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Original paper

Lovastatin effects on indirect somatic embryogenesis of petunia

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Abstract Somatic embryogenesis (SE) is one of the most challenging processes for studying plant morphogenesis as it is the best example supporting totipotency's theory published by Haberlandt in 1902. Very early events that are relevant for acquiring and expressing embryogenic competency continue to remain the main subject for present laboratory studies. However, the metabolic pathway of mevalonate, that is essential in producing secondary metabolic compounds in plants, is little studied in SE. In this regard, statins as natural inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) may be used to reveal new insights for further understanding SE. The aim of this paper is to test the effect of low levels of lovastatin (i.e. 0.1 µg/l or 0.2 nM) on indirect somatic embryogenesis of Petunia x hybrida 'Snowball'. Our results reveal that lovastatin presence into the culture medium, in certain conditions, increases the number of fully developed somatic embryos. These results support the idea that lovastatin may induce relevant effects on acquiring and expressing indirect SE in liquid culture media. The process is associated with a different pattern of peroxidases expression.

Keywords Mevalonate pathway, HGMR, lovastatin, Petunia x hybrida 'Snowball', indirect somatic embryogenesis.

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Introduction

Research on plant morphogenesis has already a long history and continues (N. PRUNET & E.M. MEYEROWITZ [1]), considering that plant morphogenesis studies started in the 17th century with the famous book *The Anatomy of Vegetables* (N. GREW [2]). The development of meristems, buds, shoots, embryos and seedlings became relevant subjects of scientific interest for the 19th century and starting with the beginning of the 20th century plant morphogenesis entered a new era, dedicated to laboratory experimentation. New concepts have been defined at the end of the 19th century that culminated with the theory of totipotency (G. HABERLANDT [3]). Different plant species are used as model plants and different culture media have been published during the last century in direct connection with species needs (O.L. GAMBORG & G. PHILLIPS [4]). Among these, *Petunia x hybrida* has proved to be an excellent model plant for morphogenesis studies (M. VANDENBUSSCHE & al [5]). If in the beginning of 1990 the scientific world was focused especially on *Arabidposis thaliana*, due to its small genome, *Petunia* x *hybrida* is recognized today as a model plant due to its responsiveness towards controlled conditions (i.e. small seeds, fast growing into *in vitro* culture on simple culture medium, repeatability and consistency of experimental results). However, to test exogenous molecules on plant tissue cultures, new questions need to be addressed regarding their exogenous concentration, timing of action and duration, as well as the consistency and repeatability of results to efficiently investigate their physiological role (T. GASPAR & al [6]).

Lovastatin is a member of the statin family, used for controlling the level of blood cholesterol (J.K. LIAO [7]; E.F. OLIVEIRA & al [8]). It was first isolated and purified from the fungus *Asperigillus terreus* (L. HENDRICKSON & al [9]). In a liquid and acid environment, this compound is hydrolysed from an inactive lactone to a beta-hydroxy-acid that is the active substance acting as a specific inhibitory substrate on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR, *R*-mevalonate: NADP+ oxidoreductase, CoA-acylating, EC 1.1.1.34). This enzyme is catalysing the reductive conversion of HMG-CoA to mevalonic acid (MVA), as a key reaction for cholesterol synthesis into animal and plant cells (S. DALE & al [10]; W. WANG & T.J. TONG [11]). Furthermore, this reaction is considered as a key controlling step in plant isoprenoid biosynthesis (J.C. GRAY [12]; T.J. BACH & al [13]) and the HMGR that is localized in the plasma-membrane (T.J. BACH & al [14]), was proved to be involved in controlling different physiological processes (K. LINDSEY & al [15]). Generally, the enzyme is encoded by more than two isoforms as it was described for different species (T.J. BACH & al [14]). Today it is well established that plant sterols with different composition and patterns of expression determine the optimisation of a wide spectrum of functions in plant cells including the control of plant pattern formation, morphogenesis and cell polarity (K. LINDSEY & al [15]; J.N. VALITOVA & al [16]).

