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**The Role of Polyamines in Growth Processes
and Stress Responses in Plants: Molecular
Investigations and Practical Implications**

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A. Abstract

Recent evidence, resulting also from our previous BARD project (BARD I-44-79) suggests that polyamines fulfill at least several criteria to be regarded as plant bioregulators, acting either directly or in a secondary manner, in growing and stressed tissues. The present research was aimed at molecular-physiological investigations of some basic problems of polyamine effects, and at their practical implications. Polyamine content, activity of biosynthetic enzymes, effects of inhibitors of polyamine biosynthesis, and localization and translocation of polyamines were studied in several plant (and fungal) systems:

- Significant changes and a role for polyamines has been established in the following stress-related aspects:

NaCl salinity in sensitive and tolerant tobacco cell lines, growth and pathogenicity of phytopathogenic fungi, cadmium toxicity, osmotic stress in protoplasts, nitrogen depletion in tobacco cell cultures.

- A role for polyamines has been established in the following growth and development processes:

Lateral root formation in corn, embryogenesis in celery cultures and organogenesis in tobacco explants, mandarine fruit growth, protoplast regeneration potential, gibberellin-induced growth in pea, morphogenesis in *Sclerotium rolfsii*.

- Few basic-molecular aspects have been studied and elucidated:

Microautoradiographic localization of ODC in dividing cells, the polyamine inhibition of restriction endonuclease activity, polyamine binding to regeneration related proteins, kinetics and inhibitor-sensitivity of polyamine biosynthetic enzymes, selection of tobacco cell lines with modifies polyamine contents.

- Some of the above are of significant practical value:

The use of enzyme inhibitors to control certain phytopathogenic fungi, improvement of *in vitro* organogenesis and embryogenesis, elucidation of pathways for secondary metabolite production (nicotine), uptake and long-distance translocation of polyamines in the xylem and phloem, possible use of tissue polyamine levels and enzyme activity as guidedlines for selection of stress-responsive genotypes.

Polyamines have been shown, in the present study, to be causally-linked to several developmental and stress processes, and significant changes in polyamine content and biosynthesis have been monitored. Some of these findings are of important practical value. Further elucidation of the role of polyamines awaits future research.

B. Objectives of the research proposal

Objectives and significance of the original research proposal:

Objectives: The proposed research was aimed at molecular investigations of some basic problems of polyamine physiology, and at their practical implications, as outlined below. In contrast to the first phase of the research (former BARD program), which dealt with screening and initial analysis, the proposed research was limited to several defined systems and problems, based on our previous findings. Thus, the main objectives of the research proposal were:

1. To study the molecular mechanisms, at the level of nucleic acid, proteins and membranes, by which polyamines retard senescence and stabilize cells under stress conditions.
2. To investigate the specific role of applied and endogenous polyamines, and their metabolites, in plant responses and adaptation to salinity and other stresses, including possible molecular mechanisms.
3. To investigate the causal relationships of polyamines to growth, by comparing polyamine metabolism and inhibitor effects in competent and incompetent protoplasts.
4. To study the distribution and localization of arginine decarboxylase and ornithine decarboxylase, the polyamine-biosynthetic enzymes, by coupling these enzymes *in situ* with specific enzyme-activated inhibitors.
5. To study the practical implications of our findings:
 - (a) The uptake and transport of polyamines and their metabolites,
 - (b) Modification of polyamine-levels and physiological activity by applied inhibitors and modulators of polyamine biosynthesis, and

- (c) The use of polyamine levels and turnover as guidelines for screening stress-tolerant varieties.

Modifications of the original research proposal:

As evident from the detailed description of results, the ten individual chapters cover all the original research objectives. The numerous data led us to limit the study of molecular mechanisms of polyamine effects (objective 1), and to devote more time to several practical implications (objective 5). This includes the translocation of polyamines, interrelationships with salt stress and nitrogen nutrition, and the effect of abiotic stress (cadmium toxicity and phytopathogenic fungi).

C. General introduction

The present research report is an extension of our previous research project (BARD grant I-44-79, A. Altman, U. Bachrach and A.W. Galston) which clearly indicated that naturally-occurring polyamines play an important role in plant growth and development under normal conditions and in response to stress. This is supported by the following findings:

- (a) the intracellular concentration of several polyamines, the activities of arginine decarboxylase and ornithine decarboxylase, and precursor incorporation were found to be dependent upon the type of tissue and growth processes. In several cases, the increase in endogenous polyamines and in the activities of their biosynthetic enzymes is correlated with growth.
- (b) Polyamines are involved in several growth processes and in plant response to growth hormones (auxins, gibberellins, cytokinins) and exogenous stimuli (light, excision), and causal relationships have been established in some cases. Thus, auxin-induced root formation, root elongation, phytochrome-controlled growth of stems and leaves, GA-induced elongation of light-grown internodes, and initiation of sprouting in potato tubers were all accompanied by a change in polyamine biosynthesis and titer. Appropriate inhibitors of polyamine biosynthesis generally produced predicted effects on growth and developmental processes.
- (c) Exogenous polyamines retard several aspects of leaf senescence. For example, polyamines have been shown to prevent chlorophyll loss, to inhibit the senescence-linked rise of protease and ribonuclease activity, and to inhibit ethylene biosynthesis. Solute leakage from cells and RNase activity of wounded tissues are also retarded by exogenous polyamines.

- (d) Polyamine accumulation is closely linked to plant response to osmotic, salt and pH stress: under stress conditions, putrescine (and other polyamines) accumulate in specific tissues as a result of preferential activation of ADC, and increased polyamine biosynthesis from their precursors arginine and ornithine. This may contribute to plant adaptation to pH and salt stress.

To summarize, polyamines are present, in various amounts, in all plant tissues and organs, and their concentration and metabolism respond rapidly to many environmental and endogenous stimuli. Polyamines are involved in growth processes and stress responses in plants, and interact specifically with nucleic acids and membranes. This is reminiscent of polyamine functions in procaryotes and animals, suggesting a biological regulatory role common to all living cells. Recent evidence suggests that polyamines fulfill at least several criteria to be regarded as plant growth regulators, acting either directly or in a secondary manner, in growing and stressed tissues.

The concept that polyamines may play an important role in plant growth and response to stress is relatively new. Our research during the first phase of the BARD program, as well as the work of several other laboratories, adds great support to this concept. Continuing investigations are critical to the basic understanding of polyamine functions in growing and senescing plants under various agricultural practices, and this may lead to important practical utilization of the basic concepts. In this respect, the involvement of polyamines seems of special importance in many agricultural and horticultural crops, where improved vegetative growth, fruit set and development, and growth under limiting (stress) conditions, are of major concern. In addition, post-harvest problems of flowers, fruit and propagation material, responsiveness of various plant tissues to hormone applications, and the selection of salt tolerant varieties – all may be intimately related to polyamine content, localization, and metabolism. These considerations

point to the scientific and practical significance of the present research.

Layout and acknowledgements:

The report is presented as ten individual, but interrelated, sections. Many of these were already published (or are being prepared for publication), and they are summarized in individual, self-containing, papers.

Sections 1 to 4 were performed in the laboratory of A. Altman at the Hebrew University, Rehovot. The collaboration of the following scientists, research students and lab technicians is greatly acknowledged:

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1. Translocation of polyamines:

Polyamines in xylem and phloem exudates of plants

Abstract

Polyamines were identified by HPLC (benzoylation) and by TLC (dansylation) in xylem exudates from stems of sunflowers (*Helianthus annuus* [L.]), mung bean (*Vigna radiata* [L.] Wilczek), grapevine (*Vitis vinifera* [L.] cv. Grenache), and orange (*Citrus sinensis* [L.] Osbeck, cv. Valencia), as well as in phloem sap (using elution into EDTA) of sunflower and mung bean plants. Putrescine was the major polyamine detected, ranging in concentration of 150 to 9200 pmol·ml⁻¹ exudate, whereas only trace amounts of spermine were detected. High amounts of putrescine and spermidine were found in EDTA eluates (possibly phloem sap) as compared with elution into water. Concentrations of putrescine and spermidine in xylem exudates were related to the physiological conditions of the plants prior to exudate collection. More putrescine was found in exudates of older than in younger sunflower plants, and salt stress applied to sunflower plants resulted in a higher concentration of putrescine and spermidine in the exudate.

The presence and abundance of putrescine and spermidine in xylem and phloem exudates indicate that polyamines may be readily translocated in plants. This long-distance translocation further supports the hypothesis that polyamines have a regulatory role in plant growth and response to stress.

Introduction

Polyamines, which occur ubiquitously in plant tissues, have recently been shown to function in growth and differentiation processes in plants (2, 5, 20, 21), as well as in plant response to stress conditions (1, 20). Similar functions are mediated by known plant hormones, with more solid evidence as to their actual

participation in growth control. It is, thus, tempting to consider polyamines as another class of endogenous growth regulators of hormonal nature, albeit rigorous evidence is still lacking at this stage. Uptake and transport of a compound is an important criterion for its regulatory function, and this is well established for plant hormones. Direct evidence for polyamine uptake and translocation is scarce, and this aspect was investigated only recently, using 3 experimental systems. Injection of ^{14}C -labeled amino acids and polyamines to cotyledons of etiolated pea seedlings resulted in only very little transport of Put and Spd to shoots and roots during 4h(23). In another study Put, Spd, and Spm uptake in excised petals of *Saintpaulia ionatha* was found to occur against a concentration gradient; the rate of uptake was linear, pH dependent, and was related to membrane potential (4, 17). Furthermore, it was found that apple fruitlets and young leaves can synthesize polyamines and translocate Put via the peduncle (3). Former experiments on the effect of salt stress on polyamines in mung bean plants indicated that NaCl induced Put biosynthesis in roots and its accumulation in leaves, suggesting root-to-shoot translocation (1, 10). Accordingly, the possibility of long distance translocation of polyamines in plants was investigated by analyzing vascular exudates of several plant species. The vascular exudates should contain the compounds transported from the root system to the shoot (normally in the xylem sap), or compounds exported from the shoot (usually in the phloem sap). Indeed, it is well established that the xylem sap contains a mixture of nitrate, ammonia, and organic nitrogenous compounds, including several amino acids (6, 14, 15, 16, 22), but polyamines were not assayed.

Materials and Methods

Plant Material and Growth Conditions. Sunflower (*Helianthus annuus* [L.]) and mung bean (*Vigna radiata* [L.] Wilczek) seeds were soaked for 24 h in running tap water, and grown in vermiculite in a controlled growth room (25°C, with a 16 h photoperiod of $150\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, or in the dark). Sunflower plants were grown for 8 d, when they had a 5.5 cm long hypocotyl and 2 fully expanded cotyledons, or for 13 d, when they had in addition a 5 cm long epicotyl and the first pair of leaves. Mung bean plants were grown for 8 days, and consisted of 8 cm long hypocotyl, 4.8 cm epicotyl and the first pair of fully expanded leaves. In one experiment, sunflower plants were watered on the 6th and 7th day with a saline solution containing 1.0, 2.5 or 5.0 g NaCl.l⁻¹ tap water, and the exudate was collected on the 8th day. Field-grown mature grapevines (*Vitis vinifera* [L.] cv. Grenache) were used at late winter, just before normal spring sprouting. Six-month old branches were excised at early spring form mature, orchard-grown, orange trees (*Citrus sinensis* [L.] Osbeck, cv. Valencia).

Stem Sap Collection. Bleeding exudates were collected directly at the stem cut-surface, of sunflower, mung bean and grapevine, as described previously (e.g. 14, 16.). Seedlings were cut below the cotyledonary node (8-day old sunflower plants) or below the first pair of leaves (13-day old sunflower and 8-day old mung bean plants). Exudates were collected continuously, for about 2–4 h, with capillary or Pasteur pipettes. Exudation rate from sunflower was ca. $55\mu\text{l}\cdot\text{h}^{-1}$ per plant (only $40\mu\text{l}$ at high salt stress, see later). About 1 ml of exudate was collected from 200 mung bean plants, during 2 to 3 h. Grapevine branches, 2 cm in diameter, were cut below a node, and bleeding sap was allowed to drip into appropriate vials. The exudation rate from one grapevine branch was ca. 1 ml per 5 min. For orange, 20 cm-long branches were introduced into a pressure chamber, with the basal cut-surface protruding on the outside. Exudate was collected from the cut surface, using a Pasteur pipette, while applying a 25 bar pressure for 3 min. A total of 1.5 ml sap was collected from 6 orange branches. All saps thus collected

are hereinafter referred to as 'xylem exudates'. These were immediately mixed with perchloric acid to a final conc. of 5% v/v, and stored at -20°C .

For phloem sap (hereinafter referred to as 'phloem exudates'), the EDTA exudation technique was used (12,13). Eight or 13-day old sunflower plants and 8-day old mung bean plants were excised, as described for xylem exudates, under a solution of 20 mM Na_2EDTA , pH 7.0. The excised shoot was placed into 0.2 ml Na_2EDTA solution in a Eppendorf vial so that 2–3 mm of the basal portion of the stem was immersed. The stems were thus maintained for 3.5 h in a humid chamber in the dark, at 25°C . The entire solution was then mixed with perchloric acid to a final conc. of 5% v/v and stored at -20°C .

Polyamine Analysis. The acidic exudates (in 5%, v/v perchloric acid) were centrifuged at 12000 $\times g$ for 15 min and the supernatant was used for polyamines analysis by HPLC or, in one case, by TLC. For HPLC, benzylation and analysis were performed as described previously (8,18), with slight modifications. One ml of 2M KOH was added to 500 μl exudate, or to 50 μl of 1mM standard solutions of Put, Spd, and Spm. The basic exudate was incubated for 20 min, at room temperature, with 5 μl of benzoyl chloride (99% AR, BDH) and then vortexed strongly with 3 ml of diethylether (BDH) and spun for 25 min at 2500 g. The upper ether phase was collected and evaporated to dryness under N_2 at room temperature. Dry samples could be stored at -20°C for several days with no quantitative or qualitative changes in the benzoylates products. Prior to analysis, samples were dissolved in 50–1000 μl of HPLC-grade methanol (Biolab). Analysis was carried out using an LKB HPLC instrument (2150 pump, and 2152 controller) equipped with a Jasco spectrophotometer (U.V. detector, 254 nm). Results were recorded and analyzed with a Hewlett-Packard model 3390 A integrator. The benzoylated products were separated with methanol:water-solution (58 to 68% of methanol) on Techsil 5 μ , 25 cm x 5 mm, C-18 column with a Merck Lichrosorb 100 RP-18 precolumn. Flow rate was adjusted to 1 ml per min, and detector attenuation was 0.04 a.u.f.s. Between injections, the column was washed with a

methanol:water gradient. For TLC, samples were dansylated and chromatographed as described previously (9), using Merck DC-Alufolien Kieselgel 60 TLC plates, and the spots were traced with a long wave UV lamp.

Results

Xylem exudates of the 4 plants species were initially analyzed by TLC, to observe the appropriate spots of dansylated polyamines (Fig. 1). Several spots, corresponding to cadaverine, Put, Spd and Spm dansylated standards, were detected, but they were not measured in view of the inappropriate separation and partly unclear identification. Further analyses were carried out by HPLC, and several representative charts are presented in Fig. 2. Put and Spd were clearly resolved and detected in all cases, in addition to several other peaks of benzoylated compounds. No attempt has been made, so far, to identify peaks other than those of Put and Spd. The polyamines thus separated were quantified, using appropriate standards, with respect to plant species, exudate volume, growth conditions and exudation technique, as described below.

High concentrations of Put and Spd were detected in xylem exudates of sunflower, mung bean and orange stems (Table I). Although different in their growth characteristics and exudation rates, all three species contained similar concentrations of Put and Spd. This is in contrast with grapevine, which has an exceptionally high exudation rate, where Put and Spd concentrations were much lower. Only trace amounts of Spm were detected in these 4 plants species. Exudation rate and exudate compositions can both be affected by the growth parameters and conditions, e.g., plant age and light regime. Xylem exudate of 13-d-old sunflower plants had a much higher Put concentration than younger plants (Table II). Spd, however, was present at high concentration in 8-d-old plants, but hardly detected in older plants. It should be mentioned that in 8-d old plants the exudate was collected at a cut surface of the hypocotyl, whereas in older plants exudation was from the epicotyl cut surface. Put concentrations in

xylem exudate of dark-grown sunflower plants was similar to that of light-grown plants. In view of the reports on stress-induced polyamine accumulation, the effect of salt stress on polyamine concentration in sunflower exudates was investigated (Table III). Salt stress applied for 48 h to roots by increasing amounts of NaCl, resulted in a considerable enrichment of polyamines in the xylem exudate. The high NaCl concentration induced a 2.7 to 5.5-fold increase in Put concentration in the xylem, and a smaller increase in Spd concentration (about 1.5-fold). The increase in Put concentration in the xylem exudate was positively correlated with the degree of media salinization.

In addition to xylem exudates, polyamines were analyzed as well in the phloem sap of sunflower and mung bean plants. Use of the EDTA technique revealed the presence of considerable amounts of polyamines, and especially of Put, in the phloem sap which exuded from 8-day old sunflower plants in the presence of EDTA was exceptionally high; none exuded into water. In contrast, Put was not detected in the EDTA solution with older sunflower stems, but some exuded into water. Spd was detected in the phloem sap of 8-d old sunflower plants and with EDTA only. Mung bean plants exuded both Put and Spd, in similar amounts, in the presence of EDTA only.

Table I. *Polyamine Content in Exudates of Several Plant Species*

Bleeding xylem exudates were collected from the cut-surface of 8-d sunflower and mung bean plants, and of 4-year-old grapevine. Sap from orange branches was obtained by the use of a pressure chamber. Only traces of spermine were detected. Data are average of 1 or 2 experiments, each one analyzed in triplicates ($\pm$ SD).

Plant	Polyamine Concentration		No. of Experiments
	Putrescine	Spermidine	
	<i>pmol ml⁻¹ exudate</i>		
Sunflower	1455 $\pm$ 230	1066 $\pm$ 43	2
Mung bean	1255 $\pm$ 147	1550 $\pm$ 70	2
Grapevine	150 $\pm$ 37	289 $\pm$ 39	1
Orange	1639 $\pm$ 215	1012 $\pm$ 62	1

Table II. *Effect of Plant Age and Light Conditions on Polyamine Content of Sunflower Exudates*

Bleeding exudates were collected (as described for Table I) from 8-d or 13-d plants grown in the light (Experiment 1), or from 8-d plants grown under light or in the dark (Experiment 2). Data are the average of two separate experiments in each case, each analyzed in duplicates ($\pm$ SD).

Condition	Polyamine Concentration	
	Putrescine	Spermidine
	<i>pmol·ml⁻¹ exudate</i>	
Experiment 1		
8 d	1200 $\pm$ 130	260 $\pm$ 83
13 d	9200 $\pm$ 550	Trace
Experiment 2		
Light	398 $\pm$ 71	Trace
Dark	435 $\pm$ 110	Trace

Table III. *Effect of Salt Stress on Polyamine Content of Sunflower Exudates*

Sunflower plants were watered on the 6th and 7th d with saline water. Bleeding exudates were collected on the 8th d as described for Table I. Averages of duplicates of 2 experiments ($\pm$ SD) are presented.

NaCl Concentration	Polyamine Concentration			
	Putrescine		Spermidine	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	<i>pmol·ml⁻¹ exudate</i>			
g·L ⁻¹				
Control	513 $\pm$ 71	560 $\pm$ 58	979 $\pm$ 66	1100 $\pm$ 60
1.25	752 $\pm$ 53		384 $\pm$ 31	
2.50	987 $\pm$ 77		845 $\pm$ 49	
5.00	1374 $\pm$ 127	3075 $\pm$ 147	1365 $\pm$ 107	1765 $\pm$ 115

Table IV. *Polyamine Content in Phloem Exudates of Sunflower and Mung Bean Plants*

The basal cut-surface of 8-d or 13-d sunflower plants, and of 8-d mung bean plants, was introduced into 200 L of 20 mM Na₂-EDTA or distilled water for 3.5 h. Polyamines were analyzed in the eluents of EDTA or water. Data are average of two experiments, each analyzed in duplicates ($\pm$ SD).

Plant	Polyamine Concentration	
	Putrescine	Spermidine
	<i>pmol·ml⁻¹ solution</i>	
Sunflower (8 d)		
EDTA	44900 $\pm$ 530	960 $\pm$ 115
H ₂ O	Trace	Trace
Sunflower (13 d)		
EDTA	Trace	Trace
H ₂ O	430 $\pm$ 78	Trace
Mung bean (8 d)		
EDTA	1255 $\pm$ 231	1550 $\pm$ 215
H ₂ O	Trace	Trace

FIG. 1. TLC of dansylated compounds in exudates of several plant species. Exudates were collected as described in Table I, dansylated and chromatographed (ethyl acetate:cyclohexane, 2:3, v/v). Lane 1, mung bean; lane 2, orange; lane 3, grapevine; lane 4, sunflower; lanes 5 to 8, polyamine standards (A, cadaverine; B, putrescine; C, spermidine; D, spermine).

FIG. 2. HPLC of benzoylated polyamine standards and plant exudates. Left panel, standards and exudates with 60% (v/v) methanol solvent system. Right panel, same, with 65% (v/v) methanol. Standard peaks represent 0.25 nmol of each standard (St). 1, 8-d-old sunflower, bleeding exudate; 2, same, phloem sap (EDTA elution); 3, mung bean bleeding exudate; 4, orange, forced exudate; 5, grapevine bleeding exudate.

Discussion

The results show that Put and Spd are present in considerable amounts in vascular exudates of the 4 different plant species studied (Figs. 1,2, Table I). The presence of polyamines in the bleeding exudate of cut stumps, which represents the ascending xylem sap (6, 14, 16), implies that polyamines can be synthesized in the root system and exported to the shoot. This is not surprising in view of the extensive information on the abundance of inorganic and organic nitrogenous compounds, including amino acids, in xylem and pholem saps (12, 14, 15, 16). It has been established that polyamines are present and are synthesized in roots of various plants (7, 19, 21), in addition to their synthesis in other plants organs.

The finding that Put concentration in xylem and pholem saps varies considerably in relation to plant age and root salt stress (Table II, III and IV) suggests that the physiological condition of the root system (where polyamines can be synthesized) and of the shoot (where they are translocated and synthesized) affects the formation and partitioning of polyamines. This consideration is well established for other nitrogenous constituents (6, 16), and, thus, may apply as well for polyamines which are synthesized via the arginine-ornithine-citrulline cycle, with the incorporation of nitrogen from glutamate (2, 20, 21). This is supported by the observations that asparagine, glutamine and arginine may comprise a major fraction of the transported nitrogen, especially in legumes and trees (16, 22). The vascular sap consists of nitrogen recently absorbed and metabolized by the roots, and of nitrogenous components arising from protein turnover and recycling of amino acids and amides between plant organs (16). Thus, polyamines, like other nitrogenous compounds, can be found in both xylem and pholem exudates (Tables I and IV). Environmental stress conditions like potassium deficiency, pH and salinity trigger Put accumulation in leaves (1, 20, 21), and in the case of salinity it has been suggested that Put is translocated from the roots to the shoot system (10). This is supported by the present findings that more Put is transported in the xylem exudate of salt-stressed plants (Table III).

Earlier studies, using specialized experimental systems and radioactively-labelled compounds, did not establish translocation of polyamines (23). This may suggest that polyamines lack an important feature to be regarded as a growth regulating compounds (11). Feeding of labelled compounds and their detection may be subject to some problems, e.g., isotopic dilution, metabolic conversions, and possible binding to cell components of the transport system. The latter may be of special significance to polyamines, which are protonated at acidic pH (which is usually the case with xylem sap, (16)), and, thus can bind to cell walls or membrane, preventing their free transport. Detection of polyamines in the vascular sap of plants, as found in the present study, may be regarded as strong evidence for their translocatability between organs. This is also supported by additional data of work in progress, including accumulation of polyamines in the bark above a girdling zone, and presence of polyamines in plant 'fluids' like flower nectar.

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2. A Role for polyamines in lateral root formation in corn (*Zea mays*).

Abstract

Polyamine content and the activity of arginine and ornithine decarboxylase were studied with respect to meristematic activity in primary roots and in developing lateral roots of *Zea mays* seedlings. Comparative localization of active ornithine decarboxylase and of meristematic activity were determined by labelling roots either with α -[5- ^{14}C] difluoromethyl ornithine or with [^3H] thymidine, respectively.

Lateral roots were formed during the 72 h post-decapitation period, accompanied by an initial decline in putrescine content and by a significant increase in spermidine content at 48–72 h. High levels of spermidine and lower levels of putrescine were found in the primary root apex as well. A marked increase in ornithine and arginine activity, as measured by $^{14}\text{CO}_2$ release, was found during the 72 h post-decapitation period of lateral root development. This increase in ornithine decarboxylase activity was confirmed also by a parallel rise in the incorporation of α -[5- ^{14}C] difluoromethyl ornithine into TCA-insoluble fractions. Microautoradiograms of longitudinal and cross sections of roots, labelled with α -[5- ^{14}C] difluoromethyl ornithine, show that ornithine decarboxylase is localized mainly in the meristematic zones, as evidenced by [^3H] thymidine incorporation. An *in situ* close correlation between meristematic activity and polyamines was demonstrated, suggesting that polyamine content and biosynthesis have a role in meristematic activity in corn roots.

Introduction

The polyamines Put, Spd and Spm, as well as their biosynthetic enzymes ADC (EC 4.1.1.19) and ODC (4.1.1.17), are ubiquitous in plant tissues and are associated with both growth and response to stress (Altman et al. 1983, Smith 1985). Extensive studies indicate that changes in polyamines are correlated with growth and cell proliferation (Bagni et al. 1982, Cohen et al. 1982b, Palavan & Galston 1982, Sarafini Fracassini et al. 1980). By contrast, less attention has been given to their involvement in specific morphogenetic events such as somatic embryogenesis in cell cultures (Feirer et al. 1984, Fienberg et al. 1984), adventitious root initiation in cuttings (Friedman et al. 1982, 1985, Jarvis et al. 1983) and tomato fruit development (Cohen et al. 1982a).

The involvement of polyamines in various growth and differentiation processes was inferred mainly from data which show correlations with polyamine levels and enzyme activity and thus provide only circumstantial evidence, as noted also in studies of animal tissues (Heby 1981). The use of specific inhibitors of polyamine biosynthesis greatly helped to indicate their role in growth and morphogenesis. However, a more complete elucidation should include also studies at the cellular level, such as polyamine localization. It has been reported that radioactively-labelled DFMO can be used successfully to localize ODC, as indeed was found in mammalian cells (Pegg et al. 1982), but as yet not in plants. This localization is based on *in situ* irreversible binding of α -DFMO, the enzyme-activated inhibitor, to active ODC within the tissue (Zagon et al. 1983).

In the following we present evidence that lateral root formation in decapitated roots of *Zea mays*, a distinct morphogenetic event, is accompanied by specific changes in Put and Spd levels and by a marked increase in ADC and ODC activity. We further show, by comparative auto-radiography of roots following incorporation of α -[5- 14 C] DFMO and [3 H] thymidine, that ODC activity is localized in meristems which give rise to lateral roots as well as in areas of active cell division in the primary root apex.

Materials and Methods

Plant material: Corn seeds, *Zea mays* var. Neve Ya'ar 170, were imbibed overnight in aerated tap water, sown in moist vermiculite and grown in the dark at 25 C. Three-day old seedlings were removed from the vermiculite, the primary root was decapitated at 5 mm from the tip and seedlings were grown for additional periods for up to 72 h (dark, 25 C) with their roots immersed in aerated tap-water. Apical (0–5 mm) and subapical (5–10 mm) segments were sampled for analyses of polyamine content, ADC and ODC activities and for histology and microautoradiography.

