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Comparing the Effects of Benzyladenine and *meta*-Topolin on Sweet Basil (*Ocimum basilicum*) Micropropagation

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Abstract

Micropropagation of aromatic plants reveals an effective way of obtaining high volume, virus-free plant material of uniform quality. The application of *meta*-Topolin (*m*T) (N^6-(2-hydroxybenzyl) adenine-9-riboside) and aromatic cytokinin as Benzyladenine (BAP) in the micro propagation of sweet basil (*Ocimum basilicum* L.) was tested for the first time and plant growth parameters assessed to determine the optimum level of these cytokinins. Additionally, the rate of root-growth inhibition due to these two cytokinins was also assessed. Our results show that 1 mg/l (4.43 μM) BAP and 0.5 mg/l (2.07 μM) *m*T produced the most favourable effects on new shoot developments. *Meta*-Topolin was shown to increase the quality of the plants and in comparison with BAP fewer distortions were observed. No significant differences in root-growth inhibition between the *m*T and BAP were detected.

Keywords: aromatic plants, cytokinins, in vitro multiplication, root-growth inhibition, shoot proliferation

Introduction

Ocimum basilicum L. (sweet basil) is an annual aromatic herb from the *Lamiaceae* family. Native to Iran, Afghanistan and India (Asghari *et al*., 2012; Saha *et al*., 2010) it is none-the-less widely cultivated all around the world (Kiferle *et al*., 2011). Some species including *Ocimum americanum* L. have insecticidal properties, while others have ornamental qualities with a particular leaf shape, size and colour, e.g. 'Purple Ruffles' (Kintzios *et al*., 2004; Phippen and Simon, 2000).

The common sweet basil is of high economic importance because of the essential volatile oil derived from its leaves (Saha *et al*., 2010; Sudhakaran and Sivasankari, 2002; Siddique and Anis, 2009). These compounds also have valuable pharmaceutical, aromatic and culinary properties (Gopi and Ponmurugan, 2006; Sahoo *et al*., 1997). Basil is a source of rosmarinic acid, but also contains caffeic acid and derivatives of lithospermic acid and lithospermic acid B, which help in healing several renal diseases (Rady and Nazif, 2005). These compounds are also considered to be important due to their stomachic, antihelminthic, antipyretic, diaphoretic and diuretic effects, as well as in the treatment of purulent discharge of the ear and diseases of heart and brain (Saha *et al*., 2010; Siddique and Anis, 2009; Singh and Sehgal, 1999). Volatiles are popular ingredients in dental and oral health care products and the leaf extract is highly effective in inhibiting carcinogen- induced tumour development (Chandramohan and Sivakumari, 2009). Dried leaves of basil are used to flavour stew, sauces, salads, soups, meat and tea (Siddique and Anis, 2008; Phippen and Simon, 2000). Due to these high-value characteristics, sweet basil is intensely cultivated and volatile compounds obtained cover cca. 100 t/year worldwide (Daniel *et al*., 2010; Begum *et al*., 2002).

Conventional propagation methods using seeds suffer several disadvantages regarding the requirement for uniform crops: seedling progeny shows high degree of variability and infection with viruses cannot be controlled (Asghari *et al*., 2012; Saha *et al*., 2010; Sahoo *et al*., 1997). *In vitro* micropropagation provides a solution for rapid mass multiplication of the elite planting material, without any seasonal constraints when growing conditions are strictly controlled. It is a viable tool for the germplasm conservation of endangered and rare plant species and eliminates variability among the progenies (Asghari *et al*., 2012; Kiferle *et al*., 2011; Saha *et al*., 2010; Siddique and Anis, 2008; Siddique and Anis, 2007).

By using several growth regulator hormones in the start-up phase of micropropagation, the process and the plant yield can be improved. Cytokinins are evincible in plants, animals and microorganisms as a component of *t*RNA. They can speed up the transcription, translation, the membrane functions (Strnad *et al*., 1997; Werbrouck *et al*., 1996) and play an important role in delaying the onset of leaf senescence (Mutui *et al*., 2012). The exogenously applied cytokinins are limited by the action of enzymes. Cytokinin oxidase helps the degradation process by splitting the oxidative side chain on the aromatic cytokinin. One of the best known cytokinins, N^6- benzyladenine (BA), used in culture media to promote auxiliary shoot production (Valero-Aracama *et al*., 2010), has shown to have some disadvantages in the acclimatization period. The accumulated derivatives have been shown to inhibit both root-growth and induced heterogeneity in growth. Therefore new products are needed to eliminate these negative effects.

