PUTRESCINE ACCUMULATION DOES NOT AFFECT RNA METABOLISM IN THE LICHEN EVERNIA PRUNASTRI

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Summary: Evnria prunastri thalli are able to accumulate exogenous putrescine from the media. This accumulation is optimal at pH 9.15 although the highest uptake rate is achieved at pH 5.0 in parallel to the highest production of endogenous diamine. The lowest values of RNase activity seems to be related to alkaline pH values rather than accumulation of free putrescine.

INTRODUCTION

Many lichen species accumulate polyamines, including putrescine (1), although their function and synthesis in these plants are poorly known (2,3). It has been observed that Evnria prunastri thalli floated on putrescine are progressively bleached and a loss of tissue consistence occurs in parallel to the loss of dry weight. This decrease represent about 50% of the initial value when incubations are carried out at nearly neutral pH values but only 20% at pH 9.15 (4). In addition to that, putrescine enhances the light-stimulated secretion of ribitol whereas it decreases the amount of endogenous mannitol which is not recovered in the incubation medium (5).

This is in agreement with the fact that putrescine causes lethality of the cyanobacterium Anacystis nidulans at extracellular pH values which stimulate a rapid diffusion of nonprotonated diamine and accumulate as the charged form (6). Lethality is caused by a depletion in the ability to fix carbon dioxide as well as by a decrease in the amounts of cellular DNA and RNA, a loss in the ability to synthesize protein and, also, a breaking-up of ribosomes (7). However, E. prunastri contains a green alga,
Trebuoxia, as photobiont and the accumulation of putrescine in free-living green algae, as Chlorella emersonii does, produces an increase in the amount of both DNA and RNA in parallel to a rise in the production of dry matter (8).

This specific effect of polyamines on DNA and RNA has not as yet been explained (9) although some studies have been reported. Spermidine increases activity of chromatin-bound RNA polymerase from soybean hypocotyls (10). Moreover, spermidine or spermine is required for the maintenance and replication of double-strand RNA plasmids of Saccharomyces cerevisiae that code for a killer toxin (11). However, because of the many steps involved in the biosynthesis of these killer plasmids, it is not possible to attribute this effect to any specific synthetic step (12).

The rise in the level of RNA in plant cells can also be due to an inhibition of RNase activity. Potato tuber discs evidence a dramatic rise in RNase activity after 24 h excision and this increase is completely blocked by the polyamines, spermidine and spermine. This effect is dependent upon concentration, and several precursors of polyamines, such as L-ornithine and L-arginine, are also effective in inhibiting the rise of RNase activity (13). A similar action has been invoked as an explanation to the stabilization of oat leaf protoplasts (14).

Thus, in this work we attempted to study the effect of putrescine on RNA level and RNase activity in E. prunastri thalli to explain the action of the diamine in lichen growth and metabolism.

MATERIAL AND METHODS

Evernia prunastri (L.) Ach. growing on Quercus pyrenaica Lam and collected in Valsain (Segovia, Spain) was used throughout this work. Air-dried thalli were stored, in the dark at 7°C, in polyethylene bags until required, but no longer than a month. Samples of 1.0 g of air-dried thallus were floated on 0.1 M sodium acetate, pH 5.0, 75 mM phosphate, pH 6.9 or 0.1 M Tris-HCl, pH 9.15 buffers for up to 8 h in the dark at 26°C or on 40 mM putrescine in the same buffers and conditions. To estimate the amount of putrescine in the thallus, as well as that remaining in the media, samples were macerated (media were mixed) with diethyl ether to remove lichen phenols. Both cellular debris and media were then dried in vacuo and macerated again with 15 ml of 5% perchloric acid to precipitate proteins. Homogenates were centrifuged at
48000 x g for 20 min at 2°C. The supernatants were neutralized with 0.1 M NaOH and filtered through Millipore GS filters (0.22 um pore diameter). Finally, supernatants were lyophilized and then redissolved in 2.0 ml distilled water.

