

# The Influence of Auxins on the Biosynthesis of Isoprene Derivatives in Callus Cultures of *Vaccinium corymbosum* var. *bluecrop*

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Callus cultures of *Vaccinium corymbosum* var. *bluecrop* were optimized for their isoprene derivatives production by supplementing Schenk-Hildebrandt (SH) medium with constant concentration of kinetin (2.32  $\mu\text{M}$ ) and two different amounts of selected auxins. Every auxin, except for IBA, used in 10-time higher concentration (2,4D, NAA, IAA, NOA) stimulated biosynthesis of  $\beta$ -sitosterol and inhibited triterpene synthesis. Quantitative analysis of isoprene derivatives in callus biomass collected on the 25<sup>th</sup> day of the experiment proved that the analyzed callus of *Vaccinium corymbosum* var. *bluecrop* synthesized the highest amount of isoprene derivatives after subculturing on SH medium modified with 22.6  $\mu\text{M}$  of 2,4D and 2.32  $\mu\text{M}$  of kinetin.

**Key words:** Callus Cultures, *Vaccinium corymbosum* var. *bluecrop*, Isoprenes

## Introduction

Tissue cultures of higher plants are increasingly seen as a rich source of valuable secondary metabolites (Dörnenberg and Knorr, 1995; Ramachandra Rao and Ravishankar, 2002), and are, therefore, more and more commonly used to set up production systems to obtain compounds which have documented biological properties. This group of compounds includes triterpenes and steroid compounds (Ramachandra Rao and Ravishankar, 2002).

Of the steroid compounds, the one most commonly used in medicine is  $\beta$ -sitosterol. Materials rich in this compound, as well as the compound itself, are used in prevention and treatment of prostatic gland hypertrophy (Dreikorn *et al.*, 2002; Batista-Miranda *et al.*, 2001). A diet supplemented with this compound contributes to an evident decrease of cholesterol level (Clifton *et al.*, 2004; Sudhop and von Bergmann, 2002).

Preliminary phytochemical research of the previously obtained callus cultures of *Vaccinium corymbosum* var. *bluecrop* have shown that they all synthesized triterpenes ( $\alpha$ -amyrin,  $\beta$ -amyrin, ole-

anolic acid, usolic acid) and, in addition, free  $\beta$ -sitosterol, a compound not present in the leaves of the intact plant (Migas *et al.*, 2005).

The differences in the biosynthesis of the particular isoprene derivatives in the *Vaccinium corymbosum* var. *bluecrop* callus, in comparison to the herb of the intact plant, encouraged the authors to conduct further biotechnological research of the same biomass with the intention to develop *in vitro* plant material rich in isoprene derivatives. The callus of *V. corymbosum* var. *bluecrop* obtained under optimized growth conditions could be used in the future to investigate the metabolic pathways of isoprene compounds under *in vitro* conditions.

The fragmentary research carried out so far in the biosynthesis of isoprene under *in vitro* conditions has indicated the key role of growth regulators in the process of formation of these compounds (Dembińska-Migas *et al.*, 1998).

That is why the authors decided to make it the main goal of this research to optimize the conditions for the biosynthesis of isoprene derivatives in the *Vaccinium corymbosum* var. *bluecrop* callus based on a growth medium supplemented with varying amounts of selected growth regulators. The previous biotechnological research into *in vitro* cultures of *Vaccinium corymbosum* var. *bluecrop* showed a direct negative influence of most cytokinins on the growth and vitality of the ob-

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Abbreviations: 2,4D, 2,4-dichlorophenoxyacetic acid; DW, dry weight; SH medium, Schenk-Hildebrandt medium;  $G_i$ , growth index; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; NOA, naphthoxyacetic acid.

served calli. With the exception of low doses of kinetin, cytokinins, commonly used after preliminary organogenesis, caused progressive necrosis of callus cultures (Dembńska-Migas *et al.*, 1998). Therefore, at the current stage of the research it was decided that the growth media would be modified with auxins.

