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ÎNMULȚIREA *IN VITRO* A PORTALTOIULUI DE PRUN 'DOCERA 6'
IN VITRO PROPAGATION OF PLUM ROOTSTOCK 'DOCERA 6'

Nikolova Viktorija, Akova Vanya, Nesheva Marieta, Malchev Svetoslav
Fruit Growing Institute Plovdiv, Bulgaria

Abstract

This paper describes research on the application of tissue culture techniques to the micropropagation of interspecific rootstock 'Docera 6'. The experimental work was carried out in the period 2017-2018, in the *in vitro* propagation laboratory of the Fruit Growing Institute Plovdiv. Axillary buds were employed as initial explantes in two different seasons (March-May; September-October). The action of the mineral medium was studied in the multiplication stage. The best result was obtained on LS medium included BAP 0.5 mg/l and IAA 0.05 mg/l. The obtained average multiplication rate is 3.08. The concentration of auxin applied to the basal medium influence the quality of the root system Treatment with high concentrations of IBA added to the rooting medium gives the best results (V5). The influence of the season on growth and development of micropropagated 'Docera 6' rootstock during ex vitro acclimatization is also part of our research. The spring acclimatization gives better results than the autumn.

Cuvinte cheie: portaltoi interspecific, *in vitro*, inmultire, inradacinare, aclimatizare
Key words: interspecific rootstock, *in vitro*, propagation, rooting, acclimatization

1. Introduction

Plum is a traditional fruit crop for Bulgaria. Due to its high productivity and good adaptability to agro-climatic conditions, it has become widespread in our country (Vitanova et al., 2014).

With the occurrence of monosort and endemic spread of *Plum pox virus*, plum production in Bulgaria is still of extensive type and needs to be optimized and intensified. This should be done in the direction of using resistant cultivars and rootstocks, the use of branch rootstocks, which are less vigorous, as widely used in our country seed coat of 'Myrobalan' is sensitive to PPV and at the same time strongly growing. The use of branch weaker rootstocks will allow the creation of denser plantations, the use of irrigation systems through which to import agrochemicals into the gardens, the application of modern tree formations, and hence obtaining stable high yields.

The production of many plants for a short period of time is possible only through *in vitro* propagation. Micropropagation offers not only quick propagation of plants, but also eliminates diseases and provides scope for development of new cultivars (Debergh and Read, 1990). Sometimes, traditional propagation of rootstock is difficult due to the weak affinity to plant rooting (Fachinello, 2000). Root cutting also does not guarantee the production of clean and disease-free material (Holtz et al., 1999). In this regard, the production of planting material by inoculating cuttings under *in vitro* conditions is an alternative for obtaining pure and healthy seedlings independent of the season. Clonal micropropagation is vegetative propagation by tissue and cell in which the plant forms are genetically identical to the mother plant. The method of *in vitro* propagation uses the unique ability of the plant cell to reproduce from itself a whole new plant. The biggest advantage of tissue culture is that it obtains a high quality, authentic, virus free plant material. 'Docera 6' *(P. domestica* × *P. cerasifera*), selected at the Technical University of Munich, Germany, by tracking the influence and on the vegetative and productive results of resistant and tolerant plum varieties, in order to assess the suitability and implementation in practice. Laboratory and field tests of 'Docera 6' define it as an extremely promising substrate for limiting the measles virus (Milusheva et al., 2015).

The aim of the present study is optimization all stages of *in vitro* propagation of 'Docera 6' rootstock.

2. Material and methods

The study was carried out in 2017-2018, in the *in vitro* propagation laboratory of the Fruit Growing Institute Plovdiv.

A successful protocol for *in vitro* propagation proceeds through a series of stages:

2.1. Initiation of aseptic culture

Mother plants, previously tested, with proven health status, were selected as starting material. Stem explants (1–2 cm long), each comprising a node and axillary bud, were cleaned by few drops of mild detergent, washed in running tap water for 15–20 min and rinsed with distilled water. The explants

were surface sterilized with 70 % ethyl alcohol for 1 minute and 5% solution of Ca-hypochlorite and time of action 3, 5 or 10 minutes. After washing 5–6 times with sterilized distilled water, the explants were shortened to 3–4 mm after the removal of leaves, dry sheaths and other external tissues. These were then cultured in MS medium (Murashige and Skoog, 1962).

