

Article

The Development of a Micropropagation System for a Rare Variety of an Agricultural and Medicinal Elderberry Plant *Sambucus nigra* ‘Albida’

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Abstract

Black elder (*Sambucus nigra* L.) is a temperate shrub with flowers and fruits that are edible after processing. This species is not yet widely known in the global agricultural sector, but its adaptability and drought tolerance may generate more interest in this crop. Our study aimed to find suitable micropropagation techniques for the black elder ‘Albida’ and compare suitable statistical methods for evaluating multiplication and rooting. For micropropagation, we tested the Murashige and Skoog (MS) growth medium with selected auxins and cytokinins. Five proliferation MS media containing 1, 2, and 4 mg/L BAP or 0.5 and 1 mg/L TDZ were tested. To induce root formation, three types of auxins were tested at a concentration of 1 mg/L in a 50% MS medium: IBA, IAA, and NAA. Data analysis was performed using different parametric and nonparametric tests to robustly capture the effects of treatments across varying distributional scenarios in developing explants subjected to the interactions of internal native and externally added plant growth regulators. The average multiplication rate ranged from 1.6 to 2.0 shoots per explant. High multiplication was recorded on the MS medium with 1 mg/L 6-benzylaminopurine. The root number per rooted explant was highly variable, ranging from 3.0 to 12.0 roots per explant. The highest average root number result was observed when 1 mg/L α -naphthalenacetic acid was used. All rooted plants were successfully acclimated to normal growing conditions. This in vitro propagation protocol allows for the production of hundreds to thousands of rooted plants from one initial explant within one year, enabling faster introduction to the agronomic sector.

Keywords: black elder; small fruit; propagation; in vitro; multiplication; rooting



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1. Introduction

Black elder (*Sambucus nigra* L.) is one of the oldest medicinal perennial plants, used for therapeutic and dietary purposes. It contains many physiologically active ingredients, primarily in fruits and flowers [1–3]. The species belongs to the Adoxaceae family and the *Sambucus* genus. The native distribution range of *S. nigra* is difficult to trace because it has been widely used by humans since ancient times [4]. Its natural continuous distribution and isolated populations are found throughout Europe, except in the northernmost regions, in northern Africa, and in western Asia, mainly in the area stretching from Asia Minor through the Caucasus to the Caspian Sea [5]. Due to its adaptability, the black elder has also been successfully introduced worldwide, including North and South America and Australia [6,7].

Under natural European conditions, *S. nigra* grows abundantly in bright and semi-shaded areas, such as the edges of forests, in damp forest clearings, on rocky places, on field borders, and along roadsides. The species is also common in populated rural areas, along fences, and in house gardens, mainly in nutrient-rich soils, especially those rich in nitrogen content, including both wet and dry fertile soils. In its natural form, the black elder grows as a shrub or small tree, reaching heights of 4–10 m, with abundant branching. Its root system is shallow and flat. The leaves are alternate, petiolate, and odd-pinnate. The inflorescence is a flat-topped cluster, 10–25 cm in diameter (Figure 1). The fruit is a glossy, blackish-purple drupe, up to 6 mm in size [5–9].



Figure 1. The inflorescence of elderberry ‘Albida’ (photo taken by Jiří Sedlák).

Numerous studies have examined the chemical composition of elderberries and their applications in the production of pharmaceuticals, beverages, and food [1–3,10–18]. Additionally, there are studies on the antimicrobial activity of black elder leaves and flowers against plant and human pathogens [19,20].

Due to its adaptability and beneficial nutritious compounds, black elder—currently cultivated only on a small scale—is considered a promising candidate for expanding commercial plantings for fruit, leaf, and flower production [21–23]. Black elder is also valued for its ornamental qualities [7,9]. However, commercial cultivation is limited to a few countries, and germplasm resources in *Sambucus* collections are scarce [7,24,25].

