were aligned using the CLUSTAL W program.

2.5. Molecular Data Analysis

The following three methods were employed for the molecular authentication of the selected *Sambucus nigra* genotypes: (i) basic local alignment search tool (BLAST) search using the nucleotide database at NCBI; (ii) the genetic divergence method using maximum-likelihood models; (iii) tree topology analysis based on the neighbor-joining (NJ) method

based on different loci in MEGAX with the K2P distance model and 500 bootstrap replications. The sequences obtained after removing the primers used for PCR amplification were deposited to NCBI-Genbank BankIt (https://www.ncbi.nlm.nih.gov/BankIt/ (accessed on 13 December 2021) under the accession numbers MK5335234 to MK5335242.

2.6. Phylogenetic Relationships

The phylogenetic relationship among different species of *Sambucus* was inferred using the neighbor-joining method [39]. The optimal tree with the sum of branch length = 0.16575206 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [40]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [41] and were in the units of the number of base substitutions per site. This analysis involved 25 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 283 positions in the final dataset allowing for phylogenetic analyses conducted in MEGA X [42].

2.7. Establishment of Mother Plant Stock Material for Propagation

The collected material from each genotype (Table 1) was preliminary tested for rooting under various external hormone application treatments of indole-3-butyric acid (IBA); cuttings were set for rooting in propagation trays with peat (Klasmann, KTS 1)–perlite at 1:3 v/v on mist bench in a plastic greenhouse at ambient temperature with relative humidity (RH) maintained >85% where they were attended weekly to assess their rooting capacity. The produced mother plants were kept ex situ at the grounds of BBGK, IPB, and GR under ambient conditions for plant establishment. The plants were watered regularly and were grown in 3 L pots using a mixture of peat (Klasmann, KTS 2) and perlite (3:1, v/v). This allowed the establishment of mother plants for each selected genotype which enabled the supply of mother plant stock material for further experimentation.

2.8. Screening Experimental Design and General Propagation Trials

The effects of two cutting types and three different concentrations of the auxin indole-3-butyric acid (IBA) (0; 2000; 4000 ppm) were assessed for each of the nine genotypes of *S. nigra* on root formation. The experiment was set up as a split-plot design having the nine genotypes as main plots and the six treatments that stem from the two cutting types and three hormone application levels as sub-plots resulting in 54 treatments in total. Each treatment consisted of four replications. Cuttings from the nine selected genotypes were taken in early summer. The cuttings were either from the top of plant stems of the same internode position which included the developing apical meristem (apical cuttings) or they were internode sections of the same stems with two lateral buds (sub-apical cuttings). In both cases, the cuttings were soft wood ones and were defoliated to two leaves per cutting. Cuttings were placed in propagation trays in a substrate of peat (Klasmann, KTS 1)–perlite (1:3 v/v) and were maintained at a mist bench in a plastic greenhouse with relative humidity > 85%. Observations on the progress of cuttings were taken weekly on each genotype and when a treatment reached 100% rooting or, at least >75%, the trays were taken out of mist and measurements were taken on rooting capacity, root number and root length per cutting. Consecutively, rooted cuttings were transplanted in 0.5 L pots with peat (Klasmann, KTS 2)–perlite (3:1 v/v) substrate and were kept for the first two weeks within a greenhouse with automated irrigation for plant establishment.

2.9. Special Propagation Trials of Genotype GR-1-BBGK-19,192

Genotype GR-1-BBGK-19,192 was selected for further experimentation based on the results of the screening experimentation. An experiment was conducted in 2019 which was repeated in 2020. Specifically, two cutting types, two different substrates and five

different concentrations of IBA (0; 1000; 2000; 4000 and 6000 ppm) on root formation were tested. The two experiments that were conducted abided by an identical design. Each experiment followed a split-plot design with substrate being the main plot and the ten resulting combinations of hormone level and cutting type being the sub-plots resulting in 20 treatments in total with eight replicate cuttings in 2019 and seven replicate cuttings in 2020. The cutting types used were the same with the screening experiment, namely, apical and sub-apical primary softwood cuttings of summer. The following two different substrate mixtures were tested: (a) substrate of peat (Klasmann, KTS 1)–perlite (1:3 v/v) and (b) peat (Klasmann, KTS 1)–perlite (1:1 v/v). Cuttings were placed in propagation trays and maintained at a mist bench in a plastic greenhouse with relative humidity > 85%. Rooting observations and measurements were conducted as described for the screening experimentation.

2.10. Measurement of Early Vegetative Growth of Genotype GR-1-BBGK-19,192

The rooted cuttings from genotype GR-1-BBGK-19,192 that stemmed from the propagation experiments were transplanted in 1 L pots in substrate of peat (Klasmann, KTS 2)–perlite (3:1 v/v) and were placed under ambient greenhouse conditions with automated irrigation. Plant height (cm) and total leaf number/plant were measured at 10-day to fortnightly intervals until 40 days after rooting (DAR). At the end of the above period, both the upper part and the rooting system of the plants were destructively harvested, and biomass accumulation assessment was carried out in terms of total plant dry weight and root dry weight.

2.11. Pilot Field Cultivation Trial of the Nine (9) Genotypes

On March 2020, all nine genotypes were established in the pilot field of the BBGK, IPB, and GR at sea level altitude. The effect of nine different genotypes and three different fertilization regimes on plant growth and development were studied. The field experiment resulted in 27 treatments in total under a complete randomized design with five replications per treatment. The fertilization treatments applied were as follows: (a) conventional crop fertilization; (b) organic crop fertilization; (c) no fertilization (control). Details of the particular fertilization regimes are given in Table 2. During the growing season all plants were drip-irrigated weekly with 1.92 mL s^{-1}. The applied fertilization regimes, in each case, were determined based on soil analysis. Plant height was recorded at 20-day intervals.

2.12. Statistical Analysis of Propagation and Field Growth Data

Root number and root length data of all propagation experiments were analyzed by multivariate GLM ANOVA across all applied treatments (genotype, substrate, IBA level and cutting type) to evaluate the effect of the applied factors. Following the experimental design, data were split, and separate Analyses of Variance were conducted for each genotype in the screening experiment to dissect specific differences between hormone levels and cutting types on the rooting variables measured for each genotype of *S. nigra*. Similarly, as appropriate in the propagation experiments of genotype GR-1-BBGK-19,192, special propagation data were split, and separate analyses of variance were conducted.

Early plant growth data of plant height and leaf number that were measured over time and treatment effects were evaluated through repeated measures ANOVA. The biomass accumulation data were subjected to analysis of variance (GLM ANOVA) to establish treatment effects.

Differences between means were evaluated by a Tukey HSD post hoc test ($p < 0.05$). Plant height data of the field experiment were analyzed through ANOVA using the Duncan test for mean comparison ($p < 0.05$). The statistical software used was IBM-SPSS 20 and graphs were drawn using Microsoft Excel.

Table 2. Different fertilization regimes on the established *Sambucus nigra* genotypes in the pilot field cultivation trial during 2020 in Thermi, Thessaloniki, Greece.

Application Period	Conventional Crop Nutrition	Organic Crop Nutrition
March -plantings	Peat (planting pit)	100 g zeolite + 50 g biocompost + 30 g P-30 + 30 g organic fertilizer (water-soluble Fe 12% *w*/*w*, water-soluble Mn 0.55% *w*/*w*, water-soluble Zn 0.49% *w*/*w*, total MgO 5.1% *w*/*w*, water-soluble MgO 3% *w*/*w*, water-soluble SO_3 37% *w*/*w*) (planting pit)
April	80 g/plant 21% N—17% P_2O_5—0.15% Zn—4% S	50 g natural product (2% organic acids, as complexes of natural aluminosilicate minerals and hydrated copper sulphate, adsorbed on the natural crystal)/10 L water
May	Fe: 20 g + Zn: 5 mL + B: 5 mL/10 L	20 g organic fertilizer (organic water-soluble N 11% *w*/*w*, organic C 40% *w*/*w*, total amino acids 69.2% *w*/*w*) + 1.5 g organic fertilizer [organic & humic compounds: 68–78% (humic acids 40% min.), nutrient inorganic elements: 5% N, 3% P_2O_5, 3–5% CaO, 0.7–1.0% MgO, 1.2 Fe & trace elements (Zn, B, Cu) in ppm] + 5 mL natural product (amino acids from vegetal organic matrixes, natural cytokinins, folic acid, humic and fulvic acids, glutamic acid, asparagine, alanine, lysine, vitamins (A, B, C, PP, K), carbohydrates, micronutrients)/10 L water
		50 g natural product (organic acids, organic calcium and boron sources and 3% of vitamins as complexes of natural aluminosilicate minerals) + 20 mL organic biostimulator (5% *w*/*w* total aminoacids, 1.5% *w*/*w* free aminoacids, 10% *w*/*w* organic carbon, 10 mg/kg natural triacontanol)/10 L water
June	80 g/plant 21% N—17% P_2O_5—0.15% Zn—4% S	20 mL organic biostimulator (5% *w*/*w* total aminoacids, 1.5% *w*/*w* free aminoacids, 10% *w*/*w* organic carbon, 10 mg/kg natural triacontanol)/10 L water
September	-	100 g natural product (2% organic acids, as complexes of natural aluminosilicate minerals and hydrated copper sulphate, adsorbed on the natural crystal 12.5% *w*/*w*)/10 L water
November	35 g/10 L 46—0—0	20 mL organic fertilizer (total N 2% *w*/*w*, organic N 1% *w*/*w*, water-soluble K_2O 9% *w*/*w*)/10 L water

