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Rapidly Induced Ethylene Formation after Wounding is Controlled by the Regulation of 1-Aminocyclopropane-1-carboxylic Acid Synthesis

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Abstract. Bean leaves from *Phaseolus vulgaris* L. var. Pinto 111 react to mechanical wounding with the formation of ethylene. The substrate for wound ethylene is 1-aminocyclopropane-1-carboxylic acid (ACC). It is not set free by decompartmentation but is newly synthesized. ACC synthesis starts 8 to 10 min after wounding at 28° C, and 15 to 20 min after wounding at 20° C. Aminoethoxyvinylglycine (AVG), a potent inhibitor of ethylene formation from methionine via ACC, inhibits wound ethylene synthesis by about 95% when applied directly after wounding (incubations at 20° C). AVG also inhibits the accumulation of ACC in wounded tissue. AVG does not inhibit conversion of ACC to ethylene. Wound ethylene production is also inhibited by cycloheximide, n-propyl gallate, and ethylenediaminetetraacetic acid.

Key words: 1-Aminocyclopropane-1-carboxylic acid – Ethylene – *Phaseolus* – Wounding.

Introduction

1-Aminocyclopropane-1-carboxylic acid (ACC)-synthase is the only enzyme of the methionine → S-adenosylmethionine → ACC → ethylene pathway which needs to be formed or activated for ethylene synthesis to occur. Appearance of ACC-synthase and of its product ACC at the beginning of ethylene biosynthesis has been demonstrated in every tissue studied (Boller et al. 1979, Jones and Kende 1979, Yu and Yang 1979), suggesting that synthesis or activation of ACC-synthase regulates ethylene biosynthesis. In all the cases studied ACC-synthase, ACC-accumulation, and ethylene formation were strongly inhibited by AVG. Since induction of wound ethylene takes less time than hormonal induction, 29 min as compared to 2–3 h in the case of etiolated pea stems (Saltveit and Dilley 1978), alternative control mechanisms have to be considered for rapid wound ethylene. One possible alternative to explain the fast onset of wound ethylene production would be storage of ACC in a compartment that desintegrates after wounding. Desintegration of the compartment sets free endogenous ACC which in turn is oxidized by constitutive ACC-oxidase (Konze and Kende 1979) to yield ethylene. Another possible source of ethylene occurring only shortly after wounding is the oxidation of unsaturated fatty acids, since unsaturated fatty acids are oxidized in wounded tissue which results in the production of ethane and ethylene, among other products (Lieberman and Mapson 1962).

We studied the time course of wound ethylene production in bean leaves in order to find out which of the three outlined possibilities, i) activation of ACC-synthase and ACC-synthesis ii) decompartmentation of ACC and iii) oxidation of lipids, is responsible for rapid synthesis of ethylene after wounding.

Material and Methods

Wounding and Incubation of Plant Material. Leaflets from the first trifoliar leaf of *Phaseolus vulgaris* L. cv. Pinto 111 were used in all experiments. The plants were grown in a greenhouse with a 15-h photoperiod at 19–23° C. Leaves were wounded as described by Hanson and Kende 1976. The leaves were placed flat side up on 4 layers of tissue paper and were pressed firmly with a lightweight file (Dr. Scholl's callus and corn remover). This treatment resulted in regular pattern of small perforations about 1.4 mm apart.

Single leaves were incubated in flat plastic chambers (∅ 9 cm, Vol. 13–18 ml) on one filter paper that was soaked with 1.6 ml H_2O or 1.6 ml of a solution to be tested. The leaves were incubated upside down and were gently pressed to the filter paper to provide close contact to the incubation medium. The incubation chamber was closed with a plastic lid featuring a neoprene O-ring seal and an opening that was closed with a serum cap.

Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid; AVG = aminoethoxyvinylglycine; EDTA = ethylenediaminetetraacetic acid

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Ethylene and Ethane Determination. For ethylene and ethane determination, 1-ml samples of the head space of the incubation chambers were taken with a hypodermic syringe. Ethylene and ethane was determined by gas chromatography with a model 1,400 Varian gas chromatograph, equipped with a flame ionisation detector, an activated aluminum column (0.32·61 cm), and a Varian CDS integrator 101. Ethylene and ethane were identified and calculated in comparison to the retention time and peak area of ethylene and ethane standards.

