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Optimizing the Rooting Process in the Propagation *in vitro* of Clonal Cherry Rootstocks

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The aim of this study was to optimize the technological cycle during the *in vitro* rooting stage of Krymsk®5, Krymsk®6, Krymsk®7, Gisela 5, Gisela 6, and Colt cherry rootstocks in order to improve micropropagation efficiency. Genotype-specific differences in shoot regeneration intensity (number of shoots per explant) were confirmed. The highest proliferation coefficient was obtained for Gisela 6, reaching 2.5 on MS medium supplemented with 0.5 mg·L^{-1} BAP. The effect of different IBA concentrations (1, 2, and 3 mg·L^{-1}) on *in vitro* rhizogenesis of stone fruit rootstocks was evaluated. The results demonstrated that genotype is the primary determinant of rooting success, whereas increasing auxin concentration showed a weak correlation (r = 0.12) and was associated with undesirable callus formation. MS medium supplemented with 1.0 mg·L^{-1} IBA was identified as optimal for most genotypes, resulting in rooting rates up to 94.3%, an average of 8.8 roots per explant, and root lengths of up to 4.5 cm, particularly in Krymsk®7 and Gisela 5. Gisela 6 exhibited the highest root elongation (5.9 cm) but showed increased sensitivity to higher auxin concentrations. In contrast, a modified protocol with 2 mg·L^{-1} IBA was optimal for Krymsk®6. Overall, optimized *in vitro* conditions enabled high rooting efficiency across the studied genotypes, confirming strong genotype dependence and demonstrating the effectiveness of the developed micropropagation protocol.

Keywords: microclonal propagation, *Cerasus*, culture media, IBA, rhizogenesis, genotypes

Introduction

Global practices in the intensive cultivation of sweet cherries (*Cerasus avium* (L.) Moench) and sour cherries (*Cerasus vulgaris* Mill) are based on the use of dwarf trees. This approach ensures high planting density and guarantees excellent crop quality (Apati et al., 2012; Holb et al., 2011). Early entry into commercial productivity, combined with optimal fruit characteristics, is achieved using dwarf rootstocks such as Gisela (Szabó et al., 2011).

The wide-scale introduction of these and other promising rootstocks into production has become possible due to advanced biotechnologies. Over the past few decades, the intensive development of tissue culture methods has fundamentally transformed approaches to *in vitro* plant propagation (Hosseinpour et al., 2015; Lal et al., 2022a). Currently, these techniques are actively employed for the mass production of a wide range of crops, including stone fruit rootstocks that were previously considered difficult to clone (Yadav et al., 2013).

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To address the propagation challenges of promising sweet and sour cherry rootstocks (Krymsk®5, Krymsk®6, Krymsk®7, Colt, Gisela 5, and Gisela 6) under field conditions, their *in vitro* micropropagation was investigated. The *in vitro* propagation of sweet and sour cherries typically utilizes MS medium for its micro- and macroelements, often with specific modifications (Sedlak et al., 2008). Specifically, the influence of various components of the nutrient medium on the culture was studied (Buyukdemirci 2008; Clapa et al., 2013; Mir et al., 2023).

The rate of micropropagation is influenced by numerous factors, such as medium composition and, most significantly, the types, concentrations, and combinations of plant growth regulators used (George et al., 2008). Axillary shoot proliferation is typically stimulated by supplementing the medium with cytokinins and auxins. To regulate physiological processes *in vitro*, cytokines such as 6-benzilaminopurine (BA), Z, Kin, and 2iP are most commonly used (Yancheva and Kondakova, 2016). The rooting of microshoots presents a particular challenge, as the outcome depends on a combination of exogenous and endogenous factors. Generally, the initiation of adventitious roots is promoted by incorporating auxins (specifically indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) into the medium, which serve as effective catalysts for rhizogenesis in the initial stages (Ainsley, 2001; Sedeghi et al., 2015). Once the required number of shoots is obtained, they are rooted *in vitro*, followed by an acclimatization stage under *ex vitro* conditions.

