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Lecture Course on

Polyamines as Modulators of Plant Development

Organized by

A. W. Galston and A. F. Tiburcio

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257 Lecture Course on Polyamines as Modulators of Plant Development

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I N D E X

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H.E. Flores - Catabolic Pathways and Secondary Metabolism of Polyamines in Plants.

N. Bagni - Transport and Subcellular Compartmentation of Polyamines in Plants.

J.A. Creus - Binding of Polyamines to Different Macromolecules in Plants.

2. **Regulation by polyamines and their biosythetic inhibitors of plant development: I. Cell division and in vitro plant regeneration**

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GENERAL DISCUSSION AND FINAL CONCLUSIONS.

INTRODUCTION

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INTRODUCTION

The polyamines putrescine, spermidine and spermine have recently been shown to be involved in a variety of plant growth and developmental processes, including cell division, vascular differentiation, root initiation, adventitious shoot formation, flower initiation and development, fruit ripening and senescence, and embryod formation in tissue culture (see recent reviews in 1-4). Many of these functions are similar to those mediated by known plant hormones such as auxins, cytokinins, gibberellins, abcisic acid, ethylene, and other miscellaneous compounds. In addition, direct and/or putative interactions of plant hormones and polyamines are also known (5). However, in spite of these facts, many plant physiologists still have some doubts about recognizing polyamines as another class of plant growth regulators. Some concerns are based on the facts that (a) polyamines exert their effects at higher concentrations than typical plant hormones usually do, and that (b) long distance transport of polyamines in plants is still a subject of some controversy. Major concerns are related to the lack of solid evidence to confirm the precise mechanisms by which polyamines exert their control in plant development. To counteract these last concerns, one could say that a very similar situation is found with regard to most of the known plant hormones. Thus, although the participation of the five major hormones has been known for many years, nowadays we do not know yet how they might be involved in plant development.

The plant polyamine field is a fast growing area which has as an important tool the possibility of comparing and sharing results with those obtained in animal and bacterial systems, in which it has been unequivocally demonstrated that polyamines are essential for growth and development. This is an advantage, with regard to specific plant growth regulators, which has probably helped to obtain rapid and significant achievements in the plant polyamine field, in spite of being a young emerging area.

This Lecture Course was organized to provide an up-to-date assessment and critical analysis of the involvement of polyamines in plant development. To introduce the subject, the first session was dedicated to analyse in detail the occurrence, distribution, biosynthesis, catabolism, secondary metabolism, transport, subcellular compartmentation, and binding of

polyamines in plants. The second session started with a review on the modulation of plant development by polyamines followed by lectures on the regulation by polyamines and their biosynthetic inhibitors of cell division and *in vitro* plant regeneration. The third session was dedicated to analyse the regulation by polyamines and their biosynthetic inhibitors of reproductive development and rooting. The mechanisms of polyamine action during plant development were also analysed in the last session.

Another aim of this Course was to obtain an active collaboration from the invited participants. Accordingly, in addition to the lectures of the invited speakers, 12 oral presentations were given by selected participants after evaluation of the posters. This was planned to allow young scientists to present recent data as well as new approaches and techniques for studying the involvement of polyamines in plant development.

Professor Arthur W. Galston was the author of a review entitled "**Polyamines as Modulators of Plant Development**" which was published in BioScience in 1983. This title was chosen for our Lecture Course in honour to Prof. Galston to recognize his important contribution to the plant polyamine field.

Finally, I want to thank Andrés González, from the Fundación Juan March, who has been a continuing help during the organization and development of this Course.

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FIRST SESSION

T. A. SMITH
R. D. SLOCUM
H. E. FLORES
N. BAGNI
J. A. CREUS

OCCURRENCE AND DISTRIBUTION OF POLYAMINES IN THE PLANT KINGDOM

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The polyamines spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$) and spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) probably occur in all animals and plants, together with putrescine ($NH_2(CH_2)_4NH_2$) which is considered to be the universal precursor for their biosynthesis. Cadaverine ($NH_2(CH_2)_5NH_2$), the higher homologue of putrescine, is found frequently, especially in members of the Leguminosae. These two diamines are the decarboxylation products of the amino acids ornithine and lysine respectively.

The guanidino-amine agmatine ($NH_2(CH_2)_4NHC(=NH)NH_2$), the decarboxylation product of arginine, occurs widely throughout the plant kingdom, together with putrescine. Potassium deficient plants accumulate large quantities of putrescine, and in extreme deficiency, putrescine accounts for up to 1.2% of the dry matter. Agmatine also accumulates in these plants, suggesting that it is the precursor of the putrescine, and the arginine decarboxylase responsible for the production of the agmatine is found to have an enhanced activity in potassium deficient plants (reviewed by Smith, 1984; 1991). However putrescine is now known to accumulate in many conditions of stress in a wide range of plants, notably with acid feeding, ammonium nutrition, osmotic shock, cold injury, desiccation, anoxia, and heavy metal, ozone and herbicide damage.

On feeding agmatine to barley seedlings, another amine is found, N-carbamoylputrescine ($NH_2(CH_2)_4NHC(=O)NH_2$), indicating that this is an intermediate in the production of putrescine from agmatine. This amine occurs only rarely as a natural product, but it has been found in *Sesamum* (Crocomo and Basso, 1974) and in sugar cane tissue cultures (Maretzki *et al*., 1969). The pathway found in barley for putrescine formation via agmatine now appears to be widespread in the Plant Kingdom. In *Lathyrus sativus* seedlings, homoarginine is decarboxylated to homoagmatine, and lysine to cadaverine (Adiga and Prasad, 1985), and there appears to be a metabolic pathway analogous to that found for arginine and ornithine.

N^6-Methylagmatine has now been detected in the seeds from a wide range of leguminous plants (Matsuzaki et al., 1990a).

In the shoots of barley, spermine was converted to diaminopropane, ($NH_2(CH_2)_3NH_2$), and diazabicyclononane, the cyclization product of $NH_2(CH_2)_3NH(CH_2)_3CHO$. These were shown to result from the activity of a polyamine-specific oxidase which is very active in the grasses, and both of these oxidation products have been shown to occur naturally. Another cyclic amine, pyrroline, formed by the activity of diamine oxidase on putrescine has been found in pea seedlings (reviewed by Smith and Barker, 1988). Diaminopropane is the precursor of pyrazole in cucumber seedlings (Brown and Diffin, 1990).

Homologues of spermidine and spermine are found quite frequently in the lower plants. For example, homospermidine ($NH_2(CH_2)_4NH(CH_2)_4NH_2$) has been demonstrated in many mosses and ferns, in algae (Yamamoto et al., 1984), and in some higher plants, notably in Santalum (Kuttan and Radhakrishnan, 1972), in the water hyacinth (Eichhornia) (Yamamoto et al. 1983), and Heliotropium (Birecka et al., 1984). In Heliotropium, homospermidine is the precursor of the pyrrolizidine alkaloids. Unusual polyamines are also found in members of the Leguminosae, notably Canavalia which contains $NH_2(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ (canavalmine), together with the penta-amines $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ and $NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ (Matsuzaki et al., 1990b) in addition to homospermidine. Homospermidine also occurs in the nitrogen-fixing nodules of pea seedlings where it is associated with the bacterium Rhizobium (Smith, 1977). Similarly, the occurrence of polyamine homologues in the nitrogen fixing fern Azolla is associated with the presence of the algal symbiont (Corbin et al., 1989). Other long chain polyamines found in the algae, mosses and ferns include nor-spermidine ($NH_2(CH_2)_3NH(CH_2)_3NH_2$) and nor-spermine ($NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$) (Yamamoto et al., 1984; Hamana et al., 1988; 1990). Nor-spermidine and nor-spermine also occur in Medicago sativa grown in conditions of drought (Rodríguez-Garay et al., 1989; Kuehn et al., 1990). The presence of these unusual polyamines suggests the activity of a polyamine oxidase, the

only known source of diaminopropane, and of a non-specific aminopropyltransferase.

Other structures related to the di- and polyamines which are found in higher plants are listed by Smith (1985).

Conjugates of the di- and polyamines as amides of hydroxycinnamic acids are found in large amounts in many plants, but especially in the leaves and flowering shoots of tobacco (Martin-Tanguy, 1985; Negrel, 1989). Amides of 2-hydroxyputrescine with hydroxycinnamic acids occur in rust infected wheat (Stoessl et al., 1969). Similar conjugates are also found in a wide range of plants in the form of complex dimers. An example of this is seen in barley seedlings which contain coumaroylagmatine, together with its antifungal dimers, known as the hordatines (Smith and Best, 1978). Other complexes of the hydroxycinnamic acids with the polyamines are found as the so-called macrocyclic alkaloids, which occur in a wide range of families (Smith et al., 1983). However the function of these is at present obscure.

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BIOSYNTHETIC PATHWAYS OF POLYAMINES IN PLANTS

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The major metabolic pathways for the biosynthesis of polyamines in plants have been elucidated (1, 2), although our understanding of biochemical and physiological mechanisms regulating polyamine biosynthesis is in its infancy. The biosynthesis of common polyamines can be conveniently divided into two main steps, i.e., synthesis of the diamine putrescine (Put), and formation of the polyamines spermidine (Spd) and spermine (Spm) resulting from propylamino addition to the Put precursor.

As is shown in **Fig. 1**, Put is synthesized via one of two main pathways in plants. It can be formed directly by decarboxylation of L-ornithine (Orn), in a reaction catalyzed by ornithine decarboxylase (ODC). This pathway represents the sole route to Put formation in animals and most fungi (3, 4). Alternatively, the decarboxylation of L-arginine (Arg) by arginine decarboxylase (ADC) leads to the formation of Put, through agmatine (Agm) and *N*-carbamoylputrescine (NCPut) intermediates. A modified version of this second pathway, in which Agm to Put conversion is carried out by a single enzyme, agmatine ureohydrolase, is found in bacteria (3).

The existence of these dual pathways is supported by a variety of studies utilizing labeled precursors and intermediates, as well as specific inhibitors of ADC and ODC. However, alternate routes to Put synthesis, via Put carbamoyltransferase (PutCT), citrulline decarboxylase (CitDC) and "Put synthase" have also been proposed in some plants. Evidence supporting the participation of these enzymes in Put synthesis will be reviewed.

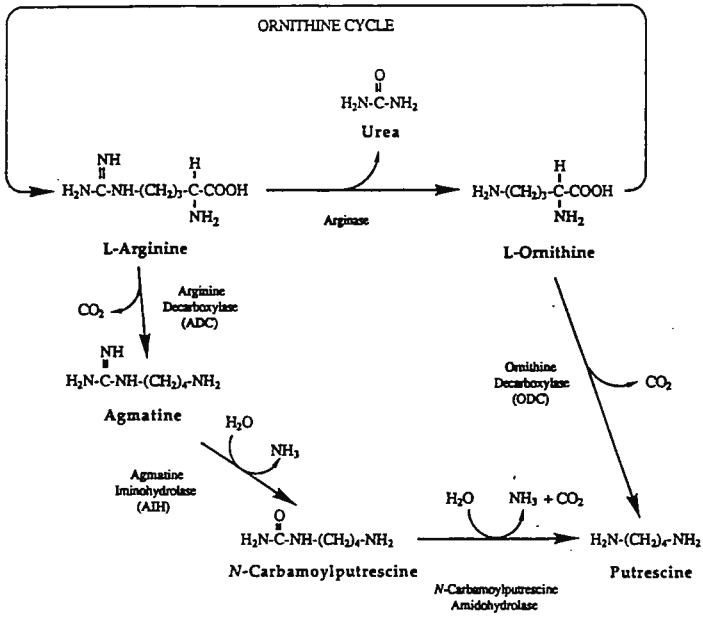

Fig. 1. Biosynthesis of putrescine.

The synthesis of Spd and Spm is carried out by addition of an aminopropyl group to one or both primary aminogroups of Put by Spd and Spm synthases, respectively (see **Fig. 2**). It is now well-documented that decarboxylated *S*-adenosylmethionine (dSAM) is the aminopropyl donor in the synthesis of these amines. Decarboxylated SAM is derived from SAM in a reaction catalyzed by SAM decarboxylase (SAMDC). SAM, in turn, is derived from L-methionine via SAM synthase.

Sincethe plant hormone ethylene is also derived from a SAM precursor, and because ethylene and polyamines promote and inhbit senescence in plant tissues, respectively, there has been considerable interest in the metabolic interactions between the ethylene and polyamine biosynthetic pathways in recent years.

All of the major enzymes involved in the synthesis of polyamines have been purified and biochemically characterized and the gene encoding oat ADC has recently been cloned (5). However, the general unavailability of antibodies to enzyme proteins, or specific DNA probes for genes encoding them, has made it difficult

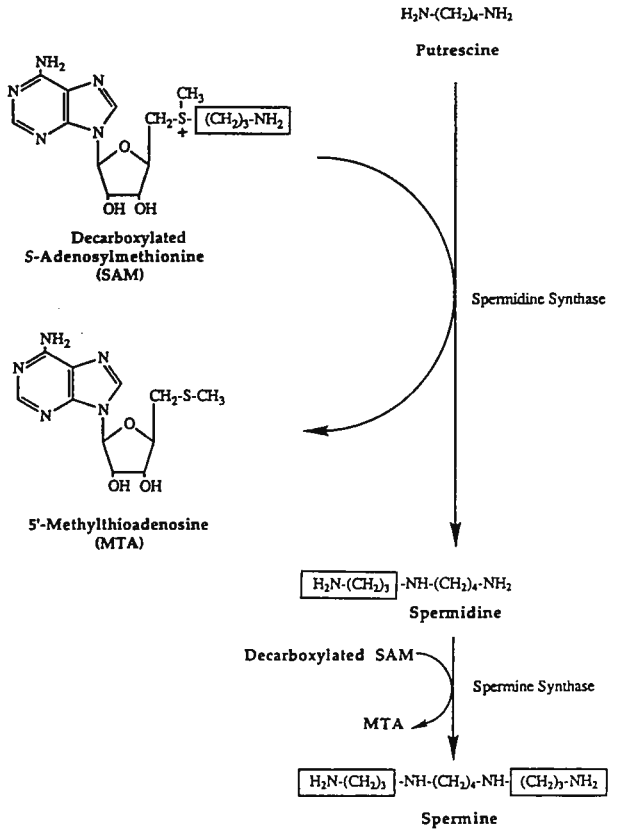

Fig. 2. Synthesis of spermidine and spermine.

to investigate various mechanisms regulating polyamine biosynthesis *in vivo*. Specific inhibitors of ADC and ODC have markedly facilitated our understanding of Put biosynthesis but analogous inhibitors of SAMDC and Spd/Spm synthases do not exist or are of limited usefulness in *in vivo* investigations. Studies addressing questions relating to the integration of polyamine biosynthesis into overall carbon and nitrogen metabolism in plants have been rare. The need for such "global" approaches in future metabolic studies will be discussed.

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CATABOLIC PATHWAYS AND SECONDARY METABOLISM OF POLYAMINES IN PLANTS

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The various functions proposed for di- and polyamines in plant cells require an understanding of the mechanisms controlling the levels and localization of these compounds. As with any other metabolites, the endogenous di/polyamine levels are controlled by the rates of synthesis, degradation, and diversion into conjugated forms. The later two mechanisms are discussed here.

