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CELL BIOLOGY AND MORPHOGENESIS

Excretion of polyamines in alfalfa and tobacco suspension-cultured cells and its possible role in maintenance of intracellular polyamine contents

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Abstract Changes in polyamines (PAs) in cells and cultivation media of alfalfa (*Medicago sativa* L.) and tobacco bright yellow 2 (BY-2) (*Nicotiana tabacum* L.) cell suspension cultures were studied over their growth cycles. The total content of PAs (both free and conjugated forms) was nearly 10 times higher in alfalfa, with high level of free putrescine (Put) (in exponential growth phase it represented about 65–73% of the intracellular Put pool). In contrast, the high content of soluble Put conjugates was found in tobacco cells (in exponential phase about 70% of the intracellular Put). Marked differences occurred in the amount of PAs excreted into the cultivation medium: alfalfa cells excreted at the first day after inoculation 2117.0, 230.5, 29.0 and 88.0 nmol g^{-1} of cell fresh weight (FW) of Put, spermidine (Spd), spermine (Spm) and cadaverine (Cad), respectively, while at the same time tobacco cells excreted only small amount of Put and Spd (12.7 and 2.4 nmol g^{-1} FW, respectively). On day 1 the amounts of Put, Spd, Spm and Cad excreted by alfalfa cells represented 21, 38, 12 and 15% of the total pool (intra- plus extra-cellular contents) of Put, Spd, Spm and Cad, respectively. In the course of lag-phase and the beginning of exponential phase the relative contents of extracellular PAs continually decreased (with the exception of Cad). On day 10, the extracellular Put, Spd, Spm and Cad still represented 11.3, 10.9, 2.1 and 27% of their total pools. The extracellular PAs in tobacco cells represented from day 3 only 0.1% from their total pools. The possible role of PA excretion into the cultivation medium in maintenance of intracellular PA contents in the cells of the two cell culture systems, differing markedly in growth rate and PA metabolism is discussed.

Keywords Arginine decarboxylase · Diamine oxidase · Ornithine decarboxylase · Extracellular polyamines · S-adenosylmethionine decarboxylase

Abbreviations

ADC	Arginine decarboxylase
Cad	Cadaverine
d	Days
DAO	Diamine oxidase
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
MI	Mitotic index
ODC	Ornithine decarboxylase
PAs	Polyamines
PCA	Perchloric acid
Put	Putrescine
SAMDC	S-adenosylmethionine decarboxylase
Spd	Spermidine
Spm	Spermine

Introduction

Polyamines (PAs) have been linked to developmental programmes, such as cell growth and division (Theiss et al.

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2002), morphogenesis (Paschalidis et al. 2001), stabilization of nucleic acids and membranes (Thomas and Thomas 2001), and biotic and abiotic stress responses (Bouchereau et al. 1999). As for other plant growth regulatory compounds, levels of PAs inside and outside cells participate in the determination of the way of development. This implies that—again similar to other plant growth substances (Davies 2004)—internal levels of PAs are precisely regulated. Generally, the content of any compound in cells can be regulated by metabolic processes (e.g., biosynthesis, conjugation and degradation) and by transport processes (uptake into and excretion from cells).

For PAs two main pathways of biosynthesis occur in higher plants. The diamine putrescine (Put) can be formed either directly from the amino acid ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17) or indirectly from arginine by arginine decarboxylase (ADC; EC 4.1.1.19). The diamine cadaverine (Cad), which is not as abundant as Put, can be formed from lysine via ODC or as a result of lysine decarboxylase activity (LDC; EC 4.1.1.18) (Bagni and Tassoni 2001). S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) is essential for biosynthesis of spermidine (Spd) and spermine (Spm). Apart from the rate of biosynthesis are the intracellular concentrations of free PAs regulated by conjugation either with small molecules, especially hydroxycinnamic acids (so-called soluble conjugated PAs; Martin-Tanguy 1985; Bagni and Tassoni 2001; Biondi et al. 2001), or with high-molecular-mass substances like hemicelluloses and lignin and, in small amounts, also with proteins (so-called insoluble conjugated PAs; Creus et al. 1991). In addition to conjugation, levels of free PAs can be down-regulated by oxidative deamination. Cytoplasmic levels of PAs can be affected also by their storage in vacuoles, mitochondria, and chloroplasts as well as by transport out of cells (Flores 1991; Bagni and Tassoni 2001). In mammalian cells cultured under normal growth conditions up to 90% of Put and 25% of Spd synthesized by the cells is excreted into the culture medium (Hawel et al. 1994). Although excretion (efflux) is an integral part of the regulatory processes responsible for controlling the intracellular PA content in animal cell cultures, information about PA excretion in plants is rather sparse. Excretion of PAs into the cultivation medium was recently studied only in connection with biotic or abiotic stresses (Messiaen and Van Cutsem 1999; Tamai et al. 2000; Königshofer and Lechner 2002).

The aim of this work was to detect and to quantify the excretion of PAs in the course of the growth cycle of suspension-cultured cells. The two cell culture systems, alfalfa and tobacco cells, differing markedly in cell multiplication and growth rates and in PA metabolism were used in this report. We focused first on the evaluation of PA conjugation, oxidative deamination and excretion in regulation of intracellular PA contents.

