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In Vitro Propagation of Commercially Used Krymsk 5® (*Prunus fruticosa* × *Prunus lannesiana*) Cherry Rootstock: Impact of Sugar Types and pH Levels

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Abstract: In the present study, the effects of different types of sugars and cultivation medium pH levels on the micropropagation of Krymsk 5® cherry rootstock were investigated. During the proliferation stage, the effects of four sugars (sucrose, fructose, glucose, and sorbitol) both separately and in two combinations were studied, along with the effects of pre-adjusted pH (4.5, 5.0, 5.2, 5.5, 5.8, 6.0, 6.2, or 6.5) on shoot proliferation parameters, growth medium's post-autoclaving and post-cultivation pH, and their relations. Similarly, during the rooting stage, the effects of four sugars (sucrose, glucose, fructose, or sorbitol) at three concentrations (1% *w*/*v*, 2% *w*/*v*, or 3% *w*/*v*) without any auxin inclusion were studied as well as the effects of two sugars (sucrose or fructose) at six pre-adjusted pH levels (4.8, 5.2, 5.8, 6.2, or 6.5), also in the absence of auxin, on rooting parameters. Explants cultivated in fructose-supplemented growth mediums exhibited superior proliferation performance, characterized by the highest values of shoots per explant, shoot length, and nodes per explant. Generally, the medium's pH decreased after autoclaving, and proliferation performance was favored by low pH values (either pre-adjusted or post-autoclaving). As far as rooting is concerned, fructose inclusion induced a higher rooting percentage (88%) compared to sucrose. The highest rooting was obtained in fructose-supplemented rooting mediums at concentrations of 2% or 3% *w*/*v* (95% rooting in both cases), in the absence of auxins. Post-autoclaving pH in fructose-supplemented rooting mediums was lower and buffered in low pH levels than in sucrose-supplemented ones, and the rooting of explants in all pH combinations with fructose exceeded 75%. In addition, rooting was negatively correlated with the post-autoclaving pH. These findings underscore the significance of both the sugar type and the post-autoclaving pH of the medium in both proliferation and rooting stages, highlighting their possible physiological, biochemical, or hormonal effects. Additionally, rooting without the use of auxin, but with the correct choice of sugar, emerges with both financial and environmental benefits, whereas fructose could be potentially used as a buffering agent.

Keywords: micropropagation; *Prunus*; sugars; pH; DKW; adventitious rooting; auxin; acid growth theory; fructose; rootstock

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1. Introduction

Krymsk 5® (*Prunus fruticosa* × *Prunus lannesiana*) is a semi-dwarf cherry rootstock suitable for heavy soils and both hot and cold climates [1,2]. Micropropagation allows the rapid clonal propagation of plant species. Especially for elite *Prunus* genotypes, such as specialized rootstocks, micropropagation is used extensively for both experimental and commercial reasons. Nutrient medium ingredients, plant growth regulators, the type of sugar used, the level of pH, and other supplemented substances are some of the factors that affect micropropagation rates [3].

Sugars act as a source of carbon and energy for the explants, regulate the explant's morphogenic response, and influence the cultivation medium's properties, such as pH

and osmotic potential [3,4]. Sucrose is widely used as the main carbon source since it is the predominant sugar in the plant phloem sap for most species, whereas it is also cheap to use on a commercial scale [3]. However, other types of sugars, mixtures, or different concentrations have been also used with sufficient or even superior results depending on the micropropagation stage and species [1,5–9] or the cultivated tissue [3]. In the micropropagation of Rosaceae species, sorbitol was superior compared to other sugars in some species during shoot proliferation [10,11], whereas in others, sucrose, fructose, or glucose were better [12]. The same was also observed during the rooting stage [6,11], where the effects of sugars without auxin inclusion are not well studied, probably because the combination of auxin with sugar is sufficient for effective rooting induction [13].

Practically, pH is adjusted before the sterilization of the cultivation medium and may differ among the species (indicatively, 5.2 for *Citrus*, 5.6–5.8 for *Prunus,* and 5.8 for olive), but it may fluctuate after autoclaving [14,15]. The extent of fluctuation is influenced by the initially adjusted levels, the nutrient medium, the type of sugar included [14], and other factors [16]. Post-autoclaving pH levels are scarcely measured even if it affects several morphogenic processes in vitro, such as adventitious bud [17] and root formation [18], and other factors, such as nutrient absorption, enzyme activity, and the medium's solidification [3]. Nonetheless, the response to the pH levels may be genotype-dependent [19].

This study aimed to assess the in vitro performance of Krymsk 5® (*Prunus fruticosa* × *Prunus lannesiana*) rootstock under various treatments of different pH levels. More specifically, in the proliferation stage several types of sugars and sugar mixtures, as well as different levels of pre- and post-autoclaving pH were tested. In the rooting stage, four different types of sugars in three different concentrations were used, along with two different sugars combined with six pH levels, in the absence of auxins. In addition, the effect of pre-autoclaving pH and sugar type on Driver Kuniyuki for walnut medium (DKW) medium's [20,21] post-autoclaving pH was examined.

2. Materials and Methods

2.1. Explant Source, Plant Material, Culture Conditions

In vitro proliferated "Krymsk 5®" mother culture, grown as described by Tsafouros and Roussos [1], comprised the source of explants. Microshoots (nodal or shoot tips), approximately 1.5 cm in length, were used as explants and cultivated in 10 mL proliferation or rooting mediums in test tubes. After their planting, they were placed in a growth chamber under a 16 h photoperiod and light intensity of 3000 lux, whereas the temperature was adjusted at 22 ± 1 °C.

2.2. Shoot Proliferation Experiments

To improve the performance of explants during the shoot proliferation stage, two experiments were conducted. More specifically, the effect of different types of sugar (experiment 1) and various pH levels (experiment 2), were assessed.

After excision from the mother culture, the explants were planted in medium A (medA), a solidified DKW medium, supplemented with 9 g L^{-1} agar, 9.6 μM benzyladenine (BA), 0.7 μM gibberellic acid (GA_3) and 0.5 μM α-naphthaleneacetic acid (NAA) (1-NAA). The sugar added as well as the pH of the medium were adjusted as indicated below (Table 1).

2.2.1. Experiment 1: Effect of Different Types of Sugar

To assess the effect of sugar type on shoot proliferation, microshoots were transplanted in medA supplemented with four different sugar types (individually added per treatment) or two sugar mixtures containing different sugar quantities. In all treatments, the final sugar concentration was 3% *w*/*v*. The treatments applied were sucrose, fructose, sorbitol, or glucose. The first sugar mixture represented an endogenous carbohydrates ratio, i.e., 2.4 g L^{-1} sucrose, 2.4 g L^{-1} fructose, 5.2 g L^{-1}, glucose and 20 g L^{-1} sorbitol, based on the previous study of Tsafouros and Roussos [1] and served as control, whereas the second one

was composed by equal quantities of each sugar, i.e., 7.5 g L^{-1} sucrose, 7.5 g L^{-1} fructose, 7.5 g L^{-1}, glucose, and 7.5 g L^{-1} sorbitol.

Table 1. Brief presentation of shoot proliferation experiments.