The aim of this study was to improve the micropropagation technology for the clonal rootstocks Krymsk®5, Krymsk®6, Krymsk®7, Gisela 5, Gisela 6, and Colt by determining the optimal medium composition for both proliferation and rooting. The effect of various concentrations of the auxin IBA on microshoot rooting parameters was experimentally verified. The relevance of this research lies in the optimization of biotechnological techniques for rootstock cultivation, to ensure the production of physiologically viable plants for successful *ex vitro* acclimatization. A key aspect of the work is a comparative assessment of the regeneration capacity of the investigated genotypes in *in vitro* culture.

Material and Methodology

Experimental conditions

The experimental studies were conducted in 2017–2018 at the Department of Virology, Health, and Propagation of Fruit and Berry Crops, Institute of Horticulture of the National Academy of Agrarian Sciences of Ukraine.

Plant material

Virus-free donor plants (Clark and Adams, 1977) of clonal cherry rootstocks Krymsk®5, Krymsk®6, Krymsk®7, Gisela 5, Gisela 6, and Colt were used as the source of primary explants. Apical and axillary buds were collected during the period of intensive shoot growth (mid-March).

Surface sterilization was performed using a 3% solution of Lysoformin 3000 (Lizoform Dr. Hans Rosemann GmbH, Germany) for 10 minutes, followed by standard rinsing procedures to establish aseptic cultures.

Culture media and conditions

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used as the nutrient base. For shoot proliferation, the medium was supplemented with 0.5 $mg \cdot L^{-1}$ 6-benzylaminopurine (BAP) and 0.1 $mg \cdot L^{-1}$ indole-3-butyric acid (IBA), along with 2% sucrose. Agar (0.8%; Agar-Agar "D19", Hispanagar, B.K.M. Services LTD) was used as the gelling agent. The pH was adjusted to 5.8 using 0.1 N HCl or 0.1 N NaOH prior to autoclaving.

Cultures were maintained under controlled conditions at 23–25 °C with 50–60% relative humidity and a 16 h photoperiod at an illumination intensity of 2,000–2,500 lx.

Subculturing was performed at 4–5 week intervals, during which shoots were excised and transferred to fresh medium.

Rooting treatments

For rhizogenesis induction, the concentration of indole-3-butyric acid (IBA) was adjusted to 1, 2, and 3 $mg \cdot L^{-1}$ in the culture medium. Rooting responses were evaluated after a 45-day culture period.

Statistical analysis

Data were analyzed using Statgraphics Plus for Windows (Version 2.1; Microsoft Corp., Redmond, WA, USA). Analysis of variance (ANOVA) was used to assess the effects of treatments (Clewer and Scarisbrick,

2001). When applicable, differences between means were evaluated, and results are presented as mean values of three independent replicates ± standard error (SE).

Results and Discussion

The efficiency of *in vitro* proliferation and rhizogenesis of microcuttings is determined by a combination of factors, among which the physicochemical parameters of the culture medium play a decisive role (Ružić and Vujović, 2008). It has been established that the critical factors determining the dynamics of these processes are the type and concentration of growth regulators (Sedeghi et al., 2015).

Building upon our previous experience with stone fruit micropropagation and taking into account the diverse physiological responses of related *Prunus* species, this study aims to refine the hormonal balance specifically for the selected high-potential rootstocks (Pakyürek and Hepaksoy, 2019).

In this regard, a group of rootstocks with diverse biological characteristics was selected for the study; their detailed morpho-biological profiles are provided in the sections below.

Colt is a english clonal rootstock (*Prunus avium* Lindl. × *Prunus pseudocerasus* L.). It has a high propagation rate via softwood cuttings and layering (up to 95%). It is characterized by poor winter hardiness, low drought tolerance, and susceptibility to root cancer. Shortened internodes complicate grafting in the first nursery's field (Long and Kaiser, 2010).

Gisela 5 is a German rootstock (*P. cerasus* L. × *P. canescens* L.) characterized by good frost resistance and tolerance to viral infections. Trees grafted onto Gisela 5 exhibit superior generative bud set, and their productivity exceeds that of other low-vigor sweet cherry rootstocks.