Once extracted the polyamines, these were tosylated according to the method of Sugiura et al. (15) modified by us. To pure acetone and 30 mg of TsCl were added and then maintained for 1 h at 70°C. After this period, samples were cooled on ice and supplied with 5 ml 2 N NaOH and twice washed with 5 ml n-hexane, which was after discarded by removing it. Then, 7.5 ml of 2 N HCl and 5 ml of chloroform were added to the aqueous phase. The organic phase was removed and dried on anhydrous sodium sulphate by vigorous shaking. This phase was recovered and evaporated. Dry residue was redissolved in 0.2 ml methanol for HPLC (Scharlau, Spain) and 10 ul of this last one were injected onto the chromatographic column. Putrescine was quantified by reverse phase HPLC using a Varian 5000 liquid chromatograph equipped with a Vista CDS 401 computer. Conditions were as follows: column, 400 mm x 3 mm i.d. packed with Micro-Pak MCH-10; mobile phase, methanol:water (65:35 v/v); flow rate, 1.0 ml/min; temperature, 25°C; pressure, 130 atm; detector, UV set at 254 nm; internal standard, 1.5 mM ethylamine. Standards were treated in the same way as above.

RNA was quantified according to Ramakrishna et al. (16) using yeast RNA as a standard but, in our work, RNA was firstly subjected to alkaline digestion with 1.5 M KOH to give nucleoside-3'-P before spectrophotometric measurements. RNase activity was assayed, according to Cherry (17) by incubating 1.0 ml of the extract with 1.0 ml of a solution containing, in a final volume of 2 ml, 0.5 M sucrose, 1.0 mM magnesium sulphate, 10 mM KCl, 2 mg yeast RNA and 1.0 mg lichen protein extracted by macerating the thalli with 0.1 M sodium acetate buffer, pH 5. Controls were the same, but without RNA. Reaction was stopped by adding 5 ml of an ice-cold solution containing 1.0 M perchloric acid and 25 mM uranyl acetate. A unit of specific activity was defined as 1.0 ug hydrolyzed RNA per mg protein and per min. Proteins were estimated, after both thalli extracts and media were dialyzed overnight, by the method of Potty (18) using bovine serum albumin as standard.

RESULTS

Tosylated putrescine and ethylamine, as internal standards, are separable through reverse phase HPLC. A typical chromatogram run at 65% methanol is shown in Fig. 1A. Two peaks are fully resolved in less than 9 min. Fig. 1B shows the calibration lines for putrescine and ethylamine and Fig. 1C, for putrescine/ethylamine ratio. Coefficient of determination (r²) with values not less than 0.99 indicates that the "goodness of fit" is almost perfect.
Fig. 1. Chromatographic identification and calibration of tosylated putrescine. A) HPLC trace of putrescine (p) where (s) is the solvent and (e) ethylamine as internal standard. B) direct calibration of (●) ethylamine as internal standard and (▲) putrescine, where a.c. represent area counts x10⁶ in relative units and mi, mass injected in μg. (●) y=0.525x+0.044; r²=0.99 and (▲) y=0.364x+0.05; r²=0.99. C) indirect calibration of putrescine as a function of the internal standard, where a.c. represent the ratio of relative area counts of putrescine to ethylamine (○) y=0.543x+0.447; r²=0.98.
Fig. 2. Time-course of remaining putrescine in the media after incubation the thalli at pH 5.0 (●), pH 6.9 (▲) and pH 9.15 (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

Fig. 3. Time-course of putrescine accumulation in thallus samples floated on A) 40mM buffered putrescine and B) buffer alone at pH 5.0 (●), pH 6.9 (▲) and pH 9.15 (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.
Fig. 4. Time-course of RNA content (A), RNase activity (B), protein content in thalli (C), protein secreted to the media (D) and dry weight (E) of the thallus samples floated on 40mM putrescine (filled symbols) or buffer alone (empty symbols) at pH 5.0 (●), pH 6.9 (▲) and pH 9.15 (■). Regression equations for each treatment are:

(A) $y = -0.004x + 0.905; r^2 = 0.97$; (B) $y = -0.004x + 0.907; r^2 = 0.88$; (C) $y = -0.007x + 0.906; r^2 = 0.97$; (D) $y = -0.014x + 0.903; r^2 = 0.90$; (E) $y = -0.017x + 0.904; r^2 = 0.92$. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.
According to this method, putrescine uptake by lichen thalli markedly depends on external pH. Putrescine is rapidly removed from the media at pH 5 since about 60% of the initial amount of putrescine disappears after 30 min incubation and, from this, the remaining 40% seems to be significantly unchanged up to 8 h incubation. A similar final value (about 35%) is obtained when incubation is carried out at pH 9.15, although, in this case, 2 h are needed to achieve the maximal initial uptake. There are no significant changes in putrescine concentration with time in media buffered at pH 6.9 (Fig. 2). This behaviour is clearly in agreement with the accumulation of putrescine in lichen thallus (Fig. 3A), although there is no correspondence, at pH 5, between diamine uptake and accumulation, since the analysis of endogenous putrescine reveals that this compound does not accumulate at concentrations higher than 15 umol/g. However, putrescine content, estimated as pre-existent (endogenous) diamine before treatments, rapidly increases in thalli floated on buffer at pH 5, whereas it slightly decreases when incubations are performed at pH values of 6.9 and 9.15 (Fig. 3B).