In vitro tissue culture technologies offer new tools for enhancing the production of horticultural and agricultural crops [26–28]. For our experiments, we selected the elderberry Albida, which could also be of interest to the ornamental horticulture sector due to its exceptional white-green fruit (Figure 2). In addition to its ornamental value, this cultivar was also identified in a study by Diviš et al. [13], along with other researchers, as a good source of major and trace elements for human diets and functional foods. The micropropagation of *S. nigra* is a largely unexplored biotechnological field; therefore, there is a great potential to accelerate the commercial production of this agriculturally versatile and environmentally significant fruit species.



Figure 2. The fruit of elderberry ‘Albida’ (photo taken by Jiří Kaplan).

A robust statistical evaluation of the possible effects of the interaction between internal native and externally added artificial plant growth regulators is necessary for interpreting multiplication and rooting experiments. This issue has been mentioned in previous studies [29,30]; yet, an appropriate statistical approach to analyze the data distribution pattern remains elusive. Data from the multiplication and rooting of *in vitro* cultures are often processed using ANOVA tests [29–31]. However, these types of data are usually represented by integers, and the values may be quite low. Narrow maximum and minimum values usually limit the range of obtained values, which may affect the distribution of the data [32]. Since plants are usually exposed to chemical compounds that increase the efficiency of the multiplication and rooting, in addition to analyzing the mean, it is necessary to characterize the distribution quality and homogeneity of the categorical variables [33,34]. The second goal of this study was to process and compare the data on multiplication coefficients and rooting using different parametric and nonparametric tests, as well as to describe the conditions under which they can be used, to evaluate *in vitro* cultures.

2. Materials and Methods

Experiments concerning the *in vitro* propagation of the black elder ‘Albida’ were conducted at the Research and Breeding Institute of Pomology Holovousy Ltd. (RBIP, Holovousy, Czech Republic). Unlike the typical black-fruited cultivars of *Sambucus nigra*, ‘Albida’ exhibits an unusual white-fruited phenotype. Micropropagation experiments with *Sambucus nigra* ‘Albida’ were initiated in January–March 2012. Following *in vitro* multiplication and rooting, plantlets were acclimatized and cultivated for two years in a greenhouse before field planting. After several years of juvenile growth, plants entered the fruiting phase. Over five consecutive years of fruit harvests, the stable white fruit color and absence of somaclonal mutations were confirmed, demonstrating that the used micropropagation protocol did not induce any genetic variation in this important trait. After this verification, statistical evaluation of the micropropagation data was subsequently performed in 2024. The genotype under study is currently a part of the gene pool collection of the national program for the conservation of genetic resources in the Czech Republic (Figure 3). It has been phenotypically verified for cultivar authenticity based on the standard morphological descriptors for *S. nigra*.



Figure 3. A low-stemmed tree of elderberry ‘Albida’ (photo taken by Jiří Sedlák).

2.1. *In Vitro* Culture Initiation

The shoots were collected during the dormancy period in winter. They were washed under clean running water. This step aims to remove part of the microbial flora that survives on the shoot’s surface in outdoor conditions. The shoots were then cut into 20 to 25 cm long segments. After making an angled cut at the bottom, the shoots were submerged in water and left to sprout for 2 to 3 weeks at a laboratory temperature of 22 °C. Shoot tips with a differentiated growth apex measuring 5–10 mm were dissected from the sprouting vegetative buds in a sterile environment using a flow box. This process removed bud scales, leaf parts, and other surface structures that could hinder sterilization. Mercuric chloride (0.15%), with the addition of the surfactant Tween 20, was used as the sterilizing agent. The sterilization time was 1 min. The sterilizing agent was then rinsed off the explants with sterile distilled water. Nine of the best morphologically differentiated explants were selected for *in vitro* culture initiation. All sterile manipulations were carried out in a laminar flow hood. The sterilized explants were placed in 100 mL Erlenmeyer flasks (3 shoots per flask), each containing 25 mL of a modified MS [35] medium (Table 1).

Table 1. Modified MS medium for the *in vitro* cultivation of *S. nigra*.