Polyamine content: Free polyamines were extracted in 4% (v/v) HClO_4 , and determined by dansylation and TLC, as described previously (Friedman et al. 1982, Seiler and Wiechmann, 1965). Dansylated derivatives were extracted in ethylacetate, and fluorescence measured in a Perkin-Elmer Fluorescence Spectrophotometer 204 (excitation at 365 nm, emission at 500 nm).

Enzyme activity: ADC and ODC activities were assayed in crude enzyme extracts as described previously (Altman et al. 1982), using DL-[1- ^{14}C] arginine hydrochloride (Commissariat à l'Energie Atomique, Gif-sur-Yvette, 35–45 mCi/mmol) or L-[1- ^{14}C] ornithine hydrochloride (Amersham, 57 mCi/mmol) as substrates, respectively. ADC and ODC activities are presented as pmole $^{14}\text{CO}_2$ released. In addition, ODC activity was assayed by measuring the incorporation of radioactively labelled α -DFMO (Zagon et al. 1983). Five root segments were incubated for 2 h, at 25 C, with α -[5- ^{14}C] DFMO (60 mCi/mmol, Amersham, 1.75 Ci/ml incubation medium), and radioactivity of the TCA-insoluble fraction was determined. Segments were washed in buffer (25 mM Tris glycine, pH 8.3 and 0.1% v/v SDS), ground in a total volume of 1.2 ml of the same buffer, using all-glass homogenizer, and 1.2 ml TCA (10%, v/v) was added. The extracts were kept overnight at 4 C, vacuum-filtered on Whatmann GFC filters and the filters

were rinsed 4 times with excess cold TCA (5%, v/v), dried, and the radioactivity counted with Permablend scintillation fluid (Packard). Blank samples of root segments were fixed for 24 h in FAA prior to incubation with radiolabelled α -DFMO.

Microautoradiography: Roots were radiolabelled with either α -[5- 14 C] DFMO or [3 H] thymidine, for localization of ODC activity and DNA synthesis, respectively. For ODC localization, 5 mm root segments were removed at various times and incubated for 2 h on a shaker (80 rpm) with α -[5- 14 C] (same as above). for DNA synthesis, intact roots were incubated for 24 h in [3 H] thymidine (Nuclear Research Center - Negev, 5 Ci/mmol, 0.5 mCi/ml). The radiolabelled root segments were fixed in FAA, dehydrated in ethyl alcohol-xylene series (Johansen 1940), embedded in Tissue-mat, and 6- μ m thick cross or longitudinal sections were cut by a rotary microtome. The sections were coated with Ilford K5 emulsion (Nuclear Research Emulsion in a gel form, diluted in water 1 : 1 w/v). α -[5- 14 C] DFMO-labelled sections were exposed for 8 weeks at 4 C and then stained with Orange G, whereas thymidine-labelled sections were exposed for 8 days at 4 C, followed by staining with Ehrlich haematoxylin.

Experimental design: For polyamine and enzyme activity determinations, 20-25 root segments were assayed in 3-4 replicates. Experiments were repeated 2-4 times and data of a representative experiment, with the standard error, are presented. For α -[5- 14 C] DFMO incorporation, 20 roots segments were labelled, of which 4 segments in 3 replicates were used for incorporation into the TCA-insoluble fraction, and the rest for autoradiograms. These experiments were repeated 4 times. Ten root segments were used for [3 H] thymidine incorporation autoradiography.

Results

Lateral root formation, polyamine content and enzyme activity

Structural changes during lateral root formation were studied with respect to changes in endogenous polyamine levels and the activity of their key biosynthetic enzymes, in the course of 72 h of root development. Twenty-four hours after decapitation, lateral root primordia could be observed in the subapical 5–10 mm root segment (Fig. 1 a-d). After 72 h the lateral roots have emerged and grow way beyond the cortex limits (Fig. 5e).

This morphogenetic course of events in the subapical zone is accompanied by considerable changes in the polyamine content and the activities of ADC and ODC. At decapitation time (0 h), the endogenous level of polyamines in the subapical segments (5–10mm) was quite different from that of the apical segment (0–5 mm), as seen in Table 1. While in the apical segment the level of Put was lower than that of Spd, in the subapical more differentiated segments the situation was reversed resulting in a major change in the Put/Spd ratio. The level of Spm in the apical segment was low, and could not be detected at all in the subapical one.

Marked changes in polyamines were observed during the 72 h post-decapitation period (Fig. 2). Put level declined considerably at the early hours, and it remained low during the entire period. In contrast, Spd level, which was lower initially, rose mainly after 48 h and the Put/Spd ratio approached that of the apical segment. Spm level in the subapical segment, which was near zero, reached the level found in the apical segment (Fig. 2).

The activity of ADC and ODC increased considerably with time after decapitation, and especially during the 48–72 h period of primordia development (Fig. 3). ODC activity in the subapical segment was similar to that of ADC throughout. The considerable and significant rise in ODC activity, as measured by the decarboxylation reaction, was substantiated by an alternative method for

assaying ODC activity, which is based on binding of α -[5- ^{14}C] DFMO into the TCA-insoluble fraction in the course of 72 h post-decapitation, during which time root primordia developed (Fig. 4), was indeed similar to the rise in ODC activity (FIG. 3). This is supported by the insignificant incorporation in roots which were killed in FAA (note star sign in Fig. 4), and by repeated experiments which all show that incorporation increases with time after decapitation (Table 2).

Localization of mitotic activity and ODC activity

Microautoradiographs of root segments, which were removed at various times after decapitation and incubated with α -[5- ^{14}C] DFMO, clearly show the appearance of intense labelling within the developing lateral root primordia at 48 h (Fig. 5c,d), with no indication of labelling immediately following decapitation (Fig. 5a,b). At 72 h post decapitation, when lateral roots already emerged, labelling is confined to the region of active meristem in the apex of the lateral root (Fig. 5e). This indicates that active ODC is localized in areas of meristematic activity which give rise to lateral root initiation and development.

The correlation between mitotic activity and ODC activity was further investigated by comparing the incorporation of [^3H] thymidine and α -[5- ^{14}C] DFMO in the apical segment. A high incorporation of [^3H] thymidine into DNA was found in the meristematic zone above the QC (Fig. 6a, 7d). Similarly, high activity of ODC, as evidenced by incorporation of α -[5- ^{14}C] DFMO, was observed in this zone (Fig. 6b, 7d'). In the QC, incorporation of both [^3H] thymidine and of α -[5- ^{14}C] DFMO was low (Fig. 6, 7c, c'). The heavy label of [^3H] thymidine as seen in the periphery of the QC exhibits the outer meristematic zone surrounding the lens shaped QC (Fig. 7c). The root cap initials, calyptragen, is characterized by high label of both thymidine and α -DFMO as seen clearly in cross sections (Fig. 7b, b').

Root cap present a mature tissue consisting of differentiated cells which exhibit low, if any, cell divisions (Phillip and Torrey, 1971). In comparing autoradiograms of this zone, there was no apparent overlapping of the thymidine labels and that of α -DFMO. While labelling by α -DFMO was weak (Fig. 6b, 7a'), which points to low ODC activity as expected, there was a considerable label of thymidine in root cap (Fig. 6a, 7a). The heavier label incorporated into the DNA can, however, be explained by the prolonged labelling period with thymidine (24 h) and the intense renewal rate of the root cap.

Fig. 1. Longitudinal 8 μ m thick sections of subapical corn root segments (5–10 mm) at various times after decapitation, showing lateral root formation. Sections stained with safranin-fast green. A, 0 h; B, 24 h; C, 48 h. LRP, lateral root primordia. Bar 0.2 mm.

Fig. 2. Changes in free polyamine content during lateral root formation in corn. The subapical (5–10 mm) segment was assayed at various times after decapitation. Polyamine content of the apical segment (0–5 mm) at time of decapitation is recorded on the right. One hundred % represents the putrescine content (nmol (20 segments)⁻¹) of the subapical segment at zero time. Zero time values of a representative experiment (nmol (20 segments)⁻¹) in the apical segment: putrescine, 52 ± 9.8 ; spermidine, 115 ± 2.2 ; spermine, 9.7 ± 2.2 . In the subapical segments: putrescine, 115 ± 15 ; spermidine, 54 ± 2.8 ; spermine, 0. Put, putrescine; Spd, spermidine; Spm, spermine.

Fig. 3. ODC and ADC activity in subapical segment (5–10 mm) during lateral root formation and in apical segment (0–5 mm) at time of decapitation (incubations were for 1 h at 37°C and pH 7.2). Δ , Fresh weight of 50 root segments.

Fig. 4. Incorporation of α -[5- 14 C]-DFMO in subapical segments (5-10 mm) during lateral root formation and in apical segments (0-5 mm) at zero time. Incorporation in the TCA-insoluble extract was determined as mentioned under Materials and methods. Blank segments (*) were fixed in FAA 24 h before labelling. Data are expressed in cpm; counting efficiency of all samples was similar.

Fig. 5. Microautoradiographs of subapical corn root segments (5-10 mm) following incorporation of α [5- 14 C]-DFMO. A, Cross section of subapical segment labelled immediately after decapitation. Hardly any label can be seen; B, Enlargement of portion of A; C, Cross section 48 h after decapitation. Note heavy label in lateral root primordia (LRP); D, Enlargement of portion of C; E, Longitudinal section 72 h after decapitation. Emerged lateral root (LR), label prominent in apex. Bar 0.2 mm.

Fig. 6. Microautoradiographs of 6 μ m thick longitudinal sections of primary root apex. A, Incorporation of [3 H]-thymidine (24 h); B, incorporation of α -[5- 14 C]-DFMO (2 h). Note, no label of either thymidine or α -DFMO in the quiescent centre (QC). a, Portion of root cap; b, root cap initials (RCI); c, quiescent centre (QC); d, meristematic zone above QC. Bar 0.2 mm.

Fig. 7. Microautoradiographs of 6 μ m thick cross sections of primary root apex following incorporation of [3 H]-thymidine (a, b, c, d) and α -[5- 14 C]-DFMO (a', b', c', d'). Cross sections (a, b, c, d) are those marked in Fig. 6: a, root cap, b, root cap initials; c, quiescent centre encircled by ring of active meristem; d, meristematic zone above quiescent centre. Note correspondence of intensity of label in the sections a, b, c, d and a', b', c', d', respectively. Bar 0.2 mm.

Discussion

Decapitated corn roots were used as the experimental system to study the interrelationship between polyamines and the meristematic activity of lateral root primordia. Decapitation of 5 mm root tips prevents further elongation of the roots, however initiation and differentiation of lateral root are not impaired (Schwartz, 1977; Schwartz *et al.*, 1983). Thus, physiological processes which accompany cell division and primordia formation can be monitored. Indeed, initiation and development of lateral root primordia (Fig. 1) is accompanied by marked changes in Put and Spd content (Fig. 2), and especially in the activity of ADC and ODC (Fig. 3). Put content in the more differentiated subapical segment is higher than that of Spd, but this is reversed as the size of lateral root meristems increases, resulting in higher Spd content. These results support those of Dumortier *et al.* (1983), and Schwartz *et al.* (1983), who also found a reverse gradient of Put and Spd along root axis of corn. Similar results were also obtained by Porter and Bergeron (1983) which indicate that Spd is required for proliferation of cultured leukemia cells. However, in some cases cell division and growth were found to be correlated with increased levels of Put, or both Put and Spd (Altman *et al.*, 1983).

The involvement of polyamine biosynthesis in root morphogenesis is further indicated by the marked increase in both ADC and ODC (Fig. 3), as was shown previously in other plant systems (Altman *et al.*, 1982, Cohen *et al.*, 1982a, 1982b, Dumortier *et al.*, 1983, Palavan and Galston, 1982). It has been claimed that ODC activity is involved in plant cell division, whereas changes in ADC activity are more typical for the stress response (Cohen *et al.*, 1982b, Smith *et al.*, 1985, Young and Galston, 1984). The present data do not support the generality of this conclusion, but rather show that both Put biosynthetic pathways may operate and respond to growth stimuli, as discussed previously (Altman *et al.* 1983). It should be monitored however, that although arginase activity was not determined in the present work, the proper labelled products of ADC activity were detected in

preliminary studies. Higher ADC and ODC activities usually correlate with higher levels of Put (Altman *et al.*, unpublished data), whereas in the present study changes in enzyme activity correlate with Spd levels. This may indicate a different response of the present experimental system or, more likely, a differential "removal" of Put and Spd to either a bound fraction or to other chemical forms, e.g. oxidation and acetylation. Relevant information on this aspect of plant polyamines is lacking.

Detection of ODC by reaction with radiactively-labelled α -DFMO was successfully achieved with mammalian ODC, based on the specificity of α -DFMO as an enzyme-activated irreversible inhibitor of ODC (Zagon *et al.*, 1983). This permits the quantitation of the active enzyme, using ODC extracts. In the present study we used *in vivo* labelling of root segments and the data (Fig. 4) clearly show that α -[5- 14 C] DFMO incorporation into macromolecules (irreversible binding to active ODC) increases with time after decapitation, similar to and supporting the data of ODC quantitation by measuring the decarboxylation reaction (Fig. 3). The reliability of the α -DFMO method for measuring active ODC is supported by findings in animal tissues, that α -DFMO penetrates readily into cells and is not metabolized significantly in other pathway (Zagon *et al.*, 1983).

One common obstacle in most of the studies concerning the interrelationship of polyamines and growth, is that the tissues sampled for polyamines analysis and biosynthesis contain several types of cells, at various stages of growth and differentiation. Recently, Henningsson *et al.* (1983) and Zagon *et al.* (1983) have demonstrated that labelled α -DFMO can be used also for autoradiographic localization of active ODC in mice kidneys. Our results show (fig. 5, 6, 7) a definite *in situ* correspondence between the site of ODC activity and the zone of cells that undergo mitosis, in the lateral roots as well as in the apex of the primary root. To the best of our knowledge, this is the first report of ODC localization in plant tissues. It is noteworthy that although the apical segment is considered to be the main meristematic zone as a whole, in fact it has been shown that there is quite a

diversity in the mitotic activity within the apex (Arzee *et al.*, 1977, Clowes, 1961). This is well reflected by the correspondence of label of thymidine and α -DFMO in the mirrored sites of autoradiograms (Fig. 6, 7). Thus, we have demonstrated a very close relationship between meristematic activity and polyamines biosynthesis.

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3. Polyamines and ornithine decarboxylase activity during growth and differentiation in *Sclerotium rolfsii*.

Abstract

Changes in polyamine content and in the activity of ornithine decarboxylase (ODC) were followed during growth and differentiation of the plant pathogenic fungus *Sclerotium rolfsii*, in submerged mycelium liquid cultures and on solid agar cultures. The various morphogenetic events (i.e. mycelium growth, sclerotia formation and maturation, and germination of sclerotia) can be controlled and synchronized in these two experimental systems.

Distinctive changes in polyamine content and ODC activity, highly specific to the morphological stages, were detected. Mycelium growth in submerged cultures was characterized by high putrescine content and ODC activity. Growth cessation, resulting from glucose exhaustion in the medium, was accompanied by a sharp decrease in putrescine content and ODC activity. Spermine, which is rarely found in filamentous fungi, was detected in high amounts after all glucose was consumed and when the fungus developed the potential for sclerotia formation. A decrease in spermidine, and especially putrescine content, and an increase in spermine content, were observed during the transition from mycelium to mature sclerotia on solid agar medium. These changes in the content of the three polyamines were reversed when sclerotia were allowed to germinate. Moreover, α difluoromethylornithine (α -DFMO), the enzyme-activated inhibitor of ODC, greatly inhibited mycelium growth, sclerotia germination and ODC activity, and this inhibition was completely reversed by the addition of putrescine. Cycloheximide delayed sclerotia germination and inhibited initially ODC activity, but ODC inhibition was relieved as soon as sclerotia began to germinate.

The data indicate that growth and differentiation of *S. rolfsii* may be causally linked with polyamine content and biosynthesis.

Introduction

Polyamines (PA) are present in various amounts in all living organisms and are involved in the control of growth and development of microorganisms, mammalian tissues, and plants (1,3,8,29,31). PA have been shown to interact specifically with nucleic acids and membranes, suggesting a basic bioregulatory role (3,8,31). In fungi, changes in the PA putrescine and spermidine have been associated with a variety of morphological changes (30). Usually, the levels of putrescine and spermidine increased when morphogenesis was accompanied by a high rate of cell division, nucleic acid and protein synthesis. This phenomenon was observed with respect to germination of zoospores of *Blastocladiella emersonii* (19), *Achlya ambisexualis* (33), spherules of *Physarum polycephalum* (20), ascospores of yeasts (4), and during morphogenesis of *Mucor racemosus* (13). However, when differentiation occurred without rapid proliferation, polyamine content decreased parallel to low synthesis rate of RNA and proteins, as found in fruiting body formation of *Physarum polycephalum* (20). An increase in the activity of ornithine decarboxylase (ODC)(E.C. 4.1.1.17), a key enzyme in polyamine biosynthesis, accompanied the increase in the rate of macromolecule synthesis and growth, in microorganisms and in mammalian tissues (14,31). Considerable changes in polyamine content and biosynthesis, including the activity of both ODC and arginine decarboxylase (ADC) (E.C.4.1.1.19), were found also to accompany growth and certain stress responses of plant tissues (1,29). Polyamines may play a role in stabilization of plant cell membranes (29) and were found to bound to membrane phospholipids in *Escherichia coli* (23). Recently, it was reported (26) that α -difluoromethylornithine (α -DMFO), the specific inhibitor of ODC, inhibited mycelial growth of four phytopathogenic fungi.

Morphogenesis in the plant pathogen *Sclerotium rolfsii* has been studied for the last two decades (7,25). Synchronous formation of sclerotia could be triggered by certain supplements to the growth medium (5,6,7,16,22), or by changing the proportions between aerial and submerged mycelium (11). However, no informa-

tion is available concerning polyamines metabolism during sclerotial morphogenesis and mycelium growth in *S. rolfsii*.

In this study we present evidence that formation and germination of sclerotia, as well as mycelium growth of *S. rolfsii*, are accompanied by marked changes in the content of endogenous putrescine, spermidine and spermine, and in the activity of ODC. Moreover, α -DFMO, the enzyme-activated inhibitor of ODC, inhibited both the enzyme and mycelium growth. This inhibition was reversed by addition of putrescine to the growth medium, indicating a possible causal relationship of polyamine biosynthesis and growth of *S. rolfsii*.

Materials and Methods

Growth of fungal material.

Solid medium: *Sclerotium rolfsii* type A ATCC 26325 (6) was grown at 30°C on a cellophane membrane in Petri dishes (8.5 cm diameter) containing 15 ml synthetic medium (22). The plates were inoculated in the center with agar discs (0.5 cm in diameter) covered with fungal mycelium which had been cut from a 3-d-old colony. Mycelium from solid medium was harvested by cutting only the marginal 1 cm mycelium. Sclerotial initials were removed from the fungal colony, and only those which had a white colour were selected. The dark brown sclerotia were defined as mature sclerotia. Samples from each morphological structure (mycelium, white initials, and mature sclerotia) were taken for determination of PA content and enzyme activity.

Submerged culture: Flasks containing 50 ml of the same medium were inoculated with 1 ml of suspension containing 10 mg dry wt mycelium $\times$ ml⁻¹ and incubated at 30°C on a rotary shaker (New Brunswick Scientific) at 150 r.p.m. for 3 d. Inoculum was prepared by homogenizing mycelium in the culture flasks with an Ultra-Turrax homogenizer (Janke & Kunkd, FRG) operated at one-third of maximum speed (34). Mycelium from liquid medium was separated from the

extracellular polysaccharide by centrifugation at 12,000 x g for 20 min. Samples of mycelium were taken for determination of PA content and enzyme activity.

Determination of biomass, glucose and protein.

Fungal material was separated from the growth medium, washed twice with cold 50mM phosphate buffer (pH 7.4) and dried at 70°C to constant weight. Glucose was determined using glucose oxidase reagent (Sigma), according to the manufacturer directions. Protein in the extracts was determined according to Lowry *et al.* (17), after precipitation with 7% (v/v) HClO_4 and hydrolysis in 1 N NaOH.

Polyamine determination.

Polyamines (PA) were analysed according to Friedman *et al.*, (10) with some modifications. Free PA were extracted by homogenizing 100 to 500 mg fresh weight of fungal material with 5 ml cold 4% (v/v) HClO_4 for 5 min in an Ultra-Turrax homogenizer. The extract was centrifuged for 25 min at 15,000 x g and the supernatant was used for PA determination by the dansyl reaction. To a 0.2-ml aliquot of the HClO_4 extract, sufficient Na_2CO_3 was added to bring it to a pH of approximately 7.0, followed by an overnight incubation in the dark (at room temperature) with 0.4 ml dansyl-chloride (30 mg/ml, in acetone). Excess dansyl chloride was removed by a 30-min incubation with 0.1 ml L-proline (100 mg/ml), after which dansylated PA were extracted in 0.5 toluene. Twenty to 50 μl aliquotes of toluene extract were spotted on activated (1 h at 110°C) TLC plates precoated with 375 μm silica gel G-60 (Merck), and co-chromatographed with dansylated PA standards. Plates were developed in ethyl acetate:cyclohexane (2:3, v/v) and measured in a Perkin-Elmer Fluorescence Spectrophotometer 204 (excitation at 365 nm, emission at 485 nm) The content of individual PA is presented as nmol x g dry wt⁻¹.

Extraction and assay of ADC and ODC.

Fungal material was harvested and homogenized in a prechilled mortar with a pestle (100–500 mg fresh weight/2 ml medium), for determination of ADC and ODC activity according to Altman *et al.*, (2). Extraction medium consisted of 10 mM phosphate buffer (pH 7.2), 0.1 mM DTT, 1 mM pyridoxal-5-phosphate, and 20 mM Na-EDTA. The extracts were centrifuged at 12,000 x g for 20 min, and the supernatant, hereafter referred to as crude enzyme, was used immediately. The enzymatic reaction was started by adding 100 μ l crude enzyme to a vial containing 150 μ l of the extraction medium and 50 μ l DL-[1- 14 C] arginine hydrochloride (Amersham, 0.125 μ Ci, 20mCi/mmol) or L-[1- 14 C] ornithine hydrochloride (Amersham, 0.125 μ Ci, 59 mCi/mmol) for ADC or ODC assay, respectively. The vials were then capped with rubber stoppers fitted with plastic center wells (Kontes Glass Co. NJ) containing 0.2 ml of Soluen-100 (Packard) on a Whatman No. 1 paper wick. The reaction was allowed to proceed for 60 min at 37°C in a shaking water bath, and was terminated by injecting 0.2 ml 4% HClO₄ into the reaction mixture. After an additional 30 min shaking, the center wells were removed and placed in vials with 10 ml toluene-PPO-POPOP scintillation mixture. Blank values were obtained by using boiled crude enzyme. ADC and ODC activities are presented as nmol liberated 14 CO₂ x h⁻¹ x mg protein⁻¹.

Results

Changes in polyamine content and ODC activity during *Sclerotium rolfsii* growth in submerged culture.

Growth of *S. rolfsii* in submerged culture continued as long as glucose was present in the medium (Fig 1A). Upon glucose exhaustion, the culture develops the potential for sclerotia formation (11). The dependence of *S. rolfsii* growth on glucose availability supports our previous results (28), and was investigated in this work with respect to polyamines and ODC activity. We have found that

fungal growth is accompanied by considerable changes in the content of free polyamines (Fig 1B). Putrescine content, which is especially high during the lag and in the beginning of the exponential phase (the first 50 h), declined dramatically during the second part of the exponential phase and reached a constant low level throughout the stationary growth phase (80–160 h). Spermidine content was constant during the entire growth. Spermine, which is rarely found in filamentous fungi (21,30), was detected only after all the glucose was consumed, and reached considerable levels thereof. ODC activity was highly correlated with putrescine content (Fig 1C). Thus, the lag and the first part of the exponential phase was characterized by high ODC activity, which later declined and reached very low levels during the entire stationary phase. ADC activity was assayed, but could not be detected during all growth stages of *S.rolfsii* growth in submerged culture.

Changes in polyamine content during sclerotia formation and sclerotia germination on solid medium.

Synchronization of sclerotia formation occurs when *S. rolfsii* colony is transferred to a solid medium supplemented with 10^{-5} M L-threonine (16). Several culture samples, which represent the various morphological stages, were collected and analyzed for PA content during the transition from mycelium to mature sclerotia (Fig 2A). High levels of the diamine putrescine (11 nmol x g dry wt⁻¹) and spermidine (13 nmol x g dry wt⁻¹) were found in mycelium grown on solid medium. The transition from mycelia, through sclerotia initials (white initials), to mature sclerotia, was accompanied by a dramatic decrease in putrescine content and a more gradual decrease in spermidine. Spermine was not detected in the fungal mycelium, but its content increased considerably towards sclerotia formation and maturation, to concentrations well above putrescine. However, a mirror image became apparent upon sclerotia germination (Fig 2B) :

putrescine and spermidine content increased rapidly during sclerotial germination and mycelium growth, while spermine decreased and could not be detected already 30 h after germination (Fig 2B).

Effect of α -difluoromethyl ornithine and cycloheximide on *S. rolfsii* growth and germination and on ODC activity.

Addition of α -DFMO to the fungal growth medium did not affect sclerotial formation, but repressed fungal linear growth (Fig 3A) and sclerotia germination (Fig 4A). The untreated mycelium reached the end of a Petri dish within 80 h, while the colony grown in the presence of 10^{-3} M α -DFMO hardly reached half the distance (Fig 3A). ODC activity was high during the entire growth period up to 80 h but was almost totally inhibited by α -DFMO during the initial 60 h (Fig 3B). After 80 h of growth, the control colony reached the rim of the Petri dish (cessation of linear growth), and this was accompanied by a marked decrease in ODC activity. The colony morphology of *S. rolfsii* was also affected by the presence of α -DFMO, thus instead of a fan-like typical growth, it was characterized by a compact and condensed mycelium (not shown).

The addition of 10^{-3} M putrescine to the growth medium completely reversed the inhibitory effect of α -DFMO on both ODC activity and the fungal growth (Table 1). Putrescine alone, at 10^{-3} M, did not affect growth rate but caused a slight increase in ODC activity. The addition of 10^{-2} M putrescine was toxic to *S. rolfsii* (Table 1).

In addition to its inhibitory effect on the growth of *S. rolfsii*, α -DFMO was found to be involved as well in sclerotial germination (Fig 4). Thus, germination in control medium was completed within 40 h, while germination in the presence of 10^{-3} M α -DFMO was delayed for almost 60 h, and only 20% of the sclerotia germinated (Fig 4A). The inhibitory effect of α -DFMO on sclerotial germination was paralleled by its marked effect on ODC activity (Fig 4B).

High rate of protein synthesis is associated with rapid biosynthesis of PA in microorganisms, as well as in plants and mammalian tissue (3,8,31). Cycloheximide, a protein synthesis inhibitor, also inhibits sclerotial germination, but its initial inhibition of ODC activity was relieved as soon as sclerotia began to germinate (Fig 4B).

Discussion

Changes in the endogenous level of PA and in the activity of several key enzymes of PA biosynthesis usually precede or accompany growth and morphological changes in many organisms, including fungi (3,8,12,29,31). Spore germination in fungi is characterized by accelerated synthesis of PA (15,18,33). The changes which were found in the endogenous levels of PA during sclerotial germination of *S. rolfsii* are similar to those occurring in spore germination of other fungi. The formation of fruiting bodies in several fungal species is characterized by a simultaneous decrease in polyamines levels and in the synthesis of macromolecules (20). Similarly, our data show that sclerotial formation in *S. rolfsii* is accompanied by a rapid decrease in PA level (Fig 2A). Indeed, Willetts (32) analyzed the evolutionary sources of sclerotia in several fungi, and concluded that fungal sclerotia are degenerated fruiting bodies.