Assay of 1-Aminocyclopropane-1-carboxylic Acid. ACC was assayed as described by Lizada and Yang 1979. One µmol $HgCl_2$ was added to the extracts or ACC solutions. The test tubes were closed with serum caps and kept in ice. Approximately 100 µl of a cold mixture of 5% NaOCl and saturated NaOH (2:1, v/v) were injected, and the tubes were agitated on a shaker for 2.5 min, after which 1.0-ml gas samples were withdrawn for ethylene determination.

Chemicals. 1-Aminocyclopropane-1-carboxylic acid (ACC) was purchased from Calbiochem, Lahn, FRG. Aminoethoxyvinylglycine (AVG) was a generous gift of Dr. Stempel, Hoffmann-LaRoche Inc., Nutley, N.J., USA. All other chemicals were purchased from Merck, Darmstadt, and Sigma, Munich, FRG.

Results

Ethylene and Ethane Production of Untreated and Wounded Bean Leaves. When bean leaves were incubated on moist filter paper in a closed plastic chamber for two hours, they formed only small quantities of ethylene. When the leaves were wounded prior to incubation at 20° C, they formed ethylene after a lag phase of 15–20 min (Fig. 1). The bean leaves hardly produced any ethane during incubation. The rate of ethane production was not altered by wounding.

Inhibition of Wound Ethylene Production by Aminoethoxyvinylglycine, Ethylenediaminetetraacetic Acid, n-Propylgallate, and Cycloheximide. When bean leaves were wounded and incubated on filter paper soaked in a solution of 0.1 mM AVG, an effective inhibitor of ACC-synthase in vivo and in vitro (Boller et al. 1979, Adams and Yang 1979), no wound ethylene was formed (Fig. 2E). Preincubation of leaves on 0.1 mM AVG for 15 min before wounding, in addition to incubation on AVG after wounding, also prevented accumulation of wound ethylene (Fig. 2F). When leaves were wounded, incubated on H_2O for 15 min, 30 min, or 45 min, and then transferred to 0.1 mM AVG, wound ethylene production was partly inhibited in the leaves which had been transferred to 0.1 mM AVG after 15 min (Fig. 2D); but it was not inhibited in leaves which had been transferred to AVG after 30 or 45 min on H_2O (Fig. 2C, Fig. 2B). Figure 2A shows ethylene production of wounded leaves continously incubated on H_2O.

AVG did not inhibit ethylene production of bean leaves which were wounded and incubated on 1.3 mM

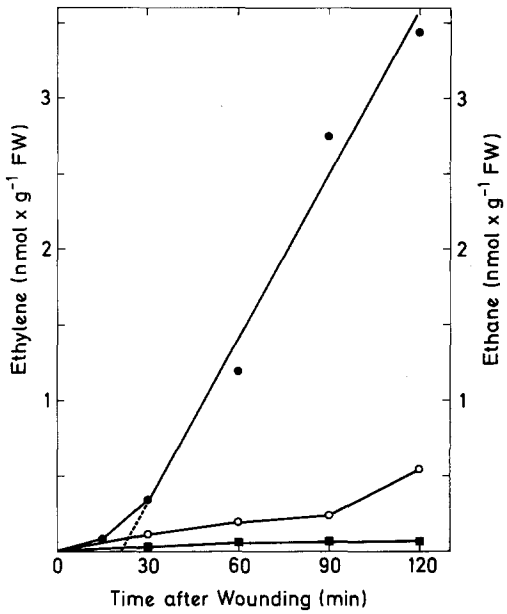

Fig. 1. Ethylene and ethane formation of wounded bean leaves. Bean leaves were wounded with a lightweight file and were incubated on H_2O in a closed plastic chamber for 2 h at 20° C. Control leaves were not wounded but directly incubated. (o – – o) Ethylene formation of control leaves; (● – – ●) ethylene formation of wounded leaves; (■ – – ■) ethane formation of control leaves and wounded leaves

