EFFECT OF CHITOSAN AND META-TOPOLIN IN MICROPROPAGATION OF VACCINIUM CORYMBOSUM

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Abstract

The culture medium is often optimised to improve growth and morphogenesis in vitro. The efficiency of chitosan (CH) and meta-topolin (mT) on in vitro growth of blueberries (Vaccinium corymbosum) cv Liberty was investigated. The explants were grown on Woody Plant Medium (WPM) supplemented with mT at a concentration of 0.2, 0.4, and 0.6 mg l^{-1} and WPM with chitosan with a molecular weight 3.33 kDa at 10, 20, and 40 ppm. WPM with the addition of 0.1 mg l^{-1} zeatin was used as a control. Among the tested combinations, the maximum mean shoot length (23.81 cm), the number of new shoots (1.34), fresh mass (42.29 g), and dry mass (14.24 g) were recorded on the control medium. The addition of lower concentrations of chitosan (10 and 20 ppm) to the WPM medium decreased shoot length (23%-24.5% of control), fresh mass (30%-40% of control), and dry mass (20%-29% of control). However, blueberries treated with 40 ppm chitosan had similar shoot lengths, but darker (112% of control) and greener (139% of control) leaves compared with the control. In contrast, mT had a negative effect on the studied traits of Liberty blueberries, regardless of the concentration.

Keywords: *chitosan, in vitro, blueberry, cytokinin*

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1. Introduction

Blueberries are horticultural plants belonging to a genetically diverse group of woody shrubs that produce a range of products of considerable value to humankind [1, 2]. Due to the high nutritional value and health benefits of blueberries, there has been a marked increase in interest in the mass production of blueberries. The production of high-quality plants requires the adoption of modern propagation techniques. The use of *in vitro* cultures eliminates the limitations associated with traditional seedling propagation, providing an alternative to faster plant growth throughout the year, which increases production efficiency and profitability [3]. The best plant growth under *in vitro* conditions can be achieved by using the right medium composition for the species and even the cultivar. Research has focussed on selecting an optimal medium and the concentration of main cytokinins to obtain high multiplication efficiency of blueberry shoots [1, 2, 4]. Most researchers recommend the use of zeatin as a general growth hormone for research and commercial purposes [4-7]. *Meta*-topolin (6-(3-hydroxylbenzylamino) purine) is an aromatic cytokinin that differs from isoprenoid cytokinins such as zeatin and 2-isopentenyladenine (2-iP) in its biochemical and biological activity. It was originally isolated from the leaves of poplar trees. Topolins have been shown to positively influence shoot proliferation, to maintain hormonal/histogenic stability, and to improve the rooting efficiency of explants [8]. According to Kruczek *et al.* [8], Bairu *et al.* [9], and Nowakowska and Pacholczak [10], the activity of *m*T may be comparable to that of zeatin.

There are fewer reports describing the application of chitosan *in vitro* for shoot multiplication of blueberry plants. Chitosan is a natural biopolymer that is completely safe for the environment. It is a polysaccharide derivative from the shell of crustaceans, insects, fish scales, or fungus. Chitosan has been recognised as a product to enhance crop production because it stimulates the growth of plants and seed germination, increasing chlorophylls and photosynthesis, enlarging chloroplasts, and reducing stress [8, 11, 12].

The present study compared the effects of 0.1 mg l^{-1} zeatin, chitosan, and *m*T on micropropagation of blueberry explants *in vitro*. Optimal medium, cytokinin requirements, and chitosan treatment were identified for multiplication of *Vaccinium corymbosum* cv. Liberty.