To investigate the role of HMGR in plants physiology statins were used as specific inhibitors, and lovastatin has proved to be an excellent growth inhibitor in plant cell cultures (D.N. CROWELL & M.S. SALAZ [17]). Thus, 1 µM lovastatin specifically inhibits cytokinin biosynthesis and consequently cell growth. Another statin, mevinolin, proved to be an inhibitor of HGMR, when tested on cell cycle progression of tobacco BY-2 cells (i.e. *Nicotiana tabacum* L.). Mevinolin at a concentration of 5 mM completely blocked the cell cycle progression, and it was associated with a partial cell cycle synchronization, based on the analysis of mitotic index (A. HEMMERLIN & T.J. BACH [18]). On the other hand, acquiring the embryogenic competency into *in vitro* plant culture is a precondition for obtaining somatic embryos and *in vitro* experiments using embryogenic callus proved to be an excellent tool for studying morphogenesis (J.L. ZIMMERMAN [19]).

The scope of this study is to reveal the effect of lovastatin on indirect somatic embryogenesis of *Petunia* x *hybrida* 'Snowball'. A low concentration of lovastatin was tested (i.e. one thousand times lower compared to previous studies, 0.2 nM), added to liquid culture media for both phases: acquiring and expressing embryogenetic competencies. The primary callus as well the tested calli were also investigated for the expression of protein peroxidases in order to correlate them with the two stages of somatic embryogenesis.

Material and Methods

Plant material

Seeds* of *Petunia* x *hybrida cultivar 'Snowball' originating from the trade market have been used for *in vitro* germination. Sterilized seeds with Domestos solution (5 %) were inoculated into Petri dishes on a MURASHIGE-SKOOG (T. MURASHIGE & F. SKOOG [20]) or MS62 culture medium without hormones for 8 weeks (Table no 1, variant V_0). After 8 weeks the resulted hypocotyls were tailored in segments of 1 cm and used as starting material for primary callus production through their cultivation on a modified MS62 culture medium supplemented with 1 mg/l 2,4-Dichlorophenoxyacetic acid (2.4D) and 6-Benzylaminopurine (BAP) 0.1 mg/l for 4 weeks at 22°C in dark conditions ($V_{1.1.}$).

Primary callus

After 4 weeks of hypocotyl fragments cultivation the resulted primary callus was used for somatic embryogenesis experiments.

Embryogenic competency acquiring

The primary calli (1 g per sample) were transferred for 14 days on a liquid culture medium MS62 supplemented with 2.4D and BAP ($V_{2.1.}$). As an experimental variant, the culture medium was supplemented with 0.1 µg/l lovastatin or 0.2 nM (Merck, Lovastatin - CAS 75330-75-5 – Calbiochem) ($V_{2.2.}$) (Table no. 1).

Embryogenic competency expressing was obtained on a liquid culture medium MS62, hormone-free, for three weeks ($V_{3.1.}$). For testing lovastatin effects, it was also added at the same concentration ($V_{3.2.}$).

Culture media

All culture media variants were based on the modified MS62 (Table no 1). A stock solution of lovastatin was prepared after hydrolysing the lactone ring in ethanolic NaOH (15% [v/v] ethanol, 0.25% [w/v] NaOH) at 60°C for 1 h (D.N. CROWELL & M.S. SALAZ [17]) (Table no. 1).

Table 1. Variants of culture media used for testing the effect of lovastatin 0,1 µg/l on petunia somatic embryogenesis

Somatic embryogenesis	Culture Media Variants	2.4D mg/l	BAP mg/l	Lovastatin µg/l	Agar g/l
Seeds germination	V0	-	-	-	8
Primary callus induction	V1	1	0.1	0	8
Competency acquiring	V2.1.	0.2	0.1	0	0
	V2.2.	0.2	0.1	0.1	0
Competency expressing	V3.1.	0	0	0	0
	V3.2.	0	0	0.1	0

Culture conditions

Petunia seeds germination and primary callus induction were realized on solid culture medium (12 ml of culture medium V_0 filled in Petri dishes of 6 cm diameter), at 22-24°C, a photoperiod of 16/8 h and a light intensity of 53.8 µmol/m^2s. For somatic embryogenesis, Erlenmeyer flasks of 50 ml filled with 10 ml culture media were used, submitted at a 60 rpm in dark conditions at the same temperature ($V_{2.1.}$, $V_{2.2.}$, $V_{3.1.}$ and $V_{3.2.}$).

