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BREAKING BUD DORMANCY IN APPLE WITH A PLANT BIOREGULATOR, THIDIAZURON

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Key Word Index—*Malus domestica*; Rosaceae; apple; breaking bud dormancy; plant bioregulator; metabolic changes; polyamines; ACC; ethylene; SAM.

Abstract—The effects of *N*-phenyl-*N'*-1,2,3,-thidiazol-5-ylurea (thidiazuron; Dropp; SN49537; TDZ) on metabolic changes in apple buds during dormancy break were determined. The data showed that thidiazuron has the capacity to release lateral buds from dormancy. Decreasing degree of bud break and bud growth with thidiazuron treatment occurred in a basipetal direction, suggesting a gradient of increasingly deep rest from shoot apex to base. The breaking of dormancy by thidiazuron is correlated with increase in DNA, RNA, protein, 1-aminocyclopropane-1-carboxylic acid (ACC), 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC), *S*-adenosylmethionine (SAM) as well as with greater polyamine formation. Polyamine and ethylene biosynthesis did not seem to be competing for SAM, their common substrate, during bud break and bud development. The release of dormancy in apple bud by thidiazuron was inhibited by cordycepine, 5-fluorouracil, 6-methylpurine and cycloheximide. Inhibition of bud break and bud growth also resulted from treatment with α-difluoromethylarginine (DFMA) and α-difluoromethylornithine (DFMO). DFMO was more inhibitory than DFMA.

INTRODUCTION

Plant hormones play a role in many aspects of growth and development. Dormancy may also be hormonally controlled [1]. Cytokinins have been shown to stimulate or accelerate release of buds from dormancy [2–8]. Recently, a plant bioregulant *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (thidiazuron; Dropp, SN49537, TDZ) has been used as a cotton defoliant [9] and was also found to exhibit cytokinin-like activity in bioassay systems [10–12] and to promote growth in cytokinin-dependent callus cultures in *Phaseolus* [13]. However, this compound was 20 times more effective in breaking dormancy in apple compared to cytokinin [Steffens and Wang, unpublished data]. In this report, we examined the metabolic changes occurring concomitantly with breaking of bud dormancy in apple by thidiazuron.

RESULTS AND DISCUSSION

Effects of thidiazuron on breaking bud dormancy and bud growth

Thidiazuron applied to buds in the upper and middle region stimulated bud break at all concentrations (Fig. 1). The optimum dosage was 100 μM for the release of dormancy and inducing subsequent growth of buds. The time course of the response to an optimum dosage of thidiazuron is shown in Fig. 2. Decreasing degree of bud break and bud growth with thidiazuron treatment occurred in a basipetal direction, suggesting a gradient of increasingly deep rest from shoot apex to base (Fig. 1). Untreated buds from thidiazuron treated plants remained dormant, indicating that thidiazuron was not translocated in apple stems (data not shown).