Materials and methods

Plant material

Cell suspension cultures of *Medicago sativa* L. were derived from alfalfa plants, genotype Derby, as described by Binarová and Doležel (1988) and maintained as described by Cvikrová et al. (1999). The tobacco bright yellow 2 (BY-2) cell line (*Nicotiana tabacum* L. cv. BY-2) was maintained as previously described (Nagata et al. 1992). The cells were cultivated at 25°C in darkness and the typical subculture periods were 7 and 10 days for tobacco and alfalfa cells, respectively. Cells were harvested daily, and samples were stored at −80°C before analyses.

Cell viability

Viability of the cultured cells in suspension was estimated by the trypan blue dye exclusion test (Phillips 1973) using 0.4% trypan blue solution (Sigma-Aldrich, St Louis, MO, USA).

Mitotic index

Samples of cultured alfalfa cells in suspension were stained according to standard Feulgen procedure (Doležel et al. 1992). Cells (1,000) were analyzed on each slide and five slides were examined in each sample.

Polyamine analysis

Cells were ground in liquid nitrogen and extracted overnight at 4°C with 5% (v/v) perchloric acid (PCA) (100 mg fresh weight (FW) tissue ml^{-1} 5% PCA). 1,7-Diaminoheptane was added as an internal standard. The extracts were centrifuged at 21,000×*g* for 15 min, and then PCA-soluble free PAs were determined in one-half volume of the supernatant. The remaining supernatant and pellet were acid-hydrolysed in 6 M HCl for 18 h at 110°C to obtain PCA-soluble and PCA-insoluble conjugates of PAs as described by Slocum et al. (1989). Standards (Sigma-Aldrich, St Louis, MO, USA), PCA-soluble free PAs, and acid hydrolysed PA conjugates were benzoylated. HPLC analysis of benzoyl-amines was performed on a Beckman-Video Liquid Chromatograph equipped with a UV detector (detection at 254 nm) and C_{18} Spherisorb 5 ODS2 column (particle size 5 µm, column length 250 × 4.6 mm) according to the method of Slocum et al. (1989).

Extraction of polyamines from the cultivation medium

To separate the PAs from the other components in the cell culture medium, the samples were subjected to ion-exchange on Bio-Rex 70 anion exchange resin (100–200 mesh, sodium form, Bio-Rad Laboratories, Hercules, CA, USA) as described in detail in Hawel and Byus (2002). Briefly, the cultivation medium was separated from the cells by careful filtration through filter paper under reduced pressure. Two separate samples of 50 ml medium were analyzed in parallel. Prior to ion-exchange chromatography, 1,7-diaminoheptane was added to the media samples as an internal standard. The samples were gradually loaded onto mini-columns and allowed to flow through Bio-Rex 70 resin by the force of gravity. The resin was then washed with 30 ml of 19 mM sodium carbonate (pH 9.3) to remove the majority of non-polyamine cations bound to the resin. Afterwards, the columns were placed to into tubes and centrifuged for 1 min at 800×*g* to remove the remaining liquid. The resin was than resuspended in 10 ml of 0.1 M NaOH and it was shaken for 1 h at room temperature. PAs were than benzoylated and HPLC analysis of benzoyl-amines was performed as described above.

Ornithine decarboxylase, arginine decarboxylase and S-adenosylmethionine decarboxylase assays

Ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19) and S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) were determined by a radiochemical method as described by Tassoni et al. (2000). Samples were extracted in three volumes of ice-cold 0.1 M Tris–HCl buffer, pH 8.5, containing 2 mM β-mercaptoethanol, 1 mM EDTA and 0.1 mM pyridoxal phosphate, and centrifuged at 20,000×*g* for 30 min at 4°C. Aliquots (0.1 ml) of both supernatant (soluble fraction) and resuspended pellet (particulate fraction) were used to determine ODC and ADC activity. Enzyme activity assays were performed by measuring the $^{14}CO_2$ evolution from 7.4 kBq L-[1-^{14}C]ornithine (1.92 GBq mmol^{-1}, Amersham Pharmacia Biotech, UK) or 7.4 kBq L-[U-^{14}C]arginine (11.5 GBq mmol^{-1}, Amersham Pharmacia Biotech UK), for ODC and ADC, respectively, in the presence of 2 mM unlabelled substrate during a 1.5-h incubation at 37°C. CO_2 was entrapped in hyamine hydroxide and the radioactivity was counted on a liquid scintillation analyzer, Tri-Carb 2900TR, Packard.

To determine SAMDC activity samples were homogenized in three volumes of 0.1 M phosphate buffer, pH 7.6 containing 2 mM β-mercaptoethanol and 1 mM EDTA, and centrifuged at 20,000×*g* for 30 min at 4°C. The supernatant and resuspended pellet (0.1 ml aliquots) were incubated separately with 3.7 kBq [1-^{14}C]*S*-adenosylmethionine (2.15 GBq mmol^{-1}, Amersham Pharmacia Biotech UK) in the presence of 2.8 mM unlabelled substrate and 3 mM Put. $^{14}CO_2$ evolution was measured for 1 h at 37°C. The radioactivity was counted on a liquid scintillation analyzer, Tri-Carb 2900TR, Packard.

