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RESEARCH ARTICLE

Biofertlizer Lumbrical improves the growth and *ex vitro* acclimatization of micropropagated pear plants

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Abstract

In vitro micropropagation of plants is highly useful for obtaining large quantities of planting material with valuable economic qualities. However, plantlets grow *in vitro* in a specific environment and the adaptation after the transfer to *ex vitro* conditions is difficult. Therefore, the acclimatization is a key step, which mostly determines the success of micropropagation. The aim of this investigation was to study the effect of the biofertlizer Lumbrical on *ex vitro* acclimatization of micropropagated pear rootstock OHF 333 (*Pyrus communis* L.). Micropropagated and rooted plantlets were potted in peat and perlite (2:1) mixture with or without Lumbrical. They were grown in a growth chamber at a temperature of 22±2 °C and photoperiod of 16/8 hours supplied by cool-white fluorescent lamps (150 µmol m^{-2} s^{-1} Photosynthetic Photon Flux Density, PPFD). The plants were covered with transparent foil to maintain the high humidity, and ten days later, the humidity was gradually decreased. Biometric parameters, anatomic-morphological analyses, net photosynthetic rate and chlorophyll *a* fluorescence (JIP test) were measured 21 days after transplanting the plants to *ex vitro* conditions. The obtained results showed that the plants, acclimatized *ex vitro* in the substrate with Lumbrical, presented better growth (stem length, number of leaves, leaf area and fresh mass) and photosynthetic characteristics as compared to the control plants. This biostimulator could also be used to improve acclimatization in other woody species.

Keywords

Anatomic-morphological analysis, biofertlizer, growth parameters, JIP test, rootstocks, vermicompost

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Introduction

In vitro micropropagation of plants is highly useful for obtaining large quantities of planting material with valuable economic qualities. However, woody plants are often recalcitrant to *in vitro* cultivation and this process is highly genotype dependent. Pear rootstocks and varieties are often difficult to cultivate *in vitro*, although some micropropagation protocols have been published (Chevreau et al., 1992; Bell, Reed, 2002; Nacheva et al., 2009; Reed et al., 2013). 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. During acclimatization, the adaptation of the plant to new environmental conditions is essential (Apóstolo et al., 2005; Ďurkovič et al., 2009). According to Brainerd and Fuchigami (1982), the low survival rate of plants when they are removed from *in vitro* culture is associated with poor stomatal functioning and excessive water loss. During *ex vitro* acclimatization, many changes can occur to the morphological and physiological state and photosynthesis due to differences in the environmental conditions. However, studies on this aspect of acclimatization are still limited (Shina et al., 2014). Natural light shading, antitranspirant treatment for reducing plant transpiration and application of different plant growth-promoting substances are often used to increase plant survival rate after transplanting. The benefits associated with inoculation of *in vitro* – raised plantlets with selected N_2-fixing bacteria and/ or arbuscular mycorrhizae fungi (AM) have been reported in several horticultural, fruit, ornamental and forest species (Rai, 2001; Kapoor et al., 2008; Singh et al., 2012).

Vermicompost is an organic fertilizer that is a result of a bio-oxidative process of organic waste, which is done by earthworms and microorganisms, with significant effects in the improvement of the soil fertility, in the crop yield and in the contribution to the agro-ecological sustainability (Broz et al., 2016; Xu, Mou, 2016). Vermicompost is a rich source of nutrients and plant growth regulators that could increase plant production (Singh et al., 2008; Lim et al., 2015).

The vermicompost Lumbrical is a product of the activity of cultivating worms of *Lumbricus rubellus.* This red California worm excreta is extremely rich in humus, containing all the substances necessary for plants. A significant number of works have shown that Lumbrical improves the growth and yields of many crops in particular vegetables: lettuce (Steffen et al., 2010), tomatoes (Gutiérrez – Miceli et al., 2007; Masheva et al., 2009), potatoes (Mrinal-Saikia et al., 1998), watermelon (Pelizza et al., 2013), as well as ornamentals, such as petunia, gladiolus (da Cruz et al., 2018), etc. It was shown that vermicompost is a suitable planting substrate for hardening of *in vitro* regenerated plants of *Tylophora indica* (Rani, Rana, 2010; Kaur et al., 2011) and banana (Fernández et al., 2016) to their field transfer.

