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EFFECT OF BIOSTIMULATOR REGOPLANT ON ACCLIMATIZATION OF MICROPROPAGATED GiSelA 6 CHERRY ROOTSTOCK IN FLOATING SYSTEM

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Abstract

Acclimatization is one of the key steps in the success of micropropagation of woody plants. Improvement of acclimatization of micropropagated woody fruit plants could be achieved in different ways. The purpose of this study was to optimize the acclimatization of micropropagated sweet cherry rootstock GiSelA 6 (Prunus cerasus 'Schattenmorelle' × Prunus canescens) through application of biostimulators of natural origin in floating system. A new generation of plant growth biostimulators Regoplant and Charkor (Agrobiotech, Ukraine) contain metabolism products of symbiotic fungus-endophyte of ginseng roots, cultivated in vitro. Micropropagated and rooted plantlets from cherry rootstock GiSelA 6 (Prunus cerasus 'Schattenmorelle' × Prunus canescens) were acclimatized in a floating system with 100 µl L^{-1} Regoplant or Charkor. As control plantlets with no additional treatments served. Multi-cell bedding plant trays filled with peat: perlite 1:1 (v:v) were used for acclimatization. Plants were grown in a greenhouse under high humidity conditions for one week and then humidity was gradually decreased. Data on some growth, physiological and biochemical parameters were collected 45 days after transplanting to ex vitro conditions. Regoplant (100 µl L^{-1}) led the highest plants survival rate (86%), also the greatest values for the growth determined parameters.

Key words*: acclimatization, pears, Regoplant, Charkor, nutrient solutions.*

INTRODUCTION

Micropropagation *in vitro* has shown promises for rapid and large scale clonal multiplication of disease-free planting material all year round. But woody plants are often recalcitrant to *in vitro* cultivation and this process is highly genotype dependent. During *in vitro* cultivation, plantlets grow under specific conditions - in small tightly closed vessels with high air humidity, low gas exchange and thus a CO_2-shortage during almost the whole photoperiod, ethylene production and relatively low light intensity, in a culture medium with a large concentration of sugar (Ziv, 1991). These special conditions result in the formation of plants with abnormal morphology, anatomy and physiology. After the transfer from *in vitro* to *ex vitro* conditions, plants have to correct the abnormalities and to acclimatize to the new environment in the greenhouse or in the field. Acclimatization is one of the key steps in the success of micropropagation of woody plants and most losses of *in vitro* plants occur when the plantlets are moved from *in vitro* to the *ex vitro* conditions.

Natural light shading and antitranspirants application for reducing plant transpiration are some of the approaches which are often used to increase plant survival rate after transplanting.

GiSelA 6 rootstock (*Prunus cerasus* 'Schattenmorelle' × *Prunus canescens*) is the most popular rootstock for new plantings in the northwest Pacific, but already sought after in other parts of the world, including in Bulgaria. GiSelA 6 is not demanding for soils and although it is a relatively vigorous rootstock, it can easily be controlled. The production of GiSelA 6 by rooting cuttings by conventional methods is a rather difficult and slow process and do not always deliver high-quality, healthy and uniform propagating material. The scale and rapid multiplication of quality plants can be achieved through biotechnological approaches through micropropagation (Nacheva & Gercheva, 2008).

Floating system is one of the most simplistic cultivation systems composed by a tank

containing the nutrient solution and floating panels where the plants are sown and grown (Pardossi et al., 2006). On the surface of the nutritive solution, there are floats made of polystyrene or other materials that sustain the plants (Sheikh, 2006). Floating systems are increasingly used for greenhouse production of fresh-cut leafy vegetables and for the cultivation of medicinal plants (Dorais et al., 2001). Several reports suggest that float hydroculture could be successfully applied in acclimatisation of *in vitro* produced plantlets, such as *Solanum tuberosum* L. (Nhut et al., 2006), *Grammatophyllum speciosum* Blume (Sutthinon et al., 2015), *Lycium barbarum* L. and other species, including cherry rootstock GiSelA 5 (Clapa et al., 2013). In these studies, hydroponics were proved to be a feasible alternative to acclimatise *in vitro* plantlets in a clean, convenient and water-saving way. However, there has been little information about an efficient method for acclimatization of *in vitro* cherry plantlets using a hydroponic system.