Enzymatic activity was expressed in pkat mg^{-1} protein.

Protein content was measured according to Bradford (1976) using bovine serum albumin as a standard.

Diamine oxidase assay

Diamine oxidase (DAO, EC 1.4.3.6) activity was assayed by a spectrophotometric method based on detection of the aldehyde with *cis*-1,4-diamino-2-butene as the substrate (Peč et al. 1991). Samples were homogenised in 0.1 M Tris–HCl buffer, pH 8.5, containing 2 mM β-mercaptoethanol and 1 mM EDTA, and centrifuged at 20,000×*g* for 15 min at 4°C. The reaction mixture contained 0.1 M Tris–HCl buffer, pH 8.5, catalase (25 μg) and 0.01 M *cis*-1,4-diamino-2-butene. The reaction was started by the addition of 0.2 ml supernatant, incubated for 1 h at 37°C and stopped by adding 1 ml of Ehrlich's reagent. The reaction mixture was incubated at 50°C for 5 min, and then chilled on an ice bath before reading the absorbance of produced pyrrol at 563 nm. Enzymatic activity was expressed in pkat mg^{-1} protein.

Statistical analyses

Two independent experiments with three parallel determinations were carried out using plant material from 2 to 4 vessels in one time-course study. The results presented here were obtained from one particular subculture, thereby allowing us to follow and compare the intra- and extracellular PA concentrations more precisely. Analogous results were obtained in both experiments. Mean ± SE of one experiment (with 3 replicates) are shown in the figures. Data were analyzed using the Student's *t* distribution criteria.

Results

Growth of cultures

After inoculation into fresh medium, the FW of cultures increased after a lag-phase of approximately 2 days to give characteristic sigmoidal growth curve (Fig. 1). Mitotic activity of alfalfa cells was characterized by 2 MI peaks occurring on day 3 and on days 5–6 (Fig. 1a). The values of MI of BY-2 cell suspension culture presented in Fig. 1b were adapted from our previous work (Gemperlová et al. 2005). The typical subculture periods were 7-days and 10-

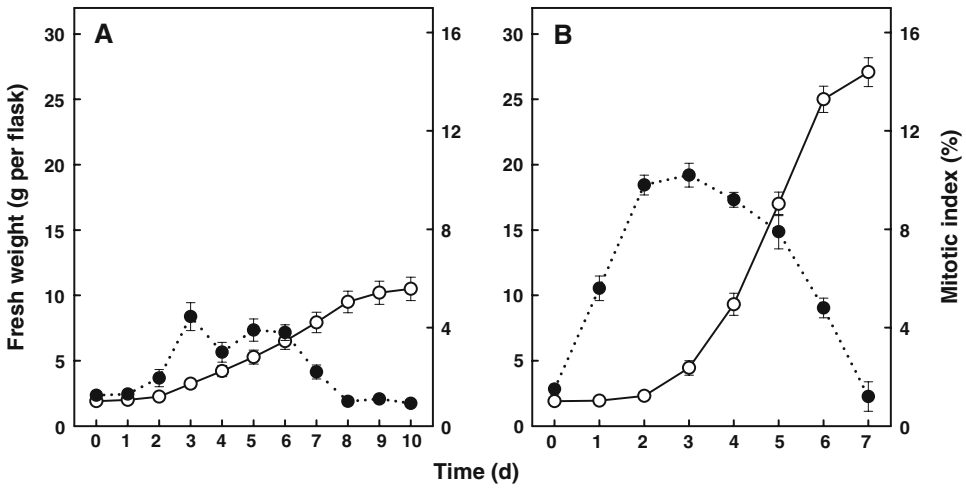

Fig. 1 Growth of cell lines of alfalfa (**a**) and tobacco BY-2 (**b**). The growth curves for both lines are expressed as fresh weight (FW, *open circles*) of cells per one flask (250 ml). The values of MI (*full circles*) of tobacco BY-2 were adapted from our previous work (Gemperlová et al. 2005). Mean ± SE of two independent experiments with three replicates are shown

