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Osmotic Stress Tolerant Cherry Rootstock: *In vitro* Propagation Features

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ABSTRACT

Background: Krymsk® 5 (VSL-2) is a semi-dwarf cherry rootstock often used to produce drought-resistant varieties with high-quality fruits. The present study aims to optimize the *in vitro* micropropagation protocol for rootstock.

Methods: At the propagation and rooting stages, growth regulators such as 24-epibrassinolide (″Epin-extra″), kinetin, 6-benzylaminopurine and indolyl-3-butyric acid were used in various concentrations. Morphological changes, pigment content, stomatal density and size and stomatal slit size for evaluation of drought resistant rootstock were quantified.

Result: Our study showed the efficiency of using preparation "Epin - extra" (1.0 ml/L) at the initial growth stage to stimulate regenerative activity, cytokinin kinetin, (0.5 mg/L) at the stage of reproduction and auxin IBA (0.5 mg/L) at the rooting stage. Application of 0.5 mg/L 6-BAP with Epin proved to be ineffective due to shoot vitrification. For the first time, the resistance of VSL-2 cherry stock to the lack of moisture was assessed using polyethylene glycol 6000 under *in vitro* conditions and resistance to osmotic stress in the studied samples was confirmed. The obtained results will hopefully allow scaling up the reproduction process of the VSL-2 rootstock *in vitro*.

Key words: Cherry rootstock, *In vitro*, Micropropagation, Osmotic stress, Plant growth regulators.

INTRODUCTION

Currently, modern technologies for growing high-quality planting material for the mass production of stone fruit crops involve the clonal rootstocks use to regulate growth and fruiting processes and to obtain high yields in different environmental conditions (Mir *et al.*, 2021).

In stone fruit crop selection, an important task is to create highly adaptive and productive varieties of a new generation for certain soil and climatic conditions in horticultural zones, taking into account modern industry requirements. For varieties of southern horticulture, winter and drought hardiness, plant resistance to diseases and productivity are important. Nikolskaya *et al.* (2023) confirm that with rootstock's help, it becomes possible to influence the quality indicators of fruits.

Among the clonal rootstocks used by various researchers (Dimitrova *et al.*, 2020; Narandi and Ljubojevi, 2022), Krymsk® 5 (cv. VSL 2; hereinafter VSL-2; *Prunus fruticosa* × *Prunus serrulata* var. Lannesiana) is promising in arid conditions. The origin country is Russia. It is an early-maturing, vigorous cherry rootstock, but it is susceptible to viruses (Long *et al.*, 2014). Suitable for stone fruit crops of elite varieties, it has an average size and reduces the fruit plant's growth by 30-50%. With its application, trees reach a height of 3-3.5 m and the frost resistance of its root system and its high productivity have been confirmed (Eremina *et al.*, 2020).

For the large quantity of high-quality, pure-grade planting material production, it is necessary to use high-tech methods for the creation of mother plantations. Micropropagation provides genetic accuracy for plants in limited time and space and plant material without pathogens and viruses (Kandha *et al.,* 2022).

Two factors in woody plant propagation are important: the carbon source and the nutrient medium, which provide energy and help the plant maintain osmotic balance (Yaseen *et al.*, 2013). A comparison of nutrient media for rooted rootstock VSL-2 *in vitro* by Bamatov and Edelgeriev (2020) has shown modified MS medium with iron chelate (15 g/L, KNO_3-20 g/L and $CaCl_2$-10 g/L) to be the most suitable.

Jafarlou *et al.* (2022) obtained the highest rates of shoot proliferation of dwarf cherry rootstocks' shoots *in vitro* (16.5 for Gisela 12 and 9.3 for Maxma 14) for WPM medium with 45 g/L sorbitol and 2 mg/L BA + 0.5 mg/L TDZ. The highest percentage of rooting *in vitro* (94.74%) for Gisela 5 cherry rootstock was given with DKW medium

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with 1 mg/L indole-3-butyric acid (IBA) (Borsai *et al*., 2020).

There is a trend towards 6-BAP (6-benzylaminopurine) and NAA (naphthaleneacetic acid) use as effective plant growth regulators (Jayusman *et al*., 2022). IBA and IAA (indolylbutyric and indoleacetic acids) are also widely used in the propagation and rooting stages. Tsafouros and Roussos (2019) used six cytokinins for propagating VSL-2 rootstocks: Synthetic auxins NAA and IBA were tested and a high percentage of rooting was achieved with their combination. The possibility of using dopamine, chlorogenic acid and quinones as possible cofactors for the rooting of VSL-2 cherry rootstock *in vitro* was shown by Tsafouros and Roussos (2022).

