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Polyamine metabolism, floral initiation and floral development in Chrysanthemum (*Chrysanthemum morifolium* Ramat.)

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Abstract

In the short-day plant Chrysanthemum (*Chrysanthemum morifolium* Ramat. variety Pavo) putrescine and spermidine conjugates appeared in the apical bud before the first observable transformation of the meristem into floral structures. These compounds accumulated on floral initiation and well before floral evocation. Spermidine conjugates were predominant during floral initiation whereas free amines did not accumulate to any significant extent. Different associations of amides were observed during floral initiation as compared with the reproductive phase. 3,4-Dimethoxyphenethylamine conjugates (water-insoluble compounds) were the predominant amine conjugates observed during flower development. These compounds decreased drastically after fertilization. In vegetative buds from plants grown in long days polyamine conjugates were very low and appeared as plants aged. We present evidence that ornithine decarboxylase (ODC) regulates putrescine biosynthesis during floral initiation and floral development. When ODC action was blocked by DFMO (α-DL-difluoromethylornithine, a specific, irreversible inhibitor of ODC), flowering was inhibited, and free and conjugated polyamines were not detected. This treatment led to a slight enhancement of ADC activity. When putrescine was added, polyamine titers and flowering were restored. A similar treatment with DFMA (α-DL difluoromethylarginine, a specific, irreversible inhibitor of ADC) did not affect flowering and the polyamine titers. The results suggest that ODC and polyamine conjugates are involved in regulating floral initiation in Chrysanthemum.

Abbreviations: ADC = arginine decarboxylase; ODC = ornithine decarboxylase; DFMA = α-DL-difluoromethylarginine; DFMO = α-DL-difluoromethylornithine.

1. Introduction

Polyamines (PAs) are now generally recognized as necessary for the orderly patterns of growth and development in plants, animals and microorganisms [1, 14, 25]. Recent studies with higher plants have also implicated PAs in such varied processes as response to stress [21], senescence [13], regulation of the cell cycle [2], embryogenesis in tissue culture [9] and floral initiation [17, 22].

Amine conjugates (polyamine and aromatic amine conjugates), covalently bound to hydroxycinnamic acids have also been found in high

levels in plants [3, 16] and are thought to be correlated with developmental phenomena. These compounds do not normally occur in leaves or other vegetative shoot tissues of plants. They accumulate in shoot apices upon floral initiation [18]. The amide appears before the first observable transformation of the meristem into a floral structure. When the plants flower, the amides are found in abundance in the inflorescence, mainly in the sex organs of the flower, but practically disappear from the leaves [18]. These compounds are absent from sterile reproductive organs, and they appear to constitute biochemical markers for pollen and ovule fertility [18]. The concentration of the amides in flowers decreases quickly and drastically following fertilization, while free amines do not accumulate to any significant extent at any time.

It appears that in tobacco plants free polyamines derived through ADC may be involved in vegetative development [7], while conjugated polyamines derived through ODC may be required for floral initiation and sexual differentiation. The irreversible suicide inhibitors DFMA [13] and DFMO [20] specifically inhibit plant ADC and ODC activities. They are not metabolized in plants.

The function of polyamines in cell division and morphogenetic processes in plant systems has been studied using two kinds of experiments: (1) seeking correlations between polyamine levels and activities of their biosynthetic enzymes on cell division or morphogenetic processes and (2) studying the effects of inhibitors of polyamine biosynthesis, with and without exogenous polyamines, on the morphogenetic process. We are presently using this approach in Chrysanthemum focusing on two morphogenetic processes in which cell division is involved: floral initiation and floral development. Chrysanthemum plants grown under 8 h light/16 h dark periods will flower, while they remain vegetative under a 16 h light/8 h dark regime in our controlled condition growth rooms.

