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Efficiency of a Double-Phase Medium in Micropropagation of Serviceberry (*Amelanchier* sp.)

Wojciech Litwińczuk *, Beata Jacek and Aleksandra Siekierzyńska

Institute of Agricultural Sciences, Environmental Protection, and Management, Faculty of Technology and Life Sciences, University of Rzeszów, Ćwiklińskiej 2, 35-601 Rzeszów, Poland; bjacek@ur.edu.pl (B.J.); asiekierzynska@ur.edu.pl (A.S.)

* Correspondence: wlitwinczuk@ur.edu.pl

Abstract

The use of double-phase medium (2F) gave beneficial effects in the propagation of woody plants belonging to the Rosaceae family. Despite this, it appears that such research has not yet been carried out in relation to (*Amelanchier* sp.). Thus, the efficiency of such a technique in micropropagation of three cultivars of serviceberry (*Amelanchier* sp.)—'Autumn Brilliance', 'Ballerina', 'Snowcloud'—was evaluated. The 2F medium was obtained by pouring the liquid MS solution (10 mL) onto the solid (1F) medium (50 mL) after inoculation of the explants. Generally, the response of the in vitro cultures to 2F medium was positive but clone-dependent. This medium stimulated, to various extents, the elongation and proliferation of the shoots. The use of 2F medium did not significantly increase hyperhydricity, whereas it lowered shoot tip necrosis frequency during the multiplication stage. However, the residual effect of 2F medium on the in vitro rooting of shoots and acclimation was adverse in the case of two out of three studied clones. Considering the efficiency of the three micropropagation stages, the use of 2F medium was only favorable in the case of one clone ('Ballerina'), yielding over 90% more acclimated plantlets in comparison to the control (1F medium).

Keywords: in vitro; DPS method; Pomoideae; Rosaceae; juneberry; small fruit crop

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Academic Editor: Valeria Cavallaro

Received: 23 October 2025
Revised: 17 November 2025
Accepted: 21 November 2025
Published: 23 November 2025

Citation: Litwińczuk, W.; Jacek, B.; Siekierzyńska, A. Efficiency of a Double-Phase Medium in Micropropagation of Serviceberry (*Amelanchier* sp.). *Agronomy* **2025**, *15*, 2694. https://doi.org/10.3390/agronomy15122694

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1. Introduction

The genus *Amelanchier* (f. Rosaceae, sf. Pomoideae) comprises more than 20 species of deciduous shrubs or small trees distributed in North America, Europe, northern Africa, and Asia [1]. The *Amelanchier* species are closely related and are often difficult to distinguish. They are tolerant of various soils and climatic conditions [2]. Most *Amelanchier* sp. plants are grown as ornamental shrubs because of their attractive foliage and fragrant, showy flowers [1,2]. The species *A. laevis* and *A.* × *grandiflora* are considered the most decorative [1]. They also bear edible fruits [2]. However, other species, such as *A. alnifolia*, *A. spicata*, *A. oblongifolia*, and *A. canadensis*, are more suitable for use as a source of fruit [1]. They are relatively new commercial fruit crops [1,3,4]. The fruits are sweet, juicy, and suitable for processing to make jam and jelly [3]. They are highly valued for their nutraceutical and antioxidant properties and can be mechanically harvested [2,4]. In the opinion of Żurawicz et al. [4], the cultivars of *A. alnifolia* could be grown successfully, and their cultivation should be easier than the cultivation of highbush blueberry (*Vaccinium corymbosum* L.) in Poland. The genus *Amelanchier* is also valuable in agroforestry.

As the popularity of the *Amelanchier* species increases, there is a greater demand for high-quality and uniform nursery plants. Problems encountered during conventional vegetative propagation (through grafting or hardwood and softwood cuttings) make them inefficient, which results in a limited supply. Micropropagation, as in the case of other woody plants, can contribute to solving this problem. Much research has already been carried out on the optimization of *Amelanchier* propagation through in vitro cultures over several decades, since 1981 [2,3,5–16]. In spite of this, some difficulties, like physiological disorders coupled with intense shoot proliferation, unsatisfactory rooting of shoots in/ex vitro, losses of plantlets during acclimation, and clone-specific reaction, still appear [4]. Therefore, further research on micropropagation is needed, for example, on the direct and indirect influence of double-phase media. The double-phase medium technique (double-phase nutrient media/double-phase culture system—DPS) is cheap and easy to use and needs no expensive instrumentation. It involves the culturing of plants in vitro on a solid medium onto which a liquid medium is poured in a small volume. The explants are inoculated at first, and then a liquid medium is applied (immediately after or after a few days or weeks).

Such a technique has been known and used for many years [17–21]. It is simple, cheap, and does not require specialized instrumentation [17,18]. To some extent, it combines the advantages of solid and liquid media. Its beneficial effects include, above all, the stimulated growth, proliferation, and/or elongation of shoots in vitro, and the improved rooting and acclimation of shoots have been proven for many woody plants [19–28]. On the other hand, it does not significantly increase vitrification and deformation of shoots compared to liquid media, including TIS (temporary immersion system). However, currently, there are few new reports on the use of double-phase media for the in vitro propagation of plants [29–32]. Only one of them concerns woody plants [32]. Nevertheless, the described effects of double-phase medium overlap with those mentioned above. However, the statement of Senapati [31] that this method reduces the time, cost, and labor of the micropropagation of *Rauwolfia serpentine* is noteworthy. To our best knowledge, there is no report concerning the micropropagation of *Amelanchier* sp. With the application of DPS. However, some reports for other species belonging to the family/subfamily Rosaceae/Pomoideae could be found for *Malus* [19,27,32], *Pyrus* [20,23,24,26], *Cydonia* [21,25], and *Aronia* [28,33]. The use of such methods produced mostly beneficial effects in those genera. However, there are reports about the improved shoot proliferation (although at the expense of increased vitrification) of serviceberry on liquid media (TIS—temporary immersion system) [6,7] as well as better and faster rooting of shoots obtained from TIS [6]. Therefore, there are some reasons to assume that the double-phase media method will provide similarly favorable results (like improved multiplication and rooting of shoots) in the micropropagation of serviceberry. Thus, the aim of the presented work was to verify this assumption and perform a preliminary evaluation of the usefulness of this method in the propagation of *Amelanchier* sp. through in vitro cultures.