Light microscopy studies

Samples of less than 0.1 g of embryogenic callus were stained with methylene-blue day solution (0.4%) and examined by light microscopy (10x4) (Canon CR-DGi). Only fully developed somatic embryos were counted.

Samples preparation for electrophoreses

Samples of 1 g of petunia callus or pellets (i.e. in case of solid medium were taken as such and in case of liquid culture medium the samples were obtained from pellets resulted upon filtration) were ground with mortar pestle in phosphate buffer 0.1 M, pH 7 at 4 °C (3:1 w/v), followed by centrifugation at 18,000 rpm, for 20 min. to obtain the cytoplasmic soluble proteins for electrophoretic analysis. The filtrates were treated for proteins precipitation by adding 90 % acetone (v/v) under continuous stirring for 2 h for. The resulted solution was centrifugated at 15,000 rpm for 15 min. The precipitate was solubilized in 50 mM Tris-HCl buffer pH 6.9 overnight at 5°C for excreted POX analysis (C. BANCIU & al [21]).

The wells are noted for each sample as follows: S1 (primary callus grown on V_1), S2 (general control), S3 (callus treated with lovastatin in first stage of somatic embryogenesis), S4 (control), S5 (callus treated with lovastatin only in the second stage of somatic embryogenesis), S6 (callus treated with lovastatin only for the first stage of embryogenesis) and S7 (callus treated with lovastatin in all stages) (Fig. 2).

The cultivation flow of each of the investigated calli for POXs expression are as following:

S1: V_1; 4 weeks;

S2: $V_1 \rightarrow V_{2.1}$; six weeks (4 weeks on V_1 + 2 weeks on $V_{2.1.}$);

S3: $V_1 \rightarrow V_{2.2.}$; six weeks (4 weeks on V_1 + 2 weeks on $V_{2.2.}$);

S4: $V_1 \rightarrow V_{2.1.} \rightarrow V_{3.1.}$; nine weeks (4 weeks on V_1 + 2 weeks on $V_{2.1.}$ + 3 weeks on $V_{3.1.}$);

S5: $V_1 \rightarrow V_{2.1.} \rightarrow V_{3.2.}$; nine weeks (4 weeks on V_1 + 2 weeks on $V_{2.1.}$ + 3 weeks on $V_{3.2.}$);

S6: $V_1 \rightarrow V_{2.2.} \rightarrow V_{3.1.}$; nine weeks (4 weeks on V_1 + 2 weeks on $V_{2.2.}$ + 3 weeks on $V_{3.1.}$);

S7: $V_1 \rightarrow V_{2.2.} \rightarrow V_{3.2.}$; nine weeks (4 weeks on V_1 + 2 weeks on $V_{2.2.}$ + 3 weeks on $V_{3.2.}$).

For excreted POXs the wells carrying samples are noted such as S4' S5' S6' S7' (Fig 2).

Electrophoretic analysis was carried out at 20 mA, for 3 h, at +4°C, using a running gel 8% polyacrylamide (PAA), a stacking gel 4% PAA and a buffer Tris-Gly 0.05 M (pH 8.3) with a Biometra vertical electrophoresis unit. Bromphenol blue dye was used as a running maker. POX activity in the gel was revealed by incubating gels into a 0.5 M acetate buffer at a pH 5 supplemented with 0.08% benzidine and some drops of 30% H_2O_2 (C. BANCIU & al [21]). Two different electrophoresis were realized by taking into account the cultivation momentum for each of the stages for the third repetition so all samples to be run in the same time for both stages of somatic embryogenesis as well as for the primary callus and the first stage of somatic embryogenesis.

