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EFFECTS OF *META*-TOPOLIN RIBOSIDE AND *META*-METHOXY TOPOLIN RIBOSIDE ON THE *IN VITRO* MICROPROPAGATION OF *Pyrus communis* L.

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ABSTRACT

The present study aimed to evaluate the effects of new *meta*-topolin derivatives *meta*-topolin riboside (*m*TR) and *meta*-methoxy topolin riboside (*mem*TR) on the multiplication and subsequent rooting and *ex vitro* acclimatization of *Pyrus communis* L. ('OHF 333'). The cytokinins *m*TR and *mem*TR were included in the nutrient medium (0 µM, 3 µM, 6 µM, 9 µM, 12 µM). In plants from three passages of three-week-old cultures grown on different nutrient media, the following parameters were evaluated: multiplication coefficient, fresh (FW) and dry (DW) weight (mg), average length of shoots (mm), average number of leaves, leaf length and width (mm). At the rooting stage, data on the rooting frequency, number of roots per rooted micro-cutting and the length of roots were recorded 18 days after the start of the experiment. In the acclimatized plants, leaf area, FW and DW, and the content of photosynthetic pigments were determined 40 days after the transfer to *ex vitro* conditions. Gas exchange rate and chlorophyll fluorescence were also evaluated for the control and the variants with 6 and 9 µM *m*TR and *mem*TR. The plantlets grown on cytokinin-supplemented media showed a higher number of leaves than the control. Plantlets grown on nutrient media with 6 and 12 µM *m*TR were distinguished by the highest FW and DW. In these variants, the shoots were of the greatest length. The plants grown on medium with 6 µM *m*TR had the highest number of leaves. Control plants had larger leaves. The highest rooting percentage (70%) was achieved in plantlets grown with 9 µM *m*TR. A higher *ex vitro* acclimatization survival rate (76–100%) was found in all plants cultured with *m*TR or *mem*TR compared to control plants (65%).

Keywords: cytokinins, plant growth regulators, shoot tips, tissue culture

Abbreviations: BA – N6-benzyladenine, *mem*TR – meta-methoxy topolin riboside, mT – meta-topolin, *m*TR – *meta* -topolin riboside

INTRODUCTION

In vitro micropropagation techniques for woody species are essential to fulfil the ever-increasing need for quality plants as well as for the environmental sustainability of the production. It is an alternative method to traditional vegetative propagation that reproduces identical pathogen-free plants. Unfortunately,

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the significant advantages of *in vitro* methods could only be partially realized in the reproduction of woody species, mainly due to the high cost of production.

Various propagation protocols for micropropagation of *Pyrus* sp. have been described [Yeo and Reed 1995, Nacheva et al. 2009, Reed et al. 2013b, Dimitrova et al. 2016, Nacheva et al. 2022, Kaviani et al. 2023].

According to many authors, pears are rather unruly regarding micropropagation due to the weak proliferation of the shoot, hyperhydricity, phenolic oxidation and irregular rooting, which are considered crucial bottlenecks [Yeo and Reed 1995, Bell et al. 2012, Reed et al. 2013b, Aygun and Dumanoglu 2015, Lotfi et al. 2019]. To overcome these challenges, cytokinins BAP or kinetin are added to semisolid agar medium with full- or half-strength Murashige and Skoog [1962] (MS) salts and vitamins [Cheng 1979, Nacheva et al. 2009, Rehman 2014, Lizárraga et al. 2017].

A few factors, such as the composition of the medium and growth conditions, may cause great variations in the obtained plants [Bairu et al. 2011]. The most widely used cytokinin in micropropagation is benzylaminopurine (BAP), but it sometimes causes some physiological disorders (e.g. hyperhydricity) or inhibits rooting. To overcome some disadvantages of BAP, a new family of endogenous aromatic cytokinins, hydroxylated analogs of BAP, i.e. *meta*-topolin (*m*T) derivatives, have been proposed in recent years as an alternative to BAP in plant tissue cultures [Werbrouck et al. 1996, Strnad et al. 1997, Bairu et al. 2007]. According to these authors, shoot multiplication, rooting, and acclimatization could be improved by substituting BAP with *m*T (a naturally occurring BAP analog) and some of its derivatives. *Meta*-topolin (N6-(3-hydroxybenzyl) aminopurine), *meta*-methoxytopolin riboside (*mem*TR) and *meta*-topolin riboside (*m*TR) are aromatic cytokinins that were first isolated from poplar leaves [Strnad et al. 1997]. Depending on the plant genotype, they can overcome difficulties such as bad rooting [Baroja-Fernandez et al. 2002, Nacheva and Gandev 2023], low regeneration [Salvi et al. 2002], chimera decomposition [Bogaert et al. 2006] and hyperhydricity [Bairu et al. 2007]. *Mem*TR and *m*TR were shown to be good alternative options for BA in the *in vitro* cultivation of the pear [Lotfi et al. 2019].

There are limited publications on the influence of *m*TR and *mem*TR on pear. Lotfi et al. [2020] reported a great increase in the proliferation rate of two Tunisian pear cultivars, 'Arbi' and 'Mahdia 6', in a liquid nutrient medium in a temporary immersion bioreactor SETIS™.

