The effect of benzothiazolium salt on spruce callus cells

Ľ. Krajnáková[1,2], D. Kákoniová[1], D. Lišková[1], E. Hlinková[3]

[1]*Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia*
[2]*NovaMed Policlinic, Banská Bystrica, Slovakia*
[3]*Institute of Cell Biology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia*

ABSTRACT

The effect of 3-(benzyloxycarbonylmethyl)-2-benzothiazolinone (SM-550) on the growth parameters, cell division, and cell polymorphism of spruce callus cells (*Picea abies* L. Karst) was investigated. These results were compared with callus parameters grown on the medium supplemented with NAA. The highest concentration (1 mmol) of SM-550 stimulated the growth process, as well as cell division, shortened the lag-phase, and had a significant effect on cells polymorphism. Its effect was demonstrated especially on long-term culture (3 subcultures – 84 days). On the other hand, the highest stimulation of growth by SM-550 in 1 µmol concentration was determined only in the first subculture. SM-550 in the lowest concentration (1 nmol) used was completely unsuitable in the third subculture, the callus was necrotic and resembled to calli growing on the medium without growth hormones.

Keywords: benzothiazolium salt; callus culture; linear cell polymorphism; *Picea abies* L. Karst

Substituted benzothiazole salts are biologically active substances with auxin-like activity (Giannella et al. 1971). Derivatives of benzothiazole were ranged by Sekerka (1984, 1988) to the analogues of traditional regulating compounds inducing callogenesis of monocots and dicots, which was tested on many model plants (Hlinková 1990, 1993, Henselová et al. 2001, 2002, Henselová 2002, 2004, Šimonová et al. 2005). Substituted benzothiazole salt – 3-(benzyloxycarbonylmethyl)-2-benzothiazolinone (SM-550) is a growth regulator from the auxinoid group with the commercial name RASTIM 30 DKV. This compound was tested for physico-chemical properties, toxicity, and biological impact on some crop harvest. Its activity was examined also on vegetative propagation of ornamental plants, forest and fruit trees, and their rhizogenesis (Henselová 2002, 2004, Henselová et al. 2002). Moreover, SM-550 was often used for various intact plants, mainly angiosperms. Its auxin-like activity was seldom monitored in media for callus cultures, suspension cells, and somatic embryogenesis (Hlinková 1990, 1991) of conifers. Experiments proved that this compound can substitute 2,4-D in culture media for *in vitro* cultures (Hlinková 1990), e.g. in the range of 1 µmol to 0.1 mmol concentrations for soybean callus cultures (Hlinková 1993). The effect of biologically active substances, including SM-550, on cell growth, callus active proliferation, mitotic activity, form and size of morphoses in angiosperms and derived calli depends on plant species, type of organ/explant, its developmental stage, concentration of the inductor used, and length of the treatment (Hlinková and Belková 1991). The growth of long-term cultured (12 years) spruce callus culture (*Picea abies* L. Karst) is characterized by a stable and slow growth. This culture is very sensitive to the presence of certain growth regulator. On culture media without growth regulators the callus cells turned necrotic and the callus stopped to grow. The aim of our study was to accelerate the callus growth during the long-term culture and simultaneously widen the knowledge on the effect of SM-550 (3-(benzyloxycarbonylmethyl)-2-benzothiazoline) on growth, mitotic activity and linear polymorphism of spruce (*Picea abies* L. Karst) callus cells.

