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An Efficient Micropropagation Protocol for the Endangered European Shrub February Daphne (*Daphne mezereum* L.) and Identification of Bacteria in Culture

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Abstract: *Daphne mezereum* of the *Thymelaeaceae* family is a medicinal shrub occurring naturally in Europe and under legal protection in Poland. In the present study, a protocol developed for mass propagation of February daphne from nodal explants is presented. Micropropagation is one of the in vitro techniques that allow the preservation of rare and valuable plants by developing efficient methods for their propagation. In the proliferation stage, explants were cultured in the Woody Plant Medium (WPM) with different cytokinins, and in the rooting stage on the semi-solid WPM medium with perlite, indole-3-butyric acid (IBA) in various concentrations was used. The maximum proliferation rate (five shoots per explant) was observed on the medium containing 4.14 µM of meta-Topolin (mT). When the regenerated shoots were rooted in vitro in the presence of IBA in various concentrations, that of 19.68 µM induced the highest number of roots per shoot (6.63) and the maximal root length (2.15 cm). It is also worth remembering that plants are often colonized by different groups of microorganisms, which also affect the diversity of the ecosystem. The endophytic bacteria inhabiting the *D. mezereum* shoots are *Mycobacterium*.

Keywords: in vitro; medicinal plant; endophytic bacteria; plant growth regulators

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1. Introduction

It is obvious that agricultural expansion is driven by the development of society and the growing demand for food. It is also natural that, for its own sake, humans are transforming ecosystems and increasing agricultural acreage. However, it is important to realize that in the 1980s and 1990s alone, the world's agricultural land area increased by more than 629 million hectares, most of which was gained from the conversion of forests, grasslands and other natural habitats [1,2]. This poses a huge threat to mature, species-rich forest ecosystems, so ways must be sought to protect them. One way is to protect the species of valuable trees and shrubs that form the basis of these habitats and to multiply them ex situ, so that they can be restored to the wild and rebuild a given ecosystem if necessary. The application of tissue culture in the propagation of medicinal plants and in the protection of plant species is most in favor of the use of tissue culture: the inefficiency of traditional propagation methods, and, a major advantage of in vitro methods, high efficiency. This is especially important in very small populations where a large number of new plants can be produced from a small amount of starting material [3,4]. *Daphne mezereum*, commonly known as mezereon or February daphne, is a shrub covered by legal species protection in Poland and also a medicinal raw material. It grows to an average height of 1–1.5 m, while its stems are stiff, upright and weakly branched. Leaves of bay laurel are seasonal, lanceolate or narrow-ovate (up to 8–12 cm long) and characteristically placed at the top of the shoot. Flowers in the species are pink, tubular, four-petaled, densely set on the shoots

and extremely fragrant. They appear on last year's shoots at the site of scars from fallen leaves, before the development of leaves [5]. If the weather is favorable, the first flowers of this shrub open in Central Europe as early as February, although the main flowering date is March–April [6]. *Daphne mezereum* is pollinated by both nocturnal butterflies (moths) and bees, or large pollinating insects mentioned in several other publications, such as the popular day-flying butterfly *Gonepteryx rhamni* or bumblebees (*Bombus* spp.). It is, therefore, an important part of the ecosystem. February daphne owes its sweet and intense fragrance to terpenes. According to research, 95% of the volatile compounds affecting this characteristic are linalool and its derivatives [7]. Its poisonous, as well as medicinal, properties were known in ancient times and were readily used [5]. In the 18th century, the February daphne was included in the Polish medical guide next to plants such as mallow, lovage, yarrow, raspberry, hops and mint. The raw material was *Daphne* leaves, and the bush was described as a wild-growing plant [8]. *D. mezereum* owes its properties (both medicinal and poisonous) to a resinous substance called mezerein and to coumarins: daphnin and daphnetin [9,10].

February daphne is a shrub whose shoot cuttings do not root, even under favorable conditions, or root in a small percentage, making this method completely unprofitable (unpublished own research). The only way to obtain new plants is by sowing seeds or using modern techniques, which include micropropagation. The main task is to ensure optimal conditions at each stage of culture for the type of explants and the plant they come from, in such a way as to unleash the greatest possible regenerative potential and obtain a good-quality final product. In general, we can divide the procedure into four main stages: initial, multiplication (in woody plants, shoot proliferation), rooting (in vitro or ex vitro) and acclimatization to external conditions [11,12]. During the first stage, it is important to choose the right concentrations of disinfectant solutions and the timing of their effect on plant fragments, so as not to destroy the initial explants. Despite the biocides used, there are often still bacterial spills in the subsequent stages, which hinders effective micropropagation. They should, therefore, be eliminated from the cultures, so that they do not compete with the plant explants. Endophytic bacteria live within plants without causing any substantial damages or without any visible effect, as they only benefit from living within plant tissues [13]. However, they may become harmful in the in vitro cultures due to fast multiplication as compared to slower plant tissue growth. Endophytes may, as well, positively affect plants and live with them in symbiosis, which has not been fully elucidated until now. It may be supposed that apart from living space, bacteria acquire from plants compounds necessary for colony growth and development [14,15].