2. Materials and Methods

2.1. Plant Material

The plant material consisted of 17-20 mm shoots of *V. corymbosum* cv. Liberty obtained from sterile stabilised *in vitro* culture. Shoots were proliferated by culturing on McCown Woody Plant Medium (WPM) [13] (Duchefa Biochemie B.V, the Netherlands) supplemented with 0.1 mg l^{-1} zeatin and regularly transferred onto fresh medium every 4 weeks for 4 months to prepare the explants for subsequent experiments. After that, the shoot explants were transferred to WPM with the addition of *m*T at a concentration of 0.2, 0.4, or 0.6 mg L^{-1} and WPM with the addition of chitosan with a molecular weight 3.33 kDa at the concentration of 10, 20, or 40 ppm. Chitosan was added to the medium before autoclaving. WPM with 0.1 mg l^{-1} zeatin and without the addition of *m*T and CH was the control. All WPM was supplemented with 3% (w/v) sucrose (Chempur, Poland), 0.8% agar (Biocorp, Poland), and 100 mg l^{-1} myo-inositol (Duchefa). After 35 days, the shoot length, the number of new shoots, the fresh and dry mass, and the colour were measured. The dry mass of explants was determined after drying in hot-air oven at 70ºC for 24 h.

The pH of all media was adjusted to 5.8. Culture jars (300 ml) with the medium (30 ml) were autoclaved for 20 min at 121°C and 0.1 MPa. All cultures were incubated in a growth room at 24 ± 1°C under a 16-h photoperiod with a photosynthetic photon flux density

(PPFD) of 40 µmol m^{-2} s^{-1} provided by a Narva lamp (Germany) emitting cool white daylight. Each combination included 32 shoots (8 replications with 4 explants per flask).

2.2. Chitosan

Chitosan was obtained from the Center of Bioimmobilisation and Innovative Packaging Materials at the West Pomeranian University of Technology in Szczecin, Poland. The products were obtained according to procedure described by Bartkowiak [14] using the free-radical degradation process. The chitosan was purified (filtration) and characterised using high-performance liquid chromatography (SmartLine Knauer, Germany; Tessek Separon HEMA-BIO 40 column, Tessek, Czech Republic). The average degree of deacetylation of the product was 85%.

2.3. Determination of Colour

The pigment (colour) of leaves (from the middle part of the shoot) was measured in transmission mode by photocolourimetric method with the CIE *L*a*b** system [15] using a CM-700d spectrophotometer (Koncia Minolta, Japan). The diameter of the measurement hole was 3 mm, the observer type 10º, and the illuminant D65. The parameter *a** describes the colour from green (-*a**) to red (+*a**). The parameter *b** describes the colour from yellow (+*b**) to blue (-*b**). The parameter *L** indicates monochromaticity from 0 (black) to 100 (white).

2.4. Statistical Analysis

All statistical analyses were performed using Statistica 13.0 (StatSoft Polska, Poland). The data were first tested to ensure it had a normal distribution and homogenous variance. Analysis of variance followed by Tukey's range test was used to compare the groups; $p < 0.5$ was considered significant.

3. Results and Discussion

A key aspect of the rapid multiplication of difficult-to-propagate species is optimisation of the composition of the growth medium. The genus *Vaccinium* is characterised by greater variability than other species in the family Ericaceae, whereby differences in multiplication medium requirements may also occur within cultivars [1]. Based on our literature survey, there is less research on the application of chitosan and/or *m*T for *in vitro* shoot multiplication of blueberry plants. Efficient *in vitro* shoot multiplication is generally strongly influenced by the presence of cytokinins (zeatin is used most often) in the medium [4, 6]. They stimulate dormant meristems to form new shoots after inhibition of apical dominance [16]. However, according to Varaporn and Kudan [12], chitosan may play a role as a bud break factor in explants grown *in vitro*.

The mean shoot length, the number of new shoots, and fresh and dry mass are presented in Table 1. Out of all the chitosan concentrations applied in the experiment, 40 ppm was the most efficient in stimulating the shoot length (Figure 1). Explants grown on WPM with 40 ppm chitosan had a similar height (21.86 cm) as control plants (23.81 cm). Plants from the other medium combinations had on average 23%-40% shorter shoots compared with the control. Chitosan had a favourable effect on new shoot formation and fresh and dry mass compared with *m*T. As the chitosan concentration increased, the number of new shoots and the fresh and dry mass increased, although the differences were not significant. However, the highest mean number of new shoots (1.34) and mean fresh and dry mass (42.29 and 14.24 g, respectively) were obtained for blueberry plants grown on the control medium (Table 1). As the concentration of *m*T in the medium increased, the number of new shoots and the fresh and dry mass decreased.