	Treatment	pH	g L^{-1}			
			Sucrose	Fructose	Glucose	Sorbitol
Experiment 1	Treat 1	5.8	2.4	2.4	5.2	20
	Treat 2		7.5	7.5	7.5	7.5
	Treat 3		30			
	Treat 4			30		
	Treat 5				30	
	Treat 6					30
Experiment 2	Treat 1	4.5	2.4	2.4	5.2	20
	Treat 2	5.0				
	Treat 3	5.2				
	Treat 4	5.5				
	Treat 5	5.8				
	Treat 6	6.0				
	Treat 7	6.2				
	Treat 8	6.5				

2.2.2. Experiment 2: Effect of Different Pre-Autoclaving pH Levels on Post-Autoclaving pH and Shoot Proliferation

In this experiment, the effect of pH on post-autoclaving pH and shoot proliferation was assessed. The treatments consisted of eight different pre-autoclaving pH levels. More specifically, pH was adjusted before autoclaving at 4.5, 5.0, 5.2, 5.5, 5.8, 6.0, 6.2, and 6.5. The level of pH after sterilization was estimated by placing a table pH meter electrode into the growth medium. Then, excised microshoots were transplanted in medA with the different treatments mentioned above, and the proliferation parameters were estimated after the cultivation period (see below). Moreover, post-cultivation pH was also measured.

2.2.3. Experimental Design and Statistical Analysis

In all experiments, the cultivation period lasted eight weeks. After this period, the mean shoot length, the number of produced shoots, and the number of nodes per explant were measured in eighteen to twenty explants per treatment. In addition, the number of nodes per shoot and the number of nodes per cm of shoot were estimated. The experiments were arranged according to the completely randomized design (CRD) with five replications and were repeated twice. Statistical analysis was performed using JMP 14 (SAS, Cary, NC, USA). The raw data were analyzed by ANOVA, whereas significant differences among means were detected using the Tukey HSD test at $p \leq 0.05$. Principal component analysis (PCA) based on raw data was performed to describe the proliferation performance of "Krymsk 5®" explants cultivated under three different arbitrary classes of the pre-autoclaving pH, i.e., low (pH 4.5, 4.8, 5.2), medium (pH 5.5, 5.8), and high (pH 6.0, 6.2, 6.5).

2.3. Rooting Experiments

Two experiments were conducted to assess the effect of the sugar type and its concentration and its interaction with pH, in the absence of any kind of auxin. In the first experiment, the effect of different type of sugar was studied, whereas in the second one the effect of different pH levels and their interaction with two different types of sugars (Table 2).

Table 2. Brief presentation of rooting experiments.

	Treatment	pH	Sugar Type	Sugar Concentration (% *w*/*v*)	Agar (g L^{-1})
Experiment 3	Treat 1	5.8	Sucrose	1	9
	Treat 2			2	
	Treat 3			3	
	Treat 4	5.8	Fructose	1	9
	Treat 5			2	
	Treat 6			3	
	Treat 7	5.8	Glucose	1	9
	Treat 8			2	
	Treat 9			3	
	Treat 10	5.8	Sorbitol	1	9
	Treat 11			2	
	Treat 12			3	
Experiment 4	Treat 1	4.8	Sucrose	2	9
	Treat 2	5.2			
	Treat 3	5.5			
	Treat 4	5.8			
	Treat 5	6.2			
	Treat 6	6.5			
	Treat 7	4.8	Fructose	2	9
	Treat 8	5.2			
	Treat 9	5.5			
	Treat 10	5.8			
	Treat 11	6.2			
	Treat 12	6.5			

Microshoots were excised from the mother culture and planted in medium B (medB), a solidified DKW medium, supplemented with 9 g L^{-1} agar. Different sugar types and sugar concentrations depending on the experiment were added, whereas pH was adjusted to 5.8 before autoclaving (unless otherwise stated). The cultivation period lasted six weeks. After this period the rooting percentage, the number, and the length of the formed roots were measured.

2.3.1. Experiment 3: Effect of Different Types of Sugars on Rooting in the Absence of Auxins

In the present experiment, the effect of sugar type and its concentration on in vitro rooting of "Krymsk 5®" microshoots in the absence of auxins was examined. Explants were planted in subB, whereas treatments consisted of the sugars sucrose, glucose, fructose, or sorbitol at 1% *w*/*v*, 2% *w*/*v*, or 3% *w*/*v*. In total 12 treatments were applied.

2.3.2. Experiment 4: Effect of Different Prior Autoclaving pH Levels on Post-Autoclaving pH and Rooting in the Absence of Auxins

In continuation of the previous experiment, the effect of prior autoclaving pH in relation to sugar used on in vitro rooting and post-autoclaving pH was examined. Microshoots were cultivated in subB supplemented with sucrose or fructose in the concentration of 2% *w*/*v*, whereas pH was adjusted to 4.8, 5.2, 5.5, 5.8, 6.2, or 6.5 before autoclaving. In total, 12 treatments were applied. Post-autoclaving pH was measured as mentioned previously (experiment 2).

2.3.3. Experimental Design and Statistical Analysis

Each experiment was repeated twice. The experiments were arranged according to a completely randomized design (CRD). The data were analyzed as two-way ANOVA with the factors being the sugar type (sucrose, fructose, glucose, sorbitol) and concentration (1% *w*/*v*, 2% *w*/*v*, 3% *w*/*v*) for experiment 3, and sugar type (sucrose, fructose) and pre-autoclaving pH (4.8, 5.2, 5.5, 5.8, 6.2, 6.5) for experiment 4. Statistical analysis

was performed using JMP 14 (SAS, USA). The raw data were analyzed by ANOVA and statistically significant differences among means were detected using the Tukey HSD test at $p \leq 0.05$. Principal component analysis (PCA) based on raw data from experiment 4 was performed to describe the rooting performance of "Krymsk 5®" explants cultivated under two different sugars, i.e., sucrose and fructose.

3. Results

3.1. Effect of Different Types of Sugar in the Proliferation Stage

The sugar type included in the growth medium affected the shoot number per explant, the shoot length, and the number of nodes per explant, whereas the number of nodes per shoot and nodes per cm were not affected (Figure 1, Table 3).

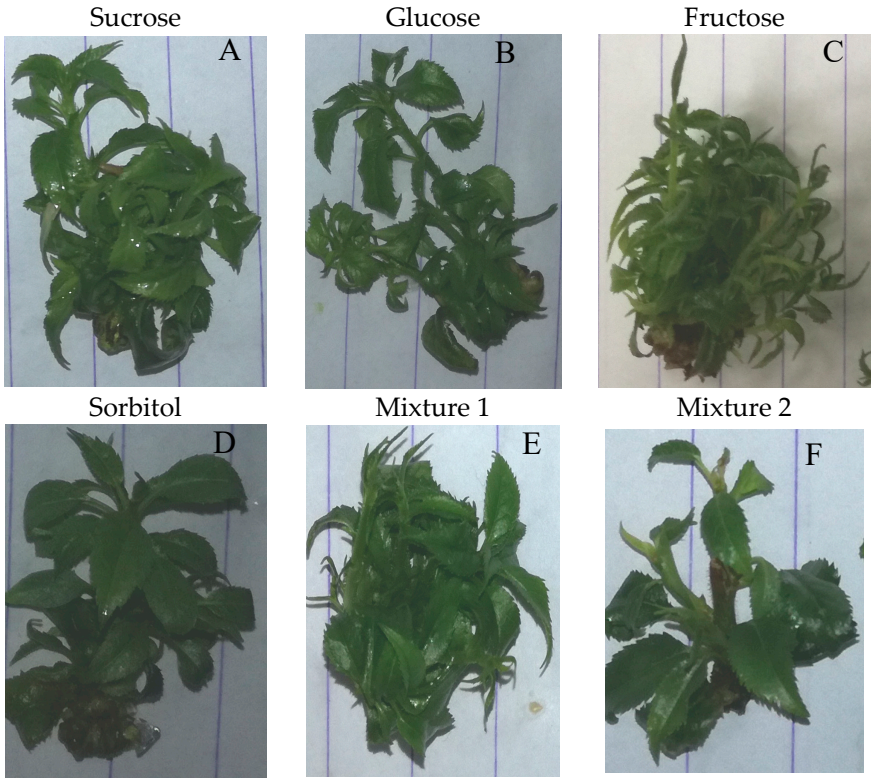

Figure 1. Proliferated explants after 8 weeks of culture (**A**) sucrose, (**B**) glucose, (**C**) fructose, (**D**) sorbitol, (**E**) mixture 1, and (**F**) mixture 2.