A new generation of plant growth biostimulators Regoplant and Charkor (Agrobiotech, Ukraine, http://www.agrobiotech.com.ua) contain metabolism products of symbiotic fungus-endophyte of ginseng roots, cultivated *in vitro*. Regoplant is composite natural plant biostimulator. Its mode of action is based on synergic effect of products of biotechnological cultivation of fungi-micromycetes from root system of ginseng and aversectin - biological product with antiparasitic action. Charkor contains a complex of amino acids, fatty acids, sugars, macro- and microelements and analogs of phytohormones. According to the authors, Charkor is more effective than indolyl-acetic and indolyl-butyric acid in rooting cuttings of a number of ornamental trees and shrubs (Ponomarenko et al., 2010). It was successfully applied for rooting of micropropagated magnolia plantlets (Gercheva et al., 2015). According to our previous results, Charkor and Regoplant stimulate growth and improve acclimatization of micropropagated pear plantlets (Dimitrova et al., 2017; Dimitrova et al., 2019).

The purpose of this study was to optimize the acclimatization of micropropagated sweet cherry rootstock GiSelA 6 (*Prunus cerasus* 'Schattenmorelle' × *Prunus canescens*) through application of biostimulators of natural origin in floating system.

MATERIALS AND METHODS

Plant material and experimental conditions

The experiment was carried out on micropropagated plantlets of sweet cherry rootstock GiSelA 6 (*Prunus cerasus* 'Schattenmorelle' × *Prunus canescens*).

The research was done in September - October, 2019 in the greenhouse at the Fruit Growing Institute - Plovdiv, Bulgaria.

Well-rooted plantlets were potted in styrofoam form pads (528x308x60 mm) filled with peat-perlite 1:1 (v:v).

The pads were placed in a plastic tank containing Knop's nutrient solution, supplemented with 36.7 mg L^{-1} iron sodium ethylenediaminotetraacetate (FeNaEDTA). Regoplant and Charkor at concentration 100 µL L^{-1} were added to the nutrient solution. Thus, three variants were formed:

1. Knop's nutrient solution - Control (C);
2. Knop's nutrient solution, supplemented with 100 µl L^{-1} Regoplant (R);
3. Knop's nutrient solution, supplemented with 100 µl L^{-1} Charkor (CH).

The nutrient solution was replaced weekly. The top of the plastic tank was covered with transparent plastic. Plants were grown in a floating system at high humidity conditions for 2 weeks and then humidity was gradually decreased.

For each experimental treatment three replications, each containing 40 plants, were tested. For biometrical analysis ten representative plants were studied. For gas-exchange and chlorophyll *a* fluorescence analysis at least 5 measurements were performed.

After 45 days in *ex vitro* conditions growth parameters, physiological and biochemical analysis have been performed.

Growth parameters

The leaves, stem and roots were separated and a specific fresh weight (FW) (g) of the relevant botanical organs, also leaf area (cm^2) were determined immediately after removing the

plants from the soil. The dry weight (DW) (g) of of the relevant botanical organs was measured after drying the material at 80°C for 48 h (Beadle, 1993).

Physiological and biochemical parameters

Gas-exchange analysis

Gas-exchange analysis was performed on the youngest fully developed leaves of 3 randomly selected plants of the respective variant. Measurements were taken with a LCpro + portable photosynthesis system (ADC, UK) on a sunny day at a light intensity of about 180 µmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD) and a temperature of 25°C. Net photosynthesis rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration intensity (E, mmol H_2O m^{-2} s^{-1}), stomatal conductivity (gs, mol H_2O m^{-2} s^{-1}) were determined.

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence analysis was performed on the youngest fully developed leaves of 5 representative plants of the respective variant. The basic parameters of rapid chlorophyll a fluorescence (JIP test) were taken with a HandyPEA portable system (Hansatech Instruments, UK). The leaves were dark adapted for 40 minutes with special clips. The main parameters of chlorophyll fluorescence were measured - minimal (F_0), maximal (Fm), and variable (Fv) fluorescence, Fv/Fm, as well as HandyPEA-specific indicators - Performance index (PI_{ABS}) on the absorption basis and total PI (PI_{total}) measuring the performance up to the photosystem I (PSI) end electron acceptors (Goltsev et al., 2010).

Photosynthetic pigments

The photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) were extracted in 80% acetone, the extracts absorbances were determined spectrophotometrically and the content (mg g^{-1} FW) calculated according to the formulae of (Lichtenthaler & Wellburn, 1983).

Determination of Total Antioxidant Activity

Free radical scavenging activity of plant samples against stable 2, 2- diphenyl-2-picrylhydrazyl hydrate (DPPH, Sigma-Aldrich Chemie, Germany) was determined spectrophotometrically. The change in colour (from deep-violet to light- yellow) was measured at 517 nm using UV-1600PC Spectrophotometer (VWR International, Europe).