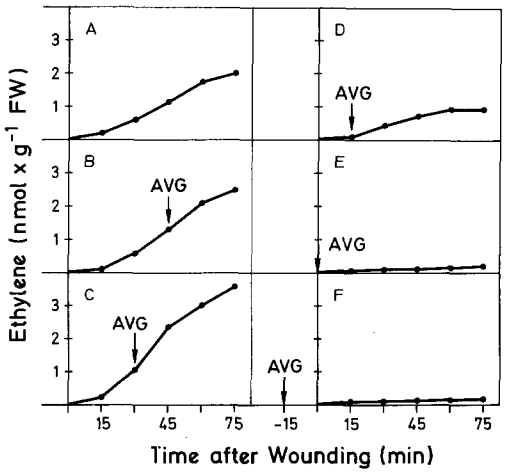

Fig. 2A–F. Effect of aminoethoxyvinylglycine (AVG) on ethylene production of wounded bean leaves. **A** Leaves were wounded and incubated on H_2O. **B** Leaves were wounded and incubated on H_2O; after 45 min they were transferred to 0.1 mM AVG. **C** Leaves were wounded and incubated on H_2O; after 30 min they were transferred to 0.1 mM AVG. **D** Leaves were wounded and incubated on H_2O; after 15 min they were transferred to 0.1 mM AVG. **E** Leaves were wounded and incubated on 0.1 mM AVG. **F** Leaves were incubated on 0.1 mM AVG; after 15 min they were wounded, and incubation on 0.1 mM AVG was continued. All incubations were performed in closed plastic chambers at 20° C

ACC (Fig. 3). Preincubation of bean leaves on filter paper that was soaked with a solution of 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM n-propylgallate, and 1 mg ml^{-1} cycloheximide for 60 min be-

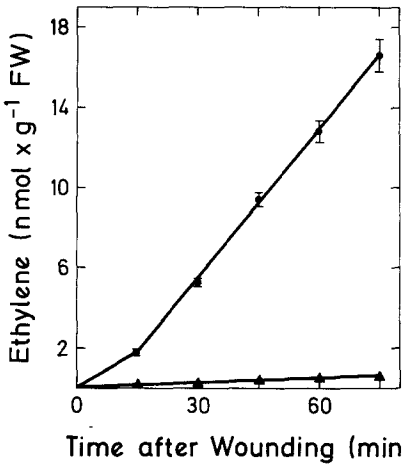

Fig. 3. Effect of aminoethoxyvinylglycine (AVG) on ethylene formation of wounded bean leaves incubated on 1-aminocyclopropane-1-carboxlic acid (ACC). Leaves were wounded and incubated on 0.1 mM AVG in the presence and absence of 1.3 mM ACC. All incubations were performed at 20° C. (●– –●) Ethylene formation of leaves incubated on a solution of 0.1 mM AVG and 1.3 mM ACC. (▲– –▲) Ethylene formation of leaves incubated on 0.1 mM AVG. The data represent means and standard deviations of four experiments

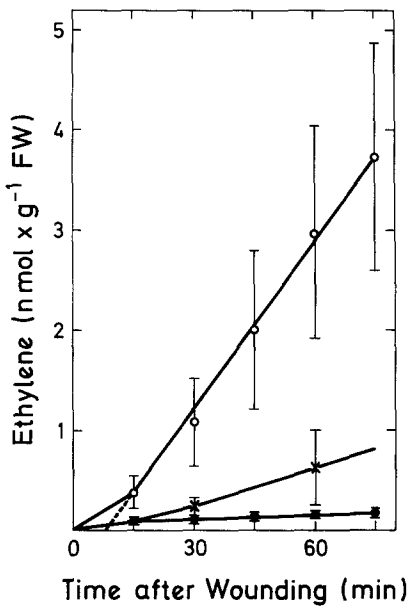

Fig. 4. Effect of ethylenediaminetetraacetic acid (EDTA), n-propylgallate and cycloheximide on ethylene formation of wounded bean leaves. Leaves were wounded and incubated on H_2O (○– –○), 1 mM EDTA (×– –×), 1 mM n-propylgallate (●– –●) and 1 mg ml^{-1} cycloheximide (●– –●). The data represent the means and standard deviations of four replicates. All incubations were performed at 28° C

fore wounding reduced the rate of wound ethylene production by about 79% with EDTA and 95% with n-propylgallate and cycloheximide (Fig. 4). The experiment shown in Fig. 4 was performed at 28° C. At 28° C wound ethylene started 8–12 min after wounding; at 20° C wound ethylene began to accumulate after 15–20 min (Fig. 1); at 15° C it was formed only after 25 min or later (data not shown).

Table 1. Influence of wounding and aminoethoxyvinylglycine (AVG) on ethylene production and ACC-content of bean leaves.

