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EFFECT OF *META*-TOPOLIN ON THE SHOOT MULTIPLICATION OF PEAR ROOTSTOCK OHF-333 (*Pyrus communis* L.)

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Abstract. This study assesses the effect of *meta*-topolin (mT), an aromatic natural cytokinin, on micropropagation of pear rootstock OHF-333 (*Pyrus communis* L.). Cultures were incubated in a growth chamber under controlled conditions. An *in vitro* culture was maintained on a modified culture medium Murashige and Skoog [1962] supplemented with *meta*-topolin (0 μM, 3 μM, 6 μM, 9 μM or 12 μM). After three weeks' growth the parameters and the physiological and biochemical analysis were investigated. The results of this study suggest that the *in vitro* culture in the absence of cytokinins does not provide a practical solution for efficient multiplication of pear rootstocks. A good multiplication rate and high quality shoots were found at 6–9 μM mT treatment. The use of *meta*-topolin resulted in improvement of the leaf gas exchange and low content of phenols, as well as in the total antioxidant activity. Hence the cytokinin *meta*-topolin in concentrations of 6–9 μM was selected as an optimum cytokinin level in the multiplication of pear rootstock OHF-333.

Key words: micropropagation, cytokinin, growth parameters, physiological changes

Abbreviations: BA – N^6-benzyladenine; CK – Cytokinin; DPPH – 2,2-diphenyl-1-picrylhy-drazyl; DW – dry weight; FW – fresh weight; *meta*-topolin, mT – 6-(3-hydroxybenzylami-no)purine; PGR – plant growth regulator

INTRODUCTION

Shoot regeneration and multiplication during micropropagation is affected by the type and concentration of the plant growth regulators (PGRs) applied, especially cyto-

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kinins (CKs) due to their importance for cell division and cell expansion in plant tissue cultures [Howell et al. 2003, Aremu et al. 2012]. The choice of CK to be used in a tissue culture is determined by its cumulative efficiency in inducing an acceptable rate of shoot multiplication, normal shoots and roots and the eventual ability of the plants to acclimatize easily. Many studies have reported suitable CK types and their concentrations for each species [Huetteman and Preece 1993]. Currently N^6-benzyladenine (BA) is the most widely used CK in the micropropagation industry due to its effectiveness and affordability [Bairu et al. 2007]. BA at a high concentration has disadvantages such as stunted growth, epigenetic and somaclonal variation in some crops [Harrar et al. 2003, Werbrouck, 2010, Smulders and De Klerk 2011]. BA is also reported to cause hyperhydricity in many species [Leshem et al. 1988, Teramoto et al. 1993, Magyar-Tabori et al. 2010]. Therefore it is imperative to try and find an alternative to BA while maintaining a reasonable multiplication rate and acceptable plant quality.

A few reports on the use of topolins indicate that this group of cytokinins could be a new source of CKs with high morphogenetic activity. Jones et al. [1996] reported the occurrence of aromatic CKs, BA, 6-(3-hydroxybenzylamino)purine (mT) and 6-(2-hydroxybenzylamino)purine (oT) in various tissues of oil palm (*Elaeis guineensis* Jacq.). Strnad et al. [1997] isolated 6-(3-hydroxybenzylamino)purine, a highly active aromatic cytokinin, from poplar leaves (*Populus* × *canadensis* Moench) and proposed the name '*meta*-topolin'. Recently monomethoxy derivatives of 6-benzyladenine and 6-benzyladenosine were also isolated and identified from several different plant sources and their high cytokinin activity has been confirmed [Tarkowska et al. 2003]. In study conducted by Podwyszyńska et al. [2012] the effects of *meta*-methoxytopolin (MemT) and its riboside (MemTR), on the micropropagation efficiency and shoot quality of smoke bush (*Cotinus coggygria* Scop.) 'Royal Purple' was studied. Their results imply that *meta*-methoxytopolons can be considered an alternative to other commonly used cytokinins in micropropagation of recalcitrant species. Using equimolar concentration (10.0 μM) Werbrouck et al. [1996] compared the types and effects of the derivatives of BA and mT in the tissue culture of *Spathiphyllum floribundum*. They found that the main metabolite of BA, 6-benzylamino-9-b-D-glucopyranosylpurine ([9G]BA), was more stable but had a negative impact on rooting and acclimatization when compared with the main metabolite of mT, the O-glucoside, which was degraded easily during acclimatization. They also compared the post *vitro* effect of different concentrations of BA and mT on rooting after an acclimatization period of four weeks. Their results revealed that plants treated with mT produced significantly higher number and greater length of roots than those treated with BA. Baroja-Fernandez et al. [2002] also reported that addition of the aromatic cytokinin 6-(3-hydroxybenzylamino)-9-b-D-ribofuranosylpurine (mTR) to the culture medium significantly improved survival in potato cultures. Bairu et al. [2007] have also noticed that mTR has a comparable effect with that of mT when applied at higher concentrations than the optimum level of mT (5 mM). In banana cv. 'Williams', the use of topolins at 7.5, 15 and 30 μM had higher shoot multiplication rates than BA [Bairu et al. 2008]. Kaminek et al. [1987] compared the activities of BA and mT in induction of growth of lateral buds in *Poinsettia* and gerbera daisy. They found that mT was nearly twice as effective as BA in the induction of shoot growth of cuttings. Studying the effect of nine CKs on shoots production in micropropagated *Curcuma longa*, Salvi et al. [2002] obtained more shoots with the use of ribosides of kinetin, N^6-(2-isopentenyl)adenine (iP) or mT (no significant difference)