3. Results

3.1. Molecular Authentication Efficiency Using Barcoding ITS2 Region

The authentication efficiency of the studied ITS2 marker in Greek native *Sambucus nigra* accessions was evaluated by applying BLAST1 and distance-based tests. The results of the BLAST1 method revealed high efficiency in the identification of species and genus. More specifically, the efficiency was 99% for the species level and 100% for the genus level. Results of the distance method using the ITS2 marker had 97% identification efficiency for the species. Figure 2 depicts the neighbor-joining (NJ) phylogenetic tree using the ITS2 barcode, showing that the barcoding method can efficiently classify all genotypes of members of *Sambucus*, and can clearly distinguish Greek native genotypes of *S. nigra* from other genotypes of different species of *Sambucus*. Furthermore, phylogenetic NJ tree discriminated Greek accessions of *S. nigra* in a distinct branch with 69% branch support. The bootstrap values confirmed the results of the NJ tree classification. Overall, our results showed that the use of ITS2 marker in combination with the barcoding technique has 100% efficiency in the classification and distinction of members of *Sambucus*. Additionally, 283 SNPs were detected which can effectively distinguish and classify Greek native *S. nigra* genotypes from other genetically close genotypes of *S. nigra* or species of genus *Sambucus*. Thus, the nine *S. nigra* accessions studied herein were fingerprinted with the application of barcoding technique with ITS2 nuclear region.

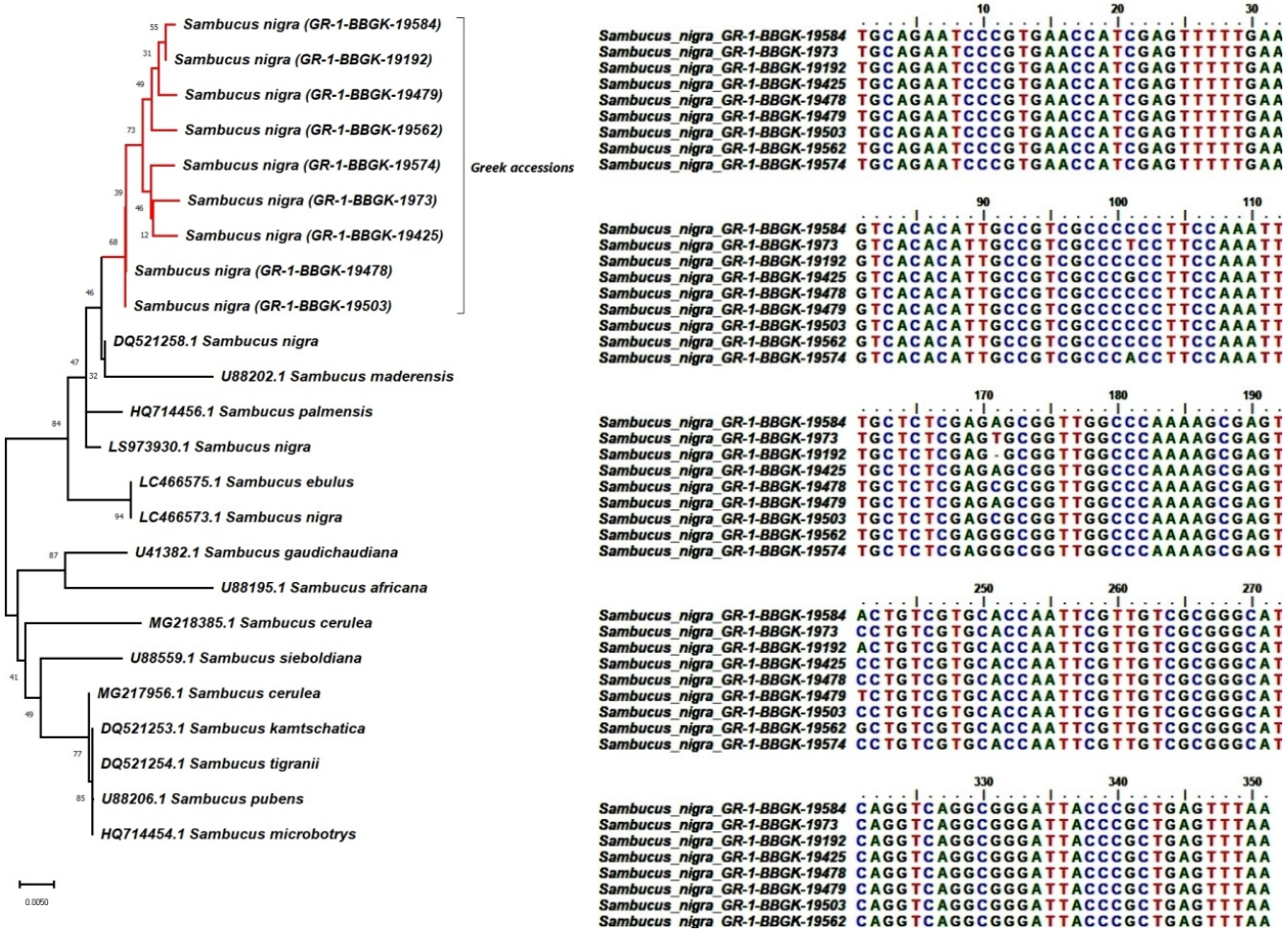


Figure 2. Phylogenetic tree (**A**) constructed on the basis of ITS2 regions of the Greek native *Sambucus nigra* accessions contrasted with other *S. nigra* genotypes and other members of genus *Sambucus* retrieved from NCBI with ClustalW alignment of the ITS2 barcode region of the genotypes analyzed in this study (**B**). Results from neighbor-joining (NJ) bootstrap analyses with 1000 replicates was used to assess the strength of the nodes. The node numbers indicated the bootstrap value of NJ.

3.2. Screening of Genotypes in Terms of Propagation Success

The propagation trial for the nine collected genotypes of *S. nigra* presented broad rooting rates of the cuttings across genotypes and treatments over a 30-day period, with the exception of GR-1-BBGK-19,192 which achieved maximum rooting rates in 15 days. The observed rooting frequencies ranged from 0% in control treatments of genotypes GR-1-BBGK-19,479, GR-1-BBGK-19,596 and GR-1-BBGK-19,637 and in the 2000 ppm IBA/sub-apical cuttings treatment of GR-1-BBGK-19,479 up to 100% in most of the samples except GR-1-BBGK-19,479 (Figure 3). Concerning root number and average root length, a relatively small number of roots with comparatively high length was measured in general throughout all samples except GR-1-BBGK-19,192 and GR-1-BBGK-19,562 ($p < 0.05$). The observed values on the eight samples except GR-1-BBGK-19,192 ranged from one root on average with mean length of 13.7 mm to 25.7 roots with mean length 31.9 mm in GR-1-BBGK-19,562 4000 ppm IBA/apical and GR-1-BBGK-19,629 2000 ppm IBA/sub-apical, respectively (Figure 3). Similarly, the root lengths (mm) that were observed ranged from 3 mm (mean root number 1.2) to 40.9 mm (mean root number 12) in GR-1-BBGK-19,562 2000 ppm IBA/apical and GR-1-BBGK-19,596 2000 ppm IBA/sub-apical, respectively (Figure 3). The rooted plants, however, did manage to survive in all cases. Genotype GR-1-BBGK-19,192 presented higher number of emerged roots with a comparatively shorter length throughout

all treatments with 2000 ppm IBA/apical cuttings presenting the highest number of roots with 27.7 roots with mean length 16.3 mm and 100% rooting capacity (Figure 3, $p < 0.05$). According to the statistical analysis of the above results an overall significant effect of genotype (population sample) was observed in interaction with hormone treatment and cutting type ($p < 0.05$). Consecutive analysis revealed that hormone treatment and cutting type, significantly affected root number and root length within each population sample (Figure 3, $p < 0.05$).