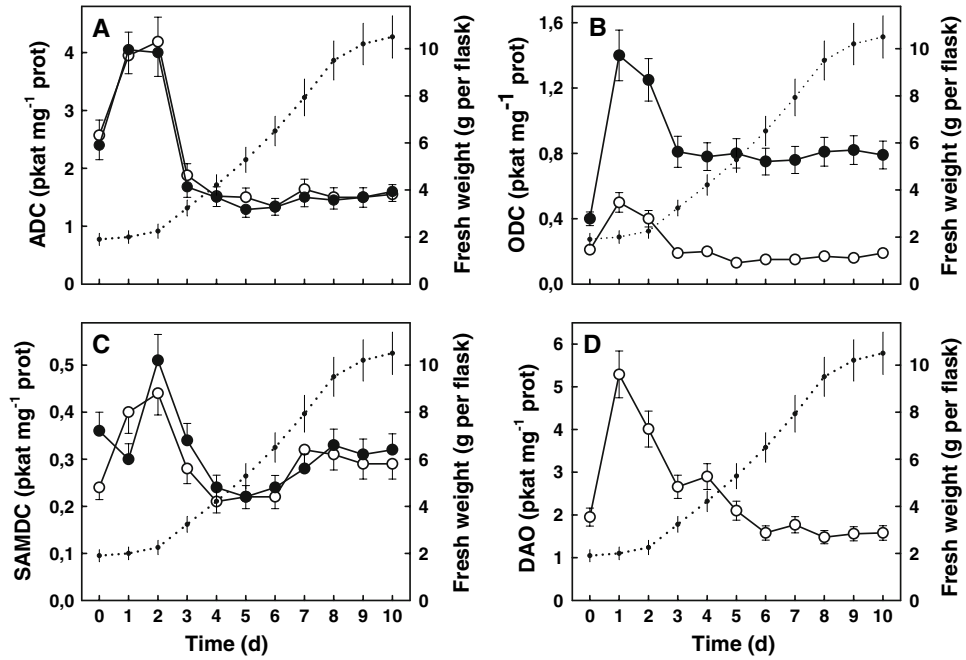

Fig. 2 Time course of enzyme activities involved in PA metabolism of alfalfa cell suspension culture. Biosynthetic enzymes: arginine decarboxylase (ADC; **a**), ornithin decarboxylase (ODC; **b**), and S-adenosylmethionine decarboxylase (SAMDC; **c**). Catalytic enzyme diamine oxidase (DAO; **d**). *Bars* represent SE of three replicates. *Bars* representing SE for SAMDC activity are partially hidden into the symbols. Activities of enzymes in soluble (*open symbols*) and particulate (*full symbols*) fractions. *Dashed line*, growth curve expressed as fresh weight (FW) of cells per one flask (250 ml)

days for tobacco and alfalfa cells, respectively. Cell viability during the whole subculture period was stable, varying between 80 and 90% of living cells in both suspension cultures.

Activities of PA biosynthetic enzymes in alfalfa cells

Activities of PA biosynthetic enzymes, measured in both soluble and pellet fractions, showed an increase in cells on the first and second days after the inoculation. ADC was the main enzyme involved in Put biosynthesis (Fig. 2a) and its activity was equally distributed between soluble and pellet fractions. The activity of ODC was much lower (Fig. 2b) and about 70% of its total activity was found in the pellet fraction. The peak of SAMDC activity coincided with the maximum of ADC and its activity was almost equally divided between soluble and pellet fraction (Fig. 2c).

Activity of diamine oxidase in alfalfa cells

The rise in the DAO activity on the first day after inoculation correlated with the decline in free Put content (Figs. 2d, 3) and coincided with the increase in the activities of both biosynthetic enzymes (ADC and ODC).

Intracellular and extracellular polyamine contents in alfalfa culture

After inoculation, the content of PAs in alfalfa cells started to increase (after a transient decline on the first day caused by a decrease in free Put content), and reached the