Rootstocks influence the reaction of the grafted plant under conditions of water stress. They show a certain resistance to abiotic stress due to the presence of adaptation and protection mechanisms, which makes them promising materials for use in breeding (Nikolskaya *et al*., 2023). Therefore, the photosynthetic pigment content and parameters of the stomatal apparatus can be used as indicators for determining the tolerance of stone fruit crops explants *in vitro* to simulated drought conditions. The study's aim is the optimization of growth regulators in an *in vitro* protocol for cherry rootstock Krymsk® 5 (cv. VSL-2) as well as assessment of its resistance to drought under simulated *in vitro* conditions.

MATERIALS AND METHODS

The research was carried out at the Laboratory of Biotechnologies, FSC of Agroecology, RAS, Volgograd, Russian Federation, in 2021-2023. Nodal segments (0.7-1.0 cm) were excised from young shoots (July 2021) from the mother tree of the VSL-2 rootstock, provided by the Laboratory of selective breeding, seed growing and nursery farming of FSC agroecology of RAS (Dubovka, Volgograd region, 49°0645′N, 44.8217′E) in the middle of the growing season and used as primary explants.

The growth regulator "Epin-extra" (24-epibrassinolide; Epin) at a concentration of 0.025 g/L (ANO "NEST M", Russia), 6-BAP (GreenAgro Lab, Russia) and kinetin (6-furfurylaminopurine) (GreenAgro Lab, Russia) were used at the reproduction stage. IBA (GreenAgro Lab, Russia) was used to speed up the rooting process. PEG 6000 (Croda Europe Limited, Great Britain) was used to create osmotic stress in the nutrient medium. Dimethyl sulfoxide, 99% (Scharlau, Spain), was used to isolate the pigments. Desinfectants "Lysoformin 3000" (Hygiene Plus, LLC, Russia) and "Belizna-econom" (PK "Rusbytkhim", LLC, Russia) were used for the explant sterilization. Full and half content of the nutrient medium MS protocol of Murashige and Scoog (1962) were used for the propagation and rooting phases.

All stages of the study were carried out under sterile conditions in a NEOTERIC laminar box (Lamsystems, Russia). Plants were grown *in vitro* at 22-24°C with a 16-h photoperiod and a light intensity of 70 umol s^{-1} m^{-2} on STELLAR-FITO LINE racks (AWTech, Russia) for 40-55 days. For treated explants culture tubes of 50 ml and MS medium with 7 g/L agar and 30 g/L sucrose without hormones as controls were used.

Growth regulators used for tree crops, including VSL-2 cherry rootstocks, were added to the nutrient medium to obtain a high multiplication factor and increase the yield of regenerated plants (Tsafouros and Roussos, 2019). VSL-2 microshoots were transplanted onto media containing Epin at a concentration of 0.2-2.0 ml/L and combined with 6-BAP at a concentration of 0.5 mg/L at the propagation stage (Zholobova *et al*., 2022). Kinetin, a plant-derived cytokinin, was also used at the breeding stage in the concentration range of 0.1-0.5 mg/L to study the effect on regeneration of VSL-2 rootstock *in vitro* culture (Tsafouros and Roussos, 2019). The qualitative morphogenesis indicators for each of the regulators were noted during the experiment.

IBA at concentrations of 0.1-1.0 mg/l to stimulate rhizogenesis, was added to full- and half-strength MS nutrient media without hormones (Kovalenko and Polivara, 2021). During the experiment, the following indicators were noted: Rooting percentage, second-order rooting percentage, number and length of roots and shoot length.

To simulate abiotic conditions *in vitro*, we used a water-soluble osmotic agent PEG MW=6000, polyethylene glycol polyester, consisting of ethylene glycol units (Mehmandar *et al*., 2023). Osmotic concentrations were 20-60 g/L (hereinafter PEG 20, PEG 40 and PEG 60) and control (PEG 0). Media with an added osmotic agent were sterilized at 121°C for 20 minutes. To assess the effect of PEG-induced stress morphological changes, pigment content (chlorophyll, carotenoids), stomata density and size, stomatal gap size were studied (Zholobova *et al*., 2024).