The similarity between the PA-related morphogenetic changes in *S. rolfsii* and other fungi, was also established by the use of α -DFMO, a specific inhibitor of ODC – the key enzyme in PA biosynthesis in fungi (30,31). Thus, sclerotia formation in *S. rolfsii* was not affected by α -DFMO while sclerotial germination and fungal growth rate were inhibited (fig 3A, 4A). The inhibitory effect of α -DFMO on the growth of several fungi was recently described (26). In *S. rolfsii*, inhibition of sclerotial germination and mycelium growth is reversed by the addition of putrescine to the growth medium, indicating that PA biosynthesis is essential for these stages in the life cycle of the fungus (Table 1). Indeed, a large

amount of experimental data show a close correlation between high rate of ODC activity, PA accumulation and macromolecular synthesis and growth in many organisms (3,8,29,31). It was suggested that PA, and perhaps ODC itself, play an important role in mammalian cell proliferation and differentiation (12,27). Several studies indicate that the half life of ODC changes throughout cell growth and differentiation, and that the enzyme may be stabilized at later growth stages (31). This may partially explain the relieve of cycloheximide-induced ODC inhibition in germinating sclerotia (Fig 4).

Changes in PA are related to the various developmental stages in the life cycle of fungi (30). Indeed, *S. rolfsii* sclerotia contain the PA spermine, which was not detected in germinating sclerotia or in the fungal mycelium when grown on a solid medium (fig 2A, 2B). Moreover, the major decrease in putrescine level, and the appearance of spermine in submerged culture of *S. rolfsii*, occurred when the fungus developed the potential for sclerotia production. Thus, it can be assumed that spermine is essential to the structure or function of sclerotia as a resting body. It is noteworthy that spermine is rarely found in filamentous fungi, and absolutely absent in bacteria and protozoa (21). This specific presence of spermine in a distinct structural unit in *S. rolfsii* is reminiscent of the presence of spermidine, another PA, as an intrinsic component of turnip yellow mosaic virus (9). Poso *et al.* (24) suggested that the presence or absence of spermine in an organism is determined by whether or not S-adenosylmethionine decarboxylase is activated by putrescine. Further work should be done to determine whether this enzyme is synthesized *de novo* by *S. rolfsii* only in relation to sclerotia formation, or is it constitutively present but activated by the changes in putrescine, or else spermine synthase is the key enzyme.

Table 1. Inhibition of *Sclerotium rolfsii* linear growth and ODC activity by α -DFMO, and its reversal by putrescine.

Addition	40 h		80 h	
	Colony diameter (mm)	ODC activity nmol CO ₂ xh ⁻¹ x mg prot ⁻¹	Colony diameter (mm)	ODC activity nmol CO ₂ xh ⁻¹ x mg prot ⁻¹
None	35	45	85	45
α -DFMO (10 ⁻³ M)	0	0	25	20
α -DFMO + Putrescine (10 ⁻³ M)	37	44	83	48
Putrescine 10 ⁻³ M	35	53	81	54
Putrescine 10 ⁻² M	0	0	5	0

Fig 1. Mycelium growth (A) polyamine content (B) and ODC activity (C) of *Sclerotium rolfii* in submerged culture. The arrow in A denotes the time of glucose exhaustion.

Fig 2. Changes in polyamine content during formation and germination of sclerotia of *Sclerotium rolfsii* grown on a solid medium. A: Maturation of mycelium and sclerotia formation; B: Germination of mature sclerotia.

Fig 3. The effect of α -DFMO on mycelium growth (A) and ODC activity (B) in *Sclerotium rolfii* grown on a solid medium.

Fig 4. The effect of α -DFMO and cycloheximide on sclerotia germination (A) and ODC activity (B) of *Sclerotium rolsii* grown on a solid medium.

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4. Polyamines in Growth and Differentiation of Plant Cell Cultures: the Effect of Nitrogen Nutrition, Salt Stress and Embryogenic Media.

Abstract

Plant cell cultures offer a unique opportunity to elucidate the role of polyamines in cell growth and differentiation. Growth and polyamine content of tobacco cell lines were investigated with inhibitors of polyamine biosynthesis. Changes in polyamine content and biosynthesis were followed in embryogenic celery cultures.

Polyamine content of tobacco cells decreased in parallel to the decrease in growth rate. Both DFMO and DFMA inhibited putrescine accumulation. DFMO, however, caused a marked increase in spermidine and spermine levels. CHA caused a dramatic increase in cellular spermidine content. A combination of low nitrogen level in the medium, together with DFMO or CHA, inhibited putrescine accumulation in cells but caused a dramatic increase in spermidine and spermine content. Cell exposure to NaCl was accompanied by an increase in cellular content of all 3 polyamines. This greatly prevented the inhibition of growth by salt stress. Salt-resistant tobacco cell lines differ considerably from sensitive cells in their polyamine content and biosynthesis. Formation of somatic embryos in celery cell suspensions was accompanied by considerable changes in polyamine content and ADC and ODC activity. Several inhibitors of polyamine biosynthesis interfere with embryogenesis. Thus, polyamines are involved in growth, as well as in morphogenetic changes in plant cell cultures.

General Introduction

In vitro culture of plant cells and tissues has an increasingly important role in the advancement of both basic and applied aspects of plant growth and development. This includes the use of plant tissue cultures for the introduction of new traits by cell selection and genetic engineering, clonal micropropagation, pathogen elimination, as well as for elucidation of several molecular and metabolic events. Controlled organogenesis and/or embryogenesis in cell and tissue cultures (i.e. regeneration of new plants) and selection of specific cell lines, are prerequisites for the practical utilization of the aspects mentioned above. Regeneration from tissue cultures is easily achieved in some plant species such as tobacco and carrot. Several agricultural crops and most woody plants are especially recalcitrant. In most cases growth of cell cultures and *in vitro* embryogenesis and organogenesis is manipulated by the use of known plant hormones only, in an empirical manner, and very little is known on the underlying mechanisms, and on the use of additional or alternative means which may regulate regeneration.

Recent investigations confirm that active cell division and treatment with plant hormones result in significant changes in polyamine (PA) content and biosynthesis, in many plant species and with respect to many developmental processes(1,2,3,4,5). Changes in hormone levels and synthesis in response to PA treatments have been studied only occasionally, primarily in relation to

ethylene(6). The interaction of PA with auxins, gibberellins and ethylene have received more attention than with cytokinins and abscisic acid, and hence more experimental data are available for the former(1). Likewise, sharp and rapid changes in PA content and biosynthesis have been documented when intact plants, or plant tissues, were exposed to stress conditions such as accelerated senescence, increased medium osmotica and adverse pH, drought and heat conditions(7,8). Data on the interaction of salinity stress and polyamines are rather scarce, and plant tissue cultures have been used only rarely for such studies(9).

Growth and differentiation of plant tissue cultures is primarily dependent on the composition of the nutrient medium, and especially on appropriate relative concentrations of hormones (auxins and cytokinins, and in some cases also gibberellins, abscisic acid and ethylene). Therefore, the interaction of PA, growth and plant hormones in tissue cultures seem to be more obvious than in other experimental systems, and plant cell cultures offer a unique opportunity to study and elucidate the role of PA in cell growth and differentiation.

Following the pioneering studies which demonstrated that PA play an important role in growth and activation of *Helianthus* tissue *in vitro*(3,10), PA have been studied recently in tissue cultures of other plant species, in relation to growth and morphogenesis(1). Thus, analysis of PA content of cells and tissues, measurement of ADC, ODC and other enzymes, and the use of specific inhibitors of PA biosynthesis have indicated that PA play a role in induction of embryogenesis in carrot cell suspension cultures(11,12,13,14), callus formation, cell growth, and organogenesis in leaf explants of tobacco(15,16), *Passiflora*(17), *Pinus radiata* cotyledons(18) and *Helianthus tuberosus*(19).

In addition to their effect on growth and morphogenesis of plant tissue cultures, PA content, biosynthesis and metabolism were studied with respect to inhibitor resistance, selection of mutants, and formation of secondary metabolites and conjugates, in suspension cultures of tobacco, carrot and Paul's scarlet

rose(20,21,22,23,24). Few additional studies were directed at elucidating the role of PA in protoplast and cell senescence of *Avena* and *Alnus*(25,26); and the response of tobacco cells to increased media salinization(9).

The above clearly indicate that a better insight into the role of PA can be gained by simultaneous studies of changes in PA and cell development in response to modification of the culture medium. In the following, we shall report data which were obtained in 3 experimental systems:

- a. Changes in polyamine accumulation in tobacco cell cultures in response to nitrogen nutrition and inhibitors of PA biosynthesis.
- b. Polyamines in sensitive and NaCl-resistant tobacco cell suspensions.
- c. Formation and growth of somatic embryos in embryogenic celery cultures.

For the sake of brevity, and since the full results are being prepared as separate publications, only specific experimental plant systems will be described. The general methodology of polyamine and enzyme analysis was presented elsewhere(5,25,28,30,33).

a. Interactions of Inhibitors of Polyamine Biosynthesis and Nitrogen Nutrition in Tobacco Callus and Cell Cultures.

Introduction

Changes in polyamine content and biosynthesis in plants are correlated with growth and meristematic activity. Likewise, stress conditions of various types have been shown to induce putrescine biosynthesis and polyamine accumulation (1-8). The cause-effect relationships have been greatly aided by the use of several enzyme inhibitors which specifically interfere with the formation of di- and polyamines. This includes, among others, α -DFMO and α -DFMA, The enzyme-activated inhibitors of ODC and ADC, respectively (putrescine biosynthesis), CHA which inhibits spermidine synthase and MGBG which inhibits SAM-decarboxylase, thus interfering with spermidine and spermine biosynthesis(27).

Unlike their effect in several mammalian tissues and microorganisms, α DFMO and α DFMA do not cause considerable polyamine depletion when applied to intact plants or excised organs and tissues. Neither do they inhibit substantially growth, although inhibition of ODC and ADC was found in many cases(29). The relatively large pools of inorganic and organic nitrogenous compounds and reserves in plant cells may be one reason for this lack of inhibitor effect on depletion of polyamines, whose synthesis is tightly linked to the glutamine - citrulline - arginine - ornithine cycle(2,28). Nitrogen content and metabolism of cell cultures can be controlled conveniently and directly by modifying medium composition (total nitrogen, and the ratio of ammonium-N to nitrate-N). Therefore, the effects of nitrogen nutrition, and polyamine inhibitors, alone and in combination, were studied in short-term (1-2 subcultures, every 8-10 days) and long-term (over 10 subcultures, or "generations") of tobacco cell suspension cultures.

Materials and Methods

Cell cultures Wild-type tobacco callus and cell suspensions (*Nicotiana tabacum* genome, with cytoplasm substituted from *N. glauca*) were obtained from Prof. L. Reinhold, Botany Dept, Jerusalem (31). Suspension cultures were grown in a Murashige and Skoog salt mixture, with the following supplements (mg/l): myoinositol, 100; thiamine HCl, 0.1; glycine, 2; nicotinic acid, 0.5; pyridoxine HCl, 0.5; 2,4-D, 1; sucrose, 20,000; and 7mM CaCl₂. The pH was adjusted to 5.8–6.0. Suspensions were subcultured every 7–8 days by transferring 10 or 20 ml cell suspension to 35 or 70 ml fresh medium, respectively (total 45 or 90 ml, in 125 or 250 ml Erlenmeyer flasks, respectively). Flasks were maintained on a gyratory shaker (110 cycles min⁻¹), in a controlled growth room (25±1°C, 16h photoperiod of cool-white fluorescent light at ca 2000 lux). Inhibitors and other constituents, as mentioned, were included in the culture medium during the entire culture period, or for a short period only. Callus cultures were grown in the same medium, supplemented with 0.8% Difco-Bactoagar, in Petri dishes.

Growth. Growth of suspension cultures was assayed by measuring the packed cell volume (PCV, %) after centrifuging a 15ml sample at 1000g for 5 min. Fresh and dry weight were determined after filtration of cells.

Analytical procedures. Protein content and polyamine content were determined as described previously, using TLC and/or HPLC(5,8,25,30).

Results and Discussion

Tobacco cell cultures exhibit continuous growth during 8–10 days, reaching a stationary phase thereof, as measured by the increase in packed cell volume, fresh and dry weight. The molar ratio of free polyamines is ca. 100 : 10 : 1 (putrescine : spermidine : spermine), and their cellular content decrease in parallel to the decrease in growth rate (Table 1, Fig 1).

The presence of inhibitors of polyamine biosynthesis throughout the short-term culture period resulted in considerable changes, specific to the inhibitors, in free PA content of tobacco cells. Thus, both α DFMO and α DFMA inhibited considerably putrescine accumulation, whereas α DFMO, but not α DFMA, brought about an increase of spermidine, and especially spermine, content (Table 2). Cyclohexylamine (CHA), at 2 mM, induced a marked accumulation of free spermidine, as evident also by the total PA content in the presence of CHA. A concentration of 0.5 mM CHA was not as effective (See also Table 7). This effect was unexpected, in view of the fact that CHA is regarded as an inhibitor of spermidine synthase(29). It can be argued that either CHA is not a specific inhibitor in this cell system, or that free spermidine is released from bound or conjugated PA, or else that it affects the overall nitrogen nutrition. MGBG, at 2 mM, caused a considerable increase in putrescine and spermidine content. When DFMO was present continuously in long-term cultures (during more than 10 generations, or subcultures), it did not affect cellular PA content, and the contents of all three PA were very similar to those of control. Moreover, cell lines which grew for several generations in the presence of DFMO evinced resistance to higher concentrations of DFMO when transferred, gradually, to 5 + 7.5 + 10 mM DFMO. This was combined with lower cellular level of putrescine, and elevated spermidine and spermine contents. This "resistance" may indicate selection of cells with modified PA metabolism, possibly overproducing cell lines, but other mechanisms should not be excluded. This aspect is currently being investigated.

The data mentioned above were obtained when cells were cultured in standard Murashige & Skoog Medium (see also Table 4) which contains 2,4-D (1mg/l) as the only hormone. Since growth of cell cultures is dependent on hormone concentrations, and since plant hormones have been shown previously to affect PA content, the effect of 2,4-D concentration was studied (Table 3). Growth was not affected by reducing 2,4-D concentration, or even when it was

omitted. This is not unexpected in the first subculture. However, 2,4-D concentration affected considerably both putrescine and spermidine content, though a direct correlation with 2,4-D concentration could not be established. This indicated that hormone concentration can affect PA content, independent of their effect on growth.

The nitrogen levels of the medium, in combination with several inhibitors of PA biosynthesis, were investigated with respect to culture growth and cellular PA content. The data for DFMO (at 5 mM) indicate several significant interactions (Table 4 and Fig 1). Growth was only slightly inhibited by DFMO, whereas combinations of DFMO and nitrogen depletion inhibited growth by ca. 50%. Depletion of ammonium nitrate (i.e. both ammonium-N and nitrate-N) resulted in a marked decrease in putrescine content, a small increase in spermidine, with no effect on spermine content. Depletion of potassium nitrate resulted in ca. 50% increase in putrescine content, and a 4 fold increase in spermidine content (Table 5, Table 6). Total nitrogen depletion was accompanied by a considerable reduction in the cellular content of all 3 polyamines. The combined effect of DFMO and nitrogen depletion (NH_4NO_3 , or KNO_3 , or both) are especially noteworthy: while the effect on putrescine content is rather moderate, a dramatic increase in spermidine content (5-7 fold of control) and spermine content (7-14 fold of control) is evident.

The results of the experiments mentioned above are summarized in the following:

1. Tobacco cells exhibit continuous growth during 8 days. Polyamine content follows, generally, cell growth. Ratio of polyamines is approximately 100:10:1 (Putrescine:spermidine:spermine).

2. Depletion of ammonium nitrate results in a marked decrease in Put, moderate increase in Spd, and no change in Spm contents.
3. Depletion of potassium nitrate (possibly a potassium effect) results in a moderate increase in Put and Spd contents.
4. DFMO inhibits Put accumulation, but increases considerably Spd, and especially Spm, contents. DFMA is more inhibitory to Put accumulation but does not affect Spm content.
5. Cells growing in the presence of CHA exhibit a dramatic increase in their Spd content, but not in Put and Spm contents. The effect of MGBG on Spd content is much smaller.
6. A combination of nitrogen depletion and the inhibitors DFMO brings about a decrease in cell Put and a dramatic increase in Spd and Spm contents of tobacco cell cultures.

The interactions of nitrogen nutrition with DFMO (Fig. 1, Tables 1-6) and several other inhibitors (Table 7), showing clear additive or synergistic effects, are now being studied. Preliminary data show that the changes in PA content involve also marked changes in ADC and ODC activity, and in the incorporation of several PA precursors. A shift in the ratio of free to "bound" PA is also possible, e.g. release of spermidine and spermine from conjugated compounds, to "compensate" for changes in nitrogen metabolism and cation/anion balance.

Table 1. Growth and free polyamine content of tobacco cell suspension cultures^a.

harvest day	PCV, %	fresh wt., g/flask	polyamines, nmol/g fresh wt		
			Put	Spd	Spm
0	10	1.4	540	25	tr.
4	21	3.0	632	56	tr.
11	63	9.0	432	27	tr.
14	64	8.8	384	18	tr.

- ^a Cells were subcultured into fresh standard Murashige & Skoog medium (see also Table 4) at a rate of 10% (v/v), and harvested at the indicated time. Growth (percent packed cell volume, PCV, and fresh weight) and free polyamine content were recorded. tr.: traces.

Table 2. The effect of inhibitors of polyamine biosynthesis on growth and free polyamine content of tobacco cell suspensions^a.

	PCV, %	polyamines, nmol/g fresh wt			
		Put	Spd	Spm	total
Control	66	288	13	2	303
α DFMO	54	38	40	10	88
α DFMA	70	70	13	tr.	83
CHA	61	288	560	6	854
MGBG	46	400	40	tr.	540

- ^a Cells were cultured for 7 days in a standard medium with the indicated inhibitors (all at 2mM). Growth (% packed cell volume, PCV) and polyamine content were recorded.

Table 3. The effect of 2,4-D concentration on growth and polyamine content of tobacco cell suspensions^a.

2,4-D mg/l	fresh wt. g/flask	PCV %	PA content, nmol/g fresh wt.	
			Put	Spd
1	9.8	62	218	32
0.25	12.0	70	120	88
0	10.8	75	173	15

- ^a Cells were cultured for 8 days in a standard Murashige & Skoog medium, with different concentrations of the auxin 2,4-D.

Table 4. The effect of nitrogen nutrition and α DFMO, alone and in combination, on growth and free polyamine content of tobacco cell cultures^a.

medium	DFMO, mM	PCV %	polyamines, nmol/g fresh wt.			
			Put	Spd	Spm	total
Control	0	69	312(100)	20(100)	3(100)	335
"	5	60	45(14)	22(111)	7(233)	74
-NH ₄ NO ₃	0	76	40(13)	22(111)	0(0)	66
"	5	50	117(38)	136(680)	42(1400)	295
-KNO ₃	0	77	448(144)	80(400)	4(133)	532
"	5	40	102(33)	128(640)	39(1300)	269
-NH ₄ NO ₃ -KNO ₃	0	49	145(47)	3(13)	0(0)	170
"	5	33	73(23)	108(540)	23(767)	204

^a Cells were cultured for 8 days in a standard Murashige & Skoog medium^b, or with modified nitrogen regimes^c, without or with α DFMO. Growth (% packed cell volume, PCV) and polyamine content are presented.

^b Control medium: Murashige & Skoog medium (macronutrients, micronutrients, vitamins, 3% w/v sucrose), containing also 2,4-D (1 mg/l).

^c Nitrogen content:

control: NH₄NO₃ (1650 mg/l) and KNO₃ (1900 mg/l)

-NH₄NO₃: - KNO₃ (1900 mg/l)

-KNO₃: NH₄NO (1650 mg/l) -

-NH₄NO₃ -KNO₃: -

*Data in brackets: Percent of control.

Table 5. The Combined Effect of Nitrogen Levels and α DFMO on Polyamine Content in Tobacco Cell Suspensions

Cells were grown for 7 days in a control (Standard Murashige & Skoog medium (MS), or with modified nitrogen content, without or with 5mM DFMO (See Table 4).

Control (MS) data: PCV: 53%

Put: 320 nmol/g.fr.wt

Spd: 20 - " -

Spm: <1 - " -

		% OF CONTROL			
		PCV	Put	Spd	Spm
MS		100	100	100	100
MS	+ DFMO	106	50	180	624
MS - KNO ₃		102	117	104	100
MS - KNO ₃	+ DFMO	118	44	740	2720
MS - KNO ₃ , 1/2 NH ₄ NO ₃		112	107	88	100
MS - KNO ₃ , 1/2 NH ₄ NO ₃	+ DFMO	103	65	880	3520
MS - KNO ₃ , -NH ₄ NO ₃		61	88	148	100
MS - KNO ₃ , -NH ₄ NO ₃	+ DFMO	66	50	800	3920

Table 6. The Effect of α DFMO Concentrations and Nitrogen Level on Polyamine Content and Growth of Tobacco Cell-Suspension

Cells were grown for 8 days in a control (Standard Murashige & Skoog medium (MS), or with modified nitrogen content, without or with DFMO or DFMA (See Table 4).

Control (MS) data: PCV: 64%

Put: 440 nmol/g.fr.wt

Spd: 32 - " -

Spm: <1 - " -

		% OF CONTROL			
		PCV	Put	Spd	Spm
MS		100	100	100	100
MS	+ DFMO, 5 mM	78	75	325	1600
MS	+ DFMA, 7.5	76	60	219	1520
MS	+ DFMA, 5	75	24	178	100
MS - KNO ₃		51	136	125	100
MS - KNO ₃	+ DFMO, 5	76	120	825	4100
MS - KNO ₃	+ DFMO, 7.5	75	102	775	4160
MS - KNO ₃	+ DFMO, A	47	22	219	100
MS - NH ₄ NO ₃		62	46	134	100
MS - NH ₄ NO ₃	+ DFMO, 5	81	42	450	2000
MS - NH ₄ NO ₃	+ DFMO, 7.5	76	3	325	3800
MS - NH ₄ NO ₃	+ DFMO, A	66	6	150	100

Table 7. The Combined Effect of Nitrogen Level and MGBG and CHA Concentrations on Polyamine Content of Tobacco Cell Suspension

Cells were grown for 7 days in a control (Standard Murashige & Skoog medium (MS), or with modified nitrogen content, without or with MGBG and CHA (See Table 4).

Control (MS) data: PCV: 52%

Put: 384 nmol/g.fr.wt

Spd: 26 - " -

Spm: <1 - " -

		% OF CONTROL			
		PCV	Put	Spd	Spm
MS		100	100	100	100
MS	+ MGBG, 0.5 mM	83	105	138	100
MS	+ MGBG, 2	67	107	146	100
MS	+ CHA, 0.5	94	83	800	100
MS	+ CHA, 2	85	92	2460	100
MS - NH ₄ NO ₃		136	38	115	100
MS - NH ₄ NO ₃	+ MGBG, 0.5	121	52	96	100
MS - NH ₄ NO ₃	+ MGBG, 2	110	47	115	100
MS - NH ₄ NO ₃	+ CHA, 0.5	129	31	585	100
MS - NH ₄ NO ₃	+ CHA, 2	135	33	1692	100

Fig. 1 Growth (PCV) and free polyamine content of wild-type tobacco cell suspensions, in a standard control medium and in a NH_4NO_3 -depleted medium, and supplemented with 5mM DFMO, i.e. $-\text{N}_4 + \text{DFMO}$, (see also legend to Table 4).

b. Polyamine Involvement in the Response to Salt Stress of Sensitive and NaCl-Resistance Tobacco Cell Lines.

Introduction

Changes in polyamine content and metabolism in plants, and especially accumulation of putrescine and induction of ADC activity, accompany and may be causally related to a variety of stress conditions. This include potassium and magnesium deficiency, ammonium feeding, media acidification, osmotic stress and accelerated senescence(2,7,8). Salinity stress has not been investigating extensively with respect to PA, or results were not conclusive. Our previous studies indicated that intact plants respond to high NaCl levels by a major increase in ODC activity and incorporation of arginine and ornithine in roots, resulting in translocation and accumulation of putrescine in leaves(28,30 and previous BARD report). Changes in PA content, in response to salt stress, were studied also in cell cultures of *Nicotina sylvestris*(9).

Selection of cell lines which are resistant to high NaCl concentration in the medium has been achieved by stepwise exposure of cell suspensions and calli of several plant species, including tobacco. The availability of a salt resistance, stable, tobacco cell lines (which grows well in the presence of 250–300 mM NaCl), and of the sensitive wild type(31), was used in our studies with respect to the content of free and bound PA, and the activity of ADC and ODC.

Materials and Methods

Cell cultures. Wild type tobacco callus and cell suspensions (*Nicotiana tabacum* genome, with cytoplasm substituted from *N. glauca*) were obtained from Prof. L. Reinhold, Batany Dept. Jerusalem (31). Suspension culturs were grown in a Mu-

rashige and Skoog salt mixture with following supplement (mg/l): myoinositol, 100; thiamine HCl, 0.1; glycine, 2; nicotinic acid, 0.5; pyridoxine HCl, 0.5; 2,4-D, 1; sucrose, 20,000; and 7mM CaCl_2 . In addition to the wild type cells, a salt resistant cell line was employed. The stable salt-resistant line was selected, over many generations exhibiting high growth rate in the presence of up to ca 300 mM NaCl(31). This cell line was maintained routinely in the medium mentioned above, with 250 mM NaCl. All cells were grown with 90 ml medium in 250 ml. Ehrlenmeyer flask, maintained on a gyratory shaker ($110 \text{ cycles min}^{-1}$), in a controlled growth room ($25 \pm 1^\circ\text{C}$, 16h photoperiod of cool-white fluorescent light at ca 2000 lux). Cultures were subcultured routinely every 7–8 days, at 10–20% dilution rate. Salt treatments included the following variations: exposure of wild-type cell to different concentrations of NaCl in the medium, either for the entire subculture period or for a short term (2–48h); gradual transfer of the resistant cell line to lower NaCl concentrations (250mM to 0mM); re-exposure of the resistant cell line to increasing NaCl concentrations (up to 250mM), either for a short period (2–48h) or for the entire subculture period (7–8 days).

Growth. Growth of suspension cultures was assayed by measuring the packed cell volume (PCV, %) after a 5 min centrifugation at 1000g. Fresh and dry weight were determined after filtration of cells.

Analytical procedures. Protein content, polyamine content and ADC and ODC activity were determined as described previously(5,8,25,30). Free amino acids were extracted by homogenizing cells in cold 80% v/v ethanol (1g fresh weight/5ml), and centrifugation at 15,000rpm for 30 min. The clear supernatant was dried at 40°C , and redissolved in citrate buffer, pH 2.2. The clean supernatant (5min centrifugation, at 12,000 rpm) was analyzed using LC 7000 Biotronics Amino Acid Analyzer.

Results and Discussion

Major differences in polyamine content of the lines, when grown in their "standard" medium (i.e. the wild type with no salt, the resistant line with 250mM NaCl) were found. Thus the contents of free putrescine, spermidine and spermine in sensitive, unadapted cells were 2-6 fold higher than in the resistant cell line, throughout the culture period (Fig. 2). The growth of both cell lines is presented in Fig. 3.

Several experimental systems were used in order to investigate the interrelationships of PA content and the response of wild type and resistant cell line to NaCl concentration in the medium. This includes:

- a. Exposure of resistant cells (which routinely grow in the presence of 250mM NaCl, but were adapted to 0 NaCl by gradual serial subcultures) and of wild type cells to 150mM NaCl for a short period (24 and 48h) or for a long period (6-10 days).
 - b. The effect of DFMO and DFMA on growth and PA content during cell exposure to NaCl.
 - c. Changes in PA during serial transfers of the resistant cell line to 0 NaCl and re-exposure to high NaCl concentrations.
1. *The response to a short-term exposure:* culture exposure to 150mM NaCl during 24 and 48h resulted in a 60-70% decrease of cell fresh weight in both wild type and resistant cell line, and in a 20-50% decrease of cell dry weight. A 24h exposure of the 2 cell lines to salt was accompanied by a considerable increase, on fresh weight basis, of all 3 PA and especially of putrescine (Fig. 4). The increase in putrescine at 24h was particularly evident in wild type

cells as compared with the resistant cell line. This difference between 0 NaCl and 150mM NaCl is reversed when PA content is calculated on a dry weight basis. It is noteworthy that exposure to NaCl resulted also in a marked increase in total free amino acid content, and especially in the sild type cells (Fig. 5). The major increase was found in proline, alanine, isoleucine, leucine and arginine.