The present study aimed to evaluate the effects of *meta*-topolin riboside (*m*TR) and *meta*-methoxy topolin riboside (*mem*TR) on the multiplication and subsequent rooting and *ex vitro* acclimatization of *Pyrus communis* L. ('OHF 333').

MATERIAL AND METHODS

Research was carried out in the Laboratory of Plant Biotechnology at the Fruit Growing Institute in Plovdiv. The study was conducted with pear rootstock (*Pyrus communis* L. OHF 333), a member of the 'Old Home' × 'Farmingdale' series created in the USA. This rootstock is known for its good compatibility with a wide range of cultivars, high productivity, and moderate resistance to fire blight [Lombard and Westwood 1987, Wertheim 2002].

In vitro culture was maintained at 3-week subculture intervals as described previously [Nacheva et al. 2009, Dimitrova et al. 2016]. The shoots were grown *in vitro* on a modified MS [Murashige and Skoog 1962] solid medium containing 50% of NH_4NO_3 and $CaCl_2$ as well as 1000 mg L^{-1} $Ca(NO_3)_2$, 3 µM *m*T, 0.005 µM indol-3-butyric acid (IBA), 30 g L^{-1} sucrose and 6.5 g L^{-1} phytoagar (Duchefa, The Netherlands). The media (pH 5.6) were autoclaved at 121 °C for 20 min.

Multiplication stage

A study was conducted to assess the effect of *m*TR and *mem*TR at different concentrations (3 µM, 6 µM, 9 µM, 12 µM and 0.01 µM IBA) in the culture medium. A treatment without cytokinins was used as a control. Plantlets were grown in glass jars (180 mL) with transparent Magenta B-Cap lids. Five shoot tips with a length of 15 mm and two leaves were set on 25 mL of culture medium in each jar. In plants from three passages of three-week-old cultures grown on different nutrient media, the following parameters were noted: fresh (FW) and dry (DW) weight (mg), average length of shoots (mm), average number of leaves, leaf length and width (mm) and multiplication index (MI). The MI was calculated as the number of proliferated shoots from one explant.

Rooting stage

Apical shoots measuring 10–15 mm in length, with two to three leaves, from each treatment in the multiplication phase were transferred to a rooting medium containing 20 g L^{-1} sucrose, 6.5 g L^{-1} phytoagar supplemented with half-strength MS macronutrients, full-strength micronutrients and vitamins, 1.5 µM IBA, with pH adjusted to 5.6. Plantlets were grown in glass jars (180 mL). After four weeks, the following biometric parameters were registered: percentage of rooted cuttings, mean number of roots per cutting, and mean root length. The cultures were incubated in the growth room at an air temperature of 22–24 °C with a 16/8-hour photoperiod produced by cool-white fluorescent lamps (40 µmol m^{-2} s^{-1} PPFD white cool-white fluorescent lamps OSRAM 40W).

Ex vitro acclimatization stage

In vitro rooted plants were removed from the agar, and their roots were washed in distilled water to remove excess medium. They were then potted in peat:perlite mixture (1:1, v/v), sprayed with a 0.1% Switch (cyprodinil + fludioxonil) fungicide and covered with mini greenhouses with a ventilation system (Romberg GmbH & Co, Germany, 38 × 24 cm) for two weeks. The humidity was then gradually lowered to 65%. The plants were grown in a growth room at a temperature of 22–24 °C with a 16/8-hour photoperiod produced by cool-white fluorescent lamps (80 µmol m^{-2} s^{-1} PPFD white cool-white fluorescent lamps OSRAM 40W).

Finally, six weeks after potting, survival rate (%), leaf area, FW and DW, and the content of photosynthetic pigments were determined. Gas exchange rate and chlorophyll fluorescence were also measured for the control and the variants with 6 and 9 µM *m*TR and *mem*TR.

Physiological and biochemical parameters

Growth parameters. The fresh weight was measured immediately after removing the plants from the culture vessels or from the substrate. The dry weight (DW) of the plants was determined by the gravimetric method [AOAC 1990] after drying to constant weight (at 105°C). At the multiplication stage, the length and width of the first fully developed leaf were measured. The leaf area of the acclimatized plants was measured by GIMP-GNU image manipulation software (www.gimp.org).

Photosynthetic pigment content. Photosynthetic pigments – chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids (Car), were extracted from the leaves in 80% (v/v) acetone, measured spectrophotometrically (Spectrophotometer UV-1600PC, VWR) and calculated (milligrams per gram of fresh leaf weight) according to the formulae of Lichtenthaler [1987]. Chl *a*/*b* and Chl/Car ratios were also determined.

Physiological parameters, including leaf gas exchange and chlorophyll fluorescence, were determined on the acclimatized plants, control plants and the four best variants from the multiplication stage (6 and 9 µM *m*TR and *mem*TR).

