In vitro Effect of Replacement Mineral Salts of Trace Elements with P-containing Chelates to Improve Rooting of Cherry Rootstock (cv. VC-13)

N V Tsirulnikova¹, E A Nikulina¹, S V Akimova²,³, V V Kirkach², A P Glinushkin⁴ and I Yu Podkovyrov⁵

¹Non-Profit Institute for Chemical Reagents and High Purity Chemical Substances of NRC «Kurchatov Institute», Bogorodsky Val., 3107076, Moscow, Russia.
²Russian State Agrarian University - Moscow Agricultural Academy named after K.A. Timiryazev, Moscow, Timiryazevskaya st., 49, 127550, Russia;
³State Scientific Institution "All-Russian Research Institute of Phytopathology", Russian Agricultural Academy", st. Institute, own 5, Bolshie Vyazemy, Odintsovo distr., Moscow reg., 143050, Russia;

Corresponding author’s e-mail: nikulina_elena@mail.ru

Abstract. In this paper, we studied the effect of modified culture medium based on Murashige and Skoog (Murashige T. Skoog F., 1962) on the in vitro rhizogenesis of clonal rootstock of stone cultures - VC-13. For the first time, the nutrient medium was modified with complexes of all incoming trace elements based on a phosphorus-containing ligand of the bisphosphonate class. The effect of 3 levels of concentrations of chelated trace elements was studied: 1.25, 2.5 and 5 ml/l without the use of increased doses of BMI, and a comparative analysis was carried out in comparison with the modification of trace elements based on EDTA. The maximum effect – 100% rooting after 30 days of subcultivation was found at a concentration of chelated trace elements of 5 ml/l. When modifying EDTA, rooting did not occur. Also, the modification of the nutrient medium by phosphorus-containing complexes contributed to a change in the nature of the formation of the root system of micro-gears. Micro-plants had from 3 to 6 short strong roots, convenient for washing from the nutrient medium and then planting at the stage of adaptation to non-sterile conditions. Repeated tests of the effect of culture medium modification on rhizogenesis in vitro on a larger scale confirmed the high efficiency of this approach. The rooting rate of micro-gears of the VC-13 clone rootstock was more than 80%. The results obtained allow us to conclude about the key role of the nature of the chelating agent.

1. Introduction

The Prunoideae, a subfamily of Rosaceae, which include many economically important species: nectarines and peaches (Prunus persica (L.), plums (Prunus domestica L.), apricots (Prunus armeniaca L.), sour cherries and cherries (Prunus cerasus L. and Prunus avium L., respectively), etc. Currently, the world production of stone fruits exceeds 30 million tons. In Russia, the total production of fruit stone crops in 2018 reached 615.6 thousand tons, and the acreage occupied by the cultivation of the latter was estimated at more than 120 thousand hectares. [1-2] Sweet cherries (Prunus avium) and sour
cherries (Prunus cerasus) have been recognized in many countries for their potential health benefits and are the favorite fruit crops of many people. In Russia, the latter are very common, and in the production of cherries, Russia occupies a leading position in the world [3].

Modern gardens, stone fruit cultures are complex and highly productive biological systems. At the same time, the first most important requirement for the quality of fruit plant seedlings is the genetic and sanitary condition of the plants, that is, the true type of genotype and the absence of pathogenic microorganisms (viruses, bacteria, fungi). Moreover, the root system of high-quality seedlings should be well developed and contain at least 3-4 well-structured roots, evenly distributed around the root neck of the plant, equipped with fouling roots (suction, conducting, transitional roots) [2,4].

The creation of highly productive gardens of intensive type of stone crops requires the development of intensive methods of accelerated propagation of pure-grade healthy planting material. [2, 5-6]. Clonal micropropagation is the most widespread method of vegetative propagation of clonal rootstocks of stone crops [4-5, 7-10]. Plants propagated in vitro are small in size and easy to transport, free of pathogenic microorganisms.

One of the best clonal cherry rootstocks used in the climatic conditions of Russia is the VC-13 rootstock (P. cerasus x (P. cerasus x P. maackii), bred by specialists of the Crimean experimental breeding station (Russia) [11]. It is characterized by high frost resistance, drought resistance, resistance to many diseases and is physiologically compatible with all varieties of cherries and cherries.

In the culture of in vitro reproduction, VC-13 experiences General difficulties characteristic of clonal micro-propagation of many stone cultures. The rooting phase of the obtained regenerating micro-plants in vitro and their subsequent adaptation to non-sterile conditions is one of the most difficult and key factors of successful reproduction [9-10, 12-14]. To solve this problem at the stage of rhizogenesis, increased doses of synthetic auxins are usually introduced into nutrient media. However, prolonged exposure to auxins first stimulates root formation, but then inhibits root growth, promotes the development of callus and tissue necrotization in regenerating plants [15]. Another approach used in practice is to apply temporary immersion of plants in a liquid nutrient medium for different times and intervals [16]. At the same time, many authors note the importance of the correct selection of the optimal composition of the nutrient medium [8, 13].