One alternative might be the N^6-(2-hydroxybenzyl)adenine-9-riboside, "Topolin" (*m*T) that was first isolated from mature poplar leaves (Strnad *et al.,* 1997; Amoo and Staden, 2011; Amoo *et al.,* 2013, Gentile *et al.,* 2014). Kamínek *et al.* (1987) has already compared in standard bioassay, the activity of several cytokinins on ornamental plants and the results proved *m*T to be more active than BA and the other cytokonins in the class of natural "aromatic cytokinins". Werbrouck *et al.* (1996) has shown that *m*T can be a suitable alternative to BA in the microprogation of *Spathiphyllum floribundum*. The whole microprogation process can be increased by interchanging BA with *m*T, because the primary metabolite degrades more quickly during acclimatization (Werbrouck *et al*., 1996; Strnad *et al*., 1997). All these effects were demonstrated on ornamentals, but no previous study has tested *m*T on aromatic plants. Therefore, two main objectives were defined: 1. to compare the effect of different concentrations of supplemented aromatic cytokinin as Benzyladenine (BAP) and *meta*-Topolin (*m*T) on *in vitro* multiplication of cultured basil plant thereby determining the optimum level of these cytokinins and 2. to compare the rate of root-growth inhibition by these two cytokinins.

Materials and methods

Experimental condition

The experiment was carried out in the Research Laboratory of the Horticulture Department, Sapientia Hungarian University of Transylvania. In the first step we obtained packed basil (*Ocimum basilicum*) virus free seeds. The seeds were surface sterilized in a laminar air-flow cabin by soaking them in 8% NaOCl solution for 20 minutes, than were then rinsed three times in sterile distilled water. Growth mediums for the seeds were created according to the recipe developed by Murashige and Skoog (1962) containing macro and micro salts (3.2 g/l), 100 ml/l NaFe EDTA complex, 10 mg/l mezo- inositol, 1 ml/l sundry vitamins, 30 g/l sucrose and 6.2 g/l agar. This medium was coded as 'MS'. The pH was adjusted to 5.8 with 1 N NaOH. The medium was sterilized using a pressure-cooker for 20 minutes.

Thereafter seeds were dried in sterile Petri dishes. After sterilization, 96.7% of seeds (271 from 280) germinated on the MS medium lacking growth regulators (Fig. 1a) in the growth chamber, where the temperature was maintained at 22 °C and 24-hour illumination was provided by cool-white and warm-white 36 W/m^2 fluorescent tubes. In the first stage of the experiment (multiplication phase) fifteen day-old germinated plants (20 plants per treatment) were transferred to several nutrient mediums: MS (Murashige and Skoog, 1962) supplemented with different concentrations of BAP and *m*T (Fig. 1b, Tab. 1). MS mediums devoid of plant growth regulators and half strength MS (coded ½ MS) containing only half quantities of the microelements served as controls. ½ MS was used in order to obtain a better determination of the optimum level of the BAP and *m*T cytokinins. Culture flasks were covered with a double layer of sterile foil and maintained in the growth chamber for 45 days. At the end of the multiplication stage the length (cm) of main shoots, number and the average length (cm) of newly formed shoots and tap-roots, as well as the number of leaves and internodes, were measured.

