ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Hortorum Cultus 8(3) 2009, 3-12*

DEVELOPMENT OF HIGHBUSH BLUEBERRY (*Vaccinium corymbosum* hort. non L.) *in vitro* SHOOT CULTURES UNDER THE INFLUENCE OF MELATONIN

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Abstract The influence of melatonin (MEL) in comparison with IAA, IBA (all at 5.71 μM), and auxin-free (0) medium on development of highbush blueberry (*Vaccinium corymbosum* hort. non L.) 'Herbert' *in vitro* shoot cultures was examined. Depending on the kind of hormone *in vitro* cultures consisted of various number of axillary (AX) and adventitious (AD) shoots. The influence of melatonin on *in vitro* cultures was intermediate but more similar to IAA than IBA. Production of axillary shoots on media supplemented with MEL and IAA was comparable and higher than on medium with IBA. In contrast to IAA melatonin reduced development of adventitious shoots. Contrary to IBA-obtained cultures AX shoots grown on 'IAA', '0', and 'MEL' media resembled AD shoots.

Keywords: micropropagation, axillary shoots, adventitious shoots, auxins, melatonin, highbush blueberry

INTRODUCTION

Numerous studies regarding blueberry (*Vaccinium* sp.) *in vitro* cultures have been published so far. In general they aimed at improvement of shoot multiplication efficiency or elaboration the method of adventitious shoot development. Most of them concerned effect of various cytokinins. Much less attention was paid to the influence of different auxins. In general media supplemented with indole-3-acetic acid (IAA) [Zimmerman and Broome 1980; Orlikowska 1986] or without auxins [Hosier et al. 1985, Debnath and McRae 2001] are used. The only Marcotrigiano and McGlew [1991] studied (and briefly described) the influence of other auxin – IBA on *in vitro* cultures of *Vaccinium* genus (in the case of *Vaccinium macrocarpon*). They mentioned that IBA was useful in the initial culture stage. Recently Litwińczuk and Wadas [2008] found that IBA facilitates micropropagation of highbush blueberry *cv.* Herbert exclusively through

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axillary shoots as, contrary to IAA, reduces development of adventitious shoots and makes them easier distinguishable from axillary shoots. Melatonin has molecular structure similar to IAA and IBA, is present in plant tissues, and may act as auxin [hernandez-ruiz *et al.* 2004; janas *et al.* 2005; arnao and Hernandez-Ruiz 2007; Posmyk and Janas 2009]. As a medicine is also not as toxic as IBA. Therefore it might be useful for *in vitro* plant culture. Thus the purpose of current study was to examine the effect of melatonin on *in vitro* axillary and adventitious shoot development of 'Herbert' highbush blueberry.

MATERIAL AND METHODS

The experiments were carried out on *in* vitro cultures of highbush blueberry (*Vaccinium corymbosum* hort. non L., syn. *Vaccinium* × *covilleanum* But. *et* Pl.) [Butkus and Pliszka 1993] *cv*. Herbert. The influence of melatonin (MEL, 5.71 µM), indole-3--acetic acid (IAA, 5.71 µM), indole-3-butyric acid (IBA, 5.71 µM), and auxin-free medium was investigated through three subsequent two-month long subcultures. The basic ZB medium [Zimmerman and Broome 1980] supplemented with N^6-[γ,γ-Dimethylallyl]adenosine (2iP, 49.2 µM), adenine sulfate (AS, 217.2 µM), L-cysteine (41.3 µM), sucrose (87.6 mM), pH 5.0, solidified with Bacto-Difco agar (9.0 $g \cdot dm^{-3}$), was applied. Light was provided by cool white fluorescent lamps (OSRAM) at approximately 22.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (1.9 klux) with a 16 hr photoperiod. Temperature was 26±1ºC. Cultures were grown *in vitro* in glass jars (350 cm^{-3}) with ventilated polypropylene twist lids, filled with medium (50.0 cm^{-3}) and with 10 nodal explants (about 6 mm long and 2–3 nodes) of axillary origin per jar. Each combination was represented by at least 5 jars (min. 50 explants). At the end of passage cultures were took out from jar and the length of the longest axillary shoot from each culture was measured as well as the number of axillary (AX) and adventitious (AD) shoots was determined. Axillary shoots were firmly attached to initial explant and after tearing off a heel was observed at the bottom of shoot (fig. 1). The adventitious shoots were separated onto three different categories. Leaf shoots (AD-L) differentiated on leaves which contacted with the medium (fig. 1). Node-adjoin shoots (AD-P) developed from upper part of explant (not immersed in the medium) from area close to axillary bud but not directly from it. As the base of AD-P shoot was not heeled it could be easily detached from initial explant (fig. 1). Base-adjoin shoots (AD-M, madshoots) developed on the explant base immersed in the medium (fig. 1). The AD-P and AD-L shoots differentiated mostly directly from shoot/leaf tissues than from callus. In the case of AD-M shoots both direct and indirect regeneration was observed. The more detailed description of adventitious shoots is given in the previous work of Litwińczuk and Wadas [2008].