The aim of this research was to study the effect of biofertilizer Lumbrical on *ex vitro* acclimatization of micropropagated pear rootstock OHF 333 (*Pyrus communis* L.).

Materials and methods

Plant material and experimental conditions

The experiment was carried out on micropropagated plantlets of pear rootstock OHF 333 (*Pyrus communis* L. 'Old Home' x 'Farmingdale'). Well-rooted plantlets were potted in plastic form pads (528 x 308 x 60 mm), filled with peat-perlite (1:1, v/v) – control or with the same substrate enriched with the organic fertilizer Lumbrical (1:16, v:v). At the beginning of the experiment, the plants were fully covered with transparent polyethylene to prevent drying and ten days later, the humidity was gradually reduced. The plants were kept in a growth chamber at 22±2°C under 16/8 h light/ dark photoperiod (150 μmol m^{-2} s^{-1} Photosynthetic Photon Flux Density, PPFD). After 20 days in *ex vitro* conditions, plant growth analysis, physiological and biochemical analysis were performed.

Chemical analysis on the peat-perlite substrate and biofertilizer

Before the experiment, chemical analysis was performed both on the peat-perlite substrate and on the biofertilizer.

The analyses were performed according to established methods, described by Tomov et al. (1999). The total nitrogen was determined titrometrically, after burning in sulphuric acid and subsequent distillation on a Parnas-Wagner apparatus. The total content of phosphorus was determined colourimetrically using the method of Egner-Riehm. The total amount of potassium was determined in a hydrochloric acid extract (2 N HCl) using the modified method of Milcheva (Tomov et al., 1999) and the measurement was made using a flame photometer.

On the 20th day after the transfer to *ex vitro* conditions, the content of the mineral elements (N, P, K) in the leaves of the plants was recorded.

Growth parameters

The fresh weight (FW) and leaf area were determined immediately after removing the plants from the soil. The dry weight (DW) of the plants was measured after drying the material at 80° C for 48 h (Beadle, 1993).

Physiological and biochemical parameters

Gas-exchange analysis

The gas-exchange analysis was performed on the youngest fully- developed leaves of five randomly selected plants of the control and plants treated with Lumbrical. Measurements were taken with LCpro + portable photosynthesis system (ADC, UK) at a light intensity of 180 μmol m^{-2} s^{-1} 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}) and stomatal conductivity (g_s, mol H_2O $m^{-2}s^{-1}$) were determined.

Photosynthetic pigments

The photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) were extracted in 85% acetone. The extracts absorbance was determined spectrophotometrically. The content of pigments (mg g^{-1} FW) was calculated according to the formulae of Lichtenthaler, Wellburn (1983).

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence analysis was performed using a Handy PEA fluorimeter (Handy Plant Efficiency Analyzer, Hansatech Instruments Ltd., UK) on the youngest native fully-developed leaves of five representative plants of the respective variant. The measured spots of the leaves were kept in darkness in a special clip for 40 minutes just before measurement. Induction curves of 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 from this test (Table 1) were interpreted and normalised according to Strasser, Strasser (1995) and Goltsev (2016).

Anatomical and morphological analysis of the stomata

The anatomical and morphological analysis of the stomata was performed with a scanning electron microscope (SEM – FEI Quanta 200) at the Dendrology Laboratory in Przelewice, Poland. Samples for analysing the anatomical structure were taken from the middle part of fully developed 1st to 4th leaves from the top to the base of the shoots. The measurements were performed on fresh plant material with out pre-treatment. The plant material was placed on an aluminium holder in a SEM chamber for measuring at magnification (1000x). For each sample, ten measurements of the stomata were made.

Statistical analysis

For each experimental treatment, three replications, each containing 40 plants, were tested. The experiment was performed twice. For growth parameters, ten representative plants were studied. For gas-exchange and chlorophyll *a* fluorescence analysis, at least five measurements on different plants were performed.

Data of different parameters were analysed statistically using one-way ANOVA in SPSS statistical software (version 13 for Windows) at a significance level between the means and the evaluated of $P \leq 0.05$ (Tukey test).

Results and discussion

The substrate used for the experiment had low to medium nitrogen content, medium phosphorus content and a high content of potassium (Tomov et al., 1999; Table 2). Unlike the control substrate, Lumbrical biofertilizer contains a sufficient amount of mineral compounds necessary for plant growth and development.