To define the pigment concentration a 99% (v/v) dimethyl sulfoxide solution was used to extract it. Leaves weighing from 3 to 6 mg were placed in a 2 mL microtube and 250 μL of dimethyl sulfoxide was added. Incubated for 15 minutes at 65°C and cooled to room temperature. The extract was photometered at 480, 649 and 665 nm on SPECTROstar NANO absorbance plate reader (BMG, Ortenberg, Germany). The pigment content was calculated by the formulas (Gan *et al*., 2022; Wang *et al*., 2022).

To assess the stomatal apparatus, lower leaf epidermis was separated with adhesive tape and observed (× 100, × 400) with a LUM 1LED microscope (Altami, Russia) and a Levenhuk M500 BASE camera (Levenhuk, USA). The data were processed using LevenhukLite × 64 software (Levenhuk, USA). The measurements were carried out using ImageJ program (USA). The obtained data were used to determine the stomatal density and the stomatal gap according to the formulas of Gao *et al.* (2021).

Each experimental stage had control samples on a medium (MS) without hormones. During the study, 34 variants of culture media, repeated twice and 720 plant samples were used at all. Statistical data processing was carried out using STATISTICA Stat Soft Inc. (USA). To determine statistically significant differences for groups with a normal distribution at the reproduction and rooting stages, the HSD Tukey test ($p \leq 0.05$), Fisher LSD ANOVA and correlation analysis were used. To assess the rootstock resistance to osmotic stress, the Mann-Whitney U test ($p \leq 0.05$) was used. The data obtained are presented as the arithmetic mean, taking into account the mean error.

RESULTS AND DISCUSSION

Growth regulators for VSL-2 rootstock at the propagation stage

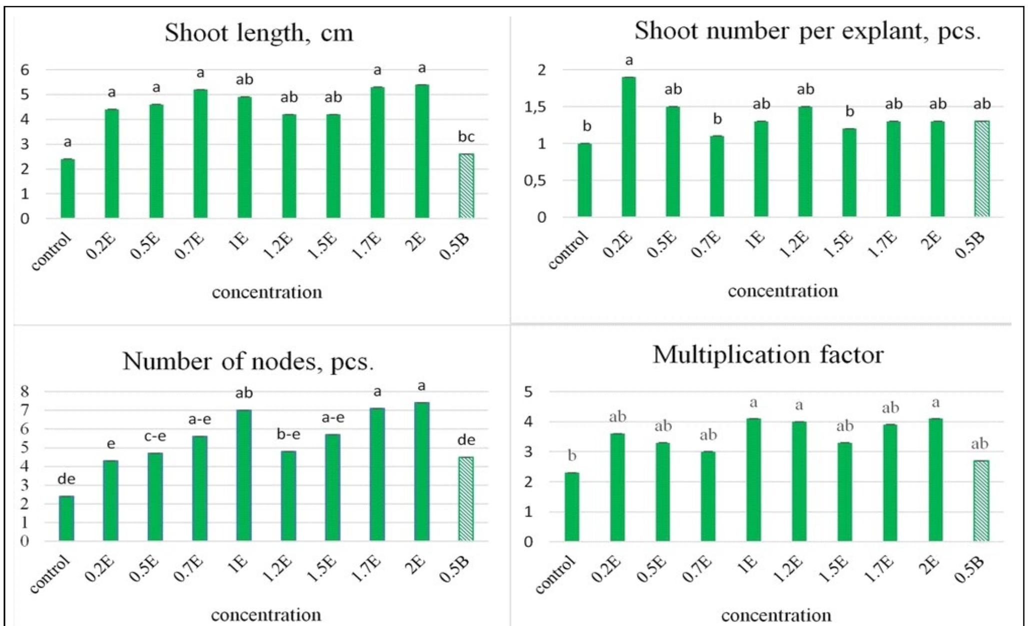
As many as 60% of viable sterile explants during the *in vitro* introduction phase were obtained. To stimulate their regenerative activity and shoot growth, media MS variants with Epin (0.2-2.0 ml/L), as well as its combination with 6-BAP at 0.5 mg/L, were used. After 8 weeks of growth, Epin and 6-BAP influence on the studied morphometric parameters and the multiplication factor in microshoots of the VSL-2 rootstock was determined (Graph 1). The correlation analysis showed dependence for the node number (r= 0.57) and the multiplication factor (r= 0.68) on the shoot length. The statistically significant differences between the shoot length in the control and the experimental variants were found (Graph 1). No significant differences between the experimental variants with Epin were found.

