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Influence of Biostimulators Regoplant and Charkor on Growth and Development of Micropropagated Pear Plants at Acclimatization Stage

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Abstract. In recent years, there has been a growing interest in biostimulants as an alternative to chemicals for safe and sustainable agriculture. The aim of this study was to analyze the effect of biostimulators Regoplant and Charkor (Agrobiotech, Ukraine) on growth and development of micropropagated pear plants (*Pyrus communis* L. 'Old Home' x 'Farmingdale') at acclimatization stage. In vitro propagated and rooted plantlets from pear rootstock OHF 333 were acclimatized in a floating system with 100 µl l^{-1} Regoplant or Charkor. Plantlets with no additional treatments served as control. Data on growth parameters, chlorophyll a fluorescence (OJIP test) and antioxidant activity were collected 45 days after transplanting to *ex vitro* conditions. Enrichment of the nutrient solution with biostimulator Charkor (100 µl l^{-1}) in floating system led to the highest survival rate (82.5%) of pear plants, the greatest stem length, number, fresh and dry mass of leaves. Combining innovative approaches such as a floating system and biostimulators would significantly improve the acclimatization and the overall process of micropropagation of fruit plants.

Key words: *in vitro* micropropagation, *ex vitro* acclimatization, floating system, nutrient solutions, chlorophyll a fluorescence, OJIP test.

Introduction

For the last few decades *in vitro* culture methods have successfully produced disease-free woody fruit plants but some limitations still remain. *In vitro*, plants grow in a special environment and rely on sugars in the nutrient medium, while in the process of adaptation to *ex vitro* conditions they must switch to autotrophic nutrition. The ability of in vitro derived propagules to withstand transplanting stress very often determines the success or failure of tissue culture operations (Nowak & Pruski, 2004). Therefore, the acclimatization is a key stage of the micropropagation process. Different approaches have been developed to increase the plant survival rate after transplanting from in vitro conditions to a greenhouse. Several authors reported 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 cherry rootstocks (Clapa et al., 2013; Dimitrova et al., 2020). In this system, 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 intensively used for greenhouse production of fresh-cut leafy vegetables and for the cultivation of medicinal plants (Dorais et al., 2001). However, there has been little information about an efficient method for acclimatization of in vitro pear plantlets using a hydroponic system.

In recent years, there has been a growing interest in biostimulants as an eco-friendly alternative to chemicals for boosting the growth of plants in stress conditions. Regoplant and Charkor are a part of a new generation of plant growth biostimulators (Agrobiotech,Ukraine, http://www.agrobiotech.com.ua) and contain metabolism products of in vitro cultivation of endophyte micromycetes of ginseng roots. Regoplant also contains aversectin – biological product with antiparasitic activity. 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,

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Charkor and Regoplant stimulate growth and improve acclimatization of micropropagated pear plantlets (Dimitrova et al., 2017; Dimitrova, et al., 2019). It was found that soaking the pear plantlets for 10 minutes in a solution of Regoplant (50 µl l^{-1}) before planting them in a soil substrate led to a significant increase in their biomass (Dimitrova et al., 2017). Also, enriching the agar nutrient medium for rooting with 0.5 ml l^{-1} Charkor stimulated rooting and had a long-term positive effect on plants growth during acclimatization (Dimitrova, et al., 2019).

The aim of this study was to analyze the effect of biostimulators Regoplant and Charkor on growth and development of micropropagated pear plants during ex vitro acclimatization in a floating system.

Materials and Methods

Plant material and experimental conditions

The experiment was carried out on micropropagated pear rootstock (*Pyrus communis* L. 'Old Home' x 'Farmingdale' 333).

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

A preliminary study with a floating system (unpublished data) showed that Knopp's nutrient solution (1865) had a better effect on the growth of pear plants than Hellriegel's (1898) and Pryanishnikov 's (1976) nutrient solutions. Therefore, Knop's nutrient solution was chosen in this experiment.