2. Materials and methods

2.1 *Plant material and growth conditions*

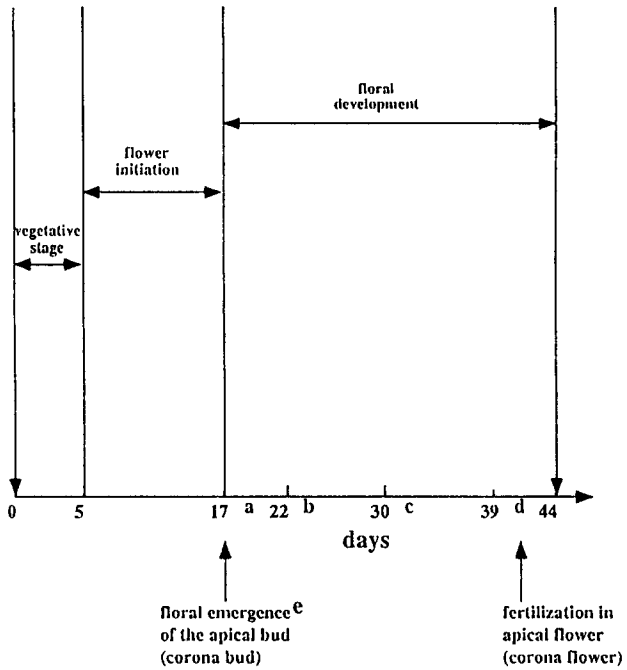
Cuttings from Chrysanthemum plants (*Chrysanthemum morifolium* Ramat. variety Pavo) cultivated under long days, were planted in clay pot containing peat and gravel and fed with a nutrient solution [8]. They were cultivated under controlled conditions; with either a 10 h or 16 h light period (300 μmol $m^{-2}s^{-1}$ PPFD from Philipps TLF 110 fluorescent tubes and 40 W incandescent lamps), 80% RH and 20 °C. When grown in short days (10 h light periods) complete development was observed and two chronological reference points were identified. The first occurred at 5 days of culture. 3-day old cuttings were grown under short days (10 h light periods), and then placed under long days (16 h light periods). These plants, never initiated flowers, but 5 day (or older) cuttings cultivated under short days and then placed under long-days flowered. Thus, the long day inhibitory effect on flowering does not exist in the latter case. The other reference point is situated at 17 days and corresponds to emergence of the floral apical bud. The different stages of development are presented in Figure 1.

The first day of treatment was designated as day 0. During the first week of treatment, inhibitors were applied to the soil of pot-grown plants three times per week in a volume of 20 ml. Controls were treated with deionized water. During the following weeks treatment was reduced to twice per week. On days of no treatment each plant received 20 ml of nutrient solution.

DFMO and DFMA were obtained from Merell Dow Research (DFMA from Cincinnati, USA and DFMO from Strasbourg, France).

2.2 *Analysis of amine content*

Amine contents and the activities of the related enzymes were quantified in shoot tips of Chrysanthemum cultivated under short and long days. A sample of the apex including the first 1 to 1.5 cm



[a] apical bud (length = 6 ± 2 mm, diameter = 5 ± 2 mm

[b] apical bud (length = 15 ± 3 mm, diameter = 8 ± 2 mm

[c] apical bud (length = 22 ± 3 mm, diameter = 14 ± 3mm

[d] apical flower before fecondation

[e] meiosis takes place during floral emergence in the corona bud

Fig. 1. Developmental stages of Chrysanthemum plants under short days.

fragment of the foliar stem before floral evocation is termed the shoot-tip.

Under short days measurements were made at different stages of floral development. In the present study we investigated changes in amines and ADC and ODC only in apical buds (corona buds) and flowers (corona flowers).

Amine analysis was as previously described [10]. Free amines were extracted in 5% v/v cold $HClO_4$, and HPLC fluorescence spectrophotometry was used to separate and quantify amines prepared as their dansyl derivatives [24]. Previously published methods [19, 23] were used for the analysis of conjugated amines. For the quantification of water-insoluble conjugates, extracts were boiled in 6 M HCl, followed by measurement of liberal free amines. Water-soluble conjugates from the aqueous extract were eluted from an Amberlite (Serva CG 50, H^+ from) column with 3 M acetic acid. The compounds were chromatographed on a Bondapak C18 reverse phase column. Amounts of free and conjugated amines are expressed relative to fresh weight. Similar results (not shown) were obtained when the data were calculated as a function of dry weight.