Leaf gas exchange analysis. The leaf gas exchange analyses were performed on the first fully developed leaf with an open photosynthetic system LCpro+ (Analytical Development Company Ltd., Hoddesdon, England). Net photosynthetic rate (A), transpiration rate (E) and stomatal conductance (g_s) were measured.

Chlorophyll fluorescence analysis. Chlorophyll fluorescence parameters were determined by Handy PEA (Handy Plant Efficiency Analyzer, Hansatech Instruments Ltd., King's Lynn, UK) on the youngest fully developed native leaf. For each treatment, the measurements were carried out on five plants. The induction curves of rapid chlorophyll *a* fluorescence (OJIP test) were recorded for 1s with 3000 µmol m^{-2} s^{-1} PPFD on the spots of the leaves previously dark-adapted in a special clip for 40 minutes. The PEA Plus Software (V1.10, Hansatech Instruments Ltd., UK) was used for data processing. The parameters of chlorophyll fluorescence (Table 1) were interpreted and normalized according to Strasser and Strasser [1995] and Goltsev [2016].

Data recording and statistical analysis. In each multiplication experiment, six jars containing five explants per jar were used for each cytokinin and concentration. The experiment was conducted in two independent replicates. Rooting trials involved 30 shoots per multiplication treatment (six jars with five explants per jar). Statistical analysis was carried out using one-way ANOVA at a 5% significance level, employing IBM SPSS Statistics version 19.

Table 1. Definitions of measured and calculated chlorophyll *a* fluorescence parameters according to 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	
$F_0 \sim F_{20\mu s}$	minimum fluorescence, when all PSII reaction centers (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
$V_J = (F_J - F_0)/(F_M - F_0)$	relative variable fluorescence at the J-step
$F_V = F_M - F_0$	variable fluorescence
quantum yields and probabilities	
$\psi_{E0} = 1 - V_J$	probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond QA^-
$\varphi_{E0} = (1 - F_J/F_M)$	quantum yield (at t = 0) for electron transport from QA^- to plastoquinone
$\delta R_0 = (1 - V_I)/(1 - V_J)$	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
$PI_{total} = PI_{ABS} \times \delta R_0/(1 - \delta R_0)$	performance index of electron flux to the final PSI electron acceptors, i.e., of both PSII and PSI

RESULTS AND DISCUSSION

Multiplication stage

All plantlets cultivated on nutrient media with the tested cytokinins had greater biomass and number of leaves than the control plantlets (Table 2).

The highest multiplication index was reported in plants cultured with 9 and 12 μM *m*TR (2.4 and 2.3, respectively). Plantlets grown on nutrient media with 6 and 12 μM *m*TR were distinguished by the highest FW and DW. In these variants, the shoots were of the greatest length. The plantlets grown on media supplemented with cytokinin showed more leaves than the control (Table 2). The plants grown on medium with 6 μM *m*TR had the highest number of leaves (16.33). Control plants had larger leaves. At high concentrations of *m*TR and *mem*TR (9 and 12 μM), leaf width was the lowest. The study by Grira et al. [2023] showed that *m*TR is effective in the micropropagation of *Handroanthus guayacan* (Seem.), and the authors recommended extending the study to other tropical woody plants. The treatments of *Merwilla plumbea* (Lindl.) with *m*TR gave the highest number of adventitious shoots when compared to thidiazuron (TDZ) and the control [Amoo et al. 2015]. According to Bairu et al. [2009], in treatments of *Harpagophytum procumbens* (Burch.) DC. ex Meisn. grown on media supplemented with *m*TR, lower percentages of necrotic shoot-tips were noted compared to treatments with BA or *m*T. The application of the aromatic cytokinin *m*TR to the culture medium significantly improved the survival of potato cultures [Baroja-Fernandez et al. 2002]. According to Amoo et al. [2015], *m*TR resulted in the highest number of adventitious shoots of micropropagated *M. plumbea* compared to thidiazuron (TDZ) and the control. Abdouli et al. [2022] reported that *m*TR was a superior substitute for BAP in pistachio (*Pistacia vera* L.) micropropagation. For most studied parameters, there was no significant difference between *m*T and *m*TR. However, for prolifera-

Table 2. Growth characteristics of pear plantlets at the multiplication stage, affected by different concentrations of *mem*TR and *m*TR

Concentrations (μM)	Fresh weight (mg)	Dry weight (mg)	MI	Length of shoots (mm)	Number of leaves	Leaf length (mm)	Leaf width (mm)
0 (control)	360^{b}	66^{b}	1.0^{c}	13.45^{b}	9.07^{a}	11.20^{a}	8.05^{a}
*mem*TR 3	500^{b}	85^{a}	1.1^{c}	15.55^{b}	11.80^{b}	9.73^{b}	6.84^{a}
*mem*TR 6	498^{b}	66^{ab}	1.3^{c}	27.75^{a}	14.40^{ab}	8.62^{bc}	4.90^{b}
*mem*TR 9	750^{a}	84^{a}	2.1^{a}	13.90^{b}	11.73^{b}	4.51^{c}	2.87^{c}
*mem*TR 12	480^{b}	55^{b}	1.8^{b}	25.90^{a}	14.87^{a}	7.35^{c}	4.12^{bc}
*m*TR 3	700^{b}	110^{a}	1.0^{c}	21.95^{b}	14.00^{ab}	10.29^{ab}	5.82^{bc}
*m*TR 6	1018^{a}	130^{a}	1.9^{ab}	33.71^{a}	16.33^{a}	10.51^{ab}	6.62^{b}
*m*TR 9	780^{b}	100^{a}	2.4^{a}	12.70^{c}	15.27^{a}	4.144^{c}	2.17^{c}
*m*TR 12	940^{a}	110^{a}	2.3^{a}	31.79^{a}	12.47^{b}	8.91^{b}	4.98^{c}

