Cytokinin Stimulation of Abscission in Lemon Pistil Explants

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Abstract. The stylar abscission bioassay was used to identify five stimulators of lemon (*Citrus limon* cv. Lisbon) abscission in pistil explants. The stimulators (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, N6-benzyladenine, kinetin, zeatin, and N6-isopentenyladenine), which are all cytokinins, accelerated the timing of explant abscission when they were added as supplements (100 μM) to the test medium. To study possible relationships between cytokinins, ethylene, and abscission, we measured accumulating ethylene concentrations in sealed cultures and endogenous 1-aminocyclopropane-1-carboxylic aicd (ACC) in explants incubated on test medium plus or minus 100 μM N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron), 100 μM N6-benzyladenine (bzl^{6}Ade), or 2 μM picloram, an inhibitor of stylar abscission. Although ethylene accumulated to similar levels in all treatments, the concentrations obtained with picloram and thidiazuron were, respectively, higher and lower than those obtained in control cultures. The accumulation of ethylene in cultures with bzl^{6}Ade, on the other hand, was not significantly different from controls. ACC concentrations in explants remained fairly constant in all treatments during the incubations, except in explants on thidiazuron, in which case the ACC concentration declined slightly. We conclude that cytokinins can stimulate *Citrus* abscission *in vitro* and that this stimulation is not accompanied by marked effects on either measurable ethylene or ACC concentrations. Our finding that 100 μM aminoethoxyvinylglycine, an ethylene biosynthesis inhibitor, counter-

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acts the stimulation of abscission by bzl[6]Ade suggests that a minimum level of ethylene production is required for the cytokinin effect. The possibility that cytokinins affect other aspects related to ethylene, such as biosynthetic rates, metabolism, or tissue retention, is not excluded by our results.

A floral explant bioassay has been developed to study the regulation of abscission in *Citrus* and to identify potentially valuable chemicals for practical applications. The bioassay is based on the observation by Goldschmidt and Leshem (1971) that excised pistils from *Citrus* flowers will undergo stylar abscission when they are incubated under appropriate conditions on a defined nutrient medium.

We have demonstrated the utility of the stylar abscission bioassay in screening for effective inhibitors of abscission for post-harvest treatment of lemon fruits (Einst et al. 1981a). We have also shown that phytohormones added to the bioassay medium regulate abscission in a manner similar to the regulation of abscission in many other systems (Addicott 1982). Several auxins, for example, effectively inhibit stylar abscission (Einset et al. 1980, 1981b), while ethylene and precursors of ethylene such as ACC[1] and 2-chloro-ethylphosphonic acid stimulate abscission *in vitro* (Sipes and Einset 1982).

For the future, important questions concerning the regulation of *Citrus* abscission include (1) identification of other phytohormones affecting abscission; (2) mechanisms involved in the interactions between different phytohormones; and (3) endogenous control of abscission *in vitro* and in the intact plant.

This paper reports on the stimulatory effects of several cytokinins, both naturally occurring and synthetic, on stylar abscission. Five new stimulators have been identified, among which the phenylurea-type cytokinin N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron) is shown to be more active than adenine-type cytokinins such as bzl[6]Ade, kinetin, io[6]Ade, and i[6]Ade.

In addition, we also report on studies of ethylene production and ACC contents in explants in which abscission is stimulated (medium supplemented with cytokinin), in control explants (test medium), and in explants in which abscission is inhibited (medium supplemented with the auxin picloram). Using a *Citrus* leaf explant system, Sagee et al. (1980) presented evidence that the stimulatory effect of ABA on abscission is mediated via induced ethylene production. In our experiments, on the contrary, we find that the stimulatory effect of cytokinins is not associated with corresponding increases in ethylene production or ACC contents of explants.