2. Materials and Methods

The research was conducted on the well-established in vitro cultures of 3 serviceberry cultivars: 'Autumn Brilliance' (AtBr) (*Amelanchier* × *grandiflora* Rehd.), 'Ballerina' (Ball) (*A.* × *grandiflora* Rehd.), and 'Majestic', previously named 'Snowcloud' (SnCl) (*A. laevis* Wieg) [1]. They were initiated two years earlier. During this time, numerous preliminary experiments were conducted to determine the composition of the media used in the present study. The results of these preliminary experiments are not presented.

2.1. Multiplication Stage (Application of 1F and 2F Media)

The cultures were multiplied through the commonly used, axillary-branching method, based on the application of cytokinins. The modified MS [34] medium with elevated (+25%) Ca^{2+} and Mg^{2+} doses, supplemented with 6-benzylaminopurine (BA), adenine hemisulphate (AdS), and 1-naphthaleneacetic acid (NAA) was used for this purpose (Table 1). The double-phase (2F) medium was obtained by pouring the liquid MS solution (5 + 5 mL/jar) on the control semi-solid medium (1F) solidified with agar (50 mL/jar) after the 1st and 2nd week after starting the subculture. The compositions of both the solid (1F) and liquid media were the same. Cultures were grown in vitro in glass jars (450 mL) with ventilated polypropylene twist lids. Eight nodal explants about 1 cm long were placed in each jar. Explants were derived separately from cultures that had previously grown for 2 passages on 1F and 2F media. Thus, the studied 2F cultures represented the third generation growing on this type of medium. Cultures were incubated at 25 ± 1.5 °C and a photoperiod of 16 h/8 h (day/night) at approximately 42 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD, provided by a combination (1:1) of fluorescent lamps: (warm white Lumilux HO 54W/830 (OSRAM, Munich, Germany) + GROLUX FHO54W/T5/GRO (SYLVANIA, Toronto, ON, Canada)). Similar temperature and light conditions were used in the following micropropagation stages. The subculture lasted 8 weeks. Thereafter, the size of callus and the presence of physiological disorders were evaluated, and the length and number of short and long shoots (length about 5–15 mm and over 15 mm, respectively) were recorded. To perform additional analyses, the jars with the medium (both fresh and used) were frozen (−26 °C). Previously, culture residues (leaves, etc.) were removed from the used medium. The jars were then thawed to room temperature. As a result, a very weakly solidified, liquid-like medium was obtained. This medium was slightly mixed/vortexed. The electric conductance (EC), acidity (pH), and refractive index (RI) measurements were then performed using standard laboratory equipment. The content of dry mass was determined by drying the homogenized liquid-like medium (1 mL) at 35 °C and then comparing the fresh mass and dry mass of the sample.

Table 1. Composition of media used for micropropagation of *Amelanchier* sp.

Media Constituents	Multiplication Stage	Rooting Stage
Macronutrients		
N salts	100% MS [1]	25% MS
K salts	100% MS	
P salts	100% MS	
Ca salts	125% MS	
Mg salts	125% MS	
Micronutrients + FeEDDHA	100% MS 10 mg L^{-1}	50% MS 5 mg L^{-1}
Vitamins	100% WPM [2]	100% WPM
Myo-inositol	100 mg L^{-1}	100 mg L^{-1}
Sucrose	30 g L^{-1}	15 g L^{-1}
BA	1.0 mg L^{-1}	-
AdS	10 mg L^{-1}	-
NAA	0.1 mg L^{-1}	-
IBA	-	2 mg L^{-1}

Table 1. *Cont.*

Media Constituents	Multiplication Stage	Rooting Stage
Agar	7 g L^{-1}	7 g L^{-1}
pH	5.8	5.8

[1] MS [34] macronutrients: NH_4NO_3 1650 mg L^{-1}, KNO_3 1900 mg L^{-1}, $CaCl_2 \cdot 2H_2O$ 440 mg L^{-1}, $MgSO_4 \cdot 7H_2O$ mg L^{-1}, 180.7 mg L^{-1}, KH_2PO_4 170 mg L^{-1}; MS micronutrients: NaFeEDTA 36.7 mg L^{-1}, KI 0.83 mg L^{-1}, H_3BO_3 6.3 mg L^{-1}, $MnSO_4 \cdot 4H_2O$ 22.3 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 8.6 mg L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg L^{-1}, $CuSO_4 \cdot 5H_2O$ 0.025 mg L^{-1}, $CoCl_2 \cdot 6H_2O$ 0.025 mg L^{-1}; FeEDDHA (Ethylenediamine-N, N′-bis (2-hydroxyphenylacetic acid) iron complex); [2] WPM [35] vitamins: glycine 2.0 mg L^{-1}, thiamine HCl 1.0 mg L^{-1}, pyridoxine HCl 0.5 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}. The used media were prepared from self-made stock solutions. All macro- and micronutrient salts (except for Fe) and sucrose (all reagents of "pure p.a." grade) were provided by Chempur (Piekary Śląskie, Poland), whereas NAFeEDTA, vitamins, and PGRs (all chemicals of "plant cell culture tested" grade) were provided by Sigma-Aldrich (Louis, MI, USA). The agar (Lab-Agar AB04) was supplied by Biocorp Poland S.A. (Lublin, Poland). PGRs: BA—6-benzylaminopurine; AdS—adenine hemisulphate; NAA—1-naphthaleneacetic acid; IBA—indole-3-butyric acid.

2.2. Rooting and Acclimation Stages (Aftermath of 1F and 2F Media)

Shoots collected separately from the 1F and 2F media under sterile conditions were trimmed (to about 2 cm long with the shoot tip) and rooted in vitro. Cultures were grown in vitro in glass jars (450 mL) with ventilated polypropylene twist lids. Ten healthy shoots were placed in each jar. The modified MS [34] medium (50 mL/jar) with reduced dose of macronutrients (−75%), micronutrients (−50%), and sucrose (−50%), supplemented with indole-3-butyric acid (IBA 2 mg/L), solidified with agar, was used (Table 1). The shoots were kept in the dark for four days and then moved to lighted shelves. Then they were grown under the same conditions as in the multiplication stage. The rooting passage lasted 6 weeks. Thereafter, the number of rooted shoots was counted, and the magnitude of the callus and root systems was evaluated. The rooted shoots in jars had been cooled (+5 °C) for 2 weeks in the dark. Then they were taken out of the jars, soaked in a solution (2.5 mL/L) of 'Florovit'® (INCO S.A., Warsaw, Poland) multi-component universal liquid fertilizer, and planted in trays filled with a semi-sterile peat-moss substrate (Jiffy TPS medium pH 5.5–6.5). The trays were covered with a transparent lid to maintain high air and substrate humidity. Fungicides were not applied. Sixty plantlets (rooted shoots) per treatment were placed in two trays. The plantlets had been grown for 4 weeks under a 16 h/8 h day/night photoperiod using fluorescent lamps (cool daylight, Lumilux L36W/865, OSRAM, Munich, Germany) at 55/90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD (with/without cover, respectively) and a temperature of 21 ± 3 °C. During the last week, the tray covers were gradually opened to reduce air humidity. The number of acclimated plants was determined 2 days after the cover was removed.