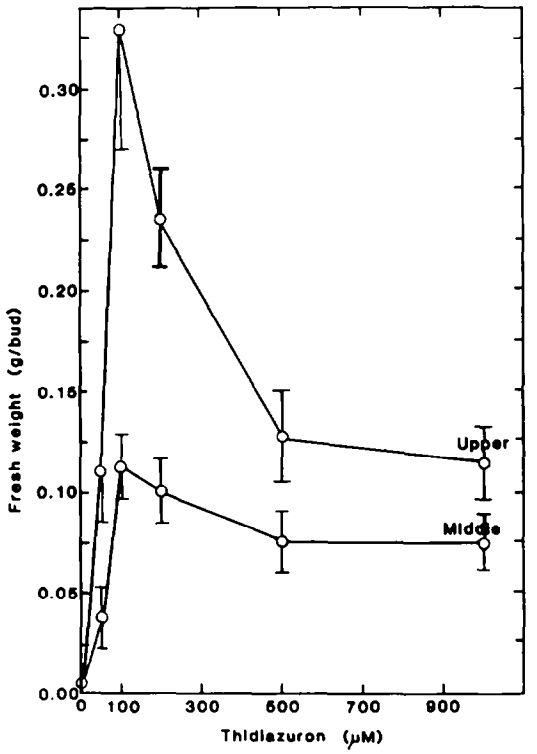

Fig. 1. Dose responses of buds in upper and middle regions of *Malus domestica* Borkh 'Gala' to thidiazuron. Measurements were made 19 days after the treatment. Thidiazuron was applied within nodes 3–20, counting from the apical tip. The treatment area was divided into three regions: upper (nodes 3–8), middle (nodes 9–14) and lower (nodes 15–20). Data of upper and middle regions are presented. Bars represent ± s.e.m.

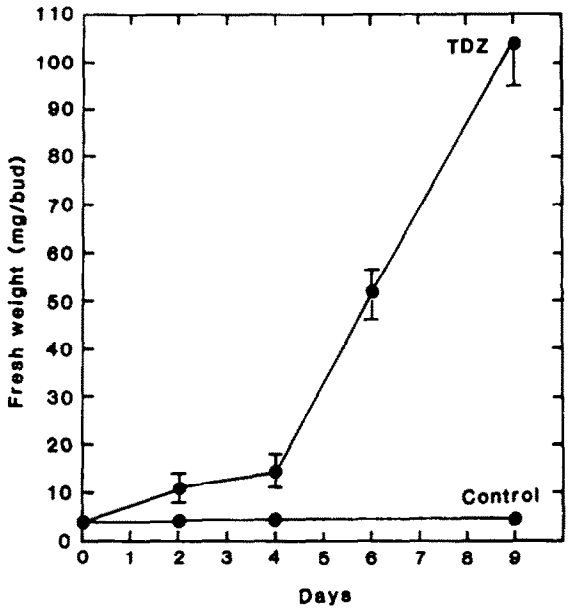

Fig. 2. Changes in fresh weight of upper region buds induced by 100 μM thidiazuron (TDZ) in *Malus domestica* Borkh 'Gala.' Bars represent ± s.e.m.

DNA, RNA and protein

Dormancy in buds involves some internal block to growth. It is under hormonal control and has a markedly low metabolic rate [1]. Thidiazuron was capable of releasing buds from dormancy. It increased DNA, RNA and protein content in apple bud (Figs 3 and 4). The release of dormancy in apple bud by thidiazuron was inhibited by cycloheximide, an inhibitor of protein synthesis [14, 15] and was also inhibited by cordycepin, 5-fluorouracil and 6-methylpurine. The degree of inhibition was proportional to the concentration of the inhibitors. The inhibition of bud break and bud growth was greater in the upper region than the lower region (Table 1).

Cordycepin has been shown to be an inhibitor of post-transcriptional polyadenylation of messenger RNA [16, 17]. 6-Methylpurine is a inhibitor of transcription [18]. It seems that cordycepin and 6-methylpurine could suppress the stimulatory effect of thidiazuron on the level of polyribosomes. A *de novo* synthesis of mRNA, as well as its post-transcriptional processing seem to be a requisite for the thidiazuron stimulation of polyribosome formation in releasing apple buds from dormancy. The inhibition of the stimulation by thidiazuron in breaking bud dormancy was also observed in the presence of 5-fluorouracil, an inhibitor of rRNA synthesis [19].

Polyamines

The breaking of dormancy by thidiazuron is also correlated with greater polyamine formation (Fig. 5). These results indicated that the transition from the dormant state to a metabolically active state also involves acceleration of synthesis of polyamines. Increased polyamine content was also observed in *Helianthus tuberosus* and potato when dormancy was broken [20–22]. The synthesis of polyamines occurred concomitantly with the synthesis of nucleic acid [22]. Our results also showed the existence of a close relationship between DNA content of the tissue and its polyamine levels on a per g fresh weight basis or on a per bud basis. Higher total polyamine levels were correlated with higher DNA content (Fig. 6).