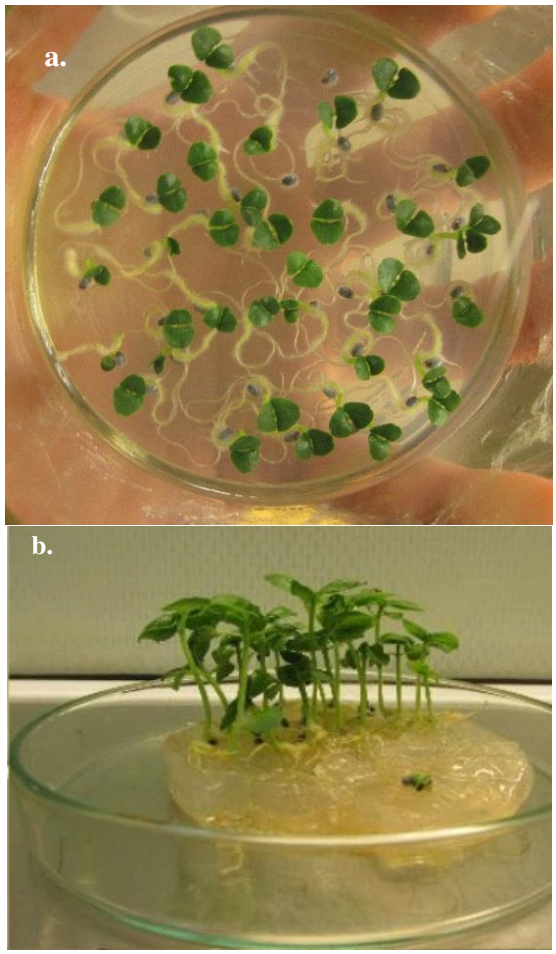

Fig. 1. *In vitro* germination of basil (*Ocimum basilicum* L.) seeds (a), 15 days old *in vitro* germinated basil (*Ocimum basilicum* L.) plants (b)

In the second stage of the experiment the root-growth phase was accomplished. This was performed in order to establish the effects of *m*T on root development. Plants obtained during the first stage of the experiment were replanted (10 plants per treatment) into MS growth mediums in the laminar air-flow cabin and transferred to the growth chamber for 15 days. The resulting new shoots were transferred to mediums with different concentrations of BAP and *m*T. To achieve the desired effect in the multiplication section, the basic medium was supplied with varying (higher/lower) concentrations of BAP and *m*T (Tab. 1). At the end of the root-growth phase the number of roots and the length (cm) of tap-roots were measured.

Data analyses

Firstly data from the multiplication phase, obtained from the different concentrations of supplemented aromatic cytokinin as Benzyladenine (BAP) and *meta*-Topolin (*m*T), were compared with controls (MA and ½ MS). The average values per plant were used for data analyses. Normality of errors and homogeneity of variances were first tested for all data. Main shoot length and the length respectively, the number of the tap-roots, showed a normal distribution, thus One Way ANOVA and post-hoc comparison Games-Howell tests were used. Data regarding main shoot numbers, the length of the side shoots,

the internode and leaf numbers did not show normal distribution, therefore the Kruskall-Wallis test and post-hoc comparison Mann-Whitney U test were used for comparison with the controls. Results are presented in figures. Data from the various cytokinin treatments obtained during the multiplication experiments were then compared in the same way and results are presented in tables. Data from the root-growth experiment were tested for normality. Top-root length data was normally distributed, therefore a two-sample T test was used to compare treatments. Data representing tap-root numbers were not distributed normally, therefore the non-parametric Kruskall-Wallis test and post-hoc comparison with Mann-Whitney U test were used to compare treatments. Results were presented in tables.

Results

Differences between cytokinin treatments and controls in the multiplication experiment

In the case of the main shoot length there were no significant differences between the plantlets cultured on MS and those cultured on half strength MS mediums, both served as controls (Fig. 2a). However, in the comparison of different BAP concentration with the control, significant differences, with the higher supplemented level of BAP in B5 (8.88 μM) and B4 (6.64 μM), were found. The shortest main shoot length was 2.46 cm cultured on B5 and the longest was 7.25 cm cultured on B1 (1.10 μM). By comparing the length of the main shoots of the control plants with the different concentration of supplemented *m*T medium cultured plant, there were significant differences only between T5 (8.28 μM), T4 (6.21 μM) and T3 (4.14 μM) (Fig. 2a). The values recorded on T1 (1.03μM), T2 (2.07 μM) did not differed significantly from the control and T5, T4, T3. The shortest main shoot length was 3.54 cm cultured on T4 and the longest was 5.4 cm cultured on T2. Comparison of the main shoot numbers revealed significant differences between control plants and B3 (1.00 μM), B2 (2.21 μM) and B1 (1.10 μM) plants (Fig. 2b). Plantlets cultured on the B3 medium were found to have formed, on average, 3 new main shoots. In the case of the *m*T supplemented mediums, all of the plants formed new main and side shoots, the highest number being recorded on T2 (2.07 μM) with an average of 6.2 shoots per plant and the lowest on T4 (6.21 μM) with an average of 4.85 new shoots per plant. The longest side shoots of the plants cultured on B3 was 2.58 mm. All of the plants cultured on *m*T supplemented mediums formed new side shoots. The longest was on the T2 (2.07μM) with 5.46 mm and the shortest on T4 (6.21 μM) with 3 mm (Fig. 2c).