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

Chlorophyll Fluorescence Parameter	Description
Measured parameters and basic JIP-test parameters derived from the OJIP transient	
F_O ~ F20μs	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 recorded 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
$PI_{total} = PI_{ABS} \times \delta Ro/(1 - \delta Ro)$	Performance index of electron flux to the final PSI electron acceptors, i.e., of both PSII and PSI

We recorded that the pear plants acclimatized *ex vitro* with the Lumbrical biofertilizer were better development than the control plants (Table 3, Fig. 1). The plants acclimatized to *ex vitro* condition on the substrate with biofertilizer were distinguished by their higher stem length, the number of leaves, leaf area, fresh and dry biomass.

The elements nitrogen, phosphorus and potassium in the plants, grown on substrate enriched with the Lumbrical biofertilizer, were higher than in the control plants (Table 4). The values were close to the optimal ones, which in pears according to Hanson (1993) are 1.8-2.5 % (on a dry mass basis) for nitrogen, 0.12-0.3 % for phosphorus, and 1.0 – 2.0 % for potassium. Other authors (Sainz et al., 1998; Tejada et al., 2007) have found similar results with vermicomposting.

The results of the gas-exchange analysis fully confirmed the data from the anatomical and morphological analysis (Table 5). The net photosynthetic rate of plants grown in a substrate with a biofertilizer was about 28% higher compared to control plants. No significant differences were found in the transpiration and stomatal conductance of the two studied groups of plants.

Although there was a tendency for a higher content of photosynthetic pigments in the plants grown with Lumbrical, the difference with the control plants was not statistically significant (Table 6).

Chlorophyll *a* fluorescence is another indicator of the functional activity of the photosynthetic apparatus of plants and along with the intensity of the photosynthesis. The

Table 2. Chemical characteristics of the peat-perlite substrate and the Lumbrical biofertilizer

Variants	NH_4-N [ppm]	NO_3-N ppm]	P_2O_5 [ppm]	K_20 [ppm]
Substrate	5.60	22.40	99.0	430.0
Lumbrical	14.0	156.8	325.0	498.0

Table 3. Growth parameters of pear plant after 20 days of *ex vitro* acclimatization

Variants	Length of the stem (mm)	Number of leaves	Leaf area (cm²)	Fresh weight (g)	Dry weight (g)
Control	35.99 b	11.00 a	46.75 b	0.34 b	0.25 b
Lumbrical	39.13 a	12.10 a	51.42 a	0.52 a	0.26 a
P=0.05*	0.397	0.668	0.637	0.051	0.034

*Tukey test
**Control – substrate without a biofertilizer; Lumbrical – substrate enriched Lumbrical

Figure 1. The pear plants (*Pyrus communis* L. OHF 333) on the 20th day after transplanting for acclimatization to *ex vitro* conditions

analysis of the induction curves of rapid chlorophyll fluorescence (OJIP test) links the structure and functionality of the photosynthetic apparatus. It allows for rapid assessment of plant viability, especially in stress conditions (Strasser et al., 2000, 2004). In the two studied variants, the rapid chlorophyll fluorescence curves had a typical OJIP shape from F_0 to F_M level with clearly separated J and I phases (Fig. 2), indicating that the pear plants, acclimatized to *ex vitro* conditions, were photosynthetically active (Yusuf et al., 2010). In both studied groups of plants, the maximal (F_M) fluorescence of two studied variants was not significantly different (Table 7).

Table 4. Content of the elements nitrogen (N), phosphorus (P) and potassium (K) in the leaves of pear plants on the 20th day after the transfer to the *ex vitro* conditions

Variants	N (%)	P_2O_5 (%)	K_2O (%)
Control	2.66	0.63	2.4
Lumbrical	3.9	0.77	2.94

Table 5. Leaf gas exchange parameters in pear plants on the 20th day after the transfer to the *ex vitro* conditions. Abbreviations: P_N – net photosynthetic rate (μmol m^{-2} s^{-1}); E- transpiration intensity (mmol m^{-2} s^{-1}); gs – stomatal conductance (mmol m^{-2} s^{-1})

Variants	P_N [μmol $m^{-2}s^{-1}$]	E [mmol $m^{-2}s^{-1}$];	g_s [mol $m^{-2}s^{-1}$]
Control	7.86 b	1.68 a	0.08 a
Lumbrical	10.07 a	1.67 a	0.10 a