2.3. Statistical Analyses

All experiments were designed in a two-factorial fashion using cultivar × treatment combinations. Each treatment was represented by 6 jars (×8 cultures) in the multiplication stage, 6 jars (×10 shoots) in the rooting stage, and 2 trays (×30 plantlets) during the acclimation stage, making 48 cultures, 60 shoots, and 60 plantlets, respectively. The jars were randomly placed on the shelves (randomized block design). Only the analyses of media were performed on bulk samples taken from individual jars. Collected data were subjected to ANOVA and $LSD_{0.05}$ mean separation test using Statistica 13.3 computer software. Data presented as percentages (number of cultures with vitrified shoots or shoot tip necrosis, number of rooted shoots in vitro, number of obtained plantlets in vivo) due to the binomial distribution were subjected to testing on the difference between two proportions. Correlation analyses were also performed to determine the relationship among the studied media and culture characteristics. They were based on the means obtained for

each cultivar × treatment combination (n = 6). In all cases, the differences were considered to be significant at α = 0.05.

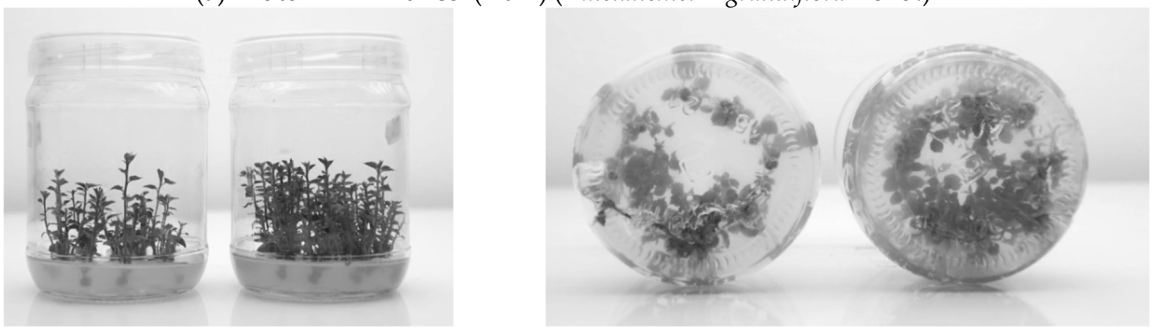
3. Results

3.1. Culture Proliferation

The growth of the studied serviceberry clones in vitro was different (Figure 1). The 'SnCl' cultures developed the most shoots, while the 'Ball' ones developed the least (Table 2). The 'SnCl' clone produced significantly more short (<15 mm) and long (>15 mm) shoots than the 'Ball' clone. The growth of shoots of both categories was intermediate in the case of the 'AtBr' clone. The differences among the studied clones in terms of elongation of long shoots were not proven. The 'SnCl' cultures developed significantly bigger callus at the explant base than the other two cultures. The symptoms of shoot tip necrosis were rarely observed. Vitrified cultures were found relatively often within 'SnCl' and 'AtBr' clones, contrary to the 'Ball' clone.



(**a**) 'Autumn Brilliance' (AtBr) (*Amelanchier* × *grandiflora* Rehd.)



(**b**) 'Ballerina' (Ball) (*Amelanchier* × *grandiflora* Rehd.)



(**c**) 'Majestic', previously named 'Snowcloud' (SnCl) (*Amelanchier laevis* Wieg)

Figure 1. Cultures in vitro of serviceberry in multiplication stage on the 1F and 2F medium (left and right jars, respectively) (phot. Adrian Baran). Jar size: height 11.5 cm; diameter 8 cm.

Table 2. Growth in vitro cultures of serviceberry clones during the multiplication stage.

Clone:	AtBr	Ball	SnCl	SL [2]
Total number (no.) of shoots (>5 mm)	7.5 ± 0.62 b [1]	5.6 ± 0.50 a	8.9 ± 0.40 c	***
No. of short shoots (5–14 mm)	1.1 ± 0.19 b	0.5 ± 0.12 a	1.4 ± 0.20 b	***
No. of long shoots (>15 mm) incl.:	6.4 ± 0.52 ab	5.2 ± 0.48 a	7.5 ± 0.35 b	**
(15–30 mm)	2.7 ± 0.31 ab	2.2 ± 0.28 a	3.2 ± 0.25 b	*
(31–44 mm)	3.2 ± 0.30 ab	2.8 ± 0.33 a	4.1 ± 0.28 b	**
(>45 mm)	0.2 ± 0.06 a	0.2 ± 0.06 a	0.1 ± 0.05 a	ns
Total shoot length [mm]	22.0 ± 1.83 ab	18.3 ± 1.72 a	25.8 ± 1.36 b	**
Mean shoot length [mm]	3.5 ± 0.10 a	3.5 ± 0.12 a	3.4 ± 0.07 a	ns
Callus size [mm]	3.4 ± 0.24 a	3.4 ± 0.13 a	4.7 ± 0.13 b	***
Percentage of cultures with vitrified shoots [%]	19.7 b	0 a	15.6 b	AF [3]: **
Percentage of cultures with shoot tip necrosis [%]	1.6 a	0 a	4.7 a	AF: ns

Statistical analyses were performed on 64 cultures per clone; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant, ***—$p < 0.001$, **—$p < 0.01$, *—$p < 0.05$; [3] AF—level of significance based on the test of difference between two proportions.

The 'SnCl' and 'AtBr' cultures used much more media ingredients than the 'Ball' ones. The medium acidity, as well as the conductance, refractive index, and dry mass content, at the end of passage were higher in the case of the 'Ball' clone in comparison to the other clones (Table 3).

Table 3. Chosen properties of the medium at the end of the subculture of the tested clones.