Collected data were subjected to ANOVA, LSD mean separation test at $P = 0.05$ significance level using Statgraphics 4.2 and Statistica 5.1 computer software. Data presented as percentage were analysed after arcsin transformation (ratio of AX shoots) or subjected to test on difference between two proportions (the number of growing cultures, number of cultures developed exclusively AX shoots, etc.). The ratio of AX shoots was calculated according to following formula: 100% × number of AX

Fig. 1. Types of shoots which develop in in vitro cultures of highbush blueberry: IE – initial explant, AX – axillary shoots, AD-L – leaf shoot, AD-P – node-adjoin shoots, AD-M – base-adjoin shoots (madshoots)

Ryc. 1. Rodzaje pędów, które rozwijają się w kulturach in vitro borówki wysokiej: IE – eksplantat wyjściowy, AX – pędy kątowe, AD-L – pędy liściowe, AD-P – pędy przykątowe, AD-M – pędy przypodstawowe (pędy szalone)

shoots/number of shoots (both AX and AD ones). Cluster analysis based on Ward's method and Euclidean distance was used to evaluate similarity of cultures grown on media supplemented with different hormones.

RESULTS

Auxins significantly affected development of blueberry *in vitro* cultures (tab. 1). In general more explants developed shoots on media supplemented with melatonin (MEL) or IAA while compared to other treatments. On the control and IBA-contained medium about 30% explants died or callused. However in such conditions the elongation of axillary (AX) shoots was intensified (tab. 1). Depending on the kind of hormone *in vitro* cultures consisted of various number of axillary (AX) and adventitious (AD) shoots. The proliferation of axillary shoots was most intense on medium with added IAA, in-

Table 1. Biometry of cultures on the media supplemented with different hormones (means from 3 subcultures)

Tab. 1. Biometria kultur otrzymanych na pożywkach z dodatkiem różnych hormonów (średnie z 3 subkultur)

Analysed traits Analizowane cechy		Hormone – Hormon			
		0	IAA 5.71 μM	MEL 5.71 μM	IBA 5.71 μM
Number of growing cultures (%) Liczba rosnących kultur (%)		69.6 a[a]	89.9 b	93.2 b	67.6 a
Axillary (AX) shoots[b]: Pędy kątowe (AX):	Mean length of the longest AX shoot (cm) Średnia długość najdłuższego pędu (cm)	2.1 b	1.8 a	1.9 a	2.2 b
	Number of AX shoots (3-15 mm) Liczba pędów kątowych (3-15 mm)	1.2 b	1.9 c	1.0 ab	0.7 a
	Number of AX shoots (> 15mm) Liczba pędów kątowych (> 15mm)	1.1 ab	0.9 a	1.3 b	1.0 a
	Total number of AX shoots (> 3mm) Łączna liczba pędów kątowych (> 3mm)	2.3 b	2.8 c	2.3 b	1.7 a
	Ratio of AX shoots in cultures (%) Udział pędów kątowych w kulturach (%)	64.0 a	64.7 a	70.6 a	91.5 b
	Number of cultures consisted of exclusively AX shoots (%) Liczba kultur złożonych wyłącznie z pędów kątowych (%)	45.0 ab	37.1 a	51.4 b	84.0 c
Adventitious (AD) shoots Pedy przybyszowe (AD)[b]:	Number of AD-L shoots Liczba pędów typu AD-L	0.0 a	0.0 a	0.0 a	0.1 b
	Number of AD-P shoots Liczba pędów typu AD-P	0.2 a	0.2 a	0.1 a	0.1 a
	Number of AD-M shoots Liczba pędów typu AD-M	1.3 bc	1.5 c	1.1 b	0.1 a
Total number of shoots (regardless their origin) Łączna liczba pędów (bez względu na ich pochodzenie)		3.7 b	4.5 c	3.5 b	2.0 a