This article aims to present a ready-made protocol for the micropropagation of *Daphne mezereum*, for which to date there is no published complete and effective procedure for obtaining new plants in vitro. In addition, attention is focused on endophytic bacteria colonizing shoot cultures of this shrub. The complete data on this subject arising from several years of our own research are presented below.

2. Materials and Methods

2.1. Medium Preparation

To initiate the cultures, the MS initiation medium according to Murashige and Skoog [16] (Duchefa Biochemie B.V, Haarlem, The Netherlands) was prepared with 2% sucrose (Diamant, Pfeifer & Langen Polska S.A., Gostyń, Poland) without any growth regulators ("0" medium). At the multiplication (shoot proliferation) and rooting stages, it was the WPM medium Woody Plant Medium [17] (Duchefa Biochemie B.V, Haarlem, The Netherlands) due to the light discoloration of the leaves that appeared during the stabilization of the cultures. Based on previous studies, it has also been shown that regenerated laurel explants on the WPM medium have a higher chlorophyll and sugar content, compared to those on the MS medium [18]. The medium pH was adjusted to 5.8 with 1 M solutions of HCl and NaOH (Chempur®, Piekary Śląskie, Poland). The source of carbohydrates was sucrose at a concentration of 3% at the stage of culture stabilization and shoot multiplication, and 2% at

the rooting stage. The solidifying ingredient was Bacto™ Agar 8 g/L (Becton, Dickinson and Company, Sparks, MD, USA), a semi-liquid medium with only 3 g/L of agar at the rooting stage. The medium was dispensed at about 50 mL each into 450 mL jars. The jars were closed with transparent twist-off caps and autoclaved at 121 °C and 1.2 kg·cm^{-2} (110 kPa) for 20 min. Each combination of the experiment consisted of 6 jars of 5 plants each (30 plants/combination).

2.2. Explant Preparation

The starting material came from labeled and described shrubs growing in a certified and reputable nursery (affiliated with the Polish Nurserymen's Association, Warsaw, Poland), which produces rare ornamental shrubs, including protected species. This provides guarantees as to the identity of the species. Plants for culture initiation came from 3-year-old shrubs growing in pots from a commercial container nursery of ornamental plants. Young shoots of about 10 cm in length were taken in spring April–May (after flowering) and then divided into smaller fragments, and all leaves were cut off (Figure 1A,B). First, the shoot fragments were rinsed twice in distilled water with 2–3 drops of Tween 20 (Sigma-Aldrich®, Merck Life Science, Darmstadt, Germany) for about 15 min. Then, they were decontaminated in 70% ethanol (Chempur®, Piekary Śląskie, Poland) for 1 min, followed by 5 min in a 3% hydrogen peroxide solution (H_2O_2) (Chempur®, Piekary Śląskie, Poland), and then for 15 min in 1% sodium hypochlorite (NaOCl) solution (Chempur®, Piekary Śląskie, Poland) with Tween 20, and finally washed in autoclaved distilled water. The material was cut into one- or two-node fragments and placed on the previously prepared initial MS medium (Figure 1C). With the appearance of bacteria in the initiated *D. mezereum* cultures, explants or already regenerated shoots were passaged onto a new medium on average every 2 weeks (Figure 1E). In the event that more bacteria flowed out of the tissues, an MS medium was made according to the above-mentioned scheme, to which either 1 mL/L PPM (Plant Preservative Mixture, active ingredients: 5-chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone) (Plant Cell Technology, Washington, DC, USA) or 1 mg·L^{-1} colloidal silver was added (alternately to prevent immunization of the bacteria).

2.3. Identification of Bacteria in Cultures

Due to recurrent problems with bacterial infection of the cultures, i.e., reduced growth and usually consequent die-off of the explants, their isolation and identification was carried out. The Difco™ Nutrient Agar medium (Avantor®, VWR Corporate, Radnor, PA, USA) was prepared to isolate bacteria from the cultures. After obtaining single colonies, the bacteria were transferred to cell wall selection media: Columbia Agar Base BBL (Sigma-Aldrich®, Merck Life Science, Darmstadt, Germany) for the growth of Gram-positive bacteria and the MacConkey medium (Sigma-Aldrich®, Merck Life Science, Darmstadt, Germany) for the growth of Gram-negative bacteria. All media were autoclaved in flasks and poured into pre-sterilized 90 mm diameter Petri dishes in a laminar airflow chamber and left until solidified. Petri dishes with the medium were stored at room temperature in a laboratory cabinet during the period of bacterial colony growth. Due to poor colony growth, the dishes were placed in a hothouse at 34 °C for a week. Despite this, the bacteria multiplied very slowly or no multiplication was observed, as at room temperature. For this reason, instead of using sterile loop, the bacteria were applied to the medium with an infected laurel explant. This caused the bacteria to multiply, and material was obtained for further study (Figure 1F). Tests to identify bacterial isolates were performed in triplicate. Genomic DNA was isolated from multiplied 3-week-old bacterial colonies using two methods:

(a) CTAB/lysozyme [19] dedicated to the isolation of genomic DNA from bacteria;
(b) According to Van Burik et al. [20], using the homogenization of material with glass balls.

