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In Vitro Propagation of *Pyracantha angustifolia* (Franch.) C.K. Schneid.

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Abstract: Narrow-leaf firethorn or pyracantha (*Pyracantha angustifolia* (Franch.) C.K. Schneid.), from the family Rosaceae, is a species of large and thorny evergreen shrub. In this study, a procedure is presented for efficient axillary shoot multiplication and root induction in *P. angustifolia* using Murashige and Skoog (MS), woody plant (WPM), and Linsmaier and Skoog (LS) culture media supplanted with 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA). The disinfection of the axillary buds was performed with a 70.23% success rate on a basal MS medium augmented with 0.5 mg·L^{-1} gibberellic acid (GA_3). Uniform and axenic explants were then cultured on MS, WPM, and LS media enriched with different concentrations of BAP, 0.3 mg·L^{-1} GA_3, and 0.1 mg·L^{-1} IBA. The highest multiplication coefficient (2.389) was obtained for the MS medium supplemented with 2.5 mg·L^{-1} BAP. After one month, newly formed micro-shoots were transferred to rooting media (MS, WPM, and LS) containing different concentrations of IBA, together with a constant concentration of 0.1 mg·L^{-1} BAP. The micro-shoots were kept in the dark for one week and then cultured in a 16/8 h light/dark regime. The MS medium supplemented with 1 mg·L^{-1} IBA was the most effective in stimulating rooting (88.76% of micro-shoots). The highest number of roots (3.5 per micro-shoot) was produced in the MS medium enriched with 1.5 mg·L^{-1} IBA. The rooted plantlets were transferred into pots filled with perlite and peat moss in a 2:1 proportion and acclimatized to ambient greenhouse conditions, with a resultant mean 92.84% survival rate. Thus, this protocol can be successfully applied for the in vitro mass propagation of *P. angustifolia*.

Keywords: culture medium; micropropagation; ornamental trees and shrubs; plant growth regulators

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1. Introduction

Pyracantha is a genus of large, thorny, and perennial evergreen shrubs in the family Rosaceae, subfamily Amygdaloideae, and order Rosales. They are native to an area extending from southwest Eastern Europe to Southeast Asia. The plants can reach up to 4.5 m in height. Their leaves are small and slender, with serrated margins and numerous thorns [1]. The name "pyracantha" is derived from the Greek words "pyr", meaning fire, and "akanthos", meaning "a thorn", hence "fire-thorn". The genus *Pyracantha* includes 11 species (*P. angustifolia*, *P. atalantioides*, *P. coccinea*, *P. crenatoserrata*, *P. crenulata*, *P. crenulata-serrata*, *P. densiflora*, *P. fortuneana*, *P. inermis*, *P. koidzumii*, and *P. rogersiana*) with small white flowers [2]. The flowers are produced during late spring and early summer. Fruit, i.e., red, orange, or yellow pomes, develop in late summer and mature in late autumn [1,3]. *Pyracantha* is commonly used in the food and medicinal industries, commercial landscapes, and as a hedge or barrier plant [4]. One of the known species of *Pyracantha*

is *P. angustifolia*, which is distinguished from the other *Pyracantha* species by its morphological characteristics of leaves and flowers [4]. Their leaves are relatively narrow, with rounded tips and entire margins, and their undersides have a density of white hair [5]. The color of its mature fruit is yellow to dark orange, and it is usually covered with white hair. The antioxidant and anti-inflammatory effects of *Pyracantha angustifolia* fruit extracts have been reported [1]. This species is cultivated as an ornamental garden plant, especially in temperate regions, as the shrub is resistant to cold and seasonal drought. Many cultivars of *P. angustifolia* are the result of hybridization with other inbred cultivars [6]. Due to the high demand for this ornamental shrub, the slow propagation pace of *Pyracantha*, and relatively low and uneven production of valuable phytochemicals under natural conditions, micropropagation is a suitable method for the mass production of this genus.