* Tukey test at 5%
**Control – substrate without a biofertilizer; Lumbrical – substrate enriched Lumbrical

Table 6. The content of photosynthetic pigments (mg g^{-1} FW) in the leaves of pear plants treated with Lumbrical 20 days after the *ex vitro* acclimatization

Variants	Chlorophyll *a* mg/g FW	Chloro-phyll *b* mg/g FW	Total chloro-phyll mg/g FW	Total carot-enoids mg/g FW	Chlorophyll *a*/chloro-phyll *b*	Total chloro-phyll/ total carotenoids
Control	2.36 a	0.63 a	3.22 a	1.05 a	2.78 b	3.05 b
Lumbrical	2.90 a	1.04 a	4.28 a	1.15 a	3.77 a	3.71 a

Although the quantum yield (Yield = F_v / F_M) of plants acclimatized with Lumbrical, that reflected the potential photochemical activity of photosystem II (PS II), was significantly higher than that of control plants (0.814 and 0.772, respectively), these values corresponded to normal values (0.750-0.830) for healthy, unstressed leaves (Bolhar-Nordenkampf, Oquist, 1993). This indicated that a normally- developed photosynthetic apparatus was functioning. However, a more in-depth analysis of the JIP test parameters revealed some characteristic features of the potential of the photosynthetic apparatus in plants, acclimatized with Lumbrical and in the control plants. The Fv / Fo ratio (Table 7.) was lower in control plants (3.38) than in those acclimatized with the Lumbrical biostimulator (4.386). According to Strasser et al. (2010), the Fv / Fo ratio reflects the efficiency of excitation energy use in PS II. Moreover, the parameter ψ_{EO} reflects the probability of electron transport outside QA. Plants acclimatized *ex vitro* with soil enriched with Lumbrical were characterised by higher ψ_{EO} as compared to the control plants. The performance index represents an absorbance basis and is used to quantify the PS II behaviour and shows the functional activity of the PS II relative to the absorbed energy (Kalaji et al., 2014a). Two times higher PI_{ABS} was found in plants, grown with Lumbrical (1.913) in comparison to the control plants (0.851), which unequivocally showed that in these plants a better structured photosynthetic apparatus functioned.

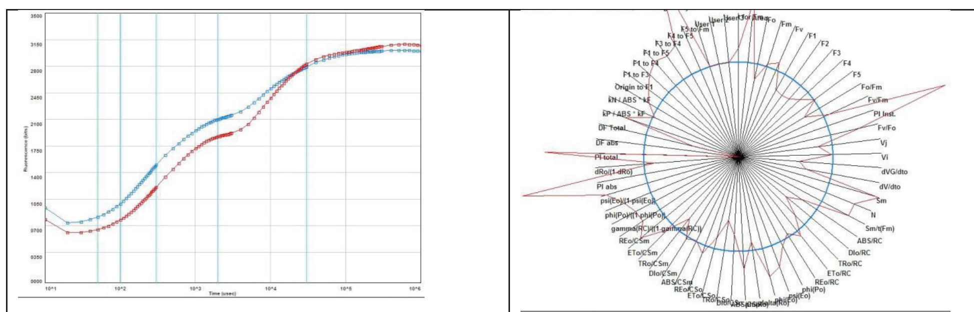

Figure 2. Induction curves (left) and basic parameters (right) of rapid chlorophyll fluorescence (OJIP test) of pear plants acclimatized to *ex vitro* conditions in the peat-perlite mixture without the Lumbrical biofertlizer (Control, blue line) or with Lumbrical (red line)

Table 7. Basic parameters of the rapid chlorophyll fluorescence (OJIP test) of pear rootstock OHF 333 plants, acclimatized to *ex vitro* conditions with the Lumbrical biofertlizer reported on the 21st day after the transfer to the *ex vitro* conditions

Basic parameters	Control	Lumbrical	
F_O	689	575	*
F_M	3018	3099	n.s.
Fv	2329	2522	n.s.
F_O/F_M	0.2283	0.1857	n.s.
Fv/F_M	0.772	0.814	*
Fv/F_O	3.38	4.386	n.s.
φ_{EO}	0.2949	0.3849	*
ψ_{EO}	0.3821	0.4726	*
δR_O	0.2337	0.2122	n.s.
PI_{ABS}	0.851	1.913	*
PI_{total}	0.2488	0.5247	*

