THE EFFECT OF CYTOKININS ON MICROPROPAGATION SUCCESS OF HIGHBUSH BLUEBERRY (*Vaccinium corymbosum* L.)

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Abstract. The aim of this work was to determine the effect of zeatin (Z), dihydrozeatin (DHZ) and 2-isopentenyl-adenine (2 iP) on shoot length and proliferation rate (PR) in three *Vaccinium corymbosum* L. cultivars: 'Blueray', 'Duke' and 'Patriot'. The culture medium used in this research was prepared from stock solution: Woody Plant Medium (WPM) supplemented with 5 mg/l 2iP, 0.25 mg/l Z, 0.5 mg/l Z, 0.25 mg/l dihydrozeatin (DHZ), 0.5 mg/l DHZ and a combination of 0.5 mg/lZ + 0.25 mg/l DHZ. The results show that the lowest PR (1.26±0.03) was recorded in 'Duke' on the medium containing 0.25 mg/l DHZ, while the highest PR (4.26±0.10) of this variety was recorded on the medium supplemented with a combination of 0.5 mg/l zeatin and 0.25 mg/l DHZ. The highest average length of the shoots (3.82±0.07) recorded was also on this medium. Among the treatments, the highest PR (3.66±0.07) induced by a single cytokinin was obtained on WPM+5 mg/l 2iP. Similar results were obtained in 'Blueray' undergoing the same treatment with hormone combinations (0.5 mg/l zeatin and 0.25 mg/l DHZ); the highest PR recorded was 4.1±0.23. On the contrary, 'Patriot' showed the highest proliferation rate (5.06±0.11) on the medium supplemented with 5 mg/l 2iP, but the average length of the shoots was slightly lower (4.21%) than that on the medium with hormone combinations.

Keywords: 2-isopentenyl-adenine, dihydrozeatin, zeatin, proliferation, blueberry

INTRODUCTION

Micropropagation is a valuable method for large scale multiplication of many plant species, but the appropriate use, type and concentration of growth regulators and the combination of culture medium salts that allows fast, efficient development of the initial explants are crucial in tissue culture techniques (Schuchovski, and Biasi, 2019).

Cytokinins are key regulators of plant growth and development (Kieber and Schaller, 2014). Most of the studies were carried out investigating the effect of various cytokinins on shoot multiplication efficiency: the use of zeatin, 2-isopentenyl-adenine, kinetin and 6-Benzylaminopurine (Eccher et al., 1986; Eccher and Noe, 1989; Gajdosova et al., 2006; Ghosh et al., 2018; Meiners et al., 2007, Ostrolucka et al, 2002; Reed and Abdelnour-Esquivel, 1991; Ružić et al., 2012, Schuchovski, and Biasi, 2019; Vescan et al., 2012). Among the cytokinins, the most efficient ones in highbush

blueberry micropropagation proved to be zeatin and 2-isopentenyladenine (Wang et al., 2019).

The aim of this work was to determine the effect of zeatin (Z), dihydrozeatin (DHZ) and 2-isopentenyl-adenine (2 iP) on shoot length and proliferation rate (PR) in three *Vaccinium corymbosum* L. varieties: 'Blueray', 'Duke' and 'Patriot'.

MATERIAL AND METHOD

The plant material used to carry out this experiment was obtained from the in vitro–regenerated plants of three highbush blueberry (*Vaccinium corymbosum* L.) cultivars ('Blueray', 'Duke' and 'Patriot'). The mini-shoots with lengths of 1.5 to 2 cm were taken from the 10-weeks-old in vitro cultured plantlets on a medium prepared from stock solutions with the following composition: WPM (Lloyd and McCown, 1980), 100 mg/l Sequestrene 138 (FeNaEDDHA 6%), 100 mg/l myo-inositol, 2 mg/l vitamin B1, 1 mg/l vitamin B6, 1 mg/l nicotinic acid, 1 mg/l zeatin and 3% (w/v) of sugar, pH=5.

In order to determine the effect of cytokinins on micropropagation success of highbush blueberry plants, 6 treatments were applied, all of them having WPM (described above) as basal medium supplemented with 2 iP, Z and DHZ in the following concentrations:

Table 1.