3.3. Field Cultivation Trial of the Collected Genotypes

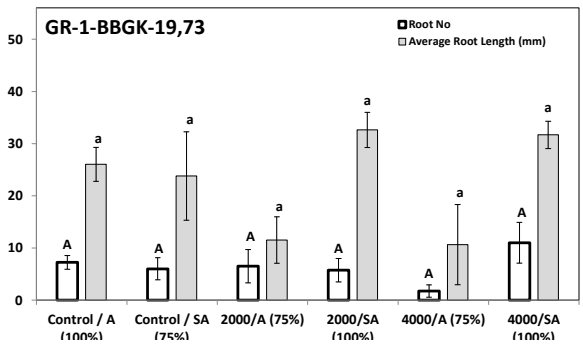
The results from the field trial showed that genotypes GR-1-BBGK-19,192 and GR-1-BBGK-19,479 showed the greatest increase in plant height compared to other genotypes of *S. nigra* ($p < 0.05$, Figure 4). The highest values of plant height observed were 212.9 cm and 214.7 cm while the lowest one was 135.9 cm for GR-1-BBGK-19,192, GR-1-BBGK-19,479 and GR-1-BBGK-19,629 *S. nigra* genotypes, respectively. Concerning the different types of fertilization for genotype GR-1-BBGK-19,192, conventional fertilization consistently presented higher values in terms of plant height in contrast with organic application and control throughout the growing season ($p < 0.05$, Figure 5).

3.4. Multifaceted Assessment of Greek Sambucus Nigra Genotypes

Based on the results of the molecular authentication (Figure 2), propagation success and rooting quality (Figure 3) coupled with the results of the cultivation trial (Figures 4 and 5) conducted concurrently, the collected genotypes of Greek native *S. nigra* germplasm were evaluated according to the fulfillment of the following three criteria: (i) successful molecular authentication (DNA barcoding), (ii) ease of propagation expressed as rooting frequency, time taken to root and rooting quality in terms of swift emergence of high number of roots, and (iii) steadfast vegetative growth under field conditions. The genotype GR-1-BBGK-19,192 was prioritized as the most promising for further experimentation and ultimately sustainable utilization as it eventually fulfilled all the above criteria which they are presented in Table 3.

3.5. Propagation of Genotype GR-1-BBGK-19,192

The rooting capacity of genotype GR-1-BBGK-19,192 within a 14-day period was high throughout, in both experiments (summer 2019 and summer 2020). On the 2019 experiment, the rooting capacity ranged from 62.5% in substrate peat (Klasmann, KTS 1)–perlite 1:1 (*v*/*v*) in apical cuttings/control to 100% in most other treatments, with 1000, 2000 and 4000 ppm IBA presenting 100% rooting in both substrates and both cutting types (Figure 6). Similarly, in the 2020 experiment, rooting capacity ranged from 71.4–100% throughout, with 1000, 2000 and 4000 ppm IBA presenting 100% rooting in both substrates in apical cuttings (Figure 7).



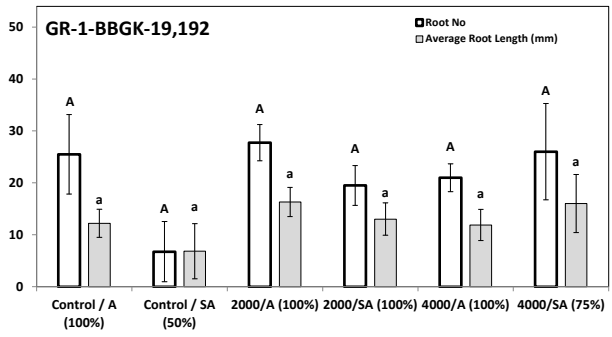


Figure 3. *Cont.*

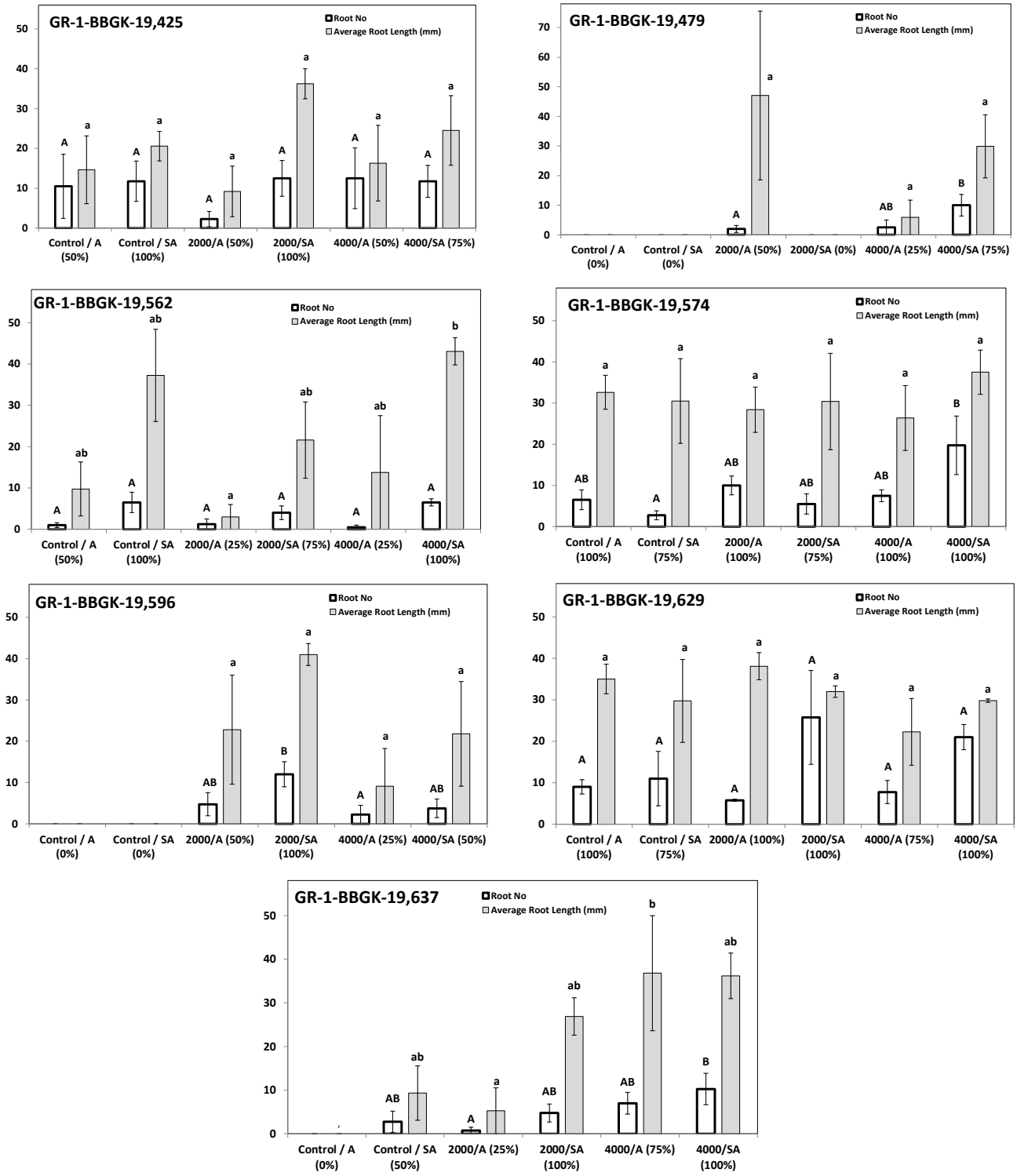


Figure 3. Mean root number (white bars) and mean root length (mm) (grey bars) for each hormone treatment (ppm IBA) and cutting type (A: apical cuttings bearing the stem's apical meristem, and SA: sub-apical cuttings including internode sections with two lateral buds) from nine Greek native genotypes of *Sambucus nigra* at 30 days of screening trials. Each graph show results obtained from different IPEN accession numbers of *S. nigra* (see Table 1). The substrate used in all trials was peat–perlite (1:3 *v*/*v*) under mist conditions. Standard errors of the means are shown on the bars ($p < 0.05$). Bars that do not share the same letter are significantly different from each other (Tukey HSD, $p < 0.05$, capital letters for root number and lowercase letters for root length). Across the horizontal axis of each graph, next to treatment name, the total rooting percentage (%) is given for each treatment.