Bean leaves were wounded and either incubated on 0.1 mM AVG or on H_2O. Control leaves were not wounded and incubated on H_2O. Incubations were performed at 20° C. After 40 min ethylene production of all leaves was determined. Then the leaves were homogenized in 70% ethanol at −18° C. The homogenates were centrifuged at 6,000 *g* for 5 miä. The supernatant was dried down under reduced pressure, extracted with H_2O and re-centrifuged. As an internal standard 500 pmol ACC was added to one of two aliquots of the supernatants. ACC in both samples and in a sample containing 500 pmol ACC was oxidized to ethylene by addition of $HgCl_2$ and NaOCl in saturated NaOH. Ethylene was assayed by gas chromatography and yields and ACC-contents were calculated by internal standards. The data represent the mean of two experiments

Treatment of the leaves	Ethylene (pmol g^{-1} FW)[a]	ACC-content (pmol g^{-1} FW)
Not wounded, incubated on H_2O	290	276
Wounded, incubated on H_2O	866	1,892
Wounded, incubated on AVG	113	223

[a] FW = freshweight

Inhibition of ACC-Formation in Wounded Leaves by Aminoethoxyvinylglycine (AVG). In several experiments we confirmed the findings of Boller et al. (1979), Jones and Kende (1979) and Yu and Yang (1979), who demonstrated that ethylene production is always dependent on the formation of ACC in the tissue. One typical example is given in Table 1. It shows ethylene production and the ACC content of leaves which were wounded and subsequently incubated on AVG or H_2O. The increase in both ethylene production and ACC content after wounding was prevented by AVG.

Discussion

Mechanical wounding of bean leaves with a metal file, as described by Hanson and Kende (1976), is a method of wounding that leads to increased ethylene production, but does not stimulate ethane formation in the tissue. This is in contrast to the effect of point freezing on sugar beet leaves. When the leaves were severely frozen, they formed more ethane than ethylene (Elstner and Konze 1976). Since ethane is an indicator for fatty-acid oxidation in the tissue that goes along with ethylene formation (Lieberman and

Mapson 1962, Elstner and Konze 1976, Konze and Elstner 1978), the possibility that lipid oxidation is responsible for wound ethylene production may be ruled out. This assumption is confirmed by our finding that wound ethylene is completely inhibited by AVG. AVG does not have an effect on radical oxidation reactions (Konze and Elstner 1976), but is an inhibitor of ACC-synthase (Boller et al. 1979, Yu et al. 1979), which is the second enzyme in the pathway of ethylene biosynthesis from methionine. Thus, ethylene produced from bean leaves after mechanical wounding is derived from methionine as is stress ethylene in tobacco leaves and morning glory flower tissue (Abeles and Abeles 1972, Hanson and Kende 1976).

Two observations confirm that wound ethylene in bean leaves is dependent on ACC synthesized after wounding and is not dependent on ACC that is stored in a compartment and released after decompartmentation. One observation is that there is a lag phase before wound ethylene starts to accumulate. Following wounding, decompartmentilized ACC should immediately start to form ethylene. The second observation is the fact that application of AVG 15 min after wounding still inhibits wound ethylene production, indicating that at 20° C most of the ACC, which is the substrate for wound ethylene, is formed 15 min or more after the stress had been applied. ACC-oxidase as the ethylene-forming system is saturated in respect to its substrate ACC within 30–45 min after wounding. Inhibition of further ACC-synthesis does not effect rates of ethylene formation anymore. If decompartmentilized ACC were the substrate of ethylene, the tissue would probably not be sensitive to AVG in the beginning and then lose its sensitivity with time. Rather, it would probably be insensitive to AVG in the beginning and become sensitive when synthesis of new ACC became necessary to keep up wound ethylene production.

Our conclusions are based on the assumption that AVG inhibits ACC-synthesis, but does not inhibit ACC-oxidation. This assumption has been verified by demonstrating the ability of wounded tissue to produce ethylene when incubated on ACC in the presence of AVG. We also demonstrated that AVG inhibits ACC accumulation in wounded leaves. The short lag phase we observed before ethylene production of leaves started, when incubated on ACC in the presence of AVG, may reflect the time the leaves needed to take up ACC.

Inhibition of wound ethylene by cycloheximide, which is an inhibitor of translation at 80 s ribosomes, suggested that synthesis of protein is part of the inductive process of ethylene formation after wounding. However, a lag phase of 8–10 min at 28° C, or maybe even shorter at higher temperatures, seems to rule out synthesis of ACC-synthase or other proteins as part of the inductive process. So the nature of the regulatory process that starts off rapid ACC-synthesis and wound ethylene production remains unclear. Its characterization may provide information about biochemical links between irritation of plants and changes in the hormonal balance of the affected tissue. In this respect it is of interest that – working with tomato slices – Boller and Kende (personal communication) found an induction of ACC-synthesis similar to the mechanism reported here.

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