Means in the column, followed by different letters are significantly different at $P \leq 0.05$. MI – multiplication index

tion rate, the most important parameter, *m*T was found to be more effective than *m*TR and it yielded a 6-fold higher value than BAP. However, the lowest number of hyperhydric usable pistachio shoots (58.9%) and callus weight (46.9%) were found in shoots treated with *m*TR. In addition, the higher anthocyanin accumulation in BA-treated shoots suggests more stress. In *H. procumbens*, a positive effect on reducing disorders was mentioned when *m*TR was used in a culture compared to BAP and *m*T [Bairu et al. 2009].

One possible explanation for the positive effects of the topolins (*m*T and *m*TR) is their less toxic effect and easily degradable metabolites as opposed to BAP. BAP has long been the most often used cytokinin in micropropagation systems due to its efficacy and low price [Holub et al. 1998]. However, its adverse impact on the growth, rooting, and acclimatization of certain recalcitrant species, as well as the induction of various physiological disorders [Aremu et al. 2012, Ahmad and Strnad 2021], have driven efforts to explore alternative solutions. It was found that the main metabolite of BAP, [9G] BA, is more stable but had a negative impact on the rooting and acclimatization of cultures of *Spathiphyllum floribundum* (Linden & André) N.E.Br. compared to the main metabolite of *m*T, an O-glucoside, which easily degraded during acclimatization [Werbrouck et al. 1996].

Rooting stage

Adventitious root formation is an important step in plant tissue culture during the micropropagation process, and according to Chevreau et al. [1992], pear rooting and acclimatization are difficult. All studied explants cultured on different proliferation media with memTR and mTR cytokinins, as well as control explants formed roots. The obtained data confirmed that the nutrient media with IBA induced the formation of roots. The most effective rooting (over 70%) was observed in plantlets cultivated with 9 μM *m*TR at the multiplication stage (Fig. 2, Table 3). A lower rooting percentage, between 5 and 20%, was reported for all plants cultivated on nutrient media with *mem*TR. The plants cultivated in the multiplication stage on a medium with 3 μM *m*TR produced the highest number of roots (5.0) (Table 3).

The longest roots were recorded in the control variant. Because excessively long roots are easily broken when planting the plants in a soil substrate, more numerous but shorter roots are preferred in micropropagation.

Some scientists reported successful rooting in European pears where the MS medium supplemented with auxins was utilized to root *Pyrus* [Chevreau and Bell 2005, Reed 2013a]. The application of IBA has been effective in stimulating the rhizogenesis of the

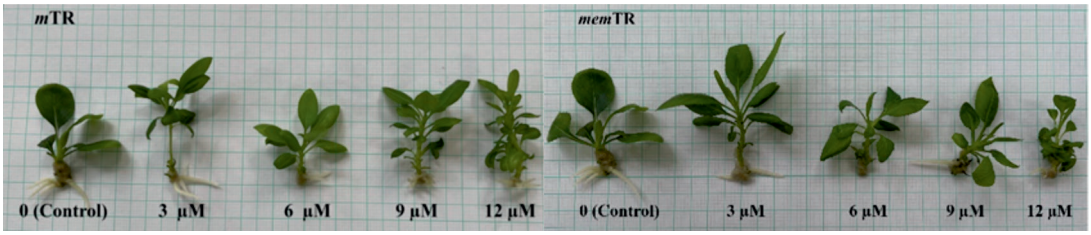


Fig. 1. Rooted pear plantlets grown at the multiplication stage on nutrient media, supplemented with different concentrations (0, 3, 6, 9 and 12 µM) of *meta*-topolin riboside (*m*TR) or *meta*-methoxy topolin riboside (*mem*TR)

Fig. 2. *Ex vitro* acclimatized pear plants cultivated at the multiplication stage on nutrient media with *m*TR or *mem*TR, respectively

Table 3. Rooting percentage, number of roots and root length (mm) of pear plantlets grown with different concentrations (0, 3, 6, 9 and 12 µM) of *meta*-methoxy topolin riboside (*mem*TR) or *meta*-topolin riboside (*m*TR) at multiplication stage

Concentrations (µM)	Rooting (%)	Root number	Root length (mm)
0 (control)	65	4.33[a]	8.54[a]
*mem*TR 3	21	1.50[a]	7.20[a]
*mem*TR 6	28	1.88[a]	6.50[a]
*mem*TR 9	20	1.50[a]	6.33[a]
*mem*TR 12	5	1.00[a]	7.33[a]
*m*TR 3	40	5.00[a]	6.51[ab]
*m*TR 6	57	3.17[a]	3.69[c]
*m*TR 9	70	4.00[a]	3.51[c]
*m*TR 12	38	2.67[a]	5.51[bc]

Means in the column, followed by different letters are significantly different at $P \leq 0.05$

obtained shoots of the *Pyrodwarf rootstock* [Ružic et al. 2011] and some other pear genotypes [Kaviani 2015].