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MATERIAL AND METHODS

Long-term spruce (*Picea abies* L. Karst) callus culture (12 years old) derived from hypocotyls of 30 days old seedlings was obtained and maintained on Z agar medium (Čierna et al. 1991) supplemented with α-naphthaleneacetic acid – NAA (2.5 µmol) under 16-h photoperiod, irradiance of 45–60 µmol/m^2/s, at 23 ± 1°C and 60% relative air humidity. Spruce callus culture was characterized by a steady growth (100% of biomass increase during 28 days of culture). The growth dynamics of this callus culture resulted from the long-term cultivation and adaptation in given culture conditions. Callus cells displayed a mitotic index of 0.5–2% and possessed 2*n* = 24 chromosomes, in some cases in metaphase, polyploidity was observed (Krajnáková, unpublished data). During the whole cultivation time the formation of tracheids was sporadically observed. Substituted benzothiazole salt – SM-550 was tested in concentrations 1 nmol, 1 µmol, and 1 mmol added into the culture agar medium and the results were evaluated after the first and third subculture. We studied growth parameters (fresh mass, dry mass, growth dynamics), mitotic index (cells were treated with 0.1% colchicine, fixed in a mixture of 96% ethyl alcohol and acetic acid – 3:1, quick squash preparations were made according to Murín (1960)), and stained with orcein (Dostál 1976), and cell polymorphism (length of cells was measured for 500 randomly selected cells and evaluated after Reisenauer (1970), cells were divided into 6 groups according to their length with algorithm 60 µm). Effect of media with SM-550 were compared with the control medium supplemented with NAA (2.5 µmol), and the negative control medium without any growth regulator. In control medium without any growth regulator the callus stopped to growth and turned necrotic im-