α-Difluoromethylarginine (DFMA) is a specific irreversible inhibitor of arginine decarboxylase (ADC), a key rate-limiting enzyme in the conversion of arginine to

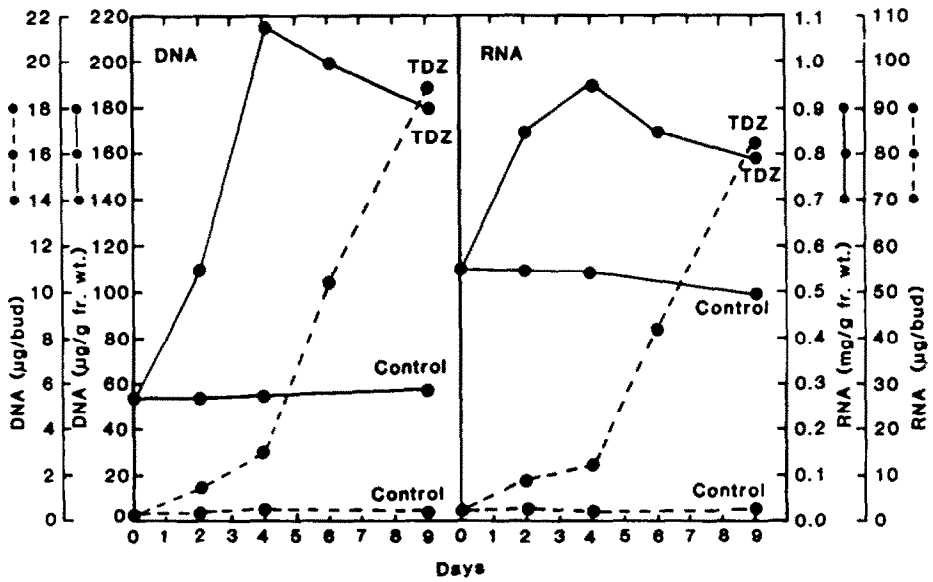

Fig. 3. Changes in DNA and RNA content during the development of upper region buds induced by 100 μM thidiazuron (TDZ). For details see Fig. 1. When expressed on per bud basis, LSD (5%) for DNA = 1.49; RNA = 5.87. When expressed as per g fr. wt basis, LSD (5%) for DNA = 26.20; RNA = 0.09.

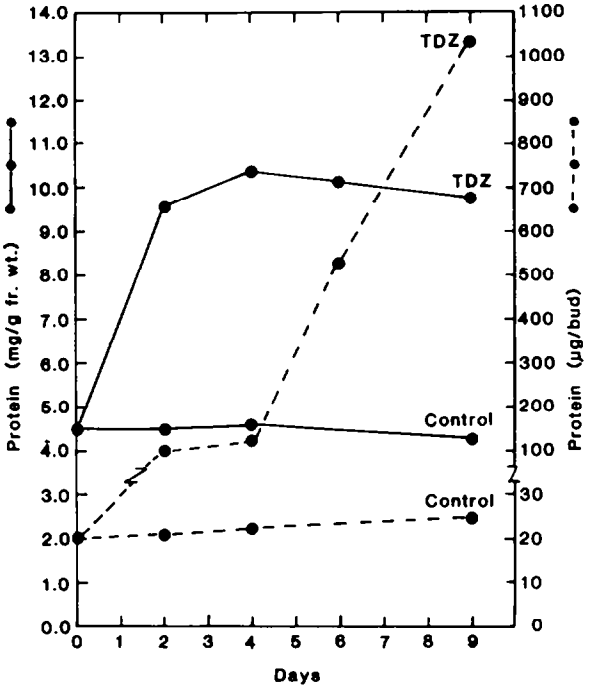

Fig. 4. Changes in protein content during the development of upper region buds induced by 100 μM thidiazuron (TDZ). For details see Fig. 1. When expressed on a per bud basis, LSD (5%) = 60.7. When expressed as per g fr.wt basis, LSD (5%) = 2.25.