No notable effect on the shoot number was recorded except the variant with Epin (0.2 ml/L). The shoot number value (1.9) was almost two times higher than the control value (1.0). The correlation analysis (r = 0.13) did not show a positive dependence of the multiplication factor on the shoot number. 6-BAP at 0.5 mg/L was ineffective; the multiplication factor was only 2.7. The combination of Epin (0.1-2.0 ml/L) and 6-BAP (0.5 mg/L) had a negative effect: It led to the shoot's conglomerate formation and their vitrification (Fig 1b).

Epin used in the nutrient media on apple rootstocks *in vitro* increased the reproduction rate to 112% and stimulated adventitious bud formation in cauliflower and coconut *in vitro* (Ali, 2019). Epin (0.5 mg/L) also contributed to an increase in the raspberry microshoots average length but had no significant effect on their number. The combined use of brassinosteroid with 6-BAP 0.5 mg/L contributed to an increased shoot number (Makarov *et al*., 2022). 24-epibrassinolide is one of the regulators that specifically modulates the response of plants to abiotic stress and can improve some indicators that affect growth, for example, in potatoes (Khalid and Aftab, 2016).

The positive effect of 6-BAP (0.5-1.0 mg/L) is mentioned by Jafarlou *et al.* (2022) during micropropagation of plants of the genus Prunus to lengthen the shoots of dwarf cherry rootstocks. In our study, such a modification of the medium gave a negative result, causing the shoot vitrification. The multiplication factor is greater than the control value by 0.4. Elongation, leaves twisting and shoots hyperhydration significantly reduced the regenerative explants capacity at the propagation stage. Using 6-BAP (0.5 mg/L) for VSL-2 didn't stimulate shoot formation (Graph 1). When combined with Epin, caused vitrification (Fig 1b), a similar response in cherry rootstocks was observed with 6-BAP (0.5 mg/L) in MS medium (Pronina and Matushkina, 2020).

Kinetin has been successfully used *In vitro* and has a stimulatory effect on the regenerants' growth and



Epin (E) concentration is measured in ml/l, 6-BAP (B) concentration is measured in mg/L. Means in a column followed by the different letter(s) are significantly different according to Fisher LSD ANOVA at $p \leq 0.05$.

Graph 1: Effect of different concentrations of Epin and 6-BAP on micropropagation of cherry rootstock VSL-2.

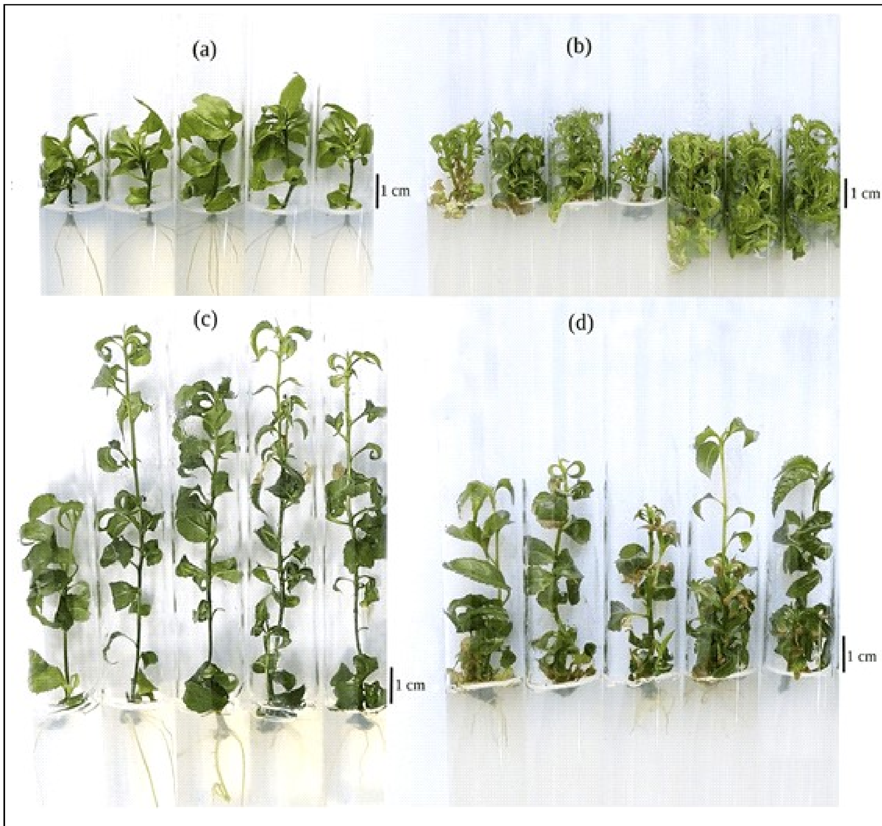
development (Prameswari *et al*., 2019). The kinetin's introduction (0.05 to 0.5 mg/L) had a positive effect on the VSL-2 regenerants at the reproduction stage. After 8 weeks of growth, the shoot length increased from 2.55 to 4.95 cm, while the node number was limited to the range of 3.16 to 6.50 per shoot. The maximum shoot length (9.12-10.31 cm) with 11.75-13.00 nodes was recorded on media with 0.45 and 0.50 mg/L kinetin (2.3 times higher than control; Graph 2). The shoot number per explant, the internode length and the leaves number were evaluated (Graph 2). The first parameter amounted to 1.0-1.5. The distance between nodes varied within 0.53-1.38 cm; at low concentrations, uneven and significant stretching of internodes and a large accumulation of nodes in the apical and basal parts of regenerated plants were noted.