The known catabolic pathways for di/polyamines operate through the oxidative deamination/cleavage catalyzed by amine oxidases. Animal amine oxidases have been well characterized in many systems, and oxidize acetylated di/polyamine preferentially, especially *in vivo* (1). In contrast, less is known about plant amine oxidases, and those which have been characterized so far act on the free protonated forms (2). Our current knowledge of di/polyamine oxidation in plant systems derives from studies of the copper-containing diamine oxidases (DAOs) of the Leguminosae, and the flavin-containing polyamine oxidases (PAOs) of the Gramineae. Di/polyamine oxidases have also been reported, and in a few cases purified from the families Compositae, Cucurbitaceae, Euphorbiaceae, Pontederaceae, and Solanaceae.

DAOs are generally dimeric glycoproteins which may represent a significant fraction of the total plant protein (for example, up to 3% in etiolated pea stems). In fact, the specific activities of plant DAOs can exceed those of the animal enzymes by more than 100-fold! Although legume DAOs act primarily on diamines (putrescine and cadaverine), the polyamines proper can also be subtrates. In all cases, DAOs act on the primary amino group, and the common byproducts of the reaction are NH_3 and H_2O_2. With the exception of the enzyme from *Oryza*, all plant DAOs known to date contain two copper (Cu II) atoms per dimer. The *Oryza* DAO resembles a plant PAO in that it contains FAD. The nature of the covalently bound cofactor of plant DAOs is still controversial. Earlier studies suggested that pyridoxal phosphate (PLP) is the cofactor, while recent reports argue for pyrroloquinolinequinone (PQQ). The present status is that PQQ has not been unequivocally identified in any amine oxidase (animal or plant). In fact, what was previously thought to be PQQ in mammalian DAOs has now been conclusively identified as 6-hydroxydopa (3). DAOs may show heterogeneity in some species, as in the case of lentil which contains at least 6 isoforms (see 2). In contrast with DAOs, plant PAOs are usually monomeric glycoproteins which contain FAD. These enzymes oxidize the secondary amino groups of spermidine and spermine. Thus, free NH_3 is not a byproduct of the reaction; instead, an aldehyde and diaminopropane are formed. As with DAOs, the reaction catalyzed by PAO also yields H_2O_2. Also in contrast with DAOs, PAOs are very specific for polyamines.

The action of DAO or PAO is only the first step in the reutilization of the carbon and nitrogen of the aliphatic amines. The fate of the NH_3 produced by DAO activity (diaminopropane in the case of PAO) is very poorly understood. We know a bit more about what happens to the other oxidation products. For

example, the 4-aminobutyraldehyde obtained by the action of DAO on putrescine (or of PAO on spermidine) can be further oxidized by a NAD-dependent dehydrogenase (4) to γ-aminobutyric acid (GABA). GABA can in turn be transaminated and the resulting succinic semialdehyde is further oxidized to succinate, a Krebs cycle intermediate (Fig. 1). This pathway thus ensures the recycling of the di/polyamine carbon skeleton, and part of the nitrogen. The amine oxidases and aldehyde dehydrogenase appear to act in a concerted manner, as both activities are always present together. Metabolic inhibitors for some of these reactions are available, providing useful probes for di/polyamine catabolic controls (4, Fig. 1).

Because of their role in the regulation of endogenous di/polyamine levels, the physiology of plant DAOs and PAOs has received a fair amount of attention. These enzyme activities appear to be influenced by numerous exogenous and endogenous factors, including light, plant hormones, and natural inhibitors. Photoreversibility experiments also suggest that the effects of light on DAO activity are mediated by phytochrome (see 2). Gradients in DAO and PAO have been observed which parallel the di- and polyamine gradients found in both monocots and dicots (see 5).

Perhaps the most intriguing aspect of DAO and PAO physiology in higher plants has little to do with the primary products of the reactions, and is instead related to the cellular localization of these enzymes. Cytochemical, immunohistochemical and biochemical evidence accumulated in the last 10 years strongly suggests that DAO and PAO are present in the cell wall. The enzymes appear to be loosely bound components of the apoplast, since they can be extracted by vacuum infiltration or by elution with low ionic-strength buffers. Particularly striking is the presence of high DAO/PAO activity in lignified cells (esp. vascular bundles). High DAO/PAO and peroxidase activities usually appear together, and gradients of both enzymes show parallel variation. This has led to the suggestion that the H_2O_2 produced by di/polyamine oxidation can be utilized for the polymerization of lignin and suberin precursors, or in the crosslinking of extensin and polysaccharide-bound phenols (2).

The pathway discussed above (Fig. 1) accounts for di/polyamine catabolism in the Leguminosae, Gramineae and a few other families. In many plant species, however, "conventional" free-di/polyamine oxidizing systems are not detectable, even though labelling experiments indicate that the amines are in fact being metabolized. This oddity has been examined in some detail in tobacco cell cultures (6). The XD line of tobacco is capable of growth in organic as well as inorganic nitrogen sources. In some cases, as when shifted from nitrate to urea, arginine and GABA, cells can adapt fairly well to the organic nitrogen source. For other sources such as putrescine, lengthy adaptation and cell selection are required. By repeated subculture and selection, we were able to obtain an XD-derived cell line which is capable of growing on putrescine as the only nitrogen source. This putrescine-utilizing line (PUT) is capable of efficiently metabolizing putrescine to GABA, as shown by labeling experiments and by blocking further GABA metabolism with a specific inhibitor of GABA-transaminase (6). However, we could not detect any putrescine oxidase activity using free putrescine as the subtrate. Recent evidence (Flores, unpubl.)

suggests that the pathway for putrescine utilization in PUT cells proceeds through conjugated intermediates. If PUT cells are labelled with ^{14}C-putrescine, the label shows up quickly in caffeoyl-putrescine and other cinnamic acid amides. Furthermore, if the label is chased, we observe an apparent turnover of the conjugates into GABA. The presence of caffeoyl-GABA in PUT cells and its absence from the control XD cells (which are not able to metabolize putrescine into GABA), is consistent with our hypothesis. It is also significant that caffeoyl-GABA is found in the plant only in flowering parts. It is this organ which can most efficiently convert labelled putrescine into GABA (Flores, unpubl.).

In addition to the oxidative pathway discussed above, the levels of di/polyamines in plant cells can be controlled by diversion of these compounds into conjugated forms or other secondary metabolites. The conjugation of di/polyamines to macromolecules is discussed by Creus at this meeting. The only low molecular weight conjugates of polyamines identified so far in higher plants are the hydroxycinnamic acid amides (HCAs). These compounds are mainly present in flowering, and generally absent from vegetative structures (with the exception of roots during certain developmental stages). HCAs can build up to very high levels, representing over 80% of the total di/polyamine pool in flowers, and a major portion of the soluble nitrogen pool. The presence of HCAs has been correlated with male fertility, and it has been suggested that HCAs may have an important developmental function (see 7). However, the evidence supporting this view is far from conclusive.

Di- and polyamines can be precursors of a wide variety of alkaloids (8). The best studied putrescine-derived secondary metabolites are the nicotine and tropane alkaloids of the Solanaceae. These two pathways share a common precursor for the synthesis of the nitrogen ring. In the case of nicotine it is well estabished that a symmetric molecule such as putrescine is incorporated into the pyrroline ring. In contrast, it had been accepted until recently that the tropine ring of hyoscyamine and scopolamine derives from an asymmetric intermediate (∂-N-methylornithine). It now appears that putrescine can also be an efficient precursor for the tropine moiety (10). The synthesis of the nicotine alkaloids of the Solanaceae, and the pyrrolizidine alkaloids of the Asteraceae (also present in Leguminosae and Boraginaceae) occurs only or mostly in the roots. The alkaloids can then be transported to the aerial parts, where they accumulate or are further metabolized (9). The ability to grow isolated roots in culture has allowed recent progress in understanding the biochemistry and molecular biology of these alkaloids and other root-specific metabolites (9,11).

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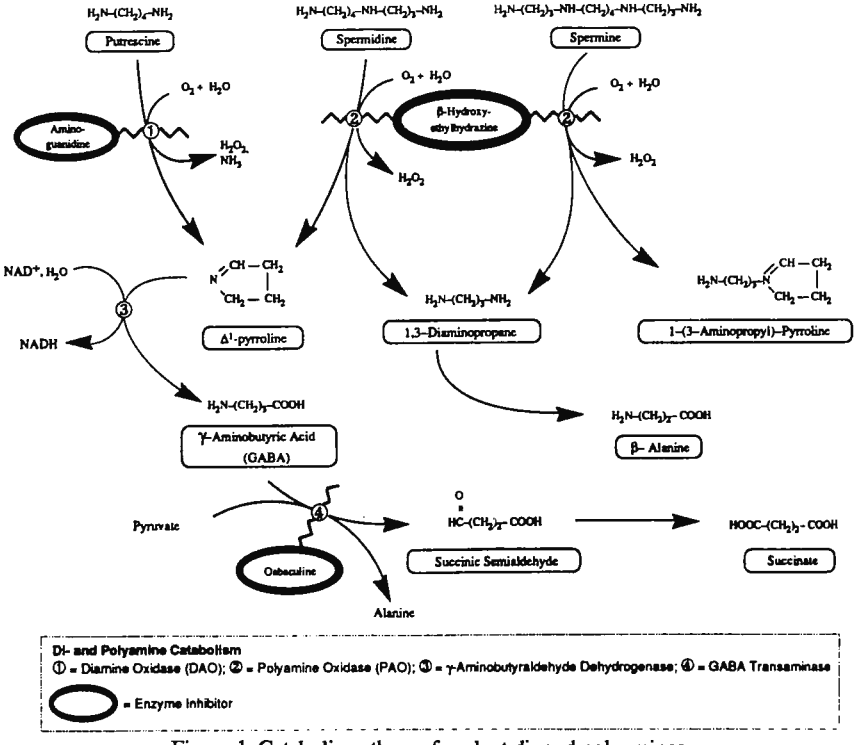

Figure 1. Catabolic pathway for plant di- and polyamines.

TRANSPORT AND SUBCELLULAR COMPARTMENTATION OF POLYAMINES IN PLANTS

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In animal and bacterial sistems it is known since many years that exogenous polyamines are transported and accumulated by cells.

In plants studies on polyamine transport were initiated only later although it represents an important point in order to consider these substances as hormones rather than as second messengers.

Long distance transport studies created the basis for the contention that polyamine transport has a physiological role. This evidence was supplied first by the presence of endogenous polyamines in xylem and phloem exudates of various plant species (1). Then we studied the characteristics of uptake of exogenous polyamines by roots of tomato and maize seedlings and their translocation. It was revealed that exogenous putrescine was taken up via roots and translocated it and its metabolites (spermidine and spermine) in the upper part within 15 - 30 minutes. Translocation was temperature and relative umidity dependent. Mainly depend by xylem transpiration, partly due to root pressure.

Basipetal transport also occurs, but to a lesser extent (2).

In further studied it was chosen different plant materials such as isolated cells in culture and subcellular organelles to have information on the cellular localization and compartmentation of absorbed polyamines as well.

Carrot cells displayed a rapid uptake with saturation after 1 to 2 minutes. which is more rapid of other system previously examined than that of isolated apple corymbs (3) and Saintpaulia petals (4 - 5). Concentration dependance showed a biphasic system for carrot cells, with two saturable components for putrescine and one saturable component for spermidine followed by a linear one (6). It has reported that polyamines con bind to cell wall constituents and that endogenous polyamines are present in this compartment. The problem arose on the localization of the absorbed molecules in the cell. It appared that most of the putrescine was in the cytoplasmic soluble fraction, while 73% and 78% of spermidine and spermine respectively were bound in the cell wall of carrot cells (6).

Owing to the large amount of polyamines (spermidine and spermine) bound to the cell wall after the uptake assay. subsequent studies were carried out using protoplasts of carrot and vacuols isolated from them so that the compartmentation analysis could be extended.

As already observed for the cell walls, polyamines binding to the membranes during the uptake experiments was also observed (8).

The uptake of all three polyamines into protoplasts and vacuoles reached a maximum within 1 to 2 minutes and did not increase further. Ca^{2+} up to 1mM, increasingly enhanced uptake of all the three polyamines.

The compartmentation analysis was performed in regard to the endogenous molecules and to the absorbed one. It revealed that 42 and 28% of the endogenous putrescine and spermidine, respectively, were localized in vacuole.

Data on the compartmentation of the exogenously supplied molecules were abtained in two different ways incubating protoplasts and isolating vacuoles from them with higher concentration of unlabelled spermidine (100 mM) or lower concentration of labelled spermidine (6.6 μM).

In the first case the spermidine content of the vacuoles increased sixfold with respect to the endogenous content, in the second case 27% of labelled spermidine it was found to be localized in the vacuoles. Leakage of the polyamine occuring during the isolation procedure could have affected both the endogenous and the exogenous data; in any case, it appears that vacuole represents a storage site, possible a temporary one, for polyamines that could explain the often observed discrepancy between the high intracellular polyamine concentration of up to 1 mM and more, and the physiological concentration needed for a proper function of the polyamines on the growth that in the case of the plants are between 10 to 100 μM.

From the previously reported data, it appears that Ca^{2+} is necessary for polyamine transport.

Further evidence was that Ca^{2+} stimulated uptake was inhibited by 50% in the presence of 10 uM FCCP. La^{3+} also stimulated spermidine uptake by fourfold, but La^{3+} -stimulated uptake was not inhibited by FCCP as well as of spermine alone. Ca^{2+} seems to act at sites on the external surface of the cell, because the ionophore A23187 did not enhance its effect and because La^{3+} which does not penetrate the plasma membrane, has a similar stimulatory effect. Nevertheless the effect of Ca^{2+} appears to be more specific than that of La^{3+} (8).

The study on the uptake and compartmentation of polyamines was extended to mitochondria from tuber of Helianthus tuberosus. The driving forces for polyamine uptake in mithocondria are clearly identified by using different ionophores, such as valinomycin and nigericin, which abolish membrane potential and pH across of membrane respectively and FCCP which causes the suppression of the total eletrochemical gradient. The complete block of spermidine uptake was obtained only with valinomycin and FCCP while nigericin only slightly affected it (9).

As Conclusive Remarks

In spite of the great number of bindings, polyamines enter into the cell and subcellular compartments and can be translocated from one part to another in the plant. The presence of polyamines stored in the vacuole, their localization in other compartments such as mitochondria, their interaction with cell wall constituents, and the existence of conjugated polyamines and di-and polyamine oxidase activities, suggest that all these events may partecipate in the regulation of the cytoplasnic levels of polyamines. In fact, dispite their high internal concentration, in the millimolar range, there is a requirement for exogenous polyamines at conc. ranging from 10 to 100 μM for cell division to occur, when endogenous polyamines are lacking or below μM. Thus, the presence of a subcellular compatmentation of polyamines, similar to that suggested for Ca^{2+}, their rapid interconversions between free and conjugated forms and their active transport into the matrix space of mitochondria and through the plasmalemma and the mediated tansport across the tonoplast are suitable explanation for the modulation of the plant response to polyamines.

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BINDING OF POLYAMINES TO DIFFERENT MACROMOLECULES IN PLANTS

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Most of studies on plant polyamines (PAs) have been conducted till now on free aliphatic PAs, mainly putrescine (Put), spermidine (Spd) and spermine (Spm). Nevertheless, these PAs represent only one of the polyamine types present in plants. At present there is an increasing interest on the other types, mainly on bounded PAs that could exert an important function in plant development.

Due to their chemical nature PAs bind easily with many different macromolecules. Bound PAs and/or the binding of them present different effects on plant physiology.

