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 (mT) (N6-(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) mT 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 mT 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 pharmacological, 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, anthelmintic, 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 Schgal, 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 caa. 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 tRNA. They can speed up the transcription, translation, the membrane functions (Stmad 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. Cytokinins oxidase helps the degradation process by splitting the oxidative side chain on the aromatic cytokinin. One of the best known cytokinins, N6- 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 N⁶-(2-hydroxybenzyl)adenine-9-ribose, "Topolin" (mT) that was first isolated from mature poplar leaves (Strnad et al., 1997; Amoo and Staden, 2011; Amoo et al., 2013, Gentile et al., 2014). Kaminek et al. (1987) has already compared in standard bioassay, the activity of several cytokinins on ornamental plants and the results proved mT to be more active than BA and the other cytokinins in the class of natural "aromatic cytokinins". Werbrouck et al. (1996) has shown that mT can be a suitable alternative to BA in the micropropagation of *Spathiphyllum floribundum*. The whole micropropagation process can be increased by interchanging BA with mT, 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 mT 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 (mT) 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 cabinet 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/m² 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 mT. To achieve the desired effect in the multiplication section, the basic medium was supplied with varying (higher/lower) concentrations of BAP and mT (Tab. 1). At the end of the multiplication 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 (mT), were compared with controls (MS and MA). 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 new shoots per plant. The longest side shoots of the plants cultured on B3 was 2.58 mm. All of the plants cultured on mT supplemented mediums formed new side shoots. The longest was on T2 (2.07 µM) 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 (1.10 µM), 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 (mT)) used during the experiment |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| Growth Regulators   | µM/l | mg/l | µM/l | mg/l | µM/l | mg/l | µM/l | mg/l |
| MS (control)        | -     | -    | -    | -    | -    | -    | -    | -    |
| ½ MS (control)      | -     | -    | -    | -    | -    | -    | -    | -    |
| BAP1 (MS+BAP)       | 1.10  | 0.25 | 2.21 | 0.50 | 4.43 | 1.00 | 6.64 | 1.5  |
| BAP2 (MS+BAP)       | -     | -    | -    | -    | -    | -    | -    | -    |
| BAP3 (MS+BAP)       | 1.03  | 0.25 | 2.07 | 0.50 | 4.14 | 1.00 | 6.21 | 1.5  |
| BAP4 (MS+BAP)       | 1.00  | 0.50 | 1.00 | 0.25 | 1.00 | 0.50 | 1.00 | 0.50 |
| BAP5 (MS+BAP)       | 8.88  | 2.00 | 8.88 | 2.00 | 8.88 | 2.00 | 8.88 | 2.00 |
| mT1 (MS+mT)         | 4.14  | 1.00 | 4.14 | 1.00 | 4.14 | 1.00 | 4.14 | 1.00 |
| mT2 (MS+mT)         | 6.21  | 1.5  | 6.21 | 1.5  | 6.21 | 1.5  | 6.21 | 1.5  |
| mT3 (MS+mT)         | 8.28  | 2.00 | 8.28 | 2.00 | 8.28 | 2.00 | 8.28 | 2.00 |
| mT4 (MS+mT)         | -     | -    | -    | -    | -    | -    | -    | -    |
| mT5 (MS+mT)         | -     | -    | -    | -    | -    | -    | -    | -    |

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 mT 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 (1.03 µM), 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 mT 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

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 and T5 (8.28 µM), 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 (1.10 µM), 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 mT supplemented mediums, the highest number of roots was recorded on T1 (1.03 µM) 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

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

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -3.24** | -5.80*** | -1.24 |
| BAP4cmT4 | 1.11*** | 4.64*** | -0.38 |
| BAP3cmT3 | 1.96*** | 4.38*** | 1.86*** |
| BAP2cmT2 | 0.76* | -3.33*** | -0.96** |
| BAP1cmT1 | -3.31*** | -4.48*** | -2.16* |

Main shoot numbers - Mann-Whitney U test

| Relation | Z  |
|----------|----|
| BAP5cmT5 | -5.80*** |
| BAP4cmT4 | 4.78*** |
| BAP3cmT3 | -1.12 |
| BAP2cmT2 | -3.33*** |
| BAP1cmT1 | -3.81*** |

Length of the tap-roots - Two Sample T test

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -3.81*** | -3.50* |

Main shoot length - Two Sample T test

| Relation | T  |
|----------|----|
| BAP5cmT5 | -5.80*** |

Discussion

No previous studies have reported the effects of mT on aromatic plants. According to our results, mT 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 mT on root development was detected during the multiplication process. The number of internodes formed on T3, T4, T5 was significantly lower than the previous studies.