Table 1. Effect of different medium compositions on morphological traits of *Vaccinium corymbosum* cv. Liberty plants under *in vitro* condition after 35 days of culture (n = 32 shoots per treatment).

Medium	Shoot length [cm]	No of new shoots	Fresh mass [g]	Dry mass [g]
WPM+0.1 mg L^{-1} zeatin	23.81a	1.34a	42.29a	14.24a
WPM+0.2 mg L^{-1} *m*T	14.97c	1.03b	18.17c	6.34c
WPM+0.2 mg L^{-1} *m*T	14.18c	1.00b	15.93c	5.68c
WPM+0.2 mg L^{-1} *m*T	14.97c	1.00b	14.81c	5.56c
WPM+10 ppm CH	18.34b	1.10ab	25.42b	10.14b
WPM+20 ppm CH	17.97b	1.16ab	27.45b	10.42b
WPM+40 ppm CH	21.86a	1.18ab	29.45b	11.73b

Note. Means followed by the same letter do not differ significantly according to Tukey's range test ($p > 0.05$).

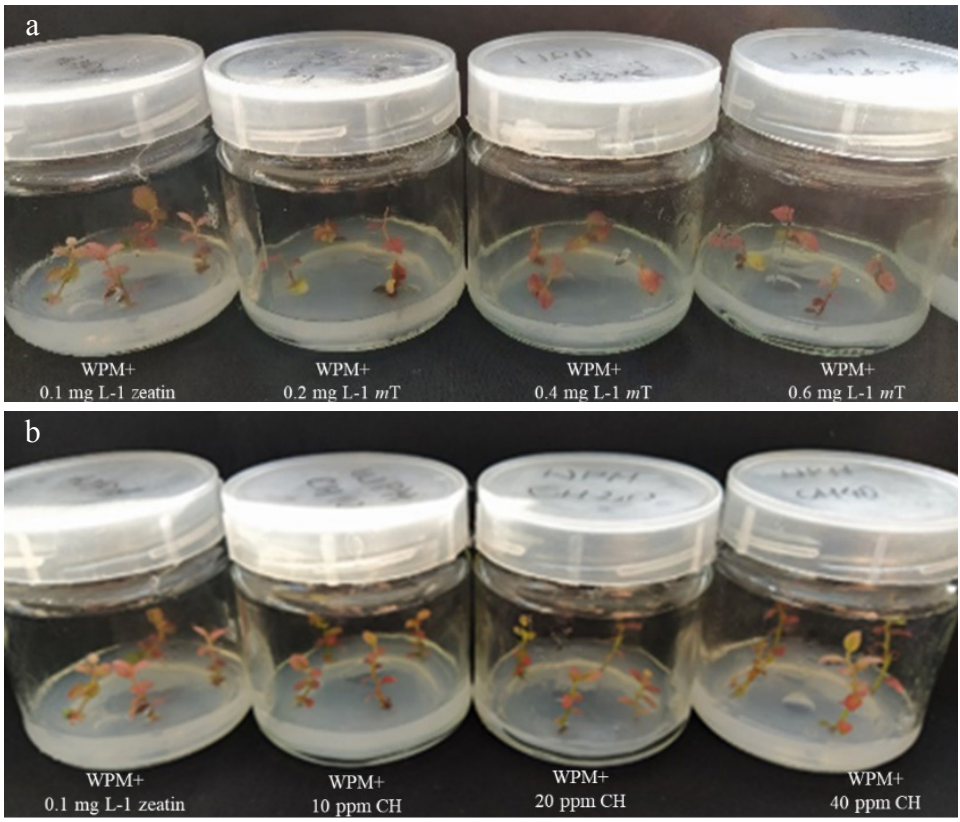

Figure 1. Shoot cultures of *Vaccinium corymbosum* cv. Liberty on different Woody Plant Medium compositions (a and b) after 35 days of culture.