2. *The response to a long-term exposure:* growth of both wild-type and selected cell lines (expressed as packed cell volume and fresh weight) was considerably inhibited by a 6 and 10 days exposure to 150 mM NaCl (Fig. 6). Growth of the selected cells in a standard control medium (without NaCl) was considerably higher than wild-type cells. Cell exposure to 150 mM NaCl during 6 days resulted in a marked increase in putrescine, spermidine and spermine (calculated on fresh weight basis) in both wild type and selected cells (Fig. 7). At day 10, NaCl-induced putrescine accumulation in selected cells was considerably higher than in wild type cells (although total putrescine content was higher in wild type cells, see also Fig. 2). Again, a two-fold increase in the content of total free amino acids was found upon exposure of both cell lines to NaCl.

3. *The effect of DFMO and DFMA:* These experiments were performed in order to find out whether DFMO and DFMA, the inhibitors of ODC and ADC respectively, will affect the NaCl-induced changes in putrescine and polyamines. Wild type cells were cultured in standard control medium, and then exposed for 24 h to 150 mM NaCl without or with a combination of DFMO and DFMA (each at 1mM, as a 2 h pretreatment and during the exposure to NaCl). The inhibitor combination did not affect cell growth during 24 h, but inhibited (by about 50%) the NaCl-induced putrescine accumulation (Fig.8). No change in spermidine content was found upon exposure to salt, but lower

spermidine content was found in the presence of DFMO+DFMA. Exposure to NaCl resulted as well in an increase in the content of total free amino acids (and especially threonine, serine and proline), and the combination of inhibitors inhibited this increase (and especially the content of glycine, histidine and ammonia) (Table 8).

Similar experiments were performed with 1 mM DFMO only, instead of a combination of DFMO and DFMA. Again, a 1 day exposure to NaCl resulted in small increase in free putrescine and spermidine content, but 1mM DFMO (alone) not only did not inhibit putrescine and spermidine accumulation, but higher contents were found in its presence (Fig. 9). Similar effects of DFMO were found during 6 day culture and with respect to free amino acid content. Cell exposure to 1mM DFMA (alone) and salt resulted in a decrease in free putrescine and spermidine content during 1 and 6 days of culture, as compared to NaCl without DFMA (Fig 10).

4. *Transfer of selected cells to 0 NaCl and re-exposure to 350 mM NaCl:* selected (NaCl-resistant) cells were routinely subcultured with 250 mM NaCl. In the following experiments, changes in growth and PA content were followed in cells which were gradually transferred from 250 mM NaCl to 0 NaCl, and in cells which were re-exposed to increasing NaCl concentration in the medium. Seven-day old cells, growing with 250 mM NaCl, were subcultured at 24 h intervals in decreasing NaCl concentrations (mM): 220, 190, 150, 120, 90, 50, 0. Free and bound PA content was determined at 150 mM (day 3), 50 mM (day 6) and 0mM (day 7). Free putrescine content increased considerably as cells were transferred to low NaCl concentrations (50 mM and no salt), whereas the content of free spermidine and spermine markedly decreased (Fig. 11). Bound PA comprise a major fraction of total PA content, and bound spermidine content was found to increase upon transfer of cells to lower NaCl concentrations.

In a second set of experiments, selected (NaCl-resistant) cells which were subcultured previously for several cell generations without NaCl, were transferred to media with 50, or 150, or 250, or 350 mM NaCl. Growth and PA content were measured 6 and 10 days after culture in NaCl enriched media. NaCl at concentrations higher than 150 mM inhibited considerably cell growth measured at day 6, but at day 10 significant growth was found even at 250 and 350 mM NaCl, indicating a "recovery" of the selected cells (Fig. 12). The contents of free PA increased considerably upon cell exposure to increasing NaCl concentration up to 250 mM, at day 6 and 10 (Fig. 13). A decrease in free putrescine content was found at the highest salt concentration (350 mM NaCl). A marked increase in bound putrescine content was also found in response to increasing NaCl concentrations after 10 days of culture. Cell exposure to high NaCl concentrations was accompanied as well by a 3-fold increase in the content of total free amino acids. This increase was especially notable with proline (200-fold) and alanine (12-fold).

5. *ADC and ODC activity:* following our studies on changes in growth and PA content in response to salt stress, initial experiments on ADC and ODC activity in the two cell lines were performed. The specific activity of ODC was generally higher than that of ADC, in both cell lines. Exposure of wild type cells to 125 mM NaCl resulted in a decrease of enzyme activity during the initial 12 h, followed by an increase at 14–48 h. NaCl stress induced a marked increase in ODC activity, whereas ADC activity was inhibited (Fig. 14). Other data will be reported elsewhere.

Fig. 2 Free polyamine content in wild type (sensitive) tobacco cells and in a salt resistant cell line.

The sensitive, wild-type, suspension cultures were grown for 14 days in a standard Murashige & Skoog medium; the salt resistant, selected, cell line was grown in the same basal medium, but with 250 mM NaCl.

Fig. 3 Growth (packed cell volume, fresh and dry weight) of wild type (sensitive) and selected (NaCl-resistant) tobacco cell lines.

Culture conditions, as in Fig. 2.

Fig. 4 The effect of short-term culture exposure to NaCl on free polyamine content of wild-type and selected (NaCl resistant) tobacco Cell lines. cells were cultured for 7 days in a Control medium, and for additional 24 and 48 h with or without 150 mM NaCl. The selected cell line was previously subcultured without NaCl.

Fig. 5 The effect of culture exposure to 150 mM NaCl on total free amino acid content of wild type and selected tobacco cell lines. Culture conditions as in Fig. 4.

Fig. 6 The effect of long term culture exposure to 150 mM NaCl on growth (packed cell volume and fresh weight) of wild type and selected tobacco cell lines. Cells were cultured for 6 and 10 days with or without 150 mM NaCl. The selected cell line was previously subcultured without NaCl.

Fig. 7 The effect of long-term Culture exposure to 150 mM NaCl on free polyamine content of wild type and selected tobacco lines.

Culture conditions as in Fig. 6

Fig. 8 The effect of a combination of DFMO and DFMA on free polyamine content of wild type cells, without or with 150 mM NaCl.

7-day old cells were exposed for 24 h to 150 mM NaCl, without or with DFMO+DFMA (each at 1 mM, present as a 2 h pretreatment and during the exposure to NaCl).

Fig. 9 The effect of DFMO on free and bound polyamine content of wild type cells, without or with 150 mM NaCl.

Culture conditions as in Fig. 8, except that cells were treated with 1mM DFMO (Without DFMA) for 1 and 6 days

Fig. 10 The effect of DFMA on free polyamine content of wild type cells, without or with 150 mM NaCl.

Culture conditions as in Fig. 9, except that cells were treated with 1mM DFMA (Without DFMO) for 1 and 6 days

Fig. 11 Free and bound polyamine content in selected (NaCl resistant) tobacco cells during stepwise subcultures in lower NaCl concentrations.

7-day old cells, growing with 250 mM NaCl, were subcultured at 24 h intervals in decreasing NaCl concentrations (mM): 220, 190, 150, 120, 90, 50, 0. Free and bound polyamines were determined at 150 mM (day 3), 50 mM (day 6) and 0 mM (day 7)

Fig. 12 The effect of re-exposure to increasing NaCl concentrations on growth of selected tobacco cells which were subcultured previously without NaCl. Cells were transferred to the indicated NaCl concentrations for 6 and 10 days, and growth was recorded.

Fig. 13 the effect of re-exposure to increasing NaCl concentrations on free and bound polyamine content of selected tobacco cells which were subcultured previously without NaCl.

Culture conditions as in Fig. 12.

INFLUENCE OF SALT SHOCK ON ADC & ODC ACTIVITIES.

Fig. 14 The effect of NaCl stress on ODC and ADC activity of wild type tobacco cell line.

seven day old cells were exposed to 125 mM NaCl for 12, 24, 36 and 48 h, and enzyme activity was assayed

Table 8. The effect of a Combination of DFMO and DFMA on the Content of Free Amino Acids in Wild Type Cells Exposed to 150mM NaCl

Culture conditions as in Fig. 8. Data on those amino acids where a major change was found are presented.

Amino Acid Content, nmol g ⁻¹ fresh wt.				
NaCl(mM)	0	0	150	150
Time(h)	0	24	24	24
DFMO+DFMA	-	-	-	+
THR	10688	11250	21000	-
SER	1436	1313	2438	-
PRO	136	151	394	366
GLY	2850	3000	2156	1219
HIS	-	263	506	206
AMMONIA	14156	12375	12188	6000
TOTAL	34872	34400	48545	28867

C. Polyamines and Somatic Embryogenesis in Celery.

Introduction

Asexual (vegetative) reproduction in plants, i.e. regeneration from somatic cells, is achieved *in vitro* by two basic morphogenetic patterns: organogenesis (regeneration and proliferation of shoots and/or roots), and embryogenesis (bipolar organization, leading to simultaneous root and shoot formation, resembling sexual embryogenesis). Somatic embryos usually arise from single cells which undergo two distinctive differentiation stages: (a) induction and initiation of embryogenic cell clumps, in the presence of auxins (mainly 2,4-D) and (b) embryo development and maturation in the absence of auxins. It has been established that several other factors are important: reduced nitrogen source in the medium, competent primary explants, and establishment of permissive condition for embryo maturation. The manipulation of somatic embryogenesis, however, is rather empirical, and the metabolic and molecular control mechanisms are only little understood (32).

Recent studies indicate that putrescine biosynthesis and PA play a role in cellular differentiation during somatic embryogenesis in carrot. Thus, a requirement for polyamines and ADC activity, in interaction with 2,4-D, was established by the use of DFMA, MGBG and CHA (12,13). It was also noted that inhibition of putrescine biosynthesis via the ODC pathway, by using DFMO, induced somatic embryogenesis in carrot even in the presence of otherwise inhibitory concentrations of the auxin 2,4-D (14). Somatic embryogenesis in celery (*Apium graveolens* L.) is, in principal, similar to that reported previously for carrot, but unlike carrot it is highly efficient, it can be synchronized, and a clear distinction can be made between the various morphological structures: cells, embryogenic clumps, aggregated embryos, and free-floating single embryos (33). The role of PA levels and biosynthesis in growth and differentiation of celery embryogenic

cell suspensions, is the subject of the present study.

Materials and Methods.

Plant material and cultures. Two week old seedlings of celery (*Apium graveolens* L. var. SB 12) were obtained from a local nursery and grown in a growth chamber at 27°C and 16 photoperiod (1700 Lux). Plants were thus maintained for 3 weeks before removal of explants. Petioles and leaves were then removed and washed thoroughly in a detergent and water for 5–8 minutes. Petiole and leaf explants, 1–2cm in length, were surface-sterilized with 95% ethanol (90 sec), followed by 1% (w/v) Na hypochlorite and 0.02% Tween 20 (10 min), and rinsed with sterile water. Aseptic explants were cultured on a standard Murashige and Skoog (MS) medium (GIBCO) containing also 2.3 μ M 2,4-D, 2.3 μ M kinetin or 4.4 μ M BA, 500 mg Casein Hydrolysate (CH), 1% agar and 3% sucrose, hereafter referred to as "callus induction medium". Cultures were maintained at 26°C and a 16 h photoperiod (1600 Lux). Initial callus appeared at about 3 weeks, and after an additional 2 weeks callused explants were transferred to MS liquid medium with 2.3 μ M 2,4-D, 0.88 μ M BA, 3% sucrose and 500 mg CH ("maintenance medium") and maintained on a gyratory shaker at 100 rpm. These initial cultures will be referred to as "primary cultures". Several cell lines were isolated. Suspension cultures were subcultured every 14–18 days. Embryogenic cultures were already observed after 3 subcultures. Complete plantlet regeneration was accomplished on "regeneration medium" consisting of MS + 2.3 μ M Kinetin + 100 mg CH.

The size of inoculum was regulated by sieving suspension cultures through a series of metal mesh screens with varying pore size from 2400 micron(μ) to 200 μ . Microscopic examination showed that cultures that had passed a 250 screen were embryo free.

In addition, explants were removed from aseptic plants which regenerated from primary cultures, and plated on callus induction media. The resulting callus was transferred to liquid media on gyratory shakers. These cultures were designated "secondary" callus cultures. Plantlets from suspension cultures were transferred to paper bridge supports supplemented with liquid "regeneration medium". Fully grown plantlets were acclimitized in a mist chamber.

Results and Discussion.

Embryogenic cell suspensions were established from petiole and leaf callus, as mentioned above, and subcultured routinely in a "maintenance medium" (with $3.2\mu\text{M}$ 2,4-D and $0.88\mu\text{M}$ benzyl-aminopurine). Development of somatic embryos occurred on a "regeneration medium" (with $2.3\mu\text{M}$ kinetin and 4% mannitol). Synchronized cultures were produced by fractionation through a series of screens. We found that induction of somatic embryos in celery, as well as the differentiation and development of somatic embryos, are accompanied by changes in the content of free PA and the activity of ADC and ODC. Moreover, several inhibitors of PA biosynthesis, and addition of certain PA to the medium, affected growth and embryo differentiation. Growth of embryogenic cultures (increase in cell volume, i.e. percent "packed" volume) continued for at least 24 days, with a lag period between the 8th and 12th day. (Fig. 15). Growth of the $200\mu\text{--}800\mu$ fraction (consisting mainly of cell clumps and globular embryos) was inhibited by $0.2\mu\text{M}$ MGBG, and more so by $2\mu\text{M}$, from the 4th day on. Spermidine at 2mM did not relieve the MGBG-induced inhibition, and improved growth in the presence of 2mM MGBG only slightly. Growth of the larger size fraction ($800\mu\text{--}1400\mu$, consisting of differentiated embryos and clumps of embryos) was completely inhibited by 2mM MGBG, but the inhibition by 0.2mM MGBG was relieved once the embryos became more mature, after 20 days in culture. Similarly, growth (fresh weight increase) of the larger size fraction

(>1400 μ) on paper bridges was also inhibited by 2 mM MGBG, but not at 0.2 mM MGBG (Fig. 16). This may indicate that maturation and later stages of embryo development are not dependent on S-adenosyl methionine decarboxylase activity, but the direct participation of PA in this process has yet to be demonstrated. The importance of PA for differentiation of celery embryos, as distinct from growth, was demonstrated in another experimental system. Plating of embryogenic clumps (removed from suspension cultures) on agar-solidified medium permits one to follow the advancement of differentiation, and to record the formation of complete plantlets, as distinct from only shoot or only root formation. MGBG was found to inhibit both growth and normal differentiation, i.e. plantlet formation. Root formation was not inhibited by MGBG, however, suggesting that shoot formation may be more sensitive to inhibition of SAM-decarboxylase activity (Fig. 17). The direct involvement of PA in this morphogenetic process is indicated by the fact that addition of spermidine to the medium dramatically promoted and enhanced plantlet formation (Fig. 17,18). Thus, the majority of the embryogenic clumps formed only shoots, and only a small number of fully-differentiated plantlets were observed in the control medium. In the presence of 0.1mM spermidine, however, almost 80% of the embryogenic clumps formed complete plantlets. In addition, the number of plantlets per clump was much higher in spermidine-enriched medium than in the control.

Fig. 15 The effect of MGBG on growth of celery embryogenic cultures

Embryogenic cell suspensions of celery were sieved through appropriate screens, yielding 2 size fraction: 200-800 μ (mainly cell clumps), and 800-1400 μ (mainly clumps of embryos and larger embryos). MGBG and spermidine were added at the start of the experiments, and growth (measured as packed volume) was recorded at 4 day intervals.

Fig. 16 The effect of MGBG on growth (fresh weight increase) of celery embryogenic cultures.

Details same as for Fig 15, except that the largest cell fraction (aggregates larger than 1400μ) was grown for 2 weeks on paper bridges.

Fig. 17 The effect of spermidine and MGBG on somatic embryogenesis in celery. The $800-1400 \mu$ cell fraction was plated on agar medium, supplemented with spermidine and MGBG

A. 1- control; 2- 0.1 mM Spd; 3- 0.2 mM Spd; 4- 1 mM Spd; 5- 0.2 mM MGBG; 6- 2 mM MGBG.

B. Normal embryo development - 0.1 mM Spd.

C. Differentiation of roots only - 2 mM MGBG

Fig. 18 Morphogenic patterns of embryogenic cell cultures of celery as affected by spermidine.

Embryogenic clumps, separated from celery cell suspensions by sieving, were placed individually on agar-solidified regeneration medium (control), or with spermidine (0.1 mM). The various developmental stages were scored for two weeks.

D. Concluding Remarks

In addition to direct evidence for changes in PA content of plants in response to hormone application, major fluctuation in PA also occur during developmental processes which are normally regulated by plant hormones. Notable, among these, are growth and morphogenesis of tissue cultures, growth of some organs, and certain aspects of senescence. The data mentioned above, and other studies, provide evidence that PA content and biosynthesis play a role in the control of plant tissue culture growth and morphogenesis in a few selected cases. As cell and tissue cultures are dependent on the presence of plant hormones in the medium, PA and plant hormones probably interact in some manner. In most cases, however, the evidence is only circumstantial, and direct evidence to a causal interaction between PA metabolism and plant hormones is lacking. In fact, it can be argued that PA change simultaneously with growth and differentiation of cell cultures, but that these processes are independent of each other. Somatic embryogenesis and cell activation may be notable exceptions.

Further elucidation of the role of polyamines in growth and differentiation of plant cell cultures should be aimed at the following issues:

1. **Timing** – do changes in polyamines biosynthesis, i.e. enzyme activation and synthesis, precede cell mitosis and differentiation? what are the mechanisms? The use of appropriate inhibitors and molecular hybridization should greatly aid answering this issue.
2. **Localization** – can specific polyamines and enzymes of PA biosynthesis be traced to specific cells, or group of cells, or tissues, during cell division and differentiation? Histochemical localization and tissue *in situ* hybridization seem to be the techniques of choice.

3. Culture media – how do growth hormones and nutritional constituents of the culture medium affect PA biosynthesis and content? The study of nitrogen metabolism with respect to PA is of special importance.
4. "Secondary" metabolism and binding – how are arginine, ornithine and putrescine diverted into alternative pathways, other than tri- and tetra- amines? Diversion into "secondary" plant metabolites, (e.g. alkaloids, cinnamoyl putrescine) and binding to insoluble cell fraction may mask the interactions between a given growth response and PA content, and should not be overlooked.

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5. Gibberellin-induced growth of pea internodes: involvement of polyamine biosynthesis

Abstract

When gibberellic acid (GA_3) is sprayed on 9-day-old light-grown dwarf Progress pea (*Pisum sativum*) seedlings, arginine decarboxylase (ADC; EC 4.1.1.9) activity increases within 3 h and peaks at about 9 h after GA_3 application. This is followed by a second lower peak at about 30h; both peaks were higher than the corresponding peaks in the controls. In contrast, no appreciable effect of GA_3 on internode length was observed until about 12 h, after which time a dramatic increase in growth rate occurred and persisted for about 12 h. Specific (DL- α -difluoromethylarginine) and non-specific (D-arginine and L-canavanine) inhibitors of ADC strongly inhibited ADC activity and to a lesser extent internode growth. The inhibition was reversed only slightly by the addition of polyamines. Actinomycin D and cycloheximide inhibited the rise in ADC activity induced by GA_3 . The half-life of the enzyme was increased by GA_3 treatment. The results suggest that part of the GA_3 -induced increase in internode growth may result from enhanced polyamine biosynthesis through the ADC pathway. Furthermore, the GA_3 induced increase in ADC activity probably requires de novo synthesis of both RNA and protein.

Introduction

We have previously reported (Dai *et al.* 1982) that the GA₃-induced increase in internode length of light-grown dwarf Progress pea (*Pisum sativum* L.) seedlings is accompanied by an increase in both ADC activity and PA levels. Since much evidence now links PAs to the control of growth and developmental processes, and since ADC is one of the key PA biosynthetic enzymes in plants (Bagni *et al.* 1981, Galston 1983, Slocum *et al.* 1984, Smith 1985), the GA-induced rise of ADC activity may be an important event in the stimulation of stem elongation by the hormone. This paper reports the effects of inhibitors of PA biosynthesis on GA₃-induced increase in ADC activity and internode growth of light-grown dwarf Progress peas. A possible relationship is shown between cell growth and hormonal stimulation of ADC activity. A preliminary report of a portion of this study has been published (Galston and Kaur-Sawhney 1982).

Materials and Methods

Plant material – Seeds of Progress pea (*Pisum sativum* L.; Asgrow Ubc.), a dwarf variety, were imbibed and sown in coarse vermiculite in controlled-condition growth rooms as reported earlier (Dai *et al.* 1982). Seedlings were sprayed either with buffer or GA₃ and/or inhibitors of PA biosynthesis.

Application of GA₃ and inhibitors – Nine-day-old seedlings were selected for uniformity and treated with GA₃ solution or 20 µl microdrops of GA₃ and/or various inhibitors were applied to the terminal bud by a micropipet. GA₃ concentration ranged from 5 to 20 µg/ml, DFMA and DFMO, 1 mM each; D-arginine 10 mg/ml, L-canavanine 2 mg/ml, Act. D. and CHI 50 µg/ml each. The inhibitors were added alone or with GA₃. All solutions were prepared in 0.03 M phosphate buffer (pH 6.4) containing 0.05% pluronic L 101 (Wyandotte Chemical Corp.) to aid penetration. For the microdrop treatment each terminal bud received two applications of 20 µl at half hour intervals. For the experiments of inhibitor

effects, the 4th and 5th internodes were harvested 9 h and 48 h after GA₃ treatment for measurements of enzyme activity and growth, respectively. In experiments for determination of the half-life of ADC, the entire fourth internodes were harvested 9 h after GA₃ treatment and incubated with 50 µg/ml CHI solution. At different time intervals, internodes were removed and used for enzyme assay.

Enzyme extraction and assay – Seven entire fourth or fifth internodes from 9-to-12-day-old seedlings were excised and homogenized in chilled mortars with 1 ml of 100 mM phosphate buffer, pH 7.0. The homogenates were centrifuged and 27,000 xg for 15 minutes at 4°C, and the supernatant fractions used as the crude enzyme. ADC activity was determined by measuring the release of ¹⁴CO₂ from [U-¹⁴C]-L-arginine as described earlier (Dai *et al.* 1982). Protein content was measured by the method of Bradford (1976) using BSA as a standard.

DNA extraction and assay – DNA content was estimated by the DAPI-method as described in a recent report (Dumortier *et al.* 1983). DNA from internodes was extracted in pH 7 Tris buffer (10mM Tris, 10 mM EDTA, 2 M NaCl) at a ratio of 100 mg fresh weight/ml buffer with a glass homogenizer. After extraction for 1 h in an ice bath, homogenates were centrifuged for 20 min at 26,000xh and the supernatant fraction was used for determination of the DNA content. Three aliquots of 10 µl each of the DNA extracts was mixed successively with 3 ml of DAPI solution (100 ng/ml, w/v) in buffer (100 mM NaCl, mM EDTA, 10 mM Tris pH 7.0) and the fluorescence values noted each time. The fluorescence of the DAPI-DNA complex was measured at 450 nm in an Aminco-Bowman spectrofluorimeter with an activation wavelength of 350 nm. Three aliquots of DNA solution (20 µg/ml, calf thymus DNA) were added successively to the same cuvette. When the fluorescence values were plotted against the cumulative volume aliquots added in the cuvette, two straight lines were obtained from which the DNA content in the extract was calculated.

The data presented are from single representative experiments of the mean $\pm$ SE of 2 to 6 experiments, each performed in duplicate or triplicate.

Results and Discussion

Effects of microdrop application of GA₃ on internode growth— GA₃ applied as microdrops to the terminal bud caused increased elongation of the 4th and 5th internodes (Table 1). Two 20 μ l drops of 10 μ l/ml of GA₃ applied at half-hour intervals to terminal buds of 9-day seedlings increased length of the 4th and 5th internodes by about 68% and 212% respectively over control treatment, while the higher concentration of 20 μ g/ml was less effective. Since microdrop application of GA₃ was as effective as the spray application reported earlier (Dai *et al.* 1982) and can be controlled quantitatively, it was adopted for study of the inhibitor effects.

Kinetics of microdrop application of GA₃ on internode growth— Earlier observations (Dai *et al.* 1982) show that GA₃ application not only caused hyperelongation of the photodwarfed pea seedlings but also induced higher ADC activity, with concomitant increases in levels of the diamine putrescine and the triamine spermidine. To determine if the increase in ADC activity is causally related to the hormone-induced hyper-elongation of internodes, the kinetics of GA₃ effect on ADC activity and internode growth were examined. The results in Fig. 1 show that ADC activity increased within 3 h of GA₃ application and peaked at about 9 h, followed by a second lower peak at about 30 h; both peaks were higher than the corresponding peaks in the controls. In contrast, no appreciable effect of GA₃ on internode length was observed till about 12 h, after which time a dramatic increase in growth rate occurred. This augmented rate continued for about 12–15 h and then declined slightly. GA₃ treatment increased the internode length by about 70% at the end of 32 h, while in controls, the internode length increased by only about 11%.

The early increase in ADC activity, which occurs much before the increase in internode growth rate, suggests a cause and effect relationship between GA₃-induced promotion of PA biosynthesis (Day *et al.* 1982) and internode growth. Similar hormonal effects on PA biosynthesis and growth have been reported in many plant and animal systems (Bagni *et al.* 1981, Galston 1983, Heby 1981, Oka and Perry 1976, Slocum *et al.* 1984). Furthermore, the biphasic induction of ADC activity by the hormone observed here resembles that for ornithine decarboxylase (ODC, the rate limiting enzyme of PA biosynthesis in mammalian cells) activity during the cell cycle noted in many animal systems (Boynton *et al.* 1980, Heby *et al.* 1976, McCann *et al.* 1975). Increased PA biosynthesis during the G₁ phase of the cell cycle, prior to the onset of DNA synthesis in dividing cells, appears to be a common phenomenon in animals (Fuller *et al.* 1977, Heby 1981) and plants (Adlakha *et al.* 1980, Cohen *et al.* 1984). Thus, the GA₃-induced enhancement of internode growth probably involves increased cell division as observed in other systems (Callebaut *et al.* 1982) and the rise in ADC activity may be one of the early effects through which GA₃ promotes internode elongation.

Effect of inhibitors on GA₃-induced increase of ADC activity and internode growth—To ascertain specifically if the GA₃-induced increase in internode length occurs via an enhancement of ADC activity, microdrips of the specific "suicide" inhibitors, DFMA and DFMO (for ADC and ODC respectively) were applied to the terminal bud of 9-day-old plants. These inhibitors have been successfully used in several plant, bacterial and animal systems (Heby 1981, Kallio *et al.* 1981, Slocum *et al.* 1984). DFMA at a concentration of 1 mM was very effective in inhibiting ADC activity and internode growth, when applied prior to the increases of both ADC peaks induced by GA₃ treatment. Data in Table 2 show that GA₃ treatment increased ADC activity by about 2-fold and internode length by about 3-fold when compared with control treatment, and DFMA inhibited the increase in ADC activity by about 60% and internode length by about 72%. Addition of putrescine (1mM) only slightly reversed the inhibition by DFMA of

internode growth. DFMA alone sharply inhibited endogenous ADC activity with no appreciable effect on internode length. Application of DFMO with GA₃ had only a small effect, while addition of DFMO with DFMA had no added effect on the inhibition of the GA₃-induced increase in internode growth (data not shown). The inhibition of growth by DFMA was also accompanied by decreased DNA content of the internodes. Treatment with GA³ increased the DNA content from 5.25 µg to 7.8µg/per internode, and DFMA completely inhibited this increase. DFMA alone had no significant effect either on internode length or on DNA content (Table 2).

