# Influence of Inhibitors of Polyamine Biosynthesis on Polyamine Levels and Growth of Plants

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*Abutilon theophrasti, Lycopersicon esculentum*, S-Adenosylmethionine Decarboxylase, Arginine Decarboxylase, Ornithine Decarboxylase, Spermidine Synthase

Inhibitors of enzymes involved in polyamine biosynthesis which stop the growth of bacteria, fungi and animal cell systems were analyzed for their potential to interfere with plant cell systems. Several compounds were found to be potent inhibitors of plant enzymes, namely the decarboxylases of ornithine, arginine and S-adenosylmethionine as well as the aminopropyltransferase. Application of enzyme inhibitors (α-difluoromethylornithine, 1-aminooxy-3-aminopropane, α-difluoromethylarginine, methylglyoxal bis(guanyl-hydrazone), cyclohexylamine) or combinations of these on whole plant systems resulted neither in a large decrease in polyamine content nor in a significant growth reduction. The lack of response cannot be explained by hindered inhibitor uptake into plants. Inhibition of single enzymes may induce alternative biosynthetic pathways.

## Introduction

Polyamines appear to play a crucial role in many cellular processes regulating growth in microorganisms, animals and plants [1, 2]. For putrescine biosynthesis, plants possess at least two pathways, namely *via* ornithine decarboxylase or arginine decarboxylase. They may synthesize putrescine also *via* citrulline decarboxylase [3]. Recent research indicates the existence of a new pathway for putrescine synthesis in bacteria which does not involve ornithine or arginine as intermediates [4].

In general, polyamine biosynthesis is more complicated in plants than in fungi and animals. Polyamines may also be supplied from storage sites, *e.g.* from cotyledons to hypocotyls and roots. Ornithine and arginine decarboxylase may be active at different stages of plant development [1]. Increased polyamine titers and rates of polyamine biosynthesis can be correlated with increased growth rates [5–13]. Inhibition of polyamine synthesis may result in inhibition of growth, which, in some cases, is reversible by the addition of exogenous polyamines [14, 15].

In a previous study inhibitors of enzymes involved in polyamine biosynthesis were evaluated [16]. Many authors described effects of single inhibitors like difluoromethylornithine on the content of polyamines of a particular plant. Here we report the influence of a series of effective inhibitors on endogenous polyamine levels and plant growth.

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## Materials and Methods

### *Plants*

Tomato seeds (*Lycopersicon esculentum* Miller cv. Supermarmande, 0.5 g corresponding to 200 seeds) were germinated in 9 cm Petri dishes containing 3 g vermiculite and 15 ml water or inhibitor solution (pH 6.5). After 10 days at 22 °C with a 14 h light period (light intensity 30 μmol $m^{-2}s^{-1}$), germination was examined and the content of polyamines in hypocotyls was determined.

Calli of *Abutilon theophrasti* Medic. obtained from stems of 21 day old plants were subcultured on agar (0.8%) containing MS medium [17] supplemented with 5 μM 2,4-D, 9 μM kinetin, 1 μM indole acetic acid, 3 μM naphthylacetic acid, 3 mM glutamine, 0.5 g/l proteose peptone (No. 3, Difco, East Molesey, Surrey, England), 58 mM sucrose (pH 5.8). Inhibitors were added to the medium before autoclaving, which did not harm their activity as enzyme inhibitors.

### *Chemicals*

The sources of chemicals have been described [16].

### *Polyamine analysis*

Polyamines were derivatized with benzoyl chloride and analyzed by high pressure liquid chromatography [18].

## Results

In this study we have examined plant species producing large quantities of putrescine and spermidine, allowing the use of standard high pressure liquid chromatography for polyamine analysis (Table I). Tomato seed germination was a suitable model because the seeds contained only small amounts of polyamines (30 nmol putrescine and 143 nmol spermidine per gram fresh weight), whereas the content was much higher after germination, especially in the hypocotyl [18]. *A. theophrasti* calli were examined, because they contained constant high levels of putrescine during the whole growth period and because calli grow in close contact with the inhibitor medium. In both species the amount of polyamines bound covalently to other constituents of the cell was negligible compared to the polyamines free in solution (Table I). Thus, there was no pool of polyamines that could not be influenced by inhibitors, except for polyamines in other parts of the tomato seedling. As was found earlier in other plant systems [19, 20], arginine decarboxylase activity was much higher than ornithine decarboxylase activity both in tomato hypocotyls and *A. theophrasti* calli (Table I). Thus, inhibitors of arginine decarboxylase might influence polyamine levels to a much higher extent than ornithine decarboxylase inhibitors.