putrescine, and α-difluoromethylornithine (DFMO) is a specific irreversible inhibitor of ornithine decarboxylase (ODC), an enzyme which decarboxylates ornithine to produce putrescine [23]. Since polyamines are synthesized in plants from arginine and ornithine, the effects of these inhibitors on the thidiazuron-induced bud break were also studied. Application of the DFMA and DFMO results in an inhibition of bud break and bud growth induced by thidiazuron (Table 2). This may indicate that polyamine formation is essential for one or more of the metabolic processes involved in bud break and growth. DFMO was more inhibitory than DFMA, indicating that ODC may play a more important role than ADC in controlling metabolic processes correlated with budbreak and bud growth. Enhanced activity of ODC has been reported when polyamine synthesis was accelerated during the sprouting of potato [21].

1-*Aminocyclopropane*-1-*carboxylic acid* (*ACC*) *and* 1-(*malonylamino*) *cyclopropane*-1-*carboxylic acid* (*MACC*)

DNA, RNA, protein, ACC and MACC also increased in buds after the breaking of dormancy with thidiazuron treatment (Fig. 7) in addition to the increase of polyamines. A marked increase in the endogenous levels of ACC, MACC and the rate of ethylene production has also been demonstrated in excised mung bean hypocotyl segments treated with the thidiazuron [24]. The ethylene-releasing chemical, [2-chloroethyl] phosphonic acid (ethephon) at 25–100 μM showed no effect in stimulating bud break when applied during the bud dormant period

Table 1. Effect of inhibitors of nucleic acid and protein synthesis on thidiazuron-induced bud break and bud growth in upper, middle and lower regions of plant

Treatment	Inhibitor concn (μM)	Fresh weight Upper	Middle (mg/bud)	Lower
Control		3.64 ± 0.79	7.34 ± 1.53	7.44 ± 0.96
Thidiazuron (TDZ, 100 μM)		297 ± 24	70 ± 12	29 ± 4
			% of thidiazuron	
TDZ + Cordycepin	25	98.0 (ns)	99.9 (ns)	100.0
	50	64.8	64.3	73.9
	100	19.1	60.2	54.9
TDZ + 5-Fluorouracil	25	89.0 (ns)	88.8 (ns)	96.1 (ns)
	50	66.0	66.2	78.5
	100	8.1	20.5	41.8
TDZ + 6-methylpurine	25	85.4	87.4	88.0
	50	70.0	76.1	85.0
	100	5.6	12.1	30.1
TDZ + cycloheximide	25	56.0	56.8	85.3
	50	40.7	50.0	66.0
	100	12.8	20.8	46.2

Significant at 5% level as compared to the thidiazuron. ns, Non-significant. Apple cv. Gala buds were treated with various inhibitor compounds at the indicated concentrations and 100 μM thidiazuron. Inhibitor which was applied directly to the buds was followed by 100 μM thidiazuron 4 hr latter. Samples were taken after 14 days.