D-Argining and L-canavanine, steric analogues of L-arginine, strongly inhibited both basal and GA₃-induced ADC activity.

The above results thus indicate that GA₃-induced enhancement of internode growth may require an increase in ADC activity and putrescine titer, both known to occur (Dai *et al.* 1982). Similar increases in ADC activity and putrescine levels in cucumber cotyledons have been reported (Suresh *et al.* 1978). Likewise, GA₃ treatment of *nana* peas increased internode length and the amount of putrescine, spermidine and spermine (Smith *et al.* 1985). Treatment with DFMA inhibited internode length and addition of putrescine to DFMA-treated plants effectively restored growth of internodes to levels of the controls. Enhancement by putrescine of GA₃-induced elongation in hypocotyls of lettuce seedling has also been reported (Cho 1983). Thus, PA biosynthesis appears to be involved in the GA₃-induced promotion of internode length.

In contrast, although the gibberellin-induced synthesis of α amylase in barley aleurone tissue can be inhibited by DFMA (Bernal-Lugo 1983), the nature of any PA involvement in this response is not yet clear. Recently, Lin (1984) reported that PA titers in aleurone layer cells do not change in response to GA₃ promotion or abscisic acid inhibition of α-amylase-activity. However, prior depletion of spermidine titer by inhibition of S-adenosylmethionine decarboxylase (enzyme involved in spermidine synthesis) by methylglyoxalbis-[guanyldiazide], de-

creased the subsequent GA₃-mediated induction of α -amylase activity. It was thus concluded that PAs, particularly spermidine, may play a contributory role.

Increase in internode length by gibberellins can be due to their effect on cell division, cell expansion or both. There is good evidence that gibberellins affect cell division in higher plants (Callebaut *et al.* 1982) and a recent report (Smith *et al.* 1985) suggests that PAs affect cell division but not expansion. However, our own data on GA-induced increase in PA biosynthesis and internode length, does not delineate whether PAs are involved in cell division or expansion.

Effect of actinomycin D and Cycloheximide- To examine whether the GA₃-induced increase of ADC requires the synthesis of new RNA and protein, seedlings were sprayed with Act. D and CHI immediately after GA₃ treatment, and enzyme activity was measured at h 9, corresponding to the first peak of ADC activity. Data in Table 3 show that Act. D inhibited ADC activity in the fourth internodes of control plants by about 36% and GA₃-treated plants by about 49%. These observations suggest that both the GA₃-induced increase in ADC and the basal ADC activity in the internode cells is dependent on the continuous synthesis of RNA. This is not unexpected in view of the relatively short half-life of ADC (discussed below).

Treatment with CHI also inhibited ADC activity in control and GA₃-treated internodes. The enzyme activity in control internodes was inhibited by about 25%, and in GA₃-treated internodes by about 43%, suggesting a requirement of de novo protein synthesis both for maintenance of the basal enzyme level and for the GA₃-increase in ADC activity (Table 3). This is in accord with the known CHI effect on cytokinin-induced increase of ADC activity in cucumber cotyledons (Suresh *et al.* 1978) and the effect on ADC activity in etiolated buds (Dai and Galston 1981). Thus, GA₃, like other growth regulators and physiological stimuli, appears to induce a de novo synthesis of the enzyme. However, the possibility that Act. D or CHI may shift the timing of the GA₃ enhancement of enzyme activity cannot be ruled out.

Half-life of ADC—Changes in the half-life of an enzyme help determine the turnover of the enzyme, and thus could be used as a marker for investigating the mechanism of enzyme induction and regulation of cell growth. The half-life of ADC as affected by GA₃ treatment was examined by adding CHI (50 µg/ml) to block protein synthesis, and then determining residual ADC activity in control and GA₃-treated tissue. The results in Fig. 2 show that the half-life of the enzyme increased from 1 h in control internodes to about 5.5 h in internodes of GA₃-treated plants. A similar increase in the half-life of ADC was reported in cucumber cotyledons treated with benzyladenine (Suresh *et al.* 1978). In several animal systems also, the half-life of ODC is increased by treatments with hormones or growth regulators. These increases corresponding with increases in ODC activity and growth (Clark 1974, Hogan and Murden 1974, Yamasaki and Ichihara 1976). Thus, the increase in half-life of ADC reported here could account for the GA₃-induced increase in ADC activity and internode growth. However, it is not yet clear from these experiments whether the GA₃ induced enhancement of ADC activity is due to increased synthesis, decreased degradation or a combination of the two.

The rapidity and nature of the GA₃-induced increase in ADC activity suggest that there is some linkage between GA₃-induced internode growth, but failure to completely reverse the inhibition with applied PAs makes this conclusion tentative. Nonetheless, the rise in ADC activity may be an important early event in the stimulation of internode elongation by the hormone.

Table 1 Effect of microdrop application of GA₃ on internode growth of light-grown Progress pea seedlings

GA ₃		% increase in internode length	
(μ g/ml)	μ g/plant	4th	5th
0	0	0	0
5	0.2	90	172
10	0.4	68	212
20	0.8	39	134

GA₃ was applied to the terminal bud on day 9 and internode growth measured on day 12.

Table 2 Effect of DFMA on GA₃-induced increase in ADC activity, DNA content and internode growth of light-grown Progress pea seedlings

Treatment	ADC activity pmol CO ₂ /h mg protein	DNA content μ g/internode	Internode length mm
Control	110 $\pm$ 4.2 (100)	5.25 $\pm$ 0.16 (100)	7.2 $\pm$ 0.18 (100)
DFMA	40 $\pm$ 0.5 (36)	5.70 $\pm$ 0.17 (108)	8.1 $\pm$ 0.20 (112)
GA ₃ ^a	198 $\pm$ 7.0 (180)	7.80 $\pm$ 0.18 (148)	21.6 $\pm$ 0.32 (300)
GA ₃ +DFMA	76 $\pm$ 5.4 (69) 59 ^b	5.25 $\pm$ 0.17 (100)	12.2 $\pm$ 0.25 (169) 72 ^b

^a GA₃ concentration is 5 μ g/ml.

^b % inhibition of GA₃-induced increase.

DFMA (1 mM) was applied 1 and 20 h after GA₃ treatment. ADC activity was measured 9 h, while DNA content and internode growth were measured 48 h after GA₃ treatment. Numbers in parenthesis represent percent relative to control.

Table 3 Effect of actinomycin D and cycloheximide on inhibition of GA₃-induced increase in ADC activity

Treatment	Relative ADC activity
Control	100
Act. D, 50 μ g/ml	64
CHI, 50 μ g/ml	75
GA ₃ , 5 μ g/ml	215
GA ₃ +Act. D	123 (49)
GA ₃ +CHI	140 (43)

Enzyme activity was measured 9 h after GA₃ treatment. Numbers in parenthesis represent percent inhibition of GA₃-induced increase.

Fig. 1 Kinetics of GA_3 -induced increase in ADC activity and internode length of light-grown Progress pea seedlings. Nine-day-old seedlings were sprayed either with GA_3 ($10 \mu\text{g/ml}$) or buffer and at different time intervals ADC activity and growth measurements were made on the 4th internode.

Fig. 2 Effect of GA_3 on the half-life of ADC activity. The half-life of ADC was determined in GA_3 -treated and control plants by adding CHI ($50 \mu\text{g/ml}$) and determining residual ADC activity at the stated times.

Fig. 1 Kinetics of GA₃-induced increase in ADC activity and internode length of light-grown Progress pea seedlings. Nine-day-old seedlings were sprayed either with GA₃ (10 μ g/ml) or buffer and at different time intervals ADC activity and growth measurements were made on the 4th internode.

Fig. 2 Effect of GA₃ on the half-life of ADC activity. The half-life of ADC was determined in GA₃-treated and control plants by adding CHI (50 μ g/ml) and determining residual ADC activity at the stated times.

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6. A role for polyamine biosynthetic enzymes in alkaloid formation in tobacco.

Abstract

The putrescine which forms a part of nicotine and other pyrrolidine alkaloids is generally assumed to arise through the action of ornithine decarboxylase (ODC). However, we have previously noted that changes in the activity of arginine decarboxylase (ADC), an alternate source of putrescine, parallel changes in tissue alkaloids, while changes in ODC activity do not. This led us to undertake experiments to permit discrimination between ADC and ODC as enzymatic sources of putrescine destined for alkaloids. Two kinds of evidence presented here support a major role for ADC in the generation of putrescine going into alkaloids: (a) A specific 'suicide inhibitor' of ADC effectively inhibit the biosynthesis of nicotine and nornicotine in tobacco callus, while the analogous inhibitor of ODC is less effective, and (b) the flow of ^{14}C from uniformly labelled arginine into nicotine is much more efficient than that from ornithine.

Introduction

Considerable evidence indicates that polyamines (PAs) and their biosynthetic enzymes arginine decarboxylase, ADC and ornithine decarboxylase ODC may regulate some aspects of plant development, senescence, and response to stress (1,2). PAs may also be metabolized into a variety of secondary plant products (2), through largely unelucidated pathways. Thus, putrescine, formed in the plant via ADC and/or ODC, is not only the precursor of the major PAs spermidine and spermine, but also of such important secondary metabolites as alkaloids. The pyrrolidine rings of tobacco alkaloids (nicotine, nornicotine), tropane alkaloids (hyoscyamine, hyoscine, meteloidine), pyrrolidine alkaloids (retronecine), *Erithroxylum coca* alkaloids (hygrine, cuscohygrine), and probably phenanthroin-

dolizidines (tylophorine) are putrescine derivatives (3,4). Putrescine, spermidine, homospermidine and spermine can also conjugate with cinnamic acids or fatty acids (5,6), and in some cases the conjugates are transformed into more complex alkaloids (7).

We have reported (8) that in tobacco callus grown on high auxin, suboptimal for alkaloids formation, putrescine and spermidine conjugates are the main putrescine derivatives, while in callus grown on or transferred to low auxin, optimal for alkaloid formation, nornicotine and nicotine are the main putrescine derivatives. The results suggested that bound putrescine could act as a pool for alkaloids formation. In addition, we found that changes in ADC but not ODC activity corresponded with altered alkaloids levels (8). This was surprising, since ODC activity has long been invoked to explain the biosynthesis of nicotine (9) and related alkaloids, and in fact, pyrrolidine alkaloids are usually classified as ornithine-derived compounds (3). To help decide between the ADC and ODC pathways, we have used two recently developed specific, enzyme-activated, irreversible inhibitors of ADC, (DFMA) (10) and ODC (DFMO), (11). We have also employed a reversible inhibitor of ADC, D-arginine (D-Arg), and have supplied the callus with the radiolabeled precursors of putrescine, L-[U- ^{14}C]arginine and L-[U- ^{14}C]ornithine, to gain additional evidence on paths of carbon flow.

Materials and Methods

Plant material. *Nicotiana tabacum* L. cv. 'Wisconsin-38' and *Nicotiana tabacum* L. cv. 'Burley-21' plants were grown in plastic pots containing vermiculite, subirrigated twice daily with a 1.2 g/l soln of Hyponex (8) and maintained in a controlled growth room under a 16 hr light/8 hr dark photoperiod (9:1 energy mixture of fluorescent and incandescent light at 1.96 W.m^{-2}) at 24° .

Callus culture and addition of inhibitors. Explants were obtained from plants grown to the 17–20 leaf stage as previously described (8,12). Callus was grown on a modified Murashige-Skoog (17) medium containing 11.5 μ M NAA, and 1 μ M kinetin at 25° in the dark (12). After 4 weeks, the calli were transferred to a medium like described above, but containing 1.5 μ M NAA (optimal for alkaloids formation) (12) in the absence (control) or presence of different concn of inhibitors. Samples for growth measurements and alkaloids analysis were harvested at the sixth week of callus grown on the lower level of NAA.

Alkaloids analysis. Alkaloids were determined according to ref. (18). Samples dried to constant weight at 60° for 24 hr were extracted with 20 vols of 25 mM K-Pi buffer (pH 7.8) at 30° for 24 hr with constant agitation. Alkaloids in the fresh tissue were extracted with 2 ml of 40% (v/v) MeOH containing 0.1% (v/v) HCl and then centrifuged at 500 g for 5 min. Both extracts were filtered through Whatman No. 2 filter paper and a 0.45 μ m Millipore filter, diluted with H₂O, and injected into the HPLC system as a 20 μ l aliquot. HPLC analysis was carried out with a programmable Altex-Beckman model 322 liquid chromatograph, using a 4.6 x 250 mm 5 μ m particle reverse-phase (C₁₈) column (Altex-octadecylsilane) and eluted with an isocratic mobile phase of 40% (v/v) MeOH containing 0.2% phosphoric acid buffered to pH 7.5 with triethylamine, at a flow rate of 1 ml/min at 254 nm. Alkaloids were quantified with a 3370 A Hewlett-Packard integrator using nicotine (K&K Lab) and nornicotine (Roth) as standards.

Administration of the radiolabelled precursors of putrescine to the callus and isolation of nicotine. NTB-21 calli grown for 2 weeks on a low NAA level (1.5 μ M) were aseptically transferred into Petri dishes (100 x 20 mm) with 6 ml of sterile H₂O containing 1 μ Ci of L-[U-¹⁴C]Arg (288 mCi/mmol; ICN) or L-[U-¹⁴C]Orn (266 mCi/mmol; NEN). After 6 hr of incubation in the dark at 30°, each callus was rinsed several times with H₂O in the shaker, dried on a filter paper and weighed. Alkaloids in the fresh tissue were extracted and analysed by HPLC as described above. From the chromatographic eluate aliquots corresponding to nicotine peaks

were taken and dissolved in 4 ml Aquasol (NEN) for measuring radioactivity in a Beckman LS 70000 scintillation counter. Aliquots from the aq. medium were also measured for radioactivity at the start and end of the incubation period to evaluate the uptake by the callus of the two radiolabelled compounds.

Determination of arginase activity. Arginase activity was determined according to ref. (19) with few modifications. NTB-21 calli (3 g) were ground in 3 ml 0.1 M Tris-HCl buffer (pH 7.0) with 0.5% polyvinyl pyrrolidone, purified as described elsewhere (8), in chilled mortars. The extract was centrifuged at 15 000 g for 20 min and the supernatant fraction was used as the enzyme source. The reaction mixture contained 33 mM L-Arg (pH 9.6); 0.3 mol MnCl_2 ; glycine-NaOH buffer (pH 9.6); and enzyme preparation in a total volume of 1.5 ml. The mixture was incubated at 37° for 60 min. The reaction was stopped by adding 1 ml 10% (w/v) TCA and the protein was removed by centrifugation. A suitable aliquot of the supernatant fraction was used for urea estimation as described in ref. (19). Urea standards were incubated with each set of assays. To evaluate possible interference, extracts of known amounts of bovine liver arginase (Sigma) were also added and the mixture analysed for enzyme activity.

Results and Discussion

Two different strains of tobacco callus were used in this study. One (NTW-38), derived from *Nicotiana tabacum* 'Wisconsin-38', contains nornicotine as the major alkaloids (12), and a second (NTB-21), derived from *N. tabacum* 'Burley-21' plants, has nicotine as the main alkaloids. Table 1 and Fig. 2 compare the growth of the NTW-38 strain callus in the absence (control) and presence of inhibitors. Addition to the medium of 0.1–3 mM DFMA promoted growth as compared with the control, while DFMO at similar concentrations inhibited callus growth (see also Table 1) and at 10 mM was severely toxic. Combination of the two inhibitors at 1–3 mM (Fig. 2), also produced inhibition of callus growth, apparently due to a predominant action of DFMO. As with DFMA, addition to the medium of 10 mM D-Arg also promoted the growth of NTW-38 strain callus (Table 1).

While these observations suggested that the ODC pathway is important for cell division and growth, the effects of the inhibitors on alkaloids biosynthesis show that ADC is more important in synthesis of pyrrolidine alkaloids. In the NTB-21 callus strain, addition to the medium of 0.1 and 1.0 mM DFMA resulted in calli with respectively, 40% and 60% less nicotine than the control (Table 1). In contrast, 0.1 and 1.0 mM DFMO produced no significant reduction of alkaloids levels in the NTW-38 callus strain; DFMO was also less effective than DFMA in reducing nicotine levels in the NTB-21 callus strain. As with DFMA, addition to the medium of 0.1–10 mM D-Arg was more effective than DFMO in depressing nicotine and nornicotine levels in the two callus strains (Table 1).

To elucidate further the preferential participation of ADC in the biosynthesis of pyrrolidine tobacco alkaloids, NTB-21 calli were fed with ^{14}C -labelled precursors of putrescine. After 6 hr of incubation the calli incorporated 15 and 17% respectively of the initial cpm fed as ^{14}C -arginine and ^{14}C -ornithine, showing therefore similar uptake of the label from the two compounds. However, callus fed with ^{14}C -arginine incorporated more of this label into nicotine than callus fed

with ^{14}C -ornithine. As indicated in Table 2, the incorporation ration for ^{14}C -arginine was about 3-fold higher than for ^{14}C -ornithine and the callus accumulated more nicotine than callus fed with ^{14}C -ornithine.

Interconversions between arginine and ornithine are illustrated in Fig. 1. In tissues with high arginase activity, arginine could be converted into ornithine and DFMA into DFMO, as recently observed (R.D. Slocum, unpublished). To test this possibility, the arginine levels of the NTB-21 callus strain were analysed. Neither detectable arginase activity nor interference with added bovine liver arginase activity were found in calli.

From the above results, we concluded that in tobacco callus, ODC is important for growth, while ADC appears to be involved in furnishing putrescine for pyrrolidine alkaloids biosynthesis (see Fig. 1, pathway I). This is in agreement with the view (2) that there appears to be a different compartmentation of the two biosynthetic enzymes for putrescine in higher plants. ODC appears to be mainly located in the nuclei and thus may regulate cell division in actively growing tissues, while ADC appears to be located in the cytosol, probably regulating cell extension and secondary process (R.D. Slocum, unpublished).

The hypothesis that ODC is the main enzyme involved in nicotine biosynthesis is based on equivocal decapitation experiments with tobacco (9,13). Following decapitation of the stems, both ODC activity and nicotine levels increased in the roots and these effects were attributed to a decrease of auxin levels in the roots (9). However, in the roots of non-decapitated plants, ADC was 4.-fold higher than ODC activity (13), and the roots of these plants also produced alkaloids. Thus, the primary role of ODC in nicotine biosynthesis remains questionable, since effects other than decreased auxin levels in the roots can result from decapitation of the plant. Yoshida (14,15) demonstrated that putrescine can be formed from either arginine or ornithine in tobacco, and that tobacco roots fed with radiolabelled arginine readily incorporated it into nicotine (16). However, from these experiments, the authors could not conclude which of the

two pathways predominated. Our observations that: (i) specific inhibitors of ADC are more effective than an inhibitor of ODC in inhibiting alkaloid formation; and (ii) label from arginine is more efficiently incorporated into nicotine than label from ornithine suggest that ADC, not ODC, is the most important enzyme in the biosynthesis of putrescine-derived tobacco alkaloids.

Table 1. Effect of the inhibitors on fresh weight and alkaloid levels in tobacco callus

Treatment	NTW-38 callus strain		NTB-21 callus strain	
	Fresh weight (g)	Nornicotine ($\mu\text{mol/g}$ dry weight)	Fresh weight (g)	Nicotine ($\mu\text{mol/g}$ dry weight)
Control	2.2 ± 0.04 (100)	85 ± 5 (100)	1.3 ± 0.09 (100)	288 ± 11 (100)
DFMO (0.1 mM)	1.5 ± 0.04 (69)**	67 ± 9 (79)	1.1 ± 0.04 (85)	199 ± 32 (69)
DFMO (1 mM)	1.4 ± 0.14 (62)*	79 ± 14 (93)	0.9 ± 0.09 (69)	167 ± 25 (58)*
DFMO (10 mM)	0.3 ± 0.08 (12)**	N.T.	N.T.	N.T.
DFMA (0.1 mM)	2.5 ± 0.07 (114)	N.T.	1.4 ± 0.03 (108)	173 ± 11 (60)*
DFMA (1 mM)	3.1 ± 0.07 (137)**	N.T.	1.5 ± 0.21 (115)	97 ± 6 (33)**
D-Arg (0.1 mM)	N.T.	N.T.	1.3 ± 0.10 (99)	175 ± 4 (61)*
D-Arg (1 mM)	2.4 ± 0.04 (107)	50 ± 6 (59)*	1.3 ± 0.25 (104)	93 ± 7 (32)**
D-Arg (10 mM)	2.6 ± 0.04 (116)*	44 ± 4 (52)*	N.T.	N.T.

Numbers represent means $\pm$ s.e.m. of at least three replicates. Figures in parentheses indicate per cent relative to control. Significantly different from control at the 5% (*) or 1% (**) levels. N.T. = not tested.

Table 2. Incorporation of the radiolabelled precursors of putrescine into nicotine in the NTB-21 callus strain

Precursor	Incorporation ratio†	dpm/ μmol nicotine	Nicotine ($\mu\text{mol/g}$ fresh weight)
L-[U- ^{14}C]Arginine	1.4	3900 ± 440 *	7.1 ± 0.9
L-[U- ^{14}C]Ornithine	0.5	1920 ± 140	4.6 ± 0.8

Numbers represent means $\pm$ s.e.m. of three replicates.

*Significantly different at the 5% level.

†Ratio in percentage of specific radioactivity of the nicotine to that of precursor fed.

Fig. 1. Schematic pathway for biosynthesis of PA and putrescine-derived alkaloids. Note that only some enzymes are indicated.

Fig. 2. Effect of the inhibitors alone or in combination on growth of the NTW-38 callus strain.

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7. Polyamine metabolism and osmotic stress in protoplasts.

I. Relation to Protoplast viability

Abstract

Cereal leaves subjected to the osmotica routinely used for protoplast isolation show a rapid increase in arginine decarboxylase activity, a massive accumulation of putrescine, and slow conversion of putrescine to the higher polyamines, spermidine, and spermine (HE Flores, AW Galston 1984 Plant Physiol 75: 102). Mesophyll protoplasts from these leaves, which have a high putrescine:polyamine ratio, do not undergo sustained division. By contrast, in *Nicotiana*, *Capsicum*, *Datura*, *Trigonella* and *Vigna*, dicot genera that readily regenerate plants from mesophyll protoplasts, the response of leaves to osmotic stress is opposite to that in cereals. Putrescine titer as well as arginine and ornithine decarboxylase activities decline in these osmotically stressed dicot leaves, while spermidine and spermine titers increase. Thus, the putrescine:polyamine ratio in *Vigna* protoplasts, which divide readily, is 4-fold lower than in oat protoplasts, which divide poorly. We suggest that this differing response of polyamine metabolism to osmotic stress may account in part for the failure of cereal mesophyll protoplasts to develop readily *in vitro*.

Introduction

Regeneration of entire plants from mesophyll protoplasts of *Gramineae* is still difficult (4), with only one authenticated report in the literature (21). By contrast, in the *Solanceae* (3) or *Leguminosae* (11, 13, 14), many species of plants have been successfully regenerated from protoplasts.

A decade ago, when we observed that PAs² could retard the rapid senescence and lysis of oat protoplasts (1, 8), we initiate a detailed study of the role of PAs in plant cells (16). We were surprised to find that the Put content of mesophyll protoplasts of oat is 5 to 10 times higher than that of the leaves from which they were derived. Later work showed that cereal leaves subjected to 0.4 to 0.6 M sorbitol exhibit a rapid increase in ADC activity, a massive accumulation of Put, and very slow changes in the higher PAs, Spd, and Spm (5, 6), which are normally formed from Put.

In the present investigation, we have studied the effect of osmotic stress in tobacco and other species that can be readily regenerated from protoplast. The results indicate that tobacco response differs from that observed previously for cereals during osmotic stress (18). To establish whether this osmotic stress response is a genral phenomenon, PA titers of two other solanaceous (*Capsicum* and *Datura*) and legume (*Trigonella* and *Vigna*) genera were analyzed in osmotically stressed leaves. Finally, we compared the Put and PA contents of protoplasts of species yielding poorly dividing protoplasts with those of species yielding readily dividing protoplasts.

Materials and Methods

Plant Materials. The following plant species were used: *Nicotina tabacum* cv Wisconsin 38 (tobacco); *Capsicum annuum* var. California Wonder (pepper); *Datura stramonium* var Chilibea; *Trigonella foenum-graecum* L.; *Vigna aconitifolia* (jacq.) Marechal var Jadia (moth bean); *Avena sativa* cv Victory (oat); and *Pisum sativum* var Progress (dwarf pea). Seeds were sown in vermuculite in plastic pots which were subirrigated twice daily with a 1.2 g/l solution of Hyponex (7-6-19 by N:P:K: analysis, Hydroponics Chemical Co., Copley, OH) and grown in a controlled chamber under a 16-h light/8-h dark photoperiod (9:1 energy mixture of fluorescent and incandescent light at 17.6 W. m⁻²) at 24°C.

Osmotic Treatment of the Excised Leaves. Median leaves from about 2-month-old tobacco, pepper and *Datura* or about 1-month-old *Trigonella* or moth bean or the first leaf of 8-d-old oat seedlings were excised. The lower epidermis was peeled off, and leaf discs (2 cm diameter) or segments (5 cm length) were floated in the dark, unless otherwise indicated, at room temperature over 10 ml 1 mM K-phosphate (pH 5.8) contained in a 100 x 15 mm Petri dish, in the absence (control) or presence of osmotica (0.6 M sorbitol) for various incubation times.

Preparation of Protoplasts. Leaf mesophyll protoplasts from seedlings of oat, moth bean, dwarf pea, and tobacco were isolated under nonaseptic conditions. After removal of the lower epidermis, the leaves were floated in the dark at 30°C, stripped side down, over the appropriate digestion medium. Oat leaves were digested for 3 h over 0.5% Cellulysin in 0.5 M mannitol (pH 5.8) (12). Moth bean leaves were digested over 1% purified Driselase in 3.1 mM Mes buffer (pH 5.8) with 0.5 M mannitol and additives (4). Dwarf-pea leaves were digested for 4 h over 0.75% Cellulase (Onozuka R-10, Kinki Yakut Manufacturing Co.) in 3.1 mM Mes buffer (pH 5.8) with 0.5 M mannitol and additives as in moth bean, except for CaCl_2 , whose final concentration was 2 mM. Tobacco leaves were digested for 5 h over 2% purified Cellulase + 2% unpurified Pectolyase Y-23 in 3.1 mM Mes buffer (pH 5.8) with 0.5 M mannitol and additives as in moth bean, except for CaCl_2 , whose final concentration was 6.4 mM (N Shehawat, personal communication). Digestion of the leaves was followed by gentle shaking at room temperature for 15 to 60 min to release the protoplasts. The released protoplasts were collected by centrifugation at 100 g for 6 min and rinsed once with the appropriate washing medium. For oat protoplasts the washing medium consisted of B-5 medium plus additives (12). For moth bean, dwarf pea and tobacco protoplasts, the wash medium was the same as the digestion medium, but without Driselase and with a higher concentration of 6 mM CaCl_2 (4). The final pellet was resuspended in 21% sucrose solution (pH 5.8) containing the same salts as the

wash medium. The suspension was then covered by a small layer of wash medium and centrifuged again at 100 g for 6 min. The intact protoplasts, concentrated at the interface, were taken up gently with a broadtipped Pauster pipette and washed once again. For PA determination, the protoplast pellet remaining after the wash with sucrose-containing medium was resuspended in an excess of a salt solution containing 2.5% KCl and 1% $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (12) and centrifuged to remove the sucrose. This is necessary, since dansylated sugars interfere with dansyl-PAs in the TLC separation. The pellet obtained after 6 min centrifugation at 100 g was resuspended in a small volume of salt solution and the number of protoplasts determined with a hemocytometer. More salt solution was added to the suspension and the protoplasts were pelleted again by centrifugation. The final pellet was used for analysis of PAs.