Treatments applied for in vitro propagation of three highbush blueberry (*Vaccinium corymbosum* L.) cultivars

Cultivar/ Treatments	'Duke'	'Blueray'	'Patriot'
1	5 mg/l 2iP	5 mg/l 2iP	5 mg/l 2iP
2	0.5 mg/l Z + 0.25 mg/l DHZ	0.5 mg/l Z + 0.25 mg/l DHZ	0.5 mg/l Z + 0.25 mg/l DHZ
3	0.25 mg/l Z	-	-
4	0.5 mg/l Z	-	-
5	0.25 mg/l (DHZ)	-	-
6	0.5 mg/l DHZ	-	-

All these six treatments were applied first to obtain preliminary results for 'Duke', and then applied for the other two cultivars ('Blueray' and 'Patriot'). In order to evaluate the efficiency of the treatments the number of proliferated shoots/explant and their lengths were recorded. Among the above mentioned treatments the longest shoots and the highest proliferation rate were recorded on the media supplemented with 5 mg/l 2iP and with the combination of 0.5 mg/l Z + 0.25 mg/l DHZ. These two treatments were further applied for 'Blueray' and 'Patriot' cultivars to evaluate the micropropagation success.

The gelling agent used in the treatments was 4 g/l of Plant agar and the pH of the medium was adjusted to 5 prior to the autoclave run.

All the chemicals were purchased from Duchefa Biochemie BV, The Netherlands.

The culture vessels used in this experiment were 720 glass jars, with a diameter of 9 cm and a height of 13.5 cm with sponge-vented screw caps. In each vessel, 100 ml of culture medium was dispensed for culturing. The in vitro cultures were incubated in growth room for a 16-h photoperiod with 32.4 $mmol \cdot m^{-2} \cdot s^{-1}$ light intensity (Philips CorePro LED tube 1200 mm 16W 865 CG (1600 lm Cool daylight) at 23 ± 3 °C and 50% ± 2% humidity.

Ten explants per jar with a length of 1.5 to 2 cm were inoculated into the culture medium in such way that two-thirds to three-fourths of the basal part of the explants to be in contact with the culture medium.

The experimental design consisted of 10 inoculated shoots/vessel and 5 vessels/ treatment. The average shoot length and proliferation rate was measured and calculated after 10 weeks of in vitro culture.

Statistical analysis. ANOVA was performed first to check if there were any differences between the means followed by Tukey's HSD test (α=0.05) to determine the statistically significant differences between the means. Values shown are means ± SE.

RESULTS AND DISCUSSIONS

Various phytohormonal regulators of plant growth have been used in tissue culture media to investigate their effect on shoot growth and proliferation rate. Among these, cytokinin is one of the growth regulators which has been used less, especially the reduced form of zeatin, the dihydrozeatin (Sirvastava, 2002). To the best of our knowledge this is the first study which investigates the effect of dihydrozeatin added to the culture medium used for micropropagation of highbush blueberry plants. DL-Dihydrozeatin (DHZ) is a naturally occuring cytokinin that is generally very active. DHZ derivatives are commonly found in plant tissues and are frequent metabolites of applied zeatin. In a bioassay, DHZ and its conjugates are equally active as their zeatin analogues. In studies where DHZ has been externally supplied to plants it appears to be more 'stable' than zeatin. This phenomenon might be explained by the fact that DHZ is not a substrate for cytokinin oxidase. DHZ may be important in the maintenance of cytokinin activity levels in an oxidative environment (https://www.duchefa-biochemie.com/product/details/number/D0933).

The results of our study show that DHZ when added to the culture medium in a quantity of 0.25 mg/l and 0.5 mg/l proliferation rates (PR) were smaller than those recorded on the medium supplemented with Z. However, the combination of 0.5 mg/l zeatin and 0.25 mg/l DHZ added to the culture media led to a higher proliferation rate in 'Duke' (Fig. 1). The lowest PR (1.26±0.03) was recorded in 'Duke' on the medium containing 0.25 mg/l DHZ, while the highest PR (4.26±0.10) of this variety was recorded on the medium supplemented with a combination of 0.5 mg/l zeatin and 0.25 mg/l DHZ. These results are in accordance with previous results reported by Doina et al. (2018) where the average shoot number recorded on WPM medium supplemented with 1 mg/l Z was 5.72 shoots/explant, indicating a high efficiency of this phytohormone in tissue cultures.