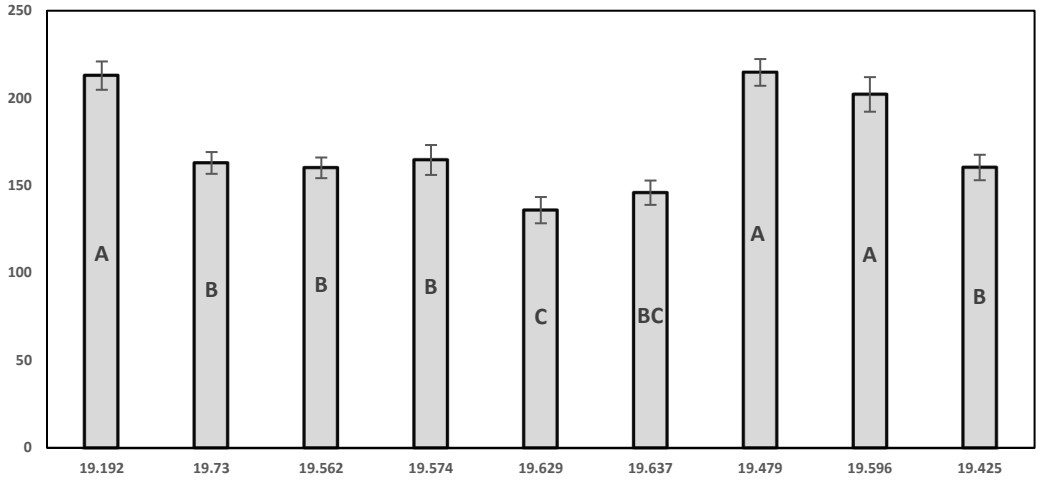


Figure 4. Average of plant height (cm) of each of the nine genotypes of *Sambucus nigra* established in the pilot field in Thermi, Thessaloniki, Greece (simplified IPEN numbers as in Table 1). Each value is the mean of five replications ± standard error for $p \leq 0.05$. Bars that do not share the same letter are significantly different from each other (Duncan test, $p < 0.05$).

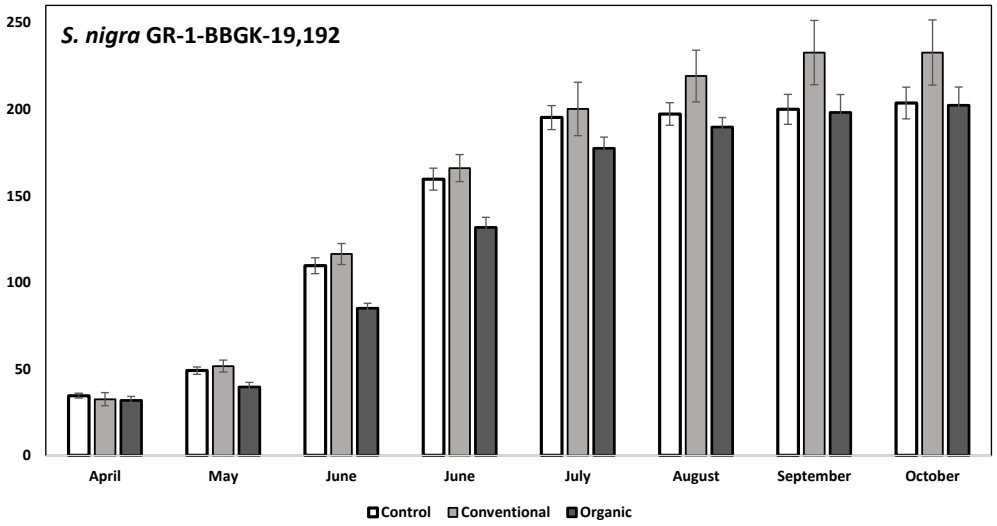


Figure 5. Effect of conventional fertilization, organic and control on the plant height (cm) of *Sambucus nigra* GR-1-BBGK-19,192. Each value is the mean of five replications ± standard error for $p \leq 0.05$.

Concerning root number and length, the statistical analysis showed a significant effect of hormone level on root number of 1:3 substrate/apical cuttings where higher values were recorded as the applied hormone level increased in 2019 (Figure 6, $p < 0.05$). The cutting type significantly affected root number and length only in control and 1000 ppm IBA treatments in both substrates. and no such effect was observed at higher hormone levels (Figure 6, $p < 0.05$). No significant differences were observed between substrates for each hormone treatment and cutting type in 2019. Comparatively, in the 2020 experiment, hormone treatments and cutting type significantly affected root number and length with particular differences in this case only detected in apical cuttings in both substrates with hormone levels >2000 ppm presenting higher values (Figure 7, Tukey HSD, $p < 0.05$). In general, in 2020 apical cuttings presented higher values of root number and length in both substrates in hormone treatments up to 4000 ppm IBA following the total rooting rates observed (Figure 7, $p < 0.05$).

Table 3. Multifaceted assessment of Greek native *Sambucus nigra* genotypes based on molecular authentication obtained (Figure 2); ease of propagation expressed as rooting frequency with hormone application (low < 50%, medium < 80% or high > 80%), speed, expressed as time (in number of days; d) taken to achieve maximum rooting frequency under the conditions studied (very high ≤ 15 d, high 16–25 d or low > 25 d) and rooting quality in terms of root number (low < 10, medium 11–20, high 21–25, very high > 26) (Figure 3); successful field vegetative establishment in terms of plant height achieved over a growing season (low < 100 cm, medium 100–200 cm, high > 200 cm) (Figure 4).

IPEN Accession Number	DNA Barcoding	Ease of Propagation *			Field Establishment
		Rooting Frequency	Speed	Rooting Quality	
GR-1-BBGK-19,73	Effective	High	Low	Medium	Medium
GR-1-BBGK-19,192	Effective	High	Very high	Very high	High
GR-1-BBGK-19,425	Effective	High	Low	Medium	Medium
GR-1-BBGK-19,479	Effective	Medium	Low	Low	High
GR-1-BBGK-19,562	Effective	High	Low	Low	Medium
GR-1-BBGK-19,574	Effective	High	Low	Medium	Medium
GR-1-BBGK-19,596	-	High	Low	Medium	Medium
GR-1-BBGK-19,629	-	High	Low	High	Medium
GR-1-BBGK-19,637	-	High	Low	Low	Medium

* The assessment is based on the highest values observed for each assessment parameter presented; in all cases where the highest value was observed, external rooting hormone application was necessary (for more details see Figure 2).