As noted before, pears are quite recalcitrant to micropropagation [Yeo and Reed 1995, Thakur et al. 2008, Reed et al. 2013b, Aygun and Dumanoglu 2015], and the rooting is one of the problem stages. Besides being dependent on the genotype, the current results showed that the pretreatment of the plants before rooting also had a significant effect. The rooting percentage varied widely on the same medium, depending on the type and cytokinin concentration in the multiplication stage.

The current research supports the hypothesis that the topolins applied at the multiplication stage will have a long-lasting positive effect on the rooting and acclimatization of woody plants to *ex vitro* conditions due to the rapid metabolism of *m*T (and their derivates) and its transport into plant tissues [Bairu et al. 2008, Amoo et al. 2011].

In line with the results of the present study, Magyar-Tábori et al. [2001] reported that pretreatment of the plantlets is very important. Applying *m*T in the multiplication medium increased the rooting of 'Red Fuji' apple shootlets compared to BA or BAR [Magyar-Tábori et al. 2001].

Ex vitro acclimatization stage

A higher survival rate was reported for all variants tested compared to the control (Table 4). The stem length and number of plant leaves from different treatments were not significantly different. A difference in the leaf area was recorded, and it was the largest in the control plants and the smallest in the variant with 12 µM *mem*TR.

The plants grown with 6 µM *mem*TR and 9 µM *m*TR had the highest fresh weight, but differences with the control were statistically insignificant (Fig. 3). Moreover, the control plants were characterized by the greatest dry weight.

The highest values of chlorophyll *a* and *b*, total chlorophyll and carotenoids were reported in the plantlets grown in the multiplication stage with the highest concentrations of *m*TR or *mem*TR (12 µM). No difference was noted in the chlorophyll *a*/*b* ratio between variants.

Chlorophyll *a* fluorescence serves as an additional indicator of the functional activity of the photosynthetic apparatus in plants, complementing measurements of photosynthesis intensity and pigment content. The light energy absorbed by plants can follow various pathways: it may be captured by photosynthetic

Table 4. Growth parameters of acclimatized pear plantlets cultivated at the multiplication stage on nutrient media with *mem*TR or *m*TR

Concentrations (µM)	Survival rate (%)	Stem length (mm)	Number of leaves	Leaf area (cm^2)
0 (Control)	65	30.98 ±3.60^{a}	11.35 ±1.70^{a}	22.70 ±2.35^{a}
*mem*TR 3	84.7	25.85 ±3.90^{a}	9.70 ±2.20^{a}	16.60 ±1.50^{ab}
*mem*TR 6	94.1	23.10 ±1.80^{a}	11.70 ±1.35^{a}	21.00 ±2.50^{a}
*mem*TR 9	71.4	25.00 ±2.30^{a}	12.25 ±1.32^{a}	20.30 ±1.63^{a}
*mem*TR 12	100	23.95 ±1.84^{b}	10.70 ±0.33^{a}	12.12 ±1.24^{b}
*m*TR 3	76.4	30.80 ±1.20^{a}	12.33 ±1.35^{a}	18.12 ±3.20^{abc}
*m*TR 6	80	26.00 ±2.35^{a}	10.33 ±0.90^{a}	13.44 ±0.82^{bc}
*m*TR 9	93.8	30.60 ±4.50^{a}	10.70 ±1.20^{a}	20.25 ±1.40^{ab}
*m*TR 12	68.8	18.95 ±4.60^{a}	11.00 ±1.50^{a}	11.19 ±2.19^{c}

Means in the column, followed by different letters are significantly different at $P \leq 0.05$