Table 3. Effect of sugar type on shoot proliferation variables.

Sugar Type	Shoots per Explant	Shoot Length (cm)	Nodes per Explant	Nodes per Shoot	Nodes per cm
Sucrose	1.79 c	1.15 b	5.3 c	2.3 a	2.15 a
Glucose	3.07 b	1 b	9.91 ab	1.99 a	2.07 a
Fructose	4.08 a	1.67 a	13.68 a	1.99 a	1.22 a
Sorbitol	2.38 bc	1.15 b	8.06 bc	1.96 a	1.77 a
Mixture 1 *	2.34 bc	1.29 ab	7.9 bc	2.4 a	1.9 a
Mixture 2 **	1.65 c	1.11 b	5.18 c	2.19 a	1.73 a

Means within the same column followed by the same letter do not differ significantly according to Tukey HSD test $p \leq 0.05$. * Mixture 1: 2.4% *w*/*v* sucrose, 2.4% *w*/*v* fructose, 5.1% *w*/*v* glucose, 20.1% *w*/*v* sorbitol. ** Mixture 2: 7.5% *w*/*v* sucrose, 7.5% *w*/*v* fructose, 7.5% *w*/*v* glucose, 7.5% *w*/*v* sorbitol.

Medium's supplementation with fructose resulted in the highest number of shoots per explant, shoot length, and nodes per explant among all treatments being twice that achieved by the addition of sucrose.

3.2. Effect of Different Prior Autoclaving pH Levels on Post-Autoclaving pH and Shoot Proliferation Variables

The effect of the pre-adjusted pH on post-autoclaving pH is presented in Figure 2A. Pre-autoclaving pH changed after autoclaving for all pH levels. Generally, pH decreased after autoclaving except for pH 4.5, which increased to 4.8 after sterilization. The higher the pre-autoclaving pH was the greater the difference between pre- and post-autoclaving pH, except for the pre-adjusted pH 5.2 and 6.0, which presented a lower difference compared to the previous pH level (Figure 2B).

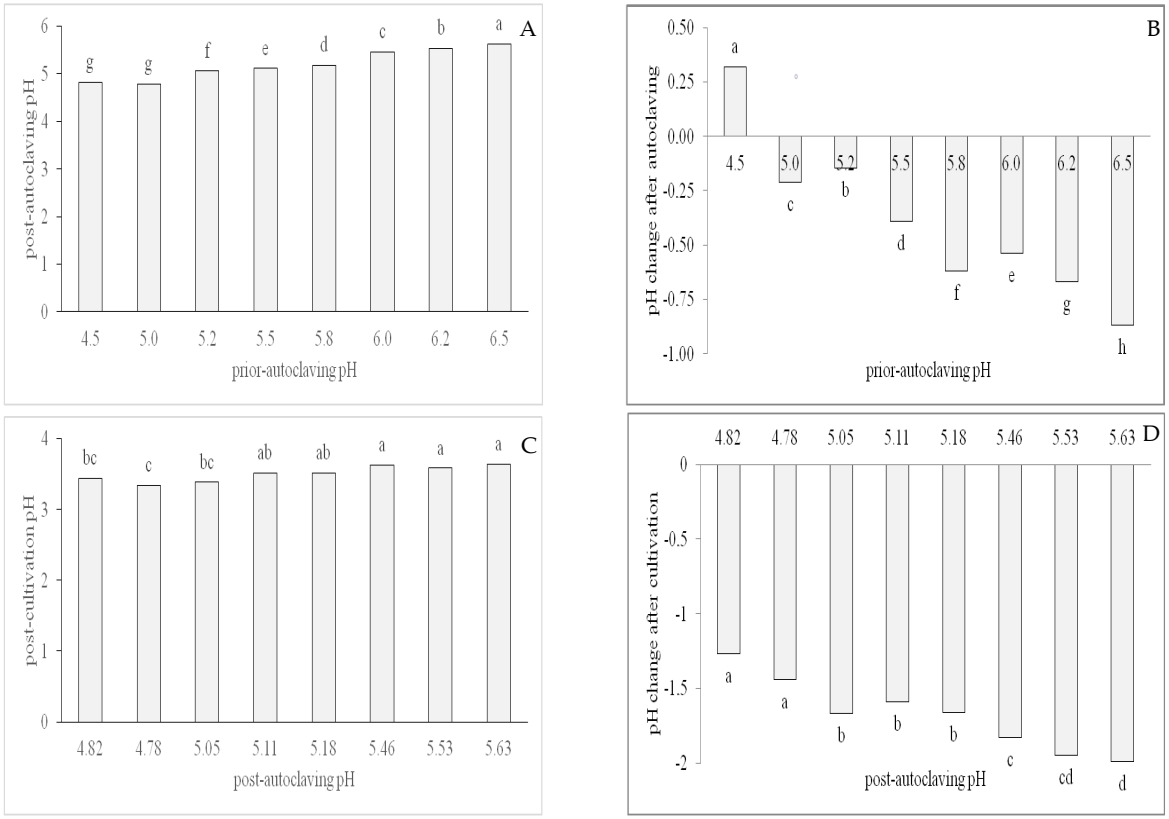

Figure 2. (**A**) Effect of different levels of prior autoclaving pH on medium's post-autoclaving pH, (**B**) prior autoclaving and post-autoclaving pH difference, (**C**) effect of medium's post-autoclaving pH on medium's post-cultivation pH, (**D**) post-autoclaving and post-cultivation pH difference. Columns followed by the same letter do not differ significantly according to the Tukey HSD test ($p \leq 0.05$). The absence of letters indicates a lack of any statistical difference according to the Tukey HSD test ($p \leq 0.05$).

The medium's pH was reduced after the explants' cultivation (Figure 2C). Post-autoclaving levels of 5.46, 5.53, and 5.63 presented higher post-cultivation pH than 4.82, 4.78, and 5.05 levels. The differences between post-autoclaving and post-cultivation pH are presented in Figure 2D. The higher the post-autoclaving pH the higher the decrease in the pH after cultivation (Figure 2D).

pH level affected the proliferation stage of explants cultivated (Figures 3 and 4).

Shoot proliferation variables were decreased from low to high pH (either pre-adjusted or post-autoclaving ones). Explants cultivated in growth mediums with low pre-adjusted pH, i.e., 4.5, 5.0, and 5.2, exhibited increased shoot numbers compared to those grown under high pH, i.e., 6.0, 6.2, and 6.5 (Figure 4A). No significant differences were observed regarding the shoot length. However, shorter shoots were observed at higher pH (Figure 4B). In addition, the number of nodes was reduced under high pH (Figure 4C). The highest number of shoots and nodes per explant were observed at pH 5.2 and 4.5, respectively, whereas the lowest was at pH 6.0.

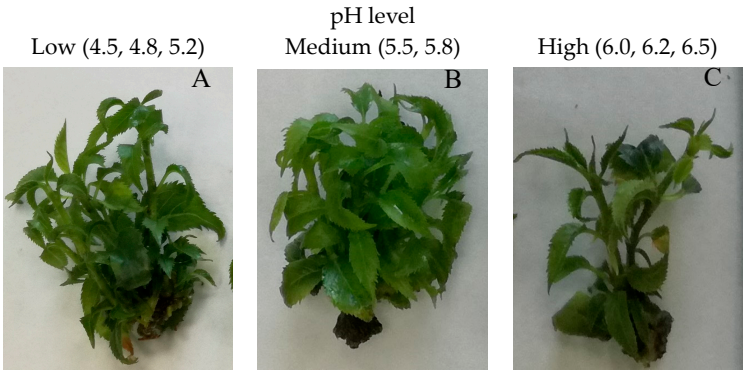

Figure 3. Proliferated explants after 8 weeks of culture at (**A**) low pH (4.5, 4.8, 5.2), (**B**) medium pH (5.5, 5.8), and (**C**) high pH (6.0, 6.2, 6.5).