Results and Discussions

Primary callus* of *Petunia x hybrida 'Snowball' was easily obtained, after four weeks of hypocotyl cultivation on a MS62 modified medium and supplemented with 1 mg/l 2.4-D and 0.1 mg/l BAP, reconfirming previous studies' results (P.S. RAO & al [22]). These calli, of 1 g per each sample were used for indirect somatic embryogenesis in two stages: [1] embryogenic competency induction and [2] embryogenic competency expression and cultivated into liquid culture media (Fig. 1).

All calli samples grew on the variant V_1 were characterized by the fully expression of four visible POXs isoforms such as the following: 1, 2, 4 and 5 (Fig. 2).

We mention that for the entire experiment, in case of petunia calli samples, were visualized eight POXs isoforms split in three groups such as: light (i.e. POX isoform band no. 1), middle (i.e. POX isoforms bands no: 2, 3, 4, 5, 6 and 7) and heavy (i.e. POX isoform band no. 8). Considering the constant expression of the 5th band that belongs to the middle POXs group it appears to be more active compared to the others for this stage.

First stage of embryogenic competency induction

A low concentration of 2.4-D, supplemented to the culture medium, was used for inducing embryogenic competency acquiring into a liquid culture medium: 0.1 mg/l or 0.455 µM (i.e. variant $V_{2.1.}$) (Fig. 1 A). In addition, to this stage it was also used an experimental variant, were the culture medium was supplemented with 0.1 µg/l lovastatin or 0.2 nM (i.e. variant $V_{2.2}$). This concentration is one thousand times lower compared to other researchers that in previous experiments used for tobacco cells a concentration of 1 µM (D.N. CROWELL & M.S. SALAZ [17]) or 10 µM (F. LAUREYS & al [23]). We preferred this low concentration because on one hand today it is established that HGMRs are integrated in the plasma membrane and on the other hand the experiment was conducted in a liquid culture medium which is maximizing the availability of statins to enzymes.

The analysis of POXs isoforms spectrum, at the end of this stage, revealed some patterns differences (Fig. 2: POX spectra 2 and 3). For the control (i.e. variant V_1), it is obvious the expression of the same POXs isoforms like in the primary calli (i.e. 1, 2, 4 and 5). However, the first three appear to be fade compared to the 5th that was also relevant for the primary callus. When calli were treated with lovastatin it is obvious that the isoforms 3rd, 4th and 5th were more expressed compared to control (i.e. developed on $V_{2.1.}$) and a new POX isoform was expressed: the 6th. It becomes obvious that lovastatin induced changes in the general pattern of POXs isoforms during the 'acquiring embryogenic competency' stage (Fig 2).

Second stage of embryogenic competency expression

After 14 days of cultivation on two different culture media (i.e. variant $V_{2.1.}$ and $V_{2.2.}$) the induced embryogenic cell suspension was transferred on a hormone-free culture medium where the cells acquiring the embryogenic competency could develop somatic embryos (i.e. variants $V_{3.1.}$) (Fig. 1 B). An additional variant was added for continuing testing lovastatin (i.e. 0.1 µg/l, variant $V_{3.2.}$). The whole experimental design, in terms of flow of cultivation, for this stage was as follows: [1] general control for lovastatin (i.e. free of lovastatin for both stages with the sequence of cell suspension growing: $V_1 \rightarrow V_{2.1.} \rightarrow V_{3.1.}$); [2] control for lovastatin in the first stage (i.e. lovastatin added only to the second phase and the sequence of cell suspension growing: $V_1 \rightarrow V_{2.1.} \rightarrow V_{3.2.}$); [3] cell suspension treated with lovastatin only in the first stage (i.e. the sequence of cell suspension growing: $V_1 \rightarrow V_{2.2.} \rightarrow V_{3.1.}$) and [4] lovastatin added to both stages (i.e. the sequence of cell suspension growing: $V_1 \rightarrow V_{2.2.} \rightarrow V_{3.2.}$).

At the end of the second stage, complete somatic embryos were counted for all four variants (Fig. 1 C).