In a previous study several compounds were found to be inhibitors of enzymes involved in polyamine biosynthesis [16]. From these inhibitors effective on the enzyme level, only the two compounds methylglyoxal bis(guanylhydrazone) and Berenil (4,4′-diaminodiazoaminobenzene) inhibited germination of tomato seeds.

The addition of 1 mM spermidine to the inhibitor solution did not reverse the inhibition of germination. As expected from their effect on S-adenosylmethionine decarboxylase, they reduced spermidine content to undetectable levels at 1 mM concentration, which was not sufficient for growth inhibition; inhibition only occurred at 10 mM concentration. All other compounds did not influence tomato seed germination substantially. 1,4-Diaminobutane and canaline (an analog of ornithine) inhibited growth of *A. theophrasti* calli slightly at 1 mM concentrations, the other compounds had no effect (results not shown).

Several compounds, which were enzyme inhibitors [16], reduced polyamine levels without affecting growth (Table II): the putrescine level in *A. theophrasti* calli by canaline and α-difluoromethylarginine, the spermidine level in tomato hypocotyls by 1-aminooxy-3-aminopropane, methylglyoxal bis(guanylhydrazone) and Berenil. Several compounds, especially inhibitors of ornithine decarboxylase, were good enzyme inhibitors, but they did not influence polyamine levels. The application of inhibitor combinations could be necessary, since plants have at least two pathways leading to putrescine. The addition of ornithine decarboxylase inhibitors to α-difluoromethylarginine, however, did not increase the effectiveness of the latter alone (Table II).

1,4-Diaminobutan-2-one, α-difluoromethylornithine, α-difluoromethylarginine and 1-aminooxy-3-aminopropane significantly increased polyamine

Table I. Content of putrescine (put), spermidine (sp), ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) in tomato seedlings and *A. theophrasti* calli (nt, not tested). To analyze covalently bound polyamines, different fractions of the extract preparation were hydrolyzed with HCl [18].

| Protein and polyamine content, enzyme activities | | Tomato seedling | | | Calli from *A. theophrasti* |
|---|---|---|---|---|---|
| | | Cotyledon | Hypocotyl | Radicle | |
| Protein content, mg per g fresh weight | | nt | 1.67 | nt | 0.61 |
| Decarboxylase activities, | | | | | 0.19 |
| nmol $CO_2$ per h and g fresh weight | ODC | nt | 0.50 | nt | 4.78 |
| | ADC | nt | 1.79 | nt | |
| Polyamine titer, nmol per g fresh weight | | | | | |
| extract supernatant, non-hydrolyzed | put | 340 | 1566 | 412 | 1242 |
| | sp | 189 | 973 | 211 | 71 |
| extract supernatant, hydrolyzed | put | nt | 123 | nt | 10 |
| | sp | nt | 5 | nt | 4 |
| pellet, hydrolyzed | put | nt | 87 | nt | 10 |
| | sp | nt | 91 | nt | 5 |

Table II. Effect of polyamine biosynthesis inhibitors on polyamine biosynthesis, on polyamine levels and growth of tomato seedlings and *A. theophrasti* calli (na, not analyzable in the cases of complete growth and germination inhibition; nd, not detectable, levels below 1 nmol per gram fresh weight; nt, not tested). Inhibitors of spermidine synthesis were not tested on *A. theophrasti* calli. Polyamine levels are given in % of control; control values for polyamine titers are listed in Table I (extract supernatant, non-hydrolyzed).