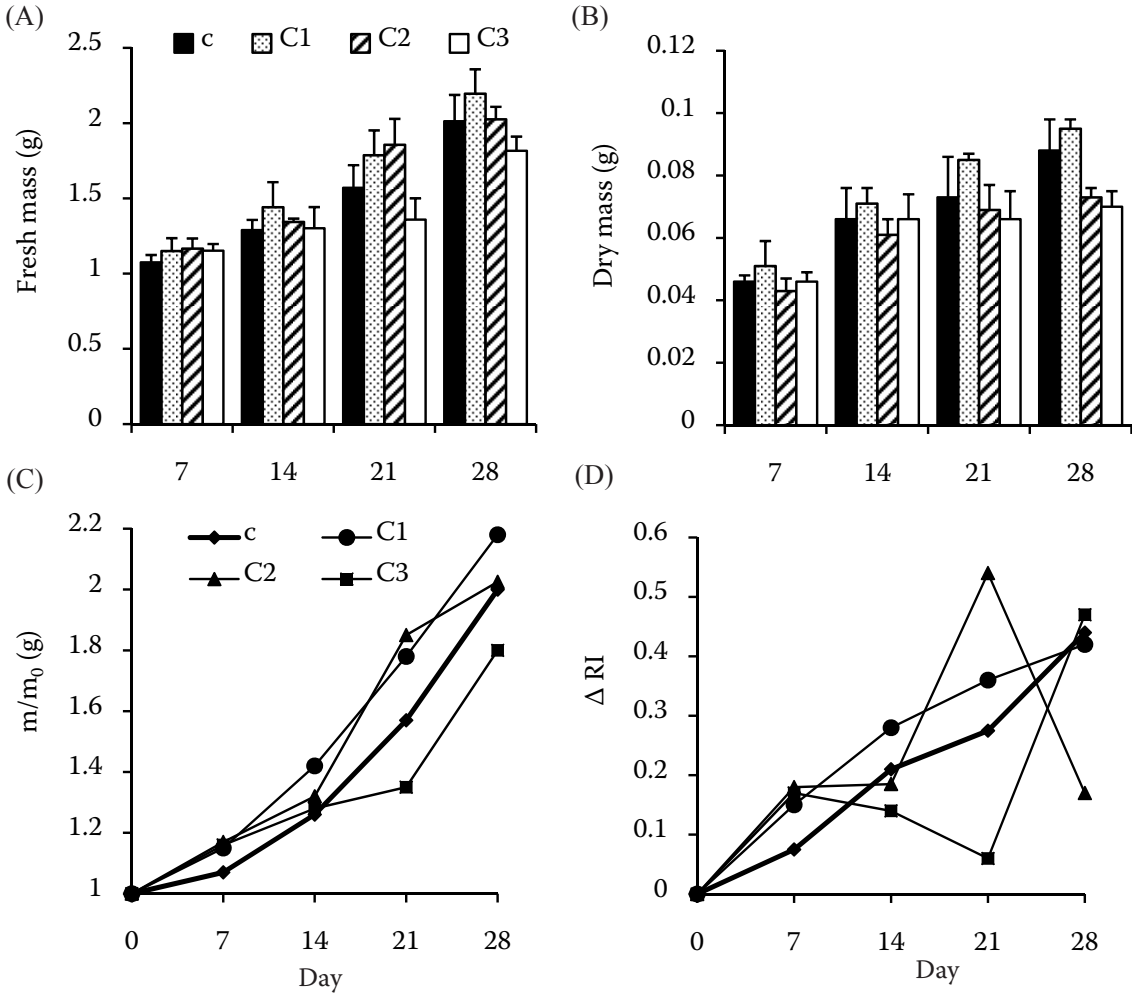

Figure 1. Effect of different concentrations of SM-550 on spruce callus culture in the first subculture. Fresh mass (A), dry mass (B), growth (C) and growth dynamics (D)

A and B: c – control with 2.5 µmol NAA (■); C1 – 1 nmol SM-550 (⚃); C2 – 1 µmol SM-550 (▨); C3 – 1 mmol SM-550 (□), C and D: c – control with 2.5 µmol NAA (♦); C1 – 1 nmol SM-550 (●); C2 – 1 µmol SM-550 (▲); C3 – 1 mmol SM-550 (■)

mediately after inoculation, therefore these results are not presented. Morphological and cytological parameters were evaluated for the studied media. All results were statistically evaluated using the STATGRAPH 5.0 system and Student's *t*-test.

RESULTS AND DISCUSSION

Spruce callus culture induced on the basal medium supplemented with NAA showed a steady growth for several years (Figures 1A–D). This growth characteristics are the result of long-term adaptation to culture conditions. The effect of SM-550 on this callus growth was dependent on its concentration and length of the treatment. In the first subculture the most effective was the concentration 1 nmol, followed by the concentration 1 µmol (Figures 1A–D). Both significantly stimulated the callus growth in comparison to the control (Figure 1C). Similar effect of SM-550 was ascertained by Hlinková (1991) in *Crepis capillaris* callus culture. The stimulation effect of both lower SM-550 concentrations used in this subculture are probably affected with the synergistic effect of endogenous level of IAA and residual concentration of NAA present in the callus cells during the first days of culture. We suppose that the effect of exo- and endogenous auxins (SM-550 vs IAA) and residual concentration of NAA affected lag-phase, but later, when the expression of aux/IAA genes participating in the exponential phase was modified by SM-550, the growth was inhibited. A benzothiazolinone ring of SM-550 molecules is open probably into the outer side of the cell wall or into the periplasmatic space were it interacts with the input channel for auxin. This channel is probably identical with the channel for 2,4-D because the opened ring of SM-550 molecules, affected with the methyltransferases, is very similar