Another study suggests (Ostrolucká et al., 2004) that 'Duke' cultivar when cultured on Anderson (1980) culture medium supplemented with 0.5 mg/l Z or 2 IP generated similar amounts of shoots/explant, their number ranging from 2 to 4.

Regarding the length of the regenerated shoots, our results show that the highest average length of the shoots (3.82±0.07) was recorded on the medium supplemented with the combination of 0.5 mg/l zeatin and 0.25 mg/l DHZ (Fig 2.) which are in accordance with confirms previous findings reported by Clapa et al. (2018) when on the same culture medium supplemented with 1 mg/l Z 'Duke' the average regenerated shoot length per explant was 3.88 cm. Among the treatments, the highest PR (3.66±0.07) induced by a single cytokinin was obtained on WPM+5 mg·L^{-1} 2iP (Fig. 1., Fig. 3.).

Similar results were obtained in 'Blueray' variety as well, undergoing the same treatments with hormone combinations (0.5 mg/l zeatin and 0.25 mg/l DHZ); the highest PR recorded was 4.1±0.23 as shown in Fig. 4. These values are higher than those reported by Ostrolucká et al. (2004) when 'Blueray' highbush blueberry cultivar was cultured in vitro on Anderson culture medium with 2 mg l-1 zeatin or 15 mg l-1 2iP when in both treatments the number of shoots/explant recorded was below 2.

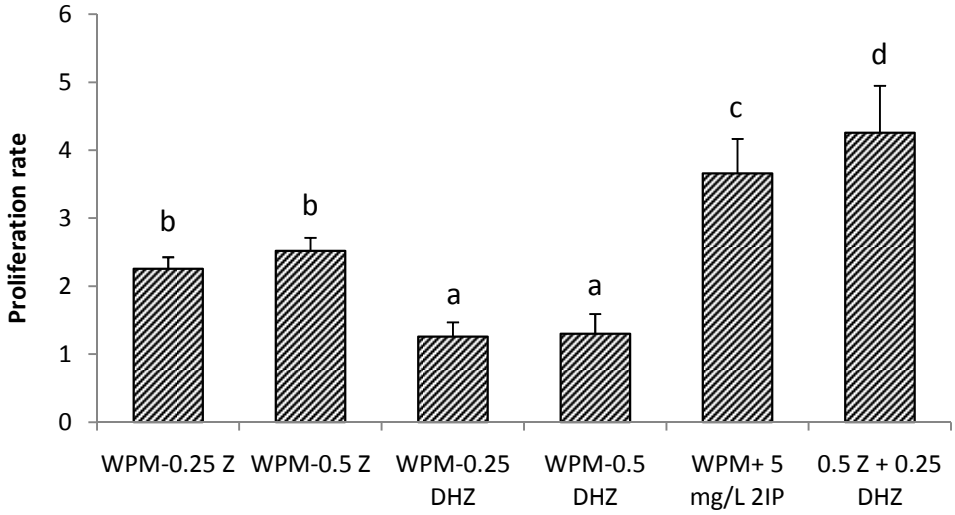

Fig. 1. The influence of growth regulators on in vitro proliferation rate in 'Duke' highbush blueberry. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences betweenthe treatments according to Tukey's HSD test (α = 0.05).

On the contrary, 'Patriot' showed the highest proliferation rate (5.06±0.11) on the medium supplemented with 5 mg/l 2iP as presented in Fig. 4. Regarding average shoot length, the result show that the medium supplemented with 0.5 mg/l Z + 0.25 mg/l DHZ induced better shoot regeneration in 'Duke' and 'Blueray', their lengths being significantly bigger than those cultured on the medium supplemented with 5 mg/l 2iP excepting 'Patriot', where no statistically significant differences were recorded between shoot lengths from the two treatments (Fig. 5, Fig. 6.).