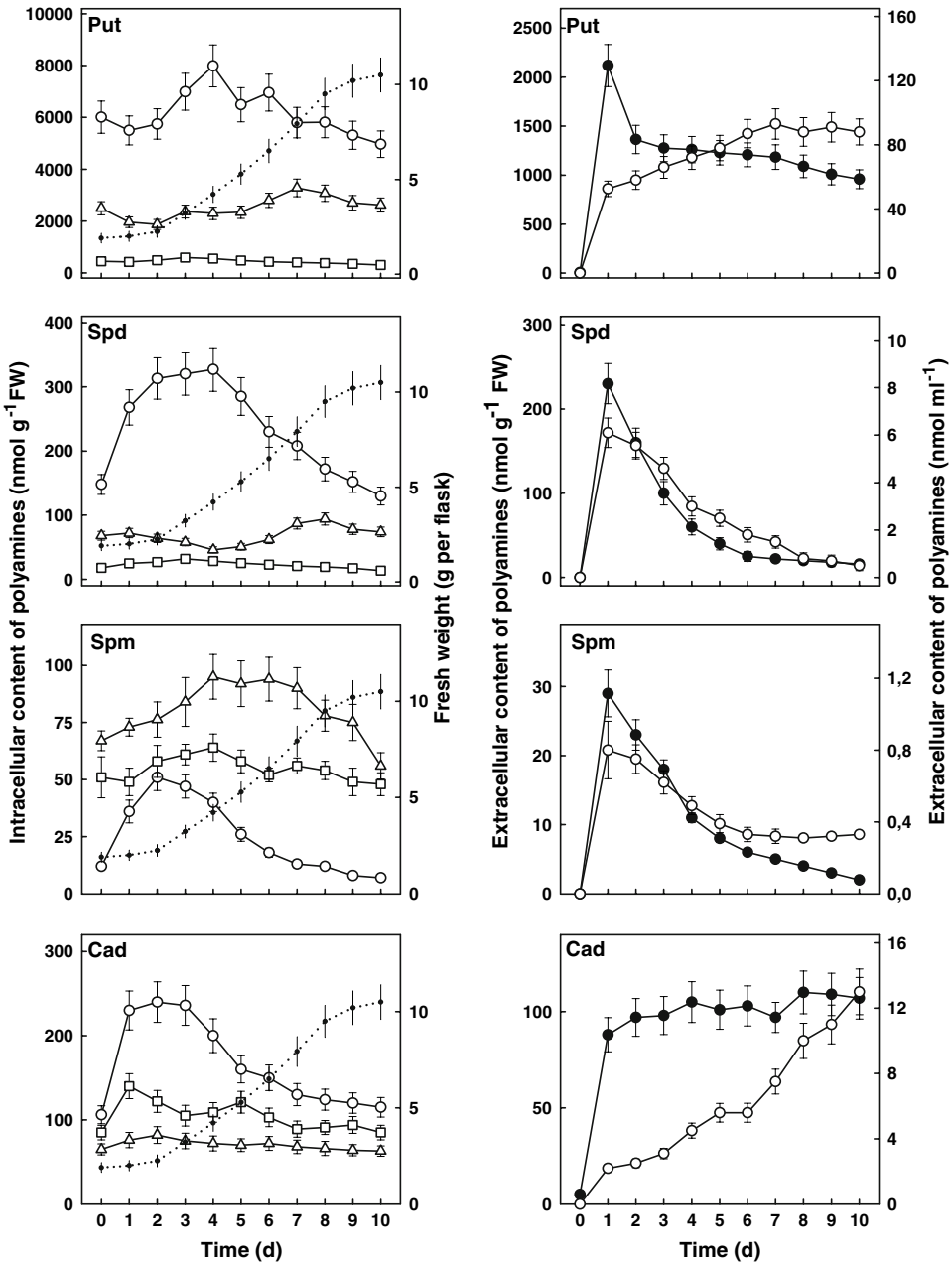

Fig. 3 Dynamics of intracellular and extracellular (i.e., excreted into cultivation medium) PAs during the growth cycle of alfalfa cell suspension culture. *Dashed line*, growth curve expressed as fresh weight (FW) of cells per one flask (250 ml). *Bars* represent SE of three replicates. Intracellular content of free Put (*open circles*), PCA-soluble Put conjugates (*open triangles*), and PCA-insoluble Put conjugates (*open squares*). Extracellular Put, expressed as nmol g^{-1} FW (*full circles*) or as nmol ml^{-1} of the medium (*open circles*). Intracellular content of free Spd (*open circles*), PCA-soluble Spd conjugates (*open triangles*), and PCA-insoluble Spd conjugates (*open squares*). Extracellular Spd, expressed as nmol g^{-1} FW (*full circles*) or as nmol ml^{-1} of the medium (*open circles*). Intracellular content of free Spm (*open circles*), PCA-soluble Spm conjugates (*open triangles*), and PCA-insoluble Spm conjugates (*open squares*). Extracellular Spm, expressed as nmol g^{-1} FW (*full circles*) or as nmol ml^{-1} of the medium (*open circles*). Intracellular content of free Cad (*open circles*), PCA-soluble Cad conjugates (*open triangles*), and PCA-insoluble Cad conjugates (*open squares*). Extracellular Cad, expressed as nmol g^{-1} FW (*full circles*) or as nmol ml^{-1} of the medium (*open circles*)

maximum at the exponential phase of growth. In the alfalfa cells free Put was by far the most abundant amine (Fig. 3). At the exponential growth phase the content of free Put represented about 65% of the total PA pool in the cells and more than 70% of the intracellular Put content. The amounts of free Spd, Spm and Cad (Fig. 3) were considerably lower. In spite of the induction of ADC and ODC activities the first day after subculture, the internal free Put level declined slightly, thereafter increasing and reaching its maximum value 2 days later than free Spd, Spm and Cad (on day 4). While the PCA-insoluble conjugates of Put and Spd represented by about 4–5% from the total content, the proportion of Cad amount in this fraction was more than 20% (it was the second most abundant PA in this fraction after Put). PCA-soluble conjugates were, with the exception of conjugated Spm, less concentrated than the corresponding free forms. The dynamics of soluble conjugate levels significantly differed from the trend observed in the free forms; the levels of soluble conjugates were more or less stable during the subculture interval in alfalfa cells (Fig. 3).

Alfalfa cells excreted considerable amounts of Put, Spd and Cad as well as small amounts of Spm into the cultivation medium (Fig. 3). The amount of total extracellular

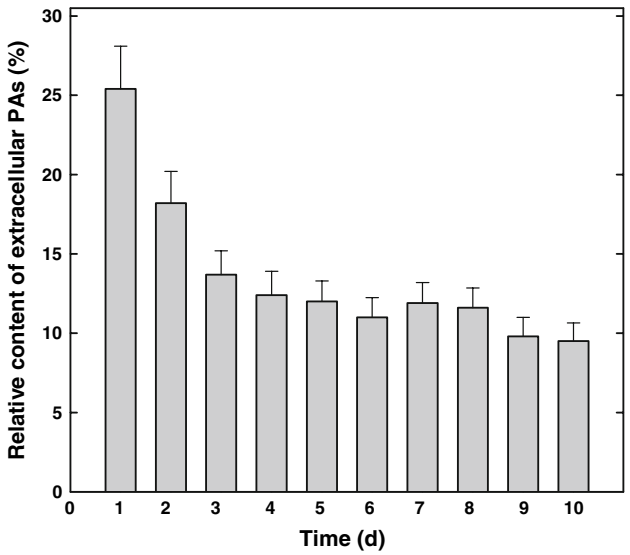

Fig. 4 The relative content (% of total intracellular PA content) of PAs excreted into cultivation medium during the growth cycle of alfalfa suspension culture. *Bars* represent SE of three replicates