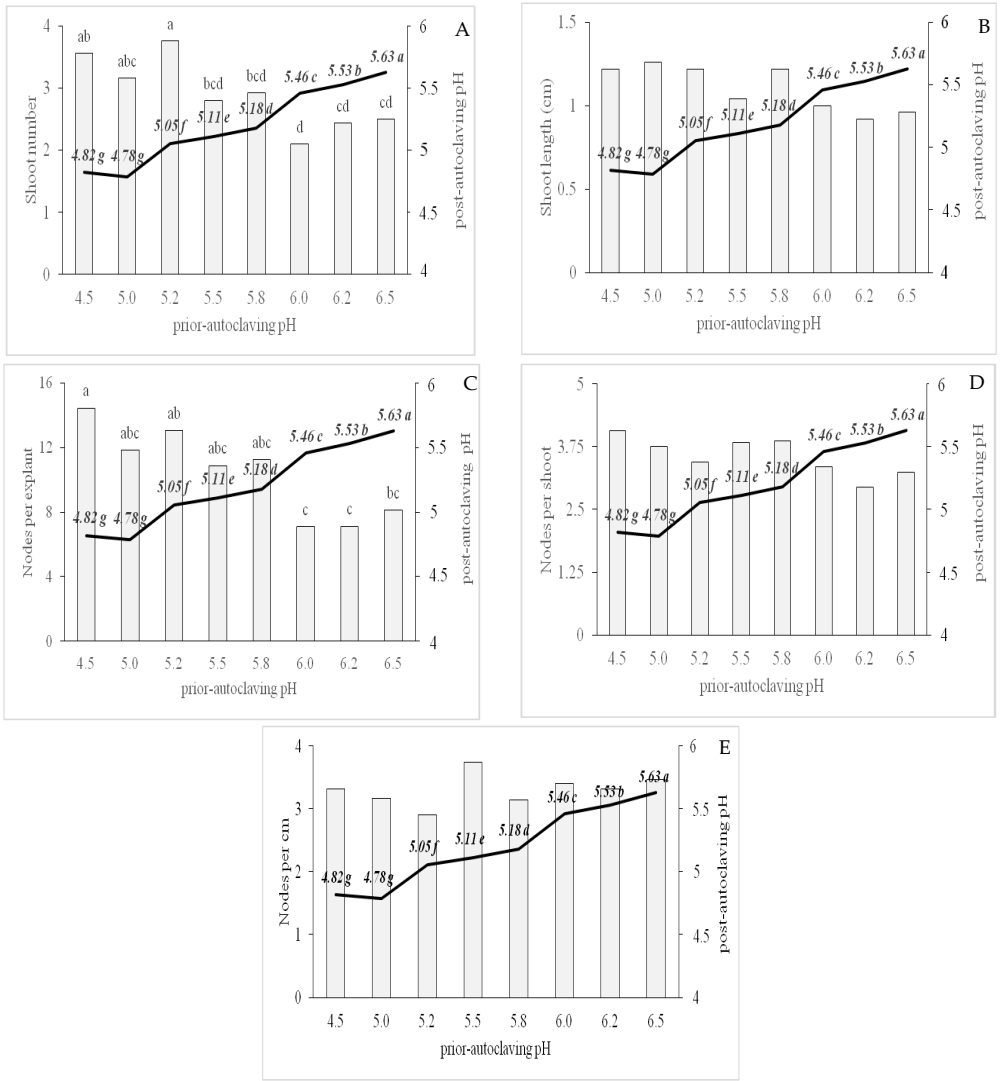

Figure 4. Effect of different levels of prior autoclaving pH on (**A**) shoot number per explants, (**B**) shoot length (cm), (**C**) node number per shoot, (**D**) node number per explant, and (**E**) node number per cm. The line represents the change level of post-autoclaving pH in relation to prior autoclaving adjusted pH. Columns followed by the same letter do not differ significantly according to the Tukey HSD test ($p \leq 0.05$). The absence of letters indicates a lack of any statistical difference according to the Tukey HSD test ($p \leq 0.05$).

With the aim of an improved visual interpretation of the effects of pH, a PCA was conducted (Figure 5). PCA revealed that post-autoclaving pH could be separated into three groups. The first group (Group I) comprised low pH levels, the second one medium pH levels (Group II), whereas the third one had the highest ones (Group III). pH classification included the following pH levels: 4.5, 5.0, and 5.2 (Group I), 5.5 and 5.8 (Group II), and 6.0, 6.2, and 6.5 (Group III). The first component of the analysis explained 57.8% of the variation and the second one an additional 23.7%, which is a total of 81.5% (Figure 5). The first component was associated with the number of nodes per explant, whereas the second one was with the number of nodes per cm. Shoot number, nodes per explant, and nodes per shoot were positively related with both components, shoot length was positively related with component 1 but negatively with component 2, whereas the opposite was observed for the nodes per cm.

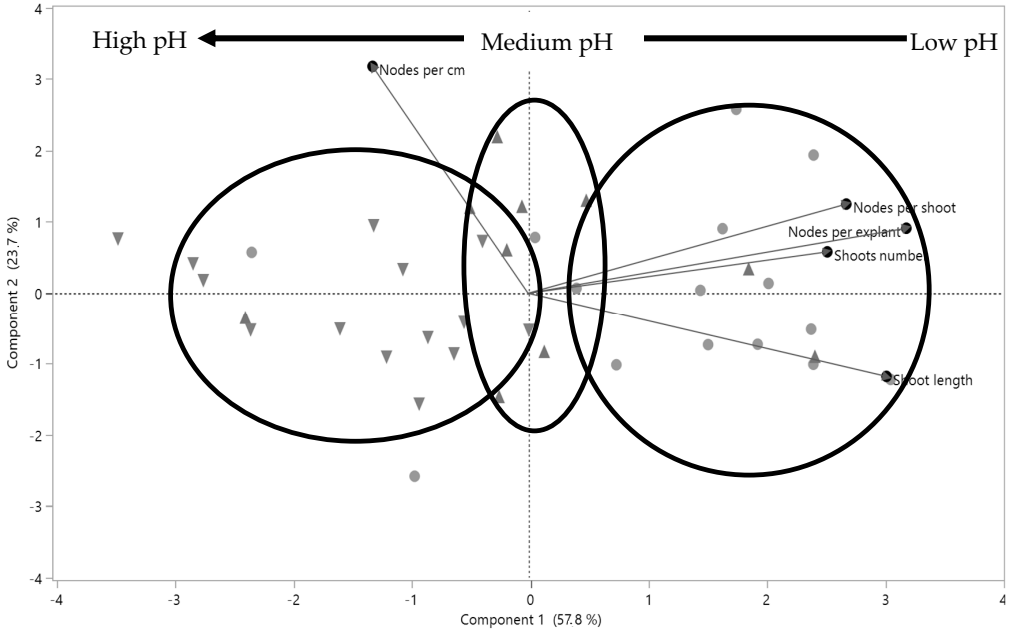

Figure 5. Biplot of the proliferation parameters (shoot number, shoot length, nodes per explant, nodes per shoot, nodes per cm) of shoots produced under different levels of pH (low pH (4.5, 4.8, 5.2) cycle marker, medium pH (5.5, 5.8) triangle marker, high pH (6.0, 6.2, 6.5) reverse triangle marker) on "Krymsk 5®" explants and pH grouping (Groups I, II and III).