An increase in the shoot length and the node number at high kinetin concentrations also contributed to a significant increase in the leaves number, which amounted to 18.75 and 22.00 on media with kinetin (0.45 and 0.50 mg/L), respectively; 2 times more than the control value (10.80 pcs). Experimental data showed a high percentage (90%) of rooted explants in the control. With an increase in the kinetin concentration, the number of rooted regenerants decreased proportionally, the minimum value being 35% with 0.50 mg/L. A positive kinetin's effect on root length was observed with 0.30-0.50 mg/L of hormone with an increase to 5.2 cm (Graph 2).

A linear relationship on the effect of low kinetin concentrations on the VSL-2 rootstock cells proliferation *in vitro* was revealed: With its increasing in the medium, the regenerative explant's ability increased, which contributed to the shoot growth and the appearance of additional nodes and leaves (Fig 1c and Graph 2). Probably, the combined kinetin and auxin use have a positive effect.

Effect of auxin IBA on *In vitro* root induction

For the rooting step, one of the most abundant auxins in numerous studies in this area (Tsafouros and Roussos, 2022; Kaviani *et al*., 2023) is indolyl-3-butyric acid, used alone or in combination with other compounds that stimulate rhizogenesis. Its addition (0.1 to 1.0 mg/L) in full- and half-strength MS media had a positive effect on the VSL-2 rooting, but statistically significant differences were not shown (Graph 3). Microshoots were rooted in 90-100% of cases on all experimental media. However, a dependence of quantity of regenerants with second-order roots on the auxin concentration was revealed. The best result was achieved on full-strength MS and IBA (0.5 mg/L); second-order root formation was 83.3%. On the half-strength MS medium and IBA (0.5 mg/L), this indicator was two times lower; there



(a) Control (MS), (b) Epin (1.0 ml/L) + 6-BAP (0.5 mg/L), (c) Kinetin (0.5 mg/L), (d) Epin (1.0 mg/L).

Fig 1: Rootstock VSL-2 cultivation on the experimental media *In vitro*.

were no second-order roots on the control medium. Significant differences in the root length in comparison with the control and a high average root number (7.5 pcs) were also observed on media with IBA (0.5 mg/L). Using the half-strength MS had no effect on the rooting and differences were observed only between the variants with IBA (0.5 mg/L); no positive effect was noted. During the increase of IBA to 1.0 mg/L, leaf curl and vitrification were fixed. The variant (IBA 0.5 mg/L) on MS medium (Graph 3) turned out to be optimal for the successful VSL-2 rooting; the maximum values of regenerants with second-order roots, the root number and length were obtained. Similar results were mentioned by Kumar *et al.* (2020).