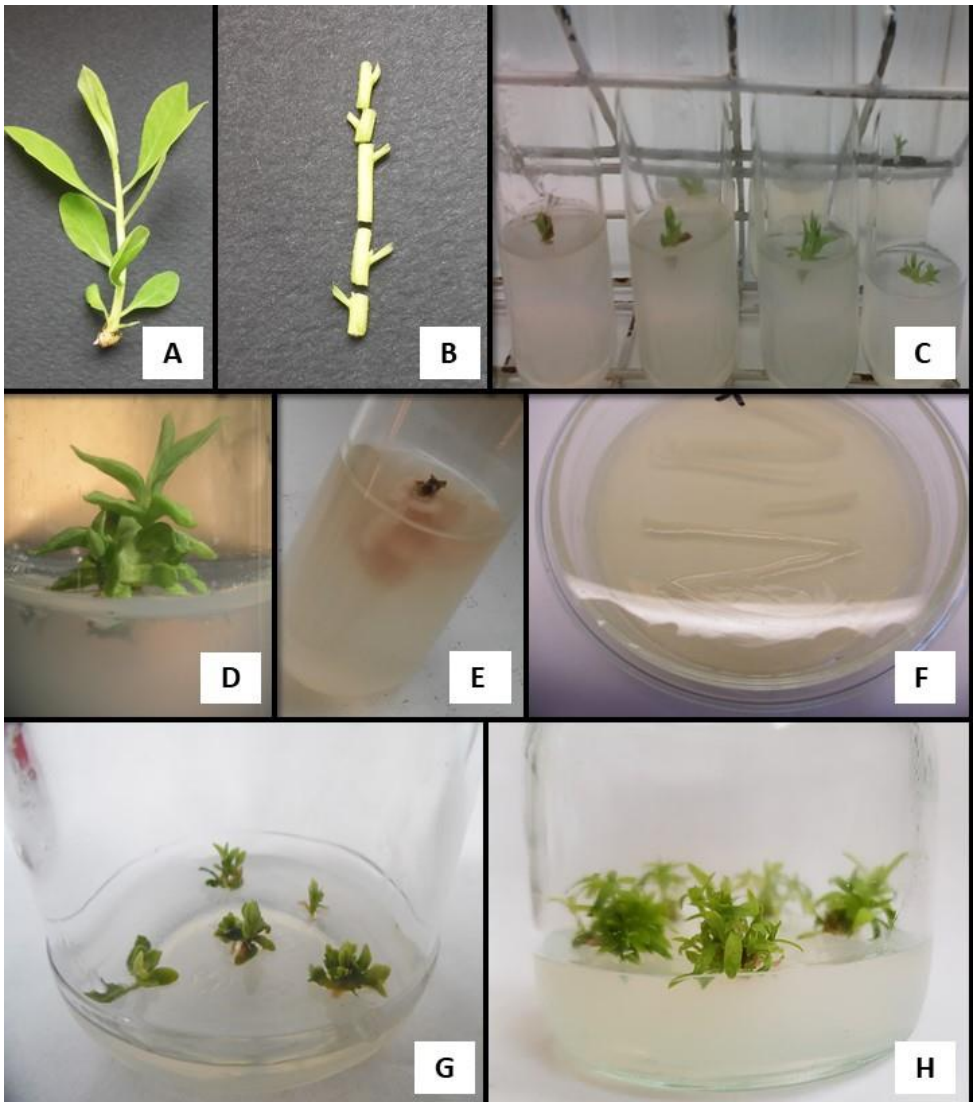


Figure 1. (**A**) Young mezereon shoots before sterilization; (**B**) Shoots devoid of leaves and cut into single explants; (**C**) The beginning of the regeneration of explants in the phytotron; (**D**) A well-regenerated explant 4 weeks after placing it on the medium; (**E**) Leakage of endophytic bacteria and dying out of explants; (**F**) Bacterial colony growth on the Columbia Agar Base selection medium; (**G**) Regeneration of explants on the WPM medium without the addition of any growth regulators; (**H**) The best regeneration of explants on the WPM medium with the addition of 4.14 μM mT.