PAs, corresponding to approximately 25% of the intracellular content, was found on the first day in the cultivation medium and the extracellular quantity of PAs exceeded 10% of the intracellular pool during the whole subculture period (Fig. 4). Unlike Put, Spd and Spm the exogenous Cad content expressed per g of the cell FW was stable from the first day during the entire growth cycle (Fig. 3). When the external Put and Cad were calculated per ml of medium, their amounts slightly increased during the growth cycle and reached 90.0 and 13 nmol ml^{-1} medium, respectively. The levels of Spd and Spm in 1 ml of cultivation medium were very low and decreased from the day 1 of subculture till the end of culture period from 6.0 to 1.0 and 1.0 to 0.3 nmol ml^{-1} medium, respectively (Fig. 3).

In view of the fact that in some ways the cell cultivation medium represents a functional extracellular compartment of cells, we compared the relative contents of individual PAs excreted into the cultivation medium in their total pool (sum of intra- and extra-cellular forms of individual PAs). On day 1 the amounts of Put, Spd, Spm and Cad excreted by alfalfa cells represented 21, 38, 12.1 and 15.6% of the total pool of Put, Spd, Spm and Cad, respectively. In the course of lag-phase and the beginning of exponential phase the relative contents of extracellular PAs (with the exception of Cad) continually decreased (Table 1). On day 7 the extracellular Put, Spd, Spm and Cad, after reaching the steady level, still represented 11.3, 10.9, 2.1 and 27% of the intra- and extra-cellular pool Put, Spd, Spm and Cad, respectively, and did not change until the end of culture (Table 1).

Table 1 Relative contents (% of the sum of intracellular plus extracellular contents) of PAs excreted into the cultivation medium of alfalfa in the course of growth cycle

Days	Put	Spd	Spm	Cad
1	21.1 ± 2.3	38.0 ± 3.9	12.1 ± 1.4	15.6 ± 1.7
2	14.3 ± 1.5	28.2 ± 2.9	7.0 ± 0.8	16.6 ± 1.8
3	11.3 ± 1.3	17.0 ± 1.8	4.2 ± 0.5	18.0 ± 2.0
4	10.4 ± 1.2	13.1 ± 1.5	2.7 ± 0.4	21.0 ± 2.3
7	11.3 ± 1.3	10.9 ± 1.3	2.1 ± 0.3	26.8 ± 2.9
10	10.8 ± 1.2	6.8 ± 0.8	2.4 ± 0.4	28.4 ± 3.1

Mean values of three replicates ± SE are presented

Intracellular and extracellular polyamine contents in tobacco BY-2 culture

Contents of intracellular PAs and activities of enzymes involved in their metabolism were studied in detail in our previous work (Gemperlová et al. 2005). In this work, in addition to the contents of extracellular PAs, the intracellular contents of Put and Spd (the only amines found in the tobacco medium) were determined. In tobacco BY-2 cells Put and Spd were the predominant amines, both occurring in free, PCA-soluble and PCA-insoluble conjugated forms (Fig. 5). The endogenous level of free Put reached its maximum on day 4, 2 days later than that of free Spd. In contrast to alfalfa, the PCA-soluble conjugates of Put were present in considerable amounts during the whole subculture interval and in their maximum on day 3, their content represented about 70% of the whole intracellular Put pool. The time course analysis of the contents of PCA-soluble conjugates of Spd showed a trend similar to that of the free form; however, their concentration was much lower. PCA-insoluble conjugates, the third determined PA fraction, were detected only in small amounts.

Contrary to high amounts of Put, Spd and Cad excreted by alfalfa cells only a small quantity of extracellular Put and Spd occurred in the medium of tobacco cells (Fig. 5). When the extracellular Put and Spd (Spm and Cad were not found) were calculated per ml of medium, the amounts were extremely low during the growth cycle (below 0.5 nmol ml^{-1} medium). The extracellular concentrations of Put and Spd (per ml of medium) in alfalfa exceeded 150–400 and 60–240 times, respectively, the amounts detected in the medium of tobacco (with the same size of inoculated cells) (Figs. 3, 5).

If the amounts of amines were expressed per cell FW equivalent, however, a small peak of excreted Put and even less marked increase in Spd were observed in the first day after inoculation (12.7 and 2.4 nmol g^{-1} FW, respectively, Fig. 5). At day 1, when the excretion was maximal, the amounts of Put and Spd excreted by tobacco cells