The longest tap-roots were formed on B1 (1.10μM), B2 (2.21 μM) and B3 (1.00 μM) with an average of 6 cm, but no significant differences from the control were detected. The shortest tap-root lengths were observed on B5 with an average

Tab 1. Different concentrations (μM/l) and levels (mg/l) of growth regulators (6-Benzyladenine (BAP) and *meta*-Topoline (*m*T)) used during the experiment

	Unit	
Growth Regulators	μM/l	mg/l
MS (control)	-	-
½ MS (control)	-	-
BAP1 (MS+BAP)	1.10	0.25
BAP2 (MS+BAP)	2.21	0.50
BAP3 (MS+BAP)	4.43	1.00
BAP4 (MS+BAP)	6.64	1.5
BAP5 (MS+BAP)	8.88	2.00
*m*T1 (MS+*m*T)	1.03	0.25
*m*T2 (MS+*m*T)	2.07	0.50
*m*T3 (MS+*m*T)	4.14	1.00
*m*T4 (MS+*m*T)	6.21	1.5
*m*T5 (MS+*m*T)	8.28	2.00

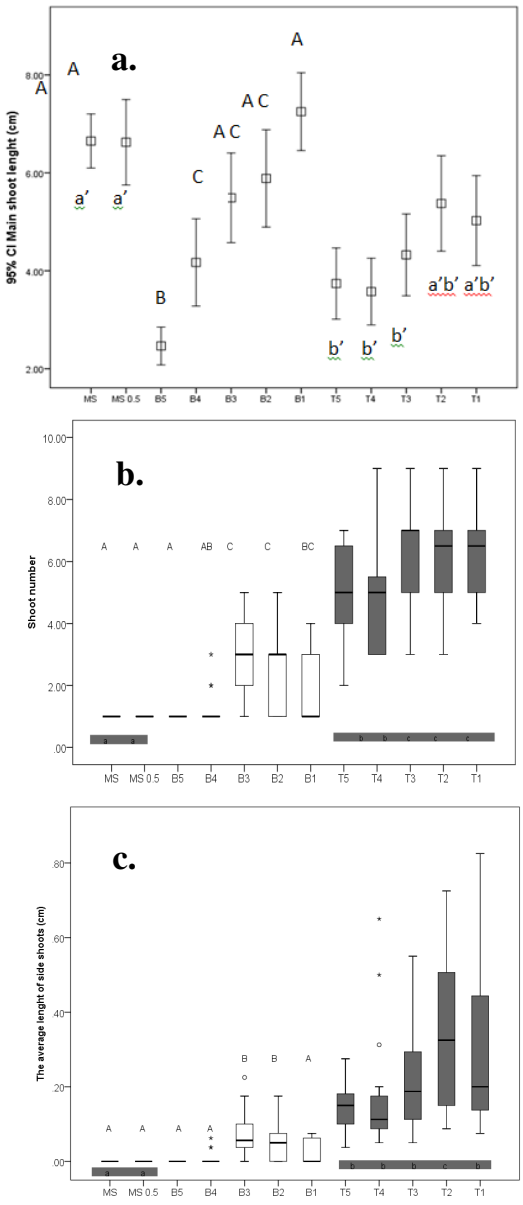

Fig. 2. Main shoot length (**a**); $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; One Way ANOVA, post-hoc comparison: Games Howell test, $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; Main shoot numbers (**b**); $N_{MS, MS1/2, B5-3, T1-5}$=20, N_{B2}=15, N_{B1}=10; Kruskall-Wallis, post-hoc comparison: Mann-Whitney U test, $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; Length of the side shoots (**c**); $N_{MS, MS1/2, B5-3, T1-5}$=20, N_{B2}=15, N_{B1}=10; Kruskall-Wallis, post-hoc comparison: Mann-Whitney U test, $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; Significant differences are represented by different characters, $p<0.05$