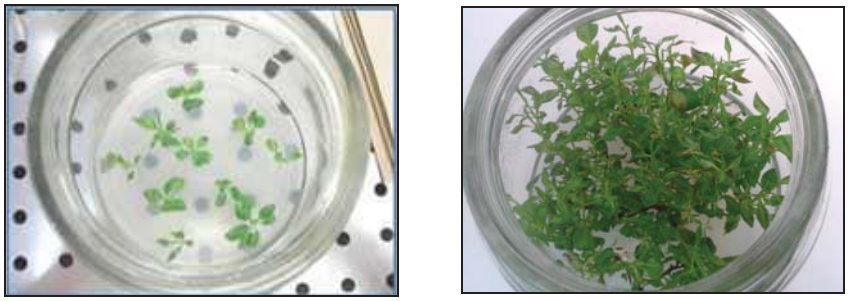
compared to either BA or other CKs used. However, regenerated shoots from mT treatment were greener and stouter than Kinetin riboside and iP-treated one. Kubalakova and Strnad [1992] compared the effects of the aromatic and isoprenoid (zeatin) cytokinins on the micropropagation and organogenesis of sugar beet cultures and found higher activity (greater number of shoots per explant) with mT. They also observed abnormal growth in BA-treated plants during subculturing, but not with mT. These results indicate that the slight structural difference between BA and mT could have a profound impact on plants during micropropagation.

Despite the availability of many media formulations, the sensitivity of the pear genotypes to *meta*-topolin has not been established. Given these reports, the effect of this cytokinin on the shoot multiplication of pear rootstock OHF-333 was explored.

MATERIALS AND METHODS

The investigation was carried out with pear rootstock OHF-333 (*Pyrus communis* L.), one of 'Old Home' × 'Farmingdale' the USA rootstock series. It was characterized by good compatibility with most of the cultivars, high yields and a moderate degree of resistance to fire blight [Lambert and Westwood 1987, Van der Zwet and Beer 1995, Wertheim 2002].

The experimental work was carried out in the Laboratory of Plant Biotechnology of Fruit Growing Institute – Plovdiv. Five-year-old stabilized in vitro shoot cultures of pear rootstock, originally established from surface sterilized shoots collected from individual mature tree, were clonally propagated at 3 week subculture intervals. An in vitro culture was maintained on a modified culture medium MS [Murashige and Skoog 1962] with ½ concentration of NH_4NO_3 and $CaCl_2$ and 1000 mg l^{-1} $Ca(NO_3)_2$, supplemented with 3 μM meta-topolin, 0.05 μM IBA, 30 g l^{-1} sucrose, 6.5 g l^{-1} Phyto agar, Duchefa.



A. B.

Fig. 1. *In vitro* shoot culture of pear rootstock at the beginning of the experiment (A) and at the end of the culture period (B) with 3 μM *meta*-topolin

For the purpose of the present experiment the same medium with different concentrations of *meta*-topolin (0 μM, 3 μM, 6 μM, 9 μM or 12 μM) was used. The study was carried out in glass jars with a volume of 600 ml.