Compound	mg/L	Compound	mg/L
KNO ₃	1900	Na ₂ MoO ₄ ·2H ₂ O	0.25
NH ₄ NO ₃	1650	CoCl ₂ ·6H ₂ O	0.025
CaCl ₂ ·2H ₂ O	440	CuSO ₄ ·5H ₂ O	0.025
MgSO ₄ ·7H ₂ O	370	Inositol	100
KH ₂ PO ₄	170	Glycine	2
Na ₂ EDTA·2H ₂ O	37.3	Nicotinic acid	0.5
FeSO ₄ ·7H ₂ O	27.8	Pyridoxine	0.5
MnSO ₄ ·H ₂ O	16.9	Thiamine	0.1
ZnSO ₄ ·7H ₂ O	8.6	Ascorbic acid	4
H ₃ BO ₃	6.2	Sucrose	30,000
KI	0.83	Agar (Difco Bacto)	7000

During the initiation phase, we added 1.5 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L indole-3-butyric acid (IBA). The pH of the medium was adjusted to 5.8 prior to adding the agar and autoclaving at 120 °C and 100 kPa for 15 min. All cultures were incubated in a growth room under a neutral-white fluorescent light provided by Sylvania F18W/840-TB tubular lamps (Germany), at a light intensity of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$, at 22 ± 1 °C with a photoperiod of 16/8 h. The lamps were positioned 30 cm above the level of the cultures. The contamination rate, survival rate, and shoot development were analyzed after sterilization. If any exudation occurred from the initial explants, they were transferred to a fresh medium. Any malformed explants were discarded. All explants were screened for microbial contamination one week after surface sterilization. Protruding *in vitro* cultures (Figure 4) were successively screened at weekly intervals for 2 months, before the explants were used in multiplication experiments (Figure 5). The developing shoots were serially subcultured onto fresh media for six consecutive 4-week passages. This provided a stock collection of shoots for multiplication studies.



Figure 4. *In vitro* culture of elderberry ‘Albida’ in the initiation phase (photo taken by Matěj Semerák).



Figure 5. *In vitro* culture of elderberry ‘Albida’ in the multiplication experiment on the MS medium with 1 mg/L BAP (photo taken by Jiří Sedlák).

2.2. Multiplication Experiment

Uniformly developing shoots (10–15 mm in length, including the apex) were detached from previously cultured explants and transferred to a fresh medium in 100 mL Erlenmeyer flasks to promote shoot proliferation. The culture conditions during the multiplication experiment were the same as those during the initiation of *in vitro* cultures. In our study,

we tested five proliferation MS media containing 1, 2, and 4 mg/L BAP and 0.5 or 1 mg/L thidiazuron (TDZ). TDZ was sterilized via filtration (25 mm, 0.2 µm Supor® Membrane, Pall Corporation, Port Washington, NY, USA) and added to the media after autoclaving.

The proliferation rate was defined as the number of shoots (>10 mm) per initial explant after 30 days of culture. Callus formation and shoot morphology were also determined for each growth regulator concentration. Twenty-five initial explants in four repetitions per treatment were used for each concentration of the respective growth regulator.

2.3. *In Vitro* Rooting and Acclimatization to Ex Vitro Conditions

After a sufficient number of in vitro shoots were multiplied in the proliferation phase, their rooting ability was tested. Single shoots (15 to 25 mm in length) were excised and planted in a modified 50% MS medium containing 1 mg/L of either IBA, indole-3-acetic acid (IAA), or α -naphthalenacetic acid (NAA) to test root induction. The culture conditions during root initiation and root growth were the same as those during the initiation of in vitro culture and the multiplication experiment. Ten shoots in five repetitions were used for each treatment, and the shoots were exposed to the in vitro rooting treatment for 30 days. Rooting was evaluated based on the percentage of rooted shoots and the number of roots per rooted explant. The rooted shoots (Figure 6) were then removed from the Erlenmeyer flasks, rinsed with water to remove the medium, and then transferred to peat ‘jiffy’ pots (Jiffy 7, AS Jiffy Products, Kristiansand, Norway), which were soaked with sterilized water. Roots extending beyond the volume of the ‘jiffy’ pot were shortened. Black elder plantlets were surface-sprayed with water to prevent wilting and to avoid desiccation during transplanting. After transfer, the Jiffy 7 pots containing the rooted plants were placed in trays under a clear transparent plastic cover in an environment with 100% air humidity. The trays were incubated at 22 °C under a 16 h light/8 h darkness cycle in a growth chamber equipped with cool-white fluorescent tubes at a light intensity of 60 µmol m⁻² s⁻¹. The developing and protruding plants were gradually acclimated by opening the covers for 14 days to reduce humidity gradually.