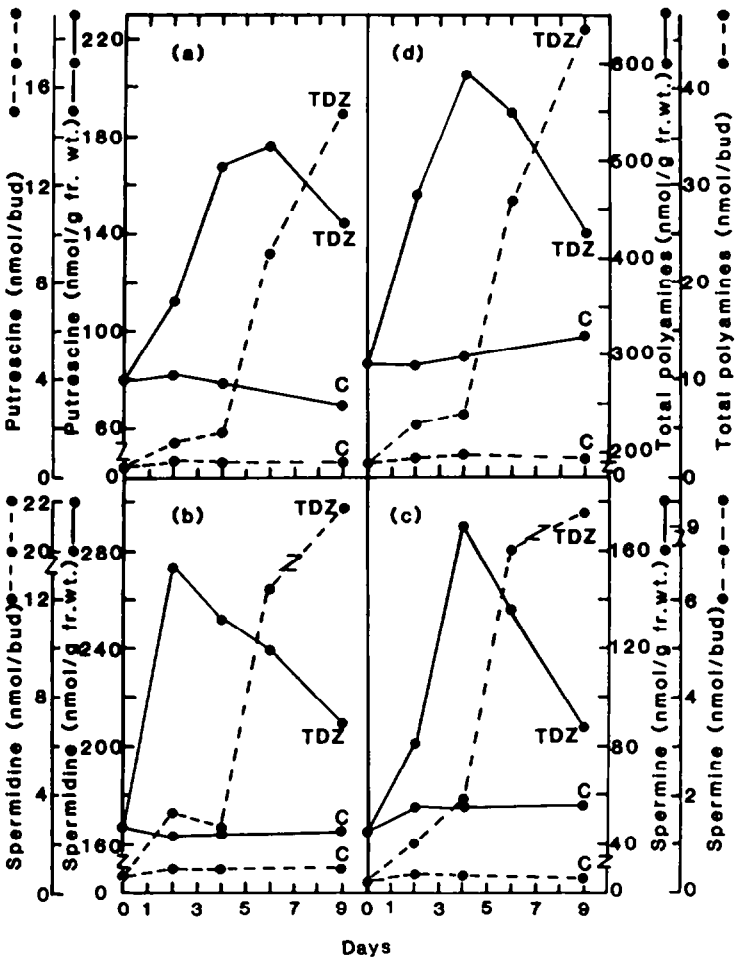

Fig. 5. Changes in content of putrescine (a), spermidine (b), spermine (c) and total polyamines (d) during the development of upper region buds induced by 100 μM thidiazuron (TDZ). For details see Fig. 1. c = control. When expressed on a per bud basis, LSD (5%) for (a) = 2.37; (b) = 2.15; (c) = 0.72; (d) = 3.83. When expressed as per g fr. wt basic, LSD 5%) for (a) = 28.5; (b) = 20.1; (c) = 30.3; (d) = 38.6.

(data not shown). Paiva and Robitaille [25] and Zimmerman *et al.* [26] also found no evidence that ethylene was involved in emergence from dormancy of buds of 'Golden Delicious' and tea crab apple. Bud break was followed by, rather than caused by, ethylene production. It was not possible to break bud dormancy in tea crab apple by addition of ethylene or ethephon [26].

S-*Adenosylmethionine (SAM) content*

SAM content followed a pattern similar to polyamine and ACC content in apple bud and increased with releasing of dormancy induced by thidiazuron (Fig. 8). Since SAM is the substrate for both ACC and polyamine syntheses, these data indicate that polyamine and ethylene biosynthesis did not appear to be competing for SAM during bud break. Similar results were also found during cherry flower bud development [27]. Adams *et al.* [28] supplied radioactive methionine to apple cell suspension culture and examined whether an inhibition of the conversion of SAM to ACC by AVG may divert the incorporation of the labeled methionine into polyamines. They found that AVG had no effect on the incorporation of the labeled methionine into polyamines. Wang and Steffens [29] also showed that SAM was maintained at a steady state even when ethylene and polyamine synthesis were active in stressed apple seedling leaves.

These studies indicate that one of the most pronounced effects of thidiazuron in apple plant is the release of lateral buds from dormancy. The breaking of dormancy by thidiazuron is correlated with increase in DNA, RNA, protein, ACC, MACC and SAM as well as with greater polyamine formation. SAM content increased during budbreak and bud development and did not seem to become a limiting factor for both polyamine and ethylene biosynthesis. The releasing of dormancy in apple bud by thidiazuron was inhibited by cordycepin, 5-fluorouracil, 6-methylpurine and cycloheximide. Inhibition of bud break and bud growth also resulted from treatment with DFMA and DFMO, suggesting that polyamine formation is linked to apple bud break and bud development. Inhibition of polyamine synthesis of ADC or ODC could account for the overall inhibitory effect. These experiments suggest that hormonal manipulation of metabolic