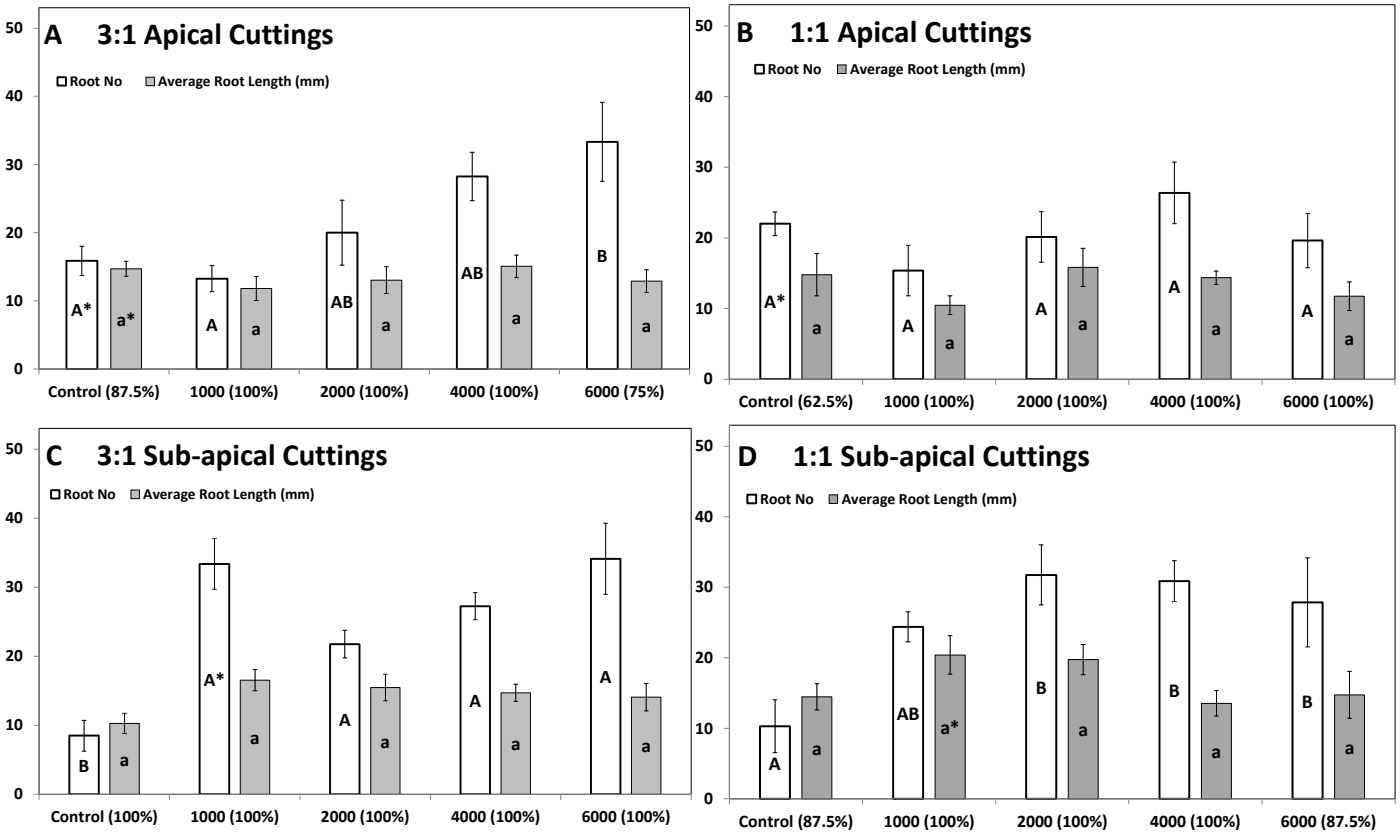


Figure 6. Mean root number (white bars) and mean root length (mm) (grey bars) for each hormone treatment (ppm IBA) across the 2019 experiment of *S. nigra* GR-1-BBGK-19,192 at 14 days after setting. The graphs are divided for the two substrates (peat–perlite (1:3 *v*/*v*) and peat–perlite (1:1 *v*/*v*)) and for each cutting type (apical cuttings that bear the stem's apical meristem and sub-apical cuttings that

were internode sections with 2 lateral buds). Standard errors of the means are shown on the graphs ($p < 0.05$). Bars that do not share the same letter are significantly different between hormone treatments within each substrate and cutting type for each graph (Tukey HSD, $p < 0.05$, capital letters for root number and lowercase letters for root length). Significantly higher values of the same hormone treatment and substrate between the two cutting types are denoted with * (comparisons between graph (**A**,**C**) for 1:3 and (**B**,**D**) for 1:1, $p < 0.05$). Across the horizontal axis of each graph, next to treatment name, the total rooting percentage (%) is given for each treatment.

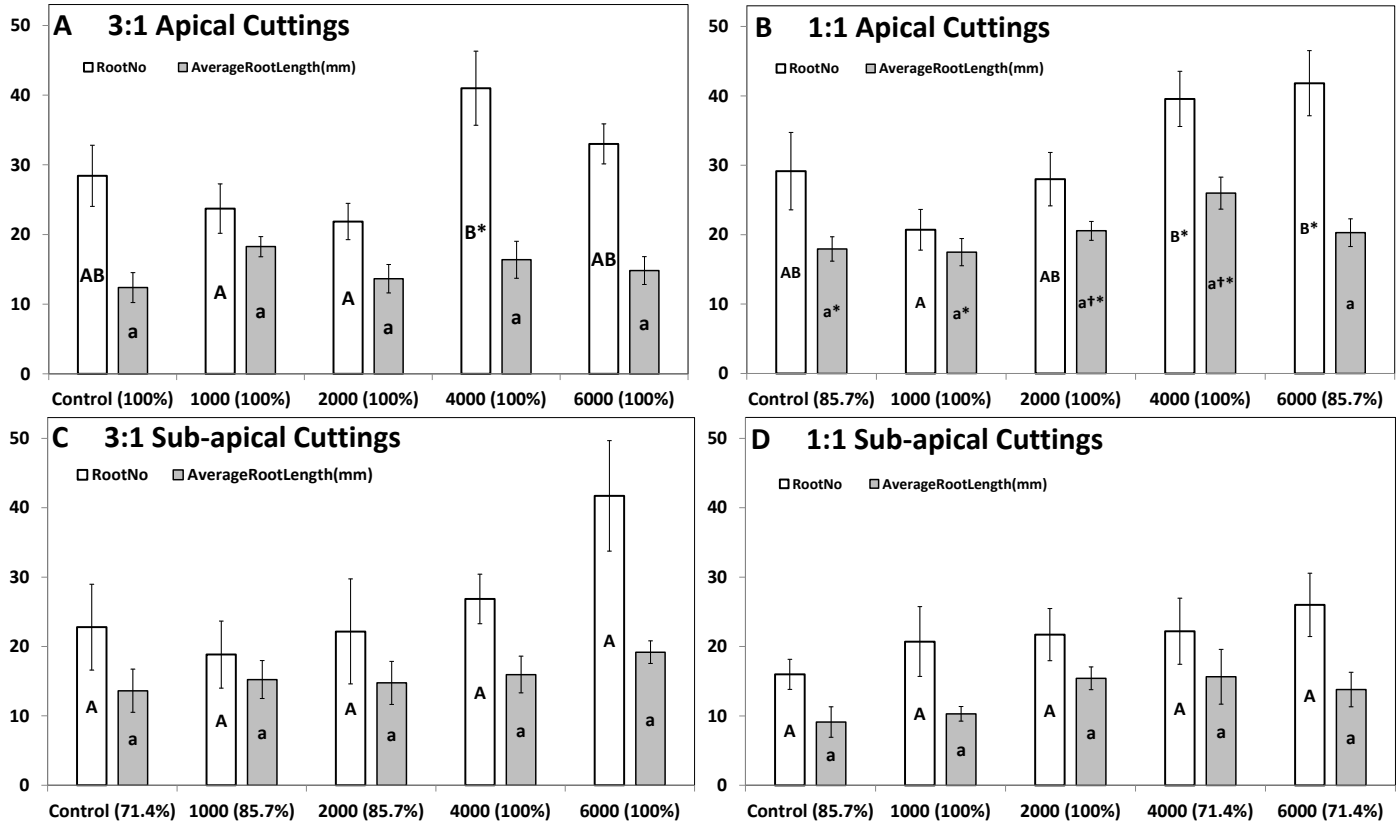


Figure 7. Mean root number (white bars) and mean root length (mm) (grey bars) for each hormone treatment (ppm IBA) across the summer 2020 experiment of *S. nigra* GR-1-BBGK-19,192 at 14 days after setting. The graphs are divided for the two substrates (peat–perlite (1:3 *v*/*v*) and peat–perlite (1:1 *v*/*v*)) and for each cutting type (apical cuttings that bear the stem's apical meristem and sub-apical cuttings that were internode sections with two lateral buds). Standard errors of the means are shown on the graphs ($p < 0.05$). Bars that do not share the same letter are significantly different between hormone treatments within each substrate and cutting type for each graph (Tukey HSD, $p < 0.05$, capital letters for root number and lowercase letters for root length). Significantly higher values of the same hormone treatment and cutting type between the two substrates are denoted with † (comparisons between graph (**A**,**B**) for apical cuttings and (**C**,**D**) for sub-apical cuttings, $p < 0.05$). Significantly higher values of the same hormone treatment and substrate between the two cutting types are denoted with * (comparisons between graph (**A**,**C**) for 1:3 and (**B**,**D**) for 1:1, $p < 0.05$). Across the horizontal axis of each graph, next to treatment name, the total rooting percentage (%) is given for each treatment.

3.6. Vegetative Growth of Genotype GR-1-BBGK-19,192

Vegetative growth patterns of plant height and leaf number directly after rooting were both affected by the applied factors on rooting ($p < 0.05$). More specifically, in the 2020 experiment, in apical cuttings that presented 100% rooting in most hormone treatments in both substrates, mean plant height after rooting was significantly affected by hormone treatment in both substrates showing a prevalence of plants that were treated with

2000 ppm IBA and to a lesser extend with 4000 ppm IBA ($p < 0.05$) (Figure 8). In comparison, across the same period, mean total leaf number per plant was also significantly affected by hormone but only in 1:3 substrate/apical cuttings and 1:1 substrate/ sub-apical cuttings ($p < 0.05$) showing a prevalence of plants treated with 4000 and 2000 ppm IBA, respectively, suggesting an interaction of substrate and cutting type with hormone affecting plant leaf number after rooting (Figure 8). However, substrate as an independent factor significantly affected leaf number after rooting only in control treatments of both cutting types and in 1000 ppm IBA treatment of sub-apical cuttings ($p < 0.05$, Figure 8). This suggests an alleviation of the effect of substrate as the applied hormone level increases, bringing about a stronger effect of cutting type at higher hormone levels affecting leaf number, which was more profound in 1:3 substrate. This was not the case in plant height, where substrate significantly affected this parameter only in 2000 ppm IBA presenting higher rates of plant height in apical cuttings compared to sub-apical cuttings ($p < 0.05$, Figure 8).