The correlation analysis of shoot variables and pH is shown in Table 4. It can be seen that shoot number, shoot length, nodes per shoot, and nodes per explant exhibited a negative correlation with prior autoclaving and post-autoclaving pH. Moreover, shoot number, shoot length, nodes per shoot, and nodes per explant were positively correlated with the difference between post-autoclaving and post-culture pH.

Table 4. Multiple regression and correlation analysis between shoot proliferation parameters, prior autoclaving pH, and post-autoclaving pH with probabilities and correlation coefficients.

		r	*p* Value
Post-autoclaving pH	Prior autoclaving pH	0.96	***
Shoot number	Prior autoclaving pH	−0.67	***
Shoot length	Prior autoclaving pH	−0.42	**
Nodes per shoot	Prior autoclaving pH	−0.41	**
Nodes per explant	Prior autoclaving pH	−0.68	***
Post-autoclaving pH	Shoot number	−0.65	***

Table 4. *Cont.*

		r	*p* Value
Post-autoclaving pH	Shoot length	−0.46	**
Post-autoclaving pH	Nodes per shoot	−0.46	**
Post-autoclaving pH	Node per explant	−0.68	***
Difference post A/C- post culture pH	Shoot number	0.59	***
Difference post A/C- post culture pH	Shoot length	0.44	***
Difference post A/C- post culture pH	Nodes per shoot	0.49	***
Difference post A/C- post culture pH	Nodes per explant	0.57	***
Difference post A/C- post culture pH	Nodes per cm	−0.08	ns

ns, not significant; A/C, autoclave; **, $p < 0.01$; ***, $p < 0.001$.

3.3. Effect of Sugars and Their Concentrations on Rooting Variables

Among the sugars tested, fructose induced the highest rooting percentage in the absence of auxins resulting in 88.3% rooted explants, resulting also in the highest number of roots and root length values (Table 5). On the contrary, sucrose was the least efficient sugar regarding the rooting efficiency. Explants cultivated under increased sugar concentration, i.e., 2% *w*/*v* and 3% *w*/*v*, exhibited a high percentage of rooted explants (Table 5). Finally, 2% *w*/*v* and 3% *w*/*v* fructose resulted in the greatest rooting percentage (95% for both treatments) and the highest number of roots and root length, respectively (Table 5). The effects of different sugar types and their concentrations on cultivated explants can be seen in Figure 6.

Table 5. Effect of sugars, concentrations, and their interactions in rooting parameters (rooting percentage, root number, and length) six weeks after planting in the absence of auxins.

	Rooted Explants (%)	Number of Roots	Root Length (cm)
Sugar			
Fructose (Fruc)	88.3 a	7.1 a	0.48 a
Glucose (Gluc)	74.3 ab	5.4 b	0.28 b
Sorbitol (Sor)	70.8 b	5.2 b	0.24 b
Sucrose (Suc)	54.9 c	4.5 b	0.18 b
Concentration (% *w*/*v*)			
1	58.2 b	5.14 a	0.25 b
2	77.6 a	6.07 a	0.24 b
3	80.5 a	5.4 a	0.40 a
Sugar × Concentration			
Fruc × 1	75 abc	6.54 ab	0.32 bc
Fruc × 2	95 a	7.97 a	0.38 b
Fruc × 3	95 a	6.85 ab	0.73 a
Gluc × 1	69 abc	4.3 b	0.28 bc
Gluc × 2	71 abc	6 ab	0.2 bc
Gluc × 3	83.3 ab	5.3 ab	0.37 bc
Sor × 1	50 bc	3.75 b	0.23 bc
Sor × 2	81.2 abc	5.05 ab	0.2 bc
Sor × 3	81.2 abc	4.58 b	0.3 bc
Suc × 1	38.9 c	6 ab	0.17 bc
Suc × 2	63.2 bc	5.28 ab	0.16 c
Suc × 3	62.5 bc	4.88 ab	0.23 bc

Means within the same column per sugar type, sugar concentration, or their combination followed by the same letter do not differ significantly according to the Tukey HSD ($\alpha = 0.05$).

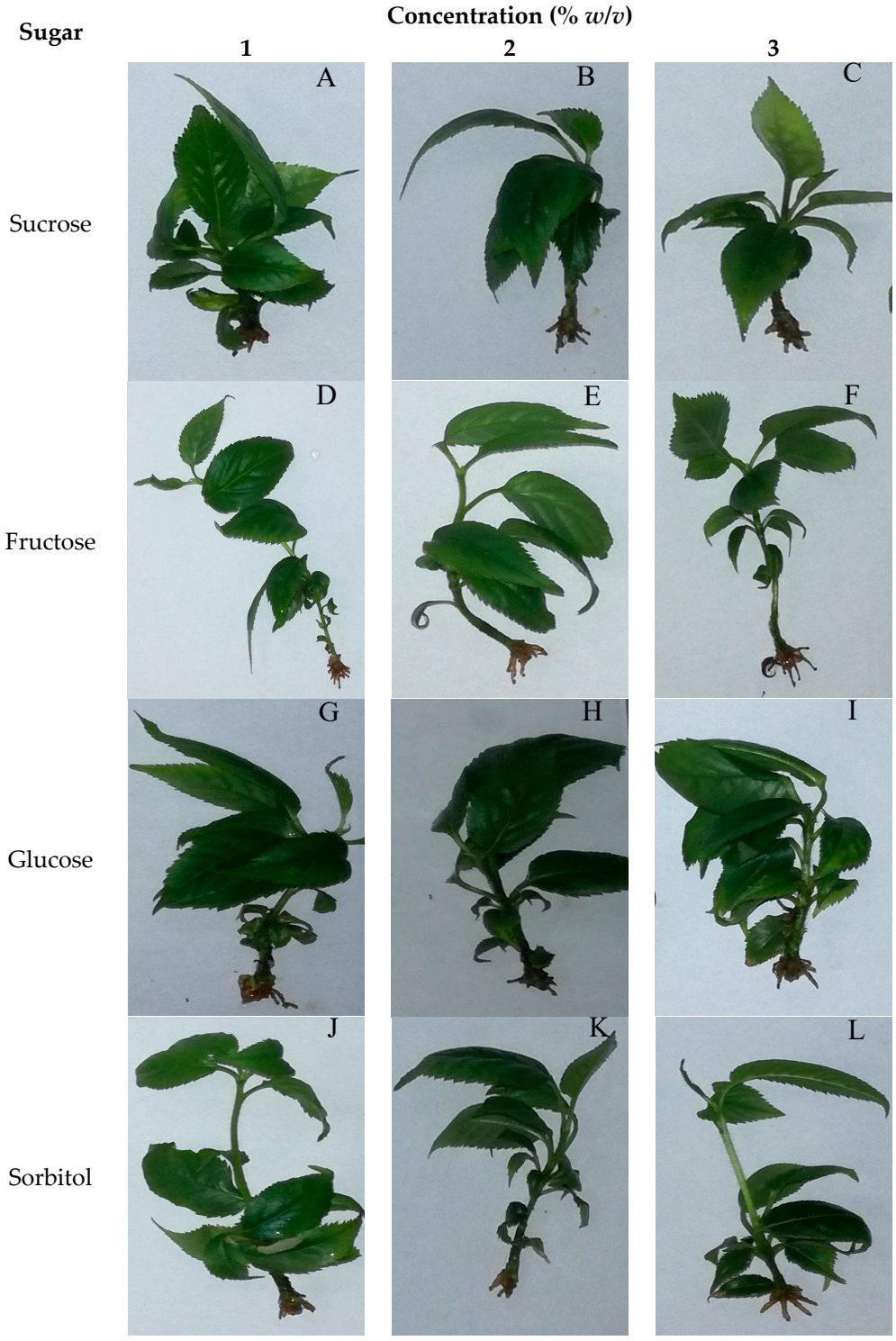

Figure 6. Rooted explants after 6 weeks of culture in different combinations of sugar type and sugar concentration. (**A**) Sucrose 1% *w*/*v*, (**B**) sucrose 2% *w*/*v*, (**C**) sucrose 3% *w*/*v*, (**D**) fructose 1% *w*/*v*, (**E**) fructose 2% *w*/*v*, (**F**) fructose 3% *w*/*v*, (**G**) glucose 1% *w*/*v*, (**H**) glucose 2% *w*/*v*, (**I**) glucose 3% *w*/*v*, (**J**) sorbitol 1% *w*/*v*, (**K**) sorbitol 2% *w*/*v*, (**L**) sorbitol 3% *w*/*v*.