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 5 l Knopp's nutrient solution (1865), 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. Plants, potted in the same way, but without nutrient solution, served as a control (conventional ex vitro acclimatization). Thus, four treatments were formed:

1. Control (C) – conventional acclimatization in peat-perlite 1:1 (v:v), without nutrient solution;
2. Acclimatization in a floating system with Knopp's nutrient solution (K);
3. Acclimatization in a floating system with Knopp's nutrient solution, supplemented with 100 µl l^{-1} Regoplant (R);
4. Acclimatization in a floating system with Knopp's nutrient solution, supplemented with 100 µl l^{-1} Charkor (CH).

In order to prevent nutrient depletion and the development of pathogens the nutrient solutions were renewed weekly.

Growth parameters

The survival rate (%) and growth analyses were made on the 45th day after transplantation of plants to ex vitro conditions. The fresh weight (FW) of leaves, stems and roots as well as the leaf area were determined immediately after removing the plants from the soil. The dry weight (DW) of the corresponding botanical organs was measured after drying the material at 80° C (±5°C) for 48 h (Beadle, 1993). The leaves were scanned and the leaf area was calculated using the software program Wisegeek.

Physiological and biochemical parameters

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence analysis was performed on the youngest native fully developed leaves of 5 representative plants of the respective variant. The basic parameters of the rapid chlorophyll *a* fluorescence were made with a HandyPEA portable fluorimeter (Hansatech

Instruments, UK). The measured spots of the leaves were dark adapted for 40 minutes with special clips. Induction curves of the rapid chlorophyll a fluorescence (JIP test) were recorded for 1 s with 3000 μmol m^{-2} s^{-1} PPFD. The primary data processing was done using the PEA Plus Software (V1.10, Hansatech Instruments Ltd., UK). The parameters measured and calculated using this OJIP test (Table 1.) were interpreted and normalised according to Strasser & Strasser (1995) and Goltsev (2016).

Table 1. Definitions of measured and calculated chlorophyll a fluorescence parameters used in the experiment (Based on Strasser and Strasser, (1995) and Goltsev et al., (2016)

Chlorophyll Fluorescence Parameter	Description
Measured parameters and basic JIP-test parameters derived from the OJIP transient	
$F_O \sim F20\mu s$	Minimum fluorescence, when all PSII reaction centres (RCs) are open; Fluorescence intensity at 20 μs
F_J	Fluorescence at the J-step (2 ms) of the O-J-I-P transient
F_I	Fluorescence at the I-step (30 ms) of the O-J-I-P transient
$F_M = F_P$	Maximum fluorescence at the P-step when all RCs are closed
$V_J = (F_J - F_O)/(F_M - F_O)$	Relative variable fluorescence at the J-step
$F_V = F_M - F_O$	Variable fluorescence
Quantum yields and probabilities	
$\psi_{EO} = 1 - V_J$	Probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond QA-
$\varphi_{EO} = (1 - F_J/F_M)$	Quantum yield (at t = 0) for electron transport from QA- to plastoquinone
$\delta R_O = (1 - V_I)/(1 - V_J)$	Efficiency/ probability (at t = 0) with which an electron from the intersystem carriers moves to reduce end electron acceptors at the PSI acceptor side
Performance indexes	
PI_{ABS}	Performance index of PSII based on absorption
$PI_{total} = PI_{ABS} \times \delta R_o/(1 - \delta R_o)$	Performance index of electron flux to the final PSI electron acceptors, i.e., of both PSII and PSI

Leaf chlorophyll content

Non-destructive measurement of leaf chlorophyll content was made using a portable chlorophyll meter (CL-01, Hansatech, UK). This device determines the relative chlorophyll content using dual-wavelength optical absorbance (620nm and 940nm wavelength) measurements from leaf and allows to estimate the Chl content in relative units - the Chl index (CI). Measurements were made on the youngest fully developed leaf without detaching from the plants. The same leaves, on which the chlorophyll fluorescence was measured, were used.