of 1.15 cm (Fig. 3a). Significant differences in tap-root lengths between the control andT5 (8.28μM), T4 (6.21 μM), T3 (4.14 μM) and T2 (2.07 μM) were detected. Values varied around 1.1 cm on T5 and 5.77 cm on T1 (Fig. 3a). On B1 (1.10μM), B2 (2.21 μM) and B3 (1.00 μM) an average of 10 roots were detected, which significantly differed from the B4 (6.64 μM) and B5 (8.88 μM), with an average of 4.1 roots per plant (Fig. 3a). The highest root number was recorded on the plants cultured on control MS and half strength MS with an average of 20 tap-roots per plant. On the *m*T supplemented mediums, the highest number of roots was recorded on T1 (1.03μM) averaging 16 per plant (Fig 3a).

On the control mediums an average of 4 internodes per plant were formed. On B1, B2 and B3 the average number of the internodes varied between 1 and 3. The number of internodes formed on T3, T4 and T5 were significantly lower than the number of internodes formed on the control (Fig. 4a). Significant differences of the newly formed leaves between control and the higher concentrations of BAP (B5, B4) were detected. No differences between control and B1, B2, B3 were found (Fig. 4b).

Differences between the two cytokinin treatments in the multiplication experiment

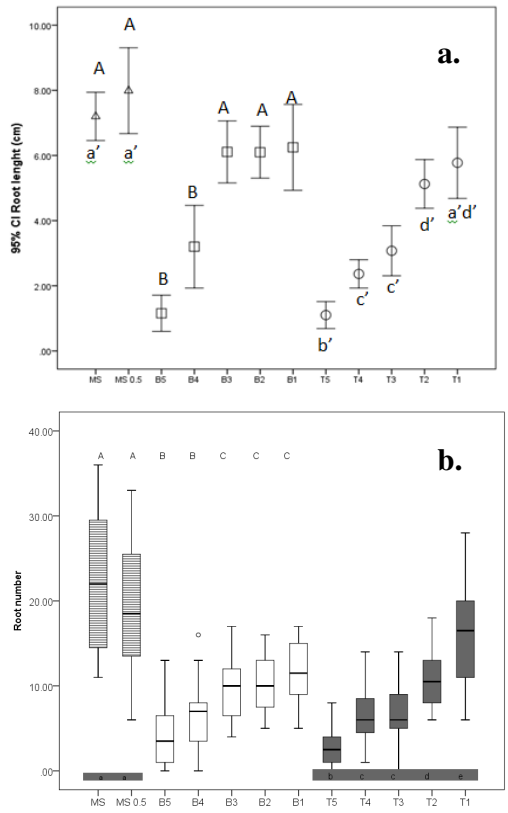

Fig. 3. The length of the tap-roots (**a**); $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; One Way ANOVA, post-hoc comparison: Games-Howell test, $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; Significant differences are represented by different characters, p<0.05. Tap-root numbers (**b**); $N_{MS, MS1/2, B5-3, T1-5}$=20, N_{B2}=15, N_{B1}=10; Kruskall-Wallis, post-hoc comparison: Mann-Whitney U test, $N_{MS, MS1/2, B5-3, T1-5}$= 20, N_{B2}=15, N_{B1}=10; Significant differences are represented by different characters, p<0.05

Statistical analysis revealed that lower concentrations of BAP and higher concentrations of *m*T formed longer main shoots (Tab. 2).Considering the main shoot numbers of different BAP supplemented mediums with the *m*T supplemented mediums there were, again, significant differences between BAP and *m*T (Tab. 2). Newly formed side shoots were recorded only in the case of the plants cultured on B4 (6.64μM), B3 (1.00 μM) and B1 (1.10 μM) (Tab. 2). Considering the length of the tap-roots, significant