Radical scavenging activity of plants was measured by method of Yen & Chen (1995). Briefly, 100mg fresh leaves were extracted with 50 ml of methanol (HPLC grade) in the ultrasonic bath for 15 minutes at 10°C.

The extract was centrifugated at 10 000 RPM for 5 minutes at 10°C. One ml of this of plant extract was mixed with 1.5 ml freshly prepared solution of DPPH in methanol (0.3 M) and 3.5 ml methanol.

The samples were kept in the dark for 15 minutes at room temperature and then the decrease in absorbance at 517 nm was measured.

The reference cuvette contained DPPH blank. The radical scavenging activity of the samples was calculated according to Rossi et al. (2003) and was expressed as percent inhibition of DPPH radical as following:

$$\text{Inhibition\%} = [(A_{control} - A_{sample}) / A_{control}] \times 100,$$

Where: $A_{control}$ was the absorbance of the control (the blank solution without plant extract) and A_{sample} was the absorbance of the sample.

Statistical analysis

Data of different parameters were analyzed statistically by using one-way ANOVA in SPSS statistical software (version 13 for Windows) and significant differences between the means were evaluated using Duncan's multiple range test at $P \leq 0.05$.

RESULTS AND DISCUSSIONS

In the floating systems, GiSelA 6 (*Prunus cerasus* 'Schattenmorelle' × *Prunus canescens)* cherry plants grew vigorously, but the differences between the plants grown with the biostimulator Regoplant were visible two weeks after planting. A relatively low survival rate (40%) was reported in the control variant (Table 1). Enrichment of the nutrient solution with Charkor led to a higher survival rate of cherry plants (56%) and the highest survival (86%) was reported in plants treated with the Regoplant. The plants of this variant were also distinguished by the greatest stem length

(111.01 mm), number of leaves, leaf area, fresh and dry mass of leaves and stems (Table 1, Figure 1). For the plants cultivated with Charkor, the biometric parameters were similar to those of the control, with the exception of the smaller leaf area.

Table 1. Growth parameters, survival rate (%) and antioxidant activity (% DPPH) of cherry plants 45 days after acclimatization on floating system with biostimulators Regoplant or Charkor

Variants	Control	Regoplant	Charkor
Stem lenght (mm)	55.32 b	111.01 a	43.09 b
Number of leaves	10.00 ab	12.60 a	9.00 b
Leaf area (cm^2)	6.48 b	8..82 a	4.33 c
FW leaves (g $plant^{-1}$)	0.611 ab	1.1991 a	0.373 b
FW roots (g $plant^{-1}$)	0.048 a	0.047 a	0.050 a
FW stem (g $plant^{-1}$)	0.025 b	0.074 a	0.029 b
DW leaves (g $plant^{-1}$)	0.1098 ab	0.2145 a	0.0793 b
DW roots (g $plant^{-1}$)	0.038 a	0.038 a	0.040 a
DW stem (g $plant^{-1}$)	0.018 b	0.076 a	0.022 b
Survival rate (%)	40	86	56
DPPH (%)	42.75 c	57.43 b	88.69 a

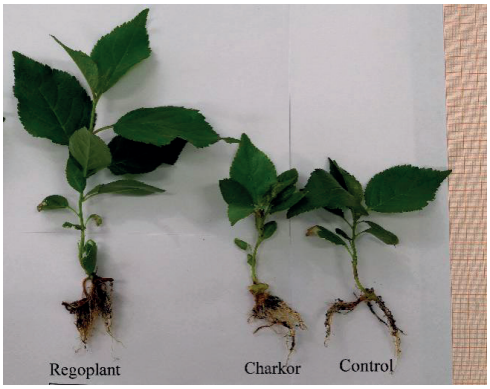

Figure 1. Sweet cherry rootstock GiSelA 6 plants (*Prunus cerasus* 'Schattenmorelle'× *Prunus canescens*) at acclimatization in floating system with biostimulators Regoplant or Charkor

The content of photosynthetic pigments in the leaves of the control plants and in those with the Regoplant did not differ significantly (Table 2). However, statistically lower values were reported for plants grown with Charkor.