In vitro propagation has been recognized as an important and efficient technique for the large-scale propagation of horticultural plants, overcoming the problems caused by heterogeneous seed production [7]. It also allows for the elicitation and more uniform production of secondary metabolites [8]. The commercial use of propagating woody plants through tissue culture is more difficult than with herbaceous plants due to the limited multiplication efficiency and plantlets' survival during acclimatization [9]. Nonetheless, several woody plant species are successfully micro-propagated in vitro [10]. The success of the in vitro propagation methods depends on several factors, such as the plant's genotype, medium type, plant growth regulators (PGRs), and explant parameters, which should be carefully optimized [11]. To date, there is no available information about the micropropagation of *Pyracantha angustifolia*. Only one paper has been published on the micropropagation of *P. coccinea* [12]. These researchers solely used the MS medium supplemented with 6-benzylaminopurine (BAP) or indole-3-butyric acid (IBA), and they found that the highest shoot proliferation rate (3.40 axillary buds) was obtained using 3/4 strong MS with 0.3 mg·L^{-1} IBA. As for the rooting step, 1/4 MS medium with 18.9 mg·L^{-1} IBA was most effective. The successful use of IBA and BAP for the shoot multiplication of some members of Rosaceae, such as *Prunus cerasifera* Ehrh. [13] and *Prunus persica* L. [14], has been reported. Likewise, studies have shown that the best auxin for rooting in several members of Rosaceae is IBA [14–16]. The results of various studies have shown that different PGR combinations and concentrations have significant roles in increasing the micropropagation efficiency of ornamental and fruit trees or shrubs belonging to the Rosaceae family [16–18]. The most frequently used explant type in these studies was the stem node.

The present study aimed to evaluate the effects of different concentrations of BAP and IBA, added individually or in combination, into the Murashige and Skoog (MS), woody plant (WPM), and Linsmaier and Skoog (LS) media on the in vitro propagation of *P. angustifolia* through direct organogenesis.

2. Materials and Methods

2.1. Plant Material

Plant material was obtained from the municipality of Tabriz city in East Azarbaijan province, Iran. East Azarbaijan province is located in the northwest of Iran at an altitude of 1800 m above sea level at 46° and 25′ east longitude and 38° and 2′ north latitude from the Greenwich meridian. This province is located in a mountainous region and has a climate with cold and long winters and mild summers. Plant samples were collected from the lateral buds of *Pyracantha angustifolia* shrubs in Tabriz city in September 2020. The samples were taken in the form of 10–15 cm-long cuttings and, to preserve moisture and prevent possible injuries, they were wrapped in a wet cloth and transported to the laboratory in an ice flask. The leaves of the cuttings were removed and then cut into approximately 2 cm-long pieces with 1 or 2 buds.

2.2. Explants Disinfection

In order to reduce surface contamination, the explants were washed carefully with dishwashing liquid and Captan 80 WDG fungicide (3 g per 500 mL water for 20 min).

Next, for better washing and removal of inhibitory substances, the explants were placed under tap water for an hour. After preliminary washing, all the explants were treated with 70% (*v*/*v*) ethyl alcohol for 1 min and then placed in sodium hypochlorite (NaOCl) at a concentration of 2% (*v*/*v*) for 5 min. All disinfection steps were performed under a laminar flow hood cabinet and 12 drops of dishwashing liquid (1 mL per 500 mL water for 20 min) were added to increase the contact surface of the disinfectant liquid with the explant. Then, the explants were washed three times in sterile distilled water, each time for 5 min. To remove the parts of the explants that were damaged in the disinfection treatments, the bases of the explants were cut off.

2.3. Establishment of In Vitro Culture

Each of the three disinfected explants were placed inside sterile Petri dishes filled with 50 mL of basal Murashige and Skoog [19] (MS) culture medium containing 3% (*w*/*v*) sucrose and 0.8% (*w*/*v*) agar (SIGMA Aldrich, Milwaukee, Brookfield, WI, USA) augmented with 0.5 mg·L^{-1} filtered sterilized gibberellic acid (GA_3) (SIGMA Aldrich, Milwaukee, WI, USA). The pH of the media was adjusted to 5.6–5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media contained in the culture bottles were autoclaved at 105 kPa and 121 °C for 20 min. In the first week of culture, the explants were kept in the dark at 24 ± 2 °C and, thereafter, at the same temperature with a 16/8 h light/dark regime with a light intensity of 50–60 µmol·m^{-2}·s^{-1} provided by cool−white fluorescent tubes. After 30 days, the newly formed micro-shoots (Figure 1A) were used for the subsequent experiments.