This paper presents for the first time the results of the impact of a wide class of exogenous auxins [2,4-dichlorophenoxyacetic acid (2,4D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA) and naphthoxyacetic acid (NOA)] on the formation of triterpenes and  $\beta$ -sitosterol under *in vitro* conditions on the basis of *V. corymbosum* var. *bluecrop* callus grown on Schenk-Hildebrandt medium.

## Experimental

### Plant material

The research used juvenile calli of *Vaccinium corymbosum* var. *bluecrop* cultivated on Schenk-Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) solidified with agar (0.7% w/v) supplemented with 22.6  $\mu\text{M}$  2,4D, 2.32  $\mu\text{M}$  kinetin and 3% (w/v) sucrose (Dembńska-Migas *et al.*, 1998).

### Callus growth conditions

Approx. 300 g of fresh weight biomass were transferred aseptically into a baby food jar containing 25 ml of solidified SH medium (0.7% w/v of agar) supplemented with the appropriate plant growth regulator and 3% (w/v) of sucrose. Callus tissue was incubated 4 weeks in baby food jars in a controlled-environment growth chamber (17 h/24 h photoperiod, 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  supplied by fluorescent TLD Philips lamps, 60% humidity, 26 °C  $\pm$  2 °C). The growth and the production of isoprene derivatives in the calli were studied in several SH media supplemented with two concentrations of various auxins – 2,4 D (2.26  $\mu\text{M}$  or 22.6  $\mu\text{M}$ ), IAA (2.85  $\mu\text{M}$  or 28.5  $\mu\text{M}$ ), NAA (2.68  $\mu\text{M}$  or 26.8  $\mu\text{M}$ ), NOA (2.47  $\mu\text{M}$  or 24.7  $\mu\text{M}$ ), IBA (2.46  $\mu\text{M}$  or 24.6  $\mu\text{M}$ ). Each experimental SH medium was supplemented with a constant amount of kinetin (2.32  $\mu\text{M}$ ). The control culture was incubated on SH medium containing only 2.32  $\mu\text{M}$  of kinetin.

Callus growth was observed after that period and the callus growth index ( $G_i$ ) was evaluated using the Klein formula (Zenkteler, 1984) employing 15 samples which were taken together. All ex-

periments were repeated 4 times. The results were analyzed statistically with t-Student's test for comparison of the mean and to assess differences. Isoprene derivatives content in all the replicates was determined after calli lyophilization for 72 h, using a freeze-dryer at –50 °C as described below.

### Quantitative analysis of isoprene derivatives

#### Sample preparation

Freeze-dried plant material (300 mg), after pulverization, was exhaustively extracted under reflux with chloroform (3  $\times$  240 ml of solvent, 60 °C), then concentrated under low pressure to form a syrup-like residue. This was diluted with a chloroform/methanol solvent mixture (2.5:0.5 v/v) and submitted to chromatographic analysis.

The stock standard solution was prepared by dissolving 1 mg of  $\beta$ -sitosterol, ursolic acid,  $\beta$ -amyrin (Extrasynthese, Genay, France) in 9 ml of a chloroform/methanol solvent mixture (2.5:0.5 v/v). The analytical grade solvents – chloroform, carbon tetrachloride, methanol and isopropanol, were purchased from POCH (Gliwice, Poland).

#### Quantitative TLC

TLC was performed on 10  $\times$  20 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with a 0.25 mm layer of Kieselgel 60 F<sub>245S</sub>. Non-diluted stock solutions of standards in increasing amount (2  $\mu\text{l}$ , 3  $\mu\text{l}$ , 4  $\mu\text{l}$ , 5  $\mu\text{l}$ , 6  $\mu\text{l}$ ) together with analyzed samples (2.5  $\mu\text{l}$ ) were spotted on the plate using a syringe. Twenty spots were applied as 3 mm lines. The plates were developed isocratically with a  $\text{CHCl}_3/\text{CCl}_4/\text{isopropanol}$  (110:5:95 v/v/v) solvent mixture in a Chrop chamber (200 mm  $\times$  200 mm; Fischer Scientific, New Delhi, India) in 8 cm distance. After separation the plates were dried in the air and visualized with vanillin reagent (Picman *et al.*, 1980). The measurements were taken 30 min after chromatogram development. The GelDoc2000 (Bio-Rad, Philadelphia, USA) image analyzing system controlled by a Quantity One program was used for quantitative and qualitative analysis (Migas *et al.*, 2002).