Figure 6. In vitro rooting on the MS medium with 1 mg/L NAA (photo taken by Jiří Sedlák).

2.4. *Statistical Evaluation of Multiplication and Rooting Experiments*

The experiment was performed using a completely randomized design. In all experiments, the independent variables were the type and concentration of externally applied plant growth regulators (BAP and TDZ for multiplication; IBA, IAA, and NAA for rooting). The dependent variables were as follows: for multiplication, the number of shoots (>10 mm) per explant after 30 days of culture and for rooting, the percentage of rooted shoots and the number of roots per rooted explant. Prior to statistical processing, the data were examined for normality of residuals and homogeneity of variance using exact Shapiro–Wilk and Levene

tests, respectively. According to the results of the observation analysis, the explant multiplication coefficient data were processed differently from the in vitro rooting data because of the categorical nature of the multiplication variable. The multiplication data were then processed using the following statistical tests: one-way ANOVA, Kruskal–Wallis, and Chi-squared (χ^2). The in vitro rooting data were processed using only one-way ANOVA. For the ANOVA and Kruskal–Wallis tests, the data were gathered as measured values for both the plant shoots and roots. Rooting percentage data were analyzed without arcsin transformation, as the normal distribution of residuals and homogeneity of variance were confirmed by Shapiro–Wilk and Levene tests, respectively. For the χ^2 test, explants were grouped according to the exact number of shoots (>10 mm) produced per explant after 30 days of culture; each such group represents a “multiplication coefficient class” (class 1 = 1 shoot, class 2 = 2 shoots, class 3 = 3 shoots, and class 4 = 4 shoots), and the frequency of explants in each class was used for the frequency distribution analysis. To compare the means among the treatments analyzed with ANOVA tests, LSD, HSD, and Duncan’s multiple-range tests were used. Results were considered statistically significant at $p < 0.05$. All statistical analyses were performed using R (R Development Core Team, 2010, version 2025).

3. Results

3.1. Initiation of Micropropagation and Multiplication

In our procedure for establishing an in vitro culture, we successfully initiated cell division and growth in all nine initial explants selected for the experiment. We observed elongation growth with a clearly differentiated growing apex. Three of the nine initial explants were visibly contaminated with microorganisms. We discarded these explants. Six uncontaminated emerging shoots were subsequently transferred to the multiplication medium, and they served as the basis for further multiplication.

Observation analyses of the multiplication confirmed that the data were subjected to χ^2 , i.e., not normal distribution (Figure 7). However, the results of parametric and nonparametric ANOVA remained similar due to the large number of observations. The only difference was in the post hoc test results, where the treatment using 1 mg/L TDZ was not distinguished from the 1 and 2 mg/L BAP treatments with higher multiplication coefficients when analyzed by HSD tests (Table 2).