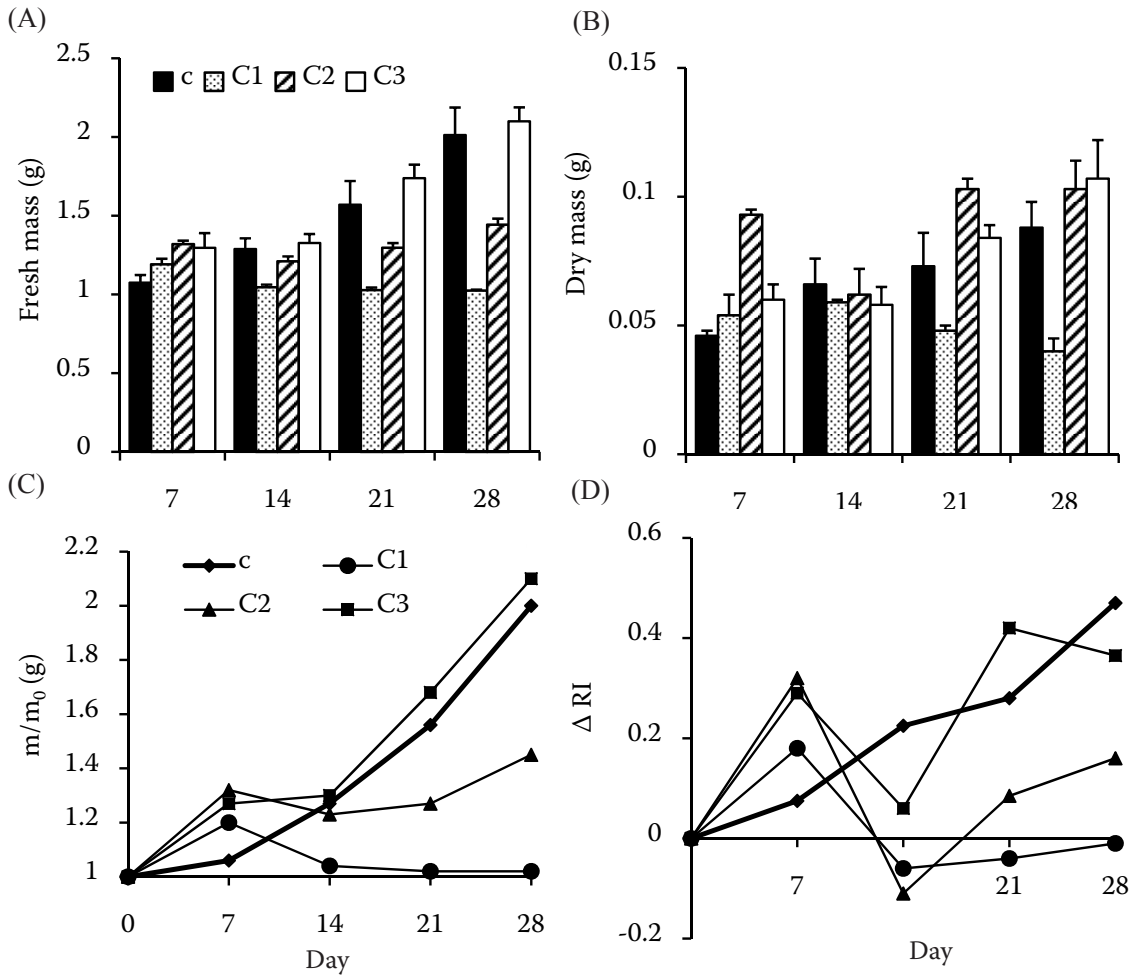

Figure 2. Effect of different concentrations of SM-550 on spruce callus culture in the third subculture. Fresh mass (A), dry mass (B), growth (C) and growth dynamics (D)
A and B: c – control with 2.5 µmol NAA(■); C1 – 1 nmol SM-550 (⚃); C2 – 1 µmol SM-550 (▨); C3 – 1 mmol SM-550 (□) C and D: c – control with 2.5 µmol NAA (♦); C1 – 1 nmol SM-550 (●); C2 – 1 µmol SM-550 (▲); C3 – 1 mmol SM-550 (■)