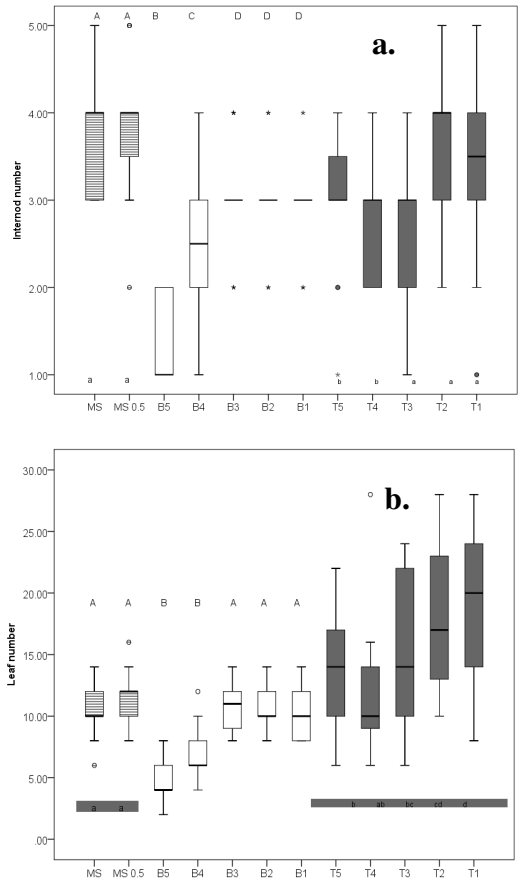

Fig. 4. Internodes numbers (a); NMS, MS1/2, B5-3, T1-5=20, N B2=15, NB1=10; Kruskall-Wallis, post-hoc comparison: Mann-Whitney U test, NMS, MS1/2, B5-3, T1- 5= 20, NB2=15, NB1=10; Leaf numbers (b); NMS, MS1/2, B5-3, T1-5=20, NB2=15, NB1=10; Kruskall-Wallis, post-hoc comparison: Mann-Whitney U test, NMS, MS1/2, B5-3, T1-5= 20, NB2=15, NB1=10; Significant differences are represented by different characters, p<0.05

differences were only detected between B3 and T3 (Tab. 3). Supplementing the different cytokinins, it decreased the number of newly formed roots. Comparing the root number of the plants cultured on different concentration supplemented BAP with those of *m*T, statistics revealed significantly higher values for B3 than for T3. The lower concentration T1 medium formed more tap-roots than the B1 medium (Tab. 3). Statistics revealed that T5 and T2 plants formed a significantly higher number of internodes than B5 and B2 plants (Tab. 4). Each concentration of *m*T yielded a higher number of leaves than supplemented BAP concentrations (Tab. 4).

Tab. 2. The plants shoot analyses between the two cytokinin treatments in multiplication experiment

Main shoot length - Two Sample T test	
Relation	T
BAP5<*m*T5	-3.24**
BAP4=*m*T4	1.11^{ns}
BAP3=*m*T3	1.96^{ns}
BAP2=*m*T2	0.76^{ns}
BAP1>*m*T1	3.31**
Main shoot numbers - Mann-Whitney U test	
Relation	Z
BAP5<*m*T5	-5.80***
BAP4<*m*T4	-5.57***
BAP3<*m*T3	-4.64***
BAP2<*m*T2	-4.38***
BAP1<*m*T1	-4.48***
Length of side shoots - Mann-Whitney U test	
Relation	Z
BAP5<*m*T5	-5.80***
BAP4<*m*T4	-4.78***
BAP3=*m*T3	-1.24^{ns}
BAP2<*m*T2	-3.33***
BAP1<*m*T1	-3.81***

Note: **p<0.001, ***p<0.0001, ns–not significant

Tab. 3. The tap-root analyses between the two cytokinin treatments in multiplication experiment

Length of the tap-roots - Two Sample T test	
Relation	T
BAP5=*m*T5	0.16^{ns}
BAP4=*m*T4	1.30^{ns}
BAP3>*m*T3	5.19***
BAP2=*m*T2	1.86^{ns}
BAP1=*m*T1	0.56^{ns}
Tap-root numbers - Mann-Whitney U test	
Relation	Z
BAP5=*m*T5	-1.06^{ns}
BAP4=*m*T4	-0.17^{ns}
BAP3>*m*T3	-2.66**
BAP2=*m*T2	-0.38^{ns}
BAP1<*m*T1	-2.16*

Note: *p<0.01, **p<0.001, ***p<0.0001, ns–not significant

Tab. 4. The internod and leaf numbers analyses between the two cytokinin treatments in multiplication experiment