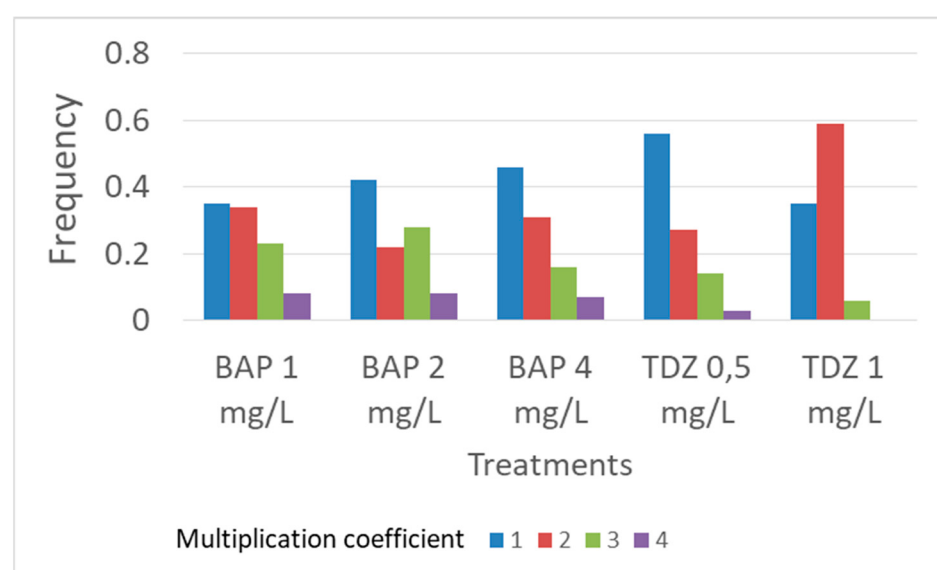


Figure 7. Frequency of shoot multiplication coefficient (1–4) distribution observed for different treatments.

Table 2. Mean number of shoots per explant for each treatment, with results from the ANOVA test combined with the Tukey HSD, Fisher LSD, and Duncan’s multiple-range test, and Kruskal–Wallis tests.

Treatment	Shoot Number/Explant	HSD	LSD	Duncan	K-W
BAP 1 mg/L	2.04	a	a	a	a
BAP 2 mg/L	2.02	a	a	a	a
BAP 4 mg/L	1.84	ab	ab	ab	ab
TDZ 0.5 mg/L	1.64	b	b	b	b
TDZ 1 mg/L	1.71	ab	b	b	b

Different letters represent significant differences among the treatments for a particular test at $p < 0.05$.

For elderberry ‘Albida’, we confirmed that adding growth regulators, cytokinins BAP and TDZ, can promote cell division in vitro, thereby inducing growth and multiplication (Table 2). The average multiplication ranged from 1.64 to 2.04. A high multiplication rate, 2.04, was noted for the MS medium with the lowest BAP concentration (1 mg/L). A comparable multiplication coefficient was observed with 2 mg/L BAP, as the difference from 1 mg/L BAP was not statistically significant. In contrast, both TDZ treatments resulted in lower multiplication coefficients.

Data analysis using the χ^2 test yielded different and more detailed results than the parametric and nonparametric ANOVA tests (Table 3). Here, the means were used to describe the multiplication coefficient instead of the median to provide a more meaningful description of the results. The results describing the occurrence of particular classes in different treatments are presented as frequencies relative to the total number of explants per treatment. The purpose of this test was to compare the distribution frequency among the treatments in different classes. When comparing treatments by all observed multiplication classes, the results showed that BAP1 and BAP2 had a higher frequency of explants with a greater number of shoots than both TDZ (BAP1:TDZ0.5, $\chi^2 = 10.111$, $df = 3$, p -value = 0.018; BAP1:TDZ1, $\chi^2 = 24.686$, $df = 3$, p -value = 1.796×10^{-5} ; BAP2:TDZ0.5, $\chi^2 = 9.4496$, $df = 3$, p -value = 0.024; BAP2:TDZ1, $\chi^2 = 39.773$, $df = 3$, p -value = 1.19×10^{-8}). TDZ1 differed from all the other treatments in frequency of the multiplication classes (TDZ1:BAP4, $\chi^2 = 21.75$, $df = 3$, p -value = 7.352×10^{-5} ; TDZ1:TDZ0.5, $\chi^2 = 22.953$, $df = 3$, p -value = 4.13×10^{-5}).

Table 3. Mean number of shoots and the results of the comparison among treatments, comparing the full set of observed classes represented by different multiplication coefficients as well as the frequency of occurrence of particular classes among the treatments using a χ^2 test.

Treatment	Shoot Number/Explant	All Classes	Class 1	Class 2	Class 3	Class 4
BAP 1 mg/L	2.04	a	35 b	34 b	23 ab	8 a
BAP 2 mg/L	2.02	a	42 ab	22 b	28 a	8 a
BAP 4 mg/L	1.84	ab	46 ab	31 b	16 ab	7 a
TDZ 0.5 mg/L	1.64	b	56 a	27 b	14 bc	3 ab
TDZ 1 mg/L	1.71	c	35 b	59 a	6 c	0 b

Different letters represent significant differences among the treatments for particular test at $p < 0.05$.