Each container contained 100 ml of culture medium, on which 10 shoot tips with a length of 15 mm were set. For each mT concentration five replications, each containing 10 shoots were tested. The cultures were incubated in a growth chamber under controlled conditions: temperature of 22 ±2ºC, photoperiod 16/8 h supplied by cool-white fluorescent lamps (OSRAM 40 W; 40 µmol $m^{-2}s^{-1}$ PPFD).

After three intervals of three weeks in the corresponding concentration of *meta*-topolin the growth parameters were measured and physiological and biochemical analyses were performed. The multiplication coefficient (MC) was calculated using the formula: MC = number of induced shoots (more than 5 mm)/total number of primary shoots. The growth of the shoots was measured including the length, fresh weight (FW) and dry weight (DW). The fresh weight of the whole clump was determined immediately after its removing from the glass jars. The dry weight of the shoots was measured after drying the material at 80°C for 48 h [Beadle 1993]. The leaf gas exchange (portable photosynthetic system Lcpro+, ADC, England) of all 10 shoot clusters in one jar was reported. The content of photosynthetic pigments in the obtained plant material was determined spectrophotometrically, calculated according to Lichtenthaler and Wellburn [1983] and expressed as mg g^{-1} FW of sample. The total polyphenols content in the methanol extracts was estimated according to Singleton and Rossi [1965] with the Folin-Ciocalteu reagent. The data was reported as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (mg GAE 100 g^{-1} FW). The antioxidant activity was determined according to the method of Yen and Chen [1995]. A 10 g of shoot sample was homogenized in 200 ml of distilled water, then was filtered and 5 ml of filtrate was diluted in 25 ml of distilled water. A 1 ml aliquot of the extract was added to 3 ml of methanol (0.79 kg l^{-1}) and 1 ml of DPPH (0.012 g DPPH 100 ml^{-1} of methanol). The mixture was shaken and left at room temperature for 10 min; the absorbance was measured spectrophotometrically at 517 nm. The percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity was calculated using the equation: $100 - [(A_t/A_r) \times 100]$ where A_t and A_r were the absorbances of the test and reference solutions respectively [Rossi et al. 2003].

The experiment was repeated three times, with 5 replications containing 10 shoots for each treatment. Statistical analyses were carried out by one-way ANOVA using the Tukey test to validate the different significance at $P \leq 0.05$.

RESULTS AND DISCUSSION

During the stage of multiplication it is important to obtain the maximum number of usable new shoots. One of the most important indicators during this period is the multiplication coefficient which represents the number of newly shoots with length more than 5 mm from one set of multiplication.

In explants maintained without *meta*-topolin no shoot multiplication rate was observed. The use of *meta*-topolin resulted in a significantly higher multiplication rate (fig. 2, 3). As the concentration increased larger numbers of shoots were recorded with the *meta*-topolin treatments and respective values of multiplication coefficient varied from 0 (control) to 14.3 (12 µM *meta*-topolin).

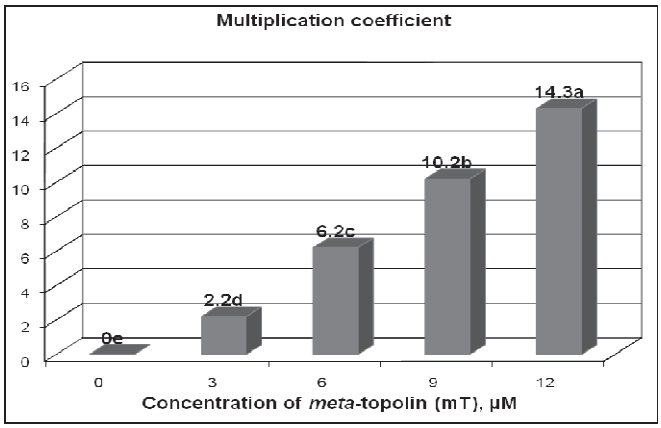


Fig. 2. *In vitro* shoot multiplication coefficient in pear plantlets in different concentrations of *meta*-topolin (mT); the values followed by different letters indicate means that are significantly different at $P \leq 0.05$