To confirm the presence of bacteria in the sample, amplification by the PCR reaction of bacterial-specific 16S rDNA fragments was performed on a DNA template isolated from bacterial colonies using primers:

27F: 5-AGAGTTTGATCMTGGCTCAG-3

1492R: 5-GGTTACCTTGTTACGACTT-3

The amplification reaction was carried out in an ABI 9700 thermocycler (Life Technologies, Carlsbad, CA, USA) using OptiTaq thermostable polymerase (EURx Sp. z o.o., Gdańsk, Poland). The PCR reaction conditions (30 cycles) were 95 °C—3 min, 95 °C—15 s, 55 °C—15 s, 72 °C—90 s, 72 °C—2 min and 10 °C—cooling. The PCR product was purified using ExoSap (Thermo Fisher Scientific, Waltham, MA, USA), and sequencing was performed using the kit BigDye Terminator Mix v3.1 (Thermo Fisher Scientific, Waltham, MA, USA) and ABI3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and specific primers. The obtained sequences (from bacterial 16S rRNA-specific primers 341F, 518R and

928F) were assembled into a contig to obtain a consensus sequence. The obtained sequences were compared with the NCBI—GeneBank database using the BLAST program.

2.4. Culture Establishment

Two or three weeks after initiating the culture, all live and visually infection-free explants (Figure 1D) were placed on the MS medium supplemented with 11.1 µM BA and 0.54 µM NAA, and those still infected with bacteria could be resterilized (as described above). The cultures were incubated for four weeks at 23 ± 1 °C in a culture room (phytotron), with a 16 h per day photoperiod and the intensity of the light from fluorescent lamps at 35 µmol $m^{-2}s^{-1}$.

2.5. Shoot Proliferation

Shoot explants, ca. 0.5 cm, were placed in the medium, with the addition of the following cytokinins (all from Duchefa Biochemie B.V, Haarlem, The Netherlands) at a concentration of 4.92 µM 2iP (2-isopentenyladenine), 4.44 µM BA (6-benzyladenine), 4.65 µM Kin (kinetin), 4.14 µM mT (meta-Topoline) and 4.56 µM Zea (zeatin). As a control combination, the WPM medium was prepared without the addition of any growth regulators (WPM "0"). After 6 weeks from the establishment of the culture, the following factors were assessed: the percentage of regeneration (%), the number of newly regenerated shoots and their length (cm).

2.6. Rooting

Due to the difficulties in rooting the February daphne, even in in vitro cultures and after using auxin in the medium [21], an experiment was carried out using perlite in combination with a semi-solid WPM medium with 3 mg·L^{-1} agar with the addition of 2% sucrose. First, jars were prepared with perlite rinsed several times in order to remove dust and other impurities. Then, it was flooded (v/v) with the WPM medium ("0") or WPM with auxin in the concentrations 4.92, 9.84 or 19.68 µM IBA (Duchefa Biochemie B.V, Haarlem, The Netherlands), and finally autoclaved. After 7 weeks, the following were assessed: the percentage of rooted cuttings, the number of roots/shoot and their length (cm).

2.7. Hardening of Micropropagated Plants

Rooted plants were taken out of the jars, and the root system was rinsed out with remnants of the medium and perlite. Then, they were placed in cuvettes filled with moist peat and perlite (2:1) and covered with plastic transparent covers. There were 2 covered vents in each cover. Half of the plants (60 plants) were treated with 3.78 µM ABA (Sigma-Aldrich®, Merck Life Science, Darmstadt, Germany) foliar spray. The rest of the plants were the control and were treated only with water. Acclimatization was also performed in 3 replications. The cuvettes were placed in a phytotron with a constant temperature of 21 °C and a 12 h photoperiod. For the first two weeks, the plants had constant, approximately 85% air humidity. After 2 weeks, the vents in the covers were discovered, and after a further 2 weeks, the covers were completely removed. After this stage, the survival rate of the plants was assessed. Plants hardened in this way were transplanted into pots (after 4 weeks from the start of acclimatization) filled with peat substrate and placed in the nursery. After 4 weeks, the survival of the plants was again assessed.

2.8. Statistical Analysis

Arcsine transformation was performed for all experimental data taken in percentages before subjecting them to statistical analysis [22]. All experimental data were subjected to a one-way factorial ANOVA conducted with the use of Statgraphics Centurion XVI (version 16.2.04 64-bit). Based on the multiple comparison test (Duncan's test), individual homogeneous groups were identified (at the significance level α = 0.05), to which the

respective average values from individual combinations were assigned by Wójcik and Laudański [23].

3. Results and Discussion

3.1. Sterilization and Identification of Bacteria in Cultures

Sterilization of plant fragments chosen for the in vitro culture is a crucial phase of micropropagation. Bacterial or fungal contaminations are the most important problems in laboratories. Contaminated plants may regenerate poorly, have problems with rooting or die due to an excess of microorganisms in the medium. Removal of all contaminations from explants during the initial culture phase is, thus, essential, although sometimes it is very difficult to obtain a sterile material for further multiplication. This problem is especially acute in woody plants, which are relative difficult to sterilize [24]. This is usually a multi-stage process and experimentally adjusted to a given plant organ and its structure, size and "resistance" to disinfectants [25]. In *D. mezereum*, such a multi-stage surface disinfection procedure of shoot parts was relatively efficient when 3% H_2O_2 and 1% NaOCl were used. A high survival rate of explants and their quick regeneration occurred, while no damages were evident and practically all fungal contaminations were eliminated. However, in 60% of explants, bacterial outflows appeared, and four passages were needed on the medium enriched with PPM or colloid silver to remove completely bacteria and to receive clean cultures. Also, Orlikowska and Zawadzka [26] claim that bacterial populations can be reduced or eliminated by different chemicals added to the media, such as silver nitrate, antibiotics, PPM (Plant Preservation Mixture), Vitrofural, essentials oils and other bacteriocides and bacteriostats. In case of returning bacterial contaminations, new trials to clean explants can be attempted.