In earlier studies, it was shown that optimal nutrient media and micro-propagation conditions strongly depend on the culture genotype [8, 15, 17]. However, the most widely used nutrient medium is Murashige-Skoog (Murashige and Skoog, 1962) [18]. It is known that stone cultures contain increased amounts of pectin substances in the tissues, which have a high affinity for trace element ions. Therefore, stone cultures are characterized by increased requirements for nutrition with trace elements. In standard nutrient media, micronutrients are used in the form of inorganic salts. This form is not optimal for plants to absorb many necessary elements, for example, Fe, Cu, Zn, Mn. The most preferred form is the chelated form. However, until now, work in the field of modification of the microelement composition of nutrient media has focused on the introduction of direct Fe complexates (usually FeEDDHA) [19-22].

The purpose of this work was to study the effect of replacing inorganic salts of all trace elements that make up the nutrient medium with chelated forms of the latter on the in vitro rhizogenesis of the clone rootstock of stone cultures - VC-13. Two types of complexons were used as chelating agents: the most widespread carboxyl – containing complexon-ethylenediaminetetraacetic acid (EDTA) and, for the first time, a phosphorus-containing complexon from the class of bisphosphonates – exyethylidene dibasic acid 1-hydroxyethylidenediphosphonic acid (HEDP).

2. Methods and materials

Objects of research: clonal rootstock of stone fruit VC-13.

At the stage of rhizogenesis (rooting), a nutrient medium with a mineral base according to Murashige and skuga (MS) was used, reduced to $\frac{1}{2}$ for macronutrients, enriched with the following
substances: (mg/l) thiamine-hydrochloride (B1), pyridoxine-hydrochloride (B6), nicotinamide (PP) - 0.5; BMI - 0.2; sucrose - 15,000, agar-agar - 6000.

The trace elements were modified into a chelated form based on EDTA and HEDP. Preparation of masterbatch solutions of chelated forms of trace elements for the nutrient medium was carried out in the laboratory of technology of complexons and complex compounds of the national Research Center "Kurchatov Institute" - Institute of chemical reagents and especially pure chemicals (IREA). The influence of 3 levels of concentrations of chelated trace elements was studied: 1.25; 2.5 and 5 ml/l.

The culture medium was poured into culture vessels (100 ml glass jars with a parafilm coating) of 30 ml each and sterilized in an autoclave lasting 20 minutes and at a pressure of 0.2 MPa. In a laminar box, 5 micro-gears with a length of 2-3 nodes were placed in each vessel. Subcultivation of experimental micro-plants was carried out in the culture room at a light intensity of 2500 Lux, a 16-hour photoperiod, and a temperature of 20-22°C. On the 30th day of subcultivation, rooting and development of regenerating plants were taken into account. Repeatability of experiments – three times 12 explants in one repetition.

At the second stage, after receiving the results of the first one, the best result is checked on a larger scale. In table.1 the General scheme of experiments is presented.

| Nutrient medium | Number of culture vessels, pieces | Number of explants (total), pieces |
|-----------------|----------------------------------|-----------------------------------|
| Macronutrients ½ by MS prescription | Trace elements in chelate form on the basis of HEDP | 5 | 3 | 12 |
| | | 2.5 | 3 | 12 |
| | | 1.25 | 3 | 12 |
| Trace elements in chelate form on the basis of EDTA | 5 | 3 | 12 |
| | 2.5 | 3 | 12 |
| | 1.25 | 3 | 12 |

3. Results and discussion
When studying the in vitro rhizogenesis of stone rootstock VC-13, it was found that the maximum effect – 100% rooting after 30 days of subcultivation was achieved at a concentration of chelated trace elements with an organophosphate ligand (HEDP) at a concentration of 5 ml/l. The results of the studies are presented in table 2. as can be seen from the above data, the best result, a 10-fold increase in rooting capacity compared to the control variant, was obtained when using a modified Murashige and skuga (MS) nutrient medium with chelated microelements based on HEDP at a concentration of 5 ml/l.

A decrease in the concentration of chelated trace elements in the medium showed a linear decrease in the percentage of rooted regenerating plants, as well as a decrease in the morphometric parameters of the formed root system. Thus, the average number of roots decreased from 5-6 to 2-3 at concentrations of 5 and 2.5 ml/l, respectively, and the average root length increased from 0.5-0.6 to 1-2 cm.