To examine the results in more detail, we determined whether the frequency distribution of multiplication coefficient classes (i.e., the number of explants producing a given number of shoots) differed among the treatments. The proportion of explants with one shoot (class 1, Table 3) for TDZ0.5 was significantly higher than that obtained for BAP1 and TDZ1. The other treatments gave intermediate results ($\chi^2 = 8.065$, $df = 1$, p -value = 0.005). The class 2 explant occurrence was higher with TDZ1 than with all the other treatments (BAP1:TDZ1, $\chi^2 = 11.577$, $df = 1$, p -value = 6.678×10^{-4}). BAP2 had a higher frequency of

class 3 explants, while TDZ1 had the lowest ($\chi^2 = 15.627$, $df = 1$, $p\text{-value} = 7.714 \times 10^{-5}$). TDZ0.5 was the only treatment with a similar frequency of class 3 explants as TDZ1. Similar results were found among treatments in class 4. However, TDZ 1 showed a pronounced skew toward class 2, with 59% of explants producing two shoots per explant.

Throughout the multiplication phase, all regenerated shoots exhibited normal morphology, with no visible abnormalities such as hyperhydricity, vitrification, or fasciation observed across any of the tested cytokinin treatments. Importantly, callus formation was not detected at the base or along the length of the explants during shoot proliferation.

3.2. In Vitro Rooting and Acclimatization to Ex Vitro Conditions

All 150 initial shoots on the rooting medium developed roots across all three treatments using the auxins IAA, IBA, and NAA. Thus, the rooting ratio reached 100%. However, the number of roots per rooted shoot varied greatly, ranging from 3.04 to 12.04 roots per explant (Figure 8). The best in vitro rooting results were observed when 1 mg/L of the auxin NAA was used.

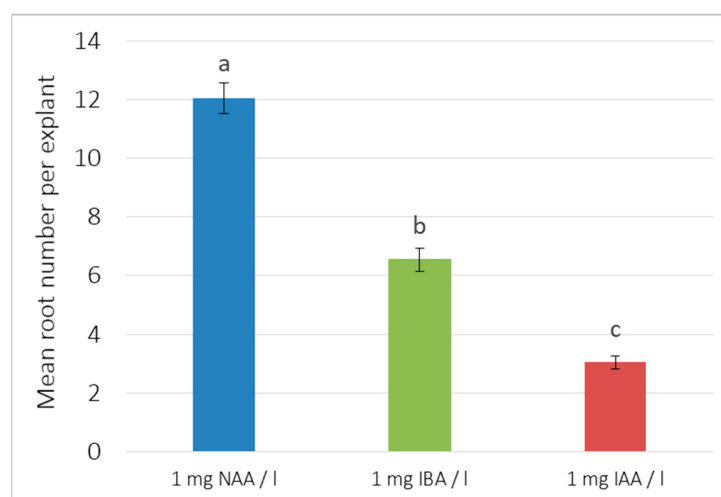


Figure 8. Comparison of the mean root number per explant analyzed by ANOVA tests. The standard deviation of the means is represented by the error bars. Different letters mean statistically significant differences according to Tukey HSD tests at $p = 0.05$, which gave the same result as LSD tests.

Observation analysis confirmed a normal distribution of the explant root numbers across all observed treatments. All treatments were clearly separated according to the statistical analysis, and the results of the HSD, LSD, and Duncan's post hoc tests were consistent. Figure 8 illustrates the mean root number per explant analyzed by ANOVA tests.

During the rooting experiments, root systems developed directly from the basal part of the shoots without any intervening callus layer between the explant base and the newly formed roots. All roots displayed a typical morphology and healthy structure, and no callus formation was observed in any treatment throughout the rooting phase. All rooted black elder plants were successfully acclimated to normal field growing conditions, demonstrating the suitability of the chosen approach. During gradual acclimatization and ambient air humidity reduction, no symptoms of wilting or yellowing shoots and leaves were observed. After acclimatization, all plants transferred from in vitro culture exhibited morphological similarity of shoots and leaves to the 'Albida' mother plants in the RBIP gene pool collection.