Polyamines bind to:

- phenols (mainly hydroxycinnamic acid) forming phenolamides (hydroxycinnamides).
- membrane phospholipids
- uronic acids and lignine in cell walls
- DNA
- RNA (mRNA, tRNA, rRNA)
- ribosomes
- proteins

Hydroxycinnamides

The most common conjugates are:

. hydroxycinnamoyl, alquilcinnamoyl and feruloyl-Put, found in *P. macrophylla, N. tabacum, P. gratissima, P. hibrida, Salix, S. subaphylla, C. paradisi, A. comosus, G. globosa* and various solanaceae and graminae especies.
. caffeoyl-Spd
. cumaroyl-Agmatine
. different combinations between several PAs (Put, Spd, Spm, tyramine) and phenolic groups (cumaril, caffeil, ferulil and sinapil) are present in many species.

In tobacco callus-cell cultures cumaroyl, caffeoyl and feruloyl-Put have been isolated. A diamine especific hydroxycinamoyl-CoA:hydroxycinnamoyl transferase using feruloyl-CoA and caffeoyl as acyl donors was present in cell suspension.

To determine conjugated PAs an acid hydrolysis is performed. In this way, PAs are liberated from cinnamic amides at 105-120 °C in a 6M.HCl solution during 24 hours. This process was originally stablished for urine samples and at present is used in plants as in animals.

A method to determine only the cinnamic amides in plants is used by Martin-Tanguy and co-workers (1978). In this method after cinnamic compounds extraction PAs are liberated by an acid hydrolysis as described above.

Membrane phospholipids

First evidences of polyamine binding to membranes were obtained observing that Spm was able to stabilize suspended cells.

Correlations between PA amounts and membrane phospholipids in marine bacteria and *E. coli* have been described. When DL-α-Difluoromethylornithine (DFMO) was supplied Ornithine decarboxylase (ODC) activity and phospholipid content decreased.

Membrane stabilization is not due to an osmotic effect because PA quantities are too small. The efect may be on phospholipid acid groups reducing the repulsion forces. The effect is specific and not only due tho the cationic forces because Ca^{+2} or Mg^{+2} are not substituts of PAs.

In senescencing *Hordeum* leaves PAs are bounded to thylacoid membranes reducing chlorophyll loss.

The binding of Spm to mytochondrial membrane prevents the membrane potential drop in saline stress. Furthermore, the binding avoid the Ca^{+2} and phosphat fluxes.

Experiments with artificial membranes show that interactions with phospholipids are different in the case of Put than in the case of Spd and Spm.

The most interesting aspect of interaction between PAs and membranes is observed during cell senescence. The first antisenescence action of PAs is the stabilization of membranes.

Cell walls

In differenciated and senescent cells PAs are bounded to lignin and uronic acids. Vallée et al. (1983) have found PAs linked to cell walls in different organs of *Nicotiana tabacum* and *Lycopersicum esculentum*. In roots of these plants linked tyramine was detected in high contents.

Nucleic acids (NA)

In the seventies' PAs were related with NA and associations were found in every cellular form studied. Most of studies were conducted by crystallography, by this way Spm - the easiest crystalizable PA - is the most used in experiences.

Two types of binding NA-PAs exist, one specific, the other not specific. Atraction between NA negative charges and PA positive charges constitutes the non specific bound. The specific binding needs specifics sequences to take place.

Polyamines and NA are also related in a biosynthetic substrate level. Carbamoyl phosphate is a substrate for Put and pyrimidin nucleotides biosynthesis. The flow to one or other product is regulated by extracellular factors that modulate aspartate carbamoyltranspherase (ACT) - nucleotide formation - or ornithine carbamoyltranspherase (OCT) - Put formation - activities. Specifically, during embriogenic stimulation OCT activity is increased allowing a fast Put biosynthesis whereas ACT levels are not affected.

Relations between PAs and NA in a biosynthetic level are also observed when a senescence process is induced.

Only partially and in some cases Mg^{+2}, a polivalent ion, can substitute PAs.

Deoxyribonucleic acid (DNA)

Thirty years ago was speculated a possible binding between positively charged aminated residues of PAs and negatively phosphate residues on opposite DNA strands. They suggested that tetramethylene chain portion of PA spans the minor groove of DNA. This model was postulated by Liquori et al. (1967) for Spm but is also useful for Spd. The model ascribes a frame rol to PA because binding confers rigidity to DNA structure.

X-ray diffraction studies showed that one Spm molecule is bounded to a specific double-stranded B-DNA dodecamer (CGCGAATTCGCG). According these observations Feuerstein et al. (1986) proposed a model in which Spm is docked into the major groove of B-DNA. Binding is stabilized by maximizing interactions between proton acceptors on the DNA and proton donors on Spm. Each amine in Spm hydrogen bonds to two sites on the DNA molecule involving either phosphate oxygen or the N^7 of guanine . This binding alters the oligomeric sugar puckering and intrastrand phosphate distances. The induced bend, 25°, is similar of the induced bend in anticodon zone of nucleotide sequence of tRNA.

Some reports imply PAs with DNA condensation and B-Z

conformation transitions. In fact, variations of Spd and Spm levels can result on B active form to Z inactive form transition.

Ribonucleic acids (RNA)

The first evidence of RNA and PAs relations was the fact that PAs estimulate RNA polymerase and subsequently RNA biosynthesis.

In activated *Helianthus tuberosus* tubers it has been determined an specific sequence (poly-A) which binds specifically Put (Serafini-Fracassini et al., 1984). The binding is stablished, as in DNA, between aminated groups of PA and ionized RNA phosphates.

Arginine and lysine increase DNA synthesis in cereal protoplasts. Nevertheless, the addition of Put, Spd, Spm stabilizes protoplasts and causes a great DNA and RNA synthesis.

In *Helianthus tuberosus* tubers PA application causes a dormancy break with NA synthesis. When canaline or canavanine are added RNA synthesis is supressed, showing the importance of PAs in the process.

Binding of PAs allows macromolecule compactation. An RNA virus posseses not interchangeable Spd that is responsable of nucleus packing.

Bound PAs neutralize negative charges of phosphate groups protecting RNA from RNAse activity.

Dormant Jerusalem artichoke tubers present low PA levels. Break of dormancy increases Put, Spd and Spm levels. Most of produced Put is found bounded to rRNA, tRNA and poly-A fractions. A protective action against RNAses and a potentiation of RNA activity has been suggested.

Crystallographic studies showed the presence of two Spm molecules bounded specifically to a tRNA molecule. The Spm bounds and Mg^{+2} presence have been stablished as necessaries for optimal tridimensional tRNA structure.

Ribosomes

Ribosome structure is completely dependent of Mg^{+2} and PAs. There is an important competition between both anions, but both of them are necessary at different physiological concentrations.

Proteins

Is well known that PAs are involved with proteins at different levels of their metabolism.

An important question is PA effects on enzymatic activity. Procariotic topoisomerases are inhibited by Spm while eucariotic topoisomerases are stimulated by PAs. The enzyme 1-aminocyclopropane-1-carboxylic synthase (ACC-synthase) is inhibited by PAs because Schiff´s basis are formed with Pyridoxal 5´-phosphate (PLP) the prostetic group of enzyme. A relation between RNAses, proteases, peroxidases, phospholipases, quinases and other minor enzymes are well documented.

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SECOND SESSION

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POLYAMINES AS MODULATORS OF PLANT DEVELOPMENT: A REVIEW

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Polyamines have been linked to a variety of growth and developmental processes in plants, including cell division, vascular differentiation, embryoid formation in tissue culture, root initiation, adventitious shoot formation, flower initiation and development, and control of fruit ripening and senescence. Each of these processes has been reviewed recently (1-6), and will be analyzed in detail by speakers coming later in this program. Accordingly, they will not be discussed further here. Instead, I would like to examine and evaluate briefly the kinds of evidence that have been advanced to adduce regulatory roles for the polyamines in plants.

One obvious suggestive approach flows from systematic analyses of individual plant parts at particular stages of development. Such an approach with tobacco led Martin-Tanguy (7) to suggest a relation, possibly causal, between high titers of polyamines conjugated to hydroxycinnamoyl acids and the onset of flowering. Such evidence is entirely correlational and suggestive, and requires further rigorous testing to validate the hypothesis.

A second approach is to apply exogenous polyamines, note effects on plant behavior, and hypothesize that polyamines limit, and thus control the process in question. This approach was used by Bagni and Serafini-Fracassini (8) to suggest that polyamines control the emergence of Helianthus tuberosus tuber cells from dormancy and by Dibble et al (9) to infer that polyamines control the senescence of tomato fruits. One should note that since different substances can produce apparently similar physiological results in plants (e.g. auxins and gibberellins on cell elongation), it is necessary to supplement such evidence with other critical experiments.

A third approach is to reduce or annul the biosynthesis of polyamines with specific inhibitors. If such treatment results in some physiological aberration which is corrected by the addition of a polyamine, then it is logical to infer that polyamine levels

may naturally control the process in question. This approach was used by Cohen et al (10) with DFMO to adduce a critical role for ODC-produced polyamines in cell division in young tomato fruits, by Feirer et al (11) with DFMA to adduce a role for ADC-produced polyamines in in vitro embryogenesis in carrot, and by Sawhney et al (12) with cyclohexylamine to adduce a role for spermidine in floral differentiation in thin-layer tobacco tissue cultures. This approach is somewhat more rigorous than the second one, but still suffers from the possibility that neither inhibitor action nor the "reversal" by applied polyamine may be as specific as asssumed.

A fourth approach notes an interaction between polyamines and another regulatory plant hormone, and conjectures that the observed effect of applied polyamine is exerted through action on or interaction with that system. This approach was used by Saftner and Baldi (13) in suggesting that the observed effect of polyamines in retarding the senescence of excised tomato fruits occurs through inhibition of the production or action of ethylene. This approach must be fortified by experiments detailing the proposed interactions in vivo, both on titer of the interactive compound, and on the physiological process.

A fifth approach involves the generation of mutants or the selection of variants with some aberration or lesion in the chain of reactions leading to polyamine biosynthesis or action. This approach has been successful in microorganisms, including yeast. The work of C.W. Tabor (14) has shown clearly that a mutation inactivating the ODC locus prevents growth, while a mutation at the spermidine synthase locus permits slow growth but not sporulation. This demonstrates clearly the essentiality of polyamines and the specific role of particular polyamines. With higher plants, Malmberg and colleagues (15) have regenerated tobacco plants from single cells resistant to inhibitors of polyamine biosynthesis by virtue of their overproduction of polyamines. The abnormal floral structure and flowering physiology of such plants supports the theory that polyamines are somehow related to flowering, but because higher plants have prominent ADC and ODC pathways, it is probably more difficult to get as definitive results with single mutants as one can get with yeast.

To achieve entirely unambiguous results, it would now seem necessary to employ the techniques of molecular biology. The use of characterized mutations or of antisense messages that damp out polyamine production should tell us about the essentiality and role of these compounds, and the production of transgenic plants should help us to understand the effects of variation in gene dosage, and therefore of polyamine titer. It would seem timely for polyamine research to move from the establishment of correlations to the delineation of precise mechanisms, and therefore of the function and significance of polyamines in the organism.

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POLYAMINE BIOSYNTHESIS AND CONJUGATION TO MACROMOLECULES DURING THE CELL CYCLE OF *Helianthus tuberosus* TUBER.

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Since polyamines (PA) are normal constituents of all cells, their function in growth can be studied not by supplying them exogenously, which can sometimes cause inhibition, but rather by depleting the cells or by supplying them to depleted cells. PA - depleted cells can be obtained by: 1) inducing mutants deficient in PA synthetic enzymes,2) inhibiting the activity of the PA synthetic enzymes, 3) utilizing temporarily deficient organs. In higher plants the occurrence of multiple pathways of putrescine biosynthesis causes great difficulties in obtaining PA- depleted mutants. The use of specific inhibitors of PA biosynthetic enzymes is easier, but also in this case there are problems. In fact a complete PA depletion of the plant cell (especially in short- term experiments) is difficult to obtain bacause there are : alternative (compensatory ?) biosynthetic pathways, large amounts of intracellular PA, PA have slow turnover rates (at least in animals) and PA can probably be stored in a bound form (undetectable with the usual methods).

In our studies, we used the last two systems, namely: inhibitors of the activities of the biosynthetic enzymes in a temporarily deficient organ, like the dormant tuber of *Helianthus tuberosus*.Plants of *H. t.* propagated by vegetative reproduction, provide a homogeneous tissue: the medullary parenchyma of the tuber. The latter, formed in late summer, becomes dormant in early winter. Its cells cease multiplying early on, enlarge and accumulate reserve substances. The cells, all in G_0 phase, are characterized by a large vacuole and a thin layer of cytoplasm which includes nuclei,adherent to the thin cell wall; their non-green plastids contain tubular complexes[1]. These parenchyma cells contain very small amounts of several growth substances and a considerable amount of inhibitors; polyamines too are present in amounts insufficient to sustain growth[2]. All the

biosynthetic enzyme activities[3], as well as diamineoxidase (DAO)[4] and transglutaminase-like (TGase-like) activities[5] are low or practically absent, while arginase (ARGase) is active[3].

Dormancy can be broken by excising slices of tuber parenchyma and by treating them with IAA, 2,4-D or PA added to the sterile growth medium[6]. The sudden switch from a state of slow metabolic activity to one characterized by the entry into a synchronous cell cycle is evidenced by the appearance of morphological and physiogical modifications[1,2]. The cell cycle is 30 hours long and the main events of its different phases are reported below.

G_1 phase.

PA biosynthesis is probably one of the earliest metabolic events induced by activation with 2,4-D since an increase in their content can already be measured at 15 minutes and the level attained after one hour is already sufficient to sustain protein synthesis. Within 1 hour many reserve substances are utilized but the very early putrescine (PU) synthesis does not appear to be clearly correlated with the ODC and ADC activities assayed *in vitro*, which are transiently inhibited by DFMO and DFMA[3,7]. However caution is necessary in evaluating the enzyme activities *in vitro*. Free PA might also be released from their bound form. At any rate, free PA continue to increase throughout the G_1 phase, also because the activities of enzymes that subtract PA from their free pool, namely DAO and Tgase-like activities, are very low[4,5]. Other cellular events take place in this phase: DNA, RNA and polysomes are very rapidly degraded but all of these are also newly synthesized: their synthesis becomes detectable after 20-30 min. PA have been found tightly-bound and non-tightly-bound to tRNA, rRNA and to a poly(A)RNA and the PA/RNA molar ratio increases in early G_1 phase[8]; this suggests that PA can be directly involved in the activation of RNAs or in protecting them from nucleases. In fact ribosomes extracted from dormant tuber cells require the addition of PA and Mg^{2+} to be active; on the contrary ribosomes extracted from dividing cells are already active, since they contain PA in sufficient amounts.

DNA synthesis peaks around 3-6 hours: mitochondria and plastids have been shown to be responsible for this early synthesis. This DNA and protein synthesis is probably related to the upcoming division of the organelles themselves or to their

functionality[1]. All three main PA as well as their precursors and their biosynthetic enzymes are also present in mitochondria, possibly in relation to mtDNA synthesis or with the enhanced respiration.

After the 6th hour, all the degradative processes observed in the first half of G_1 phase seem to end. In contrast, the biosynthesis of proteins, free PA and arginine continues. The ODC, ADC, DAO and TGase-like activities all increase conside rably[3,4,5]; the latter is probably due to a newly-synthesized enzyme.