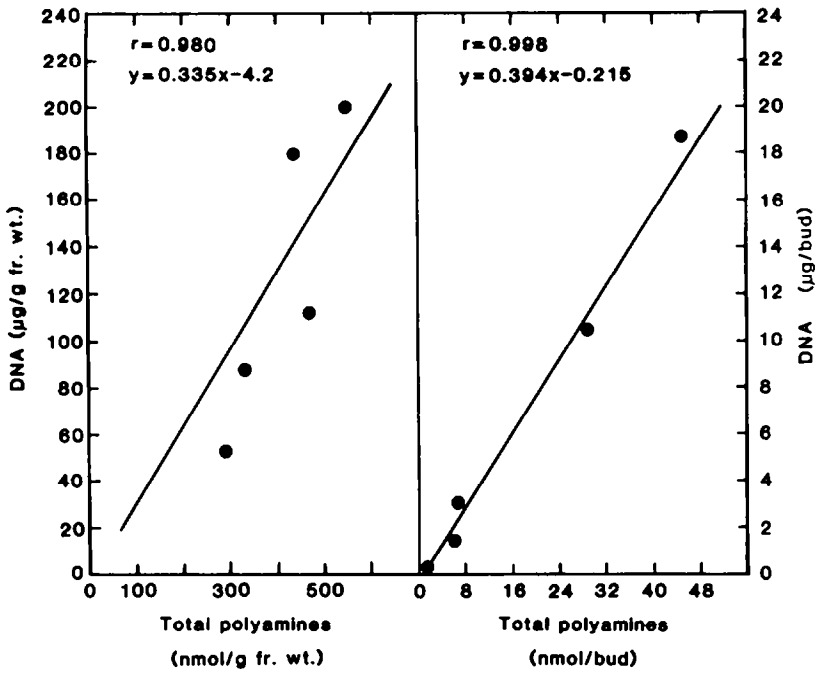

Fig. 6. Relationship between DNA content and total polyamines level during the development of upper region buds induced by 100 μM thidiazuron. For details see Fig. 1.

Table 2. Effect of the polyamine biosynthesis inhibitors, DFMA and DEMO, on thidiazuron-induced bud break and bud growth in upper, middle and lower regions of plant

Treatment	Inhibitor concn (μM)	Fresh weight Upper	Middle (mg/bud)	Lower
Control		4.82 ± 0.83	7.67 ± 1.45	7.08 ± 0.89
Thidiazuron (TDZ 100 μM)		305 ± 25	77 ± 0.5	44 ± 11
			% of thidiazuron	
TDZ + DFMA	25	59.4	89.9 (ns)	92.5 (ns)
	50	49.9	83.5	91.1 (ns)
	100	38.6	75.7	90.1 (ns)
TDZ + DFMO	25	47.0	69.0	84.4
	50	37.5	64.9	83.3
	100	8.5	35.8	81.2

Significant at 5% level as compared with the thidiazuron. ns, Non-significant. Apple cv. Gala buds were treated with DFMA or DFMO at the indicated concentrations and 100 μM thidiazuron. DFMA or DFMO were applied directly to the buds, followed by 100 μM thidiazuron 4 hr later. Samples were taken after 14 days.

changes may be an important tool in releasing dormancy in apple bud.

EXPERIMENTAL

Plant materials and treatments. Tissue culture propagated *Malus domestica* Borkh 'Gala' and 'Mutsu' grown in the greenhouse as described previously [30] were used in the expts. Plants were generally 1 m high with a single stem and at least 30 nodes. Treatments were applied only within nodes 3–20, counting from the apical tip. The treatment area was divided into three regions: upper (nodes 3–8), middle (nodes 9–14) and lower (nodes 15–20). Within each region, five buds were treated. Unless otherwise specified, results refer to the treatment of each region as a whole. All treatment solns were prepared in 2.5% DMSO plus 0.5% Tween-20 and applied directly to the buds with a brush until run-off. Buds from upper region of control and thidiazuron treated plant collected in 2 or 3 day interval in 9-day period were used for chemical analysis.