The betting of the explants was done in two stages. The first stage is from the beginning of spring (March-May) and the second stage is in the secondary growth of shoots, in early autumn (September-October).

2.2. Shoot multiplication

25-30 days after first cultivation the pure and vital plants were then cultured in new fresh medium with different chemical composition.

1. MS: macronutrients MS (Murashige and Skoog, 1962), microelements MS, vitamins MS.
2. M: macronutrients MS, microements H, vitamins MW (Morel and Wetmore, 1951).
3. LS: macronutrients LS (Linsmaier and Skoog, 1965), microelements MS, vitamins MS.
4. QL-H: macronutrients QL (Quoirin and Lepoivre, 1977), micronutrient H (Heller, 1959), vitamins MS.

All nutrient mediums containing sucrose 30 g L^{-1}, agar 5.8 g L^{-1}, pH 5.6-5.8, supplemented with the growth regulators BAP 0.5 mg L^{-1} and AA 0.05 mg L^{-1}.

The pH of the medium was adjusted to 5.7±0.2 before addition agar-agar and autoclave. The cultures were incubated at 22-24°C under photo period of 16/8 light/dark.

2.3. Rooting of micro shoots

Proliferated uniform shoots (more than 3 cm) from sub-apical part up (remove any callus), were excised and transferred to jar containing rooting medium consisting full-strength MS micro salts and vitamins, 25% macro MS, supplemented with different concentration of IBA (1.0; 1.5; 2.0) mg L^{-1}. All of nutrient media contain 20 g L^{-1} sucrose and 5.8 g L^{-1} agar-agar. In each vessel 10 shoots are inoculated.

The pH was adjusted to 5.7±0.2 before addition agar and autoclaving.

The plants were placed in a growth-chamber at 22-24ºC and photo period 16/8 light/dark.

2.4. Acclimatization

The last stage in biotechnology process is acclimatization of the micropropagated plants. *Ex vitro* acclimatization of micropropagated plants is one of the key steps for rapid and large scale up clonal multiplication of quality planting material. On the 14th day, the plants are very well rooted and, after being removed from the agar were carefully washed with warm H2O to remove traces of medium. Multi-cell bedding plant trays filled with 45 nests and peat:perlite (2:1) are used for acclimatization. In this stage, the plants were acclimated by traditional methods in two weather seasons (spring and autumn). The adaptation of the plants in external, non-sterile conditions took place in a glass-steel greenhouse. The planted plants are placed in tunnels covered with nylon. The tunnels are left covered until the tenth day after planting in order to maintain the high humidity (90-100%), which the plants initially need. Temperature was maintained around 18-27°C. After 50 days in *ex vitro* conditions growth parameters are preformed (percentage adapted, number of leaves, stem length, FW root, FW stem, FW leafs.

The results obtained are subjected to mathematical analysis using the method developed by David B. Duncan (Duncan, 1955). Software used in the study is "R-3.1.3" in combination with "RStudio-0.98" and installed package "agricolae 1.2-2" (Mendiburu, 2015).

3. Results and discussions

The results of table1 show a proven statistical difference between the explants taken in March-May and September-October. The best results were obtained for the explants taken during the period March-May and the time of treatment with calcium hypochlorite for 3 and 5 minutes. These explants were fresh, green and vibrant, showing a high level of adaptation to *in vitro* conditions. Similar results were reported by other authors (Nacheva and Ivanova, 2017), but the disinfection of plants was with 1% chlorhexidine gluconate - 5 min.

In the next stage the initial establishment of axillary buds was observed in all the medium compositions used. However LS nutrient medium gave the highest multiplicated rate. Results of testing different nutrient media show that plants grown on MS, M and LS media, with each passage passed multiplication rate growing, but that not happening with the plant cultivated on QL-H nutrient media (Fig. 1). According to other authors, rootstock 'Docera 6' on MS medium with 2.0 mg L^{-1} BAP, in the first three passages loses its regenerative ability (Vujovich et al., 2018).