Determination of antioxidant activity

Antioxidant activity was determined according to the method of Yen & Chen (1995) and DPPH percent inhibition was calculated according to Rossi et al. (2003). Briefly, fresh leaves (0.1 g) were extracted with 50 ml of methanol (HPLC grade) in the ultrasonic bath for 15 minutes. The

extract was centrifuged at 10 000 RPM for 5 minutes at 10°C. One ml of the 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. The absorbance was measured at 517 nm with spectrophotometer.

DPPH percent inhibition was calculated using the following formula:

$$\%DPPH = 100 - [(AT / AR) \times 100],$$

where: AT – absorbance of tested sample (test solution); AR – absorbance of blank sample (reference solution).

Statistics of analysis

Data was analyzed with SPSS program (13 for Window), using the analysis of variance and least significant difference means separation of Duncan test to compare for each treatment. All analyses were done at the 95% confidence level. All analyses were done in triplicate.

Results and Discussion

In the beginning of all treatments the pear plants grew vigorously, but the difference between the plants grown with the biostimulators Regoplant and Charkor were visible two weeks after transplanting to ex vitro conditions. A relatively low survival rate (35%) was reported in the floating system without biostimulators (Table 2). In the other three treatments, significantly higher values were found in the survival rate - between 72.5 and 82.5%. Enrichment of the nutrient solution with biostimulators Regoplant and Charkor led to a higher survival rate of pear plants (75% and 82.5%, respectively).

The enrichment of the nutrient solution with biostimulators led to an increase in the number, fresh and dry biomass of the leaves, as well as in stem length (Table 1, Fig. 1). The highest values of these indicators were reported in the treatment with Charkor, although the differences were statistically proven only with the plants acclimatized in a floating system without added biostimulators. There was a tendency for a larger leaf area of plants acclimatized in a floating system compared to the control, but there were no statistically proven differences.

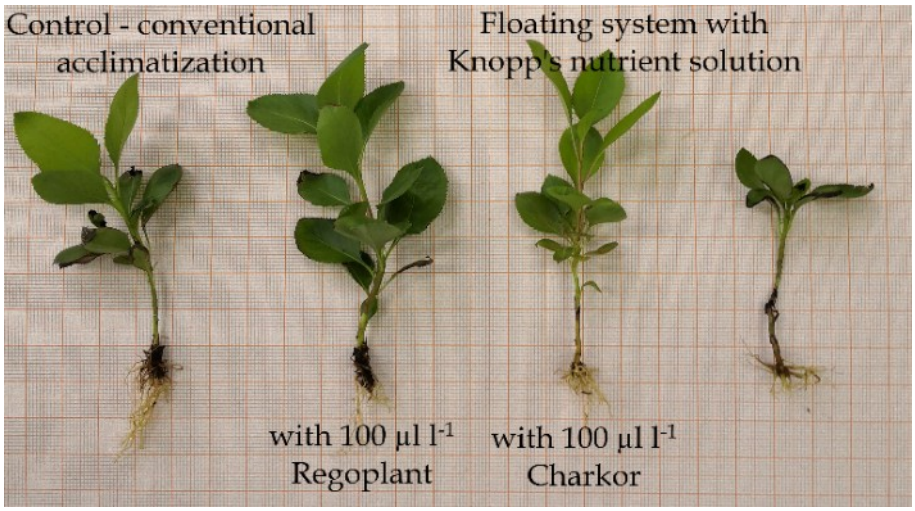

Fig. 1. The pear plants (*Pyrus communis* L. 'OHF 333') on the 45th day after transplanting for acclimatization to ex vitro conditions in a floating system. Control – conventional acclimatization in peat-perlite 1:1 (v:v), without nutrient solution.

Table 2. Growth parameters, survival rate (%) and antioxidant activity (% DPPH) of pear plants 45 days after acclimatization on floating system with biostimulators Regoplant or Charkor. Control – conventional acclimatization in peat-perlite, without nutrient solution. For each column, different letters indicate significant differences at $p \leq 0.05$.