Chemicals. ACC and bovine serum albumin (BSA) were

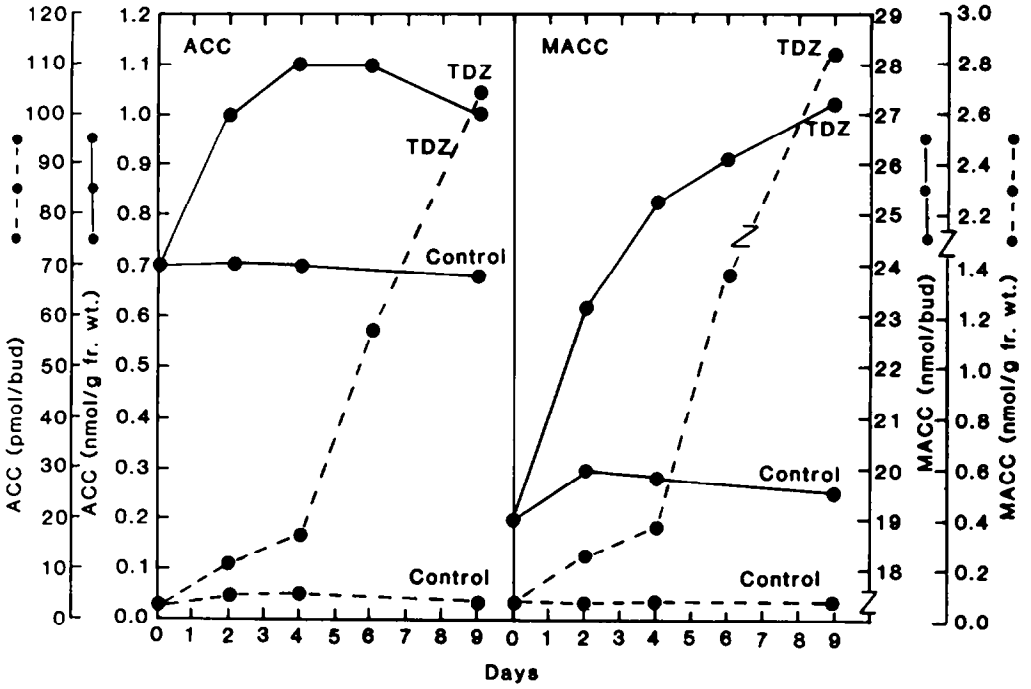

Fig. 7. Changes in ACC and MACC content during the development of upper region buds induced by 100 μM thidiazuron (TDZ). For details see Fig. 1. When expressed on a per bud basis, LSD (5%) for ACC = 10.12; MACC = 0.16. When expressed as per g fr. wt basis, LSD (5%) for ACC = 0.25; MACC = 3.49.