When modifying EDTA, rooting did not occur at any concentration. Modification of the nutrient medium with phosphorus-containing complexons contributed to the formation of 3 to 6 short strong roots, convenient for washing from the nutrient medium for subsequent planting at the stage of adaptation to non-sterile conditions.

VC-13 when modifying the culture medium Murashige and skuga (MS) with chelated forms of trace elements
Table 2. Results of rooting of micro-plants of clonal rootstock of stone Crops

| Nutrient medium | ml/l | Rooting, % | Average number of roots, pieces | The average length of the roots, sm |
|-----------------|------|------------|-------------------------------|----------------------------------|
| Macronutrients ½ by MS prescription | Control of trace elements MS | 5 | 10 | 1-2 | 3-5 |
| Trace elements in chelate form on the basis of HEDP | 5 | 100 | 5-6 | 0,5-0,6 |
| Trace elements in chelate form on the basis of EDTA | 2,5 | 50 | 2-3 | 1-2 |
| Trace elements in chelate form on the basis of HEDP | 1,25 | 0 | - | - |
| Trace elements in chelate form on the basis of EDTA | 2,5 | 0 | - | - |
| Trace elements in chelate form on the basis of HEDP | 1,25 | 0 | - | - |

A repeated experiment on the rootability of micro-seedlings of the VC-13 clone rootstock using a modified Murashige and skuga (MS) culture medium based on HEDP at a concentration of 5 ml/l on 120 explants confirmed the effective effect of this method. The rootability of micro gears was more than 80%, the average number of roots – 3-5 pieces.

In General, VC-13 is a difficult-to-root object in in vitro culture, micro-gears take root slowly and at different times, high doses of auxins (up to 1 mg/l) are required, which in turn leads to callus formation and further necrotization of micro-plant tissues. In the case of rooting individual micro-gears, as a rule, they have 1-2 roots up to 3-5 cm long, which are easily injured and broken off when removing plants from the nutrient medium, which makes further adaptation to non-sterile conditions almost impossible.

4. Conclusion

Thus, the obtained unexpected result showed that the modification of the nutrient medium with mineral macrocosmi for the recipe MS of chelated micronutrients with phosphate ligand led to a significant increase in the efficiency phase of rhizogenesis clonal rootstock VC-13. This fact is clearly related to the nature of the ligand. It is known that bisphosphonates have their own biological activity. HEDP is used as a medical substance, it is characterized by specific stereochemistry and mutual influence of phosphonic fragments [23]. The literature presents the results of numerous studies on the use of HEDP in medicine and other industries.

However, there is very little information about the role of HEDP in plant physiology. Earlier, in the works of Soviet scientists, it was reported about the retardant properties of HEDP, which were found in the cultivation of rye, barley, and buckwheat [24]. The mechanism of HEDP's retarding action has not been studied in detail. The results of this experiment show that in the case of in vitro rhizogenesis of VC-13, the effects characteristic of the action of retardants were observed: the formation of a larger number of roots, a decrease in their length and an increase in their mechanical strength. It should be noted that the use of retardants in nutrient media is practically not studied, since the mechanism of their influence is not fully understood.

Further research in this direction is of great practical importance, with the expansion of possible research objects among promising stone and other fruit and berry crops that are of great economic and economic importance. Similarly, modified nutrient media ensure the manufacturability of the clonal micro-multiplication process and increase production efficiency.

References
[1] Stone and fruit crops market in Russia – 2020. Indicators and forecasts. Marketing research (Report) https://tebiz.ru/mi/rynok-kostochkovyh-plodovikh-kultur-v-rossii
[2] Mayer N A, Bianchi V J, Feldberg N P, Morini S 2017 Rev. Bras. Frutic. V.39, n 4. Advances
in peach, nectarine and plum propagation p.355

[3] The Food and Agriculture Organization (FAO) of the United Nations http://www.fao.org/faostat/en/#data/QC

[4] Almada R, Arismendi M J, Pimentel P R, Hinchsen, P, Pinto M, Sagredo B. 2013 Tree Genetics & Genomes, Heidelberg, V.9. Class 1 non-symbiotic and class 3 truncated hemoglobin-like genes are differentially expressed in stone fruit rootstocks (Prunus L.) with different degrees of tolerance to root hypoxia. p.1051-1063

[5] Balla I and Kirilla Z. 2006 V-th IS on In Vitro Culture and Hort. Breeding Eds. M.G. Fári, I. Holb and Gy.D. Bisztray Acta Hort. Micropropagation of Peach Rootstocks and Cultivars 725.