Gisela 6 is a semi-dwarf and highly precocious rootstock, with grafted trees reaching full production by the fifth year. It requires pruning to maintain fruit quality and shows better shoot recovery than Gisela 5 (Szabó et al., 2011). This rootstock is adapted to various types of well-drained soils and does not produce root suckers.

Krymsk®5 is a rootstock from the Crimean breeding series (*P. fruticosa* Pall. × *P. lannesiana* Carr.). It is adapted to a wide range of soil types and cold climates, and exhibits moderate drought tolerance and excellent anchorage (Long and Kaiser, 2010).

Krymsk®6 (*P. cerasus* L. × (*P. cerasus* L. × *P. maackii* L.)) is less vigorous than Krymsk®5 but is adapted to similar environmental conditions. Notably, there is high to moderate root suckering.

Krymsk®7 (*Cerasus lannesiana* Carr.) is suitable for cultivation in southern fruit-growing regions, provided that irrigation is available. It exhibits moderate winter hardiness and low drought tolerance. This rootstock is resistant to waterlogged soils, root rot, cherry leaf spot (*Coccomyces hiemalis*), and root canker. The primary

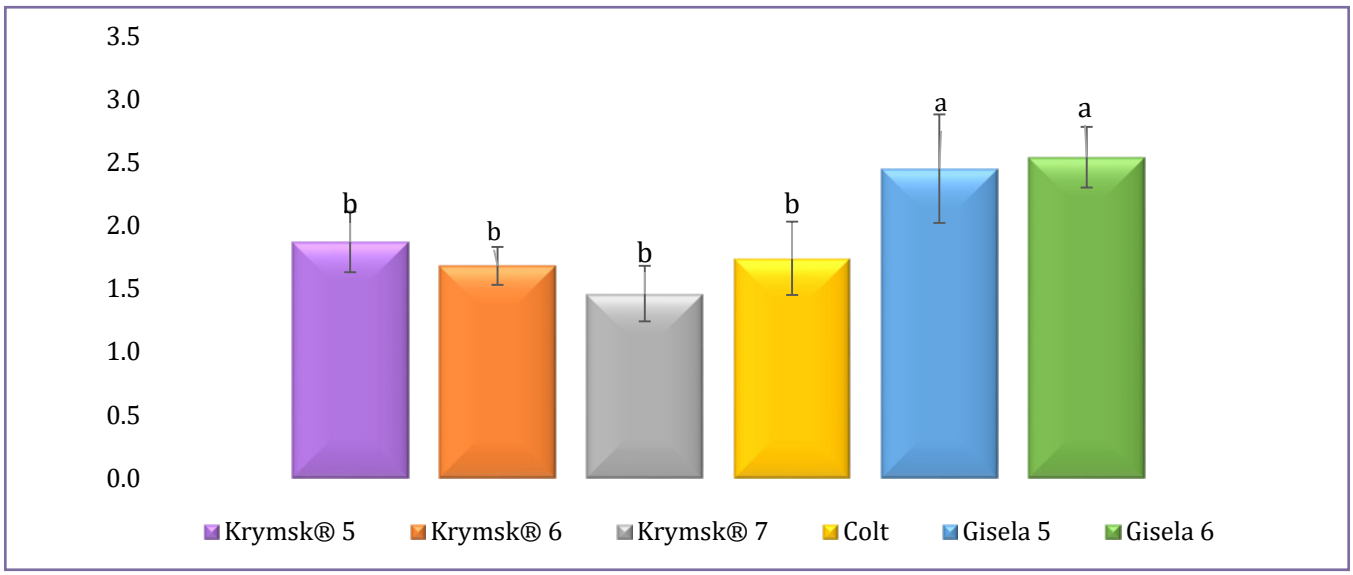

Figure 1 The average proliferation rate of the studied rootstock microshoots *in vitro*. Mean values followed by different letters are significantly different according to Tukey's test ($p < 0.05$). Error bars represent the standard error of the mean (SE)

disadvantage of Krymsk®7 is the relatively low productivity of various sweet cherry cultivars grafted onto it.

The first stage of our study involved determining the proliferation rate of various rootstock genotypes. Establishing this parameter allows for an assessment of the potential of each form for intensive propagation and provides a forecast for the yield of finished planting material. The research results indicate that the intensity of *in vitro* micropropagation is significantly influenced by the rootstock genotype (Yancheva and Kondakova, 2016; Lal et al., 2022b).