2.3 *Determination of ADC and ODC activities*

ADC and ODC activities were determined after several modifications of the procedures described earlier [4]. Samples were ground in a chilled mortar at a ratio of 1 g fresh weight ml^{-1} of 100 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 10 mM mercaptoethanol, 0.5% (w/v) ascorbic acid and 10% (w/w) activated charcoal to absorb phenolics. The extract was centrifuged 20 min at

23,000 g. The supernatant was satured at 50% with $(NH_4)_2SO_4$ for 1 h with gentle stirring. The pellet was collected after 20 min by centrifugation at 25,000 g and resuspended in a minimum of extraction buffer. This fraction was then dialysed against two changes (1 l each) of 10 mM Tris-HCl containing 1 mM EDTA and 10 mM mercaptoethanol for 8 h. All procedures were carried out between 0° and 4 °C. The dialysed extract was used to determine ADC and ODC activities [6].

To assay ODC, 50 μl of extract were mixed with 5 μl (U-$_{14}$C) ornithine (7.46 GBq/mmol, Commissariat à l'Energie Atomique, C.E.A.), 45 μl 100 mM Tris-HCl (pH 8.0), containing 10 mM mercapthoethanol, 0.1 mM pyridoxal phosphate and 5.5 mM cold ornithine [6]. To assay ADC, 50 μl of extract were mixed with 3 μl (U-^{14}C) arginine (11.1 GBq/mmol, Commissariat à l'Energie Atomique, C.E.A.), 45 μl 100 mM Tris-HCl (pH 8.0) containing 10 mM mercapthoethanol, 0.1 mM pyridoxal phosphate and 2.65 mM cold arginine. Reaction mixtures were incubated for 1 h at 30 °C. The reactions were stopped with 10 μl of 5 M acetic acid. For blanks, 10 μl of 5 M acetic acid were added at t = 0. Denatured proteins were removed after 5 min at 15,000 g centrifugation. 10 μl aliquots were analysed by thin layer electrophoresis on cellulose plates (Merck Avicel). Electrophoresis was performed in acetic acid-pyridine-water (5 : 1 : 94) for 1 h at 300 V. Unlabelled agmatine and putrescine used as standards were also spotted on the plate and developed with ninhydrin (5% w/v in ethanol). The cellulose was scraped off and transferred to scintillation vials and the radioactivity determined in a Beckman LS 1801 scintillation counter [6].

2.4 *Protein analysis*

Soluble protein was determined according to the method of Bradford [5]. Bovine Serum Albumine was used as standard.

3. Results

3.1 *Amine titers, related enzymes, floral initiation and flower development in Chrysanthemum*

The main free amines detected in Chrysanthemum var Pavo were putrescine (Put), spermidine (Spd), spermine (Spm), phenylethylamine (Phe), tyramine (Tyr) and 3,4-dimethoxyphenylethylamine (3,4-Phe). Amine conjugates were of two types: water-soluble, having a primary amine function (polyamine conjugates) and water-insoluble, showing no function that can be ionized (aromatic amine conjugates). These compounds contain hydroxycinnamic acids (p-coumaric acid and caffeic acid) and amines linked by an amide bound. Until day 9 of culture the content and distribution of free amines (i.e., polyamines, spermidine and spermine and the aromatic amines, tyramine and 3,4-dimethoxyphenylethylamine) in shoot tips of plants cultivated under short days followed a similar pattern to that observed in shoot tips of plants cultivated under long days (Fig. 2). Under both conditions the levels were similar and remained low. However, under long days a marked decrease in polyamine and aromatic amine levels was observed until day 40; amine titers then increased slightly during the later stages of culture. Under short-day conditions considerable variation was observed in the concentrations of amines during floral induction and at different stages in flower development. Putrescine, spermidine, spermine changed more or less in parallel (Fig. 2) during the 50 days of culture (Fig. 2), showing an initial increase and reaching a peak on day 14 (before the visible appearance of the flower bud). After and until day 22 a decrease was observed, followed by an increase at day 30 for spermidine and spermine and at day 44 for putrescine. After day 30 a decrease of all substances was observed during the later stages of floral development. During the whole period of development, spermidine was the predominant polyamine, representing 50 to 60% of the total polyamine pool at day 14. During floral development tyramine accumulated substantially,