For the first variant (S4), as a general control (free of lovastatin), the average number of complete somatic embryos was of 3.5 (Fig. 1 B and C). The pattern of POXs isoforms changed compared to control of the first stage. There appears a new third group of lighter POXs bands: the 7th and the 8th, that are related to the expression of somatic embryogenesis competency. Even the intensity of expression was not followed in the image appears that the first group of POXs are more expressed compared to control of the first stage (well S3) (Fig. 2).

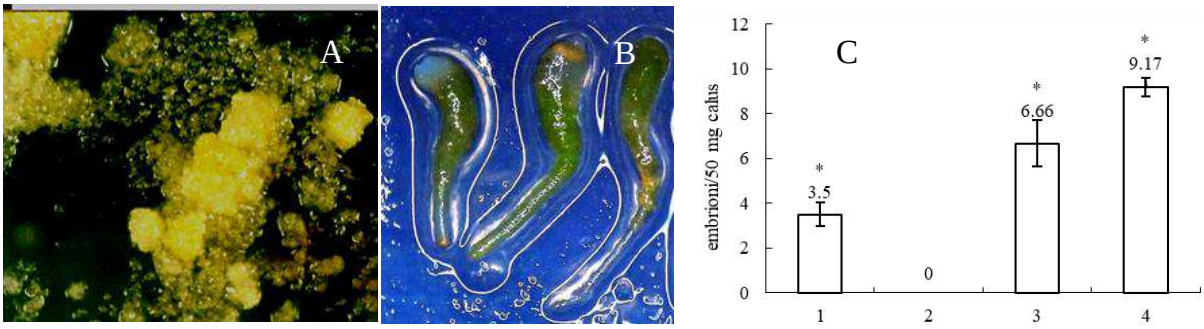

Figure 1. Somatic embryogenesis in petunia: cell pellets (**A**), somatic embryos under light microscope (x 40) (**B**), and lovastatin effect on the number of fully developed somatic embryos (**C**): 1- control (free of lovastatin, the callus was cultivated on variants V2.1. followed by V3.1.); 2- callus treated in the second phase (the callus was cultivated on variants V2.2. followed by V3.1.); 3- callus treated only in the first phase (the callus was cultivated on variants V2.2. followed by V.3.1.); and 4- callus treated for both phases (the callus was cultivated on variants V2.2. followed by V3.2.) (The error bar represents ± standard deviation (SD) of three replicates and three repetitions of each stage; The Student's t test, $P<0.05$).

For the second variant (S5), when lovastatin was added only in the last stage, no embryogenesis occurred. This result would support the idea that statins may block HMGRs activities and probably the synthesis of essential isoprenoid compounds for embryogenesis. In this case the profile of POX isoforms appears to be alike the first stage of acquiring embryogenetic competency, but more intense expressed. Taking into account this result it can be raised

the question: is it possible that lovastatin to arrest callus cells in the acquiring phase of embryogenic competency? This question is supported by the pattern of excreted POXs that is similar to the general control (i.e. POX identified into the pellet of filtrate).

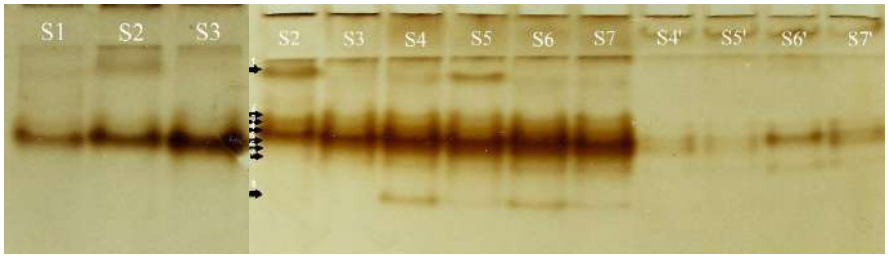

Figure 2. Lovastatin effect on POXs isoforms for indirect somatic embryogenesis: *first stage* – the induction of embryogenic competency (S1- primary callus, S2 - secondary callus as control, S3 - secondary callus treated with lovastatin – the left image) and *second stage* - the expression of somatic embryogenesis competency (wells: S4 - control secondary callus, S5 - control secondary callus sub-cultivated on lovastatin supplemented medium, S6 - callus 3 subcultivated on medium without lovastatin, S7 - callus 3 subcultivated on lovastatin supplemented medium. Wells noted S4', S5', S6' and S7' are excreted POXs, see also Material and Methods).