S phase

When the synthesis of nuclear and nucleolar DNA begins , a decrease in PA content takes place ; however the synthesis of SD and SM still continues as shown by the in vivo incorporation of arginine and by the increasing activities of ADC, ODC, SAMDC and ARGase[2,3]. ODC and ADC reach their maximum activity in mid-S phase and appear to be localized both in organelles and in the cytosol, whereas SAMDC is mainly cytosolic. The decline in PU content is probably due to a considerable DAO activity occurring up to early-S, which, thereafter, decreases sharply so that all PA start to accumulate up to the end of the S phase[2,3]. PAO activity is not detectable in *Helianthus*. PA requirement for DNA synthesis and cell cycling has been clearly demonstrated in animal cells by using inhibitors of PA synthesis or of cell division[10]. A direct interaction between PA and DNA in the *Helianthus* cell cycle has been demonstrated[11], also by in vitro interactions[12]. The in vivo effect of inhibitors (CHA, MGBG, CAV, DFMO, DFMA) is not very marked or is transitory, so that scarce information is available on the consequences of PA depletion on the *Helianthus* cell cycle[3,7]. TGase-like activity in vitro continues to increase during the S phase becoming 7- to 8- fold higher than the initial value[5]. This binding activity is partly due to a true TGase, even though it presents some characteristics different from those of animal TGase. Conjugates with PU, SM but mainly SD are formed in the cell-free extracts; PA can form intramolecular or cross-linked conjugates and the length of the PA involved determines the length of the bridges. The TGase contribution to the decrease in the free PA pool does not seem to be relevant. Different protein-conjugated PA have been found in vivo during the

cell cycle: low molecular mass conjugates (18 KDa) appear in early G_1 phase; heavy ones, but mainly very high molecular mass ones are formed during S phase and up to cell division[9]. The latter conjugates (formed both in vivo and in cell-free extracts) cannot enter the stacking gel of the SDS-PAGE[5]. It is reasonable to assume that,also in plants, PA may be involved in the formation of supramolecular cell structures. However in this phase, many enzymes involved in DNA synthesis are active: they form a multiprotein complex associated with DNA in which PA may possibly be involved.

With regard to PA bound to low molecular mass compounds peptide-PA conjugates were also found in *Helianthus* whereas hydroxycinnamoyl-PA were not detectable.

Cell division

This phase largely overlaps with the S phase; the second peak in PA accumulation and in PU synthesis from arginine, with SD synthesis remaining high, occurs in the middle of the division phase[2]. The biosynthetic activities are still high, though declining, and DFMO and DFMA become pratically ineffective[7]. The conjugation of PU continues to increase and gives rise mainly to high mm conjugates[5]. Protein accumulation, active till early D phase, begins to decline. It is also known that PA form ionic linkages with pectic substances and probably hydrogen bonds with neutral wall polysaccharides and in fact PA have been found located in the walls. Cell wall formation probably requires PA but the sharp decline in PA content observed in the last part of this phase might also result from a degradative metabolism due to DAO activity, as well as a slowing down of their synthesis[3,4].

In summary, based on studies performed at the molecular level , the following possible roles of PA during the cell cyle can be suggested: they are necessary for the synthesis of DNA and RNA; they can be involved in the "activation" of RNA and DNA or in protecting them from nucleases; PA may be necessary for the cross-linking of cytoskeletal or wall proteins (or, possibly, of enzymatic ones) via covalent linkages; PA form hydrogen and ionic linkages and are thus important in conferring to cell walls their characteristics; putrescine may be necessary, as a precursor of GABA, to furnish organic acids for the Krebs cycle and amino acids for protein synthesis.

The main metabolic features occurring in dormant tubers and in activated slices during the first cell cycle are summarized in the figure.

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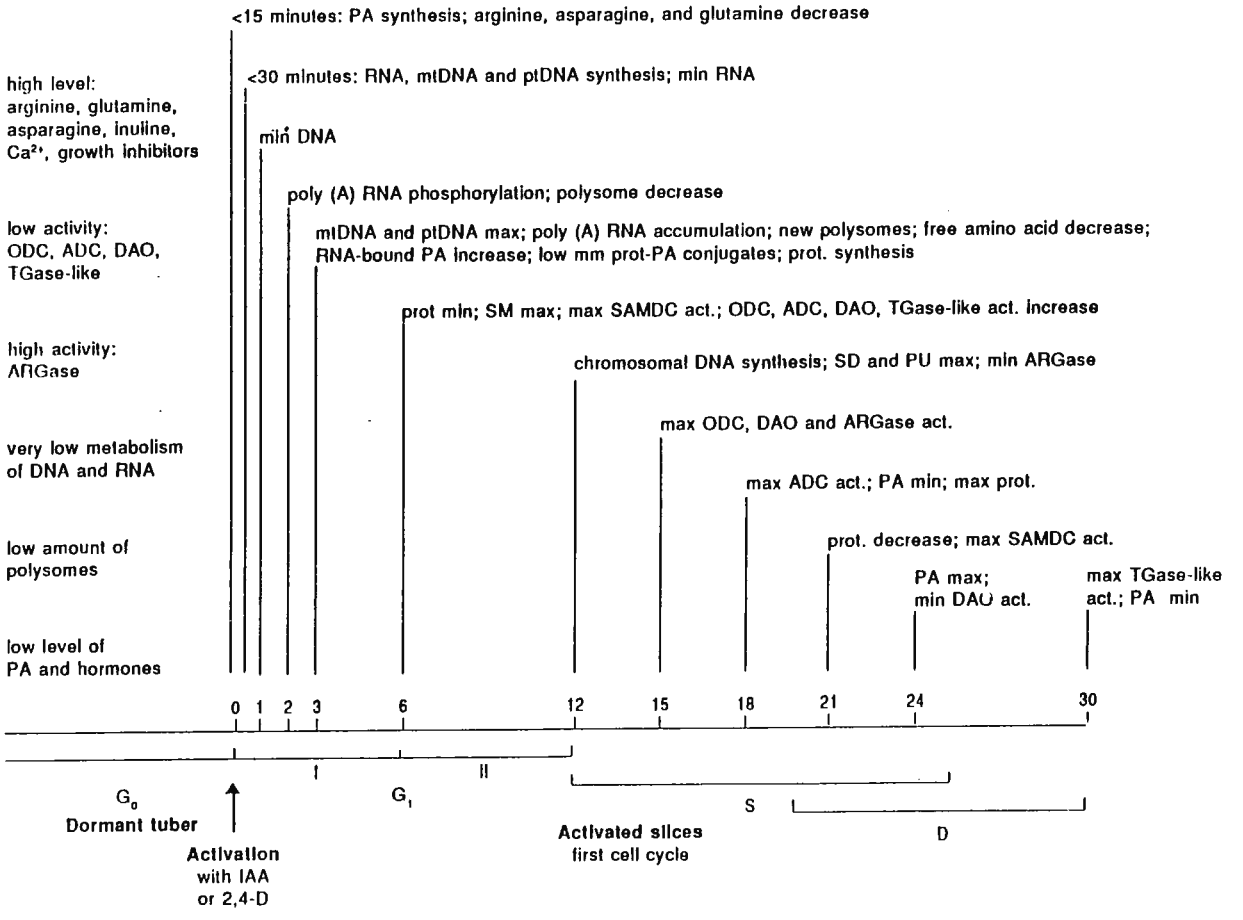

EFFECT OF POLYAMINE BIOSYNTHETIC INHIBITORS ON PROTOPLAST VIABILITY, CELL DIVISION AND PLANT REGENERATION IN CEREAL CULTURES

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It is well known that many *in vitro* cultures loss their morphogenic competence as they aged. Consequently, aging can be considered as one of the most critical factors in expressing the genetic potential of plant cells and tissues cultured *in vitro*. Although many theories have been advanced to explain aging, the precise biochemical and molecular mechanisms underlying the gradual loss of totipotency in aged tissue culture are not known yet (1).

Using different cereal model systems, we will present in this paper experimental evidences about the antisenescence properties of polyamines (PAs) as well as their relationship with the improvement of cell viability and morphogenic capacity. We will also discuss about the possible mechanisms of PA action, especially at the membrane level.

1. Model systems: leaves, protoplasts and calli

As many of the manifestations of aging are similar to senescence, the use of information from senescent herbaceous systems has served as a model for understanding aging and its related phenomena. In this respect, the *Avena sativa* leaf-system has constituted an excellent experimental model for studying biochemical and molecular aspects related with leaf senescence (2). When oat leaf segments are incubated in darkness, they undergo senescence as judged by their manifestation of several metabolic changes including an immediate rise in ribonuclease activity, followed by an increase in protease activity after 6h, and then by a gradual decline of chlorophyll (Chl) content after 24h (3). Exogenous application of PAs are able to inhibit or retard each of these events. The tetraamine spermine (Spm) is more active than the triamine spermidine (Spd), which is in turn more active than the diamines putrescine (Put) and cadaverine (Cad). Application of Spm is also more effective in preventing those senescence-related events than similar treatments with other known senescence retardants such as kinetin and cycloheximide (3).

While attempting to improve the viability of oat protoplasts, it was observed that the cells aged rapidly and this was a major obstacle to cell division and proliferation (4). Leaf pretreatment or addition to the culture medium of exogenous PAs are able (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 ribonuclease and other hydrolytic enzymes (5,6). Of those compounds, the most active are Spd and Spm, which can also induce DNA-synthesis and limited mitosis in oat mesophyll protoplasts (7).

be cautioned since exogenous PAs may exhibit non-specific effects that are perhaps unrelated to the physiological role of their endogenous counterparts (8). For instance, exogenous PAs, as Ca^{+2}, can associate with membrane lipids to induce membrane rigidification which could be an alternative mechanism that may question most of the interpretations concerning the physiological effects of PAs on plant senescence (9). Thus, in order to stablish the precise physiological role of PAs, experiments with exogenous PA application should be reinforced by parallel in-situ experiments in which endogenous PA levels are altered by using, for example, PA biosynthetic inhibitors.

To reduce endogenous PA levels, in our investigations (10-12) we have used DL-alpha-difluoromethylarginine (DFMA), an irreversible inhibitor of arginine decarboxylase activity (ADC) which is considered as the main biosynthetic enzyme involved in PA changes during leaf senescence (13). Leaf pretreatment of oat seedlings with 10 mM DFMA before a 6 hour osmotic treatment in the presence of 0.6 M sorbitol (osmotica used routinely in protoplast isolation) causes a small decrease of Put and a 2-fold increase of Spm titer. After 136 hours of osmotic stress, Put titers in DFMA-pretreated leaves show a small increase, but Spd and Spm titers increase dramatically (11). The activity of Spd synthase is also enhanced in the DFMA-pretreated leaves after osmotic treatment (12). The increase in the titers of Spd and Spm may account for the reduced Chl loss and enhanced ability of DFMA-pretreated leaves to incorporate tritiated thymidine, uridine and leucine into macromolecules (11). Furthermore, the overall viability and cell division of the mesophyll protoplasts isolated from oat leaves are significantly improved by the DFMA-preteatment, which in addition is more effective than the exogenous application of Spd and Spm (11). The pretreatment with DFMA, which produces an enhancement of endogenous Spd and Spm levels, exhibits similar antisenescence effects than the exogenous supply of these PAs. This reinforce thus the view that exogenous PAs, as their endogenous counterparts, may have a specific physiological effect on oat leaf and protoplast senescence.

Treatments with PA biosynthetic inhibitors can also influence plant regeneration from *in vitro* cultures. Thus, using callus of *Zea mays* as a model system, we have shown (14) that a prolonged treatment with DFMA for 3 consecutive months results in a dramatic decrease in the number of differentiated bud primordia and this is positively correlated with a marked decrease of total PA levels. Since decreased PA content is a characteristic step during cell aging (13,15), we have suggested that a senescence effect is exerted on the calli by the DFMA treatment which may explain the reduction of morphogenic capacity. This is further supported by the decrease in callus greening and the reduction of protein levels in the calli treated with DFMA. However, when the DFMA-treated calli are transferred to a medium of regeneration lacking the inhibitor the regenerated plants are more numerous and more developed than the untreated controls and this is positively correlated with a marked increase of protein and total PA levels. All these

results suggest that a rejuvenation effect is exerted on the calli after DFMA-removal which may explain the improvement of morphogenic competence in this mayze callus system (14). Recalcitrant cultures of other species may also benefit from this novel method. Indeed, Kaur-Sawhney et al. (15) have reported the improvement of protoplasts-derived cultures of *Vigna aconitifolia* after pretreatment with a combination of various inhibitors of PA biosynthesis, including DFMA.

2. Possible mechanisms of PA action

Most of the biological functions of PAs have been attributed to their polycationic nature, which allows electrostatic interactions with polyanionic macromolecules and with negative groups of membranes. With regard to the role of PAs on leaf senescence some mechanisms have been proposed in relation to RNA and protein metabolism. Isola and Franzoni (16) showed that both ribonuclease synthesis and activity are inhibited by PAs, although non-specific and/or indirect effects should not be discarded. Kaur-Sawhney et al (17) have shown that exogenous PAs strongly inhibited protease activity. Such inhibition is achieved by a conformational change of the enzyme induced by the binding of the PA to a site different to the substrate binding site (18).

At the membrane level, Cohen et al. (19) have proposed that PAs induce membrane stabilization through a possible binding to phospholipid components. However, it has been indicated that this effect could be (a) non-specific since PAs can also induce membrane rigidification (9) or (b) indirect since PAs can also act as free radical scavengers (20). Free radicals are implicated in plant aging and senescence phenomena since they can cause membrane damage through direct attact to membrane lipids by peroxidation (21). It has been suggested that the free radical-scavenging properties of PAs can be derived from (a) the hydroxycinnamic acid moities of PA conjugates or (b) possible inhibition by PAs of lipid peroxidation; although indirect mechanisms can not be discarded (20).

Since PAs and ethylene biosynthetic pathways may interact at the level of SAM, another possible mechanism of PA action at the membrane level could be an interference with ethylene biosynthesis (22).

We will also discuss recent data (23) concerning the effects of PA supply and DFMA-pretreatment on the stabilization of key polypeptides in the thylakoid membrane of senescent oat leaves using several recently produced antibodies probes specific for components of the Chl-protein complexes.

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THIRD SESSION

A. W. GALSTON
J. MARTIN-TANGUY
E. B. DUMBROFF

SPERMIDINE AND FLORAL DIFFERENTIATION IN THIN LAYER TOBACCO CULTURES

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Despite early evidence for the movement of a florigenic stimulus from leaves to buds in photoperiodically induced plants, the mechanisms of floral initiation remain obscure (reviewed in 1). In recent years, we have obtained evidence supporting a role for polyamines (PA's), especially spermidine (Spd), in floral bud differentiation and development in certain systems.

Previous studies indicate that PA's, in particular Spd, play an important role in reproductive differentiation in various organisms. These investigations include regulation of sporulation in yeast and various fungi (2,3), differentiation of embryoids in tissue cultures of carrot (4) and Vigna (5), abnormal flowering habits of PA mutants of tobacco (6), and the accumulation of acylated PA's in flowering, but not in vegetative, tobacco and other plants (7). Spd is biologically ubiquitous, and is known to be required for cell division and many developmental processes in plants and animals (reviewed in 8,9).

We investigated the possible role of Spd in floral bud initiation and development in thin layer tobacco tissue cultures by excising thin strips of tissue (approx. 1.5 cm x 0.4 cm) composed of the epidermis and three to five subepidermal layers from the internodes of floral branches at the green fruit stage of Wisconsin - 38 tobacco plants (*Nicotiana tabacum* L). Using Tran Thanh Van's system (10), we observed that such explants grown on Murashige-Skoog medium (11) supplemented with equimolar auxin and kinetin (1 μM) yield floral buds, but raising the cytokinin level

tenfold leads to differentiation of vegetative buds instead. Endogenous PAs increase with time of bud development, and final Spd titer in floral buds is much higher than in vegetative buds. When Spd synthesis is inhibited by cyclohexylamine, flowering decreases parallel with the decline of Spd titer; this effect is reversible by applied Spd. Exogenous application of 0.5 - 5.0 μM Spd to the vegetative cultures, on the other hand, induced flower bud formation (12). These observations indicate a possible causal relation between Spd and flowering in this system. Similar results have been obtained using the tobacco variety Samsun; however, spermine, not Spd was found to be the most effective PA (13).