Polyamine Analysis. Leaf-samples (previously washed with distilled H_2O and gently blotted on filter paper) were extracted by homogenizing in 5% (v/v) cold PCA at 100 mg fresh weight/5% PCA in ratio varying according to the species. The homogenates were kept on ice 30 to 60 min before centrifugation at 27,000 for 20 min. The supernatant was set aside and the pellet was resuspended in the original volume of 1 N NaOH by vortexing. Aliquots (200 μl each) of the pellet suspension and the original supernatant were mixed 1:1 (v/v) with 12 N HCl and hydrolyzed as previously described (19). The filtered hydrolysates (19) were resuspended in 200 μl PCA. The nonhydrolyzed PCA supernatant containing the free, soluble PAs(S), the hydrolyzed PCA supernatant (SH), and the hydrolyzed pellet (PH) containing PAs liberated from conjugates were dansylated and chromatographed as previously described (19, 22). Therefore, unless otherwise indicated, data on total PA titers represent the sum of the free (S-fraction) and PCA-soluble PAs (SH-fraction). Dansyl-PAs were separated on high-resolution silica gel TLC plates (Whatman LK6D). After development of the plates, bands were scraped into 2 ml of ethyl acetate and the fluorescence at 495 nm determined in an Aminco spectrophotofluorimeter with an activating wavelength of

350 nm.

Protein Analysis. Protein was determined according to the method of Bradford (2) in the insoluble PCA-pellet resuspended in 1 N NaOH. Bovine γ -globulin (Sigma) was used as a standard.

Determination of ADC and ODC Activities. Samples were ground in chilled mortars in a ratio of 100 mg fresh weight/ml of 100 mM K-phosphate (pH 7.5) containing 10 mM DTT, 20 mM sodium ascorbate, 5 mM EDTA, and 1 mM pyridoxal phosphate. Purified insoluble PVP was added to the extract at 0.5g wet weight/g tissue to absorb phenolics (19). The extract was sonicated as previously described (19) and pelleted for 20 min at 27,000 g. The supernatant fraction was made 60% saturated with $(\text{NH}_4)_2\text{SO}_4$ allowed to stand for 30 min, then centrifuged for 20 min at 27,000 g. The resulting pellet was resuspended in the original volume of 100 mM K-phosphate (pH 8.0) containing 1 mM DTT, 0.1 EDTA, and 0.05 mM pyridoxal phosphate, and dialyzed against the same buffer for 24 h in the dark (19). The activities of ADC and ODC were determined as described elsewhere (6, 22). For assay of ADC activity, the labeled substrate was 20 $\mu\text{Ci/ml}$ L-[U- ^{14}C]arginine (276 mCi/mmol; ICN) diluted with unlabeled arginine to give a final concentration of 10 mM. For assay of ODC activity, the labeled substrate consisted of 20 $\mu\text{Ci/ml}$ DL-[1- ^{14}C]ornithine (58 mCi/mmol; NEN) diluted with unlabeled ornithine to give a final concentration of 50 mM. Reaction mixtures were incubated for 45 min with gentle shaking at 37°C, at which time the reaction was stopped by adding 0.2 ml of 10% (v/v) PCA. Trapping of the labeled CO_2 onto the KOH-impregnated collection disc continued for 45 min at 37°C. The discs were then removed and immersed in 2 ml of Betafluor (NEN). The radioactivity liberated was determined by counting for 10 min in a Beckman LS 7000 scintillation counter. Enzyme was expressed as nmol $^{14}\text{CO}_2$ released/h/ mg protein.

Results

Osmotic Stress in Tobacco Leaves. Peeled tobacco leaves were floated over buffer (pH 5.8), in the absence (control) or in the presence of 0.6 M sorbitol and incubated in the dark (control) or presence of 0.6 M sorbitol and incubated in the dark for various times. PA titers (free and PCA-soluble or insoluble conjugates) and ADC and ODC activities were determined. In tobacco leaves, Spd and Put are the major PAs, while Spm is found only in small amounts (Fig. 1; Table V). Putrescine and Spd are present mainly in the PCA-soluble conjugate form (about 70% of the total); and Spm is equally distributed into the PCA-soluble fraction (free and bound; S- and SH- fractions). Only trace amounts of PCA-insoluble conjugated (PH-fraction) PAs were found.

Figure 1a shows that Put levels in control tobacco remained higher than in osmotically stressed leaves during the incubation. Put titers increased during the first 4 h, then declined to their lowest values after 6 h, and then increased again after 8 h incubation in both control and osmotically stressed leaves. In contrast, Spd and Spm leaves increased during osmotic treatment, reaching their maximum difference with control after 6 h of incubation (Fig. 1, b and c). The changes occurring in Put titers during osmotic stress were produced mainly in the free fraction, while the osmotically induced changes in Spd and Spm titers occurred mainly in the PCA-soluble conjugate fraction (data not shown). Thus, the ratio of Put to Spd + Spm in tobacco leaves decreased during osmotic stress, reaching its lowest value after 6 h (Fig. 1d; Table V). This response is opposite from that observed previously for cereal leaves (5, 6).

In contrast with cereals (5, 6), ADC activity of tobacco leaves does not increase after exposure to osmotic stress. ADC activity decline after 4 h of osmotic treatment and then remained constant (Fig. 2a). ODC activity also declined after 4 h, but then increased after 8 h in control and osmotic-stressed leaves (Fig. 2b).

Polyamines and Osmotic Stress in Pepper and *Datura* Leaves. Peeling the leaf had no effect on Put content in pepper, but decreased Spd titer by about 20% (Table I). In contrast with tobacco, pepper leaves contained Put as the main PA (see also Table V). Spermine titers were very low and Put was found mainly (about 80% of the total) in the form of PCA-soluble conjugates, as in tobacco leaves. However, Spd and Spm in pepper leaves were present mainly in the free PA fraction (70 and 65% of the total, respectively). Table I shows that, as in tobacco but unlike cereal, Put titers in pepper decreased during osmotic treatment while Spd and Spm titers increased in both fractions analyzed (S- and SH-fractions). As in tobacco, the maximum difference from the control occurred after 6 h of incubation in 0.6 M sorbitol. In this case, the osmotically induced changes of PA titers were produced mainly in the PCA-soluble conjugate fraction (Table I).

In *Datura*, we studied the effect on PA titers of incubating peeled leaves on 0.6 M sorbitol in dark or light. Table II shows that *Datura* leaves contained Put as the main PA, but unlike tobacco and pepper, contained Spm in relatively high quantities, approximately equal to Spd. Light did not affect the decrease in Put and increase in Spd and Spm after 6 h (Table II). Light did reduce somewhat the osmotically induced decrease of Put, and it reversed the relative rise in Spd *versus* Spm.

Polyamines and Osmotic Stress in *Trigonella* and Moth Bean Leaves. The response of PA leaves to osmotic stress in two leguminous plants which can be regenerated from protoplasts (13, 14) was investigated. Peeled leaves of *Trigonella* were floated in the dark over buffer in the presence or absence of osmoticum and PA titers analyzed after 6 h. Spd was the major PA, followed by Put and Spm (32 and 22% of the total, respectively (Table III). Putrescine and Spd were presented mainly in the PH-fraction, while Spm was concentrated mainly in the S-fraction. In *Trigonella*, after 6 h of osmotic treatment, Put titers did not increase but Spm titers increase slightly over the 6-h control (Table III).

In moth bean leaves, Spd was also the main PA, followed by Spm and Put (36 and 32% of the total, respectively) (Table IV; Table V). Polyamines were present mainly in the SH-fraction. When osmotically stressed, peeled moth bean leaves did not exhibit increased Put titers, but Spm titers increased as compared with the 6-h control (Table IV). As in *Trigonella*, osmotically stressed moth bean leaves showed an increase of Spm titers of more than 2-fold when compared with 0-h control.

Effect of Osmotic Stress on the Put/Polyamine Ratios. Table V shows the relative distribution of PAs as well as the Put/Spd + Spm ratios in various control or osmotically stressed peeled leaves. After 6 h incubation in buffer, Put titers increased in tobacco and remained almost unchanged in pepper, *Trigonella*, and moth bean leaves; spermidine titers decreased in tobacco and *Trigonella* and increased in pepper; and Spm titers decreased in pepper and increased in *Trigonella* (Table V). After 6 h of incubation in osmotica, Put titers decreased in all these dicotyledonous leaves; Spd titers (except in *Trigonella*) increased; Spm titers also increased. As a consequence of these changes, the diamine/polyamine (Put/ Spd + Spm) ratio decreased in all osmotically stressed dicot leaves. This phenomenon occurred in all the PA fractions analyzed (Tables II and III). Although for *Trigonella* and moth bean the differences are small, the ratio does not increase as in oat leaves (Table V).

Polyamine Titters in Oat and Moth Bean Protoplasts. Table VI shows the PA titers of protoplast isolated in the presence of osmotica from peeled oat and moth bean leaves. In oat protoplasts, Put was the major PA (66% of the total) and Spd and Spm represent 29 and 5% of the total, respectively. In contrast, in moth bean protoplasts Spm was the main PA (about 45% of the total) and Put and Spd represented 32 and 22% of the total, respectively. As a consequence of these differences, the Put/PA ratio in moth bean protoplast was 4-fold lower than in oat protoplast (Table VI).

Table I. Polyamine Titrers and Osmotic Stress in Pepper Leaves

Peeled leaf discs were floated over buffer or buffer + 0.6 M sorbitol in the dark and samples were analyzed for PAs after 4 h and 6 h of incubation. Numbers represent means $\pm$ SE of three replicates.

Treatment	Time of Incubation	PA Fraction	Put	Spd	Spm
	<i>h</i>		<i>nmol/mg protein</i>		
Control ^a	0	S ^b	0.76 $\pm$ 0.01	1.75 $\pm$ 0.16	ND ^c
		SH	4.10 $\pm$ 0.21	0.61 $\pm$ 0.02	ND
		Total	4.87 $\pm$ 0.09	2.3 $\pm$ 0.02	ND
Control	0	S	0.87 $\pm$ 0.24	1.25 $\pm$ 0.40	0.39 $\pm$ 0.10
		SH	3.90 $\pm$ 0.5	0.5 $\pm$ 0.18	0.22 $\pm$ 0.06
		Total	4.75 $\pm$ 0.24	1.8 $\pm$ 0.13	0.61 $\pm$ 0.06
Control	4	S	1.30 $\pm$ 0.14	1.10 $\pm$ 0.14	0.29 $\pm$ 0.12
		SH	2.40 $\pm$ 0.2	1.05 $\pm$ 0.11	0.16 $\pm$ 0.07
		Total	3.65 $\pm$ 0.14	2.16 $\pm$ 0.11	0.45 $\pm$ 0.04
0.6 M Sorbitol	4	S	1.13 $\pm$ 0.09	1.78 $\pm$ 0.2	0.64 $\pm$ 0.15
		SH	2.02 $\pm$ 0.10	1.75 $\pm$ 0.4	0.56 $\pm$ 0.11
		Total	3.15 $\pm$ 0.02 ^d	3.53 $\pm$ 0.38 ^d	1.20 $\pm$ 0.11 ^d
Control	6	S	1.10 $\pm$ 0.13	0.85 $\pm$ 0.10	0.19 $\pm$ 0.02
		SH	2.27 $\pm$ 0.24	0.85 $\pm$ 0.09	0.15 $\pm$ 0.03
		Total	3.36 $\pm$ 0.13 ^d	1.72 $\pm$ 0.09	0.35 $\pm$ 0.02 ^d
0.6 M Sorbitol	6	S	1.01 $\pm$ 0.10	1.40 $\pm$ 0.17	0.53 $\pm$ 0.11
		SH	1.70 $\pm$ 0.15 ^d	1.56 $\pm$ 0.21 ^d	0.41 $\pm$ 0.10
		Total	2.7 $\pm$ 0.13 ^e	3.00 $\pm$ 0.17 ^d	0.95 $\pm$ 0.11

^a Unpeeled control leaves. ^b S, Free, PCA-soluble PAs; SH, PCA-soluble conjugated PAs released by hydrolysis. ^c ND, not determined. ^d Significantly different from 0-h control (peeled leaves) at $P < 0.05$. ^e Significantly different from 0-h control (peeled leaves) at $P < 0.01$.

Table II. Polyamine Titrers and Osmotic Stress in *Datura* Leaves

Peeled leaf discs were floated over buffer or buffer + 0.6 M sorbitol in the dark or under light and samples were analyzed for free PAs (S-fraction) after 6 h of incubation. Numbers without $\pm$ SE are the mean value of two analyses.

Treatment	Time of Incubation	Put	Spd	Spm	Put/Spd + Spm
	<i>h</i>	<i>nmol/mg protein</i>			
Control	0	1.20 $\pm$ 0.06	0.40	0.40 $\pm$ 0.03	1.5
Control (dark)	6	2.00 $\pm$ 0.50	0.40 $\pm$ 0.04	0.30 $\pm$ 0.01	2.8
0.6 M Sorbitol	6	1.20	0.45 $\pm$ 0.05	0.40 $\pm$ 0.05	1.2
Control (light)	6	1.70 $\pm$ 0.1 ^a	0.50 $\pm$ 0.04	0.33 $\pm$ 0.03	2.0
0.6 M Sorbitol (light)	6	1.40	0.63 $\pm$ 0.10	0.47 $\pm$ 0.01	1.2

^a Significantly different from 0-h control at $P < 0.05$.

Table III. Polyamine Titrers and Osmotic Stress in *Trigonella foenum-graecum* leaves

Peeled leaf discs were incubated in the dark for 6 h as indicated in Table I.

Treatment	Time of Incubation	PA Fraction	Put	Spd	Spm	Put/Spd + Spm
	<i>h</i>					
Control	0	S ^a	0.32 $\pm$ 0.05	1.51 $\pm$ 0.03	1.08 $\pm$ 0.01	0.4
		SH	0.90 $\pm$ 0.11	1.13 $\pm$ 0.15	0.30 $\pm$ 0.05	0.6
		PH	2.35 $\pm$ 0.45	2.34 $\pm$ 0.30	0.92 $\pm$ 0.04	0.7
		Total	3.57 $\pm$ 0.44	5.00 $\pm$ 0.04	2.30 $\pm$ 0.09	0.5
Control	6	S	0.55 $\pm$ 0.06	1.65 $\pm$ 0.10 ^b	1.05 $\pm$ 0.06	0.2
		SH	1.55 $\pm$ 0.20	2.25 $\pm$ 0.08 ^b	1.30 $\pm$ 0.03 ^b	0.4
		PH	1.85 $\pm$ 0.26	0.69 $\pm$ 0.10 ^b	2.07 $\pm$ 0.10 ^c	0.7
		Total	4.00 $\pm$ 0.17	4.65 $\pm$ 0.25 ^b	4.50 $\pm$ 0.20 ^c	0.4
0.6 M Sorbitol	6	S	0.44 $\pm$ 0.69	1.00 $\pm$ 0.10 ^b	1.35 $\pm$ 0.20	0.2
		SH	0.69 $\pm$ 0.06	1.60 $\pm$ 0.25	2.15 $\pm$ 0.25 ^b	0.2
		PH	1.45 $\pm$ 0.10	0.65 $\pm$ 0.10 ^b	1.91 $\pm$ 0.30	0.5
		Total	2.60 $\pm$ 0.05	3.30 $\pm$ 0.50	5.40 $\pm$ 0.11 ^c	0.3

^a S, Free, PCA-soluble PAs; SH, PCA-soluble conjugated PAs released by hydrolysis; PH, PCA-insoluble conjugated PAs. ^b Significantly different from 0-h control at $P < 0.05$. ^c Significantly different from 0-h control at $P < 0.01$.

Table IV. Polyamine Titrers and Osmotic Stress in Moth Bean Leaves
Peeled leaf discs were incubated in the dark for 6 h as indicated in Table I.

Treatment	Time of Incubation h	PA Fraction	Put	Spd	Spm
Control	0	S*	0.14	0.41	0.35
		SH	0.23	0.71	0.62
		Total	0.57	1.12	0.97
Control	6	S	0.18 ± 0.02	0.55 ± 0.03	0.39 ± 0.04
		SH	0.62 ± 0.03	0.87 ± 0.10	0.80 ± 0.12
		Total	0.80 ± 0.02	1.42 ± 0.20	1.19 ± 0.10
0.6 M Sorbitol	6	S	0.17 ± 0.02	0.63 ± 0.04	0.86 ± 0.05 ^b
		SH	0.54 ± 0.12	0.85 ± 0.03	1.44 ± 0.07 ^b
		Total	0.71 ± 0.10	1.48 ± 0.06	2.30 ± 0.07 ^b

*S, PCA-soluble PAs; SH, PCA-soluble conjugated PAs released by hydrolysis. ^bSignificantly different from 6-h control at P < 0.01.

Table V. Relative Distribution of Polyamines (S + SH Fractions) and Put/Spd + Spm in Various Dicot Leaves, as Compared with Oat as a Representative Monocot

Peeled leaf discs or segments were incubated in the dark as indicated in Table I.

Peeled Leaf of	Treatment	Put	Spd	Spm	Put/Spd + Spm
% relative to total Put + PA (100%)					
Tobacco	0-h Control	36	59	5	0.6
	6-h Control	44	51	5	0.8
	6-h 0.6 M Sorbitol	19	73	8	0.2
Pepper	0-h Control	66	25	9	1.9
	6-h Control	62	32	6	1.7
	6-h 0.6 M Sorbitol	41	45	14	0.7
Trigonella	0-h Control	24	50	26	0.3
	6-h Control	25	47	28	0.3
	6-h 0.6 M Sorbitol	16	36	48	0.2
Moth bean	0-h Control	23	41	36	0.3
	6-h Control	24	41	35	0.3
	6-h 0.6 M Sorbitol	16	33	51	0.2
Oat	0-h Control	15	47	38	0.2
	6-h Control	17	46	37	0.2
	6-h 0.6 M Sorbitol	55	27	18	1.2

Table VI. Polyamine Titrers in Freshly Isolated Protoplasts from Peeled Oat and Moth Bean Leaves

Oat protoplasts were isolated from primary leaves of 7-d-old seedlings. Moth bean protoplasts were isolated from seedlings 8 and 12 d old in experiments 1 and 2, respectively.

Protoplasts of	Experiment No.	Put	Spd	Spm	Put/Spd + Spm
nmol/mg protein					
Oat	1	17.60	9.50	1.42	1.60
		(62) ^a	(33)	(5)	
	2	18.60	6.81	1.30	2.30
		(70)	(25)	(5)	
Moth bean	1	2.79	1.91	4.08	0.46
		(32)	(22)	(46)	
	2	2.22	1.44	3.00	0.50
		(33)	(22)	(45)	

^a Relative distribution of the PAs as % (total Put + PA is 100%).

FIG. 1. Osmotic stress and polyamine titers in tobacco leaves. Peeled leaf discs were floated over buffer (●) or buffer + 0.6 M sorbitol (○) in the dark. a, Putrescine; b, spermidine; c, spermine; d, Put/polyamine (Spd + Spm) ratio. Values are the sum of the free and PCA-soluble conjugate polyamines. Bars represent $\pm$ SEM. Points without bars are the mean value of two analyses.

FIG. 2. Osmotic stress and arginine and ornithine decarboxylase activities in tobacco leaves. Samples were taken at the indicated times and assayed for enzyme activity. a, Arginine decarboxylase; b, ornithine decarboxylase. Points represent the mean value of two analyses.

Discussion

We reported previously that mesophyll cells of cereals (oat, barley, corn and wheat) exposed to the osmotica used routinely in protoplast isolation exhibit a rapid and massive increase in Put, with minor and slow changes in Spd and Spm. The accumulation of Put results from *de novo* synthesis of ADC (5, 7). In this study we report that leaves of tobacco and other dicots, which readily regenerate from leaf mesophyll protoplast, show no such reaction to osmotic stress. In fact, Put titers decline in osmotically stressed tobacco leaves, while Spd and Spm titers increased. Thus, osmotic stress caused a 4-fold decrease of the Put/polyamine ratio in tobacco leaves in relation to the 6-h control, contrasting with the 6-fold increase of the same ratio in oat (Table V). The activities of the Put biosynthetic enzymes (ADC and ODC) also remained higher in control tobacco leaves which also contrasted with the 2-fold increase of ADC induced by osmotic stress in oat leaves (6).

The increase of higher polyamine Spd and Spm by osmotic shock in dicots contrasted with a gradual decline of Spd and Spm titers during the initial 4 h incubating oat leaves of Spd and Spm titers during the initial 4 h of incubating oat leaves on osmotica (6). The response (as indicated in tobacco and pepper) is rapid and peaks at 4 h of osmotic treatment. An eventual but not significant rise of Spd was observed in osmotically stressed oat leaves after 6 to 10 h under light (6), but decreased Spd and Spm levels were observed in the dark (Table V). A decrease Put/Spd + Spm ratio occurred in all osmotically stressed dicot leaves.

We have shown that the high Put/polyamine ratio in leaves and protoplast of species which yield poorly dividing protoplast contrasts with the low ratio in leaves and protoplast of species which yield readily dividing protoplasts. This is interesting since many investigators point to an important role of the higher PAs (Spd and Spm) in DNA synthesis and mitosis (review in Heby (10)). On the other hand, high concentration of exogenously applied or endogenous Put appear toxic for the plant (9, 15-17). Therefore, the results of this investigation suggest that

the difference in response to osmotic stress might be related to failure of cereal mesophyll protoplast to divide *in vitro*. Additional data supporting such a hypothesis are presented in the following paper (20).

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II. Improvement of Oat Protoplasts by an Inhibitor of Arginine Decarboxylase.

Abstract

We have attempt to improve the viability of cereal mesophyll protoplast by pretreatment of leaves with DL- α -difluoromethylarginine (DFMA), a specific 'suicide' inhibitor of the enzyme (arginine decarboxylase) responsible for their osmotically induced putrescine accumulation. Leaf pretreatment with DFMA before a 6h osmotic shock caused a 45% decrease of putrescine and a 2-fold increase of spermine titer. After 136 hours of osmotic stress, putrescine titers in DFMA-pretreated leaves increased by only 50%, but spermidine and spermine titers increased dramatically by 3.2- and 6-fold, respectively. These increases in higher polyamine could account for the reduced chlorophyll loss and enhanced ability of pretreated leaves to incorporate triated thymidine, uridine, and leucine into macromolecules. Pretreatment with DFMA significantly improved the overall viability of the protoplasts isolated from these leaves. The results support the view that the osmotically-induced rise in putrescine and blockage of its conversion to higher polyamine may contribute to the lack of sustained cell division in cereal mesophyll protoplast, although other undefined factors must also play a major role.

Introduction

In the preceding study (27), we have shown that the osmotically induced Put accumulation in oats, which yield poorly dividing protoplast, contrasts with the low Put content in species which yield readily dividing protoplasts. Moreover, osmotically stressed leaves of species which yield protoplasts capable of sustained growth show increased Spd and/or Spm levels, in contrast with the decrease of these higher PAs in osmotically stressed oat leaves (27). Since Spd and Spm are essential for cell division (11, 23), while Put accumulation is toxic to certain cells (9, 22, 24), we have suggested that this difference in response to osmotic stress may be one of the reasons for failure of cereal mesophyll protoplasts to divide *in vitro* (27).

To test this hypothesis, we have attempted to prevent the accumulation of Put in oat protoplast by pretreating oat leaves with DL- α -difluoromethylarginine (DFMA), an irreversible inhibitor of the enzyme (ADC) responsible for the osmotically induced Put rise in cereal (5). We have estimated the short- and long-term effects of the pretreatment with DFMA on PA levels and macromolecular synthesis in osmotically shocked oat leaves and on the viability and development of protoplasts isolated from these leaves.

Materials and Methods

Plant Material Pretreatment with DFMA. Seeds of *Avena sativa* cv Victory were sown in vermiculite in plastic pots and allowed to germinate and develop in a controlled growth room as detailed in the preceding report (27). Four-day-old seedlings (about 50 plants/pot) were sprayed, unless otherwise indicated, for 4 consecutive days with 5 ml of distilled H₂O containing 0.002% Tween-20 and adjusted to pH 8 (control), or the same solution containing 10 mM DFMA. The first leaf of 8-day-old oat seedlings was used as the experimental material.

Osmotic Treatment of Excised Leaves. The excised leaves were rinsed several times with distilled H_2O , after which the lower epidermis was removed by peeling, and peeled leaf segments (5 cm length) were floated in the dark over buffer or 0.6 M sorbitol (27) for 6 h or 136 h. In the long-term experiments (136 h), the leaves were first sterilized by immersion for 7 min in 10% Clorox containing Tween-20 (1 drop/10 ml), followed by 10 rinses with sterile-distilled H_2O . All manipulations were performed aseptically in a laminar flow hood.

Polyamine and Enzyme Analysis. Polyamine extraction, separation by TLC, and quantification by spectrophotofluorometry were performed as detailed in the preceding report (27). For ADC and ODC determinations, leaves were extracted in 100 mM K-phosphate (pH 7.6) and the extracts were sonicated twice for 1 min (26) and centrifuged at 27,000 g for 20 min. The supernatant fraction after dialysis was assayed for ADC and ODC as previously describes (6,27).

Incorporation Studies. DNA, RNA, and protein synthesis were estimated according to the method of Gulati *et al.* (10). Six peeled leaf-segments were incubated in Petri dishes (100 x 15 mm) in the dark for 6 and 136 h with 1 μ Ci/leaf segment of [methyl- 3H]thymidine (70 Ci/mmol), or [5- 3H]uridine (27 Ci/mmol); or 0.1 μ Ci/leaf segment of [4,5- 3H] leucine (59.2 Ci/mmol) in the absence or presence of osmotica (0.6 M sorbitol) (27). The long term experiments were performed under asptic conditions as described above. After incubation, the leaf segments were gently blotted on filter paper and trasferred to stainless steel perforated microcapsules, and immediately placed in chilled 10% (w/v) TCA for 2 h. The segments in the capsules were washed: three times with 5% TCA, once with 0.25% $HCOOH$ in methanol, and finally twice with a mixture of absolute ethanol and anhydrous ether (1:1, v/v); each for 15 min, consecutively. All steps were carried out at 5°C with constant shaking. The washed leaf-segments were removed from the capsules, gently blotted on filter paper, and placed in minivials containing 2 ml Betafluor with 5% (v/v) of the tissue solublizer Bio-Solv 3 (Beckman). After overnight digestion of the experimental material at room tempera-

ture, radioactivity was measured a Beckman LS 7000 scintillation counter.

Isolation and Culture of Oat Protoplasts. Leaves from control seedlings and seedlings pretreated with DFMA were rinsed several times with distilled H₂O and sterilized as described above. Protoplasts were isolated aseptically in a laminar flow hood by peeling off the lower epidermis and floating the peeled leaf surface on a filter-sterilized mixture of 1 mM phosphate buffer (pH 5.8) containing 0.5% Cellulysin (B grade, Calbiochem) and 0.5 mannitol for 2 h at 30°C in the dark (1). The released protoplasts were collected by centrifugation. The final protoplast pellet was resuspended at a concentration of about 10⁵ protoplasts/ml in a modified GS-1 medium (21) containing 25 g/L glucose, 20 mg/L arginine, and 10 mg/L ascorbic acid. This medium adjusted to pH 5.8 was previously filter-sterilized. The protoplast suspensions were cultured at about 50- μ l drops (about 5000 protoplasts) on the bottom of 60 x 15 mm Petri dishes (6 drops/dish) placed in moist chambers in the dark at 26°C. After 15 d, fresh medium with 0.2 g/L glucose and 0.2 g/L mannitol was added, and after 4 to 5 weeks the cultures were transferred to the same medium without mannitol but with 0.05 g/L glucose as microdrops or with 1% purified agar added (25). Two separate protoplast isolations and cultures were maintained up to 2 months in liquid-drop medium to follow developmental changes. Viability of protoplasts or developing cells was evaluated by microscopic observations of the cytoplasmic streaming and cell wall regeneration.