The Gisela seria rootstocks demonstrated the highest efficiency (Figure 1). Specifically, Gisela 6 showed the highest rate with a relatively low standard error (2.5 ±0.24), indicating the stability of results within the sample.

In Gisela 5, the average proliferation rate was comparable to that of the aforementioned genotype; however, a significant variability in explant response to culture conditions, particularly cytokinin concentration, was observed. Consequently, further research is required to optimize the cytokinin content in the medium for this specific genotype. It is noteworthy that the leaves of the shoots exhibited hypertrophy, especially those in contact with the medium.

The significance of differences in proliferation rates among the studied rootstocks confirmed that Gisela 6 exhibited the highest and most stable proliferation rate on MS medium.

The rootstocks of the Krymsk series demonstrated moderate yet stable results. In terms of shoot proliferation rates, Krymsk®5 (1.9 ±0.24) showed average values within the group, consistent with data describing its good adaptability to *in vitro* conditions (Tsafouros and Roussos, 2019). This trend was not observed for Krymsk®7 (1.5 ±0.22), which exhibited the lowest multiplication efficiency using this method. The Colt rootstock had a multiplication coefficient (1.7) identical to that of Krymsk®6.

Effect of auxin treatment on rhizogenis of rootstocks

The highest rooting frequency (94.3%) was recorded in rootstocks treated with 1 mg·L^{-1} IBA (Table 1). Similar observations were made regarding the positive effect of auxin on rhizogenesis (Sedeghi et al., 2015).

A direct linear relationship between auxin dose and rooting within the studied range (1–3 mg·L^{-1}) is weakly expressed. This suggests that the genotype is a significantly more potent regulator of rhizogenesis than the variation in IBA dosage within these limits. The analysis confirms the decisive role of the genetic factor in the *in vitro* rhizogenesis of stone fruit rootstocks.

The Colt rootstock demonstrated high stability in rooting intensity across a range of exogenous auxin concentrations. Muna et al. (2000) reported the highest rooting percentage for the Maxma-14 cherry rootstock at minimum IBA levels in MS medium; however, high auxin concentrations delayed root initiation by 3–5 days. Notably, a high capacity for rhizogenesis was also observed in Krymsk®5 and Krymsk®7, which showed a trend toward increased rooting with higher IBA concentrations. Conversely, the Krymsk®6 rootstock exhibited the lowest rooting level (46–63.3%), potentially indicating a requirement for a different type of auxin NAA. Both Gisela 5 and Gisela 6 occupied an intermediate position; the performance

Table 1 The effect of IBA concentration on the rooting rate (%) of clonal cherry rootstocks

Type of rootstock	Rooting of plants, %			Factor A – genotype (rootstock)
	IBA (1 mg·L^{-1} ±SD) (control)	IBA (2 mg·L^{-1} ±SD)	IBA (3 mg·L^{-1} ±SD)	
Krymsk®5	86.0 ±11.1	69.3 ±11.8	88.0 ±12.7	a
Krymsk®6	46.3 ±13.8	63.0 ±12.3	52.0 ±17.2	ab
Krymsk®7	77.4 ±11.9	75.3 ±12.7	87.3 ±7.0	ab
Gisela 5	65.3 ±23.1	57.3 ±15.2	68.0 ±12.5	bc
Gisela 6	64.0 ±15.0	73.3 ±11.3	76.0 ±15.6	c
Colt	94.3 ±3.0	85.0 ±12.6	83.3 ±6.7	d
Factor B – IBA concentration	A	A	A	–

Notes: Different lowercase letters indicate significant differences between rootstocks, while different uppercase letters denote significant differences between IBA concentrations ($p < 0.05$); SD – standard deviation

of the latter (64–76%) was statistically significant and showed a direct dependence on increasing auxin concentration.

Other authors established that maximum rooting (100%) for Gisela 5 was achieved even at a low concentration of 0.5 mg·L^{-1} IBA on MS medium (Kumar, 2020). Meanwhile, the optimal IBA concentration for Gisela 6 microshoots was determined to be 1 mg·L^{-1}, yielding the highest rooting rate of 93% (Doric et al., 2014).