Medium Parameters	Unused Medium	Clone			SL [2]
		AtBr	Ball	SnCl	
pH	5.0 ± 0.05	4.6 ± 0.32 b [1]	3.8 ± 0.13 a	5.0 ± 0.17 b	**
conductance [mS]	5.3 ± 0.13	1.6 ± 0.31 a	2.8 ± 0.34 b	0.9 ± 0.07 a	***
refractive index [%]	4.5 ± 0.10	1.3 ± 0.29 a	2.5 ± 0.34 b	1.2 ± 0.15 a	**
dry mass content [%]	4.0 ± 0.18	0.6 ± 0.16 a	2.2 ± 0.23 b	0.7 ± 0.11 a	***

Statistical analyses were performed on eight jars per clone; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ***—$p < 0.001$, **—$p < 0.01$.

The double-phase (2F) medium stimulated the proliferation of shoots, especially those longer than 30 mm (Table 4). The shoots obtained on 2F medium were significantly longer than those grown on 1F medium. The growth of the callus was also stronger. The 2F medium prevented necrosis of shoot tips. The relationship between the kind of medium and frequency of culture vitrification was not statistically confirmed (Table 4). The differences between 1F and 2F media in terms of all measured traits of the used media (pH, conductivity, refractive index, and dry mass content) were not proven after the passage (Table 5).

Table 4. The influence of medium on the growth in vitro cultures of serviceberry clones during the multiplication stage.

Medium:	1F	2F	SL [2]
Total number (no.) of shoots (>5 mm)	6.2 ± 0.39 a [1]	8.7± 0.45 b	***

Table 4. *Cont.*

No. of short shoots (5–14 mm)	1.0 ± 0.14 a	1.0 ± 0.17 a	ns
No. of long shoots (>15 mm) incl.:	5.2 ± 0.32 a	7.7 ± 0.40 b	***
(15–30 mm)	2.5 ± 0.20 a	2.9 ± 0.26 a	ns
(31–44 mm)	2.4 ± 0.21 a	4.5 ± 0.25 b	***
(>45 mm)	0.1 ± 0.04 a	0.2 ± 0.05 a	ns
Total shoot length [mm]	16.9 ± 1.06 a	27.8 ± 1.43 b	***
Mean shoot length [mm]	3.3 ± 0.08 a	3.7 ± 0.07 b	***
Callus size [mm]	3.6 ± 0.15 a	4.2 ± 0.16 b	**
Percentage of cultures with vitrified shoots [%]	9.7 a	14.8 a	AF [3]: ns
Percentage of cultures with shoot tip necrosis [%]	4.3 b	0.0 a	AF: *

Statistical analyses were performed on 96 cultures per treatment; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant, ***—$p < 0.001$, **—$p < 0.01$, *—$p < 0.05$; [3] AF—level of significance based on the test of difference between two proportions.

Table 5. Chosen properties of the studied media at the end of the subculture.

Medium Parameters	Unused Medium		Medium (Treatment)		SL [2]
	1F	2F	1F	2F	
pH	5.1 ± 0.09	5.0 ± 0.04	4.4 ± 0.18 a [1]	4.7 ± 0.28 a	ns
conductance [mS]	5.6 ± 0.08	5.0 ± 0.05	1.9 ± 0.33 a	1.5 ± 0.27 a	ns
refractive index [%]	4.5 ± 0.21	4.4 ± 0.00	1.7 ± 0.27 a [1]	1.4 ± 0.27 a	ns
dry mass content [%]	4.3 ± 0.21	3.6 ± 0.11	1.2 ± 0.28 a	1.0 ± 0.22 a	ns

Statistical analyses were performed on 12 jars per treatment; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant.

The studied clones reacted variously to the type of medium (Table 6). The reaction of the 'Ball' clone was the strongest. Shoot proliferation on 2F medium increased by 124% compared to the control (1F medium). The number of shoots (longer than 15 mm and 31–45 mm length) collected from 2F medium surpassed the control by 133% and 228%, respectively. The reaction of the 'SnCl' clone was weaker than the 'Ball' clone. The number of shoots (longer than 15 mm and 31–45 mm length) collected from 2F medium exceeded the control by 27% and 56%, respectively. The clone 'AtBr' basically did not react to the 2F medium. The number of shoots (31–45 mm length) collected from the 2F medium was significantly higher than the control (+11%). The 2F medium did not stimulate the elongation of shoots of that clone, contrary to other clones ('Ball' and 'SnCl'). The growth of callus on 2F medium was significantly stronger only in the case of the 'Ball' clone. The significant differences in the frequencies of physiological disorders (shoot tip necrosis, shoot vitrification) of the three studied clones on the two examined media were not proven (Table 6). The medium (both 1F and 2F) acidity, conductance, refractive index, and dry mass content at the end of passage were higher in the case of the 'Ball' clone in comparison to other clones (Table 7). The clone x medium interaction was not found in the case of the aforementioned traits.

Table 6. The influence of medium on the growth in vitro cultures of three serviceberry clones during the multiplication stage.

Clone:	AtBr		Ball		SnCl		SL [2]	SL_{int} [3]
Medium:	1F	2F	1F	2F	1F	2F		
Total number (no.) of shoots (>5 mm)	7.1 ± 0.74 b [1]	7.9 ± 0.97 b	3.7 ± 0.48 a	8.3 ± 0.68 bc	8.1 ± 0.52 bc	9.8 ± 0.57 c	***	*
No. of short shoots (5–14 mm)	1.2 ± 0.26 bc	1.0 ± 0.28 abc	0.4 ± 0.12 a	0.5 ± 0.24 ab	1.5 ± 0.27 c	1.4 ± 0.30 c	*	ns
No. of long shoots (>15 mm) incl.:	5.8 ± 0.62 b	6.9 ± 0.81 bcd	3.3 ± 0.45 a	7.7 ± 0.67 cd	6.6 ± 0.39 bc	8.4 ± 0.55 d	***	*
(15–30 mm)	2.9 ± 0.41 b	2.8 ± 0.48 ab	1.8 ± 0.29 a	2.8 ± 0.51 ab	3.1 ± 0.30 b	3.2 ± 0.39 b	*	ns
(31–44 mm)	2.7 ± 0.39 b	3.8 ± 0.43 cd	1.4 ± 0.29 a	4.6 ± 0.45 de	3.2 ± 0.34 bc	5.0 ± 0.40 e	***	*
(>45 mm)	0.2 ± 0.09 a	0.2 ± 0.08 a	0.1 ± 0.04 a	0.3 ± 0.11 a	0.1 ± 0.04 a	0.2 ± 0.09 a	ns	ns
Total shoot length [mm]	19.0 ± 2.03 b	24.7 ± 2.91 bc	10.6 ± 1.53 a	28.6 ± 2.01 c	21.3 ± 1.40 b	30.2 ± 2.07 c	***	*
Mean shoot length [mm]	3.3 ± 0.16 ab	3.6 ± 0.12 bc	3.2 ± 0.18 a	3.9 ± 0.13 c	3.2 ± 0.09 a	3.6 ± 0.09 bc	**	ns
Callus size [mm]	3.2 ± 0.32 ab	3.7 ± 0.34 ab	3.1 ± 0.17 a	3.8 ± 0.19 bc	4.4 ± 0.22 cd	5.0 ± 0.14 d	***	ns
Percentage of cultures with vitrified shoots [%]	13.8 b	25.0 b	0 a	0 a	15.6 b	15.6 b	AF [4]: *	-
Percentage of cultures with shoot tip necrosis [%]	3.4 a	0 a	0 a	0 a	9.4 a	0 a	AF: ns	-

Statistical analyses were performed on 32 cultures per combination; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant, ***—$p < 0.001$, **—$p < 0.01$, *—$p < 0.05$; [3] SL_{int}—level of significance of interaction (clone × medium); [4] AF—level of significance based on the test of difference between two proportions.