Plant Growth Parameters	Control	Knopp	Knopp + Regoplant	Knopp + Charkor
Survival rate (%)	72.5	35	75	82.5
Number of leaves	13.4 ± 2.97 ^{ab}	9.4 ± 2.40 ^{b}	14.8 ± 1.30 ^{a}	15.8 ± 1.79 ^{a}
Leaf area (dm^2)	1.62 ± 0.54 ^{a}	1.68 ± 0.36 ^{a}	1.72 ± 0.34 ^{a}	1.87 ± 0.36 ^{a}
Stem stem length (mm)	34.08±5.51^{b}	33.61±3.14^{b}	42.55±1.95 ^{ab}	44.2±3.07 ^{a}
FW leaves (g $plant^{-1}$)	0.059 ± 0.015 ^{ab}	0.035 ± 0.014 ^{b}	0.072 ± 0.025 ^{a}	0.082 ± 0.026 ^{a}
FW roots (g $plant^{-1}$)	0.022 ± 0.010 ^{a}	0.011 ± 0.001 ^{a}	0.012 ± 0.005 ^{a}	0.013 ± 0.011 ^{a}
FW stem (g $plant^{-1}$)	0.021 ± 0.005 ^{a}	0.017 ± 0.007 ^{a}	0.027 ± 0.010 ^{a}	0.026 ± 0.007 ^{a}
DW leaves (g $plant^{-1}$)	0.053 ± 0.014 ^{ab}	0.031 ± 0.014 ^{b}	0.058 ± 0.021 ^{ab}	0.069 ± 0.023 ^{a}
DW roots (g $plant^{-1}$)	0.016 ± 0.009 ^{a}	0.008 ± 0.001 ^{a}	0.008 ± 0.005 ^{a}	0.008 ± 0.009 ^{a}
DW stem (g $plant^{-1}$)	0.016 ± 0.006 ^{a}	0.012 ± 0.005 ^{a}	0.019 ± 0.010 ^{a}	0.019 ± 0.008 ^{a}
Chlorophyll (CI)	3.78 ^{b}	3.28 ^{b}	5.89 ^{a}	6.61 ^{a}
DPPH (%)	55.58 ^{c}	58.88 ^{c}	78.92 ^{b}	92.24 ^{a}

Antioxidant activity, determined as DPPH percent inhibition, in plants treated with biostimulators was higher than the control plants and these from floating system with Knopp's solutions without any supplements. Significant differences were reported in this indicator between plants treated with different biostimulants - 78.92% in Regoplant and 92.24% in Charkor, which could correspond to a higher resistance of plants to transplant stress. Along with better plant growth, higher values of DPPH- radical scavenging activity could be an indicator of the protective role of the two biostimulators. DPPH- radical scavenging activity is a measure of non-enzymatic antioxidant activity (Kang and Saltveit, 2002a) and higher levels of DPPH- radical scavenging activity have been correlated with enhanced stress tolerance in rice and cucumber seedlings (Kang & Saltveit, 2002a; b).

Higher chlorophyll content was measured in the leaves of plants treated with the biostimulators used, with a maximum value reported for Charkor treatment (Table 2). This could be a prerequisite for better functioning of the photosynthetic apparatus in these plants.

Chlorophyll *a* fluorescence is another nondestructive method to evaluate the functional activity of the photosynthetic apparatus of plants. 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 four variants studied, the rapid chlorophyll fluorescence curves had a typical OJIP shape from Fo to F_M level with clearly separated J and I phases (Fig. 2), indicating that the pear plants, included in the experiment, were photosynthetically active (Yusuf et al., 2010). No significant differences in the minimum (Fo), maximum (F_M) and variable (Fv) fluorescence in plants were found from the studied variants (Table 3).

The quantum yield (Yield = Fv/F_M), reflecting the potential of photochemical activity of PS II, ranges from 0.794-0.809 and corresponds to normal (0.750- 0.830) in healthy, unstressed leaves (Bolharnordenkampfh & Oquist, 1993). This indicates that in all four variants studied, the photosynthetic apparatus was functioning normally.