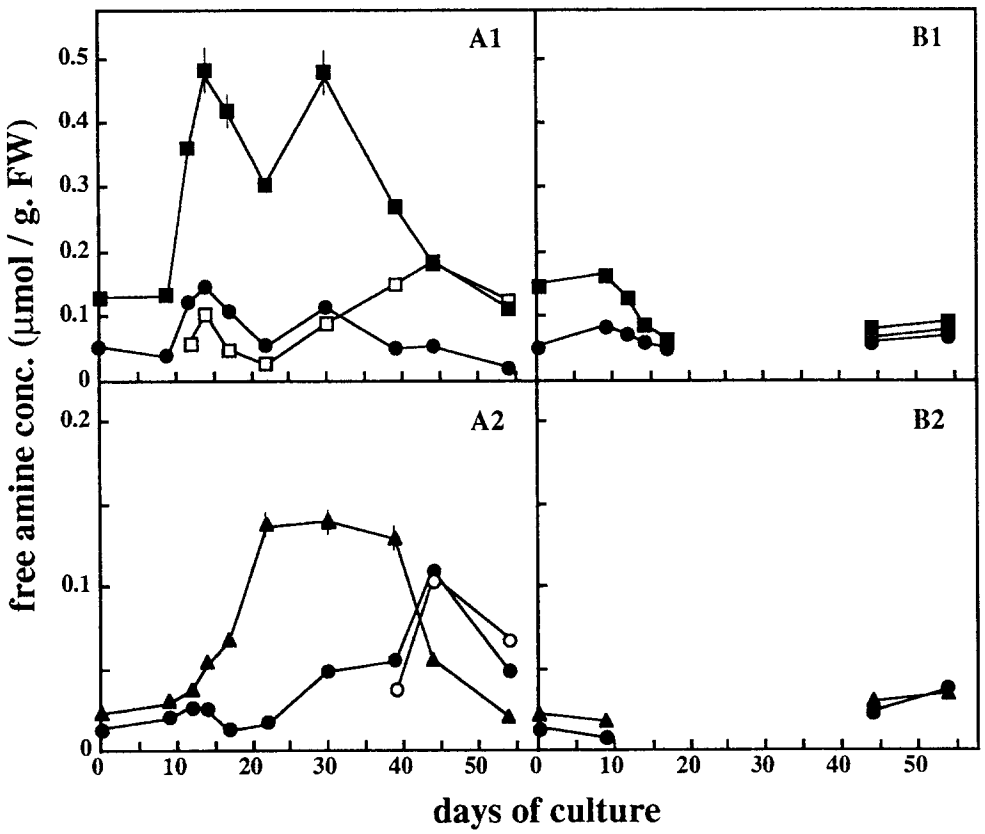


Fig. 2. Free polyamine and aromatic amine levels in the shoot tips of Chrysanthemum plants grown under short (A1, A2) and long (B1, B2) days and during flower development under short days (A1, A2). A1, B1: free polyamines; Put □, Spd ■, Spm •; A2, B2: free aromatic amines; Phe ○, Tyr ▲, 3,4-Phe •. Values expressed as µmol/g fresh weight. Means ± SD of 3 or 4 replicates each representing 10 to 20 plants.

reaching a maximum at day 22 and decreasing markedly thereafter. Phenylethylamine and 3,4-dimethoxyphenylethylamine behaved similarly, reaching a maximum at day 44, during the later stages of floral development. The levels of tyramine were higher than those of phenylethylamine and 3,4-dimethoxyphenylethylamine up to day 39.