to 2,4-D from the structural point of view. The effect of suppression was evidently eliminated in the third subculture (Figure 2D) when SM-550 in 1 mmol concentration significantly enhanced the callus growth dynamics. Both lower concentrations (1 μmol and 1 nmol) stimulated the growth only to the 7th day and afterwards a significant inhibition followed compared to the NAA control (Figures 2A–D). The 1 nmol concentration of SM-550 caused necrosis of the tissue at the end of the third subculture likewise we could see on the negative control medium without growth regulators (Figure 2D). In all cases SM-550 shortened the lag-phase which indicates partial synchronization of cells division (Figure 2D). Similarly, in *Glycine max* the 1 nmol concentration of this benzothiazolinone significantly inhibited the mitotic activity (Hlinková 1993). The values of dry mass in the first and in the third subculture were in accordance with the variations in fresh mass (Figures 1A, 1B, 2A and 2B). The dry mass of spruce callus increased during the 28-days of the first subculture in all studied variants, especially on media enriched with 1 nmol SM-550 (Figure 1B). Their changes in the third subculture were considerably different, whereas for calli cultured on media supplemented with 1 nmol of SM-550, the dry mass decreased, both other concentrations of SM-550 (1 μmol and 1 mmol) significantly increased the portion of dry mass from the 21st day of culture (Figure 2B). Media with the lowest concentration of SM-550 significantly decreased the dry mass on the 28th day in accordance with the callus growth (Figures 2C and 4) as well as mitotic activity inhibition (Figure 3B).

Long-term cultivated callus cultures are generally characterized by a low mitotic activity (MI) (Hlinková and Ružičková 2000). Cell division, biomass production, as well as the whole cell life are, besides others, affected by growth regulators and composition of culture media (Forsyth and van Staden 1986). The values of MI in long-term spruce callus culture are relatively low, as mentioned for such type of cultures (Figure 3A). However, compared to the control, the MI values achieved some enhancements during the culture period with the 1 nmol and 1 mmol concentrations of SM-550 on the 14th day and 1 μmol on the 21st day of culture. Different picture, with MI inhibition and even its stop (1 nmol on the 21st day) were ascertained in the third subculture (Figure 3B) which corresponds to decreasing growth dynamics, reduction of dry mass, and necrosis. This concentration of SM-550 in the third subculture seems to be unsuitable for spruce callus growth. It shortened the lag-phase in the third subculture and prolonged the mitotic activity of cells, which is documented by higher MI value after 28 days of cultivation, compared to the first subculture for all SM-550 concentrations tested. Some effect was observed in the third subculture for 1 μmol and 1 mmol concentrations of SM-550 (Figures 3A and 3B). Cell division did not show any changes during the whole experiment compared to callus cells growing on the medium with NAA (2.5 μmol).

Cytogenetic variability of callus cells is demonstrated also in their length and shape. SM-550 in all concentrations affected the linear polymorphism of cells. The mean length was shortened (Table 1). Most amount of cells (%) had the length 60–89.9 μm