4. Discussion

Contamination by fungi and bacteria after the initial explants were sterilized was rare, probably because the shoots that sprouted in the laboratory were not directly exposed to the field microflora. Additionally, taking explants from expanding shoots soon after bud break may have greatly aided in establishing sterile in vitro cultures.

A very limited number of research papers have been published regarding the multiplication and rooting of black elder in vitro cultures. Charlebois and Brassard examined the suitability of various medium compositions for the multiplication and rooting of five *S. canadensis* cultivars. On average, the MS medium containing 1 mg/L BAP and 5 µg/L NAA induced slightly higher multiplication rates than treatments with 2 mg/L BAP and 5 µg/L NAA. For rooting, the 50% MS medium containing 1 mg/L BAP and 5 µg/L NAA was most effective [36]. Research on *S. nigra* cultivars showed that both BAP (2 mg/L) and 0.5 or 1 mg/L *meta*-Topolin (mT) produced satisfactory multiplication results. However, mT had a better effect on shoot elongation. Explants rooted readily on the medium containing 0.5 mg/L mT and 0.1 mg/L NAA [37]. For *S. williamsii*, a combination of 3 mg/L BAP, 0.2 mg/L NAA, 0.03 mg/L TDZ, and 2 mg/L GA₃ was successfully used in the multiplication phase. The addition of 0.4 mg/L NAA and 0.2 mg/L IBA worked best for root induction [38]. Our results for elderberry ‘Albida’ are in line with these findings, as high multiplications were achieved on the MS medium supplemented with 1 mg/L or 2 mg/L BAP. Our multiplication intensity (1.64–2.04 shoots per explant) falls within the lower part of the range reported for other *Sambucus* species and cultivars, confirming the importance of genotype. Unlike studies using mT or more complex cytokinin/auxin combinations, our protocol, especially with BAP alone, proved sufficient for the effective multiplication of ‘Albida’.

Additional research has been conducted on other species within the Viburnaceae family. An MS medium containing 2.5 mg/L 6-(γ,γ-dimethylallylamino)purine and either 0.5 or 1 mg/L BAP was recommended for the multiplication of *Viburnum opulus* ‘Nanum’. During the rooting phase, a concentration of 2.5 mg/L NAA proved optimal [39]. Another representative of the species *V. opulus*, the cultivar ‘Roseum’, formed lateral shoots particularly well when either BAP (1 or 2 mg/L) or kinetin (2 or 4 mg/L) was added to the MS medium. Both auxins, NAA and IBA, proved efficient for root induction. The highest rooting percentage and average number of roots per explant were observed in the full-strength MS medium with 0.5 mg/L NAA. However, the 50% MS medium with 0.5 mg/L IBA resulted in the longest roots [40]. The same genotype achieved a high multiplication rate on the Woody Plant Medium (WPM) supplemented with 1 mg/L BAP and 0.15 mg/L IBA and rooted well on the 50% WPM supplemented with 0.3 mg/L NAA and 0.3 mg/L activated charcoal [41]. The WPM also provided good results for *V. treleasei*. Adding 0.25 mg/L BAP for shoot multiplication and 0.24 mg/L NAA for rooting was effective [42]. The WPM supplemented with 0.1 mg/L BAP and 4.85 mg/L GA₃ was recommended for the multiplication phase of *V. odoratissimum*. NAA at a concentration of 1.1 mg/L showed the highest potential for the induction of rooting. However, excessive callus formation occurred. Therefore, IBA at a concentration of 0.6 mg/L proved more suitable [43]. Similarly, in our study, the best rooting of elderberry ‘Albida’ shoots was achieved on the 50% MS medium with 1 mg/L NAA, resulting in up to 100% rooting and an average of 12.04 roots per explant, which is comparable or superior to the results reported for other Viburnaceae species. Importantly, we did not observe excessive callus formation during rooting, which is sometimes reported in the literature for higher auxin concentrations. All rooted plants were successfully acclimated, confirming the practical applicability and robustness of our protocol in comparison with previous studies on both *Sambucus* and related genera.