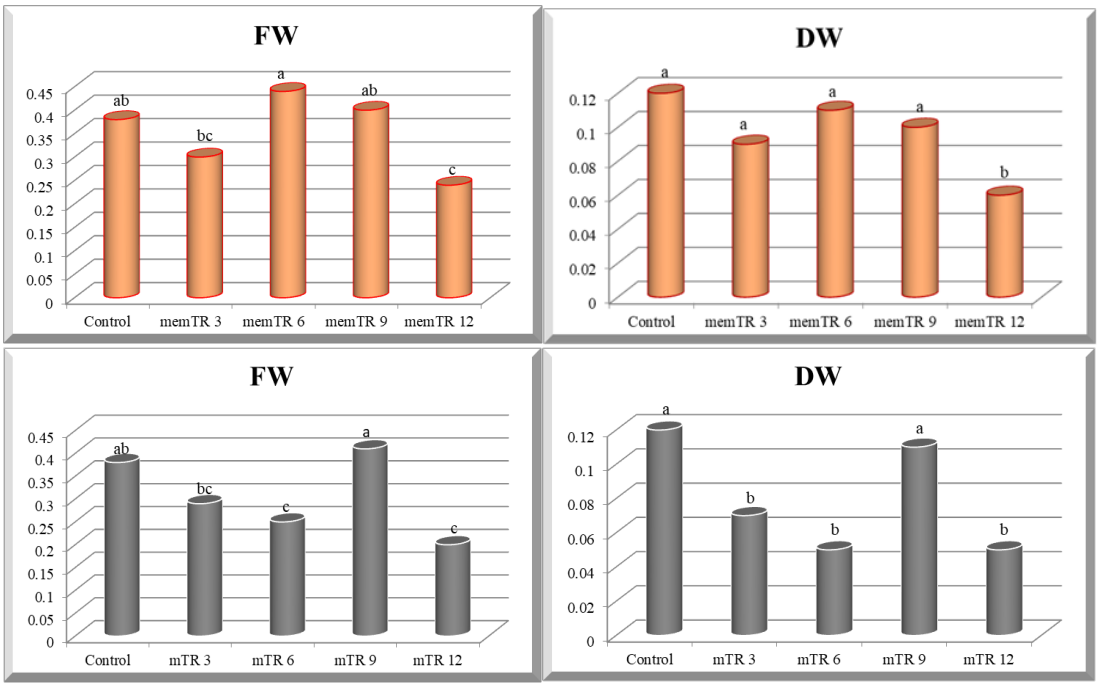


Fig. 3. Fresh weight and dry weight of *ex vitro* acclimatized pear plants cultivated *in vitro* at the multiplication stage on nutrient media with *mem*TR or *m*TR

Table 5. Photosynthetic pigment content (mg g^{-1}_{FW}) of *ex vitro* acclimatized pear plants, cultivated *in vitro* at the multiplication stage on nutrient media with *mem*TR or *m*TR

Concentrations (µM)	Chl (*a*)	Chl (*b*)	Chl (C*a* + C*b*)	Car	Chl (*a*/*b*)	Chl (*a* + *b*)/Car
0 (Control)	2.80 ±0.22^{c*}	0.83 ±0.07^{b}	3.60 ±0.29^{c}	0.98 ±0.09^{b}	3.35 ±0.00^{a}	3.67 ±0.06^{a}
*mem*TR 3	2.80 ±0.12^{c}	0.83 ±0.02^{b}	3.60 ±0.13^{c}	0.98 ±0.03^{b}	3.32 ±0.08^{a}	3.67 ±0.02^{a}
*mem*TR 6	3.30 ±0.11^{ab}	0.99 ±0.02^{a}	4.30 ±0.15^{ab}	1.13 ±0.05^{ab}	3.30 ±0.02^{a}	3.80 ±0.02^{a}
*mem*TR 9	3.20 ±0.003^{bc}	0.94 ±0.02^{ab}	4.10 ±0.02^{bc}	1.11 ±0.01^{ab}	3.35 ±0.08^{a}	3.70 ±0.02^{a}
*mem*TR 12	3.65 ±0.02^{a}	1.07 ±0.01^{a}	4.70 ±0.04^{a}	1.24 ±0.005^{a}	3.40 ±0.07^{a}	3.80 ±0.05^{a}
*m*TR 3	3.00 ±0.05^{b}	0.90 ±0.04^{b}	3.90 ±0.09^{b}	1.05 ±0.01^{b}	3.33 ±0.09^{a}	3.70 ±0.03^{b}
*m*TR 6	2.60 ±0.06^{c}	0.80 ±0.03^{c}	3.40 ±0.10^{c}	0.91 ±0.01^{c}	3.33 ±0.08^{a}	3.70 ±0.03^{b}
*m*TR 9	3.08 ±0.002^{b}	0.95 ±0.01^{ab}	4.01 ±0.01^{b}	1.07 ±0.006^{b}	3.30 ±0.04^{a}	3.73 ±0.03^{b}
*m*TR 12	3.44 ±0.04^{a}	1.05 ±0.008^{a}	4.50 ±0.03^{a}	1.20 ±0.008^{a}	3.29 ±0.06^{a}	3.85 ±0.001^{a}

*Means in the column, followed by different letters are significantly different at $P \leq 0.05$. Chl – chlorophyll, Car – carotenoids