Figure 1. In vitro propagation of *Pyracantha angustifolia*. (**A**) Shoots produced in the initiation step in the MS medium containing 0.5 mg·L^{-1} GA_3. (**B**) Preparation of the explants (nodal segments with

axillary buds) for the multiplication step. (**C**) Micro-shoots produced in the proliferation step (LS medium containing 3 mg·L^{-1} BAP (left), 2 mg·L^{-1} BAP (medial), and 2.5 mg·L^{-1} BAP (right)). (**D**) Micro-shoots produced in the proliferation step (MS medium containing 2 mg·L^{-1} BAP (left), 2.5 mg·L^{-1} BAP (medial), and 3 mg·L^{-1} BAP (right)). (**E**) Micro-shoots produced in the MS medium containing 2.5 mg·L^{-1} BAP. (**F**) Micro-shoots produced in the WPM containing 2.5 mg·L^{-1} BAP. (**G**) Rooted micro-shoots in the MS medium containing 1 mg·L^{-1} IBA. (**H**) Plantlets obtained from the MS medium containing 1 mg·L^{-1} IBA. (**I**) Acclimatization of the plantlets in plastic pots filled with perlite and peat moss (2:1) in the acclimatization room.

2.4. Shoot Proliferation

After the establishment and growth of the micro-shoots, a total of 108 nodal explants with axillary buds (Figure 1B) were used as secondary explants in the proliferation stage (Figure 1C–F). Three types of basal culture media–MS, woody plant medium (WPM) [20], and Linsmaier and Skoog (LS) [21]—containing 3% sucrose and 0.8% agar were prepared. The pH levels of the media were adjusted to 5.6–5.8 before autoclaving as described above. The media were augmented with different concentrations of BAP (0, 2, 2.5, and 3 mg·L^{-1}) for the induction of direct organogenesis. In all experimental objects, 0.3 mg·L^{-1} GA_3 and 0.1 mg·L^{-1} IBA were used for hormonal balance. Jars of jam were used as the culture vessels and 50 mL of medium was poured into each jar. All the cultures were incubated at 24 ± 2 °C and a 16 h photoperiod of 50–60 µmol·m^{-2}·s^{-1} irradiance was provided by cool−white fluorescent tubes. The produced micro-shoots were sub-cultured twice after 30 days.

2.5. Root Induction on Microshoots

The micro-shoots produced in each culture vessel, from all experimental objects, were transferred to the rooting media. Three culture media–MS, WPM, and LS—were prepared and autoclaved. The media were supplemented with different concentrations of IBA (0, 0.1, 0.5, 1, and 1.5 mg·L^{-1}) (Figure 1G). For hormonal balance, 0.1 mg·L^{-1} BAP was added to these root induction media. All the cultures were kept in the growth chamber with the same light and temperature conditions as described previously.

2.6. Acclimatization Process

The rooted micro-shoots—95 plantlets from the MS medium, 55 from the LS medium, and 45 from the WPM medium (Figure 1H)—were taken out from the culture jars and the remains of the culture medium were removed from the roots. Then, the lower leaves of the plantlets were dissected with scissors for easier cultivation of the plantlets in the pots. The plantlets were placed in the autoclaved perlite and peat moss substrate (in a ratio of 2:1) in plastic pots and watered with sterile water. A transparent plastic cup was also placed over the plantlets. The pots were placed in an acclimatization room with 70 ± 5% relative humidity under a 16/8 h light/dark regime (light intensity of 100 µmol·m^{-2}·s^{-1}) at a temperature of 25 ± 2 °C. The plantlets were fed by a syringe with nutrients containing 1 g·L^{-1} of N.P.K and 500 mg·L^{-1} of iron every 4 days from the second week of transferring to the acclimatization room. After 30 days, the plantlets were transferred to larger plastic pots containing garden soil and perlite in the proportion of 2:1 and transferred to the greenhouse (Figure 1I).