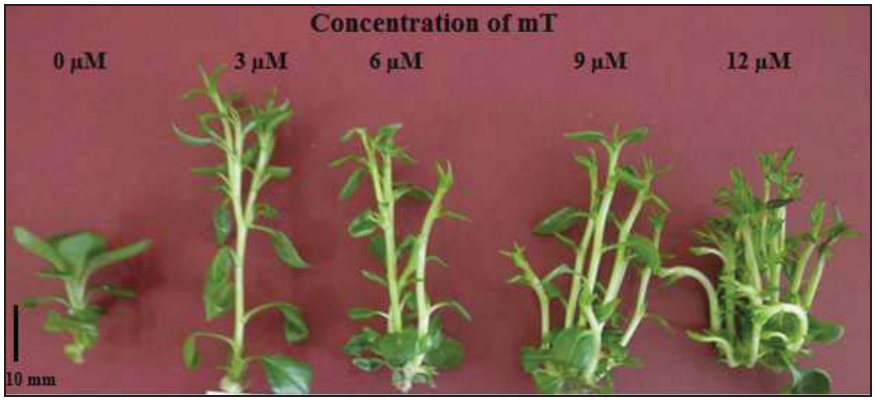


Fig. 3. Shoot multiplication of pear rootstock OHF-333 on medium containing mT at various concentrations

Shoot tips cultivated on the media with mT had a typical appearance – thick lateral shoot, short internodes, small leaves with long internodes and more narrow leaves than those grown on a medium without mT (fig. 3). The shoots revealed a different morphology already on media supplemented with different concentrations of mT – those from the medium with 3 µM mT had longer internodes and the lateral buds grew not at the base of the shoot but from the apical part of plant stem. Shoots cultivated with 6–9 µM mT were shorter than those with 3 µM, but with thicker stems. Higher concentrations of mT (more than 9 µM) resulted in manifestation of fasciation. The stems of the axial shoots were fused with axillary shoots (fig. 3).

The highest shoot multiplication coefficient was obtained on the media containing *meta*-topolin at 12 μM. However, the multiple shoots were short, having numerous tiny bud rudiments not only at the base of the shoot, but also along its entire length. These bud rudiments and shoots were less than 5 mm and were unusable for further multiplication. Therefore we did not recommend the concentration of mT higher than 9 μM for multiplication of pear rootstock. A good multiplication rate and high quality shoots were found at 9 μM mT treatment, hence selected as the optimum cytokinin level.

Table 1. Effect of mT on shoot growth parameters of pear rootstock; mT – *meta*-topolin

Parameters	Concentration of mT				
	0 μM	3 μM	6 μM	9 μM	12 μM
Fresh weight (g)	0.36d	0.72b	0.69bc	0.78b	1.65a
Dry weight (g)	0.07d	0.11b	0.09bc	0.10b	0.18a
Content of free water (%)	79.7b	84.4ab	86.4a	87.0a	89.0a
Length of main shoot (mm)	14.6d	44.1a	33.2b	30.0b	21.0c
Length of lateral shoots (mm)	0	20.4a	21.4a	21.1a	20.2a

* – values followed by different letters indicate means that are significantly different at $P \leq 0.05$.

Our investigations showed that during multiplication shoot fresh and dry weights of mT treatment were higher than the control (tab. 1). There was a strong treatment effect at all concentrations of *meta*-topolin. With an increase in concentration, a consistent increase of FW and DW was observed with mT treatment. Positive effects on *in vitro* shoot multiplication with mT have been described with other species [Werbrouck et al. 1996, Wojtania and Gabryszewska 2001, Roels et al. 2005, Bairu et al. 2007, 2008].

Data obtained showed that the content of free water in the tissues of the control plants was about 10% lower than in the variants, cultivated on media containing 9–12 μM mT. The same tendency was observed in the variant with 3 μM mT, but without a statistically significant difference. Differences among other variants with *meta*-topolin were not recorded.

The analysis of the photosynthetic pigments and leaf gas exchange aimed at estimating the shoot physiological condition. The content of both chlorophyll types, as well as the carotenoids were the highest in the shoots obtained on the medium without *meta*-topolin (tab. 2). Because of the small thin leaves the content of photosynthetic pigments was determined in the whole shoot clusters. For that reason (consequently) in the variants with more newly formed shoots and buds lower pigment content on the fresh weight basis was reported. Many authors [Fujiwara et al. 1992, Pospisilova 1996] have noted that these variations in the chlorophyll content can be attributed to the fact that in the scientific reports chlorophyll content is often expressed on a fresh weight basis, and due to the specific conditions *in vitro* ratio FW/DW varies and is always higher in the *in vitro* compared to the corresponding *ex vitro* plants. This can lead to large differences in the performance values.