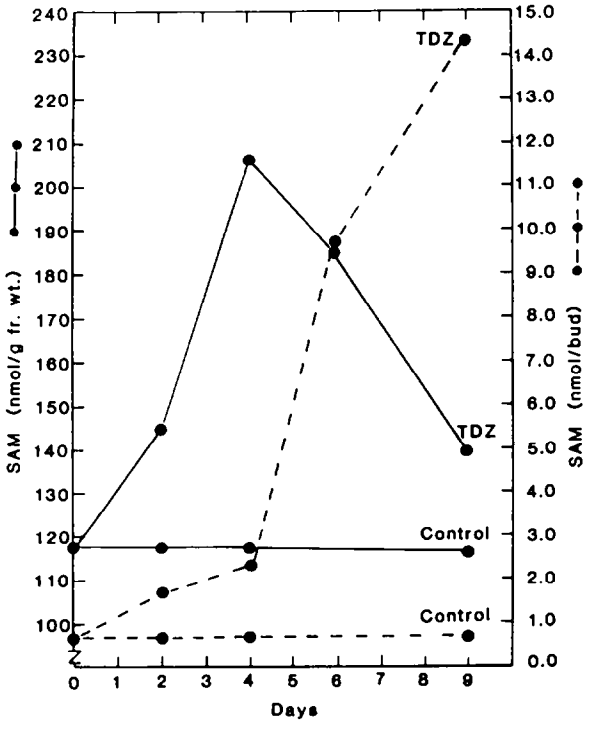

Fig. 8. Changes in SAM content during the development of upper region buds induced by 100 μM thidiazuron (TDZ). For details see Fig. 1. When expressed on a per bud basis, LSD (5%) = 0.86. When expressed as per g fr. wt basis, LSD (5%) = 20.0.

purchased from Calbiochem. Cordycepin, cycloheximide, DNA, diphenylamine, 5-fluorouracil, 6-methylpurine, orcinol, RNA and polyamines (putrescine, spermidine, spermine) were purchased from Sigma. DFMA and DFMO were gifts from P. P. McCann (Merrell-Dow Research Center). Ethephon was kindly provided by Union Carbide Agricultural Products Co. Protein assay kits were purchased from Bio-Rad Laboratory. Technical grade thidiazuron was a gift from E. Pieters of Nor-Am Agricultural Products, Inc. Other chemicals were of reagent grades.

Determination of polyamines, SAM, ACC, MACC and protein. The extraction and purification procedures have been described previously [29]. The tissues were extracted with 5% ice-cold $HClO_4$. After centrifugation, the supernatant which contained ACC, MACC, SAM and free polyamines, were subjected to further purification. Levels of the polyamines were determined after dansylation [31]. The dansylated products were then extracted with 0.5 ml C_6H_6 and separated on silica gel thin layer plates (silica gel 60, without fluorescent indicator, EM reagents) in 2 D at 5°. The dansyl-amines were separated [32] in cyclohexane–EtOAc (5:4 v/v) (solvent 1), then the plate was run in the 2nd dimension with $CHCl_3$–Et_3N (5:1 v/v) (solvent 2). The fluorescent spots were compared with dansylated standards. The spots were eluted with EtOAc and were quantified in an HTV Fluoroflow Detector V spectrophotofluorimeter, with excitation at 350 nm and emission at 495 nm. The concn of SAM was determined spectrophotometrically, assuming a molar absorption coefficient of 15 400/cm-M at 256 nm [33]. The ACC was assayed according to the method of ref. [34], which is based on the chemical conversion of ACC to C_2H_4 with NaOCl. Quantification of MACC was carried out by hydrolysis in 6 N HCl at 100° for 1 hr [35], and the resulting ACC was assayed as described above.

The perchloric pellet was rinsed twice with 80% (v/v) Me_2CO and solubilized in 1 N NaOH (60 min at 37°). Protein was determined according to ref. [36], using BSA as a standard.

Determination of RNA and DNA. The procedures of ref. [37] for estimating nucleic acids were followed. Highly purified yeast RNA, highly polymerized calf thymus DNA were carried through the procedures, and were used as standards for quantitative determination. RNA and DNA were assayed in the residue of cold $HClO_4$ extraction after alkaline and acid hydrolysis by the orcinol and the diphenylamine methods, respectively [27].

Application of inhibitors. DFMA, DFMO, cordycepin, 5-fluorouracil, 6-methylpurine and cycloheximide at various concn (25, 50 or 100 μM) were applied directly to the buds. This was followed by 100 μM of thidiazuron 4 hr later. After 2–3 weeks, the effect of various inhibitors on thidiazuron-induced bud break and bud growth were evaluated.

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