Evaluation of the stomatal apparatus in the abiotic stress conditions *In vitro*

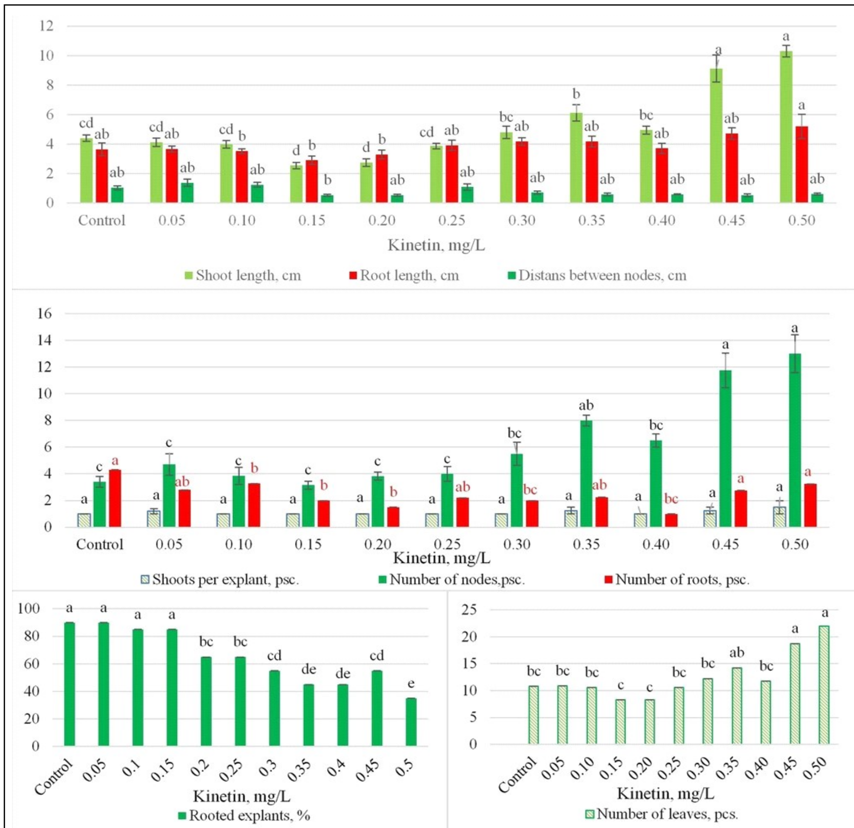
Under osmotic stress influence, there is a decrease in the leaves number, leaf plates twisting and partial drying after 6 weeks of growth of VSL-2 regenerants on media containing PEG 6000 (Fig 2). The root system development of the experimental groups' explants was inhibited. All studied regenerants were viable.

Osmotic stress adversely affected leaf weight (Table 1). The leaf area in the experimental groups decreased, but there were no statistically significant differences between the medium MS and the PEG content media. When assessing changes in the stomatal apparatus, leaves formed under osmotic stress were used as material for analysis. There were no significant differences in the leaf area of regenerated plants in the control medium and in media with PEG content (Table 1). Leaf curl in explants cultured on osmotic media is a morphological change to reduce water loss and light absorption area.

During prolonged drought, mature leaves are able to generate signals for developing leaf plates in order to form the optimal stomatal apparatus under specific conditions (Li *et al.*, 2021). At the same time, a balance must be maintained between transpiration and CO_2 uptake for the normal operation of the photosynthetic apparatus (Hasanuzzaman *et al.*, 2023).

Changes in the VSL-2 leaf plate stomatal apparatus occurred in the absence of available water (Table 1). The lowest value of stomatal density was recorded in the control group (21.79) and the highest value was in the plant leaves on PEG 20 (33.93).

The stomatal cells' area in the experimental groups decreased by 2-2.75 times. The length and width ratio of stomatal cells L_1/D_1 in the experimental groups was



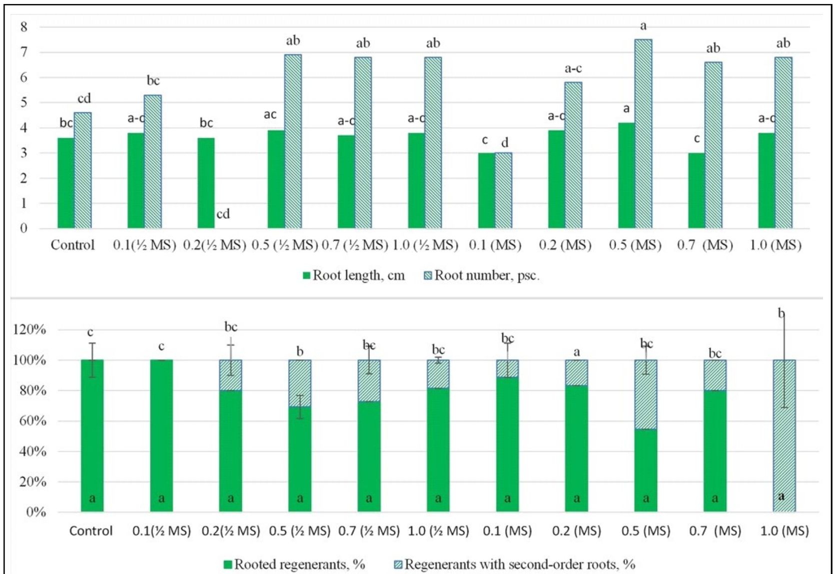
Means±SE with different letters in a column are statistically different according to Tukey HSD test (p≤0.05). Pearson correlation coefficient showed a direct and high degree of dependence between shoot length and the node number (r= 0.89).