Materials and Methods

Chemicals, Composition of Media, and Culture Conditions

The cytokinins bzl[6]Ade, kinetin, io[6]Ade, and i[6]Ade were all obtained from Sigma Chemical Co., St. Louis, Missouri, USA. The N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron) from Nor-Am Agricultural Products, Naperville, Illinois, USA, was kindly furnished by Dr. D. W. S. Mok, Oregon State University. The sources of other chemicals have been described (Sipes and Einset 1982).

The test (i.e. unsupplemented) medium contained the recommended concentration of Murashige and Skoog (1962) salts, 50 g/l sucrose, 100 mg/l *myo*-inositol, 5 mg/l thiamine·HCl, and 1% Phytagar (w/v), pH 5.6, plus or minus tested chemical(s). All constituents were added prior to autoclaving.

Pistil explants were obtained during November and December of 1982 from lemon (*Citrus limon* cv. Lisbon) trees at the Citrus Research Center and Agricultural Experiment Station of the University of California at Riverside, California, USA. Flowers at the pre-anthesis stage (pistil length approximately 1.5 cm) were picked in the field, placed on ice, and taken directly to the laboratory where they were handled aseptically as described (Einset et al. 1981b) and then planted singly in culture tubes (25 × 150 mm) containing 20 ml of medium. Cultures were covered either with serum caps (continuously sealed) or with plastic-vented closures from Bellco Glass, Inc., Vineland, New Jersey, USA, (vented), and were incubated in the dark at 27°C. In all cases, experiments were performed on at least three different occasions.

Ethylene Determinations

When the levels of accumulating ethylene were determined, 1-ml air samples from continuously sealed cultures were injected into a Varian Aerograph 1400 gas chromatograph, operated isothermally at 90°C and equipped with a flame ionization detector and a stainless steel column (0.3 × 450 cm) containing Porapak Q. The chromatograph was interfaced with a Spectra-Physics minigrator, which permitted ethylene detection as low as 0.01 ppm. Ethylene concentrations, expressed as microliters per tube, were calculated based on comparison with standards and based on the volume of air space (35 ml) in each tube. All experiments were performed on at least three different occasions.

Measurement of ACC Contents

After determining the accumulating level of ethylene in a tube, the explant was removed and weighed (approximately 0.07 g per explant). Pooled explants (three per treatment) were then homogenized in 5 ml of 0.1 N HCl using a pestle. After centrifuging homogenates at 18,000 × *g* for 20 min, the clear supernatants were applied to fresh columns (0.5 × 2.5 cm, 2 ml) of AG50W-X4 (H^+) cation exchange resin packed in Pasteur pipettes and equilibrated in 0.1 N HCl. Columns were washed with 2-ml volumes of H_2O and then eluted with 20-ml of 1N NH_4OH. The NH_3 eluates were evaporated to dryness *in vacuo*, and the residues were dissolved in 2 ml of H_2O.

To determine ACC contents according to the procedure of Lizada and Yang (1979), 0.1 ml of extract was mixed with 0.9 ml of 0.1 mM Na_3PO_4, 1.1 mM $HgCl_2$, pH 11.5, in a 10-ml Erlenmeyer flask. After sealing with a serum cap, 0.1 ml of 5% NaOCl was injected through the stopper with a needle and syringe. The flask was then incubated at room temperature with agitation for 5 min, after which time the concentration of ethylene was determined by gas chromatography. The efficiencies of ACC conversion to ethylene (50–65%) were determined for each sample using internal standards. ACC contents were

Table 1. Effect of cytokinins and picloram on stylar abscission in pistil explants of Lisbon lemon. In all cases, data are from at least 10 independent experiments (8–10 cultures per experiment).

Addition to test medium	Concentration (μ)	No. of cultures	% Abscission ± SE		
			Day 3	Day 4	Day 5
None	—	300	0	17 ± 4	55 ± 5
Thidiazuron	100	152	15 ± 4	70 ± 6	96 ± 1
bzl^{6}Ade	100	252	4 ± 1	55 ± 4	94 ± 1
Kinetin	100	92	0	34 ± 5	77 ± 6
i^{6}Ade	100	92	0	29 ± 4	71 ± 6
io^{6}Ade	100	92	0	29 ± 5	76 ± 6
Picloram	2	120	0	0	3 ± 2

calculated based on tissue weights, conversion efficiencies, and based on comparison with ACC standards.