2.7. Experimental Design and Data Analysis

The factorial in vitro experiment was conducted in a completely randomized block design with 10 replications. Each experimental unit consisted of 5 jars, and 3 explants were cultured in each jar. The effect of the culture media on the growth of the explants and the percentage of the survival of the plantlets were evaluated. The first observation was completed 15 days after cultivation and the second one was repeated 15 days later. Initially, the number of jars in each experimental unit was higher, but 5 days after culture initiation,

by removing the contaminated jars, it was reduced to 5 jars in each experimental object. This design was repeated in the multiplication (proliferation) and rooting experiments.

During the micro-shoot proliferation stage, the multiplication coefficient, micro-shoot length (cm), and share of hyperhydrated micro-shoots (%) were evaluated after 30 days of culture. The micro-shoots produced in the proliferation stage were usually suitable for rooting, but to produce more shoots, the explants were sub-cultured twice.

The rooting of micro-shoots was performed for 21 days, after which the rooting effectiveness (%), root number, and root length (mean length of all the regenerated roots per one micro-shoot; cm) were measured.

The greenhouse experiments were set in a completely randomized block design with three replicates. Data were subjected to analysis of variance (ANOVA) and the means were compared by Duncan's test at $p < 0.05$ using the SAS version 9.1 software [22].

3. Results

3.1. Micro-Shoot Establishment

After disinfection, where efficiency reached 70.23%, the explants were transferred to the initiation culture medium (MS with 0.5 mg·L^{-1} GA_3), on which narrow-leaf firethorn shoots were successfully established (Figure 1A). The share of shoot-producing nodal segments was 88.8, 83.33, and 72.23% in the MS, LS, and WPM media, respectively. Secondary explants (nodal segments) were obtained after 30 days of culture (Figure 1B). They were then transferred to the proliferation media (MS, WPM, and LS) augmented with different concentrations of BAP (Figure 1C–F).

3.2. Micro-Shoot Proliferation

The analysis of variance showed that in terms of all the studied characteristics, there was a significant effect of the tested BAP concentrations and basal culture media types, singularly and in combination (Table 1, Supplementary Figures S1 and S2). In general, the MS medium was more effective than the LS and WPM media (Supplementary Figure S1). The highest multiplication coefficient (2.389) was obtained in the MS medium augmented with 2.5 mg·L^{-1} BAP (Table 2). This coefficient was approximately two-fold higher than that of the other treatments. The lowest multiplication coefficients (0.960 and 1.021), on the other hand, were obtained in the WPM and LS media, both without BAP (control). Likewise, the highest concentration of BAP (3 mg·L^{-1}) had a negative effect on the multiplication coefficient in all three media types (MS, WPM, and LS) (Table 2).

Table 1. Analysis of variance of the effects of the different concentrations of BAP and the basal culture medium type on the measured characteristics of the *Pyracantha angustifolia* plants grown in vitro in the multiplication step.

Source of Variance	df	Multiplication Coefficient MS	Plantlet Length MS	Hyperhydricity MS
BAP	3	0.379 **	0.598 **	2.259 *
Culture medium	2	2.943 **	0.874 **	19.704 **
BAP × culture medium	6	0.173 *	0.019 **	0.148 *
Error	24	0.053	0.04	0.481
CV (%)	-	14.79	11.33	18.61

*, **: significant at the 0.05 and 0.01 probability level, respectively; CV: coefficient of variation; df: degrees of freedom; MS: mean square.

The longest micro-shoots (2.132 and 2.081 cm) were produced in the MS and LS media, both of which were supplemented with 2.5 mg·L^{-1} BAP, respectively (Table 2). The shortest lengths (1.513 and 1.523 cm) were obtained in the WPM medium containing 3 mg·L^{-1} BAP and in the control object without BAP, respectively. The evaluation of the individual effects of medium type and BAP concentration on the length of the micro-shoots also showed the superiority of the MS medium and the 2.5 mg·L^{-1} BAP concentration (Supplementary Figures S1 and S2).