## Results and Discussion

The experiment used the calli of *V. corymbosum* var. *bluecrop* which is a rich source of isoprene derivatives (Migas *et al.*, 2005).

Structural research (EI-MS,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR) of the dominating triterpenes isolated from

the leaves of the intact plant confirmed that both the natural plant and the callus synthesized ursolic acid, oleanolic acid,  $\alpha$ - and  $\beta$ -amyrin. Unlike the herb of the intact plant, callus, cultivated on SH medium modified with kinetin ( $2.32\ \mu\text{M}$ ) and  $2.26\ \mu\text{M}$  2,4D, produced significant amount of free  $\beta$ -sitosterol (Migas *et al.*, 2005).

The choice of auxins as the growth regulators which could stimulate the biosynthesis of the investigated class of compounds under *in vitro* conditions was based on previous biotechnological research and literature reports.

It has been established that some auxins generally stimulate the biosynthesis of both triterpenes (free and bound) and steroid compounds (Fernandes-Ferreira *et al.*, 1992; Brain and Lockwood, 1976; Geuns, 1975). The experiments carried out during this project showed the effect of 5 auxins and their respective concentrations in the medium on the type of the synthesized isoprene derivative and its concentration in the *V. corymbosum* var. *bluecrop* callus. Previous investigations showed that high amounts of auxins tested in the experimental media (higher than ca.  $30\ \mu\text{M}$ ) seriously decreased the growth and juvenility of *V. corymbosum* calli (Dembińska-Migas *et al.*, 1998). That is why the decision was made to use all growth regulators in the range between 2 to  $30\ \mu\text{M}$ .

Chromatographic analysis (TLC) together with image analysis proved that auxins added to the experimental medium had a substantial impact on the biosynthesis of both triterpenes (amyrines and acids) and  $\beta$ -sitosterol in the *V. corymbosum* var. *bluecrop* callus.

It was noted that the increased concentration of IBA in the medium resulted in a clear decay of the biomass, coinciding with an inhibited production of sterol, whereas the increased concentration of IAA in SH medium clearly stimulated both the growth of the biomass and the biosynthesis of the investigated steroid compound (Fig. 1). Other auxins, *i.e.* 2,4D, NAA and NOA inhibited the growth of the biomass and stimulated the production of  $\beta$ -sitosterol, in proportion to their concentrations (Fig. 1). Based on these results it seems that the course of the biosynthesis of the sterol in the *Vaccinium corymbosum* var. *bluecrop* callus depended on the type of the auxin and its concentration in the medium.

Consequently, the highest concentration of  $\beta$ -sitosterol, albeit correlated with the decay of the callus, was identified in the biomass inoculated on

SH medium supplemented with  $26.8\ \mu\text{M}$  NAA ( $497\ \mu\text{g g}^{-1}$  DW) (Fig. 1). This was probably related to NAA inducing a cycle of biochemical changes conditioning the transfer of cycloartenol to steroid compounds, rather than to amyrines and, consequently, to respective triterpene acids (Fig. 2) (van der Heijden *et al.*, 1989). The metabolic pathways of triterpenes and steroid compounds are based on the same substrates. The enzymes which participate in the formation of the particular isoprenes determine whether they are used in the biosynthesis of steroids or triterpenes (Bach, 1995; Harrison, 1990; van der Heijden *et al.* 1989). This could be the reason for the competition for substrates in the *V. corymbosum* var. *bluecrop* callus and the resulting domination of one metabolic pathway, in this case the one leading to the formation of  $\beta$ -sitosterol. It is possible that the same mechanism could be attributed to other auxins which stimulate the formation of  $\beta$ -sitosterol, *i.e.* 2,4D, NOA and IAA (Fig. 2).