* Tukey test at 5%

The total performance index (PI_{total}) reflects the functional activity of the PS II, PS I and the electron transport chain between them (Strasser et al., 2000). PI_{total} is closely related to the overall growth and survival rate of plants under stress conditions and has been described as a very sensitive parameter for the JIP test (Strasser et al., 2004). The decrease in the values of PI_{ABS} and PI_{total} observed in the control plants (C) compared to the plants cultivated with Lumbrical corresponded to the lower FW, DW, stem length, leaf area and P_N of the control plants and could be indicative of the negative effects of culture conditions on PSII and PSI activity (Yusuf et al., 2010). The acclimatization of pear plants in soil enriched with the biofertilizer Lumbrical contributed to the more active development and structuring of the photosynthetic apparatus, which is a prerequisite for more intensive photoassimilation and biomass accumulation (Fig. 1 and Tables

3, 5). These results also indicated that chlorophyll fluorescence parameters could be a reliable non-destructive method for early diagnosis of disorders in PS II functionality and growth. Martins et al. (2015) studied these parameters of JIP test to improve growth and acclimatization of micropropagated *Neoregelia concentrica* under different day light regimes.

The anatomical and morphological analysis revealed a significantly higher density of the stomata (per mm^2) in plants grown on the enriched substrate: 184.9 per mm^2, which is higher than the control (106.0 per mm^2) by 74.4 % (Table 8 and Fig. 3). At the same time, the stomata length of the control plants was greater (25.9 µm) compared to that of the plants treated with the Lumbrical biofertilizer (20.9 µm). The greater number but smaller stomata in the plants grown in the substrate with Lumbrical may be a prerequisite for a more intensive gas exchange.

There is a specific course of water vapour diffusion through very small openings, such as the stomata. According to Stefan's law (Kerin et al., 2011), the amount of water vapour that diffuses through small holes within a definite period of time is proportional to the perimeter instead of the total pore area. Alternatively, with a greater number of smaller pores (which is observed in plants grown on soils with Lumbrical) the diffusion will be more intense, as the relative share of the total length of the boundary peripheries increases and therefore the evaporation is more intense.

During the acclimatization to *ex vitro* conditions, plants are forced to switch to autotrophic carbon assimilation. For this reason, the adaptation of the plant to new environmental conditions is essential. Because of that, the acclimatization is a key stage in micropropagation (Apóstolo et al., 2005; Ďurkovič et al., 2009). The transfer of *in vitro* grown plantlets to *ex vitro* conditions is often accompanied by water stress and/ or photoinhibition (Semorádová et al., 2002; Carvalho et al., 2001, 2006). Therefore, the application of approaches to stimulate photosynthesis are extremely important for the success of acclimatization.

Vermicompost is a very useful growth medium for most crops because of the high content of many available nutrients and plant growth promoters (Arancon et al., 2004). According to Ricci et al. (1995), vermicompost provides P, Ca, Mg and S similarly to inorganic fertilizers. Additionally, several researchers have documented the presence of plant growth regulators such as auxins, gibberellins, cytokinins of microbial origin (Muscolo et al., 1999) and humic acids (Atiyeh et al., 2002; Arancon et al., 2004) in vermicompost in considerable quantities. Positive effects of vermicompost have been reported for many crops (Arancon et al., 2004; Gutierrez-Miceli et al., 2007; Berova et al., 2013) and also have been observed in forest species, such as pine trees (Lazcano et al., 2010). Possibly due to better physical properties, higher microbial and enzymatic activity and higher content of available nutrients, the vermicompost could be used as a natural fertilizer having a number of advantages over chemical fertilizers (Venugopal et al., 2010). Our results showed that Lumbrical improved the growth and photosynthetic ability of micropropagated pear plants during *ex vitro* acclimatization and they are similar to the findings obtained by other authors, e.g. to results reported by Vasane et al. (2010), concerning the increased survival of banana plants after their transfer to *ex vitro* in medium containing soil: PMC:vermicompost in a ratio of 1:1:1 (v/v/v). In

Table 8. Anatomic-morphological analysis of pear plants on day 20 after transfer to *ex vitro* conditions. The first fully- developed leave was examined (4th leaf from top to bottom)