As suggested by many authors [1, 2], WPM supplemented with zeatin was most favourable for the multiplication of *V. corymbosum* explants. The present study also confirmed that the addition of zeatin promotes the most efficient multiplication of blueberry plants. As opposed to *m*T, chitosan could be used to enhance axillary shoot growth, providing similar results to zeatin. Many authors have reported that chitosan elicits the growth of different plant species *in vitro*. Obsuwan *et al.* [17] described the stimulating effect of chitosan on the morphology of *Rhynchostylis gigantea* protocorms propagated *in vitro*. Sopalun *et al.* [18] suggested that chitosan promoted *in vitro* shoot formation of *Grammatophyllum speciosum*. Krupa-Małkiewicz and Fornal [11] confirmed that 15 ppm of chitosan (independent of its molecular weight) could function as a plant growth stimulator for petunia micropropagation. Veraplakorn and Kudan [12] described that 0.1 mg l^{-1} high-molecular-weight chitosan produced the highest shoot number with a long length of *Lantana camara* L. *in vitro*. On the other hand, Kruczek *et al.* [8] described that MS medium [19]supplemented with 20 ppm of chitosan at a molecular weight of 10 kDa was optimal for the initiation of goji explant rhizogenesis.

According to Krupa-Małkiewicz and Calomme [20], the contents of photosynthetic pigments in leaves are closely correlated to their colour. Hence, measuring the colour provides a way to assess the condition of the plants. The leaf colour was analysed in the transmitted mode using the photocolourimetric method in the CIE *L*a*b** system [21]. According to Ochmian *et al.* [22], the change in *L** is usually related to physiological attributes of the visual appearance of brightness. In this study, an increase in the *m*T or chitosan concentration decreased *L** (from 46.19 to 42.9 and from 36.1 to 35.21, respectively) compared with the control (39.84) (Figure 2a). Moreover, all blueberry explants grown on WPM containing chitosan had greener leaves (from 36% to 41% higher) compared with the control (7.36) (Figure 2b). This is shown by the value of *a**, which values colour ranging from green (negative *a**) to red (positive *a**). The leaf surface colour defined by b^* indicates the location along the axis between yellow and blue. Blueberry leaves growing in WPM containing 10 or 20 ppm chitosan had higher *b** (52% and 6%, respectively) compared with the control (10.49). Explants grown in WPM containing *m*T were bluer compared with the control. The results obtained in this study are comparable to the results reported by Kruczek *et al.* [21] in goji leaves *in vitro* (*L** from 34.57 to 42.36). However, the blueberry leaves examined in this study were less green than goji leaves *in vitro* (*a** from -38.94 to -43.56).

4. Conclusion

In terms of shoot multiplication, zeatin was superior to chitosan and *m*T. In general, all chitosan treatments were more effective than *m*T in stimulating the frequency of shoot induction. The highest concentration of chitosan (40 ppm) produced longer shoots, more shoots, and greater fresh and dry mass compared with the lower chitosan concentrations. The addition of any *m*T concentration to WPM produced the worst results. Our results have shown that using chitosan at the highest concentration of 40 ppm, as a complex of organic substances, can be successfully used as an agent for *in vitro* multiplication of Liberty blueberry plants and stimulates plant growth with a similar effect to zeatin.