Table 7. Chosen properties of the studied media at the end of the subculture, depending on the clone.

Medium Parameters	Unused Medium		Combination						SL [2]	SL_{int} [3]
			AtBr		Ball		SnCl			
	1F	2F	1F	2F	1F	2F	1F	2F		
pH	5.1 ± 0.09	5.0 ± 0.04	4.5 ± 0.66 abc [1]	4.7 ± 0.15 bc	3.6 ± 0.09 a	4.1 ± 0.18 ab	4.8 ± 0.23 bc	5.3 ± 0.21 c	*	ns
conductance [mS]	5.6 ± 0.08	5.0 ± 0.05	1.6 ± 0.44 ab	1.7 ± 0.52 ab	3.1 ± 0.42 c	2.4 ± 0.55 bc	1.0 ± 0.10 a	0.8 ± 0.05 a	**	ns
refractive index [%]	4.5 ± 0.21	4.4 ± 0.00	1.3 ± 0.51 ab	1.3 ± 0.34 ab	2.7 ± 0.41 c	2.2 ± 0.61 bc	1.3 ± 0.30 ab	1.0 ± 0.04 a	*	ns
dry mass content [%]	4.3 ± 0.21	3.6 ± 0.11	0.6 ± 0.13 a	0.6 ± 0.31 a	2.4 ± 0.35 b	2.0 ± 0.29 b	0.8 ± 0.16 a	0.6 ± 0.16 a	***	ns

Statistical analyses were performed on 4 jars per combination; the results are presented as mean ± SE. [1] means in rows marked with diverse letter are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant, ***—$p < 0.001$, **—$p < 0.01$, *—$p < 0.05$; [3] SL_{int}—level of significance of interaction (clone × medium).

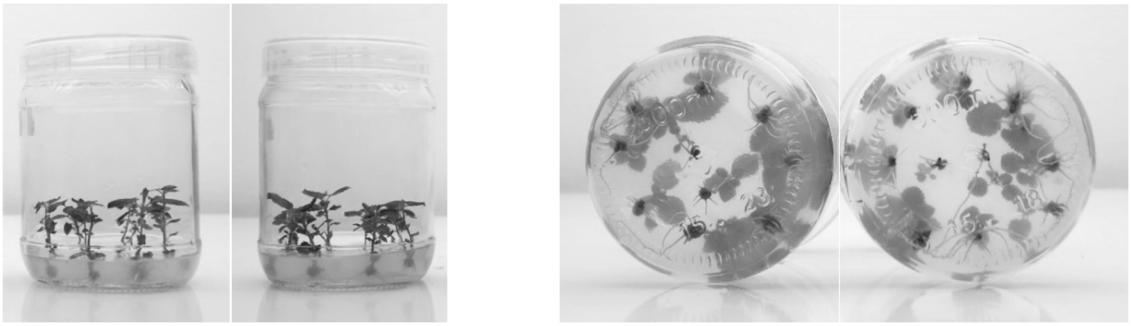
3.2. Rooting of Shoots In Vitro and Acclimation of Plantlets

The size of the obtained plantlets (rooted shoots) after 3 weeks of in vitro rooting varied depending on the clone (Table 8, Figure 2). Plantlets of the 'Ball' type had the longest shoots and medium-sized callus at the shoot base. Plantlets of 'SnCl' were the shortest and produced the largest callus. Plantlets of 'AtBr' were intermediate in size and produced the smallest callus. Contrary to other clones' plantlets, 'SnCl' produced significantly fewer roots, which were also longer. Both the in vitro rooting of shoots and the acclimation of obtained plantlets were the worst in the case of clone 'SnCl. The 'Ball' shoots rooted better than 'AtBr' shoots, while the acclimation of plantlets of both clones was similar.

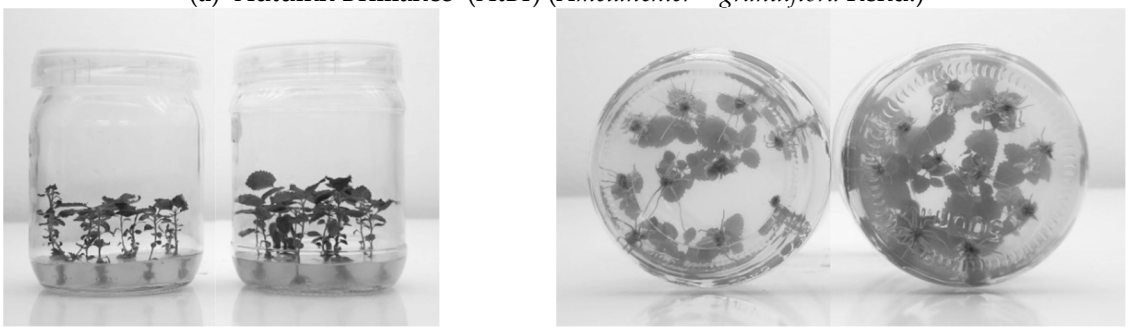
Table 8. In vitro rooting of shoots and acclimation of plantlets of three serviceberry clones.

Clone:	AtBr	Ball	SnCl	SL [2]
Mean shoot length [mm]	2.8 ± 0.06 b [1]	3.3 ± 0.05 c	2.3 ± 0.05 a	***
Callus size [mm]	3.7 ± 0.41 a	4.6 ± 0.13 b	6.1 ± 0.20 c	***
No. of roots	6.4 ± 0.41 b	6.9 ± 0.33 b	4.5 ± 0.33 a	***
Mean root length [mm]	0.9 ± 0.06 a	1.1 ± 0.06 a	2.9 ± 0.17 b	***
Percentage of rooted shoots [%]	95.5 b	99.2 c	63.5 a	AF [3]: *
Percentage of acclimated plantlets [%]	89.7 b	90.7 b	56.7 a	AF: *

Statistical analyses were performed on 120 shoots/plantlets per clone; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ***—$p < 0.001$, *—$p < 0.05$; [3] AF—level of significance based on the test of difference between two proportions.