Results and Discussion

The previous paper demonstrated that a high Put:Spd + Spm ratio is negatively correlated with protoplast survival and development. The diamine Put increases rapidly during isolation of cereal mesophyll protoplasts, due to osmotic stress activation of the biosynthetic pathway mediated by ADC (5), while conversion of Put to Spd and Spm is inhibited (5, 6). Under such stress conditions, the

ratio of Put to Spd and Spm rises (27). Simultaneous osmotic treatment of cereal leaves with DFMA, an irreversible inhibitor of ADC, was effective in preventing the rise of Put, but did not alter the Spd and Spm levels (5, 6). To prevent Put accumulation while permitting normal Spd and Spm synthesis, we opted for *in situ* leaf pretreatment with DFMA before subjecting the excised leaf segments to osmotic stress in connection with protoplast isolation.

Table I shows the effect of spraying oat seedlings with different concentrations of DFMA on ADC and ODC activities. ADC activity was progressively inhibited by increasing concentrations of DFMA, while ODC activity was only slightly affected. The highest inhibition of ADC activity (65%), which was obtained by spraying oat seedlings for 4 consecutive d with 10 mM DFMA, was used in subsequent pretreatments.

When pretreated leaves were subjected to osmotic stress by floating them on 0.6 M sorbitol for 6 h, Put titers decreased by 45%, Spm titers increased about 2-fold, and Put:Spd + Spm ratio decreased sharply as compared with nonpretreated, osmotically stressed leaves (Table II). After 136 h osmotic stress, Put titers in pretreated leaves increased by only about 50%, but Spd and Spm titers dramatically increased by 3.2- and 6-fold, respectively, in relation to nonpretreated, osmotically stressed leaves. As a consequence of these changes, the Put:Spd + Spm ratio in DFMA-pretreated leaves is only about one third as high as in untreated leaves (Table II).

The low Put:Spd + Spm ratio in leaves pretreated with DFMA corresponds with greater vigor of the leaves after osmotic stress. For example, pretreatment with DFMA causes a marked retardation of Chl loss ordinarily occurring during the incubation period. In all the long-term experiments, the control leaves floated in the dark over buffer or osmotica appeared pale yellow, while the leaves pretreated with DFMA, especially those which were osmotically stressed, appeared green even after 136 h of incubation. Previously, we have reported that oat leaves floated in the dark over buffer showed a loss of Chl of about 71% after 48 h, and

that this effect was prevented by the addition of Spd or Spm (15). To further examine the integrity of the leaves, we investigated the effect of the pretreatment of the seedling with DFMA on subsequent macromolecular synthesis as affected by osmotic stress (Table III). Incorporation of [^3H]thymidine into macromolecules continues to increase for at least 136 h, whereas incorporation of [^3H]uridine or -leucine declines from 6 to 136 h of incubation under osmotic stress in both DFMA-pretreated and control leaves. Pretreatment with DFMA increased the incorporation of all precursors into macromolecules. At 136 h of osmotic stress, there is a significant increase of thymidine and uridine incorporation in osmotically stressed, DFMA-sprayed leaves when compared with unsprayed leaves. The incorporation of leucine was, however, severely reduced in both pretreated and nonpretreated leaves after 136 h of osmotic stress. This may be due to a breakdown of the labeled leucine induced by osmotic treatment rather than to a decrease in synthetic capacity (20). This is further supported by our observation that the incorporation of thymidine and uridine into macromolecules in leaves incubated over buffer for 6 to 136 h was significantly higher than in osmotically stressed leaves (data not shown). These results suggest that (a) macro-molecular synthesis in oat leaves is inhibited by osmotic stress, and that (b) this effect can be partly prevented by pretreatment of the leaves with DFMA.

The higher macromolecular synthesis and lowered rates of senescence observed in osmotically stressed leaves pretreated with DFMA encouraged us to examine whether protoplasts isolated from such leaves will grow better in culture. Thus, we isolated and cultured protoplast from DFMA-treated oat leaves and compared them with those isolated from control leaves. Protoplasts isolated from DFMA-pretreated leaves appeared greener, with greater cytoplasmic streaming, more uniform regeneration of the cell wall, and showing less lysis than protoplasts extracted from control leaves. Although in the early stages of culture (13 d; Table IV) no significant differences were observed in the total number of viable

protoplasts, some improvement of development was observed in protoplasts isolated from leaves pretreated with DFMA. Most of the control protoplasts remained at the oval stage of development, whereas cultures obtained from leaves presprayed with DFMA showed a significant increase of 'doubles' (Table IV). With time, the pretreated cultures showed a significant increase of the total number of viable protoplasts and developing cells as compared with controls (Table IV). In 23-d-old pretreated cultures the number of doubles and 'triples' increased 2.3- and 2.2-fold, respectively, in relation to control cultures. One-month-old pretreated cultures showed an increase in all stages of protoplast development when compared with the control cultures (Table IV). Some of the cultures maintained in liquid droplets for up to 2 months were transferred to solid medium. These cells still show active cytoplasmic streaming and continuing development, as indicated by the occurrence of doubles, triples, and occasionally 'multiples', while cells from control cultures had senesced. Many protoplasts from pretreated cultures regenerated complete cell walls, as opposed to the usual patchy, deposition of cellulose (17). Although DFMA pretreatment significantly improved the overall viability of the protoplasts, this effect is not striking, indicating that other undefined factors must also play a major role in oat protoplast viability.

Oat mesophyll protoplasts are labile and usually lyse within 24 h of incubation in various osmotica and nutrient media (3,4). They senesce rapidly upon isolation, do not regenerate a uniform cell wall, and do not divide consistently (14,17). Leaf pretreatment or addition to the culture medium of senescence retardants such as cycloheximide, kinetin, PAs, and their precursors arginine and lysine has been reported (a) to stabilize oat protoplasts against spontaneous or induced lysis, (b) to increase the incorporation of amino acids and nucleosides into proteins and nucleic acids in leaves and protoplasts, (c) to retard Chl breakdown in leaves and protoplasts, and (d) to decrease or prevent the postexcision and senescence induced rise of RNase and other hydrolytic enzymes (1, 8, 13-15). Of those

compounds, the most active are the tri- and tetraamines Spd and Spm (15, 16), which can also induce DNA-synthesis and limited mitosis in oat mesophyll protoplasts (16). We now show that the pretreatment of oat leaves with DFMA reduces the osmotically induced rise of the diamine Put and prevents the arrest of the conversion of Put to Spd and Spm. The increase of the endogenous levels of Spd and Spm may thus be related with the increased macromolecular synthesis and the decrease of Chl loss observed in DFMA-sprayed leaves during osmotic stress, as well as with the improved viability and development of the oat protoplasts extracted from these leaves.

Genetic factors and injuries (excision and wounding) sustained during isolation of mesophyll protoplasts have been emphasized in the past as the main reasons for lack of sustained cell division and failure of plant regeneration in cereals (4). For example, it has been suggested (7, 13) that morphological instability and deterioration of oat protoplasts may be related to a complex of senescence-induced changes, especially increased R Nase activity (13, 28) following leaf excision and protoplast isolation. However, tobacco protoplasts which readily divide also show a dramatic increase (12- to 15-fold) of RNase activity after 24 h of isolation (18), and this increase has been shown to be due mostly to the osmotic stress rather than the injury caused during isolation of protoplast (19, 20). We have presented evidence suggesting that the accumulation of Put and the arrest of Spd and Spm biosynthesis may be one of the reasons for failure of sustained cell division in mesophyll cereal protoplasts cultured *in vitro*.

Table 1. *Effect of DFMA on Decarboxylase Activity (ADC and ODC) in Oat Seedlings*

Numbers represent the mean $\pm$ SE of three replicates. Numbers without $\pm$ SE are the mean value of 2 replicates.

DFMA Concentration	Decarboxylase Activity	
	ADC	ODC
	<i>nmol CO₂/h·mg protein</i>	
0	4.99 $\pm$ 0.15 (100)	1.30 $\pm$ 0.05 (100)
1 ^a	3.30 (66) ^b	1.15 (88)
5 ^a	2.61 (52)	1.10 (85)
10 ^a	1.76 $\pm$ 0.32 ^d (35)	1.14 $\pm$ 0.08 (88)

^a Four-d-old oat seedlings were sprayed once with DFMA (1 and 5 mM). ^b Figures in parentheses express activity in percent relative to control (-DFMA).

^c Four-d-old oat seedlings were sprayed for 4 consecutive d with 10 mM DFMA as described in "Materials and Methods." The first leaf of 8-d-old seedlings was used as the experimental material. ^d Significantly different from control at $P < 0.05$.

Table II. *Effect of Pretreatment with DFMA on PA Titer in Oat Leaves Subjected to Osmotic Stress*

Numbers represent the mean of 2 replicates from a single experiment.

Pretreat- ment	Osmotic Treatment ^a	Put	Spd	Spm	Put/Spd + Spm
	<i>h</i>	<i>nmol^b/mg protein</i>			
-DFMA	6 ^c	4.00 (100)	2.00 (100)	1.30 (100)	1.21
+DFMA	6	2.20 (55) ^d	2.10 (105)	2.70 (208)	0.45
-DFMA	136	4.70 (100)	0.90 (100)	0.60 (100)	3.13
+DFMA	136	7.10 (151)	2.90 (322)	3.60 (600)	1.09

^a Oat leaves were floated over 0.6 M sorbitol in the dark for 6 or 136 h as indicated in "Materials and Methods." ^b PA titer are the sum of the S- and SII-fractions (27). ^c The experiment at 6 h was repeated twice. ^d Percent in relation to controls (-DFMA).

Table III. *Effect of the Pretreatment with DFMA on Macromolecular Synthesis in Oat Leaves Subjected to Osmotic Stress*

Numbers represent the mean $\pm$ SE of 6 replicates.

Pretreatment	Osmotic Treatment	[³ H]Thymidine	[³ H]Uridine	[³ H]Leucine
	<i>h</i>	<i>dpm/mg protein</i>		
-DFMA	6	694 $\pm$ 36 (100)	2885 $\pm$ 785 (100)	803 $\pm$ 100 (100)
+DFMA	6	1090 $\pm$ 190 (157)	4037 $\pm$ 278 (140)	1212 $\pm$ 167 (151)
-DFMA	136	7469 $\pm$ 746 (100)	700 $\pm$ 110 (100)	86 $\pm$ 16 (100)
+DFMA	136	11,566 $\pm$ 1,072 ^a (155)	1743 $\pm$ 227 ^b (249)	110 $\pm$ 26 (128)

^a Significantly different from control (-DFMA, 136 h) at $P < 0.05$. ^b Significantly different from control at $P < 0.01$.

Table IV. *Effect of Pretreatment with DFMA on Oat Protoplast Viability and Development*

Pretreatment	Age of Cultures ^a	No. of Drops Examined	Oval ^b	'Doubles'	'Triples'	Total
	<i>d</i>	<i>No. of viable^c protoplasts or developing cells/drop</i>				
-DFMA	13	11	31.5 $\pm$ 2.1	1.5 $\pm$ 0.4	0	33.0 $\pm$ 1.9
+DFMA	13	11	32.7 $\pm$ 2.7	3.4 $\pm$ 0.3 ^d	0	36.1 $\pm$ 3.0
-DFMA	23	11	20.1 $\pm$ 1.0	2.5 $\pm$ 0.3	0.4 $\pm$ 0.2	23.0 $\pm$ 1.2
+DFMA	23	12	20.3 $\pm$ 1.0	5.8 $\pm$ 0.5 ^e	0.9 $\pm$ 0.2	27.0 $\pm$ 1.2 ^f
-DFMA	30	11	14.9 $\pm$ 0.9	5.9 $\pm$ 0.3	1.0 $\pm$ 0.2	21.8 $\pm$ 0.9
+DFMA	30	11	20.5 $\pm$ 1.7 ^f	7.4 $\pm$ 0.5 ^f	1.5 $\pm$ 0.3	29.4 $\pm$ 1.8 ^d

^a Oat protoplasts from leaves pretreated (+DFMA) or not (-DFMA) were isolated and cultured as described in "Materials and Methods." ^b Oval: initial stages of protoplast development. Many of these cells from the cultures pretreated with DFMA showed complete cell wall regeneration as indicated by staining with Calcofluor White and plasmolysis test; 'Doubles' and 'Triples' (3) are cells undergoing karyokinesis and showing 2 or 3 nuclei, respectively, as indicated by staining with Carbolfuchsin. Some of these cells showed completion of cytokinesis as indicated by the presence of calcofluor-stained cross walls. ^c Viability of protoplasts or developing cells was evaluated as described in "Materials and Methods." ^d Significantly different from control at $P < 0.01$. ^e Significantly different from control at $P < 0.001$. ^f Significantly different from corresponding control (-DFMA) at $P < 0.05$.

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8. Control of phytopathogenic fungi by inhibitors of polyamine biosynthesis: kinetic studies on the control of the bean rust fungus (*Uromyces phaseoli* L.) by DFMO.

Abstract

α -Difluoromethylornithine (DFMO), a specific and irreversible inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase, effectively inhibits mycelial growth of several phytopathogenic fungi on defined media *in vitro* and provides systemic protection of bean plants against infection by *Uromyces phaseoli* L. race O (MV Rajam, AW Galston 1985 Plant Cell Physiol 26: 683-692; MV Rajam *et al.* 1985 Proc Natl Acad Sci USA 82: 6874-6878). We now find that application of 0.5 millimolar DFMO to unifoliolate leaves of Pinto beans up to 3 days after inoculation with uredospores of *U. phaseoli* completely inhibits the growth of the pathogen, while application 4 or 5 days after inoculation results in partial protection against the pathogen. Spores do not germinate on the surface of unifoliolate leaves treated with DFMO 1 day before infection, but addition of spermidine to the DFMO treatment partially reverses the inhibitory effect. The titer of polyamines in bean plants did not decline after DFMO treatment; rather, putrescine and spermidine contents actually rose, probably due to the known but paradoxical stimulation of arginine decarboxylase activity by DFMO.

Introduction

It has been established, through the use of genetic mutants and specific chemical inhibitors, that polyamines are essential for optimal growth and development in bacteria and fungi (9), higher plants (5,8), and mammals (3). The formation of PA in bacteria and higher plants may proceed through either of two

enzymes, *e.g.* ADC or ODC, while only the ODC pathway operates in many fungi. DFMA and DFMO which specifically and irreversibly block ADC (1) and ODC (4), respectively, have been used to specify the initial route of Put biosynthesis in many organisms. Since Spd and Spm are formed from Put, effective blockage of Put formation can deprive the organism of all PA. The presence of but a single Put biosynthetic pathway in fungi (via ODC) suggested a possible approach to the use of DFMO as a protectant or possibly a chemotherapeutic agent in the prevention of certain types of fungal pathogenesis in higher plants.

In growth experiments on defined media with several phytopathogenic fungi (6), we observed that DFMO is an effective inhibitor of mycelial growth, and that such inhibitions are completely reversed by application of Put or Spd to the culture medium. In a recent paper (7), we reported on the remarkable efficacy of DFMO as a protectant of bean plants against infection caused by uredospores of *Uromyces phaseoli* race O, the common bean rust fungus. Not only could we obtain complete protection by as little as 400 μ l of 0.5 mM DFMO applied to a single unifoliate leaf, but DFMO was found to confer protection as well on unsprayed parts of treated plants, indicating the translocation of some protective effect from the sprayed areas. The present work extends that study reporting on the kinetics and reversibility of the DFMO effect.

Materials and Methods

Unifoliate leaves of 10-d-old greenhouse-grown bean seedlings (*Phaseolus vulgaris* cv 'Pinto') were sprayed with DFMO or DFMA in 0.01% Tween-20 at pH 7.0 before or after inoculation with uredospores of *U. phaseoli* L. race O. Control plants were sprayed similarly with Tween-20 without inhibitor. In experiments involving the reversal of DFMO effects, we utilized 0.05 mM DFMO (which yielded 50% inhibition of uredial development) together with 0.01, 0.1 and 1.0 mM Put or Spd. After inoculation with rust uredospores (25 mg/100 ml of 0.01%

Tween 20), all plants were placed in dew chambers (100% RH) for 16 h at 19°C in total darkness, as previously described (6). Plants were returned to the greenhouse and arranged randomly following exposures to inhibitor and uredospores. Disease severity was evaluated 7 d after inoculation by counting the lesions on the leaf.

PA analysis was performed on leaf samples collected from inhibitor-sprayed bean plants. Leaf samples were ground in prechilled mortars with 10% (w/v) HClO_4 at a ratio of 200 mg fresh weight leaf per ml HClO_4 . Homogenates were centrifuged at 26,000g for 20 min at 4°C. The clear supernatant fractions were used for dansylation according to a procedure previously described (4).

Results

We reported previously that DFMO at 0.5 mM or higher gives complete protection against the pathogen in both pre- and post- inoculation exposures when inoculation and treatment are separated by 24 h (7). In the present work, we determined the effect of varying delays in post-inoculation application of DFMO on the extent and course of pathogenesis. Test plants were exposed to 1.0 mM DFMO either 1, 2, 3, 4 or 5 d after inoculation with uredospores of the pathogen. Complete protection against the pathogen was found when DFMO was first applied up to 3 d after inoculation (Fig. 1). disease symptoms occurred when the interval between inoculation and DFMO application was 4 or 5 d, although the number of lesions was significantly reduced below control (unprotected) values (Table I).

In vitro, uredospore germination starts 30 min after incubation at 19°C in the light, and the germ tube reaches an effective size for leaf penetration within 3 h in the presence of 10 mM Mes (pH 7.0), 3 mM CaCl_2 , 2 mM MgSO_4 , and 1% purified agar. We attempted to visualize spore germination on the surface of the unifoliolate bean leaf with crystal violet, 1 d after spore inoculation. The plants

had been sprayed with DFMO at 0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM 1 d before inoculation. Germination was normal on control leaves, completely inhibited on leaves treated with more than 0.5mM DFMO, and partially inhibited at lower concentrations of DFMO.

To determine whether the effect of DFMO was related to inhibition of PA biosynthesis, we conducted experiments to determine the effects of PA application, alone or in combination with 0.05mM DFMO (which yielded ~ 50% inhibition of uredial formation), on the severity of the disease. There were generally no significant differences between controls and PAs alone except at 1 mM Put, which reduced the number of lesions by about one third. This is probably due to the well known toxic effect of mM Put (8,9). The number of lesions was reduced by 70% in plants exposed to 0.05 M DFMO alone; when Spd was supplied 1 h after DFMO, the inhibition conferred by DFMO was substantially reduced (Table II).

The effect of DFMO and DFMA sprays (0.01, 0.1 and 1.0 mM) on PA titers was also examined. Unifoliolate leaves were sprayed in the usual way and examined 1 and 3 d later, while the first trifoliolate leaves, unexpanded at the time of spray and themselves unsprayed, were examined 8 d later. There were no significant reductions of PA levels in any of the treated leaves; in fact, Put and Spd titers were increased by the highest concentration of DFMO (MV Rajam, LH Weinstein. AW Galston unpublished data). This appears to be due to the paradoxical promotion of ADC activity by DFMO (8). There was no effect of DFMO or DFMA sprays on the growth of the plants.

Thus, in the absence of any depression in the PA titer of the host plant, we reason that the protective effect of DFMO against the fungus results in part from its persistence on the surface of the leaf, where fungal spores germinate and initiate growth. Since rust uredosporelings contain progressively higher Spd as their growth and differentiation progresses (2), it is reasonable to believe that PA deprivation caused by DFMO would inhibit both processes. The translocatability

of the protective effect of DFMO (7) indicates that this substance may also initiate internal protective metabolic changes that are not reflected in the PA titer of the host plant.

FIG. 1. Kinetics of pathogen inhibition following postinoculation exposures to 1.0 mM DFMO. Unifoliolate leaves of controls (A, C, E, and G) and DFMO treatments (B, D, F, and H) on 1, 2, 3, and 4 d after inoculation with uredospores of *U. phaseoli*, respectively.

Table 1. Kinetics of Inhibition of Pathogenesis Resulting from Postinoculation Application of DFMO to Unifoliolate Leaves of Bean Plants after Different Intervals of Time

Unifoliolate leaves were inoculated with uredospores and then, at daily intervals, a different group of plants was sprayed with 0.01% Tween 20 lacking or containing 1.0 mM DFMO.

Time between Spore Inoculation and DFMO Spray	DFMO Concentration	Lesions	
d	mM	per cm ²	per leaf ^a
1	0	74 ± 6	4673 ± 373
	1.0	0	0
2	0	69 ± 6	4568 ± 398
	1.0	0	0
3	0	64 ± 4	3172 ± 276
	1.0	0	0
4	0	65 ± 6	4419 ± 461
	1.0	41 ± 5 ^b	2330 ± 211 ^b
5	0	69 ± 3	3962 ± 217
	1.0	53 ± 4 ^b	3278 ± 326

^a Each value is Mean ± SE, based on six replicates (one leaf per plant). ^b Significant difference at 5% level.

Table II. Effect of Postinoculation Application of DFMO, and of FAs, Alone or in Combination, on the Severity of Bean Rust Disease on Unifoliolate Leaves

Treatment	Lesions	
mM	per cm ²	per leaf ^a
Control	64 ± 4	4104 ± 187 (100) ^b
Put, 0.01	57 ± 2	4013 ± 264 (98)
Put, 0.1	54 ± 5	3189 ± 478 (78)
Put, 1.0	44 ± 9	2698 ± 260 (66)
Spd, 0.01	56 ± 5	3209 ± 360 (78)
Spd, 0.1	58 ± 6	3416 ± 457 (83)
Spd, 1.0	50 ± 10	3926 ± 358 (96)
DFMO, 0.05	22 ± 4	1223 ± 268 (30)
DFMO, 0.05 + Put, 0.01	30 ± 5	1823 ± 289 (44)
DFMO, 0.05 + Put, 0.1	24 ± 4	1440 ± 337 (35)
DFMO, 0.05 + Put, 1.0	32 ± 2	2009 ± 129 (49)
DFMO, 0.05 + Spd, 0.01	29 ± 3	1678 ± 240 (41)
DFMO, 0.05 + Spd, 0.1	38 ± 7	2366 ± 488 (58)
DFMO, 0.05 + Spd, 1.0	39 ± 3	2334 ± 245 (57)

^a Each value is mean ± SE, based on six replicates (one leaf per plant). ^b Percent of control values are in parentheses.

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9. Cadmium-Induced Accumulation of Putrescine in Oat and Bean Leaves

Abstract

The effects of Cd^{2+} on putrescine (Put), spermidine (Spd), and spermine (Spm) titers were studied in oat and bean leaves. Treatment with Cd^{2+} for up to 16 hours in the light or dark resulted in a large increase in Put titer, but had little or no effect on Spd or Spm. The activity of arginine decarboxylase (ADC) followed the pattern of Put accumulation, and experiments with α -difluoromethylarginine established that ADC was the enzyme responsible for Put increase. Concentrations of Cd^{2+} as low as 10 micromolar increased Put titer in oat segments. In bean leaves, there was a Cd^{2+} -induced accumulation of Put in the free and soluble conjugated fractions, but not in the insoluble fraction. This suggests a rapid exchange between Put that exists in the free form and Put found in acid soluble conjugated forms. It is concluded that Cd^{2+} can act like certain other stresses (K^+ and Mg^{2+} deficiency, excess NH_4^+ , low pH, salinity, osmotic stress, wilting) to induce substantial increases in Put in plant cells.

Introduction

One response of plants exposed to certain environmental stresses is an increase in the diamine, Put, and its biosynthetic enzyme, ADC. K^+ -deficiency, low pH, a surplus of NH_4^+ ion in the nutrient medium, osmotic stress, wilting, and salt stress have been reported to increase both Put titer and ADC activity (5, 10, 18-21, 23). Other stresses, such as heat shock (20), deficiencies of phosphorus, sulfur and nitrogen (18, 19), excesses of fluoride and sulfite salts (LH Weinstein, AW Galston, unpublished data) do not elicit important changes in PA titer in plants.

Cd is an important environmental contaminant, not only because of its phytotoxicity, but also because its uptake and accumulation in plants may introduce it into the food chain. Cd is emitted to the atmosphere from coal-fired power plants, steel mills, metal smelting and roasting operations, incineration of wastes, and electroplating processes (3, 6, 12). In soils, Cd accumulates due to deposition from the atmosphere, from phosphate fertilizers, and from sewage sludge (13, 14).

The purpose of this paper is to establish the relationship of Cd accumulation to the putrescine titer and to describe some of the metabolic changes in the pattern of PA biosynthesis in plants caused by Cd administration.

Materials and Methods

Plant Material. *Avena sativa* L. cv Victory (The General Swedish Seed Co., Svalov, Sweden) and *Phaseolus vulgaris* L. Pinto (Haney Seed Co., Twin Falls, ID) were used in all experiments. Except in dose-response studies, seeds were germinated and grown in a controlled-environment growth room in plastic dishes containing washed coarse-grade vermiculite and subirrigated twice daily with a 1.2 g/L solution of Hyponex (7-6-19; Hydroponics Chemical Co., Copley, Oh). Plants were grown under a 16-h photoperiod provided by fluorescent and incandescent lights with an energy level of about $200 \mu\text{E s}^{-1} \text{m}^{-2}$. For dose-response studies, oat seeds were germinated and grown in peat-vermiculite (Cornell mix plus "A" (1)) in a temperature-controlled greenhouse at 25/18°C day/night temperatures, a photoperiod under sunlight or high pressure sodium vapor lamps of 16 h and a light intensity of about $700 \mu\text{E s}^{-1} \text{m}^{-2}$ or higher.

Conditions of Treatment. Bean leaf discs, 14 mm in diameter, were removed with a cork borer from the first trifoliolate leaf on both sides of the main vein from 10- to 12-d-old plants. Six discs were floated, abaxial side down, in Petri dishes containing 20 ml of 1 mM K-phosphate (pH 5.8) with or without added CdCl_2 . In oats, the lower epidermis was removed from excised first leaves (11). Six to eight medial sections (50 mm each) were randomly selected and floated

peeled side down on 1 mM K-phosphate (pH 5.8). Cd^{2+} concentrations ranging from 0.01 to 0.25 mM were used in a dose-response study, and based on the responses measured, 0.1 mM Cd^{2+} was used for other experiments. Treatment times varied from 1 to 16 h. Triplicate samples were harvested for measuring PA titer or enzyme activity, and each experiment was repeated at least once. Representative data are presented in the tables.

A dose-response study was conducted by exposing intact oat plants to several concentrations of CdCl_2 . Eleven-d-old seedlings were transferred from Cornell mix to half-strength Hoagland solution. After 2 d, the nutrient solution was replaced with fresh solution containing 0, 0.1, 0.5, 1, 5, and 10 mM CdCl_2 . After 24 h of exposure, triplicate samples, each containing six first leaves, were harvested.

At harvest, leaf samples were rinsed three times in glass-distilled H_2O and either frozen and stored in liquid N_2 or processed immediately. Where DFMA or Put were added, they were incorporated into the floating buffer solution or in the enzyme reaction mixture at a concentration of 0.1 mM.

Polyamine Analysis. Leaf samples were homogenized in cold 10% (v/v) PCA at a ratio of 100 mg/ml. The supernatant fraction was collected after centrifugation at 12,000 g for 20 min. Dansyl derivation (4) of the PAs, their separation by TLC, and analyses were carried out as described earlier (4).

The amount of PA conjugates in oat was very low and no quantitative estimations were made. Therefore, PCA-soluble and -insoluble PA conjugates in beans were determined after hydrolysis of the PCA-soluble and pellet fraction in 6 N HCl at 100°C for 15 to 20 h. After evaporation of the HCl, samples were dissolved in a known volume of 10% PCA, filtered if necessary, dansylated, and separated by TLC.