| Treatment | Concentration [mM] | Polyamine levels (% of control) Calli from *A. theophrasti* | Tomato hypocotyl | |
|---|---|---|---|---|
| | | Putrescine | Putrescine | Spermidine |
| Control | | 100 | 100 | 100 |
| 1,4-Diaminobutan-2-one | 10 | | 340 | 100 |
| | 1 | 94 | 70 | 102 |
| α-Methylornithine | 10 | | 124 | 100 |
| | 1 | 70 | | |
| Canaline (analog of ornithine) | 1 | 13 | 109 | 131 |
| | 0.1 | 112 | | |
| α-Difluoromethylornithine | 10 | | 99 | 308 |
| (DFMO) | 1 | 77 | 93 | 157 |
| 1-Aminooxy-3-aminopropane | 10 | | 268 | 21 |
| | 1 | 69 | 120 | 95 |
| | 0.1 | 115 | 80 | 132 |
| α-Difluoromethylarginine | 10 | | 73 | 470 |
| (DFMA) | 1 | 33 | 85 | 203 |
| | 0.1 | 68 | 81 | 184 |
| Methylglyoxal bis(guanyl- | 10 | | na | na |
| hydrazone) | 1 | nt | 143 | nd |
| | 0.1 | | 56 | 79 |
| Berenil | 10 | | na | na |
| | 1 | nt | 76 | nd |
| Cyclohexylamine | 10 | | 132 | 115 |
| | 1 | nt | | |
| Aminopropylcadaverine | 10 | | 83 | nd |
| | 1 | nt | 109 | 107 |
| DFMO + DFMA | 1 | 48 | 68 | 143 |
| | 0.1 | 99 | 64 | 118 |
| 1,4-Diaminobutan-2-one + DFMA | 1 | nt | 123 | 87 |
| α-Methylornithine + DFMA | 1 | nt | 75 | 139 |

levels (putrescine or spermidine) in tomato hypocotyls (Table II).

## Discussion

Substantial amounts of polyamines were synthesized during tomato seed germination and growth of *A. theophrasti* calli. The influence of inhibitors of enzymes involved in polyamine biosynthesis on growth and germination was not consistent. Only Berenil, methylglyoxal bis(guanylhydrazone) and, to a minor extent, aminopropylcadaverine inhibited tomato germination. A significantly reduced spermidine level may cause inhibition of germination. These compounds could also influence other metabolic pathways than polyamine biosynthesis, because rather high concentrations were applied. Most of the very effective ornithine decarboxylase inhibitors did not reduce polyamine levels to a great extent. This was not surprising as ornithine decarboxylase seems to contribute less to the synthesis of putrescine than arginine decarboxylase. α-Difluoromethylarginine and 1-aminooxy-3-aminopropane, the latter being an inhibitor of both ornithine and arginine decarboxylase, did not influence polyamine levels to a signifi-

cant extent. The combined use of ornithine and arginine decarboxylase inhibitors did not decrease polyamine levels. This result suggests, that either a third pathway can lead to putrescine or plants can compensate the reduced availability of enzymes by increased enzyme production. With certain compounds even an accumulation of polyamines was found. This was not expected because these inhibitors should rather block the synthesis of putrescine, the precursor of spermidine. 1-Aminooxy-3-aminopropane, an inhibitor of all decarboxylases involved in polyamine biosynthesis, should not cause any increase of putrescine or polyamines in general. Uptake of most inhibitors into plants was not a problem, their presence was obvious because they influenced polyamine levels, although not in a way expected from their mode of action. A reasonable explanation for the failure to reduce polyamine levels with these inhibitors may be found in the highly regulated nature of polyamine biosynthesis and the possibility to synthesize polyamines through alternative pathways. On the other hand, we have reasonable evidence that polyamines are transported from cotyledons into hypocotyls and roots [21]. This would allow compensation of reduced levels of polyamines in hypocotyls and roots.

Several compounds reduced spermidine to undetectable levels, which was obviously not lethal to plants. It is doubtful, whether a reduction of all polyamines below 1 nmol per gram fresh weight would be lethal, because many plants are known to contain very small amounts of polyamines [18] and because it is not yet clear, whether certain plant species need higher levels for growth.

Generally, direct inhibition of enzymes involved in polyamine biosynthesis did not result in growth inhibition. More sophisticated approaches are needed to inhibit plant growth by influencing polyamine biosynthesis. Some compounds block the conversion of putrescine to spermidine only partially and cause an increase in ornithine decarboxylase activity and striking accumulation of putrescine, which may be toxic for cells [22]. The enzyme regulation in polyamine biosynthesis can be distorted by polyamine analogs [23–25]. When polyamine biosynthesis (spermidine biosynthesis) is shut down by treatment with α-difluoromethylornithine, the cells incorporate spermidine at an accelerated rate relative to control cells. This encouraged some research groups to consider the spermidine uptake apparatus as a means of delivering modified spermidine or its homologues to these cells, hoping them to be cytotoxic [26].

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