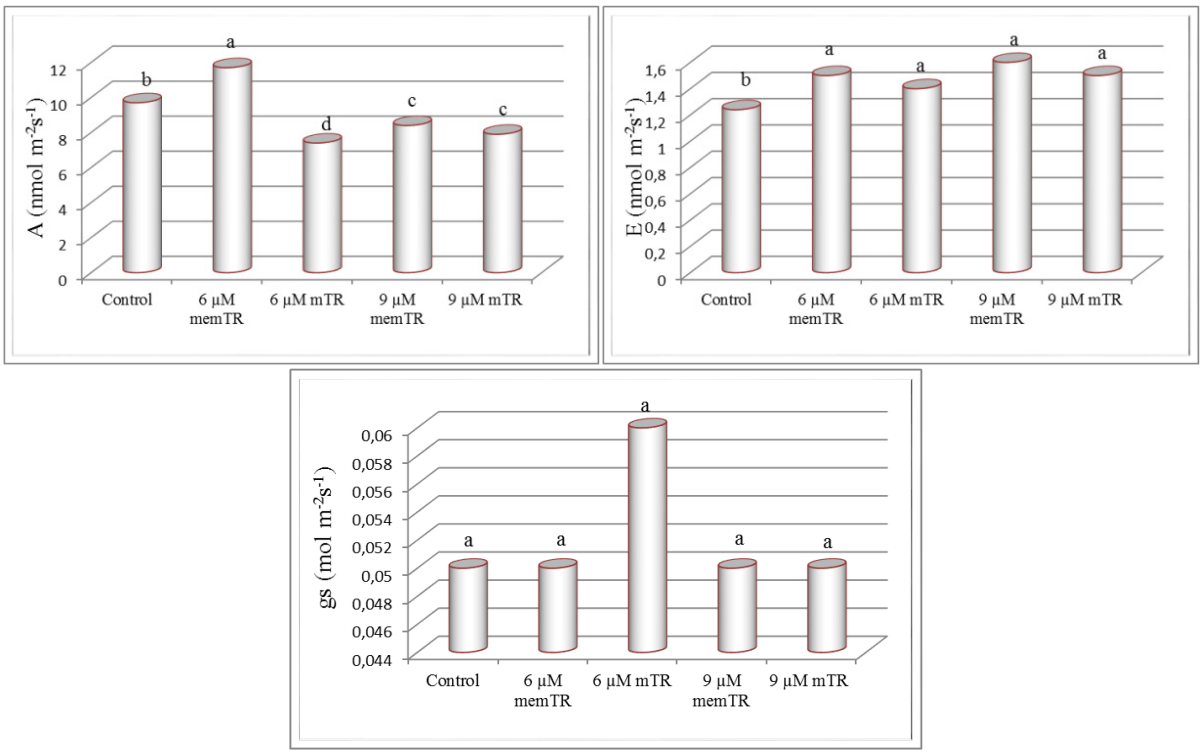
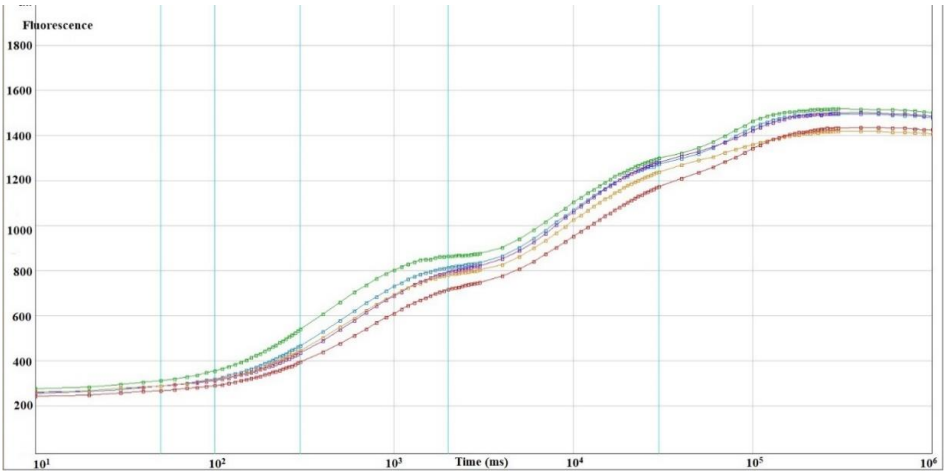


Fig. 4. Net photosynthetic rate (A, µmol CO_2 plantlet^{-1} s^{-1}), transpiration rate (E, mmol H_2O plantlet^{-1} s^{-1}), stomatal conductance (g_s, mol H_2O plantlet^{-1} s^{-1}) of *ex vitro* acclimatized pear plants, cultivated *in vitro* at the multiplication stage on nutrient media with *m*TR or *mem*TR

Table 6. Basic chlorophyll fluorescence parameters (JIP test) of the leaves of pear plants at the acclimatization stage. At the multiplication stage, they were grown on nutrient media with different concentrations of *mem*TR and *m*TR