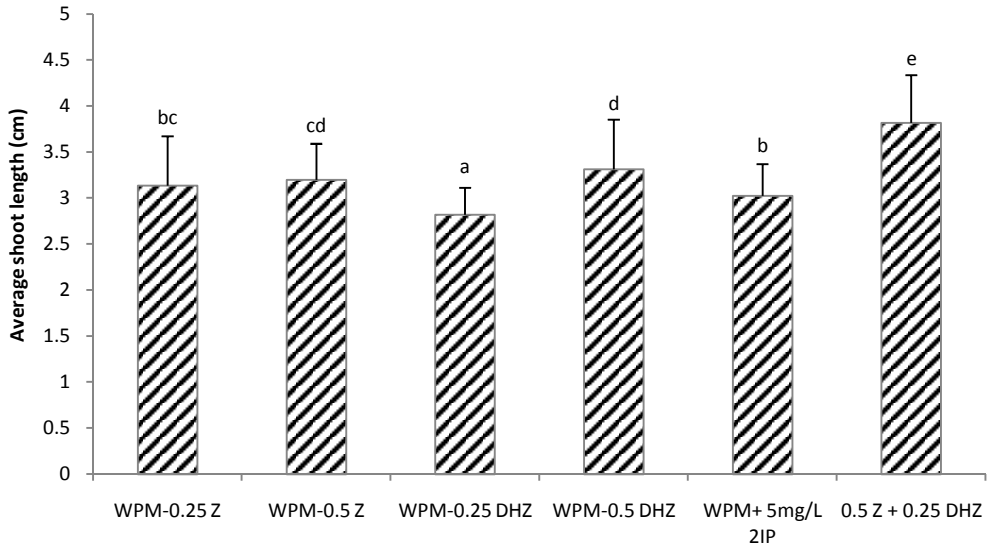

Fig. 2. The influence of growth regulators on shoot length of in vitro propagated 'Duke' highbush blueberry. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the treatments according to Tukey's HSD test (α = 0.05).

Fig. 3. In vitro cultured *V. corymbosum* L. 'Duke' plants from the six treatments applied: WPM medium + 0.25 mg/l DHZ (A), WPM medium + 0.5 mg/l DHZ (B), WPM medium + 0.25 mg/l Z (C), WPM medium + 0.5 mg/l (D), WPM medium + 5 mg/l 2iP(E), WPM medium + 0.5 mg/l Z + 0.25 mg/lDHZ (F)

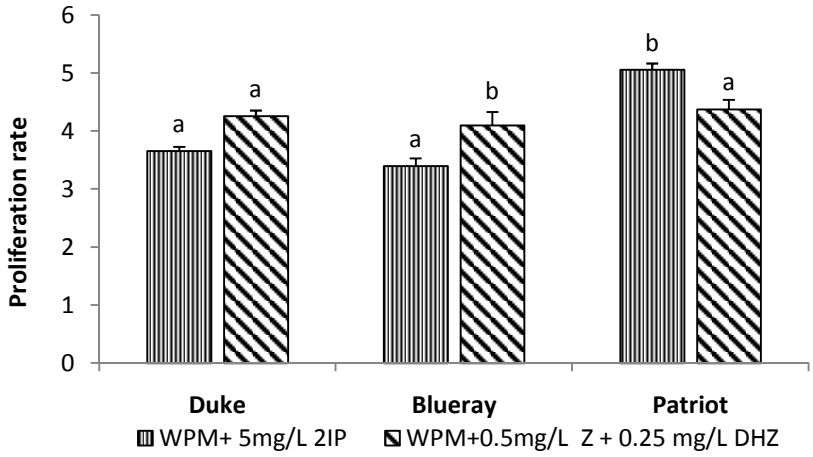

Fig. 4. The influence of 2iP, Z and DHZ on the proliferation rate in the three highbush blueberry cultivars. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the treatments according to Tukey's HSD test (α = 0.05).