In cultures of woody plants, endogenous bacteria that remain in the lethal or latent state in explant tissues are problematic. They can multiply and appear even after several or a dozen passages of the proliferation phase, but sometimes even during the rooting of tree or shrub microcuttings [26,27]. Endophytes discovered in daphne cultures kept appearing during individual passages and considerably contaminated the medium with outflows from plant tissues. In many cases, they multiplied so fast that the explants were dying because of their excess. In spite of an efficient surface decontamination in the initial phase, bacteria reappeared after the successive material sterilizations; therefore, they must have been localized inside shoots. Usually, endophytes occupy spaces near the possible points of entry or exit from plant; they may be inside cells, in the spaces between them or in conductive bundles, which enables their easy and quick translocation within the plant [13]. Therefore, each passage during which an explant base is recut provides an excellent opportunity for bacterial outflow into the medium.

Problems with bacteria multiplication that appear on media isolated from *D. mezereum* cultures can indicate that they result from a lack of certain substances, which, on natural stands, come from daphne mother plants. This was confirmed by the author, as bacteria did not multiply when they were placed on the medium with a sterile inoculation loop, while if new cultures were begun with a piece of stem, a rapid bacteria multiplication was observed. To detect and identify bacteria appearing in tissue cultures, the sensitive molecular methods based on DNA analysis, especial polymerase chain reaction (PCR) using specific starters, are recently used [28]. The analysis of amplification products of the fragment 16S rDNA with changing sequence by sequential analysis and comparing its results with the gene bank (Table 1) did not allow for univocal identification of endophytic bacteria species appearing in daphne cultures. The comparison of the sequences with data from the NCBI-GenBank determined 98% similarity. These bacteria have earlier been described as a contamination source in the culture of the ornamental plants *Syngonium* and *Spathiphyllum* [29], as well as endophytes of *Pogonatherum paniceum* [30], *Prunus avium* [31] and *Pinus sylvestris* [32]. Assessment of bacteria colonies present in stem cultures of *D. mezereum* on selection media confirmed that they are Gram-positive: they did not show any growth on the MacConkey's medium, which is a selection medium for Gram-negative bacteria, while on Columbia Agar

Base, the colony growth was evident, which confirms that they are Gram-positive. Also, according to the literature, *Mycobacterium* sp. are Gram-positive bacteria, acid-proof and a little sensitive to unfavorable environmental conditions, which enables them to colonize most habitats [33]. Furthermore, Quambusch et al. [31], using in vitro cultures of sweet cherry, concluded that *Mycobacterium* sp. bacteria are closely associated with woody plants.

Table 1. The results of genetic similarity of the tested sample with the GenBank database.

Assigned Species of Bacteria	Similarity	Sequence Coverage
Mycobacterium vanbaalenii strain PYR-1	98%	100%
Mycobacterium peregrinum strain CIP 105382		
Mycobacterium peregrinum strain ATCC 14467		

3.2. Shoot Proliferation

Having obtained clean, stabilized cultures of daphne, its explants were placed onto the WPM medium for the proliferation phase. During the last 100 years, numerous media have been developed for micropropagation of different plant groups. The most popular is the medium of Murashige and Skoog (MS) [16]. For woody plants, WPM [17]—the medium with a more balanced nutrient composition—is more frequently used. It contains fewer salts, especially nitrogen and potassium. Studies on a proper medium for other daphne species were carried out by Noshad et al. [34], who regenerated stem explants on five media without any growth regulators: WPM, MS, B5 [35], LS [36] and SH [37]. After 4 weeks of culture, they found that five out of seven species (*D. tangutica*, *D. laureola*, *D. caucasica*, *D. retusa* and *D. giraldii*) regenerated and survived better on MS. Two other species, *D. cneorum* and *D. jasminea*, regenerated better on WPM.

For two parameters evaluated during the multiplication phase, i.e., shoot number per explant and explant length, WPM or MS gave better results, depending on the species and the experiment. Nowakowska et al. [38] showed that stem explants of *D. mezereum* 'Alba' were dying on MS without growth regulators, especially at the end of culture. This was not observed on WPM, probably because of lower macroelements contents, which slowed down maturing and senescence. The nutrient composition of a medium may be modified, for example, macroelements of WPM mixed with the other ingredients of MS, as it was performed during the first trials to develop a micropropagation protocol for *D. mezereum* [21].