Results

Stimulators of Abscission

Experiments demonstrating the stimulatory effects of several cytokinins on stylar abscission in Lisbon lemon pistils are summarized in Table 1. Although each cytokinin was tested at three different concentrations (100 μM, 20 μM, 4 μM), only the results for concentrations giving a significant response are reported.

Stimulation of abscission by cytokinins is evident by a significantly higher percent abscission on days 3, 4, and 5 compared to test medium. By this criterion, thidiazuron was the most potent stimulator of abscission, followed by bzl^{6}Ade as the next most active chemical. The cytokinins kinetin, i^{6}Ade, and io^{6}Ade, although less active than thidiazuron and bzl^{6}Ade, were about equally effective to each other in stimulating abscission. The auxin picloram, as has been shown (Goldschmidt and Leshem 1971, Einset et al. 1981b), inhibited abscission.

Abscission, Ethylene, and ACC in Sealed Cultures

Because of the activity of ethylene and ethylene precursors in stimulating stylar abscission (Sipes and Einset 1982), additional investigations on cytokinins and ethylene in relation to abscission were conducted. These experiments utilized continuously sealed cultures used in Table 1. By sealing the cultures, we were able to obtain increased sensitivity in our measurements of ethylene production. Moreover, because no differences in the time course of abscission in sealed versus vented cultures were found for any treatment (Fig. 1), we feel that meaningful comparisons can be made using continuously sealed cultures.

Figure 2 shows the effects of thidiazuron, bzl^{6}Ade, and picloram on abscission, accumulating ethylene, and ACC contents of explants. As indicated, thidiazuron and bzl^{6}Ade stimulated and picloram inhibited abscission relative to controls (test medium). By contrast, culture tubes with these chemicals and
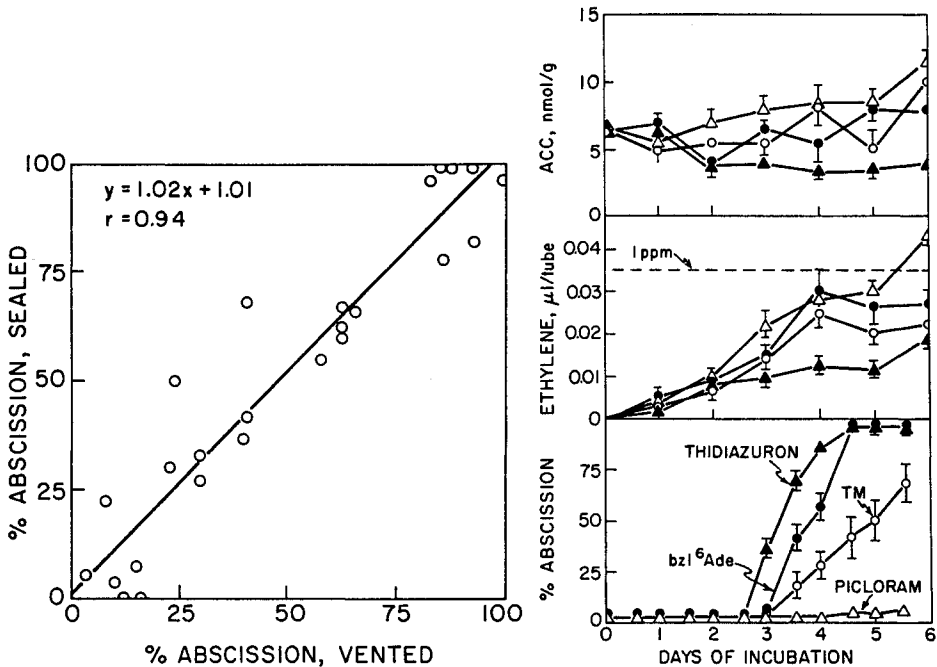
Fig. 1. Effect of closure type (continuously sealed versus vented) on the time course of abscission in pistil explant cultures. Each point represents % abscission at a specific time between 3 and 7 days in at least 40 sealed cultures and 40 vented cultures containing the same medium. The results summarize data for all media indicated in Table 1.