Time-courses of RNA content and RNase activity are shown in Figs. 4A and 4B, respectively. RNA concentration oscillates around a mean value of 75 ug/g dry weight for all the treatments and an appreciable effect of putrescine supplied from the media was not observed, except at pH 6.9, where RNA content is increased at the first 2 h of incubation. Further, exogenous putrescine diminishes the amount of RNA for the first 6 h incubation at pH 9.15, when the accumulation of diamine is the highest (Fig. 3A), although the final values at 8 h, obtained by floating the thalli on buffer alone or buffered putrescine at this pH value, are almost identical (Fig. 4A).

On the other hand, there exists a rapid increase in RNase activity of thalli floated on buffer with or without putrescine at pH values of 5.0 and 6.9. However, RNase activity is progressively nullified when incubations are performed at pH 9.15, although this effect cannot be explained on the basis of putrescine accumulation (Fig. 4B).
In all the cases, the concentration of thalli protein is maintained along the time of incubation (Fig. 4C). Fig. 4E shows the increase of dry weight. In parallel, a secretion of proteins to the incubation media at pH 6.9 and 9.15 is observed, whereas it does not exist at pH 5.0 (Fig. 4D).

DISCUSSION

HPLC quantification of putrescine in lichen thalli seems to be an accurate method, since linearity is maintained for a wide range of diamine concentrations, as is shown in Fig. 1. By using this method, it has been found that putrescine uptake by E. prunastri thallus is achieved at pH values near 5 and 9, in agreement with that reported by Bagni and Pistocchi (19) for Saintpaulia petals. In the range of pH 8.5 - 10.5, Guarino and Cohen (6) also reported an exponential increase of putrescine uptake, a fact that is in agreement with the intake of the diamine at pH 9.15, shown in Fig. 2.

Although the disappearance of putrescine from the incubation media is similar to the two pH values, there is not a high accumulation of this compound at acidic pH values. This can be due to new synthesis or activation of diamine oxidases, which break down the excess of putrescine (2). However, this apparent synthesis is not achieved at pH 9.15 and thus, free-putrescine accumulates in the cells. Another explanation could be the binding of the diamine to certain components of the cell wall, as demonstrated by Vallée et al. (21) for Nicotiana tabacum and Lycopersicum esculentum. Finally, most of the polyamines, which behave as highly-charged cations at cellular pH values, might bind to anionic cell constituents, such as ribosomes, DNA, and membranes (22, 23) and even to aromatic compounds, such as phenols (24).

The rapid and massive increase in valuable putrescine when incubations are performed at pH 5.0 (Fig. 3B) has also been found in leaves of Avena sativa (25). There, it is due to an increase in the activity of L-arginine decarboxylase, whereas L-ornithine decarboxylase activity decreases after acidic stress. E. prunastri
thallus contains both enzymatic activities (26, 3), but L-ornithine decarboxylase necessitated that L-arginine, accumulated in lichen thalli, was first hydrolyzed by both inducible (27) and constitutive (28) arginases that cannot be synthesized at pH values below 7.0 (29). This indicates that putrescine accumulation by low pH values follows identical metabolic pathways in higher plants and lichens, namely, the arginine decarboxylase pathway.

At a physiological level, the loss of dry weight found after incubation of lichen thalli at alkaline pH values is not related to endogenous content of putrescine. However, a rapid increase in the amount of extractable RNA is shown up to 30 min incubation on putrescine at pH 9.15, in disagreement to that found in thalli floated on buffer alone, in which RNA content initially decreases in parallel to RNA inactivation. Considering the loss of dry weight shown in Fig. 4E, the entrance of putrescine into the lichen cells gives values of RNA concentration lower than those obtained when putrescine accumulation does not take place at pH 6.9 (Fig. 4A) independent of RNase activity levels. We can hypothesize that this effect is primarily due to the relative inactivity of RNase at pH 9.15, and putrescine bound to RNA (3) at this pH would, therefore, show some increase of RNA content. Moreover, the chemical instability of polyamine-bound RNA has not, as yet, been reported (12).

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REFERENCES

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