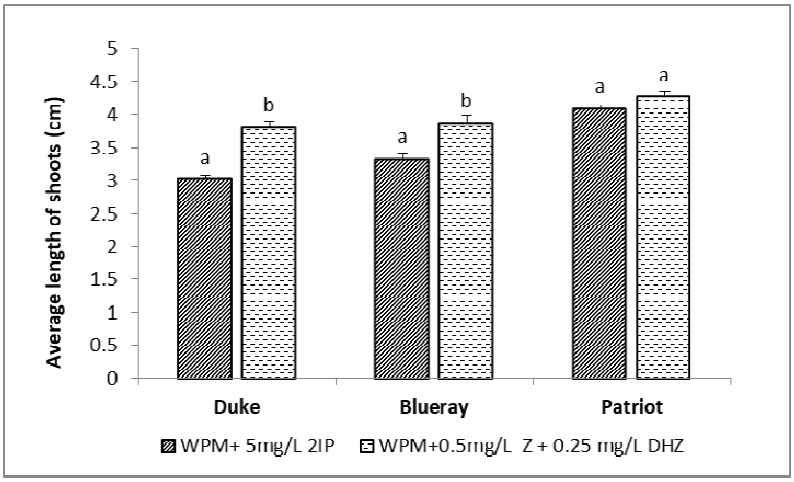

Fig. 5. The influence of 2 iP, Z and DHZ on in vitro shoot length in 'Blueray', 'Duke' and 'Patriot'. The values shown are means ± SE. Different lowercase letters above the bars indicate significant differences between the treatments according to Tukey's HSD test (α = 0.05)

CONCLUSIONS

In conclusion our results show that dihydrozeatin in combination with zeatin can successfully be used in the micropropagation of some highbush blueberry varieties such as 'Blueray' 'Duke' but in 'Patriot' the application of 2iP lead to a higher proliferation rate (5.06±0.10) than those obtained on hormone combinations (4.38±0.16).

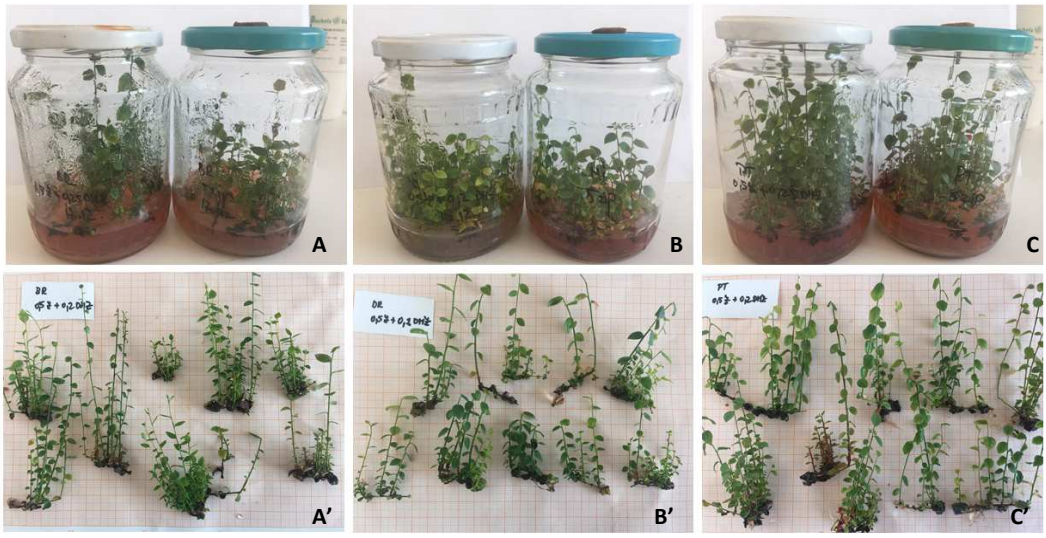

Fig 6. In vitro cultured *V. corymbosum* L. 'Blueray' (A-A') 'Duke' (B-B'), and 'Patriot' (C-C') on the WPM medium + 0.5 mg/l Z + 0.25 mg/l[1] DHZ

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