3.4. Effect of Sugar and Prior Autoclaving pH Levels on Rooting Variables and Post-Autoclaving pH Levels

All rooting parameters and post-autoclaving pH differed significantly between the two sugars. Explants cultivated in fructose-supplemented rooting mediums exhibited

higher rooting percentage, root number, and root length, whereas post-autoclaving pH was higher in sucrose-supplemented ones (Figure 7, Table 6).

Figure 7. Rooted explants after 6 weeks of culture in different combinations of sugar type and pH level. (**A**–**F**) Sucrose and pH at 4.8, 5.2, 5.5, 5.8, 6.2, and 6.5, respectively, (**G**–**L**) fructose and pH at 4.8, 5.2, 5.5, 5.8, 6.2, and 6.5, respectively.

Prior autoclaving adjusted pH affected the number and length of roots formed and the level of post-autoclaving pH. The highest and the lowest number of roots were observed at pH 4.8 and 6.2, whereas the longest and shortest roots were measured at pH 5.8 and 6.2, respectively (Table 6). The highest post-autoclaving pH corresponded to the highest prior autoclaving pH, i.e., 6.5, whereas the lowest to 5.2.

All pH combinations with fructose presented an increased rooting percentage exceeding 75%. Indeed, fructose inclusion combined with a pre-adjusted pH of 4.8 induced the highest percentage of rooted explants and the highest number of roots (along with pH 5.8) among all treatments. On the other hand, rooted explants did not exceed 57% in any sucrose treatment, whereas the least effective treatment was the combination of pH 6.5-sucrose, which resulted in only 10% rooting and the lowest number of roots, i.e., 2.5 roots. As far as post-autoclaving pH is concerned, all fructose treatments presented a post-autoclaving pH lower than 5 and ranged from 4.62 to 4.92. Also, pre-adjusted pH levels at 4.8, 5.2,

and 5.5 exhibited the same post-autoclaving pH, i.e., 4.62, which was the lowest among all treatments of both sugars. Finally, the post-autoclaving pH of sucrose treatments ranged from 4.79 to 5.71. The latter value corresponded to a pre-adjusted pH of 6.5 and was the highest of the experiment.

Table 6. Effect of sugars, prior autoclaving pH, and their interactions on post-autoclaving pH and rooting parameters (rooting percentage, root number, and length) six weeks after planting in the absence of auxins.

	Rooted Explants (%)	Number of Roots	Root Length (cm)	pH Post
Sugar				
Sucrose (Suc)	35.6 b	4.2 b	0.25 b	5.24 a
Fructose (Fruc)	86.0 a	5.8 a	0.41 a	4.73 b
pH pre				
4.8	66.2 a	6.6 a	0.35 ab	4.78 e
5.2	69.8 a	5.8 ab	0.49 ab	4.71 f
5.5	67.5 a	4.9 ab	0.28 ab	4.90 d
5.8	57.5 a	5.2 ab	0.47 a	5.00 c
6.2	55.0 a	3.4 b	0.22 b	5.21 b
6.5	49.1 a	4.0 ab	0.29 ab	5.32 a
Sugar × pH pre				
Fruc × 4.8	95.0 a	7.3 a	0.36 a	4.62 i
Fruc × 5.2	83.3 ab	5.5 ab	0.57 a	4.62 i
Fruc × 5.5	85.0 ab	5.3 ab	0.57 a	4.63 i
Fruc × 5.8	90.0 ab	7.0 a	0.54 a	4.70 h
Fruc × 6.2	75.0 abc	4.0 ab	0.24 a	4.9 f
Fruc × 6.5	88.2 ab	5.6 ab	0.44 a	4.92 ef
Suc × 4.8	37.5 cde	5.9 ab	0.32 a	4.94 e
Suc × 5.2	56.3 bcd	6.2 ab	0.22 a	4.79 g
Suc × 5.5	50.0 b–e	4.5 ab	0.23 a	5.16 d
Suc × 5.8	25.0 de	3.5 ab	0.40 a	5.31 c
Suc × 6.2	35.0 de	2.7 b	0.20 a	5.51 b
Suc × 6.5	10.0 e	2.5 b	0.15 a	5.71 a

pH pre, prior autoclaving pH; pH post, post-autoclaving pH; x, denotes interaction. Means within the same column per sugar type, prior autoclaving pH, and their combinations followed by the same letter do not differ significantly according to the Tukey HSD ($\alpha = 0.05$).

The rooting of explants was negatively correlated with post-autoclaving pH and positively correlated with root number and root length. Surprisingly, no significant correlation was established between rooted explants and pre-adjusted pH (Table 7). Finally, the root number was negatively correlated with prior and post-autoclaving pH, whereas the root length was only with the post-autoclaving pH (Table 7).

Table 7. Multiple regression and correlation analysis between rooting parameters, prior autoclaving pH, and post-autoclaving pH with probabilities and correlation coefficients.

		r	*p* Value
Post-autoclaving pH	Prior autoclaving pH	0.59	***
Rooted explants %	Prior autoclaving pH	−0.23	ns
Rooted explants %	Post-autoclaving pH	−0.79	***
Rooted explants %	Root number	0.52	***
Rooted explants %	Root length	0.31	*
Root length	Prior autoclaving pH	−0.08	ns
Root length	Post-autoclaving pH	−0.34	*
Root length	Root number	0.14	ns
Root number	Prior autoclaving pH	−0.42	***
Root number	Post-autoclaving pH	−0.59	***

ns, not significant; *, $p < 0.05$; ***, $p < 0.001$.

To obtain an improved visual interpretation of the sugar effect a PCA was conducted (Figure 8). PCA revealed that sugars could be separated into two groups. Group I consisted of fructose, whereas the second one (Group II) of sucrose. The first component of the analysis explained 57.4% of the variation and the second one an additional 18.7% is a total of 76.1% (Figure 8). The first component was associated with the percentage of rooted explants, whereas the second one was with the length of roots. Root length and the percentage of rooted explants were positively related with both components, root length was related positively with component 1 but negatively with component 2, whereas the opposite was observed for pre-adjusted pH and post-autoclaving pH.