Internod numbers - Mann-Whitney U test	
Relation	Z
BAP5<*m*T5	-4.56***
BAP4=*m*T4	-1.40^{ns}
BAP3=*m*T3	-1.14^{ns}
BAP2<*m*T2	-2.15*
BAP1=*m*T1	-1.75^{ns}
Leaf numbers - Mann-Whitney U test	
Relation	Z
BAP5<*m*T5	-5.33***
BAP4<*m*T4	-4.12***
BAP3<*m*T3	-2.03*
BAP2<*m*T2	-4.07***
BAP1<*m*T1	-3.50***

Note: *p<0.01, ***p<0.0001, ns–not significant

Differences between the two cytokinin treatments in the root-growth experiment

The average tap-root length of the plants varied between 4.05-5.5 cm in BAP and between 4.75 and 6 cm in *m*T. In pairwise comparison of the same concentration supplemented BAP and *m*T no significant differences between values were found (Tab. 5). However, the lowest number of roots were detected on plants pre-treated with BAP (6.8 per plant), while the average number of tap-rots in *m*T varied between 9 and 10.25, no differences between treatments were detected (Tab. 5).

Discussion

No previous studies have reported the effects of *m*T on aromatic plants. According to our results, *m*T provided more and longer shoots and also increased the quality of the plants by increasing shoot numbers and side shoot length. No significant effect of *m*T on root development was detected during the multiplication process. The number of internodes formed on T3, T4, T5 was significantly lower than the number of internodes formed on the controls and significant differences in the newly formed leaves between control and the higher concentrations of BAP (B5, B4) were detected. In previous studies Bairu *et al.* (2007) demonstrated that *m*T induced shoot multiplication enhanced root-growth, reduced hyperhydricity and stimulated the acclimatization in the case of the *Aloe polyphylla*. Mutui *et al.* (2012) compared the effects of *m*T with thidiazuron (TDZ) on post-harvested *Pelargonium x hortorum* cuttings: leaves treated with *m*T and TDZ contained higher chlorophyll amount than the controls. They observed that *m*T slightly reduced the root length, the root surface area and the total volume of the roots, however the TDZ severely inhibited root formation. In our results, significantly higher numbers of main shoots and leaves were formed with *m*T and the lengths of the side shoots were also higher in most cases utilizing *m*T. With previous studies Valero-Aracama *et al.* (2010) observed that a high concentration of *m*T has an inhibitory effect on the root-growth process and influences acclimatization of sea oats

Tab. 5. The tap-root analyses between the two cytokinin treatments in rooting experiment

Tap-root length - Two Sample T test	
Relation	T
BAP5g=*m*T5g	1.02^{ns}
BAP4g=*m*T4g	0.90^{ns}
BAP3g=*m*T3g	-2.00^{ns}
BAP2g=*m*T2g	-1.93^{ns}
BAP1g=*m*T1g	-0.96^{ns}
Tap-root number -Mann-Whitney U test	
Relation	Z
BAP5g=*m*T5g	-1.98^{ns}
BAP4g=*m*T4g	-1.25^{ns}
BAP3g=*m*T3g	-0.99^{ns}
BAP2g=*m*T2g	-1.30^{ns}
BAP1g=*m*T1g	-0.72^{ns}

Ns – not significant

(*Uniola paniculata*). In our experiments, no deleterious effects due to *m*T on the sweet basil root-growth process were detected. In the case of *m*T treatments, the T2 medium - with 2.07 μM concentration and 0.5 mg/l level of supplemented *m*T - proved to be the optimal solution for sweet basil.

Conclusions

Comparing the two cytokinins we can conclude that less distortion was observed on plants treated with *m*T than on plants treated with BAP. According to the results, 1 mg/l (4.43 μM) BAP and 0.5 mg/l (2.07 μM) *m*T had the highest effects on sweet basil development. No significant effects on root development were detected between the two cytokinins. *Meta*-Topolin increased the quality of the plants and in comparison with BAP fewer distortions were caused. No significant differences in root inhibition between the *m*T and BAP were detected. Further research is needed to establish the effects of *m*T on sweet basil acclimatization, which is a key step in successful micropropagation.

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