Amine conjugates were not present at day 0 (Fig. 3) Under short days putrescine and spermidine conjugates increased rapidly in the shoot tips and reached a peak at day 9 before floral emergence of the shoot tip (Fig. 3). A decrease was then observed from days 9 to 14, followed by a slight increase. Peaks were observed at days 39 and 44 for spermidine and putrescine, respectively, followed by a decrease in the content of both compounds. Diaminopropane conjugates appeared at day 39, then increased up to day 44. Spermidine conjugates constituted 50% of the polyamine conjugate pool at day 9. Under long days putrescine and spermidine conjugates appeared at day 44, but remained at low levels during the later stages of culture (Fig. 3). Aromatic conjugates (tyramine, 3,4-dimethoxyphenylethylamine) were not found in the shoot tips during floral initiation (Fig. 3) but increased gradually after day 16 to reach a maximum at day 39, and decreased thereafter. 3,4-Dimethoxyphenylethylamine conjugates were the predominant amine conjugates observed during the flower development, accounting for about 50% of the total by day 39. In plants culti-

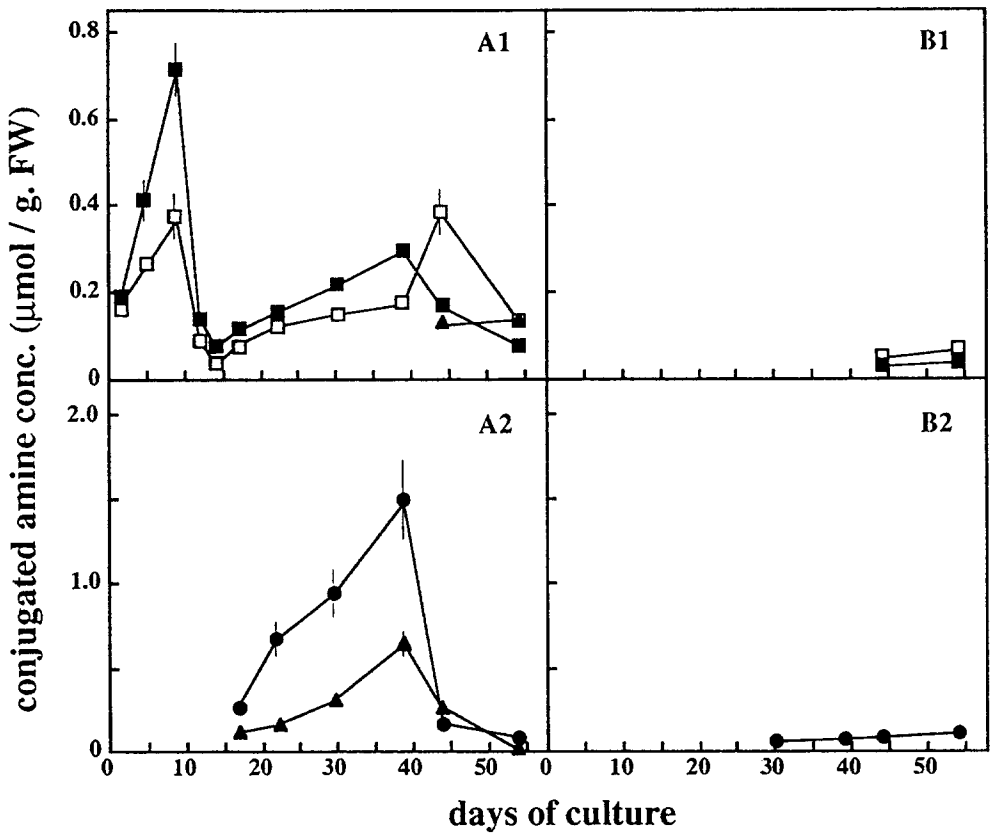


Fig. 3. Conjugated polyamine and aromatic amine levels in the shoot tips of Chrysanthemum plants grown under short (A1, A2) and long (B1, B2) days and during flower development under short days (A1, A2). A1, B1: conjugated polyamines; Put □, Spd ■, Dap ▲; A2, B2: conjugated aromatic amines; Tyr ▲, 3,4-Phe •. Values expressed as µmol/g fresh weight. Means ± SD of 3 or 4 replicates each representing 10 to 20 plants.

vated under long day conditions, very low concentrations of 3,4-dimethoxyphenylethylamine appeared at day 39, and remained low as the plants aged (Fig. 3).