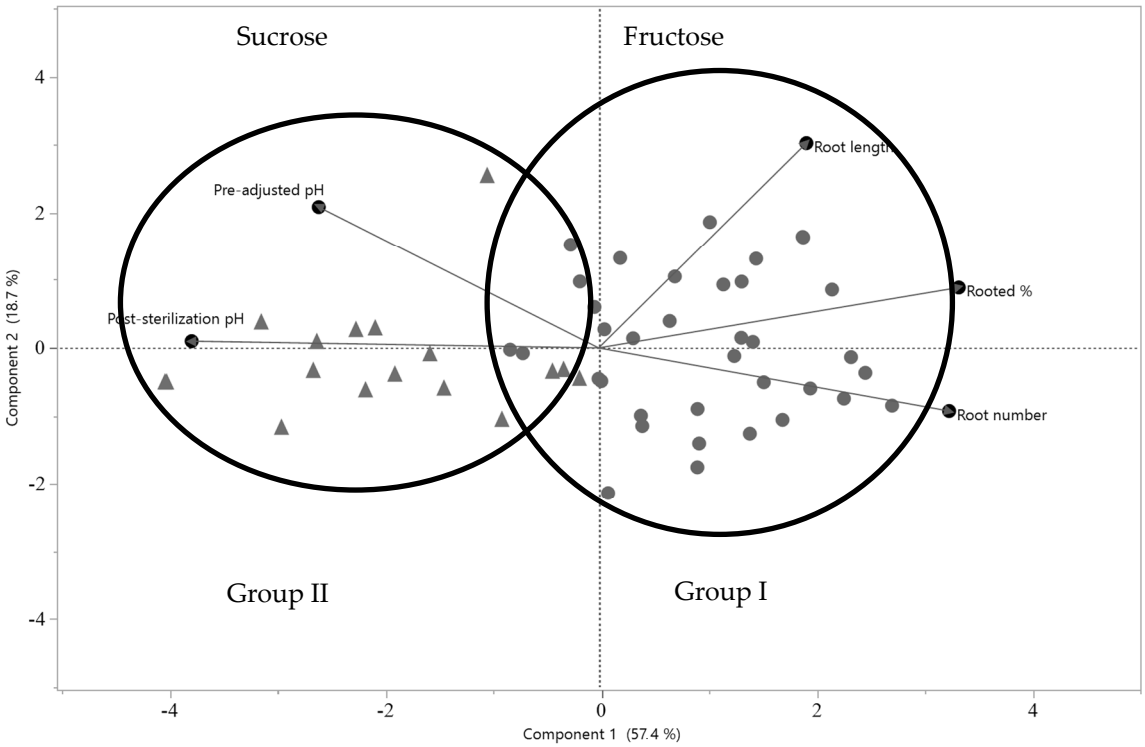

Figure 8. Biplot of the post-autoclaving and pre-adjusted pH and rooting parameters (rooted explants, root number, and root length) of explants cultivated in rooting mediums supplemented with fructose (cycle marker) or sucrose (triangle marker). Sugar grouping (Groups I and II) is also presented.

4. Discussion

4.1. Effects of Sugars in Krymsk's 5 In Vitro Propagation

Sucrose is commonly used as the primary carbon source in micropropagation [1,3]. Other sugars have also been used or assessed depending on the species studied and the desired morphogenetic response [1,4,22,23]. However, the effects of different sugar inclusions on in vitro *Prunus* sp. explant response remain poorly studied [1,24].

Sugar mixtures or sugars other than sucrose have been found effective for in vitro proliferation of many *Prunus* species [1,11,12,25]. Additionally, Hammat et al. [26] suggested that the inclusion of sugars at the endogenous ratio may improve multiplication stage performance. In Krymsk 5® rootstock this is partially confirmed since the "Mixture 1" treatment—consisting of sugars in the endogenous ratio determined during the rapid shoot growth—improved proliferation parameters compared to sucrose. This result could suggest that the presence of multiple sugars may enhance shoot proliferation [1]. Nevertheless, the ratio of sugars included seems to be more important than the presence of each sugar per se. Equal sugar contribution ("Mixture 2") presented no differences compared to sucrose (Table 3), whereas in *Prunus cerasus* equal amounts of sucrose, fructose, and glucose resulted in the lowest propagation rate [25].

Sorbitol is suggested as the preferable sugar source in many Rosaceae species during the in vitro shoot proliferation stage, such as pear [27], apple [28], the interspecies hybrid peach rootstock GF 677 (*P. amygdalus* × *P. persicae*) [29], apricot [11], and others, since it is the main sugar translocated in their phloem [29] along with sucrose [30]. Nevertheless, other authors suggest sucrose [31] or even glucose [32] as a sugar source to obtain better results. Fructose is not typically used in *Prunus* in vitro propagation. However, Cheong and An [12] discovered that the inclusion of fructose and glucose improved shoot induction rate (%) under in vitro conditions in *P. tomentosa* and *P. salicina* species but sorbitol was not included in the study. Additionally, medium supplementation with fructose inhibited hyperhydration during the in vitro propagation of almonds [33]. Likewise, in "Krymsk 5®" (*P. fruticose* × *P. lannesiana*), the inclusion of fructose yielded superior results regarding the number of shoots per explant, shoot length, and nodes per explant compared to the other sugars tested (Table 3).

Rooting performance during the rooting stage was influenced by the type of sugar in the absence of auxins, and the rooting performance was also affected by the sugar concentration (Table 5). Sucrose or sorbitol inclusion has been observed to produce the greatest rooting percentage, root number, and length in most Rosaceae species [8,10,22,29,34]. Fotopoulos and Sotiropoulos [6] concluded that glucose is equally effective as sucrose. However, Marino et al. [11] reported that sorbitol potentially inhibits rooting and root length in apricots. Interestingly, the effects of fructose on in vitro rooting of *Prunus* have not been adequately studied. Moreover, it is significant to note that auxin inclusion is a prerequisite for rooting as absence resulted in the suspension of rooting [35]. In certain *Prunus* rootstocks, such as Krymsk 5® [1,36] and Krymsk 86® [9], moderate rooting induction can occur even in the absence of auxins under preadjusted pH at 5.8. The replacement of sucrose with fructose, without including auxins, enhanced the rooting ability of "Krymsk 86®" explants [9]. Similarly, in the present study, the percentage of rooted "Krymsk 5®" explants reached 95% (Table 5) under the same treatment. The noteworthy rooting potential observed in the absence of auxins exemplifies the impact of sugar inclusion and underscores the importance of proper sugar selection for in vitro rooting.

The effects of fructose in the in vitro performance of "Krymsk 5®" explants could be partially attributed to endogenous factors, such as the regulation of enzymes, or environmental ones, such as pH or osmotic changes in the medium [3]. Indeed, sugars and their metabolic products may participate in signal transactions, regulating plant morphogenic responses [37], whereas sugars and plant hormones interplay depending on the morphogenetic and developmental stage of the plant [38]. Increasing sucrose concentrations may be linked with tuber formation, while exogenously applied glucose and fructose may promote adventitious root formation in *Arabidopsis* [37]. However, sugars can influence the developmental processes via multiple pathways, resulting in a more complex system [37]. In the developing tissues of Rosaceae plants, sorbitol is primarily transformed into fructose by sorbitol dehydrogenase (SDH) and subsequently enters the central metabolism [39]. Consequently, the direct supply of fructose could conserve sources and energy that may be redirected to other processes, such as shoot proliferation and adventitious rooting. SDH is linked to growth as it demonstrates elevated activity in the meristematic sections while declining in the mature ones [30]. High concentrations of hexoses are related to extensive meristematic activity and developmental processes in *Prunus* species. These processes include bud break in peaches [40], blooming in sweet cherries [40], and sugar utilization in young apricot leaves [41]. Moreover, studies on *Arabidopsis* suggest that fructose can promote root growth and leaf differentiation [42]. Additionally, in in vitro propagation, hexoses have been used effectively in various species such as *Bambusa nutans* or *Eucalyptus globulus* [43,44] improving rooting or multiplication parameters, whereas high sucrose concentration may lead to hyperhydration [45]. Thus, it can be postulated that fructose and, to a lesser extent, glucose inclusions may maintain the explant's tissues in an active condition, thereby enhancing the effects of plant hormones. Conversely, sucrose exhibited poor efficacy in both stages when compared to other sugars (Tables 3 and 5). This could be

ascribed to the energy-demanding process of hydrolyzing sucrose into fructose and glucose via invertase [3]. Since direct inclusion of the hydrolysis product is more energy efficient, it is preferred for supporting explants and adequately promoting morphogenesis.