Table 2. Content of photosynthetic pigments in leaves (mg g^{-1} fresh weight) of GiSelA 6 plants at acclimatization on floating system with biostimulators Regoplant or Charkor

Variants	Chloro-phyll a (mg g^{-1}FW)	Chloro-phyll b (mg g^{-1} FW)	Chloro-phyll (a + b) (mg g^{-1} FW)	Carotenoids (mg g^{-1}FW)	Chl a/b ratio	Chl (a+b)/ Carotenoids ratio
Control	1.246 a	0.360 a	1.603 a	0.467 ab	3.461 a	3.433 a
Regoplant	1.373 a	0.397 a	1.768 a	0.496 a	3.459 a	3.568 a
Charkor	0.656 b	0.194 b	0.849 b	0.275 b	3.379 a	3.087 b

The results were similar for net photosynthetic rate, transpiration and stomatal conductance, in which no significant differences were observed between the plants cultured with Regoplant and the control variant (Table 3).
In the variant with Charkor, lower values of gas exchange parameters were reported.

Table 3. Effect of biostimulators Regoplant and Charkor on net photosynthesis rate - (A) (µmol CO_2 m^{-2} s^{-1}); transpiration intensity - (E) (mmol H_2O $m^{-2}s^{-1}$) and stomatal conductance - (gs) (mol H_2O $m^{-2}s^{-1}$) at 180 µmol m^{-2} s^{-1} PPFD

Variants	Net photosynthetic rate (A)	Transpiration (E)	Stomatal conductance (gs)
	(µmol CO_2 $m^{-2}s^{-1}$)	(mmol H_2O $m^{-2}s^{-1}$)	(mol$H_2O$$m^{-2}s^{-1}$)
Control	**4.250 ± 0.28 a**	**1.032 ± 0.21 a**	**0.062 ± 0.017 a**
Regoplant	**4.233 ± 0.62 a**	**0.934 ± 0.11 a**	**0.067 ± 0.014 a**
Charkor	**2.568 ± 0.67 b**	**0.531 ± 0.10 b**	**0.025 ± 0.005 b**

Chlorophyll *a* fluorescence is another indicator of the functional activity of the photosynthetic apparatus of plants along with the intensity of photosynthesis.
The analysis of the induction curves of rapid chlorophyll fluorescence (OJIP test) links the structure and functionality of the photosynthetic apparatus and allows for rapid assessment of plant viability, especially in stress conditions (Strasser et al., 2000, 2004).
In the three variants studied, the rapid chlorophyll fluorescence curves had a typical OJIP shape from F_0 to F_m level with clearly separated J and I phases (Figure 2), indicating that the cherry plants, included in the experiment, were photosynthetically active (Yusuf et al., 2010).

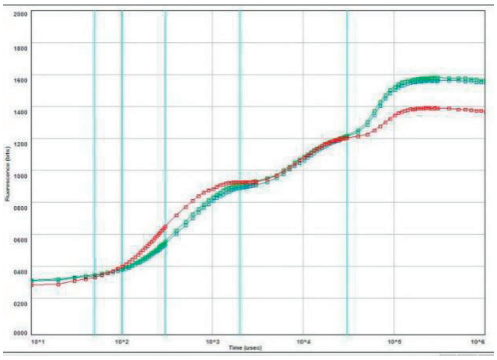

Figure 2. Induction curves of rapid chlorophyll a fluorescence (OJIP test);
(----) Control without biostimulators; (----) nutrient solution, supplemented with100 µl L^{-1} Regoplant (R); (----) nutrient solution, supplemented with100 µl L^{-1} Charkor (CH)

The minimal (F_0) and maximal (F_m) fluorescence of the control plants and plants treated with Regoplant did not differ significantly (Table 4).
In plants grown with Charkor in the nutrient solution F_0 and F_m were the lowest, and the difference was statistically proven (Table 4).

Table 4. Basic parameters of the JIP test of cherry plants 45 days after acclimatization on floating system with biostimulators Regoplant or Charkor

Variants	Control	Regoplant	Charkor
t for Fm	313.33 a	280.00 a	240.00 a
Area	30079.67 a	29691.00 a	19002.00 b
Fo	331.33 a	335.00 a	290.33 b
Fm	1573.00 a	1590.00 a	1404.67 b
Fv	1241.67 ab	1255.00 a	1114.33 b
Fo/Fm	0.211 a	0.210 a	0.207 a
Fv/Fm	0.789 a	0.789 a	0.792 a
PI Inst.	2.134 a	1.959 a	0.923 b
Fv/Fo	3.760 a	3.747 a	3.839 a
Sm	24.123 a	23.693 a	17.000 b
N	32.443 a	32.346 a	33.075 a
psi(Eo)	0.530 a	0.520 a	0.408 b
phi(Eo)	0.418 a	0.410 a	0.324 b
delta(Ro)	0.528 a	0.551 a	0.406 b
PI abs	2.561 a	2.351 a	1.108 b
PI total	2.850 a	2.882 a	0.776 b