Graph 2: Effect of low kinetin concentrations on VSL-2 regenerants' parameters.

statistically significantly higher than on PEG 0 by 15%. When assessing changes in the stomatal apparatus caused by osmotic stress, the smallest changes in this parameter were recorded.

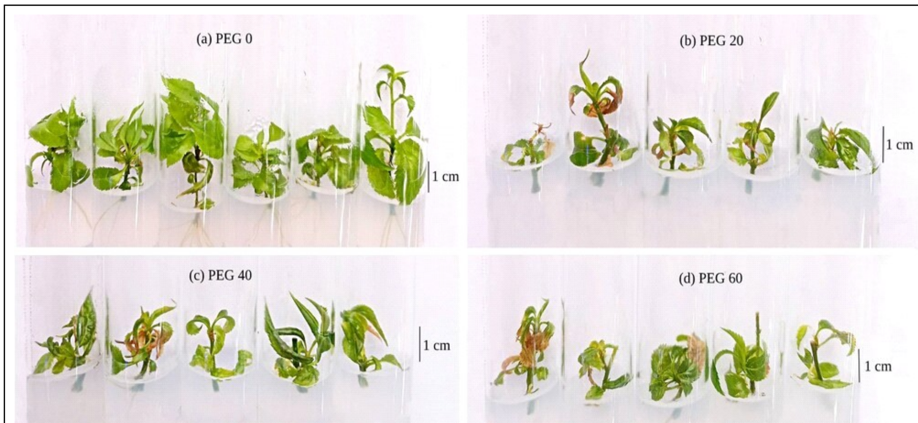
The stomatal gap area under osmotic stress decreased by 5-10 times. The ratio L_2/D_2 (Table 1) change indicates a stomatal cell's shape modification. Statistically significant differences in L_2/D_2 to control (1.59) were observed on PEG 20 (1.9) and PEG 60 (2.4). When there is a scarcity of water, increasing stomatal density in VSL-2 explants (Table 1) results in higher conductivity, which increases carbon uptake but decreases water use efficiency (Hasanuzzaman *et al.*, 2023). The observed disproportionate decrease of the stomatal fissure area size (Table 1) from the total stomatal cell area in the control from 15.5% to 3.6-6.1% on PEG-content media occurs in accordance with the hydropassive and hydroactive mechanisms of stomatal gap narrowing. (Wankmüller and Carminati, 2022). These changes characterize the rootstock VSL-2 as a xerophytic plant (Babaei *et al.*, 2021).

No significant difference was observed between the control and PEG 20 examples when analyzing the total leaf plate chlorophyll content (Graph 4). When comparing the mean values of the control group, PEG 40 and PEG 60, the obtained data were in the uncertainty zone. The hypothesis put forward about the significance of the differences was not confirmed. We noted a statistically significant increase in the carotenoids in the experimental groups (2.77-3.15) compared with the control (1.89). When comparing the chlorophyll *a* to chlorophyll *b* ratio no statistically significant



Means±SE with different letters in a column are statistically different according to Tukey HSD test (p≤0.05).

Graph 3: Effect of various IBA concentrations (mg/L) on rooting of VSL-2 regenerants.



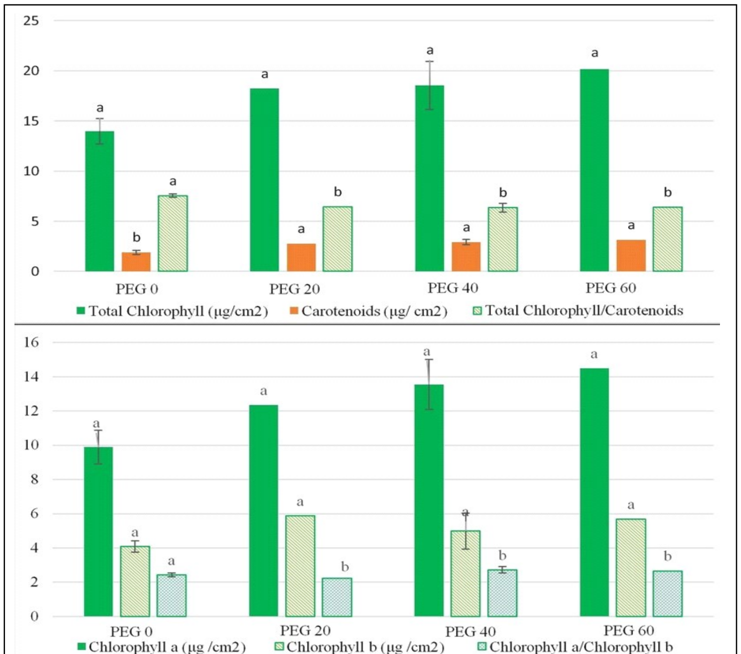
(a) PEG 0; (b) PEG 20 (c) PEG 40 (d) PEG 60.