Under short days, the activity of ODC was much higher than that of ADC at all stages of development, reaching maximal activity between days 14 and 22. At day 14 ODC activity was approximately 7 times higher than that of ADC (Fig. 4). ADC activity was not found during floral initiation (Fig. 4). ADC and ODC activities remained low in shoot tips cultivated under long days (Fig. 4), with ODC detected only after 14 days of culture.

3.2 *Effects of polyamine biosynthesis inhibitors on development, polyamine titers, and related enzymes in Chrysanthemum cultivated under short-days*

Treatment with DFMO at 2 mM inhibited flowering under a short day regime (Table 1), and produced plants having short internodes, wrinkled leaves and developed axillary buds (data not shown) (Table 1). Simultaneous treatment with DFMO plus putrescine at 2 mM did not produce these phenotypic changes (data not shown); flowering and did not produce phenotypic alterations.

Free polyamines (spermidine and spermine) and conjugated polyamines (spermidine and spermine conjugates) were not detected after treatment with DFMO (Table 2), but agmatine

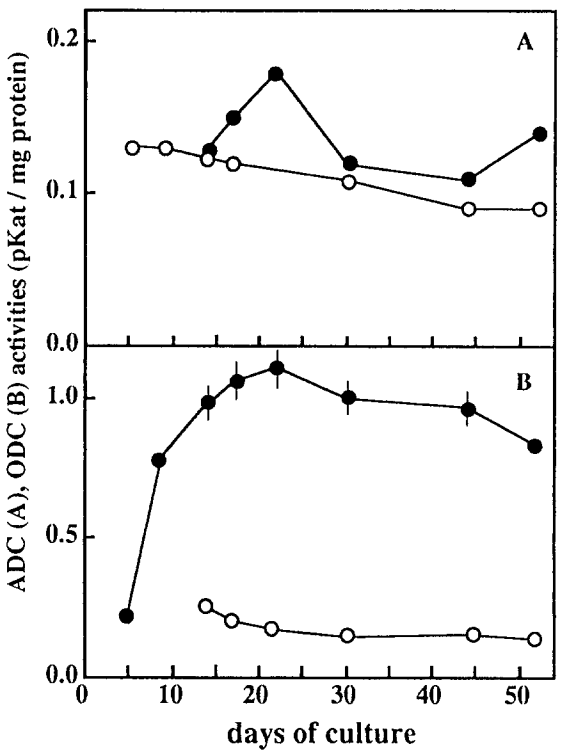


Fig. 4. Changes in ADC (A) and ODC (B) activities (pKat/mg protein) in the shoot tips of Chrysanthemum plants grown under short (•) and long (o) days and during flower development under short days (•). Means ± SD of 3 or 4 replicates each representing 10 to 20 plants.

accumulated in the shoot tips (Table 2), but agmatine accumulated in the shoot tips (Table 2). A combination of DFMO + putrescine increased free polyamines (spermidine and spermine) and polyamine conjugates (putrescine and spermidine); spermine conjugates also appeared. Free and conjugated polyamine titers were not lowered by DFMA at 2 mM.

No activation of ODC was detected after treatment with DFMO but ADC actively was promoted (0.30 pKats/mg protein) after 9 days of culture.

4. Discussion

The relation between amines and flowering in a higher plant can be best studied in a strictly photoperiodically determinate plant. In the short-day plant, Chrysanthemum (*Chrysanthemum morifolium* Ranat.) we observed that amide accumulated on floral initiation and well before floral evocation, i.e., before the first observable transformation of the meristem into a floral structure. Of these water-soluble polyamine conjugates, spermidine conjugates were predominant during floral initiation whereas no significant free amines accumulated during this period. Different associations of amides were observed during floral initiation as compared to fully reproductive tissues, where polyamine and aromatic amine conjugates were found. 3,4-Dimethoxyphenylethylamine conjugates (water-insoluble compounds) were the predominant amine conjugates observed during flower development, but these decreased rapidly after fertilization. Considerable changes in free amines were observed during flower development. In plants cultivated under long days amine conjugates appeared at very low levels during the last stages of culture.