Spd-mediated floral bud formation is dependent on the time and duration of Spd application; Spd is required for about three weeks from the start of the cultures, which is roughly the time needed for bud initiation and development. Spd cannot replace kinetin in cultures, but once the buds are initiated in the presence of kinetin, addition of Spd greatly increases the number of floral buds (14). The increase in Spd is accompanied by a concomitant increase in arginine decarboxylase (ADC) activity and a decrease in ornithine decarboxylase (ODC) activity (15). Application of the ADC suicide inhibitor DL-α-difluoromethyl arginine (DFMA) inhibits bud initiation while the corresponding inhibitor of ODC, DL-α-difluoromethyl ornithine (DFMO) does not. DFMO, however, inhibits the subsequent development of the newly initiated floral buds, but DFMA does not. Thus, PAs derived through ADC are most likely to be involved in the initiation of floral buds and PAs derived through ODC in their development.

The Spd-mediated floral bud formation on explants grown in vegetative medium appears to be limited to tissue derived from plants previously programmed to flower. When explants (thin layer, internode tisssue or leaf disc) obtained from non-flowering plants are cultured in the presence

of Spd, there is no switch from vegetative to floral buds, indicating that Spd is not itself florigenic, but is likely part of a complex of substances interacting in floral initiation and development (14). A similar conclusion was reached for *Spirodela punctata*, a quantitative LDP (16). With this plant, Spd biosynthesis inhibitors decrease flowering; this inhibition is reversed by exogenous application of Spd, but direct application of Spd to uninhibited plants did not affect flowering.

Some reports do not find any connection between PAs and the control of flowering in the thin cell layer tobacco system. Torrigiani et al (17) found no increase in endogenous Spd in flower buds compared to vegetative buds in the variety Samsun, and, Wyss-Benz et al (18,19) found no differences in hydroxy-cinnamic amides in cultures obtained from induced and uninduced plants or between photoperiodically determinate and indeterminate species of tobacco. A further complication in determining the role of Spd in flowering is that such diverse substances as cytokinins (10), oligosaccharins (20) and ethylene (21) have also been found effective in the control of flowering in the thin cell layer system. Clearly, a definitive role of PAs in flowering will require studies on whole plants in which flowering is controlled by photoperiod or other precise stimulus. Such experiments are being conducted.

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Effects of Ri- T-DNA from Agrobacterium rhizogenes and the inhibitors of putrescine synthesis on growth, organogenesis and polyamine metabolism in tobacco.

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INTRODUCTION

Floral induction in plants is a paradigm for signal perception, transduction, and physiological response. The introduction of root-inducing, left-hand transferred DNA (Ri T-DNA) into the genomes of several plants results in modifications of flowering (1), including a delay in flowering in tobacco (*Nicotiana tabacum*), partial sterility, wrinkled leaves, shorter internodes and increased branching.

Conjugated amines (polyamines, aromatic amines) are markers for flowering in numerous species of plants. In tobacco, their accumulation is correlated with the onset of flowering (2). These compounds are absent from sterile reproductive organs in several species of plants; they appear to constitute biochemical markers for pollen and ovule fertility (3).

Using tobacco, we have explored the possibility of a correlation between the expression of Ri T-DNA and changes in amine metabolism. We made use of two levels of phenotypic change, designated T and T' (4), that retard flowering by 5 to 10 and 15 to 20 days, respectively.

T' is an exaggerated form of T, e.g. in tobacco internode distance is further shortened ; leaf wrinkling is dramatically increased; flowering is more retarded. The other alterations in reproductive physiology that affect tobacco plants of T phenotype are also exaggerated in T' plants ; flower size, number and seed production are reduced.

Our objective was to identify the biochemical basis for the transformed phenotype induced by Ri T-DNA

RESULTS AND DISCUSSION :

We have demonstrated an inverse relationship between the level of phenotypic change and the level of amines (polyamines, aromatic amines) and their conjugates : as phenotypic change increases amines decrease (5). Furthermore, the appearance of the polyamine conjugates, previously shown to be markers for flowering, is delayed in direct proportion to the delay observed in flowering. The changes in amine metabolism we have described are correlated with changes in phenotype induced by Ri T-DNA in both quantitative and temporal fashions.(Figure 1)

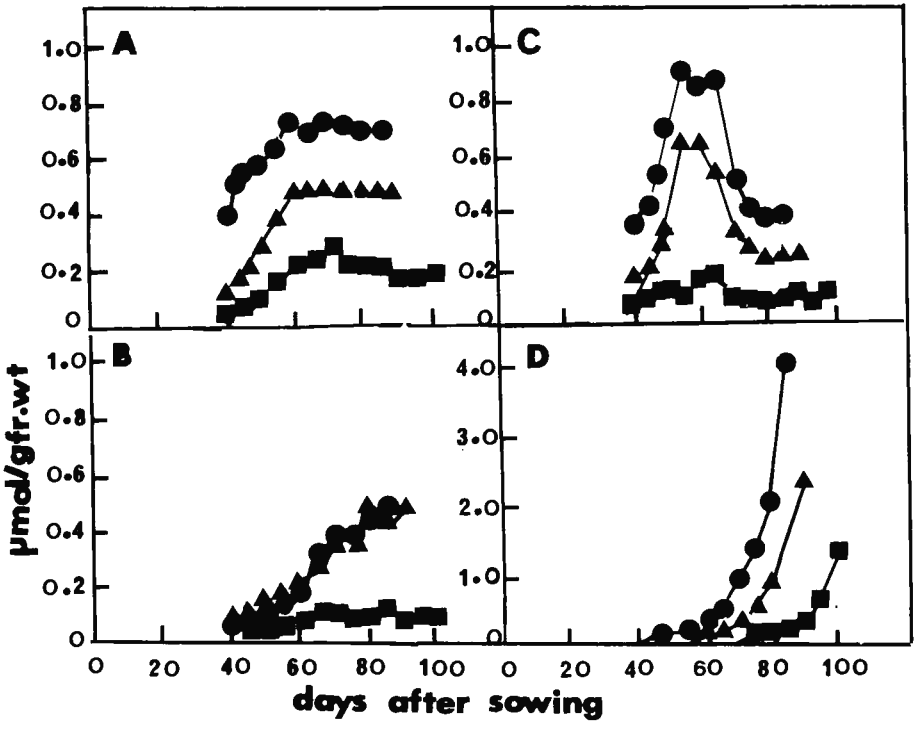

Figure 1 : The accumulation of amines : polyamines (putrescine + spermidine + spermine) (A), tyramine (B), phenethylamine (C) and conjugated polyamines (D), in the shoot tops of N ( ), T ( ) and T' ( ) tobacco plants. Amine content is expressed as a function of time after sowing. Flowering occurred at 80-85 days in normal plants (N) ; at 90-95 days in T plants and at 100-110 days in T' plants.

DFMO (specific irreversible inhibitor of ODC) but not DFMA (specific irreversible inhibitor of ADC) induced a phenotype in normal plants (N) that resembles the transformed phenotype attributed to the Ri T-DNA : flowering is retarded or inhibited, leaves are wrinkled (6). Similar treatment of T plants causes accentuation of the transformed phenotype (T') (6). Simultaneous treatment with DFMO and putrescine did not produce these phenotypic changes, although slight wrinkling was sometimes observed. The morphological alterations induced by DFMO were correlated with the expected changes in free and conjugated polyamines (6). Free and conjugated polyamine titers were lowered by treatment with DFMO, but not by DFMA. In plants of all three phenotypes (N, T and T') putrescine restored conjugated polyamines to normal titer. It thus seems likely that Ri T-DNA acts, at least in part, by depressing putrescine synthesis through interference with the ODG pathway.

Inhibition of spermidine synthesis (CHA or MGBG) interferred with sexual differentiation, but did not clearly mimick the transformed phenotype (6). The time of flowering was normal, but flowers aborted falling off the plant. This defect was reversed by spermidine. In addition CHA treatment caused malformation of anthers, including lack of pollen, heterostyly, replacement of anthers by petals and infertility (6). MGBG treatment caused leaf clearing (reversible by spermidine), and sometimes led to the absence of pollen, blackening of the stigma and low fertility.

While these results strengthen the correlation between Ri T-DNA expression and changes in polyamine metabolism, they still do not establish a direct relationship. There are 18 ORFs (ORF = open reading frame, or an extensive region in the nucleotide sequence devoid of stop codons ; thus this region has a high probability of being a gene) encoded in the Ri T-DNA and any one gene could have pleiotropic effects. For instance, it has been suggested that ORF 10 encodes a nucleic acid binding protein. A further complication arises from the observed redundancy in the Ri T-DNA, eg ORF 8, 11, 12, 13 and 14 contain sequences of probable common origin, and ORFs 15 and 17 plus 18 are

clearly a duplication (7). Preliminary results show that the expression of single genes (ORF 10, ORF 12) from this T-DNA leads to interference with amine biosynthesis (putrescine, tyramine) in plants (manuscripts in preparation).

It can be hoped that the molecular dissection of the Ri T-DNA, in a reversal of the coevolution through which it originated, will provides precise tools for establishing the importance of polyamines in the diverse developmental processes in which they are implicated.

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MECHANISMS OF POLYAMINE ACTION DURING PLANT DEVELOPMENT

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The ubiquitous nature and physiological importance of polyamines in living systems have been clearly established by a large number of correlative and functional studies at the biochemical and biophysical levels. Indeed, a wide array of experimental strategies has confirmed the involvement of polyamines in several cellular processes that are reflected in a diverse range of physiological phenomena, including embryogenesis, cell division, flowering, fruit development, mechanisms of stress resistance and modulation of plant senescence. However, the precise mode of polyamine action at the sub-cellular level remains unclear, but current evidence suggests that, in plant systems, four principal mechanisms may be involved, viz. (a) a contribution to cell homeostasis via regulation of pH, (b) physical effects on the conformation and stability of membranes and macromolecules, (c) their possible function as free radical scavengers and (d) an indirect mechanism that can modulate ethylene synthesis via competition between the ethylene and polyamine biosynthetic pathways for a common precursor (1).

A proposed contribution of polyamines to the buffering capacity of plant cells rests largely on observations that putrescine levels rise sharply in K^+ deficient plants and during treatment-induced acid stress in developing tissues (2,3). Although polyamines are relatively strong bases, the likelihood that they act as homeostatic entities that buffer against a rise in proton concentration is a moot point. The high pKa values (approx. 9-11.5) of common polyamines indicate that at least 99% of their nitrogen groups will be protonated under normal physiological conditions, and an even higher percentage of protonation could be expected under conditions of acid stress. Once protonated the amines would buffer against a large rise in pH but not against its decline. However, their conjugate bases, principally HCO_3^- and HPO_4^{2-} would provide substantial

buffering potential against an increase in proton concentration, but polyamines as such would have little effect. Moreover, the possibility that a stress-induced increase in acidity would be moderated by the rapid synthesis and subsequent protonation of polyamines should be viewed with caution since essentially all amino acid precursors and subsequent intermediates would also be protonated within the range of physiological pH.

Physical interaction of polyamines with sub-cellular components may constitute the principal mechanism by which these protonated alkylamines influence cellular functions. Although little information exists concerning the precise nature of this mode of action, available data suggest that polyamines may control a diverse range of biochemical events that depend on specific changes in or maintenance of molecular conformation. A number of enzymes involved in protein and nucleic acid synthesis appear to be regulated by charge-charge and charge-dipole interactions and/or by covalent bonding with polyamines (4). In addition, many of the physiological functions ascribed to polyamines may derive from their stabilizing potential, including the maintenance of membrane integrity and their modulation of ribonuclease activity and losses of protein and chlorophyll during stress and senescence (3,5). These antisenescence effects apparently result, in part, from the capacity of polyamines to rigidify membranes, thereby delaying the purely structural modifications that are characteristic of membrane deterioration during senescence. Studies with paramagnetic and fluorescent lipid-soluble probes have confirmed the effects of polyamines on the fluidity of microsomal membranes isolated from bean leaves, and they have demonstrated that surface probes were more strongly immobilized by physiological concentrations of the amines than probes that partitioned deep into the membrane bilayer (6). Spermidine and spermine were more effective than putrescine, and at equimolar concentrations were as effective as Ca^{2+} in their stabilizing effects. Like Ca^{2+} the polyamines were bound more strongly to membrane surfaces than

to interior portions of the bilayer, presumably by electrostatic interaction with the negatively charged phospholipid head groups. When fluorescence polarization values were obtained with a surface probe using native membranes, heat-denatured membranes or liposomes prepared from lipid extracts of membranes, each value was raised to a similar extent by 20 mM spermine, suggesting that interactions with proteins are not essential for expression of the stabilizing effect of polyamines on membrane systems.

The potential for polyamines to act as free radical scavengers may explain, in part, some of their physiological functions, including their suppression of lipid peroxidation in aging membranes and their inhibition of the terminal step in ethylene biosynthesis which may involve a free radical intermediate. Drolet et al. (7) have shown that polyamines could effectively scavenge superoxide radicals (O_2^-) generated in both chemical and enzymatic systems. At concentrations of 5 to 50 mM, they also strongly inhibited the production of O_2^- by senescing microsomal membranes and the superoxode-dependent conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene. The capacity of polyamines to scavenge O_2^- generated photochemically eliminated the possibility that the amines were simply inhibiting enzyme activity. Scavenging efficiency was concentration dependent, with the tri- and tetraamines (spermidine and spermine) consistently more effective than the diamines (putrescine and cadaverine). The highly reactive hydroxyl radical ($OH^\bullet$) was also scavenged by spermidine but only at high concentrations.

The actual mechanisms by which polyamines act as free radical scavengers have not been resolved, but superoxide is conceivably dismutated via disproportionation using polyamines as a source of protons in an overall reaction whereby: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Although their high pKa's and the availability of protons from other sources would seem to mediate against the importance of polyamines in the dismutation reaction, about 1% of

the amine groups are presumably not protonated at physiological pH, thereby providing a small but relatively constant source of protons for the dismutation of O_2^-.

A straightforward mechanism by which polyamines might scavenge $OH^\bullet$ without producing secondary radicals is not readily apparent. Moreover, data from Bors et al. (8) indicate that polyamines are not effective scavengers of the hydroxyl radical.

Competition between the ethylene and polyamine biosynthetic pathways for S-adenosylmethionine (AdoMet) may provide an effective but indirect mechanism by which synthesis of polyamines can influence developmental processes in plants. However, the degree of linkage between the two pathways is not well-defined and appears to vary with species and to change with physiological stage of development. Nevertheless, several studies have shown that blocking specific steps in one pathway will shunt AdoMet into the alternate pathway and thereby increase the synthesis of either ethylene or polyamines. When ethylene synthesis in carnation flowers was inhibited at the ACC synthase step, the incorporation of [^{3}H]methionine into spermidine increased, and a large rise in the concentration of spermine was observed in the petals (1). In contrast, inhibition of polyamine synthesis at the AdoMet decarboxylase or arginine decarboxylase steps increased ethylene production and hastened the onset of flower senescence by several days. In addition to the regulatory effect that polyamine synthesis may have on the ethylene pathway, polyamines may directly limit ethylene production by binding, via mechanism b, to ACC synthase or to cell membrane with consequent distortion of the binding site on the ethylene forming enzyme (1).