Following the above growth rates after rooting, plant biomass accumulation in terms of total plant dry weight and root dry weight, was significantly affected by hormone levels but only in interaction with substrate and cutting type ($p < 0.05$). More specifically, similarly to the results on plant height at 2000 ppm IBA, plants deriving from apical cuttings showed significantly higher values of total plant dry weight and root dry weight in 1:3 substrate 40 days after rooting ($p < 0.05$, Figures 9–12). However, the same hormone level presented significantly higher total plant dry weight and root dry weight in sub-apical cuttings compared to apical cuttings within the 1:1 substrate ($p < 0.05$, Figures 9–12).

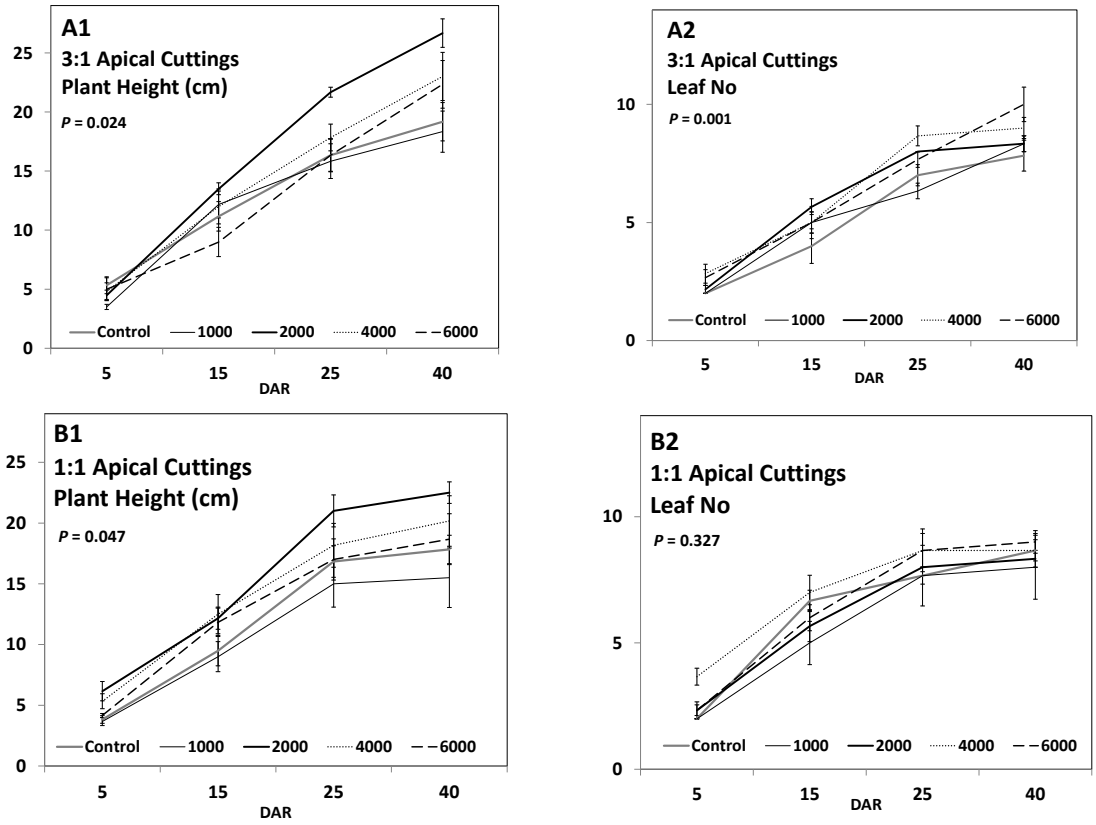


Figure 8. *Cont.*

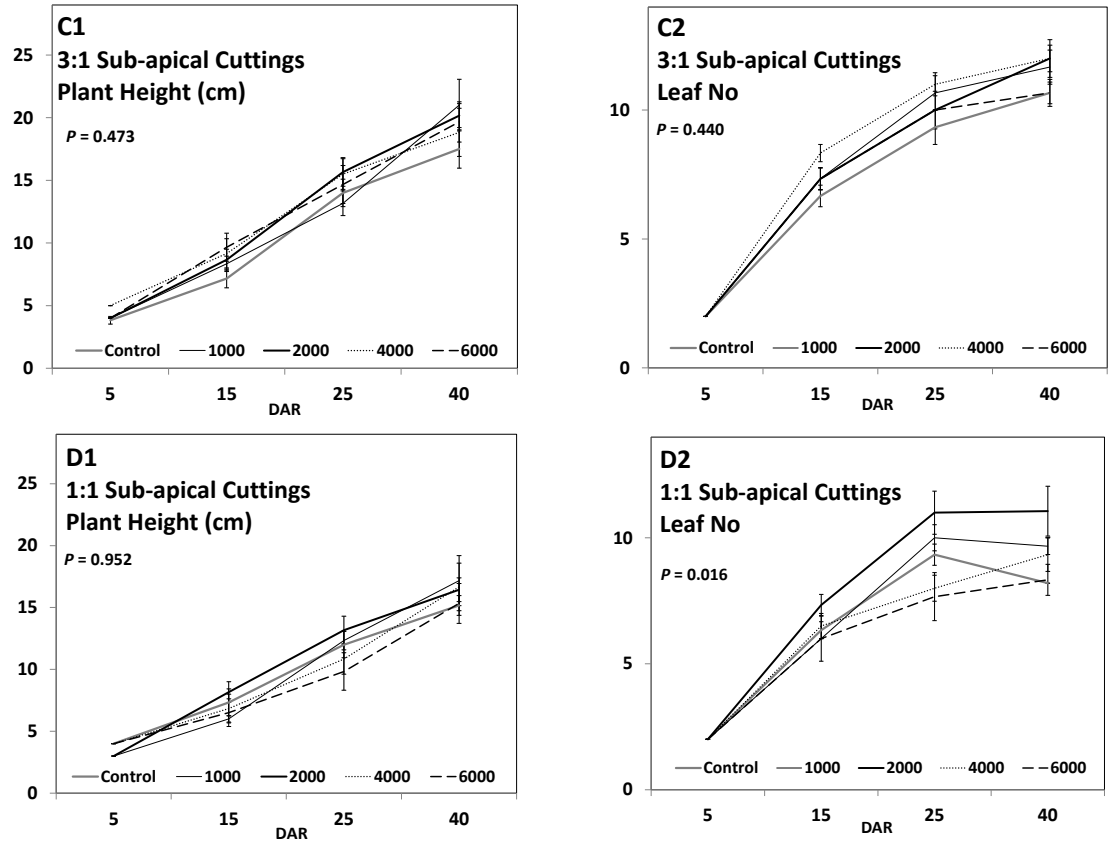


Figure 8. Plant growth patterns after rooting (DAR: days after rooting) across the 2020 experiment of *S. nigra* GR-1-BBGK-19,192. (**A1**–**D1**): Mean plant height (cm) for each hormone treatment (ppm IBA) presented for the two substrates (peat–perlite (1:3 v/v) and peat–perlite (1:1 v/v)) and for each cutting type (apical cuttings that bear the stem's apical meristem and sub-apical cuttings that were internode sections with 2 lateral buds). (**A2**–**D2**): Mean number of leaves for each hormone treatment (ppm IBA) presented for the two substrates and for each cutting type. Standard errors of the means are shown on the graphs ($p < 0.05$) as well as the respective p values of a repeated measures ANOVA conducted on hormone treatment effects (between-subjects effects) on each measured variable for each combination of substrate and cutting type presented in each graph across the measured dates ($p < 0.05$).

Figure 9. Representative photos of cutting propagation results of the prioritized Greek native genotype

of *Sambucus nigra* GR-1-BBGK-19,192 across the five hormone (indole-3-butyric acid, IBA) level treatments in 2020. (**A**) Apical softwood cuttings in peat–perlite (1:3, v/v); (**B**) Sub-apical softwood cuttings in peat–perlite (1:3, v/v); (**C**) Apical softwood cuttings in peat–perlite (1:1, v/v) and (**D**) Sub-apical softwood cuttings in peat–perlite (1:1, v/v). Leaf discoloration can be seen in the 6000 ppm IBA treatment in sub-apical cuttings. Bars in photos represent 5 cm.