No significant differences were observed between the treatments in the other three important indicators of the OJIP test - φ_{EO}, ψ_{EO}, PI_{ABS} and PI_{total}. The parameter ψ_{EO} reflects the probability of electron transport outside QA. The performance index (PI_{ABS}) shows the functional activity of the PSII 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 rate under stress and is considered to be a very sensitive indicator of the JIP test.

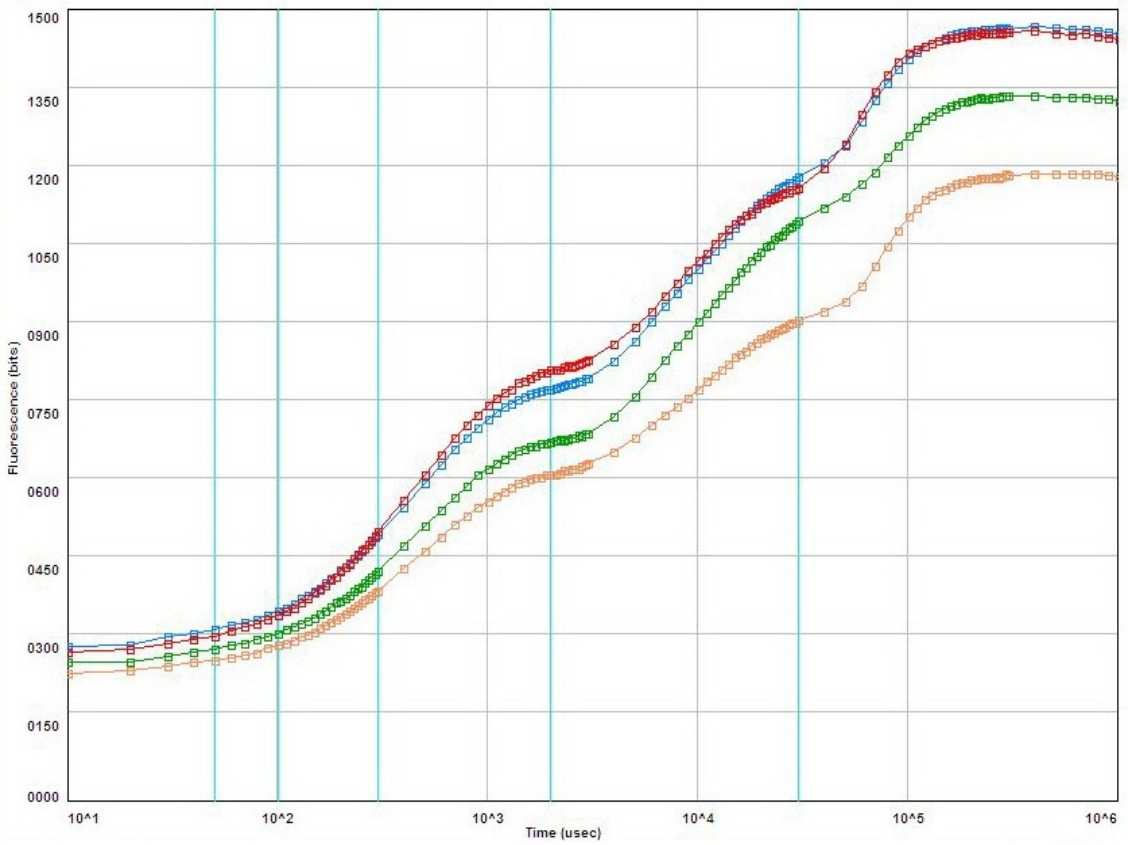

Fig. 2. Induction curves of rapid chlorophyll fluorescence (OJIP test) of pear plants acclimatized in a floating system - (--) Control, conventional acclimatization in peat-perlit; (--) Knopp's nutrient solution; (--) Knopp's nutrient solution, supplemented with 100 µl l^{-1} Charkor; (--) Knopp's nutrient solution, supplemented with 100 µl l^{-1} Regoplant.

Table 3. Chlorophyll fluorescence parameters (OJIP test) of the pear plants 45 days after acclimatization on floating system with biostimulators Regoplant or Charkor. Control – conventional acclimatization in peat-perlite, without nutrient solution. For each column, different letters indicate significant differences at $p \leq 0.05$.