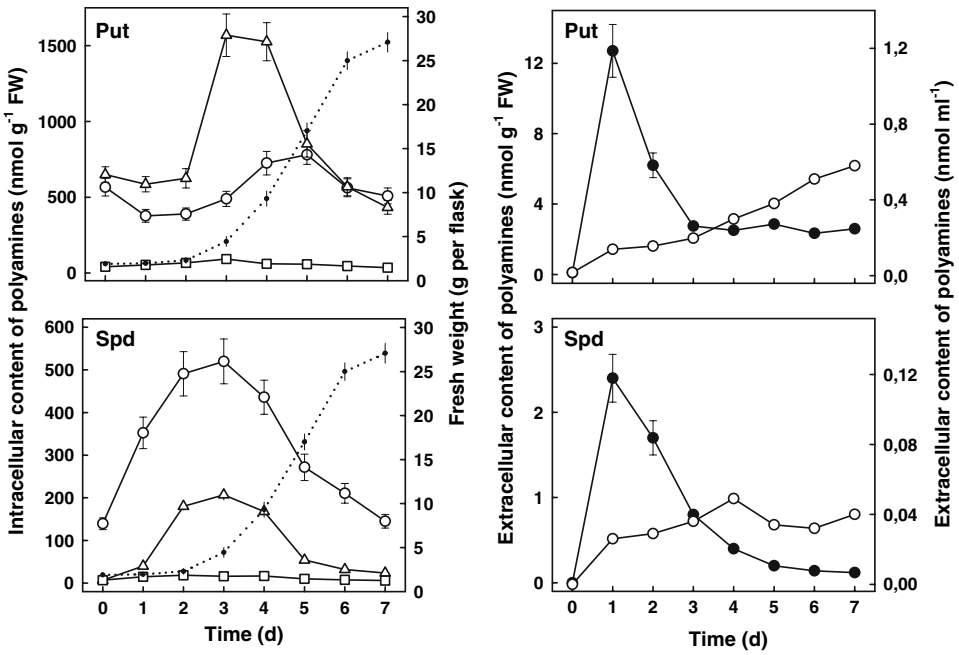

Fig. 5 Dynamics of intracellular and extracellular PAs during the growth cycle of tobacco BY-2 cell suspension culture. *Dashed line*, growth curve expressed as fresh weight (FW) of cells per one flask (250 ml). *Bars* represent SE of three replicates. Intracellular content of free Put (*open circles*), PCA-soluble Put conjugates (*open triangles*), and PCA-insoluble Put conjugates (*open squares*). Extracellular Put, expressed as nmol g^{-1} FW (*full circles*) or as nmol ml^{-1} of the medium (*open circles*). Intracellular content of free Spd (*open circles*), PCA-soluble Spd conjugates (*open triangles*), and PCA-insoluble Spd conjugates (*open squares*). Extracellular Spd, expressed as nmol g^{-1} FW (*full circles*) or as nmol ml^{-1} of the medium (*open circles*)

represented only 1.4 and 0.65% of the total (intra- and extra-cellular) pool of Put and Spd, respectively.

Discussion

In the two distinct cell culture systems we have evaluated the alterations in PA metabolism which included the activities of biosynthetic and catalytic enzymes and the mutual relationship between intra- and extra-cellular levels of PAs, a phenomenon which has hardly been investigated in plants. As we referred in our previous work the time courses of biosynthetic and catalytic enzyme activities in BY-2 cells were similar to that determined in alfalfa cells in this experiment, however, the activities were higher (Gemperlová et al. 2005). High titres of free Put in alfalfa and, on the contrary, the high content of soluble Put conjugates found in tobacco cells contributed substantially to differences in PA metabolism between tobacco and alfalfa cultures (Figs. 3, 5). These differences further included: the size of the total content of PAs (at the time of inoculation nearly 10 times higher in alfalfa), the activities of PA biosynthetic enzymes (significantly higher in tobacco), the activity of PA-catalytic enzyme (2 times higher in tobacco), and finally the extent of PA excretion into the cultivation medium (much higher in alfalfa).

Inoculation of plant suspension cultures into the fresh medium results in activation of various processes of cell metabolism (Hahlbrock 1976). An increase in PA levels belongs to the first events accompanying the induction of cell division (e.g., Pfosser et al. 1990; Bezold et al. 2003). In spite of the higher rate of PA biosynthesis in BY-2 cells after inoculation and more than doubled amounts of PAs at the beginning of the exponential phase (related to the value at the time of inoculation, Gemperlová et al. 2005), the amount of PAs remained many times lower due to the initial high level in alfalfa cells. It is not necessarily the presence of the high level of free PAs itself, however, which is required for induction of cell division. It seems that it is an adequate Put/Spd ratio (Faure et al. 1991; Hunter and Burrit 2005) which is needed for the induction of morphogenic event and/or its change. Due to extremely high free Put in alfalfa cells the ratio of Put/Spd was probably already too high for active cell division. The pronounced rise of free Spd content and its higher level in the onset of the exponential phase in BY-2 cells (compared with alfalfa, Figs. 3, 5) is in good agreement with the observed different mitotic activities of alfalfa cell suspension culture (MI 4–5%) and tobacco BY-2 cells (MI 10%).