Similar tendency was observed in regard to chlorophyll b and carotenoids. No significant deviations from the norm have been observed in the relationships between the photosynthetic pigments.

Table 2. Photosynthetic pigment content (mg g^{-1} DW) in *in vitro* cultivated pear shoots in multiplication stage on medium containing mT at different concentrations

Parameters	Concentration of mT				
	0 µM	3 µM	6 µM	9 µM	12 µM
Chl a	1.90a	0.53 c	0.99 ab	0.85 b	0.65 bc
Chl b	0.54 a	0.27c	0.35 bc	0.41ab	0.28 c
Chl (a+b)	2.63 a	0.87c	1.45 b	1.38 b	1.01 bc
Carotenoids	0.82 a	0.18 cd	0.41 b	0.29 c	0.21 cd
Chl (a/b)	3.49 a	2.00 c	2.82 b	2.23 c	2.48 bc
Chl (a+b)/carotenoids	3.23 c	4.85 ab	3.50 bc	4.83 ab	5.16 a

* – values followed by different letters indicate means that are significantly different at $P \leq 0.05$

The experiment showed that *meta*-topolin treatment improved the leaf gas exchange compared to the control. In the shoots cultivated in a medium supplemented with 6–12 µM *meta*-topolin the photosynthesis rate was higher compared with the control mT (fig. 4). The transpiration intensity in the variants treated with 6 and 9 µM mT was also higher than the control (fig. 5).

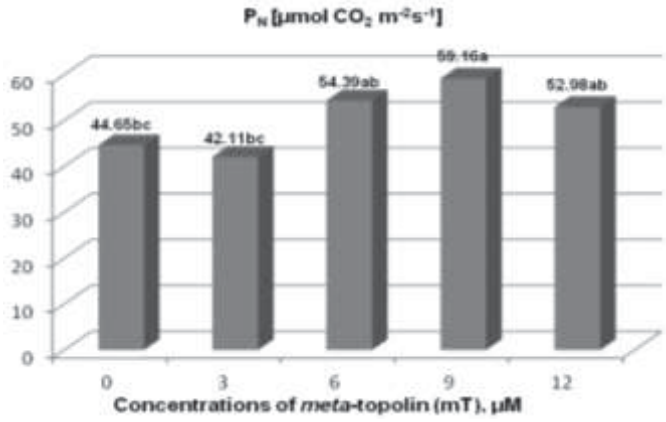


Fig. 4. Net photosynthetic rate – P_N (µmol CO_2 $m^{-2}s^{-1}$) of all pear shoots in the container during multiplication stage; Values followed by different letters indicate differences at $P \leq 0.05$

According to several authors [Parthier 1979, Fletcher et al. 1982, Caers and Vendrig 1986] the application of CKs promotes photosynthetic activity mainly by increasing the chlorophyll content, accelerating the conversion of etioplasts into chloroplasts, or modifying other components of photosynthesis, such as CO_2 assimilation capacity and activity of the photosynthetic enzymes. In our study the increased activity of photosynthesis accompanied by a reduced content of photosynthetic pigments showed that the changes occurring under the influence of *meta*-topolin can be attributed primarily to stomatal limitation (tab. 2, fig. 4). On the other hand, these results support the view of a number of authors that the qualitative composition and the quantity of photosynthetic pigments are not limiting factors for assessing the photosynthetic ability of the *in vitro* plants [Fujiwara et al. 1992, Pospisilova 1996].

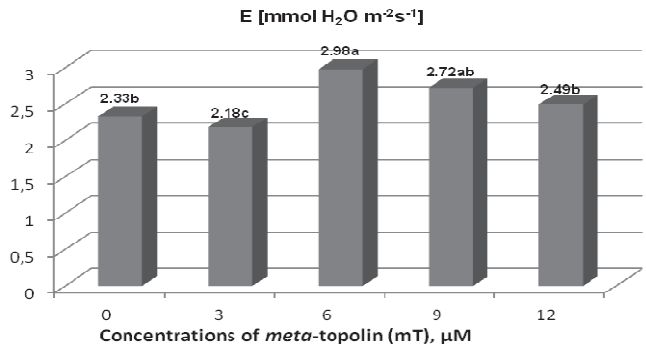


Fig. 5. Transpiration rate – E [mmol H_20 $m^{-2}s^{-1}$] during multiplication stage; Values followed by different letters indicate significantly differences at $P \leq 0.05$

The extracts of *in vitro* cultures were characterized by a low phenols content (tab. 3). This was most likely due to the fact that the secondary metabolites serve as a natural defence of plants to oxidative stress, protect the biological molecules from oxidation and the plants from microorganisms, insects and herbivores [Sengul et al. 2009], and these environmental factors were minimized or totally absent in the plants grown *in vitro*.