The only auxin which inhibited the biosynthesis of  $\beta$ -sitosterol in the *V. corymbosum* var. *bluecrop* callus was IBA. All other auxins clearly induced the formation of  $\beta$ -sitosterol and the process was accompanied with a reduction in the quantity of the determined triterpenes in the tissue material (Fig. 2).

The quantitative determination of the total amount of  $\alpha$ - and  $\beta$ -amyrin in the respective calli of *V. corymbosum* var. *bluecrop* showed that the biosynthesis of these compounds in the biomass is directly related to the amount of the auxin used in the medium, and is less dependent on the type of the auxin. The higher concentrations of auxins clearly stimulated the formation of amyrines (Fig. 2).

Thus, the calli collected from the media supplemented with higher concentrations of auxins had, on average (except for IAA), approx.  $70\ \mu\text{g}$  of total amyrines per 1 g of dry tissue weight. The biomasses grown on media supplemented with smaller amounts of the above growth regulators were characterized by varied, lower amounts of amyrines, from  $0\ \mu\text{g g}^{-1}$  DW ( $2.85\ \mu\text{M}$  IAA) to  $53\ \mu\text{g g}^{-1}$  DW ( $2.46\ \mu\text{M}$  IBA) (Fig. 2).

The direct proportion between the level of amyrin biosynthesis and the amount of the auxin supplement in the medium is particularly clear in the case of SH medium modified by supplementing with 2,4D ( $2.26\ \mu\text{M}$ , 2,4D –  $20\ \mu\text{g g}^{-1}$  DW of total amyrines;  $22.6\ \mu\text{M}$  2,4D –  $78\ \mu\text{g g}^{-1}$  DW). It is also worth noting that the medium supplemented with