Because of their fundamental role during organogenesis, cytokinins are essential for micropropagation during the regeneration and multiplication phase. In woody plants, they stimulate shoot proliferation, and when used in a low concentration in the presence of auxin, they provoke spontaneous development of adventitious roots [39]. In the present experiments, the regeneration of daphne explants was satisfactory; however, depending on cytokinin differences in the growth of new shoots, their appearance and sometimes leaf color were evident. Practically all explants regenerated in the presence of cytokinin 100%. The lowest percentage of regenerated explants and the lowest number of new shoots were observed on WPM without growth regulators, while the significant effect of cytokinins in this medium was evident after 6 weeks of culture. The highest number of new shoots appeared on the WPM medium enriched with mT, where almost five shoots were produced by a single explant, i.e., almost twice as many as on the control medium (Figure 1G,H). Usually, the cytokinin used successfully during shoot proliferation is BA, as shown, for example, in studies on *Anethum graveolens* L. [40]. Hanus-Fajerska et al. [41] studied the micropropagation of three *Daphne* species: *D. jasminea* Sibth. & Sm., *D. caucasica* Pall. and *D. tangutica* Maxim. On MS supplemented with 4.92 µM 2iP and 0.54 µM NAA, the highest propagation ratio was only 2.8 shoots/explant, obtained for *D. jasminea.* In our

experiment on *D. mezereum*, this ratio is generally much higher, as also media with BA and Zea-stimulated proliferation, producing nearly four shoots per explant. In the presence of 2iP or Kin, 2.5 shoots per explant were produced, i.e., 47% less than with mT (Table 2).

Table 2. Effect of cytokinins on shoot induction in nodal explants of *D. mezereum.*

WPM Medium with the Cytokinin [μM]	% Regeneration of Explants	Average Number of Shoot/Explants	Shoot Length (cm)
0	91.5 b *	1.99 d	0.71 c
4.92 2iP	96.6 a	2.81 c	0.73 c
4.65 Kin	98.3 a	2.63 c	0.81 bc
4.56 Zea	100 a	3.89 b	0.97 a
4.44 BA	100 a	3.91 b	0.90 ab
4.14 mT	100 a	4.85 a	0.93 ab

* Means in the column followed by the same letter do not differ significantly at $\alpha = 0.05$.

Every species and sometimes a cultivar differ in their response to a given cytokinin and its concentration, and this is easily observed on the taxonomically close plants. For shoot proliferation in *D. gnidium*, L. BA is the most suitable cytokinin [42], while for *D. jasminea*, 2iP [43]. In *D. mezereuem* 'Alba', the highest number of shoots, after 6 weeks of culture, was produced on MS medium with 4.44 μM BA and 0.54 μM NAA [38]. Also, the length of regenerated shoots can differ depending on a culture medium. The shortest shoots of daphne were obtained on control medium and in the presence of 2iP or Kin. The longest were developed on media with 4.56 μM Zea, as well as in the presence of BA and mT (Table 2). In the white variety ('Alba'), the longest shoots were produced on MS supplemented with BA or Zea [38]. During the first studies on *D. mezereum*, generally longer shoots were obtained: from 1 cm in the presence of 4.44 μM BA, up to 2 cm on the medium with mT [21].

These differences probably result from using the WPM medium modified according to MS in the contents of microelements and vitamins. The medium composition seems decisive for the basic parameters of multiplication. Apart from the shoot length, also the multiplication ratio differs significantly. Here, it is high, i.e., 5 shoots per explant, while in 2019 (also on the modified medium—WPM with MS microelements and vitamins [21]), only 1.25 in the best treatment with mT.

3.3. Rooting

Rooting is usually difficult in woody plants, which is often critical for the success of micropropagation. Rooting parameters evaluated in this work, such as root number and their length, are decisive for the plant ability to begin growth in the ex vitro conditions. February daphne is a species extremely difficult to root. So far, even in the in vitro cultures, no satisfactory results have been obtained. In studies carried out in 2019, IBA or NAA in several concentrations were added to the culture medium. The highest rooting percentage was obtained with 19.68 μM IBA (62.9%) or 21.48 μM NAA (38.8%) [21]. To improve the rhizogenesis medium, modifications may be tried or using of its semi-liquid versions. In this experiment, the significant effect of IBA added to the WPM medium mixed with perlite was shown on the rooting of daphne microcuttings (Table 3, Figure 2A). Microcuttings rooted in the mixture of the medium with perlite rooted in 45%, even without auxins. IBA increased this percentage to 66–70% when used in the concentrations 4.92 and 9.84 μM, and to 86% in the concentration 19.68 μM. Microcuttings rooted without auxin formed 2.2 roots on the average, while supplementing the medium with IBA in any concentration positively affected the root number (Figure 2B). The highest root number was produced in the presence of 19.68 μM IBA—three times higher than in the control. Without auxin, cuttings produced 0.6 root per shoot, while IBA in the concentration 4.92 or 9.84 μM doubled this number. The auxin also affected root length, confirming that IBA stimulates root elongation. In the presence of 19.68 μM IBA, the root length reached over 2 cm, significantly more than in the control (Table 3).