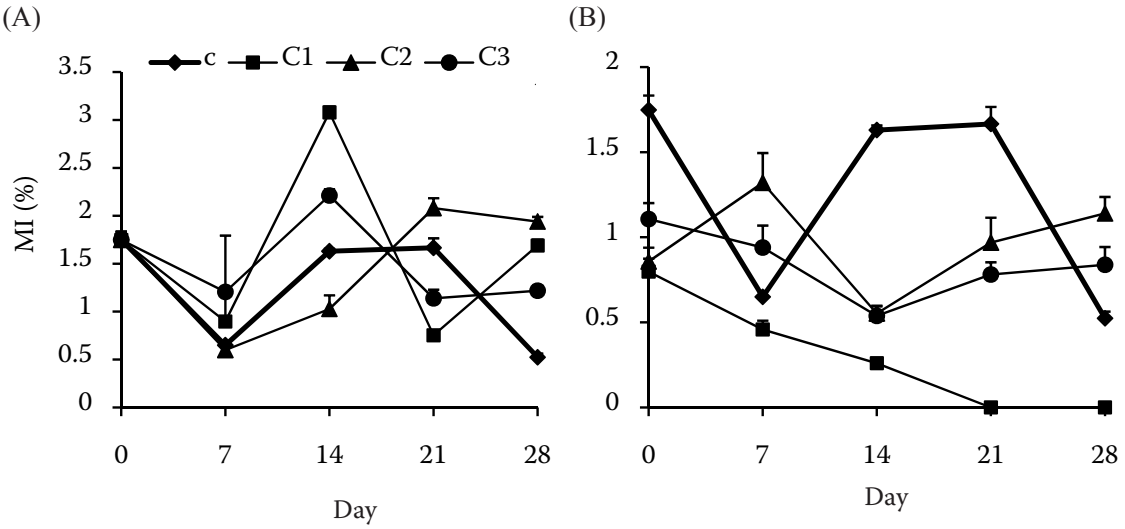

Figure 3. The mitotic index of spruce callus culture in the first subculture (A) and in the third subculture (B)
c – control with 2.5 μmol NAA (♦); C1 – 1 nmol SM-550 (■); C2 – 1 μmol SM-550 (▲); C3 – 1 mmol SM-550 (●)

Table 1. Cell polymorphism (expressed in %) affected by SM-550 in different concentrations (C) on day 0 and 28 of culture; 1st and 3rd subculture

	No. of cell category	%		No. of cell category	%
Control with 2.5 µmol NAA day 0	1	1.33	C1 – 1 nmol day 0, subculture 3	1	3.66
	2	39.66		2	56.00
	3	37.00		3	31.33
	4	16.00		4	7.33
	5	6.00		5	1.66
	6	0		6	0
Control with 2.5 µmol NAA day 28	1	2.66	C2 – 1 µmol day 0, subculture 3	1	10.00
	2	36.33		2	63.33
	3	37.66		3	26.33
	4	17.00		4	3.33
	5	5.00		5	0
	6	1.33		6	0
C1 – 1 nmol day 28, subculture 1	1	4.66	C3 – 1 mmol day 0, subculture 3	1	28.66
	2	55.33		2	62.33
	3	27.33		3	9.00
	4	10.00		4	0
	5	2.66		5	0
	6	0		6	0
C2 – 1 µmol day 28, subculture 1	1	3.66	C2 – 1 µmol day 28, subculture 3	1	8.33
	2	42.00		2	61.00
	3	40.66		3	24.00
	4	11.00		4	6.33
	5	2.66		5	0
	6	0		6	0
C3 – 1 mmol day 28, subculture 1	1	11.00	C3 – 1 mmol day 28, subculture 3	1	31.66
	2	61.33		2	64.00
	3	23.00		3	4.33
	4	4.66		4	0
	5	0		5	0
	6	0		6	0

Division of cells according to their length based on different concentrations of SM-550; day 0 and 28 of cultivation; C – concentration of SM-550. C1 – 1 nmol in the 3rd subculture after 28 days was necrotic. Cell category according to the cell length (µm): 1 – 30 to 59.9 µm; 2 – 60 to 89.9 µm; 3 – 90 to 119.9 µm; 4 – 120 to 149.9 µm; 5 – 150 to 179.9 µm; 6 – 180 to 209.9 µm

(control cells – 36.33%, 1 nmol – 55.33%, 1 mmol – 61.33%). The maximum (76.66%) of spruce callus cells grown on control medium supplemented with NAA are inserted into two length intervals from 60 to 89 and 90 to 119.9 µm. The cells percentage (73.99%) belonging to these intervals did not change essentially during the 28-day subculture, which is probably also the result of long-term adaptation of spruce callus cells to culture medium used (Table 1). The effect of SM-550 in 1 mmol concentration on cells shortage was the most intensive. This phenomenon is shown on the percentage of cells length 30–59.9 µm (28.66% on day 0 of the third subculture, 31.66% on day 28 of the third subculture), and especially in the increase of cell percentage within the length interval 60–89.9 µm. Hlinková (1991) ascertained that in *Crepis capillaris* and *Haplopappus gracilis* cultures SM-550 caused an increase of young isodiametric cells in comparison to the control.