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POSTER SESSIONS

RELATIONSHIP BETWEEN LONGEVITY AND POLYAMINE CONTENT IN SOME CLIMACTERIC SYSTEMS.

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Senescence of climacteric structures is characterized by an increase in ethylene production and 1-aminocyclopropane-1-carboxylic acid (ACC) metabolism. However, the involvement of polyamines in such an important process has been little investigated, in spite of the fact that they share a common precursor with ACC: S-adenosylmethionine (SAM).

Occasionally, varieties have been described showing a different behaviour in terms of the climacteric peak of ethylene. For instance, there are strings of tomato which are considered as "long life fruits" because of their better storage properties. They lack an ability to produce ethylene or instead show a much smaller peak of this hormone than normal strings. Likewise, there are varieties of carnation, another typical climacteric system, which do not produce ethylene. In both cases, the result is an increase in the longevity of these commodities.

The aim of this work was to investigate a possible relationship between the extended longevity shown in these two cases and the content of free polyamines.

The free polyamine content was analyzed in two varieties of carnations: one showing a climacteric ('Arthur') and the other a non-climacteric ('Killer') behaviour and also in two tomato varieties, 'Lorena' and 'Novy', the latter showing long-life properties.

Appreciable differences were found in the polyamine accumulation pattern between 'Arthur' and 'Killer' carnations. Putrescine (Put) content in 'Arthur' increased continuously from 20 nmols/gFW at day 1 of senescence to about 270 in a very advanced stage of senescence. However, Put content in 'Killer' remained almost steady at about 30 nmols/gFW. Spermidine (Spd) content was initially higher than Put in 'Arthur', but decreased until day 6 when it increased again until the end of senescence. The opposite situation was found in 'Killer' carnations, where Spd content was always about 3 times greater than Put. No significant variations in Spd level were found in this carnation variety (Serrano et al., 1990).

In 'Lorena' tomato fruits, the major polyamine was Put, with a concentration of around 100 nmols/gFW throughout ripening. Spd content, however, was in the order of 11 nmoles/gFW. In 'Novy' tomato fruits Put was also the major polyamine with a content 2-3 fold higher than in 'Lorena', while Spd showed no significant variations during ripening, being around 65 nmols/gFW, which was a 5-8 fold higher content than that found in 'Lorena' tomato fruits. (Casas et al., 1990).

Neither in senescing carnations nor in ripening tomatoes was the presence of free espermine detected.

The results found in both systems show that longevity in carnation flowers is linked to the maintainance of the Put/Spd ratio found at the preclimacteric stage, while in tomato fruits the more extended longevity is related to a higher level of Spd.

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IS THERE LONG DISTANCE TRANSPORT OF POLYAMINES? A POINT FOR DISCUSSION.

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As a product of amino acid catabolism, polyamines (PAs) are ubiquitous in plant tissues.

Recent findings showed a possible role of polyamines in growth regulation (Evans & Malmerg, 1989). However, it is not known whether they act as hormones or only modulate, *in situ*, some of the enzymes involved in protein metabolism.

To prove that PAs can function as hormones, it must be shown that they are transported within the plant to a target tissue where they act as a signal.

Attempts have been made to support a hormonal function of PAs. Friedman et al. (1986) found high concentrations of putrescine and spermidine in xylem and phloem exudates but they did not show a directional translocation of these substances. Although local uptake of [^{14}C]putrescine, [^{14}C]spermidine and [^{14}C]spermine by petals of *Saintpaulia ionantha* has been studied by Pistocchi et al. (1986), the involvement of xylem or phloem transport in the uptake of labelled polyamines by petals was not demonstrated. In etiolated pea seedlings, Young & Galston (1983) reported the transport of polyamine precursor amino acids from sites of storage to points of active growth, where they are converted to PAs. However, they could not show a direct polyamine transport. Feray et al. (1990) found radioactivity in apex, roots and tubers after feeding leaves with [^{14}C] putrescine, but the chemical nature of the radioactive compounds was not identified.

None of these studies have proved the existence of long-distance transport of PAs.

A systematic approach should be developed to follow the path of labelled polyamine molecules from a source to a target tissue. The use of [^{14}C] or fluorescent polyamine molecules would be very convenient since it would also allow the analysis of derived compounds in the target tissue. Mature plants, where phloem and xylem vessels are well-developed, would be necessary to show if there is translocation of PAs. The recent development of radioimmunoassay for serum spermidine (Fan, Zhen-Fu, et al. 1988) could be adapted to a histochemical immunoassay for identification of phloem or xylem paths. Aphid stylet phloem-sap collection for monitoring specific phloem transport may also be useful.

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Effect of Polyamines on Stabilization of Molecular Complexes in Thylakoid Membranes of Osmotically-Stressed Oat Leaves.

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Introduction

Polyamines (PAs) are essential to tobacco and tomato ovary and fruit development and implicated in root initiation and early differentiation events in the floral meristem (see review 1). Also exogenous PAs retard senescence which may be related to PA binding and stabilization of membranes (2). Support for the binding mechanism is that addition of Ca^{2+} counteracts the ability of PAs to prevent chlorophyll loss from thylakoid membranes in senescing leaves (3). The location of spermidine synthase in chloroplasts (4) suggests an important role for spermidine (Spd) and spermine (Spm) in this organelle.

While it is known that exogenous PAs stabilize the photosystem complex in thylakoid membranes of senescing tissue (5,6) more detailed analysis has not been possible due to lack of specific antibody probes to these membrane proteins.

In this report we have investigated the effects of PA supply and the inhibitors of PA metabolism on the stabilization of key polypeptides in the thylakoid membranes of osmotically-stressed oat leaves using several recently produced antibody probes. Those used were site-directed antibodies to the reaction centre polypeptides of photosystem II (D1 and D2) (7) and monoclonal antibodies to cytochrome f in the

cytochrome b_6f complex. Polyclonal antibodies to the large subunit (LSU) of Rubisco (8) were also used to assess degradation and loss of this protein from the leaf tissue.

Materials and Methods

Nine-day-old oat seedlings (*Avena sativa* cv. Victory) were developed in the light. The first leaf was excised and the lower epidermis removed, then floated on 100 mM K-phosphate containing 0.6 M sorbitol and either 1 mM Spd, 1 mM Spm or 1 mM Diaminopropane (DAP). One set of plants was pretreated with 10 mM difluoromethylargenine (DFMA) for 4 days before osmotic treatment. Leaves were then incubated in the dark at 27°C for up to 48 h. Crude thylakoids were prepared according to (7) and the polypeptide composition determined by SDS-PAGE after solubilizing in 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.4 for 5 min in boiling water. Gels were stained with Coomassie blue. For immunodetection of membrane polypeptides, washed thylakoids were solubilized as above, subjected to SDS-PAGE, electroblotted to nitrocellulose, challenged with antibodies and detected with anti-rat or anti-rabbit peroxidase. For the determination of LSU an homogenate of whole-leaf extract was used.

Results

In the first experiment leaves were compared with or without pretreatment with DFMA and subjected to 0.6 M sorbitol in the dark for up to 48 h. The polypeptide composition of the thylakoids was determined (Fig. 1). The major protein loss occurred in the thylakoids of the untreated leaves between 24 h and 48 h while the +DFMA treated samples showed only small losses over 48 h. After 24 h and 48 h total chlorophyll levels were 21% and 18% higher, respectively, in the DFMA treated

leaves compared with the values in the –DFMA material. Without DFMA pretreatment the levels of D1, D2 and cytochrome f were all lower after 48 h, D1 showing the greatest loss. The stromal protein LSU was also considerably less after 48 h in the –DFMA leaves. PAs, especially Spd and Spm, increase in oat leaves when they have been pretreated with DFMA (9) and may have been responsible for the protection of the thylakoid protein complexes under osmotic stress.

In a second experiment, exogenous Spd, Spm or DAP was therefore applied to the osmotically-treated leaves to determine their effects. All applied PAs were effective in retarding the loss of D1, D2 and cytochrome f from the thylakoid membranes as well as LSU and chlorophyll from the leaf tissue. However, most of the added PAs were converted to DAP within 6 h of application, the endogenous levels rising 8- or 9-fold above the value in control tissue (Fig. 2). With longer incubations with exogenous PAs the leaves accumulate Spd and Spm (9). This suggests that although both DFMA-pretreatment and PA applications have similar antisenescence effects, the mechanisms of action may be different. Pretreatment with DFMA acts on Spd and Spm synthesis (9,10) while PA application may affect endogenous PA degradation. Added PAs rapidly converted to DAP may inhibit PA oxidase, the main catabolic pathway for PAs in cereals, resulting in less degradation of endogenous Spd and Spm. Experiments are in progress to test this last hypothesis.

Conclusion

Stability of thylakoid polypeptides requires a well developed and integral thylakoid membrane (11). This study shows osmotically-stressed oat leaves incubated in the dark lose key polypeptides from the thylakoid membranes which leads to a reduction in chlorophyll and ultimately to loss of viability of the cells (or protoplasts). Application

of PAs to such tissues retards the loss of specific membrane proteins which suggests a direct protection of thylakoid membranes by these PAs or by their metabolites. Retention of the stromal polypeptide LSU in leaf tissue indicates the action of certain PAs in preserving the integrity of the chloroplast envelope/plasmalemma.

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POLYAMINES, CONJUGATED PUTRESCINES, ADC, ODC AND PAL ACTIVITIES IN LEAF EXPLANTS CULTIVATED IN VITRO

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Foliar explants of Nicotiana tabacum cv Xanthi n.c. were cultivated *in vitro* in three different media: (1) basal medium plus benzyladenine; (2) basal medium plus benzyladenine and 2,4 dichlorophenoxyacetic acid (2,4 D; (3) basal medium plus indolyl acetic acid.
Addition of benzyladenine caused the formation of buds on the explants without callus formation, while benzyladenine plus 2,4D caused callus formation and proliferation. IAA induced roots formation.The levels of amines and hydroxycinnamoylputrescines, ADC, ODC and PAL(phenylalanine ammoniac lyase) activities were determinated in the three types of explants. In callus cultures the levels of amines and hydroxycinnamoylputrescines were higher than those found in bud cultures The formation of buds was accompanied by significant changes in putrescine and hydroxycinnamoylputrescines levels.In the roots a transitory accumulation was observed.

In all the cultures an increase of ODC activity occurred after a few days of culture. An increase of ODC activity was observed in the young roots. ADC activity was observed in medium containing cytokinine. PAL activity is important during the first days of culture and clearly mimick ODC Activity .

These results shows that ODC is the main enzyme involved in putrescine synthesis in tobacco leaf explants cultivated *in vitro*. The similitude between ODC and PAL activities suggests that conjugated putrescine synthesis is carried out via ornithine decarboxylation.
There is a correlation betweenADC activity and the presence of a cytokinine in the medium.

The results suggest that ODC, ADC and PAL are involved in cell multiplication and differentiation process.

POLYAMINES AND THE FLOWER TRANSITION IN SOYBEAN

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Free and conjugated polyamines were analyzed during the first 9 days of photoperiodic flower induction in order to study their implication in the flowering process in soybean.

Plants were grown in controlled growth chambers under non-inductive long days (LD: 9 h fluorescent + incandescent light plus 3 h incandescent light in the middle of the 15 h dark period) until the V_2 phenological stage. From this time, inductive short days (SD: 9 h fluorescent + incandescent light plus 15 h darkness) began. Putrescine (Put), cadaverine (Cad), spermidine (Spd), and spermine (Spm) contents of terminal stem buds were determined by HPLC as 1) soluble fraction (S); 2) acid-soluble conjugated fraction (SH: bound to sugars, phenolics, alkaloids ...); and 3) acid-insoluble conjugated fraction (PH: bound to proteins, nucleic acid, cell wall, membrane fractions, ...). In other experiment, particulated leaf peroxidase was tested as an indicator of membrane state during photoperiodic flower induction and after external Put and Spm applications.

The results showed that during the first 3 days of SD flower induction there were no significant changes in Put, Spd nor Spm, although Cad-PH and SH fractions showed a transient decrease during the 1st inductive day. Just from the 3rd to the 9th inductive day, PA contents began to differ between treatments in a discontinuous way. On the 3rd inductive day, a decrease in Put-SH and an increase in Put-S were observed, with a parallel decrease in Cad-PH and an increase in Cad-SH and S. On the 6th inductive day, both Spm-S and Put-PH contents reached significant lower values with respect to non-inductive LD treatments. On the 9th day, nearly all PA contents were the same between treatments, though Spm-S maintained the low content under inductive SD.

In order to corroborate the increase in Put-S content and the decrease in Spm-S during SD flower induction, applications of 200 uM Put on LD-treated leaves and 200 uM Spm on SD-treated leaves were done. In effect, Put + LD mimicked SD-peroxidase activity and Spm + SD mimicked LD-peroxidase activity.

These results demonstrate that even PAs are related to the flowering process, their inherence appears not to be bound to the first step of the flower induction but to the direct change of the morphogenetic meristem condition.

CHANGES IN POLYAMINE CONTENT OF *Arabidopsis thaliana* AFTER UV-C IRRADIATION

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There is a widespread concern about the present changes of the ozone content in the atmosphere which may result in increased exposition of living beings to short wave UV irradiation, in particular plants which are the most exposed organisms to enviromental changes. Thus we have studied in the past the effect of UV-C irradiation (254 nm) on various aspects of plant development such as growth, pigment composition, levels of specific mRNAs, pattern of soluble proteins, alkaloid content, etc. (1,2).

Since it is well known that polyamine (PA) levels are altered under several plant stress conditions (3), we decided to investigate the effects of UV-C treatment on PA metabolism using *Arabidopsis thaliana* L. plants as an experimental model system. We have found dramatic changes in PA composition during the first two hours after irradiation. These changes can be detected even after a few minutes. Pretreatment of plants with DL-α-difluoromethylarginine (DFMA), an irreversible inhibitor of arginine decarboxylase activity, resulted in an increased sensitivity to UV-C irradiation, and no changes in the PA composition were observed.

These results suggest that PA content increases in plants as a defense mechanism against UV irradiation and represents one of the fastest responses detected so far after UV irradiation. The PA levels also represent an early indicator of UV induced stress.

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AN INCREASE IN THE LEVEL OF SPERMINE PRECEDES SENESCENCE AND A DECREASE IS INDUCED BY PLANT GROWTH REGULATORS DURING FRUIT SET AND DEVELOPMENT IN OVARIES OF PISUM SATIVUM

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We have investigated the level of polyamines (putrescine, spermidine and spermine) during the natural senescence of unpollinated pea ovaries and during fruit set and development induced either by natural pollination or by application of plant growth regulators (auxin, gibberellin, and cytokinin). Senescence is triggered in unpollinated pea ovaries about 3 days post anthesis without any manipulation or treatment of the ovaries. A decrease in the level of putrescine and spermidine and an increase in the level of spermine was observed after the day of anthesis. However a decrease in the level of spermine was observed after the application to the ovaries of plant growth regulators that induce fruit set and development. Also natural pollination of the ovaries resulted in low spermine level. Ovaries in explants cultured in spermine showed a reduction of the growth induced by the application of gibberellic acid associated with an increase of the level of spermine in the ovaries. All these results indicate that changes in the level of spermine (and not in that of putrescine or spermidine) are involved in the control of the alternative processes in pea ovaries: senescence or development.