(**a**) 'Autumn Brilliance' (AtBr) (*Amelanchier* × *grandiflora* Rehd.)



(**b**) 'Ballerina' (Ball) (*Amelanchier* × *grandiflora* Rehd.)



(**c**) 'Majestic', previously named 'Snowcloud' (SnCl) (*Amelanchier laevis* Wieg)

Figure 2. Rooting in vitro of serviceberry shoot obtained from 1F and 2F medium (left and right jars, respectively) (phot. Adrian Baran). Jar size: height 11.5 cm; diameter 8 cm.

Plantlets obtained from shoots collected from '2F' medium were shorter and developed smaller callus in comparison with the control (plantlets derived from '1F' shoots). The number and length of roots remained unchanged—the shoots collected from 2F medium

rooted worse than '1F' shoots. The acclimation of plantlets in '2F' was impaired as well (Table 9).

Table 9. The influence of studied media on in vitro rooting of shoots and acclimation of plantlets.

From Medium:	1F	2F	SL [2]
Mean shoot length [mm]	2.9 ± 0.05 b [1]	2.7 ± 0.06 a	*
Callus size [mm]	5.2 ± 0.14 b	4.4 ± 0.17 a	*
No. of roots	6.2 ± 0.29 a	5.9 ± 0.33 a	ns
Mean root length [mm]	1.4 ± 0.09 a	1.5 ± 0.11 a	ns
Percentage of rooted shoots [%]	88.9 b	82.5 a	AF [3]: *
Percentage of acclimated plantlets [%]	89.3 b	66.9 a	AF: *

Statistical analyses were performed on 180 shoots/plantlets per treatment; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant, *—$p < 0.05$; [3] AF—level of significance based on the test of difference between two proportions.

Plantlets obtained from shoots collected from '2F' medium were shorter in the case of two clones ('SnCl', 'AtBr') than the control ones (Table 10). This was not observed for the clone 'Ball'. Differences in callus size were found only in the 'AtBr' clone—'2F' shoots developed a smaller one. The type of medium used during shoot multiplication did not significantly affect the size of the root system in the case of all the tested clones. Shoots derived from examined media rooted in vitro in similar numbers in each clone. However, the acclimation of the plantlets to '2F' was significantly worse than the control ('1F') ones in the case of all clones (Table 10).

Table 10. The influence of studied media on in vitro rooting of shoots and acclimation of plantlets of three serviceberry clones.

Clone:	AtBr		Ball		SnCl		SL [2]	SL_{int} [3]
Medium:	1F	2F	1F	2F	1F	2F		
Mean shoot length [mm]	2.9 ± 0.07 c [1]	2.6 ± 0.08 b	3.3 ± 0.09 d	3.3 ± 0.06 d	2.6 ± 0.06 b	2.0 ± 0.07 a	***	**
Callus size [mm]	4.4 ± 0.15 b	2.8 ± 0.29 a	4.7 ± 0.21 b	4.4 ± 0.14 b	6.5 ± 0.25 c	5.7 ± 0.30 c	***	*
No. of roots	6.7 ± 0.56 c	5.9 ± 0.60 bc	6.7 ± 0.42 c	7.1 ± 0.50 c	4.8 ± 0.46 ab	4.1 ± 0.48 a	***	ns
Mean root length [mm]	0.8 ± 0.05 a	1.1 ± 0.11 ab	1.2 ± 0.10 b	1.0 ± 0.07 ab	2.8 ± 0.20 c	3.0 ± 0.28 c	***	ns
Percentage of rooted shoots [%]	98.3 bc	92.0 b	100 c	98.3 bc	68.3 a	58.8 a	AF [4]: *	-
Percentage of acclimated plantlets [%]	100 d	76.6 bc	100 d	83.1 c	70.0 b	43.3 a	AF: *	-

Statistical analyses were performed on 60 shoots/plantlets per combination; the results are presented as mean ± SE. [1] means in rows marked with diverse letters are significantly different at $\alpha = 0.05$; [2] SL—level of significance of differences among variables; ns—not significant, ***—$p < 0.001$, **—$p < 0.01$, *—$p < 0.05$; [3] SL_{int}—level of significance of interaction (clone × medium); [4] AF—level of significance based on the test of difference between two proportions.

3.3. Relationship Between Media and Culture Traits

Correlation analysis revealed some clear relationships between the studied traits (Table 11(A,B)). The used medium traits were significantly and strongly correlated. The pH of the medium was negatively correlated with its conductance, refractive index, and dry matter content (Table 11(A)). None of the media traits were correlated with callus growth (in II and III stages) and the frequency of shoot necrosis. However, medium refractive index and dry mass content were strongly and negatively related to vitrification frequency. The traits of all used media were strongly correlated to shoot proliferation but not to their elongation in the multiplication (II) stage (Table 11(B)). In the case of medium pH, such a relationship was negative, contrary to other traits. The traits of all used media were also strongly correlated with the length of shoots in the rooting stage (III) but not with the size of roots (Table 11(B)). The vitrification frequency was not related to any studied trait of the cultures (Table 11(B)). The growth of callus in the multiplication (II) stage did not influence the properties of cultures in that stage, but significantly and negatively affected shoot rooting and acclimation, with the exception of root proliferation (Table 11(A,B)). Such a phenomenon was not confirmed for callus growth in the rooting stage (III).

Table 11. Chosen correlations between media and culture traits.