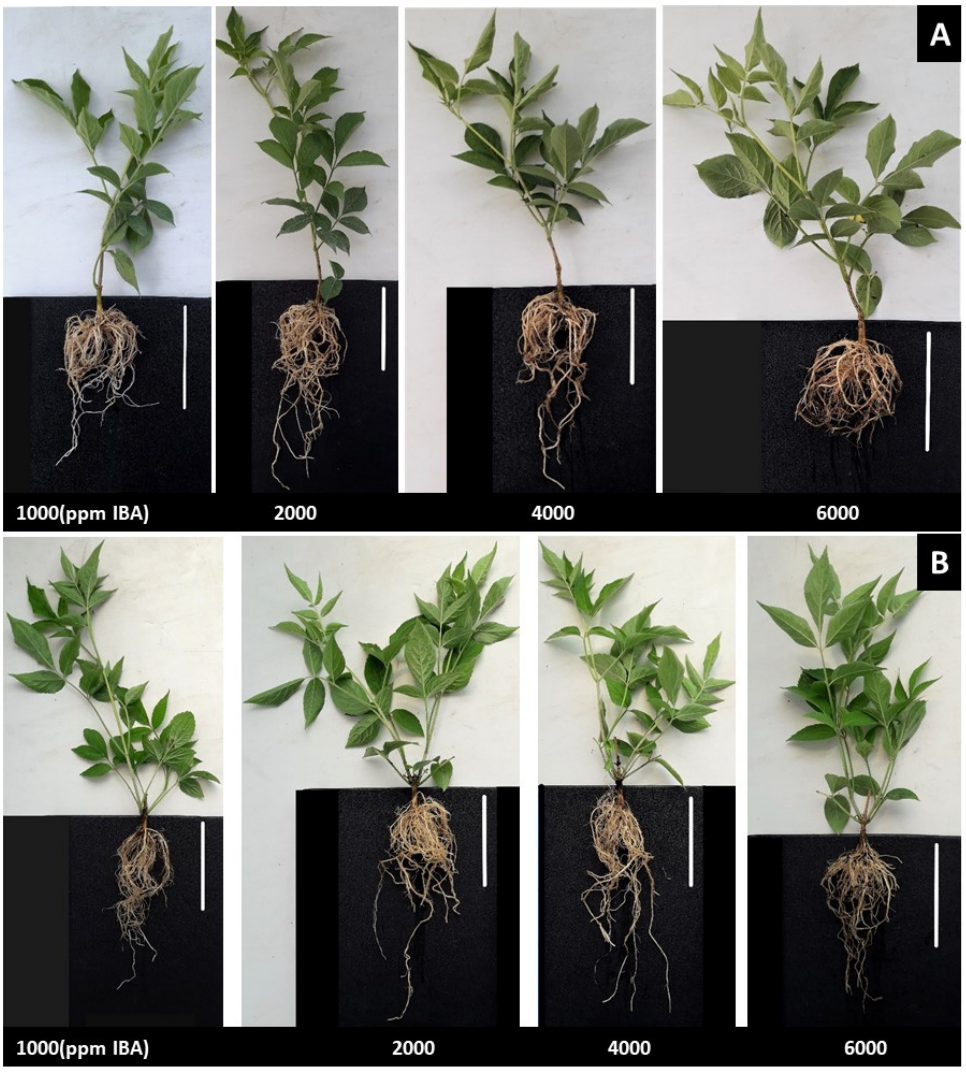


Figure 10. Representative photos of the successfully established plants at 40 days after rooting across treatments that hormone (indole-3-butyric acid, IBA) was applied from the 2020 propagation experiment of the prioritized Greek native genotype of *Sambucus nigra* GR-1-BBGK-19,192. Photos taken prior to destructive harvest for biomass accumulation assessment via dry weight measurements. Photos are grouped according to propagation treatments: (**A**) Apical softwood cuttings in peat–perlite (1:3, v/v); (**B**) Sub-apical softwood cuttings in peat–perlite (1:3, v/v). Higher root length/volume is discernible in hormone treatments of 2000 and 4000 ppm IBA in both cutting types in peat–perlite (1:3, v/v). Bars in photos represent 10 cm.

Figure 11. Representative photos of the successfully established plants at 40 days after rooting across treatments that hormone (indole-3-butyric acid, IBA) was applied from the 2020 propagation experiment of the prioritized Greek native genotype of *Sambucus nigra* GR-1-BBGK-19,192. Photos taken prior to destructive harvest for biomass accumulation assessment via dry weight measurements. Photos are grouped according to propagation treatments: (**A**) Apical softwood cuttings in peat–perlite (1:1, *v*/*v*) and (**B**) Sub-apical softwood cuttings in peat–perlite (1:1, *v*/*v*). For the 6000 ppm IBA treatments in sub-apical cuttings, plants in the photos are representative of those that managed to overcome the early leaf discoloration during rooting (Figure 9). Bars in photos represent 10 cm.

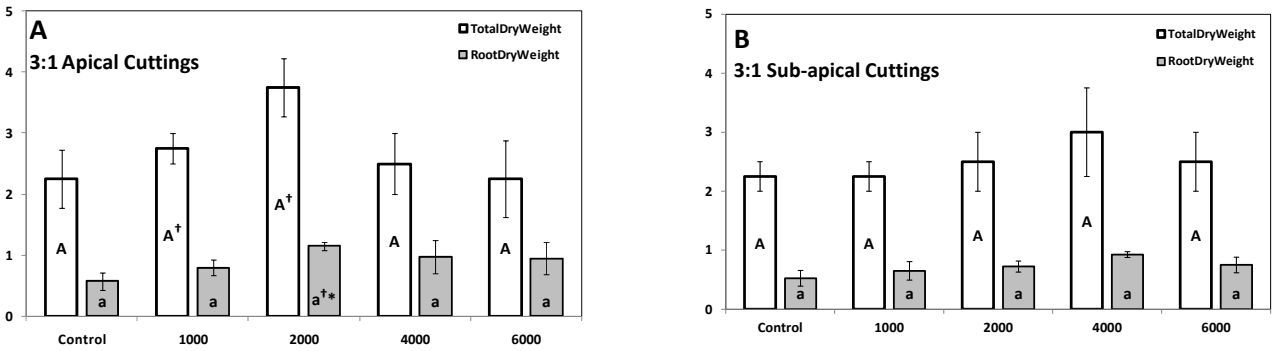


Figure 12. *Cont.*

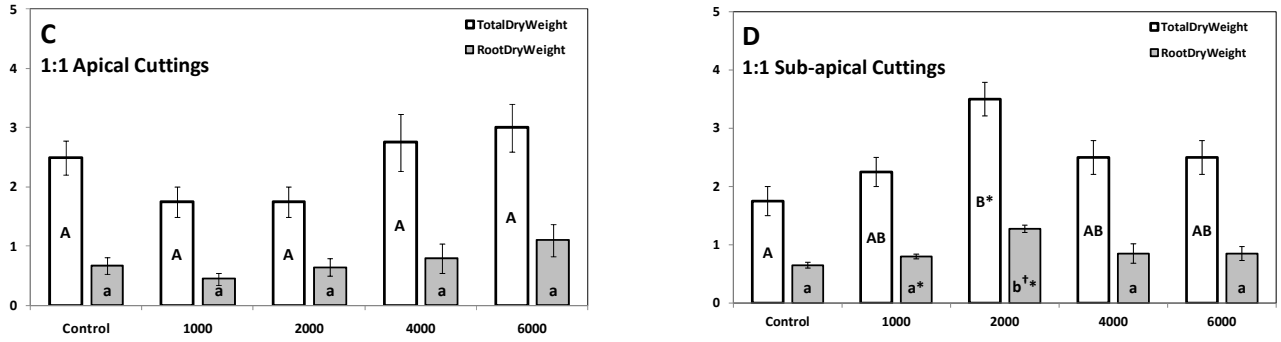


Figure 12. Plant biomass accumulation for each hormone treatment (ppm IBA) in terms of total plant dry weight (g) (white bars) and root dry weight (g) (grey bars) at 40 days after rooting within the 2020 experiment of *S. nigra* GR-1-BBGK-19,192. (**A**): peat–perlite (1:3, *v*/*v*) substrate with apical cuttings (that bear the stem's apical meristem). (**B**) peat–perlite (1:3, *v*/*v*) substrate with sub-apical cuttings (that were internode sections with two lateral buds). (**C**): peat–perlite (1:1, *v*/*v*) substrate with apical cuttings. (**D**): peat–perlite (1:1, *v*/*v*) substrate with sub-apical cuttings. Standard errors of the means are shown on the bars ($p < 0.05$). Bars that do not share the same letter are significantly different between hormone treatments within each substrate and cutting type for each graph (Tukey HSD, $p < 0.05$, capital letters for total plant dry weight and lowercase letters for root dry weight). Significantly higher values of the same hormone treatment and cutting type between the two substrates are denoted with † (comparisons between graph A and C for apical cuttings and B and D for sub-apical cuttings, $p < 0.05$). Significantly higher values of the same hormone treatment and substrate between the two cutting types are denoted with * (comparisons between graph (**A**,**B**) for 1:3 and (**C**,**D**) for 1:1, $p < 0.05$).