Concentrations (µM)	Control	6 µM *mem*TR	6 µM *m*TR	9 µM *mem*TR	9 µM *m*TR
F_O	265 ±5^a	273 ±6^a	278 ±13^a	256 ±11^a	271 ±6^a
F_M	1502 ±30^a	1506 ±37^a	1524 ±65^a	1441 ±22^a	1426 ±21^a
Fv	1236 ±29^a	1233 ±31^a	1246 ±54^a	1185 ±15^a	1155 ±19^a
F_V/F_M	0.823 ±0.004^a	0.819 ±0.001^a	0.818 ±0.004^a	0.823 ±0.005^a	0.810 ±0.003^a
φ_{E0}	0.548 ±0.012^{ab}	0.571 ± 0.007^{ab}	0.522 ±0.029^b	0.603 ±0.035^a	0.550 ±0.015^{ab}
ψ_{E0}	0.451 ±0.011^{ab}	0.467 ±0.006^{ab}	0.427 ±0.026^b	0.497 ±0.031^a	0.445 ±0.013^{ab}
δR_0	0.327 ±0.005^{bc}	0.308 ±0.007^{bc}	0.336 ±0.006^{ab}	0.366 ±0.010^a	0.283 ±0.037^c
PI_{ABS}	3.77 ±0.44^b	4.53 ±0.35^b	2.80 ±0.69^b	6.64 ±0.54^a	3.55 ±0.27^b
PI_{total}	1.83 ±0.22^b	2.03 ±0.20^b	1.42 ±0.36^b	3.74 ±0.78^a	1.46 ±0.37^b

Means in the column, followed by different letters are significantly different at $P \leq 0.05$



Control, 6 µM *m*TR; 9 µM *m*TR; 6 µM *mem*TR; 9 µM *mem*TR

Fig. 5. Induction curves of rapid chlorophyll fluorescence (JIP test) of *ex vitro* pear plantlets cultivated *in vitro* at the multiplication stage on nutrient media with *m*TR or *mem*TR

pigments, dissipated as heat, or emitted as fluorescence [Stirbet et al. 2018]. The analysis of the rapid chlorophyll fluorescence induction curves (OJIP test) provides insights into the structure and functionality of the photosynthetic apparatus. It allows a rapid assessment of plant viability, mainly in stress conditions [Strasser et al. 2000]. The parameters of chlorophyll *a* fluorescence in leaves of plants could be affected by light [Stirbet 2011, Kalaji et al. 2012], plant nutritional status [Li et al. 2012, Živčak et al. 2014], and environmental stresses [Schansker et al. 2014, Kalaji et al. 2016, Kalaji et al. 2018, Stirbet et al. 2018]. The curves of chlorophyll *a* rapid fluorescence of the observed plants had a typical OJIP shape from F_0 to F_M level with clearly separated J and I phases (Fig. 5), indicating that *ex vitro* acclimatized pear plants had active photosynthesis [Yusuf et al. 2010].

The minimum (F_0), maximum (F_M) and variable (F_v) fluorescence of the control plants and those grown with *m*TR or *mem*TR at the multiplication stage did not differ significantly (Table 6). The quantum yield (Yield = F_V/F_M), which represents the potential photochemical activity of PS II, ranged from 0.810–0.823 and corresponded to the typical photochemical activity (0.750–0.830) in the leaves of unstressed healthy plants [Bolhar-Nordenkampf and Oquist 1993]. These results indicated that the applied cytokinins *m*TR and *mem*TR did not negatively affect the normal function of the photosynthetic apparatus.

The highest quantum yield (F_V/F_M) value of 0.823 was reported for the leaves of the control plants and plants cultivated *in vitro* with 9 µM *mem*TR, although there were no statistically significant differences between the other plants.

The ψ_{E0} parameter presents the probability of electron transport outside Q_A. The plants subjected to the 6 µM *m*TR treatment had the lowest ψ_{E0}, but the parameter values significantly differed from those in the 9 µM *mem*TR treatment. The values of the parameter φ_{E0} followed the same trend.

The performance index (PI_{ABC}) can assess the PSII state and functional activity in respect of the amount of absorbed energy [Kalaji et al. 2014]. The current study revealed the highest PI_{ABC} in plants cultivated *in vitro* with the application of 9 µM *mem*TR. The functional activity of the PSII and the PSI, as well as the efficiency of the electron transport chain between them, is presented by the total performance index (PI_{total}). The PI_{total} is strongly correlated with the overall growth rate and plant survival under stress, and it is recognized as a highly sensitive and reliable indicator in the OJIP test. In the current study, the highest PI_{total} value was recorded in the plants cultivated *in vitro* with 9 µM *mem*TR. This accurately corresponded

to the highest value of fresh biomass accumulation in these plants (Fig. 3) and the highest net photosynthetic rate (Fig. 4).

Research shows that the application of cytokinins during *in vitro* stages affects the acclimatization process, production of secondary metabolites and pharmacological activity of micropropagated plants [Magyar-Tabori et al. 2001, Szopa and Ekiert 2012, Baskaran et al. 2014, Amoo et al. 2015].

CONCLUSIONS

The plantlets cultivated on nutrient media with *m*TR or *mem*TR (3–12 µM) had greater biomass and number of leaves than the control plantlets without cytokinins. The highest MI was reported in plants cultured with 9 and 12 µM *m*TR (2.4 and 2.3, respectively). The most effective rooting (over 70%) was observed in plantlets cultivated with 9 µM *m*TR at the multiplication stage. A higher *ex vitro* acclimatization survival rate (76–100%) was found in all plants cultured with *m*TR or *mem*TR compared to control plants (65%). Based on the results obtained, it can be concluded that *m*TR and *mem*TR could be a suitable substitute for BA for the shoot multiplication of pears (*Pyrus communis* L. 'OHF 333').

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