Table 3. Effect of semi-solid WPM medium with perlite and auxin on *D. mezereum* shoot rooting.

WPM Medium with Perlite and IBA [μM]	% Rooting of Shoots	Average Number of Root/Shoot	Root Length (cm)
0	45.00 c *	2.30 d	0.64 c
4.92	70.00 b	3.60 c	1.07 b
9.84	66.67 b	4.62 b	1.21 b
19.68	86.67 a	6.63 a	2.15 a

* Means in the column followed by the same letter do not differ significantly at $\alpha = 0.05$.

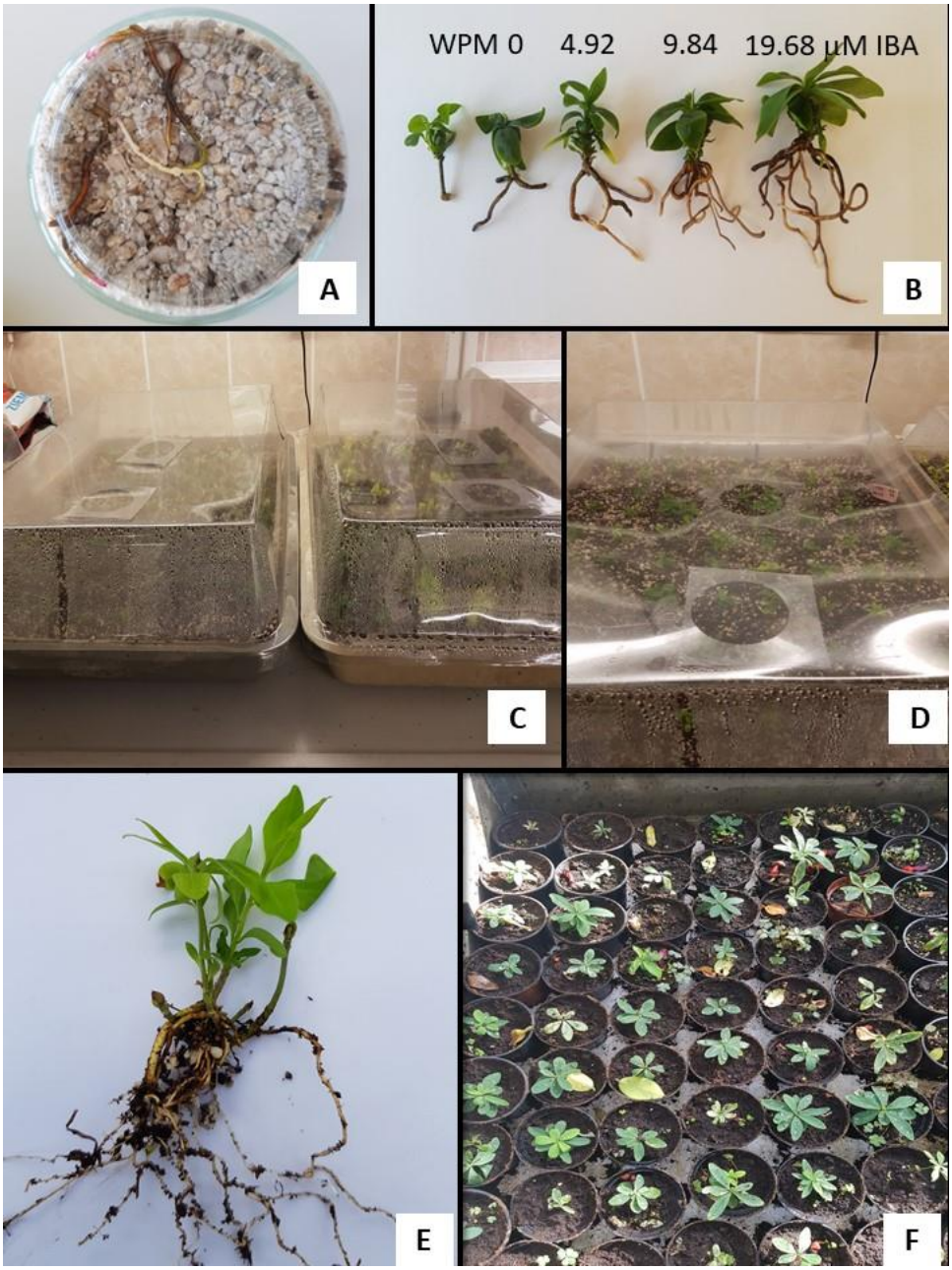


Figure 2. (**A**) Shoots rooted in perlite with WPM medium with the addition of IBA; (**B**) Rooted cuttings 7 weeks after starting the experiment (from the left two seedlings with WPM 0, then one for each IBA concentration: 4.92, 9.84 and finally 19.68 μM IBA); (**C**,**D**) Cuvettes with acclimatized seedlings placed in the phytotron; (**E**) Extended root system of seedlings after 6 weeks of acclimatization in cuvettes in the phytotron; (**F**) Acclimatized *Daphne mezereum* growing outdoors in pots.