For the third variant (S6), when lovastatin was added only in the first stage of acquiring embryogenic competency, the number of embryos doubled compared to control (i.e. 6.66/50 mg, pellet of cell suspension with a variation of ±1). It appears that the presence of lovastatin in the first stage, by blocking the HMGR in the presence of hormones will further enhance the rate of somatic embryos formation in the later stage. In this case, the POXs isoforms look like the general control. However, the POXs isoforms such as 3rd, 4th, 5th and 6th appear to be intense coloured. The expression of the 8th POX band, the lighter POX is clearly correlated to embryos generation. The analysis of excreted POXs appear to have a different pattern compared to controls, the 5th and 7th isoforms being more intense. Based on these results, it can by hypothesised that lovastatin may act synergistically with auxins in acquiring embryonic competency in the first stage.

In the fourth case (S7), when lovastatin was added in both subsequent culture media, the number of embryos tripled compared to control (i.e. 9.16 embryos/50 mg of callus). In this latter case lovastatin seems to support on one hand the first stage of acquiring embryogenic competency as well as the expression of this competency for the subsequent stage. The POXs isoforms analysis reveals a similar pattern with the general control callus as well but very interesting the 3rd and the 7th POX isoforms are much sharper compared to control. Excreted POXs appear to be more intense for the 5th, 6th and 7th isoforms being slightly different compared to the callus treated with lovastatin for the first stage and different compared to the others.

The broader analysis of these results would further support the hypothesis that by adding statins in the first embryogenesis stage it will create a better micro-environment for the acquisition of embryogenic competency. Some questions can be raised in line with current theoretical background of today: is it possible that statins to decrease the sterols levels and patterns due to the specific inhibition of HMGR? If so is it possible to associate this effect to the increase of oxidative stress that would be responsible for inducing POXs isoform expression? A direct correlation between ROSs levels and ascorbate peroxidase on one hand and glutathione peroxidase on the other hand was already proved for somatic embryogenesis in cotton (T. ZHOU & al [24]).

Based on these results it appears that some new analysis need to be realized to prove that there might be a direct or indirect link between changing the sterols synthesis and ROSs production as it was already suggested by other authors working on *Arabidopsis* (H.B. KIM & al [25]) or cotton (T. ZHOU & al [24]).

When lovastatin was added in both stages it induced a significant increase in the number of somatic embryos compared to general control and control for lovastatin. This is associated with a sharper increase in two POXs isoforms. From these results it appears that low levels of lovastatin supplemented to liquid culture medium clearly affect the POXs isoforms expression. However, when lovastatin is added in the second stage for embryogenesis competency expression, somatic embryogenesis cannot be expressed and studying the expression profiles of POXs isoforms they look similar with that of the first stage (i.e. fig. 2 the wells S2, S5 and S5'). The increased expression of POXs may be associated with an increased level of ROSs, but further studies are needed.

The analysis of these results raises new questions related to signalling pathways upon the inhibition of HGMR due to statins that are essential for totipotency expression in plants.

Conclusions

Indirect somatic embryogenesis may be modulated for its expression by different exogenous factors such as supplemented signalling molecules or by changing *in vitro* cultivation conditions. This study emphasized the relevance of lovastatin, a well-known HGMR inhibitor, in stimulating somatic embryogenesis in petunia. Based on the analysis of these results, it can be considered that lovastatin is able to increase the embryogenic competency

expression when it is supplemented in the first stage of acquiring embryogenic competency. The number of somatic embryos was doubled which is positively associated with the expression of different patterns of POXs. By adding lovastatin at both stages, it induced a three times higher number of somatic embryos compared to control. However, by adding lovastatin only in the last stage, somatic embryogenic competency is blocked, and it is supported by the revealed POXs pattern. These results suggest further that HGMR may play a pivotal role in somatic embryogenesis: acquiring and expression phases.

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