Table 2. Effects of the different concentrations of BAP and the basal culture medium type on the measured characteristics of the *Pyracantha angustifolia* plants grown in vitro in the multiplication step.

Treatment		Multiplication Coefficient	Plantlet Length (cm)	Hyperhydricity (%)
Culture Medium	BAP (mg·L^{-1})			
MS	0	1.213 ^{d} ± 0.220	1.624 ^{cd} ± 0.316	3.351 ^{cd} ± 0.751
	2	1.516 ^{c} ± 0.075	1.645 ^{c} ± 0.119	3.269 ^{d} ± 0.635
	2.5	2.389 ^{a} ± 0.488	2.132 ^{a} ± 0.414	2.053 ^{f} ± 0.332
	3	1.061 ^{de} ± 0.193	1.614 $^{c–e}$ ± 0.329	3.422 $^{b–d}$ ± 0.564
WPM	0	0.960 ^{e} ± 0.174	1.523 ^{g} ± 0.296	3.541 $^{a–c}$ ± 0.489
	2	1.082 ^{de} ± 0.196	1.543 ^{fg} ± 0.342	3.716 ^{a} ± 0.722
	2.5	1.971 ^{b} ± 0.358	1.838 ^{b} ± 0.157	2.741 ^{e} ± 0.533
	3	1.041 ^{e} ± 0.189	1.513 ^{g} ± 0.294	3.615 ^{ab} ± 0.702
LS	0	1.021 ^{e} ± 0.185	1.553 $^{e–g}$ ± 0.302	3.532 $^{a–c}$ ± 0.687
	2	1.617 ^{c} ± 0.294	1.594 $^{c–f}$ ± 0.409	3.564 $^{a–c}$ ± 0.692
	2.5	2.002 ^{b} ± 0.364	2.081 ^{a} ± 0.391	2.132 ^{f} ± 0.414
	3	1.092 ^{de} ± 0.198	1.564 $^{d–g}$ ± 0.537	3.553 $^{a–c}$ ± 0.690

Means ± standard deviations with different letters in the same column are significantly different ($p < 0.05$) based on the Duncan's test.

3.3. Micro-Shoot Hyperhydricity

The highest shares of hyperhydrated shoots (3.716 and 3.615%) were reported in the WPM medium supplemented with 2 and 3 mg·L^{-1} BAP, respectively. On the other hand, the lowest rate of hyperhydricity (2.053%) was found in the MS medium supplemented with 2.5 mg·L^{-1} BAP. In general, the micro-shoots produced in all three culture media (MS, WPM, and LS) enriched with 2.5 mg·L^{-1} BAP showed the lowest rates of hyperhydricity (Table 2). These findings were also confirmed during the individual evaluations of each of the factors (Supplementary Figures S1 and S2).

3.4. Micro-Shoot Rooting

Statistical analysis of the data showed that there was a significant influence of the studied factors, singularly and in combination, regarding rooting effectiveness (Table 3, Supplementary Figures S3 and S4). There was also a significant difference between the various concentrations of IBA and the culture medium type regarding the root number. Moreover, the type of basal culture medium affected the elongation of the roots (Table 3).

Table 3. Analysis of variance of the effects of the different concentrations of IBA and the basal culture medium type on the measured characteristics of the *Pyracantha angustifolia* plants grown in vitro in the rooting step, as well as the acclimatization effectiveness.

Source of Variance	df	Rooting Effectiveness MS	Root Number MS	Root Length MS	Acclimatization MS
IBA	4	6066.93 **	325.30 *	0.490 ^{ns}	425.80 **
Culture medium	2	845.48 **	14.40 *	7.941 **	26.63 ^{ns}
IBA × culture medium	8	159.98 *	23.50 **	0.189 *	169.95 **
Error	30	38.96	15.8	0.191	15.7
CV (%)	-	15.67	16.2	13.11	8.34

*, **: significant at the 0.05 and 0.01 probability level, respectively; ^{ns}: not significant at $p < 0.05$; CV: coefficient of variation; df: degrees of freedom; MS: mean square.