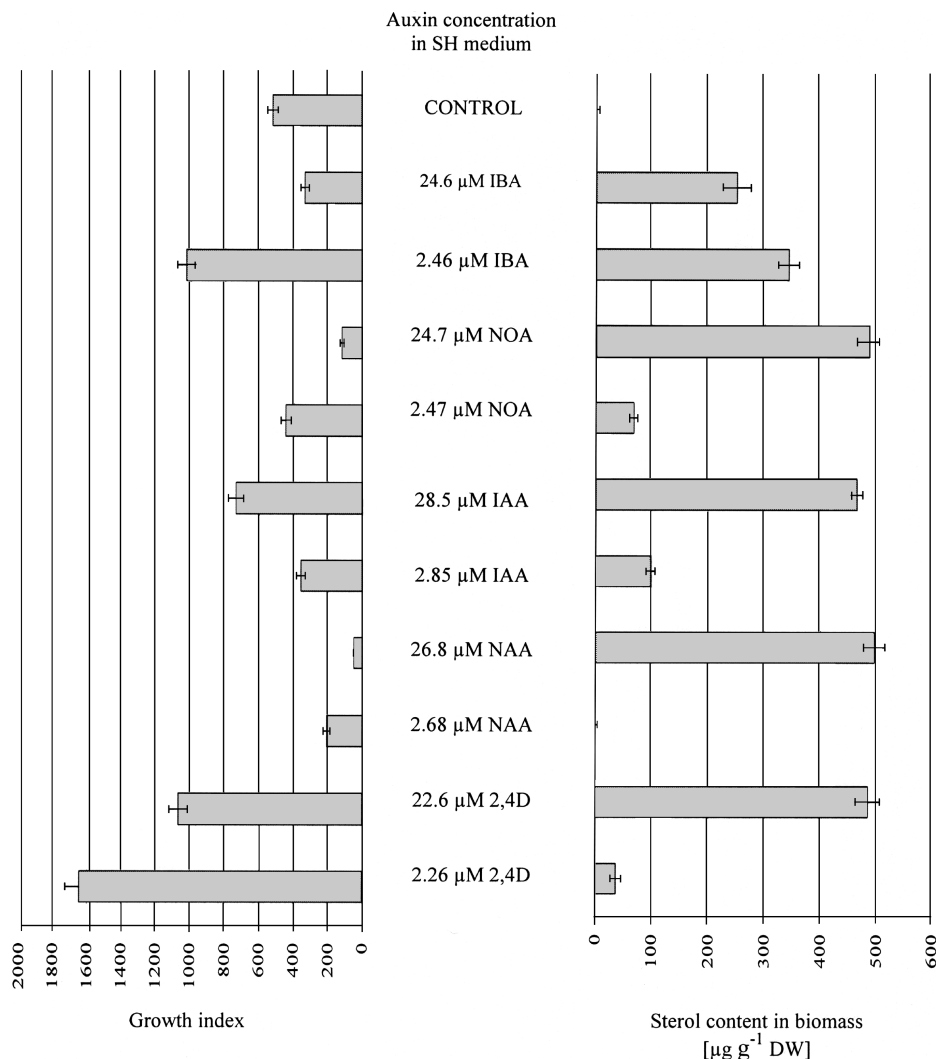


Fig. 1. Sterol content and the growth index of *Vaccinium corymbosum* var. *bluecrop* callus culture cultivated on SH medium modified with different concentrations of auxins. Culture conditions: 4 weeks cultivation in baby food jars in a controlled-environment growth chamber (17 h/24 h photoperiod,  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  supplied by fluorescent TLD Philips lamps, 60% humidity,  $26^\circ\text{C} \pm 2^\circ\text{C}$ ).

NAA, conditioning the maximum concentrations of  $\beta$ -sitosterol, also provided for an optimum biosynthesis of amyrines in the biomass  $\sim 79 \mu\text{g g}^{-1}$  DW (Fig. 2).

In the case of *V. corymbosum* var. *bluecrop* callus the reverse relation between the amount of the auxin and the total concentration of amyrin in the investigated biomass could be related to the influence of the analyzed growth regulators not only on the cycle of changes leading from cycloartenol to sterol or amyrines, but also to the inhibition

of the formation of amyrines from the respective triterpene acids. The quantitative determination of the total amount of triterpene acids showed an inversely proportional relation between the amount of the supplemented auxin and the total amount of triterpene acids in the *Vaccinium corymbosum* var. *bluecrop* callus (Fig. 2).

IBA had the highest effect on the formation of triterpene acids. Ten times higher concentration of this auxin in the medium resulted in an almost three-fold decrease of the amount of triterpene ac-