The absence of a significant difference in IBA concentrations (Group A for all doses) and a weak positive correlation (r = 0.12) indicates that for most studied forms, the control concentration of 1 mg·L^{-1} IBA is sufficient to initiate root primordia. A further increase in dosage to 3 mg·L^{-1} does not result in a proportional increase in rooted plant yield but may increase the risk of callusogenesis, which requires further investigation.

It also becomes evident that the rootstock genotype influences the explant's response to auxin concentration and may account for the results obtained for the Colt treatment in comparison with those observed in other *Cerasus* genotypes. This interpretation is further supported by the study of Dolcet-Sanjuan et al. (2004), who, working with walnut, reported that the rooting response depends on multiple factors, including the type of auxin used, the applied concentration, the species, and even the specific clone within a species.

Not only the percentage of rooted plants, but also the number of roots, and their length are important indices of the effective clonal rootstock micropropagation. Therefore, the influence of the growth regulator on these parameters was additionally evaluated. The number of roots is an important factor influencing plant survival rates during the acclimatization phase and is considered a reliable indicator of rhizogenesis (Roussos et al., 1999; Dolcet-Sanjuan et al., 2004).

A control concentration of 1 mg·L^{-1} IBA is sufficient for effective rooting across most studied rootstocks, establishing it as the optimal dosage for this stage (Table 2).

In most rootstocks (Krymsk®7 and Gisela 6), increasing the IBA concentration from 1 to 3 mg·L^{-1} reduced in the number of roots, suggesting a potential phytotoxic effect of high auxin doses on these genotypes. A similar inverse relationship between doses and rhizogenesis was also observed in Krymsk®5 (Figure 2).

Krymsk®7 exhibited the highest rhizogenic capacity, maintaining consistently high performance (8.8 ±0.63 roots per explant) at the minimum IBA concentration. A similar trend was observed in Gisela 5, allowing both genotypes to be classified as easy-to-root. Conversely, Krymsk®6 showed an atypical response: it recorded its lowest rooting (1.6 ±0.37 pcs) at 1 mg·L^{-1}, followed by an increase at higher doses, suggesting a higher auxin sensitivity threshold for this rootstock.

An elevated dose of IBA did not lead to an increase in root number in the rye rootstocks studied (with the exception of the last one), in contrast to the results of studies conducted with PR 204/84 (Fotopoulos and Sotiropoulos, 2005) and Gisela 5 (Fallahpour et al., 2015).

The statistically significant superiority of 1 mg·L^{-1} IBA (up to 8.8 roots) over higher concentrations (4.13–5.26 pcs) indicates that low auxin levels serve as optimal triggers for root primordia initiation in most studied stone fruit rootstocks. This aligns with previous findings on the *in vitro* propagation of various cherry rootstocks (Buyukdemirci, 2008; Šiško, 2011; Xu et al., 2016). Further increases in concentration lead to

Table 2 The effect of IBA concentration on rhizogenesis (number of roots) of cherry clonal rootstocks

Type of rootstock	Number of roots (pcs)			Factor A – genotype (rootstock)
	IBA (1 mg·L^{-1} ±SD) (control)	IBA (2 mg·L^{-1} ±SD)	IBA (3 mg·L^{-1} ±SD)	
Krymsk®5	7.4 ±0.53	5.1 ±0.34	4.4 ±0.47	b
Krymsk®6	1.6 ±0.37	4.3 ±0.52	4.7 ±0.57	c
Krymsk®7	8.8 ±0.63	5.6 ±0.65	7.3 ±0.57	a
Gisela 5	8.5 ±0.37	4.1 ±0.5	6.7 ±0.83	ab
Gisela 6	7.7 ±0.64	3.0 ±0.44	2.2 ±0.61	bc
Colt	6.9 ±0.46	2.7 ±0.64	6.1 ±0.83	b
Factor B – IBA concentration	A	B	B	–

Notes: Different lowercase letters indicate significant differences between rootstocks, while different uppercase letters denote significant differences between IBA concentrations ($p < 0.05$); SD – standard deviation

Figure 2 Rooted plants on MS medium supplemented with: A –1 mg·L^{-1} IBA; B –2 mg·L^{-1} IBA; C –3 mg·L^{-1} IBA after 30 days of culture

growth inhibition, as evidenced by a moderate negative correlation (r = -0.42).