[a] different letters indicate significant differences at $P < 0.05$
odmienne litery wskazują na istotne różnice przy $P<0,05$;

[b] description of shoot types – see Material and Methods and Fig. 1
charakterystyka typów pędów – patrz Materiał i Metody oraz rycina 1

termediate on 'MEL' and '0' media, and lowest on IBA-contained medium. However majority of axillary shoots grown in the presence of IAA were short (3–14 mm) whereas on 'MEL' and 'IBA' media prevailed long (> 15 mm) axillary shoots. Spontaneous development of adventitious (AD) shoots was different on studied media. In general IBA greatly suppressed whereas IAA stimulated development of adventitious (AD) shoots with the exception of shoots emerged form leaves. The development of adventitious shoots on the other media ('0', 'MEL') was intermediate. However, contrary to auxin-free medium, 'MEL' medium significantly weakened initiation of adventitious shoots while compared to IAA treatment.

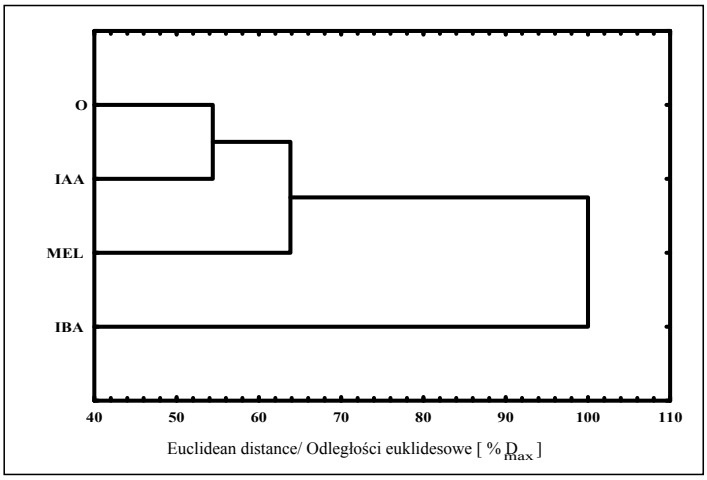

Fig. 2. Similarity of blueberry *in vitro* cultures obtained on media supplemented with different hormones

Ryc. 2. Podobieństwo kultur *in vitro* borówki otrzymanych na pożywkach z dodatkiem różnych hormonów

Cluster analyses revealed that the development of *in vitro* cultures grown in the presence of melatonin was intermediate among IBA and IAA, however more similar to the last one (fig. 2). Cultures obtained in the presence of IBA consisted mainly from axillary (AX) shoots which had relative long (3–6 mm) internodes and rigid, well developed leaves (fig. 3). The adventitious shoots, especially base-adjoin (AD-M) ones, were easily distinguishable as they were more thin and fragile, more or less vitrified, and had shorter internodes and smaller, sometimes unfolded leaves (fig. 3). In contrast to axillary shoots obtained on media with IBA, the AX shoots grown on media free of auxins or supplied with IAA and melatonin were smaller and had shorter internodes (fig. 3).