The existence of two alternative routes for Put biosynthesis in plants may reflect the necessity of specific regulation of different processes affected by Put during

growth and development (Koetje et al. 1993; Galston et al. 1997; Paschalidis and Roubelkis-Angelakis 2005). It has been suggested that a pathway via ADC is generally linked to stress responses and morphogenic processes, whereas ODC is involved particularly in the regulation of cell division (Galston et al. 1997). The results presented here show that in alfalfa cells played the main role in Put biosynthesis ADC, and its activity was equally divided between soluble and pellet fractions (Fig. 2a). The activity of ODC was much lower and about 70% of its total activity was found in the pellet fraction (Fig. 2b). As we observed in our previous work, in tobacco BY-2 cells, contrary to alfalfa, ODC was the main enzyme involved in Put biosynthesis and it was the only enzyme found in pellet fraction (approximately 30% of ODC total activity) and ADC activity remained low during the whole subculture interval. The activity of the third PA biosynthetic enzyme—SAMDC—was also much higher in tobacco than in alfalfa. Furthermore, in alfalfa cells the activity of Put down-regulating enzyme, DAO, was rather low (Fig. 2d), it represented only one half of that found in BY-2 cells (Gemperlová et al. 2005). The markedly lower activities of PA biosynthetic enzymes may look paradoxical in relation to high intercellular levels of PAs during the whole subculture period in alfalfa (Fig. 3). The extremely high content of free Put maintained in alfalfa cells is probably a constitutive property of this culture and the very high levels of PAs at the time of inoculation are optimal to maintain a standard growth cycle, including cell division (cf. Königshofer and Lechner 2002). Consequently, there is no need for very high activity of PA biosynthetic and/or catalytic enzymes.

Due to the critical role of PAs in various cellular functions, multiple pathways seem to regulate precisely their intracellular concentrations. Control of cellular PAs by rapid degradation of PA-biosynthetic enzymes ODC and SAMDC constitutes an important feedback regulatory mechanism (Gandre et al. 2002; Hu et al. 2005).

Conjugation of free PAs with hydroxycinnamic acids represents at least in some plant species, beside the oxidative deamination, another way of regulation of endogenous PA levels (Martin-Tanguy 1985; Creus et al. 1991; Bagni and Tassonni 2001; Biondi et al. 2001). The high levels of PCA-soluble conjugates of Put detected in BY-2 cells (about 70% of the intracellular Put pool at the day 3, Fig. 5) seem to be the common phenomena in *Nicotiana* and have been reported by several authors (Martin-Tanguy 1985; Pfosser et al. 1990; Gemperlová et al. 2005). Unlike what is seen in tobacco, in alfalfa cells soluble PA conjugates were less concentrated than the corresponding free PAs and the formation of hydroxycinnamic amides probably was not involved in the regulation of PA homeostasis there (Fig. 3).

Relatively low levels of PCA-insoluble conjugates detected in both BY-2 and alfalfa cells are in agreement with other data presented in plant tissues (Pfosser et al. 1990; Cvikrová et al. 1999). In alfalfa, but not in tobacco, the relatively high proportion of Cad was found in the fraction of insoluble conjugates (Fig. 3). In animal tissues Cad does not normally participate in any particular metabolic process, and it is completely excreted from cells into the culture medium (Hawel et al. 1994; Hawel and Byus 2002). In alfalfa cells Cad is probably incorporated into the insoluble fraction and/or excreted, because the levels of both free and soluble conjugated forms are low.

It is well documented that the cell culture medium represents a functional extracellular compartment of cells, which might correspond to the apoplast of differentiated plants (Wink 1994). Cells obviously secrete polysaccharides and a number of secondary compounds, such as peptides and hydrolytic and oxidative enzymes (for review, see Barz et al. 1990). The excretion of cytokinins into the cultivation medium was first described in detail in suspension-cultured tobacco cells (Petrášek et al. 2002). Thus, there may be another factor influencing the level of intracellular PAs. The excretion of PAs into the medium the first day after the subculture might represent one of the symptoms of stress response in cells after transfer into fresh medium (Figs. 3, 4). However, after the stimulation of PA biosynthetic activities in the lag-phase together with the relatively low activity of DAO (when compared with the activity in tobacco) and stable level of PCA-conjugated forms of PAs, the excretion of newly synthesized PAs probably represents an alternative, supplementary mechanism of regulation of intracellular PA level in alfalfa cells (of course in addition to metabolic control).

When we investigated the mutual relationship between intra- and extra-cellular PAs in detail, the complete picture of this data correlation was quite different in alfalfa and tobacco BY-2 cells. Contrary to the small quantity of excreted Put and Spd by tobacco cells (generally up to tens nmol g^{-1} FW and the first day only, Fig. 5), alfalfa cells excreted considerable amounts of Put, Spd and Cad (at least one order of magnitude higher than in tobacco in the course of the whole subculture, Figs. 3, 4). In principle, intracellular PAs could accumulate in the medium as a result of cell breakage, excretion, or a combination of both processes. Our results support the excretion hypothesis. Cell viability during the whole subculture period was stable varying between 80 and 90% of living cells in both suspension cultures. Therefore, any possible release of PAs from dead or injured cells is unlikely to have significantly influenced the final data. The presence of PAs in the medium may influence the cell behavior and re-uptake of excreted PAs cannot be excluded.

Based on the detailed studies of the two cell culture systems, differing markedly in cell multiplication and growth rate and in PA metabolism, we have shown that in the cells where PA-down-regulating metabolic processes (conjugation and oxidative deamination) are very active, PA excretion into the cultivation medium is negligible. However, in those cells, where the PA conjugation and activity of catalytic enzymes is low, secretion into the cultivation medium might represent, in addition to metabolic control, an important alternative mechanism controlling the intracellular PA levels.

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