Part A	**pH**	**Cond.**	**RI**	**DMC**	**Call.II**	**Vitr.**	**Shoot Necr.**	**Call.III**
medium pH	x	−0.97 *,[1]	−0.93 *	−0.89 *	0.78	0.67	0.33	0.41
medium conductance (cond.)	−0.97 *	x	0.95 *	0.91 *	−0.79	−0.76	−0.44	−0.40
medium refractive index (RI)	−0.93 *	0.95 *	x	0.99 *	−0.59	−0.87 *	−0.33	−0.14
medium dry mass content (DMC)	−0.89 *	0.91 *	0.99 *	x	−0.49	−0.91 *	−0.36	−0.07
culture vitrification (vitr.)	0.67	−0.76	−0.87 *	−0.91 *	0.35	x	0.24	−0.18
callus size II stage (call.II)	0.78	−0.79	−0.59	−0.49	x	0.35	0.22	0.60
callus size III stage (call.III)	0.41	−0.40	−0.14	−0.07	0.60	−0.18	0.63	x

Part B	**stage II**							**stages III + IV**			
	shoot number				**shoot length**			**shoot**	**root**		
length [mm]:	**<5**	**15–30**	**31–45**	**>45**	**total**	**mean**	**R% [2]**	**length**	**length**	**number**	**A% [3]**
medium pH	0.93 *	0.89 *	0.60	0.03	0.62	0.08	−0.81 *	−0.92 *	0.69	−0.80	−0.76
medium conductance (cond.)	−0.95 *	−0.91 *	−0.58	0.05	−0.61	−0.05	0.83 *	0.92 *	−0.72	0.83	0.77
medium refractive index (RI)	−0.93 *	−0.86 *	−0.51	−0.07	−0.55	−0.07	0.64	0.87 *	−0.49	0.68	0.61
medium dry mass content (DMC)	−0.92 *	−0.81 *	−0.42	−0.04	−0.46	−0.01	0.56	0.82 *	−0.41	0.61	0.51
culture vitrification (vitr.)	0.76	0.60	0.29	−0.08	0.33	−0.01	−0.43	−0.74	0.29	−0.55	−0.44
callus size II stage (call.II)	0.62	0.73	0.73	−0.09	0.72	0.25	−0.95 *	−0.82 *	0.91 *	−0.90 *	−0.97 *
callus size III stage (call.III)	0.44	0.30	0.04	−0.50	0.03	−0.42	−0.69	−0.32	0.79	−0.58	−0.41

[1] * significantly important at $\alpha = 0.05$; [2] percentage of shoots' rooting; [3] percentage of plantlets' acclimation.

3.4. Total Efficiency of the Three Micropropagation Stages

After three stages of micropropagation, the 2F medium was more effective only in the case of the 'Ball' clone, which produced over 90% more acclimated plantlets compared with

the control (Table 12). The remaining two clones ('SnCl' and 'AtBr') produced significantly fewer plants after application of '2F' medium (−34% and −14%, respectively).

Table 12. The influence of studied media on the efficiency of in vitro propagation of three serviceberry clones.

Clone:	AtBr		Ball		SnCl	
Medium:	**1F (Control)**	**2F**	**1F (Control)**	**2F**	**1F (Control)**	**2F**
A. Stage II Number of long shoots (>15 mm) [1]	5.8	6.9	3.3	7.7	6.6	8.4
B. Stage III Percentage of rooted shoots [%]	98.3	92.0	100	98.3	68.3	58.8
C. Stage IV Percentage of acclimated plantlets [%]	100	76.6 c	100	83.1	70.0	43.3
B × C [%]	98.3	70.5	100	81.7	47.8	25.5
D. Number of obtained plantlets (A × B × C)	5.7	4.9	3.3	6.3	3.2	2.1
E. Micropropagation efficiency [%] [2]	100	86	100	190.9	100	65.6

[1] chosen results from Tables 7 and 10; [2] percentage of the number of obtained plantlets compared with the control (1F).

4. Discussion

Serviceberry (*Amelanchier* sp.) is a comparatively young crop [1,3,4]. As interest in these plants grows, so does the demand for healthy and uniform nursery plants. Micropropagation is far more efficient than other conventional cloning methods; thus, it should improve the breeding and rapid propagation of new, valuable serviceberry cultivars [2,3,5–16]. There are some reports of the successful reproduction of *Amelanchier* sp. involving in vitro cultures. Nevertheless, some problems like physiological disorders coupled with intense shoot proliferation, unsatisfactory rooting, and the acclimation of shoots still exist [4,14]. However, micropropagation is an expensive method. Therefore, each increase in its efficiency or reduction in costs facilitates its use in breeding and nurseries. The double-phase method may contribute to achieving such a goal. It is simple and does not require expensive and specialized equipment [17,18]. In the opinion of Senapati [31], it is even cheaper than the control, based on the usage of solid media. The genus *Amelanchier* belongs to the subfamily Pomoideae. Lineberger [5] found a similar response of in vitro cultures of *Amelanchier laevis* and other Rosaceae woody plants to the tested factors/conditions. Probably, some solutions that gave beneficial results in the micropropagation of other related genera, like *Malus*, *Pyrus*, *Cydonia*, and *Aronia*, will also bring good results in the case of serviceberry. One of them is the application of a double-phase medium, which generally gave beneficial effects in those genera [19–21,23–28,32,33]. Any reports concerning the influence of double-phase media on cultures of *Amelanchier* sp. have not been found in scientific databases.

In the present study, the response of the in vitro cultures in the multiplication stage was positive. The shoots obtained on 2F medium were significantly longer than those grown on 1F medium. The double-phase (2F) medium stimulated the proliferation of shoots longer than 15 mm, and especially those longer than 30 mm, suitable for rooting both in and ex vitro (Table 4). The response of serviceberry culture is consistent with previous reports of increased proliferation and/or elongation of shoots on other Pomoideae genera [19–21,23–28,32,33] and other woody plants [18,21]. It should be noted, however, that the reaction of clone 'Ball' was the strongest, while that of clone 'AtBr' was only slight