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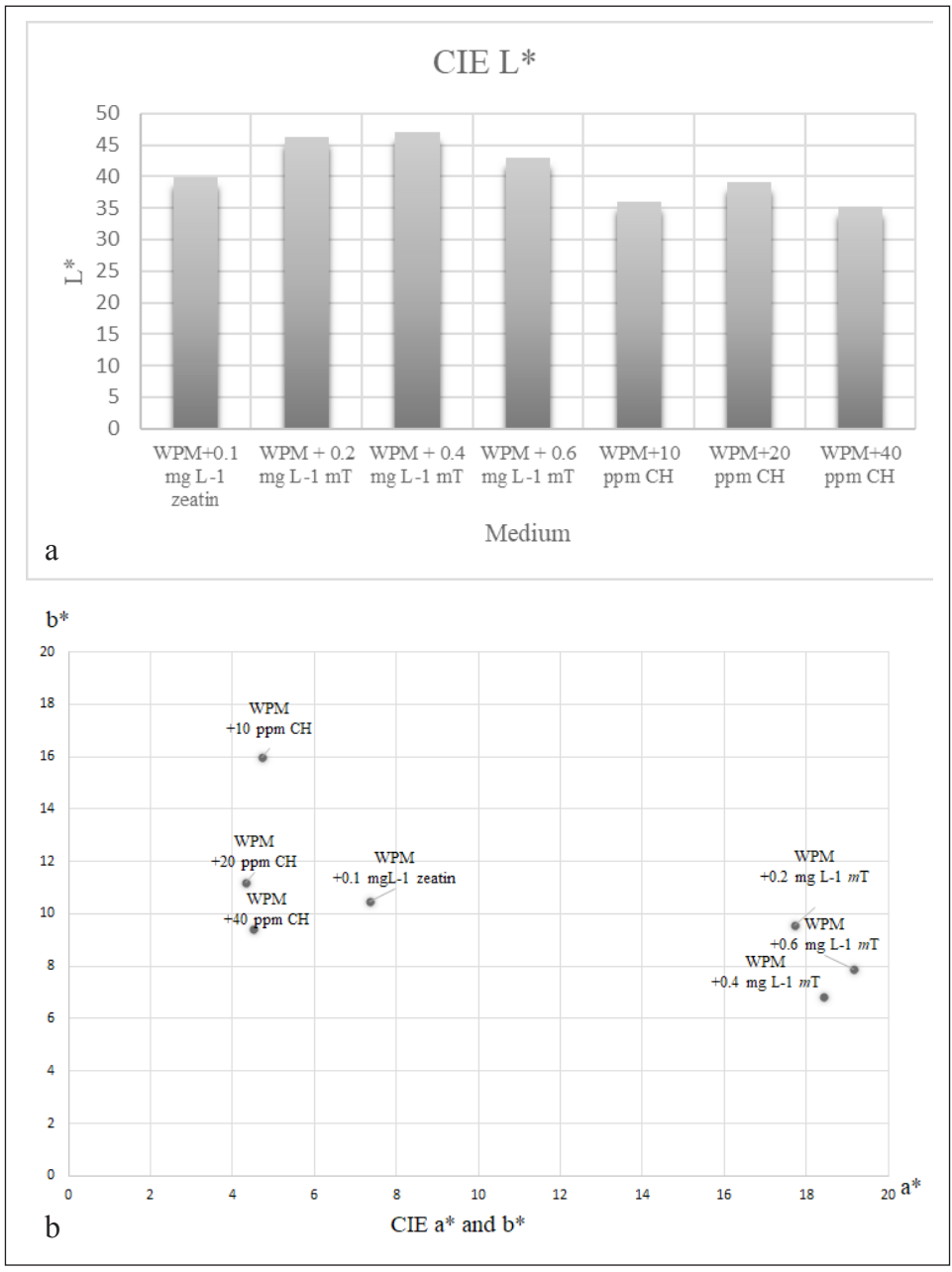

Figure 2. Effect of different medium compositions on the leaf colour of *Vaccinium corymbosum* cv. Liberty plants (n = 32 shoots per treatment), using the CIE *L*a*b** system. The graphs present *L**, the lightness coefficient (a), and *a** (green colour) and *b** (yellow colour) (b) at the end of the experiment.

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