The highest and the lowest production of roots were observed in the MS and WPM media, respectively (Supplementary Figure S3). The maximum rooting efficiency (88.76%) was found in the MS medium with 1 mg·L^{-1} IBA. On the other hand, the minimum rooting effectiveness levels of 32.71% and 35.59% were reported for the control WPM medium and that with 0.1 mg·L^{-1} IBA, respectively (Table 4).

Table 4. Effects of the different concentrations of IBA and the basal culture medium type on the measured characteristics of the *Pyracantha angustifolia* plants grown in vitro in the rooting step, as well as the acclimatization effectiveness.

Treatment		Rooting Effectiveness (%)	Root Number	Root Length (cm)	Acclimatization (%)
Culture medium	IBA (mg·L^{-1})				
MS	0	73.30 ^{d} ± 13.34	0.875 ^{e} ± 0.14	3.790 ^{b} ± 0.639	96.29 ^{ab} ± 4.17
	0.1	76.09 ^{cd} ± 13.85	1.75 ^{d} ± 0.34	3.840 ^{b} ± 0.447	96.29 ^{ab} ± 3.17
	0.5	80.10 ^{bc} ± 14.58	2.25 ^{bc} ± 0.36	3.952 ^{b} ± 0.367	97.2 ^{ab} ± 3.27
	1	88.76 ^{a} ± 16.16	2.625 ^{b} ± 0.57	4.297 ^{a} ± 0.725	97.2 ^{ab} ± 5.27
	1.5	81.21 ^{b} ± 11.78	3.5 ^{a} ± 0.39	4.155 ^{a} ± 0.704	100 ^{a} ± 2.56
WPM	0	32.71 ^{j} ± 5.95	0.375 ^{i} ± 0.11	1.817 ^{i} ± 0.307	84.08 ^{e} ± 1.88
	0.1	35.59 ^{ij} ± 6.48	0.5 ^{h} ± 0.12	1.962 ^{hi} ± 0.132	86.11 ^{de} ± 4.04
	0.5	39.69 ^{hi} ± 10.22	0.61 ^{g} ± 0.14	2.162 ^{gh} ± 0.364	86.11 ^{de} ± 4.04
	1	44.88 ^{g} ± 4.17	0.625 ^{fg} ± 0.11	2.397 ^{f} ± 0.405	88.10 $^{c–e}$ ± 2.14
	1.5	41.59 ^{gh} ± 7.39	0.68 ^{f} ± 0.13	2.225 ^{fg} ± 0.377	91.11 $^{b–d}$ ± 2.33
LS	0	41.76 ^{gh} ± 9.60	0.625 ^{fg} ± 0.12	3.172 ^{e} ± 0.596	93.46 ^{bc} ± 3.42
	0.1	42.70 ^{gh} ± 6.78	0.725 ^{ef} ± 0.12	3.215 ^{e} ± 0.440	93.46 ^{bc} ± 6.41
	0.5	49.91 ^{f} ± 9.09	1.75 ^{cd} ± 0.26	3.335 ^{de} ± 0.665	94.46 $^{a–c}$ ± 3.53
	1	55.03 ^{e} ± 10.01	1.80 ^{cd} ± 0.31	3.540 ^{c} ± 0.390	94.46 $^{a–c}$ ± 5.52
	1.5	50.82 ^{ef} ± 8.25	1.84 ^{c} ± 0.33	3.440 ^{cd} ± 0.481	95.82 ^{ab} ± 4.23

Means ± standard deviations with different letters in the same column are significantly different ($p < 0.05$) based on Duncan's test.

Likewise, the highest and lowest root numbers were produced in the MS and WPM media, respectively (Supplementary Figure S3). The highest number of roots (3.5 per micro-shoot) was produced in the MS medium supplemented with 1.5 mg·L^{-1} IBA. On the other hand, the lowest root number (0.375 per micro-shoot) was produced in the WPM medium without IBA (Table 4).

The longest and shortest roots were found in the MS and WPM media, respectively. The longest roots (4.297 and 4.155 cm) were produced in the MS medium with 1 and 1.5 mg·L^{-1} IBA, respectively. The shortest roots (1.817 and 1.962 cm) were regenerated in the control WPM medium and that with 0.1 mg·L^{-1} IBA, respectively (Table 4).