The WPM—reduced to 1/3 nutrient composition and mixed with perlite—was tested earlier for *D. caucasica, D. jasminea* and *D. tangutica.* Only for the two first species, the results of rooting were comparable (83.3%) to those presented here, and this was obtained when the high IBA concentration (29.53 μM) was used [41]. A lower rooting percentage was also obtained by Noshad et al. [34] for *Daphne tangutica* (59%) on the medium containing both IBA and NAA. However, in *D. jasminea*, 85% of shoots were rooted, confirming the differences in the rooting potential of individual daphne species and their different preferences for the auxin type and concentration in the culture medium.

3.4. Hardening of Micropropagated Plants

Microcuttings from in vitro can easily be damaged after transfer to ex vitro due to sudden environmental changes. If hardening is not conducted properly, a substantial part of plant material can be lost. Usually, under shade and gradually decreasing humidity, they need several weeks to acclimatize to new conditions. Often, wilting of microcuttings occurs, indicating an improper water balance. During culture, explants remain under nearly 95% humidity, so they do not need to develop any mechanisms to protect them against water loss, for example, they do not produce cuticular wax on the leaf surface, as it happens in plants on natural stands [44,45]. In turn, light intensity is lower in vitro than in field conditions, which, combined with high air humidity, leads to the atypical development of stomata, which are, thus, unable to close completely after the transfer of microcuttings to ex vitro. Lack of stomata response to environmental factors, like temperature, humidity or light, is the most common problem after the transfer of cuttings to ex vitro [46]. Intensive transpiration results in cuttings' dying [46,47]. To reduce transpiration and improve the acclimatization rate, cuttings may be treated with exogenous abscisic acid. The treatment results in accumulation of H_2O_2 in guard cells, which causes stomata closing [48]. Therefore, ABA plays a key role in many physiological processes, including the response to abiotic stresses, and is used during acclimation to increase plant survival [49]. Four weeks after planting microcuttings of *D. mezereum* into cuvettes, only 70% of them survived in the best treatment, where spraying with ABA was applied, while in the control treatment sprayed with water, the survival rate was lower (45% acclimatized cuttings). The positive effect of ABA on acclimatization was also observed in *Ulmus minor*, where the authors showed how, by decreasing water losses, the foliar ABA application reduced stress related to the plant transfer to ex vitro conditions [50]. In *Fraxinus excelsior*, microcuttings were acclimatized similarly, as described here for *D. mezereum*. After 4 weeks in cuvettes, they were transferred into the greenhouse. The longer shoots (3 cm) survived in the culture chamber and acclimatized in 76% [51].

After 4 weeks of acclimatization in the phytotrone (under reduced humidity and a photoperiod shorter by 4 h than in vitro), plants of *D. mezereum* developed their roots (Figure 2E). Potted and transferred outdoors, they showed 55% survival after 4 weeks of growth. In most protocols, the survival rate is evaluated after 4–6 weeks after planting into soil. This is also when it is usually higher. However, it is the subsequent weeks and the external conditions—not just the greenhouse conditions, where we, nevertheless, protect the plants, such as from winds—that should ultimately be evaluated.

4. Conclusions

The aim of this study was to develop an effective micropropagation protocol for the endangered shrub *Daphne mezereum*. In addition, the isolation and identification of bacteria colonizing in vitro cultures of this species, which disturbed the multiplication of shoots, were carried out. Our research has shown that it is possible to achieve an effective propagation protocol for this valuable plant.

To reduce costs during initiation, Murashige and Skoog (MS) medium is recommended (with 2% sucrose without growth regulators and 8 g/L agar). To reduce or eliminate the appearance of bacteria in the cultures, it is recommended to add 1 mL/L PPM to the medium. In case of the appearance of leaks of cream bacteria, despite the use of PPM, an antibiotic

against *Mycobacterium* should be added to the medium through filters (after medium autoclaving). For the multiplication, we recommend Woody Plant Medium (WPM) containing 3% sucrose, 8 g/L agar and 4.14 µM mT. It allows for obtaining 100% regeneration and about five new shoots/explants. The best rooting of *D. mezereum* microshoots (more than 86%) is achieved by using semi-liquid WPM medium with perlite (medium:perlite 1:1 *v*/*v*) and the addition of 3 g/L agar, 2% sucrose and 19.68 µM IBA. We have shown that successful acclimatization can be carried out in two stages: in cuvettes in the phytotron, where plants are treated with an aqueous solution of 3.78 µM ABA, and in pots in the nursery (4 weeks).

This protocol will allow for the propagation of good-quality February daphne shrubs and limiting harvesting plants from their natural habitat.

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