Phenotypic alterations in rolA-transgenic Arabidopsis plants: A possible link to polyamine metablism?

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Conjugated polyamines are markers for flowering in numerous plant species. In tobacco their accumulation is correlated with the onset of flowering (Cabanne et al., 1981). A delay in flowering is part of the complex nature of phenotypic alterations caused by the introduction of T_L-DNA of Agrobacterium rhizogenes into the genome of tobacco plants (Tepfer, 1984). Two observations suggest a possible link between T_L-DNA expression and polyamine metabolism (Martin-Tanguy et al., 1990): The delay in flowering is correlated with a reduction in polyamine accumulation and with a delay in the appearance of conjugated polyamines. Secondly, preliminary experiments indicate that phenotypic pertubations similar to those caused by the T_L-DNA can be induced by specific inhibitors of polyamine biosynthesis.

A single T_L-DNA locus, the rolA gene causes late flowering in transgenic tobacco plants (Schmülling et al., 1989; personal communication by Dr. T. Schmülling); suggesting by the criteria discussed above, that the activity of the rolA gene product might be the proposed link to polyamine metabolism. To test this hypothesis we are currently using Arabidopsis as a genetic model system (for review see Meyerowitz, 1987). Arabidopsis plants transgenic for the rolA gene display a distinct phenotype including epinastic leaf growth, inhibition of leafstalk elongation, reduction in internode length, and delayed flowering. Preliminary experiments indicate that a (nearly) phenocopy of the rolA phenotype can be induced on wild type seedlings by the polyamine biosynthesis inhibitor Methylglyoxal-bis(guanylhydrazone) (MGBG). To identify targets of the rolA gene product activity, transgenic Arabidopsis plants are mutagenized and screened for second site suppressor mutations. Assuming that rolA expression directly affects polyamine metabolism, such suppressor mutations might influence polyamine metabolism as well. The approach and the recent state of the project will be discussed on a poster.

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CONJUGATION OF POLYAMINES TO PROTEINS DURING THE CELL CYCLE

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A characteristic trend in free polyamine content is observed during the cell cycle in both plants and animals. During the synchronous cell cycle of Helianthus tuberosus parenchyma, the pattern of free polyamines can be only in part related to the activity of their biosynthetic enzymes. The decline in free polyamine titer occurring in S phase and after mid-division phase suggests that they are utilized, as such or as derivatives, in some fundamental processes: they can be oxidized to γ-aminobutyric acid or bound to nucleic acids, protein and other compounds. *In vivo* polyamines were found linked mainly to proteins and their conjugation can be attributed to a transglutaminase-like activity detected in *in vitro* assays. This activity,which is temperature - sensitive, after an early decrease in mid-G_1,rises 7-fold in late G_1/early-S phases and then remains constant. A TLC analysis after acid hydrolysis of the *in vitro* conjugates shows that the main bound polyamine is putrescine. Other derivatives in different percentages were also found. SDS-PAGE fluorograms show that heavy molecular weight conjugates, obtained both *in vivo* and *in vitro*, cannot enter the 12% running gel, but with an appropriate decrease of the percentage of the acrylamide, some of them can be separated. The velocity of methionine incorporation in protein is progressively increasing during G_1 phase and the labelled proteins are still of high molecular weight. Cycloheximide strongly inhibits the activity after mid- G_1 phase; on the contrary it is apparently ineffective when it is supplied during early G_1 phase. Infact it seems that the enzyme is synthetized in early G_1 phase, but that it becomes active after 6 h of the cell cycle.

Effect of Polyamine biosynthetic inhibitors on Somatic embryogenesis in *Hevea brasiliensis*.

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Hevea brasiliensis, like other ligneous plants, have been considered for a long time as recalcitrant with regard to somatic embryogenesis. Nevertheless, exogenous polyamines (PA) (putrescine: Put, spermidine: Spd, spermine: Spm) added individually or together to the culture medium constitute one of many factors favorising the embryogenic capacity of calli.

Inhibitors of enzymes involved in polyamine biosynthesis like DL-difluoromethylarginine (DFMA), DL-difluoromethylornithine (DFMO), methylglyoxal bis(guanylhydrazone) (MGBG), cyclohexylamine (CHA), and dicyclohexylamine (DCHA), are usually used to demonstrate that PA are required for normal growth and development. In *Hevea brasiliensis*, application of these inhibitors, at concentrations which did not affect callogenesis, resulted in a large decrease in somatic embryos formation. The inhibition was, in only some cases, partially reversed by addition of Spd (50 µM).

Nevertheless, the effect of inhibitors on PA levels was opposite to actual knowledge on pathways for PA synthesis. MGBG,an inhibitor of S-adenosylmethionine decarboxylase (SAMDC), decrease largelly Put level while accumulation of this diamine should have increased by this treatment. DFMO, an inhibitor specific of ornithine decarboxylase (ODC), paradoxically increase Put. The accumulation of Spd and Spm by DFMA treatment, appears also paradoxical because DFMA, as a specific inhibitor of arginine decarboxylase (ADC), should have produced a decrease in both PA.

These results suggest that inhibitors of PA biosynthesis could perturbate other metabolic pathways. It is not excluded that other pathways can lead to Put synthesis and inhibition of one pathway can stimulate another. Elsewhere, treatments by PA synthesis enzyme inhibitors may increase the production by calli of *Hevea brasiliensis*, of other enzymes which can allow the synthesis of PA.

QUANTIFICATION OF SOLUBLE BOUND POLYAMINES

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Most of work on polyamines has been conducted till now on free polyamine levels, mainly Putrescine, Spermidine and Spermine. At present, is well acepted that bound polyamines could exert an important function in plant development in different ways.

An important fraction of bound polyamines corresponds to the soluble fraction present in the acid supernatant of the extraction process. Most of the bound soluble polyamines are polyamides, the product of conjugation between polyamines and phenolic acids (mainly cinnamic acids). The ratio between bound soluble and free polyamines in a plant differs from especies in a wide range.

To determine plant soluble bound polyamines an acid hydrolysis is performed. In this way, polyamines are libered from cinnamic amides at 105-120 ºC in a HCl.6M solution during 24 hours. This process was originally stablished for animal (urine) samples by Seiler and is used in plants as in animals.

A method to determine only the cinnamic amides in plants is used by Martin-Tanguy and co-workers. In this method after a long process of cinnamic compounds extraction polyamines are libered by an acid hydrolysis as described.

When acid hydrolysis is used in plant samples some problems are present. The most important cause is the non cleaning extraction process used. In the usually process -homogeneization and precipitation by perchloric or trichloracetic acid- plant sample presents many substances other than polyamines. Carbohydrates, mainly starch, are always present in the sample. In the strong conditions of mentioned acid hydrolysis a loss of polyamines can occurs. The formation of Schiff's basis is possible between carbohydrates and polyamines -in the mentioned conditions- resulting a non hydrolysable products. This produces incorrect quantification of polyamines when dansylating procedure is subsequently used.

In present work we have tried to minimize the polyamines loss using an ion-exchange Dowex colum. The elimination of starch, glucose and saccharose and the recovery of polyamines using Dowex column are studied on synthetic and plant samples.

'Genetic analysis of resistance to methylglyoxal-bis(guanylhydrazone) in tobacco following T-DNA insertional mutagenesis'

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Agrobacterium mediated transformation of plants has now become a routine procedure and the versability of T-DNA based vectors is such that they can also be used in gene tagging. T-DNA is an ideal gene tag as it has the potential to insert in any part of the genome and recently it has been shown to insert into regions of the genome which are potentially transcribed.

We have developed a strategy to carry out T-DNA mutagenesis in such a way that following insertion into the plant genome flanking plant genes are overexpressed. In order to this we have engineered T-DNA in such a way that it contains multiple enhancer sequences able to activate the expression of flanking plant DNA sequences. Marker genes based on antibiotic resistance allow identification of transgenic clones. Our T-DNA vectors contain a bacterial origin of replication as well as a bacterial marker gene which enables us to rescue the T-DNA physically linked to tagged plant-DNA sequences from plant tissue. Agrobacterium containing these vectors have been used in cocultivation of tobacco protoplasts. In this way we can regenerate large numbers of transformants.

Methylglyoxal-bis(guanylhydrazone)(MGBG) is a potent inhibitor of S-Adenosyl Methionine Decarboxylase (Sam-DC) which plays a key role in the synthesis of the polyamines Spermine and Spermidine. We have developed a system to induce and to identify mutants which express resistance to MGBG in a dominant manner following T-DNA mutagenesis using our overexpression vectors. By this, we hope to identify genes coding for Sam-DC or any regulators of its expression level. Suitable selection schemes resulted in a number of MGBG resistant calli, which were regenerated to shoots. These shoots were subsequently cultured to form roots and normal plants have been formed. From a population of transgenic plants individuals have been found to be able to form roots on media containing MGBG at concentrations where control non-transgenic plants are unable to do so. Moreover, transgenic individuals have increased levels of MGBG activity. Southern analysis indicates that transgenic plants contain T-DNA integrated in the genome.

The analysis of the genetic linkage of the T-DNA and observed phenotype of transgenic plants as well as the rescue of plant DNA flanking the T-DNA in *E.coli* is under current investigation.

FIRST EVIDENCE ABOUT THE EXISTENCE OF A CARRIER FOR PUTRESCINE IN LICHENS.

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The existence of a carrier for putrescine in Evernia prunastri thallus has been investigated by using ^{14}C-putrescine. Depending on the pH of the incubation media, putrescine can be taken up, trapped by negative charges in the cell wall or metabolized outside the thallus by the action of polyamine oxidases. At very alkaline pH values, putrescine is taken up by a carrier. This protein has been kinetically characterized and behaves as michaelian with a K_m value of 5.3 mM for putrescine and a v_{max} value of 12.5 µmol putrescine·h^{-1}. Spermidine and spermine behave as competitive inhibitors of putrescine transport. I_{50} values for these polyamines have been estimated as 2.17 and 1.0 mM respectively. Some aminoacids such as glutamic acid, ornithine, arginine or lysine and lichen phenolics, evernic and usnic acid, do not compete for the same carrier of putrescine.

Because of the characteristic morphology of the thallus and the distribution of hyphae on the upper cortex, a high proportion of putrescine can be adsorbed onto the fungus. This phenomenon is also analyzed.

MODIFICATION OF AN 18 KD POLYPEPTIDE BY SPERMIDINE IN RICE CELL SUSPENSION CULTURES

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A lysine-rich mutant cell line recovered from *in vitro* inhibitor selections in rice with lysine plus threonine and S(-2 aminoethyl) cysteine (4C) and the control cell line (Calrose 76) were regenerated in to whole plants. Mature embryos were reisolated from seeds and grown on callus for more than 5 monthly subcultures. Cells in liquid suspensions were started from actively growing callus and sized thru stainless steel sieves at each subculture. Cell suspension cultures of both mutant and control have similar growth rates as well as cell size. These cell lines are good sources for biochemical and molecular studies. Rice cell suspension cultures of Calrose 76 show increased levels of Put as compared to the lysine-rich 4C mutant. However, 4C cells show elevated levels of the higher polyamines, Spd and Spm, than Calrose 76, suggesting slightly better conversion of Put to Spd and Spm in lysine-rich mutant cells. When cell suspension cultures were incubated in the presence of [^{3}H] Spd the label was stably incorporated in an 18 kD protein in both Calrose 76 and the 4C mutant. Furthermore, when inhibitors of polyamine biosynthesis, i. e., DFMA and DFMO were incorporated into the growth medium, free polyamine levels declined, albeit variably from experiment to experiment, relative to that in untreated controls of both cell types, and efficiency of incorporation of [^{3}H] label into the 18 kD polypeptide increased. The incorporation of label into the 18 kD polypeptide was inversely related to the endogenous polyamine levels in both Calrose 76 and lysine-rich mutant 4C cell suspension cultures. Various chemical treatments (Triton-X-100, SDS, and high salt) did not remove the label from the 18 kD polypeptide indicating a strong non-ionic attachment of the label to the protein. In cells in which protein synthesis was arrested by cycloheximide, no label was detected in the 18 kD polypeptide, suggesting a requirement for *de novo* protein synthesis. A coupled translation/modification biogenic pathway seems compatible with this data. Presently, we are in the in the process of large scale isolation and purification of the labeled 18 kD protein to determine the nature of this modification and its biochemical characterization.

CULTURE AND CHARACTERIZATION OF AGROBACTERIUM RHIZOGENES STRAINS IN RELATION TO POLYAMINE PATTERN AND BIOSYNTHESIS

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Agrobacterium rhizogenes is a gram negative soil bacterium. Its ability to induce the "hairy root" disease in many dicotiledonous plants is determined by a virulence plasmid, known as Ri plasmid, similar to the Ti plasmid found in Agrobacterium tumefaciens. In an earlier work polyamine content and biosynthesis in Agrobacterium tumefaciens was studied, and since the mechanisms of transformation in both bacteria seem to be similar, these data prompted us to analyze the involvement of polyamines also in the growth of two different Agrobacterium rhizogenes strains (p Ri 1855 and p Ri 8196).

Homospermidine [N-(4-aminobutyl)-1,4-diaminobutane] was the major polyamine in these strains, both on yeast-mannitol medium and on "199" synthetic medium (Flow Lab.); homospermidine is an "unusual" polyamine found in many microorganisms, such as Thermus thermophilus and some cyanobacteria. It is produced from a Schiff-base complex of putrescine with the aminobutylaldehyde which is formed by oxidative deamination of putrescine. A significant peak of homospermidine, coinciding with the exponential phase of growth, was observed in both strains grown on "199" medium; putrescine was also present in bacteria coming from both culture conditions, while spermidine and spermine were undetectable in bacteria grown on "199" medium. The presence, in the yeast extract, of significant amounts of putrescine, spermidine and spermine could account for these differences in polyamine patterns.

Arginine- (ADC) and ornithine-decarboxylase (ODC) activities, measured at the exponential phase of growth, were present in both strains. Generally ODC was higher than ADC activity both in the supernatant and in the pellet. No differences were found in the pellet with and without sonication of the extract used for the enzyme assay. ADC and ODC activities were significantly higher in Agrobacterium rhizogenes strain grown on "199" polyamine-free medium. This is in agreement with other data concerning the inhibition of ADC and ODC activities by the presence of polyamines in the culture medium. The possibility that a high arginase activity might have affected the evaluation of ADC and ODC activities was checked.

ACTIVITIES OF ARGININE. ORNITHINE AND S-ADENOSYLMETHIONINE DECARBOXYLASES DURING TOMATO FRUIT DEVELOPMENT

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INTRODUCCION

Polyamines (PAs) are considered as a new natural system of plant growth regulation, with different functions and whose mechanisms are not completely understood. Some papers point out a possible role of these compounds in climateric fruit maduration, probably by competition with ethylene biosynthesis. It is not unreasonable to think they are involved in non climateric fruit maduration too. The simultaneus study of ethylene and PAs metabolic pathways is being done in our research program called Phytohormones and enzymatic systems involved in ripening and senescence. In this poster we show the results of arginine decarboxylase (ADC), ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) activities during tomato fruit development.

MATERIAL AND METHODS

PLANT MATERIAL Tomato plants (Lycopersicon sculentum Mill. cv Robin) grown in a sheltered place under natural light conditions. Fruit at various stages of development was harvested.

ENZYME EXTRACTION Fruit tissues were homogenized in 0.1M phosphate buffer (pH7) at 0.1g fresh weight/ml. with a **OMNIMIXER**. The homogenates were centrifuged at 27.000g for 20 min at 0-4 C° and the supernatants were used as the cell free extracts. Protein concentrations were determined according to Bradford(1976).