4.2. Effects of Sugars and Prior Autoclaving pH on Post-Autoclaving pH Levels

The pH can be influenced by various factors. Several authors [3,14,16] have reported differences in the pH levels before and after the sterilization process, as demonstrated in the current study. Fructose incorporation was found to result in decreased pH levels in comparison to sucrose, under specific pre-autoclaving pH levels (Table 6). This finding is consistent with prior research demonstrating that fructose inclusion led to a reduction in pH levels in other nutrient media, including Murashige and Skoog (MS), White (WH), B5, Nitsch and Nitsch (NN), Schenk and Hildebrandt (SH), and Woody Plant Medium (WPM) [14]. Owen et al. [14] further noted that fructose resulted in the lowest post-autoclaving pH level compared to glucose, maltose, and sucrose, whereas media supplemented with sucrose had the highest pH, supporting the current study's findings. Sucrose also raised the pH levels after sterilization at a pH of 4.8 (Table 6), similar to what was observed in the WH medium [14]. This confirms that sterilization lowers the pH levels in the DKW medium, but it can be affected by the pre-adjusted pH and the type of sugar used. Furthermore, the addition of fructose in the DKW medium acts as a buffer and maintains the pH between 4.62 and 4.92 irrespective of the pre-adjusted pH (Table 6). On the other hand, when sucrose is included, the greater the pre-adjusted pH was, and the larger the difference with the final pH (Table 6) in accordance with Selby et al. [46].

4.3. Effects of pH in "Krymsk 5®" In Vitro Propagation

Different pH levels can affect in vitro morphogenesis by enhancing or inhibiting tissue formation or development [47]. In "Krymsk 5®", proliferation parameters were grouped based on pre-autoclaving pH levels (Figure 5) and correlated with pre-autoclaving and post-autoclaving pH levels during both proliferation and rooting stages (Tables 4 and 7).

Notably, shoot formation for "Krymsk 5®" explants increased when the post-autoclaving pH was below 5.05 (Figure 4). Accordingly, Pasqua et al. [17] reported that post-autoclaving pH ranging between 4.0 and 5.0 favored adventitious bud formation in tobacco, whereas higher values inhibited adventitious bud formation and shoot number as observed in the present study. Additionally, Cherve et al. [48] reported that low pH levels were more efficient for chestnut bud formation, whereas Anderson and Ievinsh [49] concluded the same for *Pinus sylvestris*. Similarly, in the present experiment, the positive correlation among proliferation parameters and the difference between post-autoclaving and post-culture pH indicated that lowering the pH even more is beneficial for explants. Indeed, explants modify their environment by releasing protons acidifying the substrate, which is favorable for nutrient absorption [3]. Adventitious root formation in vitro can be affected by the rooting medium's pH [3,50]. In the present study, no differences were found among different initial pH levels and rooting performance even though autoclaving affected post-autoclaving pH (Table 6), indicating that prior autoclaving pH per se is not crucial for "Krymsk 5®" explants' rooting. This is further supported by the results of some treatments, such as sucrose, 4.8 pH, and fructose, 6.5 pH, which had similar post-autoclaving pH levels but different rooting percentages, highlighting the significance of the sugar type included (Table 6). This effect is further emphasized in Figure 8, where fructose and sucrose explants were discriminated with regard to their effect on rooting parameters and pH levels for rooting mediums. This suggests that the pre-adjusted pH does not significantly affect the rooting capacity for a given type of sugar. The interaction between sugar and pH during sterilization appears to be more critical. In addition, autoclaving sugar may lead to hydrolysis and caramelization [3], the generation of substances with unknown activity [51], and changes in the medium's composition [14]. However, after sterilization, pH levels below 5 resulted in higher percentages of rooting compared to higher pH levels, thereby confirming the notion that low pH levels are beneficial for in vitro rooting [18,50,52].

The regulation mechanisms of in vitro morphogenesis by the pH level of the medium remain unclear. Generally, the medium's pH may influence cellular and apoplastic pH, gene expression, ion uptake, and cellular growth even regulating the activity of the enzymic factors [49,53]. In the present experiment, low pH values favored the shoot proliferation stage, whereas decreasing post-autoclaving pH was linked to increased rooting capacity in sucrose-supplemented cultivation mediums (Tables 4 and 7). The pH could have affected the explants' response in various ways, such as anatomically, nutritionally, or hormonally. Indeed, lowering the apoplastic pH levels may cause cell wall loosening, expediting the morphogenic changes [54,55]. In accordance, treatments with H_2O_2 that provoke cell wall creep have been used to induce responses, such as adventitious rooting [56].

Acid growth theory posits a positive correlation between apoplast acidification and the presence of auxin, while exogenously applied or endogenously derived auxin can reduce apoplastic pH [54,55]. In the case of rooting, the presence of auxin is crucial to stimulate the formation of rooting primordium [57]. The low medium pH levels after autoclaving could have facilitated or amplified the processes related to rooting, creating a chemical environment similar to that of auxin-derived. Indeed, it has been observed that low pH values may influence the uptake of IAA at the rooting zone [50], which is supported by the findings of Hasenstein and Rayle [58] who reported that IAA uptake is affected by pH. Accordingly, the computational model of Steinacher et al. [59] suggests that auxin-induced apoplast acidification can cause the passive transport of auxin into the cell's cytoplasm [54]. Indeed, according to chemiosmotic theory, in low pH levels, such as those at the acidic extracellular space (pH 5.5), IAA is dissociated (dissociation constant pK_{IAA} 4.7) and can easily enter the cells [60]. Hence, the presence of endogenous auxin combined with the reduction in apoplastic pH and the increased IAA intake from cells could be responsible for the improvement of the rooting performance.

Additionally, reduced pH could provide an improved efficient sugar supply for the cultivated explants. According to Roitsch and Gonzalez [61], cell wall invertase activity increases at a pH range between 3.5 and 5.0, whereas its activity can also be boosted by auxin [62]. Furthermore, significant levels of cell wall invertase activity have been reported during initial rooting stages in the cutting's stem base [63,64], callus proliferation or induction [65], and in rapidly growing tissues [66]. Therefore, a high rate of sucrose hydrolysis and increased production of fructose can be anticipated in low pH mediums supplemented with sucrose explaining the negative relation between pH and rooting or shoot proliferation. Therefore, the utilization of fructose, as described above, can more effectively support the growth and dedifferentiation processes in "Krymsk 5®" explants, thereby explaining the performance of sucrose-cultivated explants depending on the pH level. In any case, applicable anticipation for stable pH would be the readjustment of medium after autoclaving or the use of buffering agents like 2-(N-morpholino)ethanesulfonic acid (MES) [50] or even fructose, which exhibited quite an impressive buffering capacity, since pH levels never surpassed post-autoclaving the level 5.0, even when the pre-autoclaving pH was adjusted to levels above 6.0 (Table 6).

5. Conclusions

In conclusion, the present findings indicated that fructose inclusion positively influenced shoot proliferation, demonstrating superior performance compared to other sugars, especially against the widely used sucrose. Additionally, the effects of pH, both pre- and post-autoclaving, on shoot formation and rooting capacity were noted. Briefly, lower post-autoclaving pH levels were associated with increased rooting percentages and shoot proliferation, supporting the notion that low pH levels are beneficial for improved in vitro propagation. Moreover, the interaction between sugar type and pH level after the autoclaving was highlighted. Fructose exhibited the ability to maintain lower post-autoclaving pH levels, providing a favorable environment for explant growth. In summary, the findings underscore the significance of careful sugar selection, considering type, ratio, and pH conditions, in optimizing the in vitro propagation of Krymsk 5® cherry rootstock. The

study contributes valuable insights for refining micropropagation protocols, enhancing the efficiency of this important horticultural process.

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