The data on explant multiplication did not follow a normal distribution. As Cochran and Cox [34] described, the assumptions of ANOVA, i.e., the normal distribution of residuals, homogeneity of the variance, and independence of observations, need to be met before data analysis. Although Fisher [44] describes one exception that should compensate for the normal distribution of the residuals when the dataset has a sufficient number of observations per treatment (so called asymptotic theory), Lehmann [45] pointed out that this method may compromise the accuracy of the test results because this approximation is not distribution-free but, instead, depends both on the sample size and on the underlying distribution. Therefore, in cases in which the results of parametric and nonparametric tests are similar, the parametric alternative could be a better option. However, both the parametric and nonparametric analyses should be tested before making a decision. In this case, our parametric and nonparametric ANOVA provided consistent results, and parametric ANOVA was preferred. Among the ANOVA post hoc tests, the least significant difference test and Duncan's multiple-range test should provide adequate consideration of the differences among the analyzed treatments due to similar results compared to the Kruskal–Wallis test. In contrast, processing the data with the χ^2 test allows us to identify differences in the frequency of occurrence of explants in specific multiplication categories. This is particularly important for explants with an unknown concentration of internal native growth regulators that affect the number of newly produced shoots in a growing medium with the external addition of different concentrations of artificial phytohormones antagonistically or synergistically. The χ^2 test thus allows us to further characterize the uniformity of the multiplication pattern in proliferation experiments. A dominant frequency in a single category can be highly advantageous because it yields a uniform cohort of explants with predictable shoot numbers. This, for example, simplifies downstream automated handling.

Our results show that the in vitro cultivation protocols for elderberry 'Albida' are similar to other Viburnaceae representatives. The potential of 1–2 mg/L BAP for lateral shoot formation was confirmed. However, the multiplication coefficients remained relatively low compared to some other studies and showed only modest variation in response to different cytokinin concentrations. With a subcultivation interval of one month, even a multiplication coefficient of 2 would theoretically yield around 1000 shoots from a single initial explant within 10 months. The genotype also exhibited outstanding rooting capacity, particularly in response to NAA, which is consistent with previously published studies on *Sambucus*.

Our laboratory protocol enables the rapid micropropagation, rooting, and growth of the selected genotype. In addition to facilitating highly effective mass propagation and the production of healthy plant material, in vitro culture enables the safe exchange of plant material across countries and continents [46–48]. This can also be a very useful tool for gene bank curators to enrich their collections of genetically valuable plant resources in the agricultural sector [49]. For these reasons, the results of the present study are expected to contribute to faster in vitro propagation for commercial fruit growing and ex situ techniques for conserving *S. nigra* biodiversity.

5. Conclusions

The established micropropagation protocol enables efficient and steady multiplication of black elder, also applicable to rare or valuable genotypes, supporting their conservation and long-term maintenance in gene banks and germplasm collections. Moreover, having enough initial planting material could help this lesser-known species be integrated more quickly into the global agricultural business. Cultivated varieties of black elderberry are economically significant because these cultivars provide disproportionately higher

yields per hectare than wild elderberry. Unlike harvesting from wild stands, cultivated varieties provide consistent quality and reliability, making them more attractive to food or pharmaceutical processors. Biotechnological studies on the in vitro culture systems would benefit mass and future *S. nigra* breeding programs aimed at improving fruit quality and yield.

The discrete nature of shoot and root number data inherently reflects the stochastic behavior of meristem activation and cell proliferation. Thus, the concurrent use of parametric (ANOVA) and nonparametric (Kruskal–Wallis) tests is necessary to robustly capture treatment effects across varying distributional scenarios. This complex and integrated statistical framework (including the χ^2 test), which describes the frequency distribution analysis of the number of newly formed shoots during multiplication, enhances the biological relevance and reproducibility of in vitro propagation protocols, paving the way for scalable micropropagation systems.

Currently, RBIP is investigating methods for propagating additional species and genotypes of lesser-known and horticulturally promising fruits. This includes techniques for rooting and transferring them to standard field or greenhouse cultivation conditions. The aim is to diversify the portfolio of cultivated crops in the agricultural sector.

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