3.5. Acclimatization Efficiency

It was found that 84.08–100% of the plantlets survived transplantation and acclimation to the greenhouse conditions (Figure 1I). The composition of the culture medium, particularly the auxin concentration (Supplementary Figure S4), used during rooting affected the survival of the plantlets (Tables 3 and 4). The highest survival rate was reported in the 1.5 mg·L^{-1} IBA treatment in the MS culture medium and the lowest in the WPM control object (0 mg·L^{-1} IBA).

4. Discussion

Production of true-to-type plants within a short time is the main goal for successful in vitro propagation. Many woody ornamental species are recalcitrant to vegetative propagation by conventional cutting methods. Thus, in vitro propagation techniques have been developed [12]. The present study reports a mass propagation system for *Pyracantha angustifolia*, an economically valuable ornamental shrub. The optimum concentration of cytokinins for maximum shoot multiplication is different for each species. This might be due to the different contents of endogenous phytohormones. Moreover, the response to exogenous PGRs varies between cultivar and explant type [11,17,18]. Several studies have reported that the use of 0.1–2 mg·L^{-1} BAP is optimal for shoot proliferation in tree and shrub species [13,23,24]. The current study confirmed that BAP is effective for the proliferation of micro-shoots in *P. angustifolia* when used in moderately high concentrations (2.5 mg·L^{-1}),

as higher contents of this cytokinin inhibited callus induction. Research on several species of the Rosaceae family has revealed that the maximum shoot multiplication rates were obtained in media supplemented with both BAP and IBA [16,20,25]. Several researchers have evaluated the effect of other cytokinins such as zeatin (ZEA), thidiazuron (TDZ), N6-(Δ^2-isopentenyl) adenine (2-iP), and meta-Topolin (mT) on the shoot multiplication rate of some woody plants [26,27], although BAP was the most cost-effective [26].

Jagiełło-Kubiec et al. [26] found a positive effect of gibberellic acid (GA_3) on shoot proliferation in ninebark (*Physocarpus opulifolius* L. Maxim.). In our study, GA_3 was used for hormonal balance in the multiplication step. Similar results were reported for other members of the Rosaceae family [14,17]. In peach (*Prunus persica* (L.) Batsch.), BAP (0.5 mg·L^{-1}) together with IBA (0.01 mg·L^{-1}) and GA_3 (0.5 mg·L^{-1}) was found to be the optimal combination treatment for shoot initiation (100%) from nodal explants. Likewise, 2 mg·L^{-1} BAP together with 0.01 mg·L^{-1} IBA and 0.5 mg·L^{-1} GA_3 was the best treatment for shoot proliferation (7.67) in *Prunus persica* (L.) Batsch. 'Garnem' [14]. Zare Khafri et al. [16] demonstrated that the highest number of lateral shoots induction in three Iranian apricot cultivars (*Prunus armenica* L.) was obtained in a WPM medium supplemented with 4 mg·L^{-1} GA_3 and 1 mg·L^{-1} BAP.

The proliferation of axillary shoots is strongly influenced by cytokinins, and the improper concentration of these PGRs often causes hyperhydricity of the tissues [12], as observed in the present study. Hyperhydricity (previously known as vitrification) is a morphological and physiological disorder of plants that results in excessive hydration, low lignification, impaired stomatal, and reduced mechanical strength of plants vegetatively propagated in vitro [28]. It can be a source of serious financial loss to commercial laboratories and ought to be avoided. The relatively low share of hyperhydrated shoots in the present study (2.053–3.716%) confirms the suitability of the described protocol in the micropropagation of firethorn.

In the present study, among the three basal media types used, the MS medium was most effective both for shoot multiplication and rooting. Likewise, in the dwarf rootstock of apple (*Malus domestica* Borkh 'Gami Almasi'), MS was better than Chu's N6 basal medium [29] during shoot proliferation [30]. As for *Prunus africana* (Hook f.) Kalkman and *Mandevilla guanabarica* Casar. ex MFSales, an endemic plant from Brazil with pharmacological and ornamental potential, the WPM medium was more effective than the MS medium [31,32]. The addition of coconut water or other natural supplements could further increase shoot formation in *P. angustifolia,* as reported in a study on sandalwood by Solle and Semiar [33].