Variants	Number of stomata [mm^2]	Length of stomata [µm]
Control	106.00 b	25.90 a
Lumbrical	184.90 a	20.90 b
P=*0.05**	2.436	1.113

*Tukey test
**Control – substrate without a biofertilizer; Lumbrical – substrate enriched Lumbrical

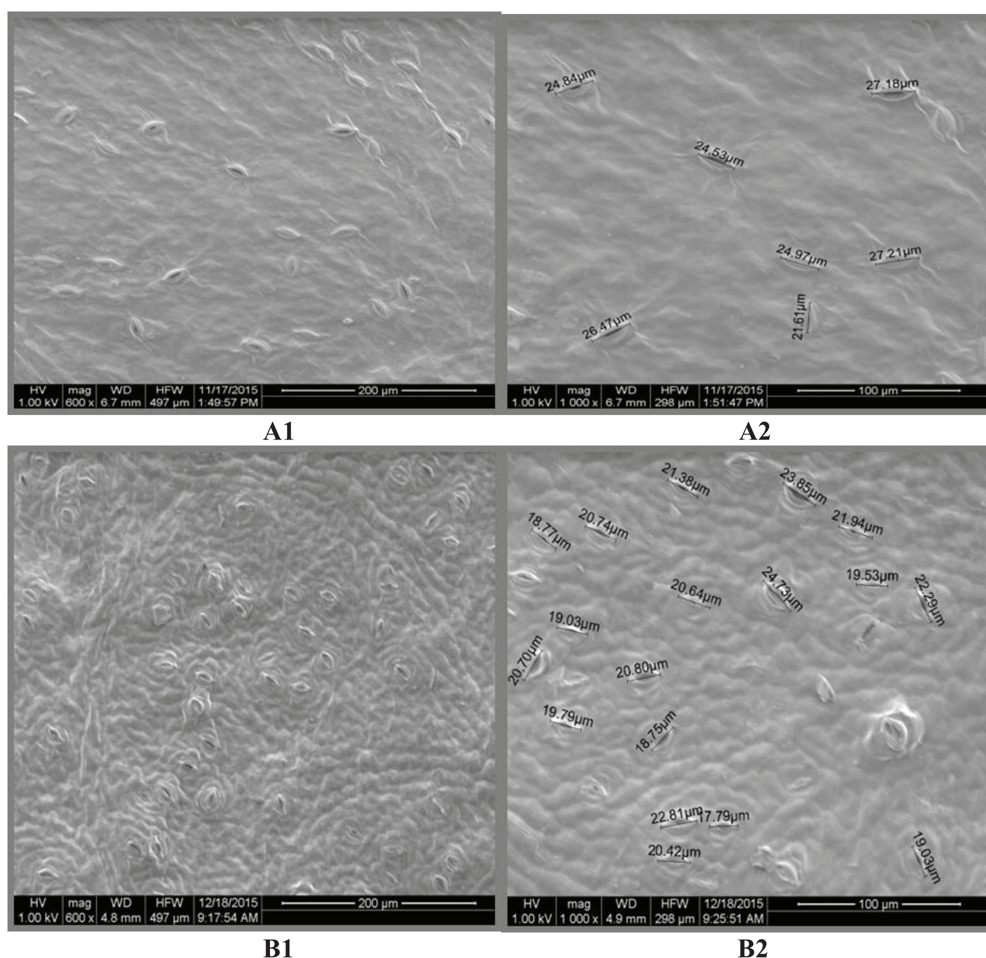

Figure 3. The lower epidermis of pear plants on day 20 after transfer to *ex vitro* conditions;
A1 – Control – Number of stomata [mm^2]; **A2** – Control – Length of stomata [µm];
B1 – Lumbrical – Number of stomata [mm^2]; **B2** – Lumbrical – Length of stomata [µm]

addition, the results presented in this study are in accordance with the positive effect of vermicompost humic acids (40 mg L^{-1}) on banana plants in the acclimatization stage established by Fernandez et al. (2016).

Conclusions

In conclusion, environmental conditions during transfer to *ex vitro* conditions influenced the growth and acclimatization of *in vitro* propagated pear plants. The biofertilizer Lumbrical improved the vegetative growth, photosynthetic ability and *ex vitro* acclimatization of micropropagated pear plants. These results showed that the Lumbrical biofertilizer could be useful in acclimatizing other woody species and large-scale commercial production of high- quality plants.

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