The PA band designated as Unk in oat occurred between Spd and Spm, and was assayed because it appeared to vary in response to treatments as did Put. It was 'quantified' by assuming that it was a triamine, using Spd as the standard.

Enzyme Assays. ADC and ODC were assayed after extraction from fresh or frozen (liquid N₂) leaf tissues. Leaf samples, at a ratio of 100 to 200 mg/ml of extraction medium, were ground in 100 mM K-phosphate (pH 7.8) containing 10 mM DTT, 5 mM EDTA, and 1 mM PLP. The extracts were further disrupted by sonicating twice 2 min each time in a salt-ice mixture, and were dialyzed against 100 mM K-phosphate buffer (pH 7.6)(15, 16). Preliminary assays for ODC in oat using 100 mM Tris (pH 8.0) showed enzyme activity no greater than that obtained in phosphate buffer.

The enzyme assays were carried out as described earlier (11). Protein was determined by the method of Bradford (2) using bovine γ -globulin as a standard.

Results

Effect of Cd²⁺ on PA Titers in Bean and Oat Leaves. The PCA-soluble fraction from bean leaf discs exposed to 0.1 mM Cd²⁺ for 16 h contained 4 to 5 times more Put than did controls. The increase in Put titer was accompanied by a relatively small but significant decrease (about 25%) in Spd and no significant change in Spm.

Oat leaves were much more responsive in terms of Put accumulation to the Cd²⁺ stress effect than were beans. For example, in oats treatment with 0.1 mM Cd²⁺ for 16 h resulted in a greater than 20-fold increase in Put titer as compared to the controls (8.7 versus 200 nmol/g fresh weight). Put accumulation was not accompanied by important changes in Spd or Spm, despite the normal conversion of Put to these compounds by enzyme-mediated transfer of aminopropyl groups from SAM (10). An unknown PA, which had an R_F value between Spd and Spm, responded in a manner similar to Put.

Because oat leaves had very low concentrations of PCA-soluble (SH) and -insoluble (PH) conjugates, the effects of Cd²⁺ on these fractions in bean leaves was examined. Cd²⁺ treatment induced a 2-fold increase in the soluble Put titers. Soluble Spd was decreased by 25% and Spm remained unchanged. After acid

hydrolysis, conjugated Put in the soluble fraction of Cd^{2+} -treated discs was about 2.5 times greater than the controls. Soluble conjugated Spd was slightly lower with Cd^{2+} treatment, but there were no apparent changes in Spm. Conjugated PAs were also found in the insoluble pellet, but there were no significant differences in the titers of Put or Spd between control and Cd^{2+} treated discs in this fraction. Spm was not detected. Thus, the response of PCA-soluble free and conjugated PAs responded similarly.

Dose-Response to Cd^{2+} in Oats. In the range of Cd^{2+} from 0 to 0.25 mM ([a] in Table I), Put reached a maximum titers at 0.10 mM. At 0.25 mM, the Put titers was lower, indicating that a concentration of Cd^{2+} somewhere between 0.10 and 0.25 mM became inhibitory to Put accumulation.

The regression equation for Spd was significant, with the greatest accumulation occurring at 0.01 and 0.025 mM. The maximum Spm accumulation was found at 0.10 mM. Other dose-response experiments concentrated on effects of Cd^{2+} levels between 0.10 and 0.25 mM ([b] in Table I). The titers for Put, Spd, and Spm are represented as functions of Cd^{2+} concentration by appropriate linear and quadratic regression equations in Table I which indicate that the maximum Put titers was attained at 0.175 mM Cd^{2+} . However, a concentration of 0.1 mM Cd^{2+} was used for subsequent experiments because it represented a level at which the responses could be expressed or interpreted as first-order effects.

In intact oat plants, uptake of Cd^{2+} induced increases in Put of up to 15-fold (Table II); a nutrient concentration of Cd^{2+} as low as 0.1 mM (11.2 ppm) resulted in a 2-fold increase in Put. The Spd titers was relatively constant. Nutrient concentrations of 5 and 10 mM Cd^{2+} elicited an increase in Spm of up to 2-fold.

DFMA and the Stress Response in 'Victory' Oats. The effects of Cd^{2+} on PAs and their biosynthetic enzymes was studied by using DFMA, an enzyme-activated, irreversible inhibitor of ADC. Addition of 0.1 mM DFMA to the floating buffer reduced ADC activity by about 90% with or without Cd^{2+} (Table III). The Put titers was significantly increased by addition of Cd^{2+} and DFMA re-

duced its accumulation by 75 to 80% in both cases. ODC activity was much lower than ADC activity and was unaffected by Cd^{2+} or DFMA treatment (Table II). Cd^{2+} induced a modest increase in ADC activity. Later experiments with inhibitors confirmed that ADC is in the primary pathway of Put formation under stress conditions.

Although Cd^{2+} treatment increased the Dap content about 2-fold over the controls, this change was not statistically significant. However there was a DFMA-mediated increase in Dap titers of about 3-fold relative to comparable treatments without DFMA, and in a view of the known origin of Dap from Spd and Spm breakdown, it appears that the presence of DFMA may lead to increase decomposition in PAs. Spd content was significantly reduced by treatment with Cd^{2+} , and the addition of DFMA induced further decreases in control and Cd^{2+} -treated tissues. Spm content was very low, but there was a significant increase in titers upon Cd^{2+} treatment, which was eliminated by DFMA. Unk was unchanged in the presence of Cd^{2+} with or without DFMA as compared to controls. In controls, however, treatment with DFMA resulted in a lower Unk titers. Results for the response of ADC, ODC and PAs to DFMA provided persuasive evidence that Cd^{2+} -induced Put biosynthesis is mediated through the ADC pathway.

Response Kinetics of Cd^{2+} Toxicity in 'Victory' Oats. The time-course kinetics of PA response of Cd^{2+} are shown in Table IV. Even after incubation on buffer containing 0.1 mM Cd^{2+} for 1 h, there was a measurable increase in Put content in Cd^{2+} -treated tissues, but only after 4 h was the content of Put in Cd^{2+} -treated tissues statistically significant. After 16 h, when controls normally showed a decline in Put titers, treatment with Cd^{2+} induced a 10-fold increase in Put. Spd titer was unaffected by Cd^{2+} treatment or time. Spm was higher in Cd^{2+} -treated tissues after 8 and 16 h, but the difference was due more to a decrease in controls than an increase in Cd^{2+} treated tissues. Dap, a breakdown product of Spd and Spm, formed by a PA oxidase (10), was present but no clear pattern of

change was evident. The unknown PA responded like Put with large increases in titers after 8 and 16 h.

Table 1. *Effect of Cd²⁺ on the PA Titer in Detached Oat Leaf Segments*
Oat leaf segments were treated for 16 h in the dark.

Concentration Cd ²⁺	Put	Spd	Spm
<i>mM</i>	<i>nmol/g fresh wt ± SEM</i>		
(a) 0	35.7 ± 0.6	62.4 ± 2.4	14.0 ± 0.8
0.01	58.9 ± 5.4	84.9 ± 2.8	23.5 ± 0.7
0.025	85.4 ± 4.5	86.2 ± 3.7	22.1 ± 1.0
0.065	211.6 ± 4.4	55.6 ± 3.1	27.1 ± 1.1
0.10	383.3 ± 36.9	69.1 ± 2.4	41.2 ± 1.1
0.25	315.9 ± 27.8	55.9 ± 3.2	38.0 ± 1.1
(b) 0	26.7 ± 1.3	45.7 ± 1.1	8.2 ± 1.0
0.10	193.9 ± 3.5	37.5 ± 1.2	13.2 ± 0.4
0.13	224.0 ± 2.5	34.5 ± 0.9	11.6 ± 0.6
0.1625	241.0 ± 3.1	43.5 ± 1.5	11.9 ± 1.2
0.20	230.3 ± 1.7	45.8 ± 0.7	9.5 ± 0.7
0.25	212.8 ± 2.2	38.7 ± 1.0	18.6 ± 1.5

Estimates of Parameters				
Polyamine	Intercept	Cd ²⁺ Concentration		Correlation coefficient
		Degree of polynomial		
		1	2	
(a) Put	8.18	4793.1587	-14191.4593	R ² = 0.90
Spd	76.06	-82.79		r = 0.54
Spm	16.02	314.6100	-903.3820	R ² = 0.85
(b) Put	26.80	2326.7889	-6368.9727	R ² = 0.94
Spd	0.05			NS
Spm	8.19	28.4219		r = 0.50

Table II. *Effects of Cd²⁺ on the PA Titer of Leaves of Intact Oat Seedlings*
Seedlings were exposed to Cd²⁺ in nutrient culture for 24 h.

Nutrient Concentration of Cd ²⁺	Put	Spd	Spm
<i>mM</i>	<i>nmol/g fresh wt ± SEM</i>		
0	17.4 ± 3.7	92.7 ± 7.5	14.0 ± 2.1
0.1	31.8 ± 1.6	92.2 ± 1.3	9.4 ± 0.4
0.5	79.0 ± 12.0	108.9 ± 7.4	11.2 ± 1.1
1.0	90.9 ± 15.3	92.0 ± 10.3	7.2 ± 2.2
5.0	219.9 ± 18.3	79.4 ± 17.5	16.7 ± 3.2
10.0	249.7 ± 23.7	105.2 ± 6.3	35.0 ± 4.0

Estimates of Parameters				
Polyamine	Intercept	Cd ²⁺ concentration		Correlation coefficient
		Degree of polynomial		
		1	2	
Put	32.52	55.6264	-3.4037	R ² = 0.93
Spd	95.07			NS
Spm	10.86	-0.5132	-0.2954	R ² = 0.83

Table III. Effects of DFMA and Cd²⁺ on the PA Titer and ADC/ODC Activity in Oat Leaves

Oat leaf segments were treated with 0.10 mM Cd²⁺ for 16 h in the dark. DFMA was added to the floating buffer or incorporated into the enzyme reaction.

Treatment	Put	Spd	Spm	Dap	Unk ^a
nmol/g fresh wt ± SEM					
Control	54.2 ^b ± 4.2	130.1a ± 7.8	0.07b ± 0.05	16.9b ± 12.7	15.5a ± 2.4
Control + DFMA	13.0b ± 1.0	62.8c ± 15.2	0.0b	52.9a ± 14.8	8.1b ± 2.6
Cd ²⁺	228.2c ± 58.6	97.9b ± 7.1	2.2a ± 1.5	30.7b ± 10.9	20.1a ± 3.0
Cd ²⁺ ± DFMA	45.0a ± 9.4	59.5c ± 7.8	0.2ab ± 0.06	111.6a ± 27.7	1a ± 3.0
ADC			ODC		
nmol ¹⁴ CO ₂ /h·mg protein ± SEM					
Control	9.57a ± 0.3		0.82a ± 0.3		
Control + DFMA	0.66b ± 0.02		0.64a ± 0.04		
Cd ²⁺	12.03c ± 1.0		0.61a ± 0.06		
Cd ²⁺ + DFMA	1.45b ± 0.02		0.56a ± 0.04		

* Calculated as a triamine. ^b Values in a column followed by the same letter are not significantly different at P < 0.05.

Table IV. Kinetics of Cd²⁺ on Free Polyamine Titer in Oat Leaves

Treatment	Time	Free PAs				
		Put	Spd	Spm	Dap	Unk ^a
	<i>h</i>	<i>nmol/g fresh wt ± SEM</i>				
Control	0	37.5 ± 2.1	54.5 ± 11.7	2.7 ± 0.9	12.1 ± 1.5	8.8 ± 1.7
Control	1	39.0 ± 3.2	80.1 ± 0.3	11.3 ± 5.4	15.1 ± 2.5	12.1 ± 2.1
Cd ²⁺		45.1 ± 1.3	78.8 ± 9.5	10.5 ± 5.5	10.2 ± 1.2	8.9 ± 1.6
Control	2	44.7 ± 5.3	63.0 ± 1.5	7.8 ± 1.4	7.8 ± 1.2	10.2 ± 0.7
Cd ²⁺		57.6 ± 5.1	74.8 ± 3.9	5.9 ± 1.0	7.7 ± 0.1	6.3 ± 2.0
Control	4	62.1 ± 7.8	81.3 ± 3.9	7.3 ± 0.7	9.1 ± 0.7	6.5 ± 0.1
Cd ²⁺		91.8 ± 6.4	74.4 ± 2.0	6.0 ± 0.8	11.7 ± 1.3	10.0 ± 1.8
Control	8	65.5 ± 1.8	77.3 ± 2.0	4.4 ± 2.3	6.4 ± 0.7	5.9 ± 2.1
Cd ²⁺		151.8 ± 5.2	76.1 ± 0.2	9.0 ± 1.0	5.4 ± 0.5	19.0 ± 1.0
Control	16	25.1 ± 6.0	73.7 ± 0.9	0.5 ± 0.2	6.9 ± 0.8	4.2 ± 0.9
Cd ²⁺		227.3 ± 5.1	72.8 ± 1.0	5.5 ± 0.6	7.6 ± 0.4	22.7 ± 1.9

Estimates of Parameters

Polyamine	Treatment	Intercept	Time		Correlation coefficient
			Degree of polynomial		
			1	2	
Put	Control	33.83	8.4926	−0.56468	R ² = 0.97
	Cd ²⁺	38.77	12.25		r = 0.99
Spd	Control	71.65			NS
	Cd ²⁺	71.90			NS
Spm	Control	7.80	−0.419		r = 0.65
	Cd ²⁺	6.60			NS
Dap	Control	11.46	−0.373		r = 0.63
	Cd ²⁺	9.11			NS
Unk	Control	10.02	−0.406		r = 0.80
	Cd ²⁺	6.61	+1.088		r = 0.90

* Calculated as triamine.

Discussion

Our results support the view that Cd^{2+} can be added to the list of generally unrelated stresses such as K^+ or Mg^{2+} deficiency, excess NH_4^+ , low pH, salinity, osmotic stress, and wilting, as an agent that can induce a massive accumulation of Put in plant cells. In these experiments, Put increased in cells of detached leaf segment exposed directly to Cd^{2+} or of intact leaves of plants exposed through the roots in nutrient culture. There was also a clear relationship between Put accumulation and ADC activity in Cd^{2+} -treated tissues (Table III) (5, 17).

The results presented were achieved with concentrations of 'available' Cd often present in only mildly polluted environments. About 1 ppm (0.01 mM) Cd^{2+} in the floating buffer for only 6 h significantly increased the Put titers in oat leaf segments (Table I). Similarly, about 10 ppm (0.10 mM) Cd^{2+} added to nutrient culture for 24 h resulted in about the same Put accumulation in leaf tissues of intact seedlings (Table II), demonstrating that the two systems gave comparable results. Although the concentrations used in leaf segments and intact seedlings are somewhat higher than those normally associated with polluted soils (3, 6, 12-14), the effects of Cd in the ecosystem are judged over the seasonal period of the plant's growth, not over 1 d or less. Furthermore, the amounts of Cd^{2+} leading to Put accumulation are within the range of effects noted by Fuhrer (7-9) for promoting ethylene formation in oats and beans.

The ADC requirement for Put biosynthesis in oat leaves was demonstrated *in vitro* and *in vivo* by addition of DFMA to the ADC reaction mixture or to oat segments on floating buffer, both of which nearly completely inhibited ADC activity and Put accumulation but had no effect on ODC activity (Table III). The decrease in Put titer by DFMA was also accompanied by a decrease in Spd and Spm. This suggests that Spd and Spm synthase activities were reduced either by direct inhibition from accumulated Put (4), or because the concentration of Put was below the K_m for enzyme (5). It was recently found that osmotic stress induced an increase in Put titer and a decrease in Spd synthase in oat leaves (22).

Inhibition of Spd or Spm synthase activities by accumulated Put could account for the modest increase in ADC activity found.

Cd^{2+} -induced accumulation of Put in Pinto bean leaf discs was less pronounced than in oat leaf segments, perhaps because of a lower uptake of Cd^{2+} by unpeeled bean leaf discs. In both species, Spd and Spm were relatively unresponsive to Cd^{2+} administration, as they are to other stress stimuli (19, 23). In bean also, soluble conjugated Put increased proportionately with soluble free Put, suggesting that the synthetic pathways for Put-containing conjugates also responded to Cd^{2+} treatment. This was not the case, however, for Spd-containing conjugates. Evidence that PA conjugates may serve as a storage form of PAs and may exchange with free PA pools has been presented (15, 16).

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10. Other studies

Uptake and long-distance translocation of polyamines

Polyamines, and especially putrescine, are readily taken-up by intact root systems or through a cut-surface of the stem, and translocated to a long distance to all other plant organs. Part of the translocated polyamines is metabolized while part remains unchanged. Uptake and translocation of polyamines is affected by environmental conditions (light, temperature, humidity) and by the type and age of the tissues. In addition, putrescine, spermidine and spermine were identified in xylem and phloem exudates of several plant species. The presence and abundance of polyamines in translocated plant fluids further supports the hypothesis that polyamines have a regulatory role in plant growth and response to stress.

(A. Altman)

Interactions of polyamines, plant DNA, and restriction endonucleases.

Previous studies suggest that polyamines may act in stabilizing and modulating nucleic acid and membrane structure and functions, by binding to their charged phosphate groups. In order to better elucidate one aspect of the role of polyamines in these systems, we investigated the *in vitro* effect of polyamines on the restriction pattern of DNA from several sources. DNA (total chloroplast DNA from maize and spinach, plasmids harboring the gene(s) for the "32 kd" thylakoid membrane protein and for the enzyme RuBPCase of maize chloroplasts, and pBR322 DNA) was incubated with various restriction endonucleases under standard conditions in the presence of 0.1 to 10 mM spermidine or spermine, and the restriction pattern was detected using agarose gel electrophoresis.

Data show that spermidine and spermine inhibit the activity of DNA restriction enzymes to various degrees, depending on the DNA source and the type of endonuclease. Spermine concentrations which inhibit restriction endonucleases were 5-10 times lower than those of spermidine. This inhibition seems to be general rather than site-specific, since no changes in the band pattern were observed so far.

The involvement of polyamines in the response to stress seems to be a rather general phenomenon in plants, procaryotes, and animal tissues. Nucleic acid conformation may be one common site for polyamine action, and these results may be of a general significance.

(A. Altman)

Polyamine content and activity of ADC and ODC in developing Fruit tissues of "Murcott" mandarine.

Arginine decarboxylase (ADC), ornithine decarboxylase (ODC), putrescine (Put) and spermidine (Spd) contents were analysed in growing 'Murcott' mandarin fruits from bloom to full development. Enzymes of polyamine synthesis and, to a lesser extent, polyamine contents roughly followed patterns of growth activity in fruit tissues, with a maximum at the early stages, a minimum at mid-summer and intermediate values at full fruit development. ADC/ODC and Put/Spd ratios were 2-8 and 2-30, respectively. Possible links between polyamine metabolism and citrus fruit set and development are suggested.

(A. Altman)

Polyamines and organogenesis in thin-layer epidermal cultures of tobacco

Thin-layers of epidermis removed from the flowering peduncles of tobacco plants can be cultured under auxin-cytokinin ratios, which will yield either vegetative buds, floral buds or callus. We found that floral buds invariably had spermidine as the main polyamine, while vegetative buds contained putrescine as the main polyamine. Putrescine peaked early in vegetative buds, while spermidine peaks late in floral buds. This coincides closely with the time of determination of the individual bud type. DFMA at low concentration inhibited the development of buds in general but of floral buds in particular. At critical concentrations, it was able to reverse bud determination, that is, buds normally destined to become reproductive are now vegetative. DFMO by contrast, affected growth greatly and increased the number of bud primordia formed. It thus appears that polyamines are related to patterns of differentiation, and that specific enzyme systems functions in either growth or organogenesis.

(A.W. Galston)

E. Description of Cooperation

The long and good cooperation between the two research groups, which was established in our first joint BARD project (I-44-79), was continued during the present BARD project.

Each group devoted its efforts and time to specific, but interrelated, research areas, as indicated in this report:

A. Altman (Hebrew University, Rehovot): chapters 1-4

A.W. Galston (Yale University, New Haven): chapters 5-9

Data and conclusions resulting from the specific research areas were exchanged, either by mail or when the investigators met on several occasions. This helped considerably in planning and modifying specific experiments, and in preparing the annual reports.

The two principal investigators met for thorough discussions, and presented invited and submitted lectures on the work which was supported by BARD, on the following occasions:

1. 12th International Conference on Plant Growth Substances, Heidelberg, FRG, August 26-31, 1985.
2. International Conference on Polyamines in Life Sciences, Lake Yamanaka, Japan, July 14-18, 1986.
3. 1988 International Symposium on Polyamines in Biochemical and Clinical Research, Sorrento, Italy, June 13-17, 1988.

In addition, A. Altman actively participated in the 1985 and 1987 Gordon Conference on Polyamines, New Hampshire, USA.

This cooperation between the two research groups, supported by the BARD grant resulted in several important achievements which are reflected in the present report and the list of publications.

F. Evaluation of the research achievements

In our original proposal to this project we mentioned that "As a result of our earlier joint work (BARD I-44-79), polyamines are now considered as endogenous modulators of plant development and response to stress, including important practical areas such as senescence, salt stress and osmotic and pH stress. As we learn more about polyamine biochemistry and physiology in plants, we will be able to formulate conjectures and experiments concerning possible practical uses of polyamines in agriculture."

The main aims of the original research proposal included the following (see also part B of this report):

1. To study basic molecular mechanisms by which polyamines interact with senescence and stress, and with growth. Related to it are effects of applied and endogenous polyamines, as well as inhibitors of polyamine biosynthesis.
2. To study the distribution and localization of important polyamine biosynthetic enzymes.
3. To investigate the practical implications of our findings with respect to polyamine uptake and transport, modification of polyamine levels and physiological activity, and related applied aspects.

We also mentioned in the research proposal that "Continuing investigations are critical to the basic understanding of polyamine functions in growing and senescing plants under various agricultural practices, and this may lead to important practical utilization of the basic concepts."

In retrospect, after 3 years of intensive experimental work (and our previous BARD project), we feel that a major part of the original research aims has been achieved, and that polyamines significantly affect plant growth and response to stress and are causally linked (at least in some cases) to plant development. Moreover, several basic and applied aspects of polyamine physiology have been stu-

died and elucidated:

- A role for polyamines has been established in the following stress-related aspects:
NaCl salinity, growth and pathogenicity of phytopathogenic fungi, cadmium toxicity and osmotic stress, nitrogen depletion.
- A role for polyamine has been established in the following growth and development processes:
lateral root formation, embryogenesis and organogenesis in tissue cultures, fruit growth, protoplast regeneration potential, gibberellin-induced growth.
- Few basic-molecular aspects have been studied and elucidated: microautoradiographic localization of ODC in dividing cells, the polyamine inhibition of restriction endonuclease activity, polyamine binding to regeneration-related proteins, kinetics and inhibitor-sensitivity of polyamine biosynthetic enzymes, selection of cell lines with modified polyamine contents.
- Some of the above are of significant practical value: the use of enzyme inhibitors to control certain phytopathogenic fungi, improvement of *in vitro* organogenesis and embryogenesis, elucidation of pathways for secondary metabolite production (nicotine), uptake and long-distance translocation of polyamines, possible use of tissue polyamine levels and enzyme activity as guidelines for selection of stress-responsive genotypes.

Needless to say, not all the aims have been fully elucidated during the 3 years of the present research, and much is still left to be investigated. As with other research areas, many of the observed facts are, at this stage, good "correlations" rather than true cause-effect relationships. Yet, polyamines have been shown, in the present research to be causally-linked to several processes (mainly by the use of enzyme-activated inhibitors). Thus, we can conclude that polyamines are "true" endogenous modulators of plant development and response to stress.

We expected to do more on the molecular aspects of polyamine physiology and metabolism at the membrane and DNA level. Some has been achieved, but much is left to be done. We think that now, after most of the needed physiological and biochemical background information is available, the time is ripe for basic molecular work. This will certainly affect the practical utilization of polyamines. Cloning of genes for polyamine biosynthesis in plants is now possible (as found with animals and microorganisms), and this should be the major aim in future projects of this type.

G. List of Publications

1. Altman, A. *et al.* (1986). Polyamines in plants: hormonal role in differentiation and stress. Inter, Conference of Polyamines in Life Sciences, Japan (Abstract).
2. Altman, A., N. Levin, P. Cohen, M. Schneider and B. Nadel (1988). Polyamines in growth and differentiation of plant cell cultures: the effect of nitrogen nutrition, salt stress and embryogenic media. In: *Advances in Polyamine Research Vol. 5*, V. Zappia *et al.*, eds. Plenum Press (in press).
3. Altman, A. (1989). Polyamines and Plant Hormones. In: U. Bachrach and Y.M. Heimer, eds. *The Physiology of Polyamines*, CRC Press (In press).
4. Friedman, R., A. Altman and U. Bachrach (1985). Polyamines and root formation in mung bean hypocotyl cuttings, II. Incorporation of precursors into polyamines. *Plant Physiol.* 79: 80-83.
5. Friedman, R., N. Levin and A. Altman (1986). Presence and identification of polyamines in xylem and pholem exudates of plants. *Plant Physiol.* 82:1154-1157.
6. Kaur-Sawhney, R. and A.W. Galston. 1986. The role of polyamines in the regulation of proteolysis, in: *Plant Proteolytic Enzymes*, Vol. I, Michael J. Dalling, ed. CRC Press I: 141-148.
7. Kaur-Sawhney, R., Yao-ren Dai and A.W. Galston. 1986. Effect of inhibitors of polyamine biosynthesis on gibberellin-induced internode growth in light-grown dwarf peas. *Plant Cell Physiol.* 27:253-260.

8. Nathan, R., A. Altman and S.P. Monselise (1984). Changes in activity of polyamine biosynthetic enzymes and in polyamine contents in developing fruit tissues of "Murcott" mandarin. *Sci. Hortic.* 22:359-364.
9. Rajam, M.V., L.H. Weinstein and A.W. Galston. 1986. Kinetic studies on the control of the bean rust fungus (*Uromyces phaseoli* L.) by an inhibitor of polyamine biosynthesis. *Plant Physiol.* 82:485-487.
10. Schwartz, M., A. Altman, Y. Cohen and T. Arzee (1986). Localization of ornithine decarboxylase and changes in polyamine content in root meristems of *Zea mays*. *Physiol. Plant.* 67:485-492.
11. Tiburcio, Antonio F. and A.W. Galston. 1986. Arginine decarboxylase as the source of putrescine for tobacco alkaloids. *Phytochemistry* 25:107-110.
12. Tiburcio, A.F., R. Kaur-Sawhney and A.W. Galston. 1986. Polyamine metabolism and osmotic stress. II. Improvement of oat protoplasts by an inhibitor of arginine decarboxylase. *Plant Physiol.* 82:375-378.
13. Tiburcio, A.F., M.A. Masdeu, F.M. Dumortier and A.W. Galston. 1986. Polyamine metabolism and osmotic stress. I. Relation to protoplast viability. *Plant Physiol.* 82:369-374.
14. Viterbo, A., M. Dekel, B. Nadel and A. Altman (1988). Regulation of growth and differentiation in embryogenic celery cultures by polyamines, 2nd Int. Congr. of Plant Molecular Biology, Jerusalem (Abstract).

15. Weinstein, L.H., R. Kaur-Sawhney, M.V. Rajam, S.H. Wettlaufer and A.W. Galston. 1986. Cadmium-induced accumulation of putrescine in oat and bean leaves. *Plant Physiol.* 82:641-645.

In preparation (manuscripts) and submitted:

1. Shapira, R., A. Altman, Y. Henis and I. Chet (1988). Polyamines and ornithine decarboxylase activity during growth and differentiation in *Sclerotium rolfsii* (submitted).
2. Altman, A. and B. Cohen (1988). Polyamines inhibit restriction endonuclease activity towards plant DNA.
3. Cohen, P. and A. Altman (1988). Polyamine content in wild-type and resistant tobacco cell lines, and in response to NaCl stress.