Fig. 2. Effect of chemicals added to test medium (TM) on stylar abscission, ethylene, and ACC in cultures continuously sealed with serum caps. Concentrations of additives were: thidiazuron, 100 μM; bzl⁶Ade, 100 μM; picloram, 2 μM. Abscission data are based on 14 independent experiments, 10 cultures per treatment ± SE. Ethylene and ACC data are means, respectively, of 12 and 4 independent determinations ± SE.

control cultures had similar levels of accumulating ethylene during the time course of the experiment. It was found, however, that cultures with thidiazuron on days 4 and 5 had a significantly lower ethylene level than controls. In addition, cultures with picloram on days 5 and 6 had a small but significantly higher ethylene level than controls. This stimulation of ethylene production in *Citrus* by auxin concentrations that effectively inhibit abscission is consistent with earlier reports using the stylar abscission bioassay (Sipes and Einset 1982) and also using leaf and fruit explants (Lewis et al. 1968).

ACC analyses of explants from control, cytokinin, and picloram cultures (Fig. 2) showed only small variations in ACC concentrations during the course of the experiment. A significant effect, however, was seen with thidiazuron, which slightly depressed the ACC content of explants. This decrease in ACC concentration in the presence of thidiazuron relative to controls was associated with a corresponding inhibition of ethylene production in a manner which is similar to several other ethylene-biosynthesizing systems (Lieberman 1979, Yang et al. 1982).
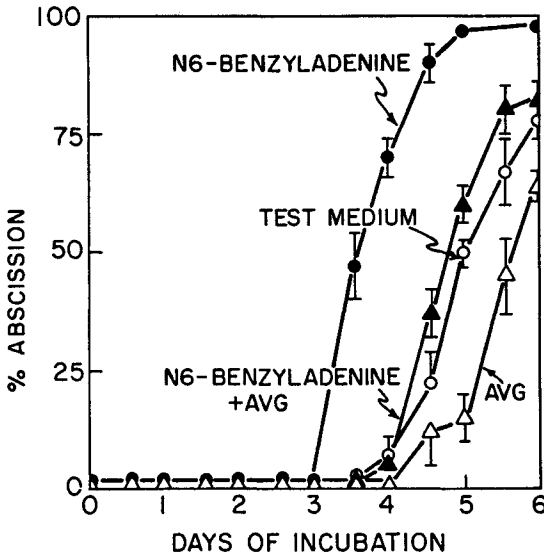
Fig. 3. Effect of 100 μM bzl⁶Ade, test medium (TM), and 100 μM AVG on stylar abscission in excised lemon pistils; means of 8 independent experiments with 10 cultures per experiment ± SE.

Interaction Between bzl⁶Ade and AVG

Based on the results above, it was apparent that stimulation of abscission in explants by thidiazuron and bzl⁶Ade was not associated with higher levels of accumulating ethylene or with higher ACC concentrations compared to controls. Because of this, we studied the effect of bzl⁶Ade on abscission in the presence and abscence of an ethylene biosynthesis inhibitor.

As has been shown (Sipes and Einset 1982), AVG, which is an effective inhibitor of ACC synthase (Boller et al. 1979, Yang et al. 1980), also inhibits ethylene production and abscission in *Citrus* pistil explants. When 100 μM AVG was used in combination with 100 μM bzl⁶Ade, the time course of abscission was essentially the same as in the controls, indicating that these two chemicals counteract each other in regulating abscission.