(Table 6). A similar observation was made by Litwińczuk [27] on 'M 26', 'MM 106', and 'P 14' apple rootstocks. A different reaction to 2F medium was also noted in the event of two *Aronia* clones, but it concerned only the elongation of shoots, not branching [28,33]. It is noteworthy that the 2F medium completely inhibited the dieback of shoot tips. This disease occurred in cultures of two clones ('SnCl' and 'AtBr') on control (1F) media (Table 6). Such an effect was previously described by Vinterhalter [25] in *Cydonia* cultures. Liquid medium prevented such disease also in the case of *Pyrus* sp., but in the TIS (temporary immersion system [36] in comparison to solid media. They also did not observe the other physiological disorder, i.e., hyperhydricity (vitrification) in TIS, contrary to in liquid media. The occurrence of hyperhydricity was reported in the case of cultures of *Amelanchier* sp. grown both in the solid and liquid media [7,8]. However, on the latter medium, the disease was much more severe [7]. In the present study, the vitrification was found in cultures of two ('AtBr', 'SnCL') of the three studied serviceberry clones (Table 2). However, the relationship between the kind of medium and frequency of this disorder was not statistically confirmed (Tables 4 and 6). The 2F medium did not change the frequency of hyperhydricity over the three subsequent passages in our study. This was similar for apple rootstock cultures [27] and several other plants [19]. Viseur [20] even suggested that 2F limited this disorder. However, this did not work in the case of mulberry cultures, where such a medium caused severe vitrification [37]. Contrary to the previous studies on *Malus* and *Aronia* [27,28], the differences in the acidity and conductance changes, as well as refractive index and dry mass content of the tested media, were present (lower values for 2F medium) but not statistically confirmed (Tables 5 and 7). This may indicate that the nutrient preferences of the studied *Amelanchier* clones were similar on solid and double-phase media, and that the availability of electrolytes did not play a major role in the growth of cultures. However, the results of correlation analysis shed additional light on these issues. All used medium traits (acidity, conductance, refractive index, dry mass content) were strongly correlated to shoot proliferation but not to their elongation in the multiplication (II) stage (Table 11(B)). This supports the well-known opinion that the thin liquid layer over the solid medium facilitates the faster diffusion of cytokinins (stimulators of branching, inhibitors of shoot elongation). Cell divisions stimulated by cytokinins increased the demand for carbohydrates and other nutrients. This could be fulfilled by facilitated diffusion of sucrose, its breakdown, and the uptake of hydrolysis products, as well as other mineral media ingredients. It could also prevent shoot tip necrosis (Table 4). We made similar observations in our previous studies on *Malus*, *Aronia*, and *Morus* [27,28,37]. Severe depletion of the medium with carbohydrates and other nutrients could result in a strong reduction in the medium's osmolarity and result in a less controlled water supply and consequently increase hyperhydricity. The ANOVA results do not clearly confirm such a thesis (Tables 2, 4 and 6), contrary to the results of the correlation analysis. The refractive index and dry mass content of the media used were strongly and negatively related to vitrification frequency (Table 11(A)).

It seems that in the case of the micropropagation of the serviceberries, the unsatisfactory efficiency of stages III and IV (among others, weak and long-lasting rooting of shoots, post-rooting dormancy, transplantation losses, clone-specific response) is a greater problem than the problems encountered in stage II [2,3,11,14]. Some studies regarding the optimization of the last stages of serviceberry micropropagation were conducted. Hunková et al. [15] evaluated many factors that contribute to effective in vitro rooting and acclimatization of micropropagated shoots of *Amelanchier* sp. The greatest number of actively growing plants was recorded after rooting the shoots on 1/2 MS solid medium supplemented with 1 mg L^{-1} NAA, followed by a spray treatment with 1 mg L^{-1} BA. Lineberger [5] recommended 1/4 strength, whereas Alosaimi and Tripepi [14] even recommended the 1/8 strength MS. Clapa et al. [12] used a new, interesting technique—a direct ex vitro rooting in floating

perlite—with success. It seems that despite relatively many experiments, no proven and universal method of rooting shoots for various species and cultivars of *Amelanchier* has been developed. We have also experienced this in our study. In our preliminary research on in or ex vitro rooting, we obtained the best result for 1/4 strength MS (data are not presented). Therefore, we used such a medium in the presented study (Table 1). However, unfavorable effects occurred, such as excessive callus growth. This began rather after root development and did not adversely affect shoot rooting or acclimation. It was confirmed by the results of correlation analysis (Table 11(B)). Therefore, further research should be carried out. Maybe a two-step in vitro rooting process and the application of vitamin B_2 or other chosen amino acids will bring profitable results.

The subsequent effect of the 2F medium used during culture multiplication on shoot rooting has not been determined so far. Nevertheless, there are some reports [21,27,32] that shoots of the Pomoideae species (mainly *Malus* sp.) originated from the 2F medium rooted better or at least as well as the control ones. On the other hand, Harris and Mason [6] reported that shoots of *Amelanchier alnifolia* produced in a liquid medium (TIS) were longer and rooted better and more quickly than those produced on semi-solid media (8 compared to 24 days, respectively). The determination of the residual impact of 2F medium was therefore justified. Unfortunately, it was clearly unfavorable in the case of all three tested clones, both for in vitro rooting and for the acclimation of the obtained plantlets (Table 10). Nevertheless, the response of the three cultivars was varied. The shoots of the clones that proliferated strongly in vitro ('SnCl', 'AtBr') rooted much worse than the shoots of the 'Ball' clone (Tables 3 and 6 vs. Table 10). This is likely due to the vitrification that the 'SnCl' and 'AtBr' cultures underwent in both media types, contrary to the 'Ball' cultures (Tables 2 and 6). However, our correlation analysis did not confirm a direct relationship between the occurrence of hypehydricity and rooting and the acclimation of shoots (Table 11(A)). It may be explained by a negative selection of vitrified shoots before rooting. Perhaps another, difficult-to-detect physiological problem has occurred (excess cytokinin contents in shoots, osmotic stress). This may be indicated by the strong, negative correlation between callus growth during culture multiplication (stage II) and shoot rooting and acclimation (Table 11(B)). Supposedly, the callus size may be a marker of changes unfavorable to shoot rooting and a signal to terminate the subculture early. The negative, subsequent reaction of the 'Ball' clone to the previously used 2F medium was therefore relatively the weakest (Table 10). However, such a medium significantly and strongly improved the growth of 'Ball' cultures during stage II in comparison to the other clones (Table 6). Thus, the total efficiency of the three micropropagation stages was significantly improved for that clone as a result of using 2F medium (Table 11). The micropropagation efficiency of the remaining clones ('SnCl', 'AtBr') was clearly deteriorated. Therefore, there is still a need to find solutions that can limit losses in the last stages of micropropagation.

5. Conclusions

To summarize, serviceberries are still recalcitrant in micropropagation. The different response of cultivars to culture conditions, known for the genus *Amelanchier* and other plant species [3,27], was confirmed in this study. Despite the beneficial effect of the double-phase medium on the multiplication of serviceberry cultures, the assessment of its usefulness is ambiguous. Considering the efficiency of the three micropropagation stages, the usage of 2F medium only worked very favorably in the case of the 'Ball' clone, yielding over 90% more acclimated plantlets. In the current state, such a solution was not beneficial for the other two cultivars. Research into further optimization of all stages of micropropagation is still necessary.

Author Contributions: Conceptualization, methodology, establishment of in vitro cultures and care, statistical analyses, manuscript writing: W.L.; preparation and plant measurements, data input: B.J.; preliminary experiments, analyses of media: A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Polish Ministry of Science and Higher Education research project within the University of Rzeszów, PB/ZFiBR/2023-5.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: We are grateful to Adrian Baran for taking photos.

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

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