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

| Relation | T  |
|----------|----|
| BAP5cmT5 | -4.38*** |

| Relation | Z  |
|----------|----|
| BAP5cmT5 | -3.48*** |

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -5.57** | -3.50* |
| BAP4cmT4 | 1.40*** | -1.12*** |
| BAP3cmT3 | -1.14*** | -2.00*** |
| BAP2cmT2 | 0.07*** | -2.03*** |
| BAP1cmT1 | -1.52*** | -3.50*** |

Leaf numbers - Mann-Whitney U test

| Relation | Z  |
|----------|----|
| BAP5cmT5 | -5.33*** |

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -5.33*** | -2.00*** |
| BAP4cmT4 | -1.14*** | -2.00*** |
| BAP3cmT3 | 0.07*** | -2.03*** |
| BAP2cmT2 | -1.52*** | -3.50*** |

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

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -5.57** | -3.50* |
| BAP4cmT4 | 1.40*** | -1.12*** |
| BAP3cmT3 | -1.14*** | -2.00*** |
| BAP2cmT2 | 0.07*** | -2.03*** |
| BAP1cmT1 | -1.52*** | -3.50*** |

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

Discussion

No previous studies have reported the effects of mT on aromatic plants. According to our results, mT 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 mT on root development was detected during the multiplication process. The number of internodes formed on T3, T4, T5 was significantly lower than the previous studies.

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

| Relation | T  |
|----------|----|
| BAP5cmT5 | -3.81*** |

Tap-root number - Mann-Whitney U test

| Relation | Z  |
|----------|----|
| BAP5cmT5 | -3.50* |

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -5.57** | -3.50* |
| BAP4cmT4 | 1.40*** | -1.12*** |
| BAP3cmT3 | -1.14*** | -2.00*** |
| BAP2cmT2 | 0.07*** | -2.03*** |
| BAP1cmT1 | -1.52*** | -3.50*** |

Leaf numbers - Mann-Whitney U test

| Relation | Z  |
|----------|----|
| BAP5cmT5 | -5.33*** |

| Relation | T  | Z  | ns  |
|----------|----|----|-----|
| BAP5cmT5 | -5.33*** | -2.00*** |
| BAP4cmT4 | -1.14*** | -2.00*** |
| BAP3cmT3 | 0.07*** | -2.03*** |
| BAP2cmT2 | -1.52*** | -3.50*** |

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

Discussion

No previous studies have reported the effects of mT on aromatic plants. According to our results, mT 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 mT on root development was detected during the multiplication process. The number of internodes formed on T3, T4, T5 was significantly lower than the previous studies.

References

1. Bairu et al., (2007) demonstrated that mT induced shoot multiplication enhanced root-growth, reduced hyperhydricity and stimulated the acclimatization in the case of Aloe polyphylla, Mutui et al., (2012) compared the effects of mT with thidiazuron (TDZ) on post-harvested Pelargonium x hortorum cuttings: leaves treated with mT and TDZ contained higher chlorophyll amount than the controls. They observed that mT 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 mT and the lengths of the side shoots were also higher in most cases utilizing mT. With previous studies

Valero-Aracama et al., (2010) observed that a high concentration of mT has an inhibitory effect on the root-growth process and influences acclimatization of sea oats...
(Uniola paniculata). In our experiments, no deleterious effects due to mT on the sweet basil root-growth process were detected. In the case of mT treatments, the T2 medium - with 2.07 µM concentration and 0.5 mg/l level of supplemented mT - 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 mT 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) mT 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 mT and BAP were detected. Further research is needed to establish the effects of mT on sweet basil acclimatization, which is a key step in successful micropropagation.

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