The variability of the Colt rootstock is of particular interest: it showed a decline at 2 mg.·L^{-1} followed by a secondary increase at 3 mg·L^{-1}, which may indicate non-linear hormonal regulation. Finally, the root length data (Table 3) highlight the high specificity of each rootstock's response to exogenous of IBA concentrations.

The highest root system development was observed in Gisela 6 at 1 mg·L^{-1} IBA, reaching 5.9 ±0.55 cm, which is significantly higher than in other treatments (Table 3). However, this rootstock also proved to be the most sensitive to increased growth regulator levels: doubling the IBA concentration led to a sharp 3.9-fold inhibition of root growth. Similar results for Gisela 6 at 1 mg·L^{-1}IBA (root length up to 7.5 cm) were reported by Doric et al. (2014).

Notably, Krymsk®6 exhibited atypical dynamics, with the highest root length (4 ±0.56 cm) observed at 2 mg·L^{-1}. In contrast, the general trend for Gisela 5, Colt, and Krymsk®7 was an optimal response to the minimum concentration (1 mg·L^{-1}), aligning with studies on *Prunus* spp. (Šiško 2011; Canli and Demir, 2014; Sharma et al., 2017). Hossini et al. (2010) reported that an increase in IBA concentration significantly reduced in root growth in Gisela 6, similar to that observed in our study.

Correlation analysis revealed a weak negative relationship (r = -0.28) between IBA concentration and rhizogenesis intensity. This pattern stems from the non-linear response of most genotypes to exogenous

Table 3 The effect of IBA concentration on rhizogenesis (root length) of cherry clonal rootstocks

Type of rootstock	Average root length (cm)		
	IBA (1 mg·L^{-1} ±SD) (control)	IBA (2 mg·L^{-1} ±SD)	IBA (3 mg·L^{-1} ±SD)
Krymsk®5	2.8 ±0.54^{a}	1.9 ±0.47^{b}	2.3 ±0.45^{ab}
Krymsk®6	1.5 ±0.33^{b}	4.0 ±0.62^{a}	1.8 ±0.33^{b}
Krymsk®7	3.7 ±0.94^{a}	1.9 ±0.34^{b}	2.2 ±0.46^{b}
Gisela 5	4.5 ±0.69^{a}	3.0 ±0.58^{b}	4.0 ±0.61^{ab}
Gisela 6	5.9 ±0.55^{a}	1.5 ±0.38^{c}	3.4 ±0.44^{ab}
Colt	4.0 ±0.56^{a}	2.3 ±0.36^{b}	3.4 ±0.69^{ab}

Notes: Different letters (a, b, c) denote statistically significant differences between concentration treatments for each rootstock independently; SD – standard deviation

stimulation. Evidently, the factor of rootstock remains the primary driver of root system variability.

To optimize root system quality *in vitro*, a differentiated approach is recommended: while the control medium (1 mg·L^{-1} IBA) is optimal for Gisela 5, Gisela 6, and Colt, increasing the concentration to 2 mg·L^{-1} is more effective for Krymsk®6.

Conclusions

The results demonstrate that rooting efficiency in clonal cherry rootstocks is strongly genotype-dependent. Among the tested treatments, MS medium supplemented with 1.0 mg·L^{-1} IBA provided optimal conditions for root induction in most genotypes, while higher auxin concentrations did not significantly improve rooting and may induce callus formation. Genotype-specific responses were observed in all evaluated parameters, indicating that uniform protocols may not be equally effective across different rootstocks. The modified IBA concentration proved beneficial for certain genotypes, confirming the need for tailored approaches in micropropagation systems. The developed protocol can be applied for efficient *in vitro* propagation of Krymsk®5, Krymsk®6, Krymsk®7, Colt, Gisela 5, and Gisela 6, ensuring high rooting performance and suitability for subsequent acclimatization.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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