SM-550 (3-(benzyloxycarbonylmethyl)-2-benzothiazolinone) as a new growth stimulator was

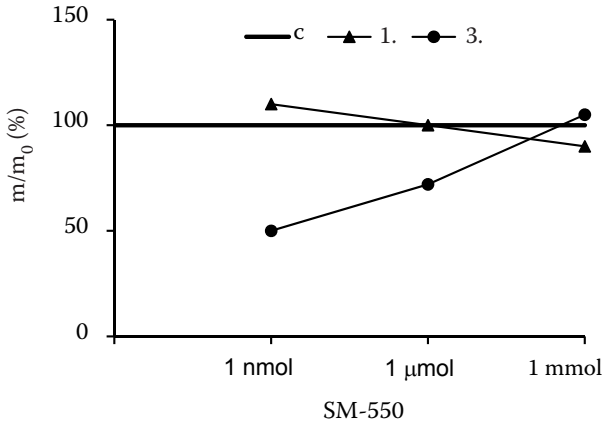

Figure 4. Growth of spruce callus culture in the 1. (▲) and 3. (●) subculture on media supplemented with SM-550 in concentrations: 1 nmol, 1 μmol, 1 mmol related to the control with 2.5 μmol NAA (represents 100 %)

developed in the Research Institute for Chemical Technology in Bratislava (Slovakia) and registered in the final formulation as dispersible concentrate for vegetative propagation of decorative plants as Rastim 30 DKV. SM-550 is a plant growth regulator with a wide spectrum of biological effects (Henselová et al. 2001) similar to those of auxins (Klíčová et al. 1994). This substance induced *in vitro* differentiation in tissues as well as dedifferentiation (callus formation) similarly as auxin-like substances – NAA and 2,4-D (Havel et al. 1994). The authors ascertained that SM-550 in lower concentrations showed analogy with NAA. This substance and its mixtures stimulated root system development, increased the total number of rooted cuttings, as well as vigorous growth of rooted cuttings *ex vitro* (Henselová 2002, 2004, Henselová et al. 2002). Šimonová et al. (2005) ascertained that their derivatives increased the formation of adventitious roots of mung bean hypocotyl cutting, as well as stem elongation and production of fresh and dry mass of buckwheat. The optimal concentration of SM-550 on the formation of root system is specific for individual plant species. In the herb cuttings lower concentrations were more effective, while in the lignified ones it was higher concentrations. In weakly rooting species this substance significantly increased the percentage of rooted cuttings and simultaneously it affected the quality of the root system (Henselová 2002). The derivatives of SM-550 increased the formation of adventitious roots of mung bean hypocotyl cuttings, as well as stem elongation and production of fresh and dry mass of buckwheat. These compounds also influenced the yield and qualitative technological parameters in spring barley, winter rape, sugar beet, potato (Zahradníček et al. 1993, Zrůst and Henselová 1998). Derivatives of SM-550 may be thus characterized as biologically active substances with dominant auxin-like growth promoting activity (Šimonová et al. 2005). In our case the highest concentration (1 mmol) of SM-550 stimulated the growth process, as well as cell division, shortened the lag-phase, and had a significant effect on cells polymorphism. Its effect was demonstrated mainly after the third subculture (84 days) of long-term cultivated callus. SM-550 used in the lowest concentration (1 nmol) was completely unsuitable in the third subculture, the callus was necrotic and resembled to calli growing on the medium without growth hormones. The absence of auxin, and in this case also the low concentration of SM-550, a substance with auxin-like properties, is probably responsible for growth deceleration and callus necrosis. It seems that SM-550 in dependence on the concentration is able to substitute auxin in the medium for long-term spruce callus culture.

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Corresponding author:

RNDr. Daniela Kákoniová, Ph.D., Institute of Chemistry SAS, Dúbravská cesta 9, 845 38 Bratislava, Slovakia
phone: + 421 259 410 291, fax: + 421 259 410 222, e-mail: chemdaka@savba.sk