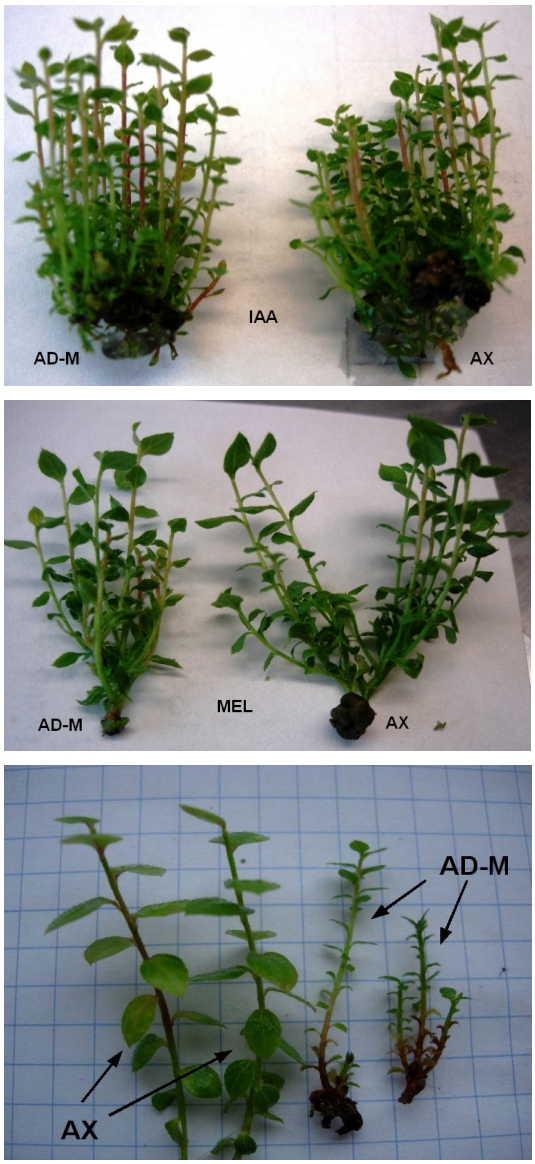

Fig. 3. Base-adjoin adventitious (AD-M) and axillary (AX) shoots obtained on the media supplemented with different hormones

Ryc. 3. Pędy przypodstawowe (AD-M) i pędy kątowe (AX) otrzymane na pożywkach z dodatkiem różnych hormonów

The appearance of melatonin-obtained cultures was intermediate but it tended to become alike to IAA-achieved cultures during successive passages. The axillary and adventitious shoots originated from 'IAA' and 'MEL' media resembled each other (fig. 3).

DISCUSSION

The method of micropropagation of blueberries (*Vaccinium* sp.) was elaborated about thirty years ago [Cohen and Elliot 1979; Zimmerman and Broome 1980]. Authors of numerous studies regarding it usually used more or less modified method described by Zimmerman and Broome [1980], based on subculturing of shoot explants or shoot clumps on the medium supplemented with IAA (2.85–22.8 μM) and 2iP (49.2–73.8 μM) as well as stimulation of shoot elongation on the auxin- and cytokinin-free medium. However, micropropagation carried out through such routine method causes in fact the propagation of blueberries through highly habituted AD-M adventitious shoots (madshoots) [Litwińczuk and Wadas 2008]. Adventitious shoots which develop *in vitro* in cultures of many species are suspected to be the main source of somaclonal variation [De Klerk 1990] and should be therefore eliminated from micropropagation process. Some reports describing differences in bush grow, flowering and yielding among cutting-derived and micropropagated blueberries [Grout et al. 1986; Lyrene and Perry, 1988; Read et al. 1989; Pliszka and Rojek 1994; El-Shiekh et al. 1996; Gustavsson 1999, Litwińczuk 2001; Litwińczuk et al. 2005] might be explained by the adventitious, not axillary origin of in vitro obtained plants.

Recently Litwińczuk and Wadas [2008] found that IBA facilitates micropropagation of highbush blueberry *cv.* Herbert exclusively through axillary shoots as it reduces development of adventitious shoots and makes them easily distinguishable from axillary shoots. It was confirmed again in the present study. However, IBA causes more often dying or callusing of explants and sometimes weakens proliferation of axillary shoots which decreases efficiency of micropropagation. Murch et al. [1997] found that melatonin is present in tissues of some medicinal plants. It is known now that many other plants contain melatonin [Murch and Saxena 2002, Arnao and Hernandez-Ruiz 2007, Janas et al. 2005]. Murch and Saxena [2002] suggested that melatonin together with serotonin might play a role in the morphogenesis of *in vitro* plants. It has also been suggested that MEL is an independent plant growth regulator, probably its action is analogous to IAA and it may mediate the actions of other plant growth regulators [Posmyk and Janas 2009]. Posmyk et al. [2009] found that melatonin improved germination of cucumber seeds.