Lower F_m values may indicate that the photosynthetic object is in a state of stress and not all electron acceptors in PS II can be completely reduced. Maximal fluorescence is a complex parameter that is determined by a number of factors but also depends on the chlorophyll content of the tissues examined. Indeed, the lower Fm values for Charkor treated plants correspond to the measured lower content of total chlorophyll a, total chlorophyll and net photosynthetic rate in these plants (Tables 2,3 and 4).
Despite fluctuations in the initial, maximum, and variable fluorescence, the quantum yield (Yield = F_v/F_m), reflecting the potential of photochemical activity of PS II, ranges from 0.789-0.792 and corresponds to normal (0.750-0.830) in healthy, unstressed leaves (Bolharnordenkampfh & Oquist, 1993). This indicates that in all three variants studied, a normally developed photosynthetic apparatus was functioning. This is confirmed by the slight differences in the measured values of the rate of net photosynthesis in control and Regoplant treated plants. However, a more in-depth analysis of the parameters of the JIP test revealed some characteristic features of the potential of the photosynthetic apparatus in plants treated with biostimulators and control plants. Characteristic differences between plants grown with and without Regoplant were not reported in the other three important parameters of the JIP test - psi (Eo), phi (ψEo), the performance index (Plabs) and the total performance index (Pl total). For plants grown with Charkor, the four parameters were statistically lower than the control plants and those cultivated with Regoplant. Parameter ψEo reflects the probability of electron transport outside Q_A. The performance index (PIabs) shows the functional activity of the PS II relative to the energy absorbed, and the total performance index (PI total) reflects the functional activity of the PS II, PS I and the electron transport chain between them. PI_{total} is closely related to the overall plant growth and survival under stress and is considered to be a very sensitive indicator of the JIP test. The highest PI_{total} of the GiSelA 6 plants treated with Regoplant clearly showed the effectiveness of the applied treatment. Treatment with Regoplant contributed to the

more active development and structuring of the photosynthetic apparatus, which in turn is a prerequisite for more intensive photoassimilation and biomass accumulation (Table 1).

The results reported by other authors regarding the acclimatization of cherry rootstocks of the GiSelA series are quite controversial. Vujovic et al. (2012) reported a high acclimatization rate (up to 100%) in GiSelA 6 plants in plastic pots containing sterile soil substrate on a "mist' bench in greenhouse, while in GiSelA 5 (*Prunus cerasus 'Schattenmorelle'* × *Prunus canescens*) 61.8% acclimatization was reported under the same conditions. The results obtained by classical acclimatization method (by using solid substrates and high air humidity) of GiSelA 5 were similar with low survival percentages-36.4% (Šiško, 2011).

In experiment for *ex vitro* acclimatization in float hydroculture of GiSelA 5 plantlets rooted *in vitro*, Clapa et al. (2013) reported the lowest survival percentages obtained by this method (58%) among studied species. They achieved higher survival rate (67.24%) when planting the *in vitro*-rooted GiSelA 5 plantlets into the layer of floating perlite.

According to Yepes and Adwinckle (1994), lack of vascular connections between roots and shoots was implicated in low survival of *in vitro* rooted apple plantlets after transfer to the soil. Similarly, indirect *in vitro* rhizogenesis through callus formation can be one of the reasons for low percentage of acclimatization in GiSelA 6 in control variant, as was shown in pear cultivar Bartlett (Bommineni et al., 2001).

Results indicate that during the acclimatization of micropropagated GiSelA 6 (*Prunus cerasus* 'Schattenmorelle' × *Prunus calescent*) plants in floating system conditions, 100 µl L^{-1} Charkor increased plant survival compared to the control, although showing some depressing effect on leaf development and photosynthetic pigment content.

The floating system provides easier maintenance of the air humidity, especially in the conditions of autumn acclimatization and is a valuable approach, especially in the conditions of hot and dry autumn as reported in Bulgaria in 2019. The results of the presented experiment would serve as a basis for the acclimatization of other woody species under floating system conditions.

CONCLUSIONS

Enrichment of the nutrient solution with biostimulator Regoplant (100 µl L^{-1}) in floating system led to the highest survival rate (86%) of GiSelA 6 (*Prunus cerasus* 'Schattenmorelle' × *Prunus canescens*) plants, the greatest stem length, number of leaves, leaf area, fresh and dry mass of leaves and stems.

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EX·TERRA·AVRVM