Discussion

Five cytokinins have been shown to stimulate stylar abscission in lemon pistil explants when they are added as supplements (100 μM) to the bioassay medium. Among these compounds, thidiazuron, a phenylurea-type cytokinin, is the most potent stimulator and bzl⁶Ade is the next most active chemical. Kinetin, i⁶Ade, and io⁶Ade, which are about equally effective in stimulating abscission, were found to be less active than either thidiazuron or bzl⁶Ade. Thidiazuron has been shown to be as active or more active than several adenine-type cytokinins in promoting the growth of cytokinin-dependent callus cultures of *Phaseolus lunatus* cv. Kingston (Mok et al. 1982). It is currently being tested for possible effects on fruit and leaf abscission in *Citrus* groves and on the release and growth of grafted buds in the nursery (Nauer et al. 1979).

The effects of cytokinins on abscission vary considerably depending on species and the site of application. In several plants (e.g. muskmelon, apple, grape, *Coleus*), cytokinin applications inhibit fruit abscission (cf. Addicott 1982). With pears, on the other hand, cytokinins stimulate flower abscission (Pierik 1980), and in beans (Chatterjee and Leopold 1964) and lupines (Carr and Burrows 1967) cytokinins either inhibit or stimulate leaflet abscission depending on the site where they are applied.

By analogy with the effect of ABA on leaf abscission in explants of Shamouti orange (Sagee et al. 1980) and the stimulatory effect of cytokinins on ethylene production in several systems (Lau et al. 1977, Lieberman 1979), we investigated the possibility that cytokinin stimulation of stylar abscission is mediated via effects on ethylene production and/or metabolism. To do this, we measured the levels of accumulating ethylene in sealed cultures and the endogenous concenterations of ACC in explants. Thidiazuron slightly inhibited and bzl[6]Ade had no significant effect on ethylene accumulation compared to controls. Similarly, thidiazuron slightly depressed the endogenous ACC concentration of explants and bzl[6]Ade had no significant effect compared to controls. We conclude that these cytokinins do not stimulate *Citrus* abscission via measurable stimulatory effects on ethylene production or ACC contents. Although it is plausible that the stimulation of ethylene production by cytokinin is so localized as to be undetected by our methods, this seems unlikely to us. The possibility that cytokinins affect other aspects related to ethylene such as biosynthetic rates, metabolism (Beyer and Blomstrom 1980), or retention (Hall et al. 1982) is not excluded.

Although no marked effects of cytokinins on ethylene or ACC were detected, we did find that the stimulation of abscission caused by bzl[6]Ade could be counteracted by AVG, which inhibits ethylene production in explants by approximately 90% (Sipes and Einset 1982). Inasmuch as AVG would be expected also to inhibit several other enzymes involved in amino acid metabolism, the interpretation of this experiment is equivocal. One reasonable hypothesis is that bzl[6]Ade stimulates abscission independent of marked effects on ethylene, but that a minimum level of ethylene production is still required. This hypothesis considers both ethylene and cytokinin as essential components of the stimulated process of abscission.

The results to date can be summarized in a model for the control of abscission in which three classes of phytohormones regulate stylar abscission in the *Citrus* pistil bioassay. These phytohormones are auxins which inhibit, ethylene which stimulates, and cytokinins which stimulate the process. In the absence of added auxin or cytokinin, the timing of abscission appears to be regulated to endogenous ethylene or, in the case of supplements, by added ethylene or precursors (Sipes and Einset 1982). In the presence of an auxin such as picloram, or cytokinins such as thidiazuron and bzl[6]Ade, effects on abscission appear to be independent of dramatic effects on detectable ethylene production or on ACC concentrations in explants. Stimulation of *Citrus* abscission by cytokinins, therefore, contrasts with the stimulation caused by ABA in leaf explants (Sagee et al. 1980) and by several commercial chemicals (cf. Addicott 1982) which appear to be mediated via direct, measurable effects on ethylene.

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