Visually, it can be seen that (Fig. 2) the plants of the nutrient media MS, LS and M are viable, with well-formed leaves and newly formed shoots. Plants cultivated on QL-H nutrient medium have the formed so-called tuft, but at the same time it shows signs of vitrification (glazing), whence comes the low propagation rate.

Overall, the table 2 shows that the nutrient medium with high concentration of IBA (V5- 2.0 mg/L^{-1})

recorded to be the best medium giving 100% root induction. The other root characters like average number of roots per cultured shoot, an average root length, height of plants and number of leaves have been influenced by the concentration of IBA in basal medium. However, in terms of the length of the roots of the plants in medium V3, as well as the indicators percentage rooting and height of the plants with V4, there is no statistical difference with the plants in medium V5

Figure 3 shows the rooted plants of the three variants of rooting media. Plants rooted in V3 and V5 have long, slender roots. The plants are vital, with green leaves and stems and suitable for planting. Plants rooted in V4 have thickened, yellowed roots with accumulated callus. The plants are vital, with fresh green leaves and stems.

The results presented in Figure 4 show the rate of acclimatization of plants

The result revealed that plants grown in the spring show over 80% survivals. Accordingly, between spring and autumn acclimatization, very significant differences were obtained for the fresh weight of shoots, leaves and roots. Obviously, spring acclimatization is more effective, which shows higher averages of all growth parameters. Therefore, there is a significant difference in stem height and number of leaves, which together with the roots are a decisive factor for survival. Before being planted in the field, the plants are transferred to individual containers until they reach the required development and size (Fig. 5).

4. Conclusions

The time for selection of quality explants for further work with them is in March-May.

Plants grown on MS, M and LS media, with each passage passed multiplication rate growing, but that not happening with the plant cultivated on QL-H nutrient media.

Percent of rooting plants cultivated in V4 and V5 nutrient media is 100%. In terms of number of roots and number of leaves the best results are obtained on V5.

The acclimatization of the plants in the spring months is better than in autumn.

Acknowledgments

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Tables and Figures

Table 1. Initiation of aseptic culture

Time of taking the explants	Time of action	Number of cultivated explants	Number of clean and vital plants	Percentage of clean and vital plants
March-May	3 min	20	13[a]	65[a]
March-May	5 min	20	14[a]	70[a]
March-May	10 min	20	7[b]	35[b]
September-October	3 min	20	6[b]	30[b]
September-October	5 min	20	0[c]	0[c]
September-October	10 min	20	0[c]	0[c]

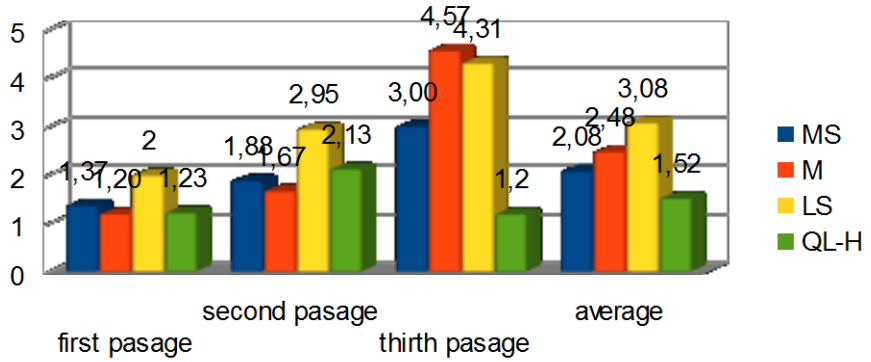

Fig. 1. Effect of sub culturing on multiplication parameters of rootstock

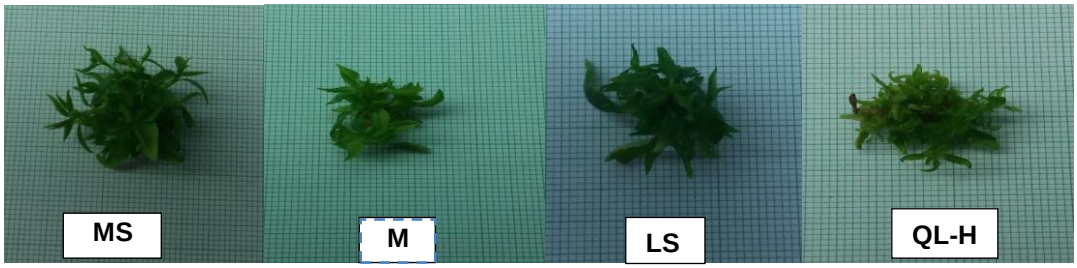

Fig. 2. Plants in multiplication phase

Table 2. Effect of different auxin concentration on in vitro root induction in microshoots of 'Docera 6'