[6] Zubkov A V, Antonenko V V 2020 Bulletin of agrarian science Assessment of the phytosanitary status and problems of protection of perennial plantings in horticulture № 1 (82) p 20-29

[7] Battistini A, De Paoli G. 2002 5-th IS on Peach Eds. R.S. Johnson & C.H. Chrisosto Acta Hort Large Scale Micropropagation of Several Peach Rootstocks 592

[8] Zilkah S, N. Zamiri N and Ziv M. 2006 6-th Intl. Peach Symposium Ed. R. Infante Acta Hort Potrescine and Hydrogen Peroxide Improve the Rooting of ‘GF-677’ Rootstock in Woody Cuttings and Tissue Culture Shoots 713

[9] Fotopoulos S and Sotiropoulos T E 2005 New Zealand Journal of Crop and Horticultural Science, V 33 In vitro propagation of the PR 204/84 (Prunus persica × P. amygdalus) rootstock: Axillary shoot production and rhizogenesis p.75-79

[10] Vaez-Livari B and Salehi-Soghadi Z. 2006 IV-th IS on Pistachios and Almonds Eds.: A. Javanshah et al. Acta Hort In Vitro Rooting of Hybrid GF677 (Prunus dulcis × Prunus persica) 726

[11] Eremin G V, Kehein V K, Pronorchenko A V, Podorojniy V K 2003 Scientific and technical progress in horticulture, VSTISP Ways to intensify the production of stone fruit crops in the Krasnodar region P 65-71

[12] Tsipouridis C, Thomidis T, Bladenopoulou S 2006 Scientia Horticulturae V.108. Rhizogenesis of GF677, Early Crest, May Crest and Arm King stem cuttings during the year in relation to carbohydrate and natural hormone content p. 200–204

[13] Guo-Qing Song 2015 Agrobacterium Protocols V. 2 Third Edition Edited by Kan Wang. Book. Springer New York Heidelberg Dordrecht London Chapter 12. Cherry. p.133-142 DOI 10.1007/978-1-4939-1658-0

[14] Cos J, Frutos D, García R, Rodríguez J and Carrillo A. 2004 I-st IS Rootstocks – Decid. Fruit Eds. M.Á. Moreno Sánchez and A.D. Webster Acta Hort In Vitro Rooting Study of the Peach-Almond Hybrid Mayor 658

[15] Perez-Tornero O Lopez J M, Egea J and Burgos L. 2000 Journal of Horticultural Science & Biotechnology V. 75(3) Effect of basal media and growth regulators on the in vitro propagation of apricot (Prunus armenica L.) cv. Canino p.283-286

[16] Damiano C, La Starza S R, Monticelli S, Gentile A, Caboni E, Frattarelli A. 2005 In: HVOSLEF-EIDE A.K. AND PREIL W. (Ed.). Liquid culture systems for in vitro plant propagation. Netherlands: Springer Propagation of Prunus and Malus by temporary immersion. p.243-251.

[17] Ghasheem N, Stánica F, Peticilă A G, Venat O 2018 Scientific Papers. Series B, Horticulture. V. LXII In vitro effect of genotype, growth season and cytokinines on peach varieties (Prunus persica (L.) Batsch)

[18] Murashige T. and Skoog Physiol. Plant., vol. 15, pp. 437–497, (1962)

[19] Licea-Moreno R J, Contreras A, Morales A V, Urban I, Daquinta M., Gomez L. 2015 Plant Cell, Tissue and Organ Culture Vol.123. Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA p.143–154

[20] Molassiotis A N., Dimassi K, Therios I, Diamantidis G. 2004 Biol. Plant. V.1. Fe-EDDHA
promotes rooting of rootstock GF-677 (Prunus amygdalus 9 P. persica) explants in vitro p. 141–144

[21] Zawadzka M, Orlikowska T. 2006 Plant Cell, Tissue and Organ Culture. Vol.85. The influence of FeEDDHA in red raspberry cultures during shoot multiplication and adventitious regeneration from leaf explants p. 145-149

[22] Van der Salm T P M, Van der Toorn C J G, Hanish ten Cate C H, Dubois L A M, De Vries D P, Dons H J M. 1994 Plant Cell, Tissue and Organ Culture Vol.37. Importance of the iron chelate for micropropagation of Rosa hybrida L. ‘Moneyway’. p.73–77

[23] Matkovskay T A, Popov K I, Yureva E A. 2001 M.: Chemistry Bisphosphonates, properties, structure and application in medicine p.6-74

[24] Diatlova N M, Lavrova O Yu, Temkina V Y, Kireeva A Yu, Seliverstova I A, Rudakova G Ya, Cirulnikova N V, Dobrikova E O 1984 Overview of ser. "Reagents and highly purified substances" NIITEKHIM The use of chelating agents in agriculture p.31