The current study confirmed the positive effect of IBA on root induction and growth in *P. angustifolia*. IBA is usually the more effective and more frequently used auxin for rooting than other auxins, as reported for several other woody species [34–37]. However, root formation can be significantly affected not only by the auxin type, but also by its concentration [12]. Some investigations have demonstrated that the optimum root initiation and development were obtained when actively growing axillary shoots were cultured on a medium enriched with a high concentration of IBA [37,38]. Zare Khafri et al. [16] showed that the highest rooting percentage in three Iranian apricot cultivars was obtained in half-strength QL medium [39] supplemented with 4 mg·L^{-1} of IBA. In peach, IBA at a concentration of 1.5 mg·L^{-1} induced the maximum rooting rate (42.86%), the maximum root number (6.33 per shoot), and the longest roots (7.17 cm) [14]. These findings were similar to our research regarding root number.

Our study showed that the maximum root induction and root length in *P. angustifolia* were obtained in the MS medium supplemented with moderately low IBA concentrations (1 and 1.5 mg·L^{-1}). Likewise, in ninebark, the best rooting was at 1 mg·L $^{-1}$ IBA in half-strength MS medium [26]. It is worth mentioning that in the study by Dong et al. [12], an increased rooting percentage from 0% to 77% in 1.5 months was reported for *P. coccinea* by adding 18.9 mg·L^{-1} IBA to the culture medium. In the present study, we were able to obtain successful rooting of this species by using much lower concentrations of PGRs, which

makes the here-described micropropagation protocol more suitable for both scientific and commercial laboratories. Differences between the species and the content of endogenous PGRs in each cultivar are the most important factors responsible for this phenomenon. For example, some researchers have reported successful rooting after using a combination of auxin and cytokinin [11]. The present study revealed that BAP treatment is effective for inducing root formation when applied with IBA. On the other hand, in some historical roses, optimal rooting was found on half-strength control MS medium, without IBA [40]. Therefore, the optimal concentration of PGRs for root production and growth is different between tree and shrub species. The obtained high acclimatization efficiency (84.08–100%), which is one of the most critical steps in the micropropagation protocol [32], confirms the functionality of the root system and the good quality of the micro-shoots. It is also worth mentioning that since the micro-shoots were produced from a meristematic explant without a callus phase, their genetic fidelity should be maintained [31]. Ex vitro rooting, including the treatments with IBA and abscisic acid (ABA), could significantly shorten the production time, as reported by Jagiełło-Kubiec et al. [26], and could be considered in future studies with *P. angustifolia*.

5. Conclusions

The in vitro propagation of woody plants is a difficult task. Therefore, more research in this area is necessary. This maiden study aimed to establish and propagate *P. angustifolia* in vitro by using single-node segments (axillary buds) as explants, as well as BAP and IBA as PGRs through the direct organogenesis method. The highest micro-shoot proliferation, rooting efficiency, and root number rates were obtained in MS medium augmented with 2.5 mg·L^{-1} BAP, 0.3 mg·L^{-1} GA_3, and 0.1 mg·L^{-1} IBA (caulogenesis), and in MS medium with 1 or 1.5 mg·L^{-1} IBA together with 0.1 mg·L^{-1} BAP (rhizogenesis). The produced plantlets were successfully acclimatized to the greenhouse conditions. The here-presented micropropagation protocol allows for the large-scale (re)production of *P. angustifolia*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8100964/s1, Figure S1: effect of different culture media types on the measured characteristics of *Pyracantha angustifolia* plants grown in vitro in the multiplication step; Figure S2: effect of different concentrations of BAP on the measured characteristics of *Pyracantha angustifolia* plants grown in vitro in the multiplication step; Figure S3: effect of different culture media types on the measured characteristics of *Pyracantha angustifolia* plants grown in vitro in the rooting step; Figure S4: effect of different concentrations of IBA on the measured characteristics of *Pyracantha angustifolia* plants grown in vitro in the rooting step.

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