Rooting of plants					
Nutrient media	**Percentage rooting**	**Height of plants (mm)**	**Leaves number**	**Number of roots**	**Root length (mm)**
V3 (1mg. L^{-1})	66,67 [b]	32,2 [c]	11,01 [b]	6,03 [c]	42,6 [a]
V4 (1,5mg. L^{-1})	100 [a]	52 [a]	9,12 [c]	8,89 [b]	27,4 [c]
V5 (2.0 mg. L^{-1})	100 [a]	48,1 [a]	13,17 [a]	11,11 [a]	42 [a]

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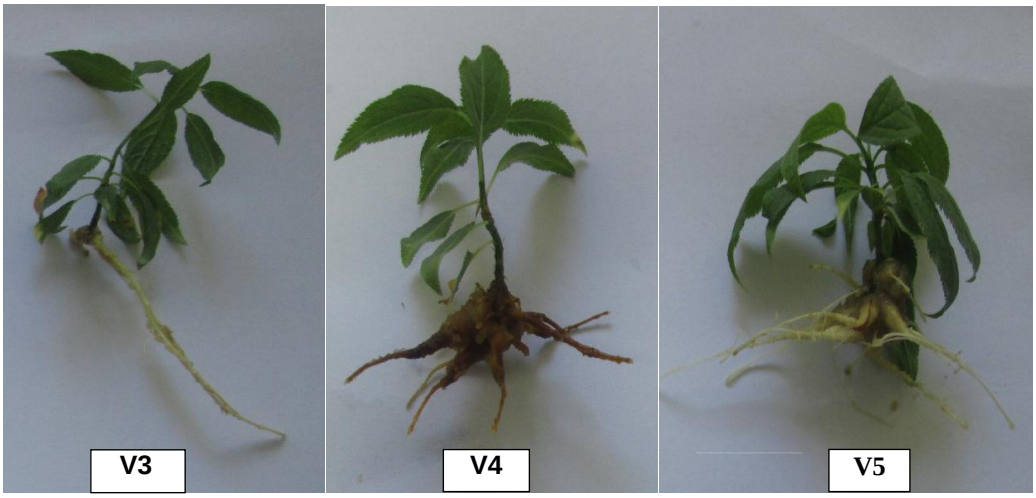

Fig. 3. Rooting plants of rootstock 'Docera 6'

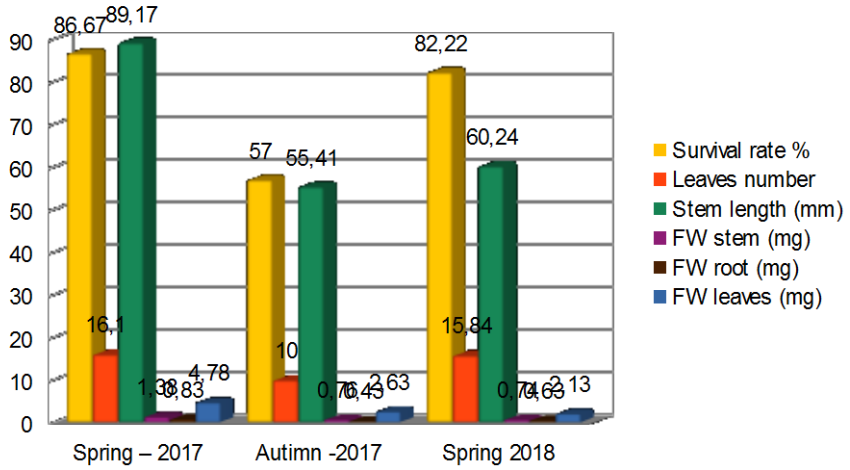

Fig. 4. Acclimated of plants in two in two weather seasons (spring and autumn)

Fig. 5. Acclimatized plant moved to a larger container for rearing