4. Discussion

4.1. Molecular Authentication of Greek Native Genotypes of Sambucus nigra

DNA barcoding is a valid technique for the discrimination of *Sambucus nigra* genotypes since it is not affected by the stage of plant development and may further enhance the classical morphological identification, offering insight regarding phylogenetic relationships of closely related species [28]. The study herein represents the first-ever report regarding the molecular authentication of Greek native germplasm of *Sambucus nigra*. The NJ (neighbor-joining) tree classification resulting from the use of barcoding technique in conjunction with the ITS2 gene was in accordance with internationally accepted phylogenetic relations, and it allowed the distinction of specific genotypes within the species *S. nigra*. Using the ITS2 barcoding region, the nine Greek native *S. nigra* genotypes evaluated herein were fingerprinted, and they were clearly discerned from other genotypes of *S. nigra* which do not originate from Greece or other members in the genus *Sambucus*. However, to confirm the application of this barcoding technique using the ITS2 sequence, different species of the genus Sambucus from different habitats in different regions of Greece and abroad should be further evaluated. Hence, the ITS2 gene can be an effective and valid marker for the identification of the species and of different genotypes of *S. nigra*.

4.2. Propagation of Greek Native Genotypes of Sambucus nigra

Asexual propagation via cuttings was conducted for Greek genotypes of *S. nigra* for the first time in the current study providing interesting data. High rates of rooting capacity among genotypes of softwood leafy cuttings taken in summer with the use of Indole-3-butyric acid (IBA) were observed, which are well above the commercially accepted threshold for propagation [43]. This observation is in agreement with similar work on this species elsewhere [31,44]. The rooting rates achieved during the summer were high, compared to similar studies on this species during the winter with hardwood cuttings [33], probably due to the vigor of growth during the summer. However, our results showed that the way maximum rooting capacity was achieved, varied between cuttings from different genotypes. The results of the screening experiment suggest that the genetic makeup of stock

plants seems to interact with external hormone application and cutting type to achieve successful rooting. The effect of genotype on rooting of cuttings has been shown to affect root number and length in interaction with external hormone application in other perennial minor crops [45,46], whereas in fruit species such as members of genus *Prunus*, genotype has been shown to affect both rooting of cuttings and plant establishment [47]. In another trial of a broader spectrum among different species, the genotype also affected rooting capacity in interaction with hormone [48]. For three out of nine different genotypes tested here, 100% rooting was achieved through apical cuttings between 2000 and 4000 ppm IBA, whereas the other six achieved 100% rooting through sub-apical cuttings between 2000 and 4000 ppm IBA. As such, the external hormone application between 2000–4000 ppm IBA seems to warrant consistency in high rooting rates of softwood cuttings according to the current results which probably stems from the interaction of external hormone with the cutting's internal activity of Indole-3-acetic acid (IAA) for cambium differentiation and rooting induction [49,50]. Rooting rates and quality in terms of root number and length has also been shown to be enhanced by external IBA application in other Greek endemic herbaceous perennials [51]. In addition, differences in rooting quality in terms of root number and length were observed between the genotypes tested, with one genotype in particular (GR-1-BBGK-19,192) presenting higher number of roots, which is considered a valuable trait [52].

The genotype GR-1-BBGK-19,192 was prioritized by being assessed against multi-faceted criteria which included effectiveness of molecular authentication (DNA barcoding) and ease of propagation in terms of rooting frequency, rooting quality and plant early establishment [43,52]. In addition, when the collected genotypes were established in field conditions, the superiority of GR-1-BBGK-19,192 was confirmed through higher plant growth rates that were observed.

Further and broader experimentation on propagation of genotype GR-1-BBGK-19,192 across a two-year period confirmed the benefit of external hormone application. Particularly, IBA levels at 2000–4000 ppm achieved 100% rooting irrespective of substrate or cutting type in 2019 but with a slight predominance of apical cuttings in 2020. Apical cuttings in woody perennials bear the highly active apical meristem, which affects internal and external hormone translocation and uptake [53,54]. This may be the reason why apical cuttings in 2020 presented higher rooting quality in terms of root number and length [43]. However, this was not the case in 2019, even though donor plant in both years were of similar developmental stage. The effect of the developmental stage of donor plants in rooting of cuttings is known for woody perennials with some species' younger plants presenting a more vigorous rooting of cuttings and early plant growth [55,56].

In addition to the above observations, significant leaf discoloration of sub-apical cuttings during rooting were observed in higher hormone levels of 4000 and 6000 ppm IBA in 2019 and in 6000 ppm IBA in 2020 (Figure 9), an effect which adversely affected plant survival and was significantly more profound in peat–perlite 1:1 (v/v) substrate, which contained less perlite and more peat than 1:3 (v/v). When perlite concentration increases when mixed with peat, it improves the physical (especially aeration) and hydraulic characteristics of the media [57,58]. This, in turn, may improve root development and plant growth [59]. However, further investigation with leaf sap analysis is suggested to shed light on this matter. As a result, plant growth patterns after rooting of 2019 for GR-1-BBGK-19,192 were not included in the analysis of the current data since significant effects on the results were encountered due to cuttings' leaf loss.

Concerning the consequent vegetative growth after rooting, the superiority of apical cuttings observed in 2020 rooting rates and quality in peat–perlite 3:1 (v/v) substrate reflected on the consequent plant height rates after rooting, which may stem from an apical dominance effect and/or the earlier and steadfast development of new roots. The prevalence of apical cuttings in terms of plant height was also observed in peat–perlite 3:1 (v/v) substrate of the 2019 experiment (data not shown). Sub-apical cuttings on the other hand, presented high leaf number rates in general, which coincided with the emergence of

lateral buds that was observed. Leaf emergence is affected by the presence of meristematic tissue on the system, but it is also affected by the differentiation level of morphological features closer to the base of the donor stem, such as phloem and xylem vessels [60,61]. However, further research may be required to investigate this connection in propagation via cuttings. The ability of newly rooted cuttings to produce large and vigorous plant bodies in a prompt manner is considered a crucial trait for successful adaptation ex situ, which was achieved in the current work within 40 days after rooting in both types of softwood cuttings used.

Considering all the above, the use of external hormone up to 4000 ppm IBA is proposed for successful propagation of *S. nigra* by softwood, leafy cuttings, coupled with a substrate with improved physical properties through the use of perlite under mist. IBA is a readily available substance of relatively low cost which provides good results, even though other hormonal substances such as 1-Naphthaleneacetic acid (NAA) have also been shown to induce rooting on this genus [62]. Cuttings should be taken during the summer from healthy, actively growing donor plants. The suggested protocol is considered fast, reliable, easy to implement and economically viable.

5. Conclusions

The current study focused on assessing the potential of wild-growing elderberry germplasm within the context of domestication of phytogenetic resources for sustainable utilization and commercial exploitation of neglected and underutilized species, reporting first-time data on the multifaceted assessment of Greek native *S. nigra* genotypes with first-ever molecular fingerprinting using the ITS2 sequence as molecular marker. Consecutively, the study herein presented the asexual propagation potential of this germplasm via cuttings after screening of various genotypes, highlighting the importance of external factors involved in their rooting, such as hormone application (IBA) and substrate, but also outlining internal factors such as genotype and cutting type. The study proposed a propagation protocol for wild *S. nigra* germplasm that is considered fast, reliable, easy to implement and economically viable with high potential for commercial production of plant material in high volumes. Finally, the establishment of an exemplary pilot cultivation system for Greek *S. nigra* germplasm is being attempted for the first time, aiming at screening the different genotypes under field conditions, thus setting the basis for domestication and sustainable utilization of this species of high pharmaceutical and nutritional value. Certainly, more multidisciplinary research is needed in agronomical and phytochemical aspects prior to any phenotyping and genetic improvement of the prioritized genotypes of Greek native *S. nigra*.

Author Contributions: Conceptualization, N.K. and I.G.; methodology, O.D., E.M., E.K., I.G., A.K., K.P., D.K., P.Y., N.N., G.P., I.G. and N.K.; software, I.G. and D.F.; validation, O.D., E.K., I.G., A.K., K.P., D.K., P.Y., N.N., G.P. and D.F.; formal analysis, O.D., E.K. and I.G.; investigation, E.K., O.D., I.G., A.K., K.P., D.K., P.Y., N.N., N.K., E.M. and G.P.; resources, E.M. and I.G.; data curation, O.D., N.K., E.K., K.P., I.G., N.N. and N.K.; writing—original draft preparation, E.K., O.D., I.G., D.F. and N.K.; writing—review and editing, O.D., E.M., E.K., I.G., A.K., K.P., D.K., P.Y., N.N., G.P., D.F. and N.K.; visualization, N.K., E.K., O.D., I.G. and D.F.; supervision, E.M. and G.P.; project administration, E.M. and G.P.; funding acquisition, E.M., N.N. and G.P. All authors have read and agreed to the published version of the manuscript.

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