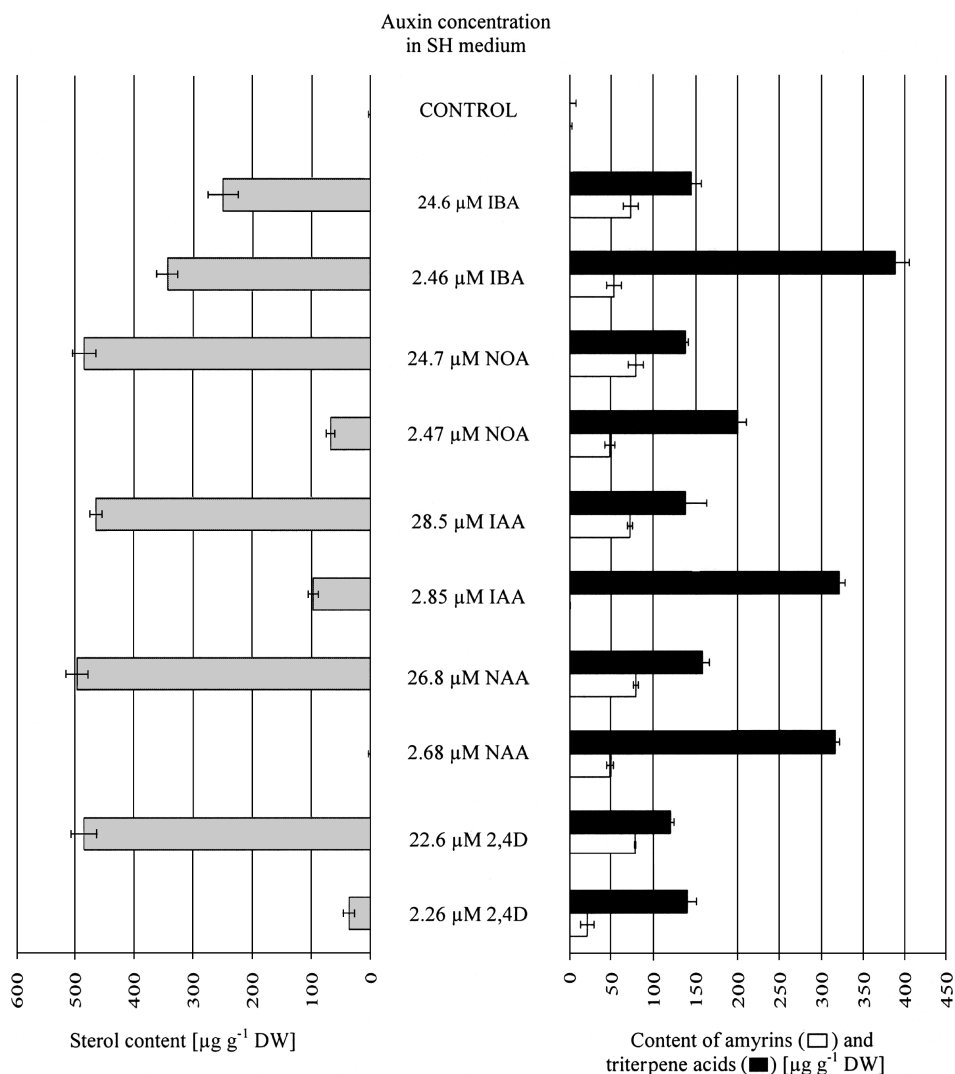


Fig. 2. Isoprene derivatives content in biomass of *Vaccinium corymbosum* var. *bluecrop* callus culture cultivated on SH medium modified with different concentration of auxins. Culture conditions: 4 weeks cultivation in baby food jars in a controlled-environment growth chamber (17 h/24 h photoperiod,  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  supplied by fluorescent TLD Philips lamps, 60% humidity,  $26^\circ\text{C} \pm 2^\circ\text{C}$ ).

ids determined in the biomass. 2,4D had the lowest effect on the formation of triterpene acids. The increased concentration of this growth regulator from  $2.26 \mu\text{M}$  to  $22.6 \mu\text{M}$  resulted in a slight decrease of the total amount of triterpene acids (Fig. 2).

Summing up, by modifying the SH medium by supplementing it with growth regulators of the auxin group, a triterpene-rich callus culture was produced, which also was a source of  $\beta$ -sitosterol, accumulated in amounts which were approx. 10-

times higher than for triterpene acids and approx. 6-times higher than for amyrins. It is worth noting at this point, that this compound has not been identified in the leaves of the intact plant of *V. corymbosum* var. *bluecrop* (Migas *et al.*, 2005). The optimum medium, ensuring both a satisfactory growth of *Vaccinium corymbosum* var. *bluecrop* callus and considerable level of biosynthesis of selected secondary metabolites, proved to be SH medium modified by supplementing  $22.6 \mu\text{M}$  2,4D,  $2.32 \mu\text{M}$  kinetin, containing 3% (w/v) of su-

crose and 0.7% (w/v) of agar. This medium produced a lively green, moist parenchymatic tissue with a high growth rate ( $G_i = 1067.40$ ), which constituted a valuable source for isoprene deriva-

tives  $-120 \mu\text{g g}^{-1}$  DW of total triterpene acids (oleanolic acid and ursolic acid),  $78 \mu\text{g g}^{-1}$  DW of total amyrines ( $\alpha$ - and  $\beta$ -amyrines) and  $485 \mu\text{g g}^{-1}$  DW of  $\beta$ -sitosterol (Figs. 1 and 2).

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