ENZYME ASSAYS ADC,ODC and SAMDC activities were determined by incubating 0.19 ml aliquots of the supernatant with 0.01 ml of the respective labelled substrate: L-[1-^{14}C]-arginine (46mCi/mmol) diluted (1:5) with 10mM unlabeled arginine,DL-[1- ^{14}C] -ornithine (50.3mCi/mmol) diluted (1:5) with 50mM unlabeled ornithine and [S-carboxyl-^{14}C]- adenosylmethionine (47.8mCi/mmol).The reaction mixture was incubated in a shaker water bath at 37 $^{\circ}$C for 45 min.After measuring the released $^{14}CO_2$,decarboxylase activities are expressed as pmol $^{14}CO_2$-mg protein^{-1}.h^{-1}.

COLOUR Measure using a Minolta CR-200 colorimeter calibrated with a standard white tile (L=97.83,a=-0.48,b=1.96). Colour was expressed in terms of 1000.a/L+b.

RESULTS AND DISCUSSION

Figures 1 and 2 present ADC, ODC and SAMDC activities with increasing tomato fruit age (days after full bloom) as related to fresh weight.. Figures 3 and 4 show those enzymatic activities at various stages of fruit ripening.

All activities exhibit similar trends : at the early stages of fruit development they increase sharply, with a maximum near the fifth day after full bloom and then, there is a decrease.(fig.1 and 2). When maturation starts, the activities increase again, although only slightly.This fact seems to coincide with the colour change associated with the climacterium (fig.3 and 4).

The analysis revealed that ODC activity was significantly greater than that of ADC and SAMDC, mainly at the logarhitmic phase of growth. This findings could be interpreted as another evidence for an important role of this enzyme in actively dividing tissues.

Additional studies on PAs, ACC and ethylene levels and decarboxylases and EFE activities will be done before conclusions can be fully established.

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PURIFICATION AND PROPERTIES OF PUTRESCINE HYDROXYCINNAMOYL TRANSFERASE EXTRACTED FROM TOBACCO CELL SUSPENSIONS.

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The enzyme hydroxycinnamoyl-CoA putrescine N-hydroxycinnamoyl transferase (PHT) is very active in tobacco callus cultures, in which it occurs together with another transferase (SHT) conjugating specifically spermidine to hydroxycinnamic acids, mainly p-coumaric acid [1, 2]. To further investigate the properties of PHT, the transferase was purified 1000 fold in 19% yield from tobacco cell suspensions (*Nicotiana tabacum* TX1 cell line [3]) to a final specific activity of 112 nkat/mg protein. The purification procedure involved ammonium sulphate fractionation, anion exchange chromatography, gel filtration, hydroxylapatite chromatography, and chromatography on caffeoyl-cysteamine-Sepharose. After this procedure, PHT was further purified to near homogeneity, but in low yield, by chromatofocalisation (pHi=8). The apparent molecular weight mesured by SDS-PAGE and high performace gel filtration was 50.000. After purification, PHT still catalysed the conjugation of several aliphatic diamines (putrescine, cadaverine and diaminopropane) to cinnamoyl-CoA derivatives (mainly caffeoyl-,feruloyl- and cinnamoyl-CoA). Several aliphatic diamines with carbon-chain length ranging from C_2 to C_8 were added to the culture medium of the cell suspensions to try to induce PHT activity. Even at high concentration (10 mM) these amines were not toxic and did not alter the growth of the cell suspensions. PHT activity was measured 3 days and 1 week after subculture in the diamine containing medium. No differences were found between treated cells and controls. Thus in tobacco cell suspensions PHT which exhibits the properties of a true diamine hydroxycinnamoyl transferase, is not inducible by diamines, whether they are used as substrates or not.

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RELATION BETWEEN CHEMICAL REGULATORS AND PHOTOPERIOD IN 'IN VITRO' POTATO TUBERIZATION

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Short photoperiods detected by potato plant leaves are thought to trigger an inducing response of hormonal nature, resulting in tuberization.

Searching for tuber-inducing chemical stimuli under photoperiodic non-inducing conditions, such as long (16h) photoperiods or contiuous darkness, the effect of several potential regulators on in vitro tuberization of stem sections was studied.

Kinetin (2.5 mg/l), paclobutrazol (2.5 mg/l) and acetic acid (6 mM) strongly promoted tuberization in the dark, while $CaCl_2$ (6mM) or putrescine (25mg/l) only led to a 30 to 50% tuberization. A lower percentage was obtained under long photoperiods: 30 to 35% tuberization for kinetin, paclobutrazol or acetic acid; none for $CaCl_2$ or putrescine.

Kinetin, paclobutrazol and acetic acid, but neither $CaCl_2$ or putrescine, effectively decreased explant length and root development, both under long photoperiods or in the dark.

POLYAMINE LEVELS IN FILBERT (Corylus avellana L.): EFFECTS OF TISSUE CULTURE, MATURATION STATE AND SEASONAL CHANGES.

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Tissue culture in woody species is affected by physiological changes related with phase change and maturation, being responsible for the decline in cloning ability and morphogenic potential observed. Partial reappearance of juvenile characteristics, including rapid growth and recovery of morphogenic competence, was achieved during tissue culture of adult filbert. Also, severe pruning of mature clones of filbert allowed to obtain trees in different maturation stages, in order to preserve plants in a responsive physiological state (in vivo rejuvenation) for micropropagation purposes. We tryed to determine the physiological basis of the maturation effect on filbert tissues, in order to gain knowledge for attempting to reduce the loss of morphogenic potential in this species.

In view of the relationship of polyamine levels and metabolism with senescence-related and growth-related physiological processes in plants, we study the endogenous levels of polyamines in filbert plant material from different sources, in order to know if the age of the material, as well as possible rejuvenation techniques, could change the polyamine metabolism. The results would allow us to make a true interpretation of the data obtained through tissue culture, and perhaps to propose polyamines as an "index" for the degree of juvenility of the plant material used in our conditions.

Polyamines were extracted in PCA from filbert leaves, dansylated along with pure standards, and chromatographed using TLC silica gel plates (Whatman Int. Ltd., Maidstone, England) with concentrating zone. As the solvent was used chloroform/triethylamine (25:2, v/v). Identification and quantification were done by comparison of chromatograms with those of pure standards, using a Cliniscan II densitometer (Helena Labs., Beaumont, Texas 77704, USA).

The plant materials tested were: mature, juvenile, forced outgrowth and in vitro tissues. On the other hand, they were also analyzed leaves from once, twice and unpruned trees, collected in spring and autumn.

Quantitative differences among the above materials were observed. Thus, polyamine levels appeared to change in response to the treatments assayed. Differential levels in relation to the age of the material with respect to the in vitro cultured tissues were observed. In addition, polyamine levels appear reflect the effectiveness of the pruning treatments in changing the physiological status of the trees.

These results can be correlated with protein patterns after electrophoretic analysis, and with the methylation status of the genomic DNA for the assayed plant materials, both as an indication of the level of gene expression during aging and rejuvenation in filbert.

PHYSIOLOGICAL STUDIES ON AMINE OXIDASE FROM *LENS CULINARIS*

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Copper containing Amine oxidases (AOs) are widespread among eukaryotes. They oxidize only primary amines, producing the corresponding aldehyde, NH_3 and H_2O_2.

Lentil seedlings amine oxidase (LSAO) is an homodimer having a Mr of 140 KDa; it contains two copper ions and an organic cofactor per molecule.

Plant AOs have been found mainly in seedlings and, though they have been purified and characterized from many sources, their physiological role is still poorly understood. The substrate specificity of these enzymes suggests that they may be involved in regulating the concentration of diamines and polyamines. Since they act as growth regulators or as antagonists of various types of stress, it seems possible an involvement of AOs in these processes.

In order to get an insight into the physiological role of AOs we started to study the expression of LSAO during the germination of *Lens culinaris* seeds.

LSAO is undetectable in the resting seed and its activity starts abruptly after imbibition. The specific activity reaches a peak after 6 days and it is much higher when the seedlings grow in the dark. There is a strong correlation between growth rate and LSAO activity both in light-grown and dark-grown seedlings. The same is true for the LSAO content, assayed by antibodies, and its mRNA level. These data indicate that the regulation of enzyme activity in the dark condition was achieved by changes in the concentration of LSAO mRNA, but when a possible phytochrome regulation was investigated by delivering to dark-grown seedligs pulses of red and/or far red light of different lenght and intensities, no changes in the LSAO activity could be observed. The same was seen with blue light irradiation, indicating that light is only indirectly involved in the LSAO activity control.

Besides the absence of light, also cytokinin (CK) stimulated LSAO activity. This is not surprising because CK is a well known stimulator of the plant growth. Anyway CK acts through a different mechanism since it does not influence the protein or mRNA content.

ARE MODIFICATIONS IN FLOWER DEVELOPMENT ASSOCIATED WITH CHANGES IN MITOCHONDRIAL GENOME ORGANIZATION AND EXPRESSION UPON SOMATIC HYBRIDIZATION IN CYTOPLASMIC MALE STERILE LINES OF *NICOTIANA*?

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Somatic hybrid/cybrid plants were produced by one-to-one electrofusion of defined selected protoplast-protoplast and protoplast-cytoplast pairs (microfusion) of male fertile *N. tabacum* and different alloplasmic cms mutant lines (with cytoplasms of *N. bigelovii* and *N. debneyi*) followed by microculture of the fusion products obtained. Analysis of the transmission genetics of organelles was performed using chloroplast (Str $^{r/s}$) and mitochondrial (mtDNA RFLPs) markers. In addition, variation in flower morphology, developmental histology and SEM studies on floral bud formation were carried out for cybrids from cms + cms' and cms + mf fusion combinations. Changes in mitochondrial genome organization as well as mitochondrial gene expression studied at the level of *in organello* translation products were detected. Correlative evidences supporting an association of the observed changes with the homeotic floral phenotypes (petalody and feminization of stamina) will be discussed.

EFFECT OF D-L-ALPHADIFLUOROMETHYLORNITHINE ON THE DIFFERENTIATION PROCESS IN MERISTEMATIC CALLI OF MAIZE

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The aim of this work was to determine the effect of increasing dosis of D-L-alphadifluoromethylornithin (DFMO) over the "in vitro" differentiation of maize meristematic calli. This type of callus is an organized tissue obtained from cauline meristem after culture in medium with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). It is derived from the hypertrophic development of a meristem caused by the action of the auxin in a specific phase of its development. (Torné et al.1984; Santos et al.1984). Meristematic calli can be perpetuated from long time and they regenerate plants via organogenesis. (Torné and Santos,1987). The DFMO (Metcalf et al.1978) is an irreversible inhibitor of the plant activity of the enzyme ornithine decarboxilase (ODC) responsable of putrescine (Put) synthesis from ornithine (Slucum and Galston, 1987) (Tiburcio et al.1989).

During the experiment of inhibition, calli were cultured in a maintenance medium (Torné et al.1984) containing 2mg/l of 2,4-D and suplemented by 0,1,3,5 or 10mM of DFMO respectively. Ten calli were used for each treatment. The increment of fresh weight, polyamine (PAs)(TLC analisis; Tiburcio et al.1985) and aminoacid (HPLC analisis) contents of the calli were measured.

In differentiation tests, previously inhibited calli were subcultured during two months in medium containing low auxin concentration whith the addition of 0mM or 1mM Put. The final number of regenerated buds or

plantlets were measured for all the treatments.

In the light of these results, it can be stated that: a) Calli fresh weight decrease with DFMO increment in the medium, b) Total PAs decrease with DFMO concentration, although a light increment of them from 5mM DFMO o 10mM DFMO can be observed, c) There are no significative changes in the aminoacid (free and structural) content of the calli.

When differentiation response was studied, it could be seen that in general the regeneration process was improved by adding to the medium 1mM Put. Inhibitor concentrations of 3mM and 10mM increased from 4 to 6 times the number of regenerated structures over the control.

As a conclusion, we can state that pretreatment with DFMO can stimulate the differentiation process of maize calli by modulating their "in vitro" response. It can be suposed that this effect is a combinate action between endogenous and exogenous putrescine contents which can induce stimulation of the calli rejuvenation process in the differentiation medium. Moreover, this effect is demonstrated to act in a dosis-dependent manner.

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CONCLUSIONS AND PERSPECTIVES

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CONCLUSIONS AND PERSPECTIVES:

The conference on "Polyamines as Modulators of Plant Development" began just as the world was plunged into the uncertainties of war in the Persian Gulf area. As a consequence, five of the originally scheduled speakers (Altman, Davies, Kaur-Sawhney, Malmberg, Roubelakis-Angelakis) could not attend. This loss of speakers was more than compensated, however, by an invitation to twelve of the poster-presenters to deliver 15-minute talks. They were organized into two sessions, one dealing with molecular and biochemical data (Mehta, Besford, Burtin, Negrel, Rossi, Fritze) and the other with developmental data (Del Duca, Caffaro, Dehio, Acosta, Carbonell, Campos-Lopez). These predominantly younger speakers richly augmented the presentations of the originally scheduled program.

It is now clear that the significance of polyamines in plants is becoming more widely accepted and recognized. The era of the necessary establishment of correlations has now ended and is being supplanted by a new period of analytic experimentation and molecular biology. We should expect these new studies, especially as they deal with specific inhibitions, purified enzymes, mutations, cloned genes and transgenic plants, to bring new definition to this field.

The first session dealt with the chemistry, metabolism, transport and compartmentation of polyamines, as presented by Smith, Slocum, Flores, Bagni and Creus. This led logically to more physiological considerations of regulation by polyamines and their biosynthetic inhibitors, analyzed by Galston, Serafini-Fracassini, Tiburcio and Martin-Tanguy. Mechanisms of action were considered by many of the speakers, and a summary talk on this subject was presented by Dumbroff. Polyamines are polycations at cellular pH's, and it is most likely that some aspects of their function depends on association with cellular polyanions, especially the phosphate groups of DNA, RNA and membrane phospholipids. In this way, polyamines could affect such vital processes as DNA replication, transcription, translation and permeation through membranes. Other possibilities include post-translational covalent attachment to proteins through the action of transglutaminase, and the quenching of free radicals, especially in membranes. Experimental evidence can be found to support each of these suggested mechanisms.

The discussions revealed general agreement that polyamines can play important roles in stress and senescence, as well as in cell division, cell

extension, and certain aspects of morphogenesis including flowering. In addition, there is considerable evidence that the T-DNA transferred from *Agrobacterium rhizogenes* to tobacco includes a gene (rol A) that down-regulates certain aspects of polyamine biosynthesis, since the phenotypic expression of this gene can be mimicked by DFMO, a specific, suicide inhibitor of ornithine decarboxylase. This underscores evidence presented in other papers that polyamine aberrations are frequently found during microbial infection.

The meeting was efficiently and agreeably organized and hosted by the Juan March Foundation, through Andres Gonzalez and his excellent staff. The meeting size and room were appropriate, the time for discussion adequate, the posters well-displayed, the food and accommodations excellent and the ambiance near optimal. Conferees were also treated to a private showing at the Foundation of a new Picasso exhibit featuring portraits of the artist's wife Jacqueline, and a concert of baroque music featuring recorder, viola da gamba and lute. By any measure, this was a remarkable three days, for which the conferees enthusiastically expressed their thanks to the Foundation.

LIST OF INVITED SPEAKERS

Lecture Course on
POLYAMINES AS MODULATORS OF
PLANT DEVELOPMENT

List of Invited Speakers

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253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
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J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P. Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

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