Basic parameters	Control	Knopp	Knopp + Regoplant	Knopp + Charkor
F_O	291 ± 9 ^{a}	243 ± 65 ^{a}	260 ± 5 ^{a}	276 ± 5 ^{a}
F_M	1465 ± 51 ^{a}	1190 ± 356 ^{a}	1338 ± 129 ^{a}	1460 ± 135 ^{a}
Fv	1173 ± 49 ^{a}	947 ± 291 ^{a}	1078 ± 124 ^{a}	1183 ± 139 ^{a}
Fv/F_M	0.801 ± 0.007 ^{a}	0.794 ± 0.010 ^{a}	0.805 ± 0.016 ^{a}	0.809 ± 0.020 ^{a}
φ_{EO}	0.465 ± 0.006 ^{a}	0.479 ± 0.008 ^{a}	0.491 ± 0.029 ^{a}	0.437 ± 0.045 ^{a}
ψ_{EO}	0.581 ± 0.013 ^{a}	0.604 ± 0.017 ^{a}	0.611 ± 0.042 ^{a}	0.539 ± 0.042 ^{a}
δR_O	0.411 ± 0.0373 ^{ab}	0.483 ± 0.031 ^{a}	0.362 ± 0.007 ^{b}	0.469 ± 0.046 ^{ab}
PI_{ABS}	3.052 ± 0.243 ^{a}	3.364 ± 0.215 ^{a}	3.774 ± 0.857 ^{a}	2.894 ± 1.160 ^{a}
PI_{total}	2.164 ± 0.468 ^{a}	3.149 ± 0.413 ^{a}	2.149 ± 0.526 ^{a}	2.480 ± 0.554 ^{a}

At a first glance, the established higher values of this indicator contradict the weaker growth of plants acclimatized to *ex vitro* conditions in a floating system with Knopp's solution alone. The plants from the control variants in conventional acclimatization and those from the floating system enriched with biostimulators continue to grow actively. Therefore, the first fully developed leaves from the top (on which the fluorescence was measured) are physiologically younger and less mature than the top leaves in the treatment of the floating system without additives. This could explain that despite the greater increase in height and number of leaves, the PI_{total} values of plants grown with Knopp's solution alone were higher than those of the other treatments.

The results of this study confirmed the beneficial effect of the floating system in the acclimatization of micropropagated OHF 333 rootstock, but in which the Regoplant biostimulator (100 μl l^{-1}) had a better effect.

Rooting and acclimatization of pear plants are difficult (Chevreau et al., 1992). Resumption of plant growth at acclimatization, which is often difficult, can be improved by foliar sprays of gibberellic acid (100–200 ppm).

The low survival rate of plants when they are removed from *in vitro* culture is associated with poor stomatal functioning and excessive water loss (Brainerd and Fuchigami, 1982). During *ex vitro* acclimatization, many changes can occur to the morphological and physiological state as well as to the photosynthesis due to differences in the environmental conditions (Shin et al., 2014). 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 climate.

Biostimulants have been a popular subject of interest in sustainable agriculture because their application activates several physiological processes that enhance nutrient use efficiency, stimulating plant development and allowing the reduction of fertilizer consumption (Kunicki et al. 2010). Many biostimulants are also able to counteract the effects of biotic and abiotic stresses, enhancing quality and crop yield by stimulating plant physiological processes (Ziosi et al. 2013). But, the effect of the biostimulants is not always consistent among the plant species.

Combining innovative approaches as a floating system and biostimulators could help plants overcome the stress of transfer provides an alternative for acclimatizing *in vitro* propagated plants in a clean, convenient and water-saving way.

Conclusions

The results of this study demonstrate that the enrichment of the nutrient solution with biostimulator Charkor (100 μl l^{-1}) in a floating system could improve the acclimatization of *in vitro* micropropagated pear plantlets to *ex vitro* conditions. The beneficial effect of Charkor, expressed in better survival rate and better growth could support the acclimatization of other woody species in floating conditions.

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