Fig 2: VSL-2 regenerants after 6 weeks growth on MS medium with different PEG content.

Table 1: Leaf plate mass, area and stomatal apparatus (stomatal cell and stomatal gap) quantitative traits in osmotic stress conditions *in vitro*.

Quantitative trait	PEG 6000 added to MS medium, g/L			
	PEG 0	PEG 20	PEG 40	PEG 60
Leaf plate mass, mg	$6.46^{a}\pm0.6$	$4.33^{a}\pm0.4$	$4.14^{a}\pm0.5$	$4.17^{a}\pm0.6$
Leaf plate area, mm^2	$0.58^{a}\pm0.1$	$0.45^{a}\pm0.02$	$0.44^{a}\pm0.05$	$0.41^{a}\pm0.05$
Mean stomatal cell length (L_1), µm	$27.66^{a}\pm0.7$	$17.87^{c}\pm0.6$	$20.9^{b}\pm0.7$	$20.81^{b}\pm0.5$
Mean stomatal cell width (D_1), µm	$25.34^{a}\pm0.5$	$14.32^{c}\pm0.3$	$17.16^{b}\pm0.6$	$16.87^{b}\pm0.5$
Mean stomatal cell area, mm^2	$0.55^{a}\pm0.02$	$0.20^{c}\pm0.01$	$0.28^{b}\pm0.02$	$0.28^{b}\pm0.01$
L_1/D_1 ratio	$1.10^{b}\pm0.03$	$1.26^{a}\pm0.04$	$1.23^{a}\pm0.04$	$1.26^{a}\pm0.04$
Mean stomatal gap length (L_2), µm	$13.81^{a}\pm0.6$	$4.6^{b}\pm0.3$	$6.05^{b}\pm0.5$	$5.44^{b}\pm0.4$
Mean stomatal gap width (D_2), µm	$8.81^{a}\pm0.2$	$2.46^{c}\pm0.1$	$3.87^{b}\pm0.3$	$2.46^{c}\pm0.2$
Mean stomatal gap area, ìm^2	$86.11^{a}\pm5.2$	$8.31^{c}\pm10.$	$17.52^{b}\pm2.8$	$10.1^{c}\pm1.4$
L_2/D_2 ratio	$1.59^{c}\pm0.1$	$1.90^{ab}\pm0.1$	$1.67^{bc}\pm0.1$	$2.40^{a}\pm0.2$
Stomatal cells number, pcs./0,1 mm^2	$21.79^{c}\pm1.4$	$33.93^{a}\pm1.8$	$27.08^{b}\pm1.7$	$26.99^{b}\pm1.6$

Means SE with different letter values are statistically different according to the Manna-whitney U criterion ($p\leq0.05$).



Means SE with different letter values are statistically different according to the Manna-Whitney U criterion ($p\leq0.05$).

Graph 4: Pigment content of rootstock VSL-2 leaves.

differences between experimental and control groups were noticed. At the same time, the total chlorophyll to carotenoids ratio in the experimental groups was statistically significantly reduced in relation to the control. The carotenoid concentration increases in the experimental groups (Graph 4), it confirms the plant photosynthetic apparatus stability under a lack of available water (Salsinha *et al.*, 2021).

CONCLUSION

To optimize *In vitro* conditions for the establishment of VSL-2 cherry rootstock we recommend "Epin - extra" (1.0 ml/L) to stimulate the regenerative microshoot activity. Adding kinetin (0.5 mg/L) is effective at the reproduction phase. Auxin IBA (0.5 mg/L) is optimum for successful rhizogenesis of VSL-2. A decrease in the stomatal cells' area and an increase in their number per unit leaf area characterized the rootstock for cherry Krymsk 5 (VSL-2) as a xerophytic plant. The present results can be successfully used both in experimental studies and for scaling up this rootstock *In vitro*.

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Conflict of interest

All authors declare that they have no conflict of interest.

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