Kępczyńska et al. [2004] found that melatonin stimulated better than IBA regeneration of adventitious shoots and retarded callus growth in *in* vitro cultured of miniature rose. However, they didn't describe the influence of melatonin on the growth of axillary shoots. Hernandez-Ruiz et al. [2004] and Arnao and Hernandez-Ruiz [2007] gave some evidence that melatonin may work as auxin. Such effect was confirmed in the current study. It is worth mentioning that in comparison with IBA melatonin decreased dying or callusing of explants. Thanks to it the efficiency of production of axillary shoots was much higher and comparable to IAA-usage. Melatonin is also not as toxic as IBA. It is also valuable that in contrast to IAA melatonin reduced development of adventitious shoots. However, in both cases adventitious shoots resembled axillary shoots contrary to IBA-obtained cultures. Thus the risk of confuse axillary and adventitious shoots during culture passage is higher in the case of IAA- and melatonin- than IBA- usage. Melatonin is also more expensive than aforementioned auxins. Thus at present it cannot be

stated whether the application of melatonin will be beneficial in blueberry propagation and breeding using *in vitro* cultures. Such statement demands more detailed and complex study (among others, on the influence of melatonin in different concentrations and its interaction with cytokinins).

Cluster analyses revealed that the development *in vitro* highbush blueberry cultures was similar on media free from auxins and supplemented with IAA and melatonin whereas different in the presence of IBA. It may suggest that both IAA and melatonin are more quickly decomposed in plants and/or destroyed by autoclaving in comparison to IBA. Thus their influence is short-lived. Therefore, it is possible that use of other natural auxins or substances which prevent degradation of IAA as well as other methods of auxin sterilisation or treatment might have benificial effect to cloning blueberries *in vitro*.

To sum up, in the current study it was confirmed that melatonin works as auxin in *in vitro* cultures of highbush blueberry. However, the evaluation of its usefulness blueberry micropropagation and breeding needs more detailed studies.

CONCLUSIONS

1. Depending on the kind of hormone *in vitro* cultures of highbush blueberry consisted of various number of axillary (AX) and adventitious (AD) shoots.
2. Melatonin (MEL) works as auxin in *in vitro* cultures. The influence of melatonin was intermediate and more similar to IAA than IBA.
3. The efficiency of production of axillary shoots on media supplemented with MEL and IAA was comparable and higher than on medium with IBA.
4. In contrast to IAA melatonin reduced development of adventitious shoots.
5. Contrary to IBA-obtained cultures AX shoots grown on ‘IAA’, ‘0’, and ‘MEL’ media resembled AD shoots.

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ROZWÓJ PĘDOWYCH KULTUR *in vitro* BORÓWKI WYSOKIEJ (*Vaccinium corymbosum* hort. non L.) POD WPŁYWEM MELATONINY

Streszczenie W pracy badano wpływ melatoniny (MEL 5.71 µM) na rozwój pędowych kultur *in vitro* borówki wysokiej (*Vaccinium corymbosum* hort. non L. 'Herbert'). Odniesienie stanowiły pożywki zawierające IAA, IBA (5.71 µM) lub wolne (0) od auksyn. W zależności od rodzaju hormonu kultury *in vitro* składały się z różnej liczby pędów kątowych (AX) i przybyszowych (AD). Wpływ melatoniny na kultury *in vitro* był pośredni, choć bardziej podobny do wpływu IAA niż IBA. Wytwarzanie pędów kątowych na pożywkach z dodatkiem melatoniny i IAA było zbliżone i większe niż na pożywce z IBA. W przeciwieństwie do IAA melatonina osłabiała rozwój pędów przybyszowych. Pędy kątowe otrzymane przy użyciu IBA wyraźnie różniły się od pędów przybyszowych. Na pozostałych pożywkach ('IAA', '0' i 'MEL') wygląd pędów kątowych i przybyszowych był podobny.

Słowa kluczowe: mikrorozmnażanie, pędy kątowe, pędy przybyszowe, auksyny, melatonina, borówka wysoka

Accepted for print – Zaakceptowano do druku: 21.08.2009