The total polyphenols content for fresh shoot extracts of *meta*-topolin treated variants was 639,1–792.2 mg 100 g^{-1} gallic acid equivalent (GAE) compared to the control which was 933,7 mg 100 g^{-1} GAE. The total antioxidant activity (DPPH) exhibited 20,4–26,9% for 3–12 µM *meta*-topolin and 30,7% for the control respectively. The data for both the total polyphenols content and the antioxidant activity show that GAE values as the DPPH values decreased in treated shoots which suggesting a correlation between these two indicators.

Table 3. Total polyphenols content (mg gallic acid – GAE 100 g^{-1} FW) and antioxidant activity against DPPH radical scavenging capacity in extracts from *in vitro* cultivated pear shoots in multiplication stage

Concentration of mT	GAE mg 100 g^{-1} FW	DPPH (%)
0 µM	933.7 a	30.7 a
3 µM	760.0 b	26.6 b
6 µM	780.0 b	26.9 b
9 µM	792.2 b	23.8 b
12 µM	639.1 c	20.4 bc

– values followed by different letters indicate means that are significantly different at $P \leq 0.05$

Although BA is reported to be among the most effective and affordable CKs used in micropropagation techniques including many species and cultivars in the genus *Pyrus* [Bell and Reed 2002], some investigations showed that other cytokinins are more effective [Ruzic et al. 2011].

A few reports on the use of topolins indicate that this group of cytokinins could be a new source of cytokinins with high morphogenetic activity. On the basis of the results

of our multiplication experiment, as the concentration increased larger numbers of shoots were recorded with the *meta*-topolin treatments. Results of the analysis showed that treatment means were significantly higher than the control for most mT levels tested. The multiplication rates increased with an increasing in the concentration up to 12 μM. Similar results reported Bairu et al. [2007] with *Aloe polyphylla*. The use of mTR for improving survival of potato cultures has been reported [Baroja-Fernandez et al. 2002]. The use of mT and its derivatives has been recommended, by some researchers [Werbrouck et al. 1996, Bogaert et al. 2006, Bairu et al. 2007] as a potential replacement for BA in the micropropagation industry.

CONCLUSIONS

The results of this study demonstrate that *meta*-topolin in a concentration 6–12 μM improves the multiplication of *in vitro*-grown pear rootstock OHF-333. Cytokinin *meta*-topolin could be used in the micropropagation of other genotypes of pear (*Pyrus communis* L.), especially in case of rooting problems.

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WPŁYW *META*-TOPOLINY NA MIKROPROPAGACJĘ PODKŁADKI GRUSZY OHF-333 (*Pyrus communis* L.)

Streszczenie. W badaniu oceniono wpływ naturalnej, aromatycznej cytokininy *meta*-topoliny (mT) na mikropropagację podkładek gruszy OHF-333 (*Pyrus communis* L.). Hodowlę eksplantatów przeprowadzono w komorze hodowlanej w kontrolowanych warunkach. Eksplantaty wykładano na pożywkę Murashige i Skooga [1962] uzupełnioną *meta*-topoliną (0 µM, 3 µM , 6 µM, 9 µM lub 12 µM). Po trzech tygodniach określono parametry wzrostu, wykonano analizy fizjologiczne i biochemiczne. Wyniki tego badania wskazują, że w hodowli *in vitro* brak cytokininy nie zapewnia praktycznego rozwiązania dla skutecznego mikrorozmnażania podkładek gruszy. Zastosowanie 6–9 µM *meta*-topoliny spowodowało polepszenie współczynnika rozmnażania oraz otrzymanie pędów o wysokiej jakości. Użycie 6–9 µM *meta*-topoliny spowodowało również poprawę wymiany gazowej liści. Dlatego cytokinina *meta*-topolina w stężeniu 6–9 µM została wskazana jako optymalny poziom cytokinin przy mikrorozmnażaniu podkładek gruszy OHF-333.

Słowa kluczowe: mikrorozmnażanie, cytokinina, parametry wzrostu, zmiany fizjologiczne

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