The results presented indicate that ODC might regulate putrescine biosynthesis during the floral initiation and flower differentiation. When DFMO inhibited ODC, flowering was suppressed but

Table 1. Effects of DFMO and DFMA at 2 mM on the development of Chrysanthemum var Pavo plants cultivated under short day conditions. Results are given as means ± SD of three replicates, each representing to 6 plants

Treatment[a]	Emergence of floral bud (days)	Final height (cm)
No treatment	17 ± 2	35 ± 4
2 mM DFMO	n.f[b]	17 ± 2
2 mM DFMA	17 ± 2	35 ± 5
2 mM DFMO + 2 mM putrescine	13 ± 2	37 ± 5

[a] treatments began at d 0 of culture

[b] n.f: no flowers formed, plants exhibited short internodes, wrinkled leaves and development of axillary buds.

Table 2. Effects of DFMO and DFMA at 2 mM on free and conjugated polyamine titers in the shoot tips of Chrysanthemum var Pavo after day 9 of culture under short days. Results are expressed in ±mol/g fresh weight, as the means ±SD of 3 to 4 replicates each representing 3 to 5 plants

	No treatment	+ 2mM DFMO[a]	+ 2mM DFMO[a] and 2mM put	+ 2mM DFMA[a]
Free polyamine titers				
Agm	nd	0.80 ± 0.04	nd	nd
Put	nd	nd	nd	nd
Spd	0.18 ± 0.04	nd	0.25 ± 0.04	0.20 ± 0.04
Spm	0.06 ± 0.04	nd	0.20 ± 0.04	0.10 ± 0.04
Conjugated polyamine titers				
Put	0.42 ± 0.04	nd	0.40 ± 0.04	0.45 ± 0.04
Spd	0.85 ± 0.04	nd	1.20 ± 0.04	0.80 ± 0.04
Spm	nd	nd	0.60 ± 0.04	nd

[a] treatments began at d 0 of culture.
nd: not detected

a similar treatment with DFMA did not affect flowering. Simultaneous treatment with DFMO plus putrescine led to reversal of the effects of DFMO alone. The floral inhibition induced by DFMO was correlated with the expected changes in free and conjugated polyamines. Polyamine titers were lowered by treatment with DFMO but not by DFMA; this effect of DFMO was reversed by putrescine. DFMO treatment led to a slight enhancement of ADC activity (accumulation of agmatine). These results suggest that ODC and polyamine conjugates are involved in regulating floral initiation in Chrysanthemum. Considerable evidence now indicates that both ADC and ODC are active in plant tissues and that their relative contributions to putrescine and polyamine biosynthesis are dependent upon the type of tissue and the developmental process [11].

In *Xanthium strumarium*, a short-day plant, exposure of leaves to successive inductive nights resulted in a rise in conjugated polyamines, especially of spermine [12], per unit protein nitrogen. Spermine conjugates rose sharply after one inductive long night, remained high during the second cycle, then declined rapidly during the third and fourth inductive cycles. Spermidine and to a lesser extent, putrescine, followed the same trend. About eight days after the initial buds, which also correlated well with the behaviour expected of a floral stimulus. The relationship between polyamine metabolism and flowering needs further investigation and analysis of vascular sap could provide some relevant information. It was proposed that during the early events of flowering in *Xanthium strumarium*, a movement of polyamine from young, expanding leaves to the buds and developing inflorescence. Such a mechanism would bee consistent with the data obtained from studies on tobacco [8, 18] and recent studies on polyamine translocatability [2] and follows the general rules for florigen transport [26].

The availability of molecular clones for the major genes involved in polyamine biosynthesis will make it possible to manipulate the endogenous levels of polyamines in transgenic plants using tissue-specific or inductible promoters.

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