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Rapidly Induced Wound Ethylene from Excised Segments of Etiolated *Pisum sativum* L., cv. Alaska

I. CHARACTERIZATION OF THE RESPONSE[1]

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ABSTRACT

A rapidly induced, transitory increase in the rate of ethylene synthesis occurred in wounded tissue excised from actively growing regions of etiolated barley, cucumber, maize, oat, pea, tomato, and wheat seedlings. Cutting intact stems or excising 9-mm segments of tissue from near the apex of 7-day-old etiolated *Pisum sativum* L., cv. Alaska seedlings induced a remarkably consistent pattern of ethylene production. At 25 C, wound-induced ethylene production by segments excised 9 mm below the apical hook increased linearly after a lag of 26 minutes from 2.7 nanoliters per g per hour to the first maxium of 11.3 nanoliters per g per hour at 56 minutes. The rate of production then decreased to a minimum at 90 minutes, increased to a lower second maximum at 131 minutes, and subsequently declined over a period of about 100 minutes to about 4 nanoliters per g per hour. Removal of endogenous ethylene, before the wound response commenced, had no effect on the kinetics of ethylene production. Tissue containing large amounts of dissolved ethylene released it as an exponential decay with no lag period. Rapidly induced wound ethylene is synthesized by the tissue and is not merely the result of facilitated diffusion of ethylene already present in the tissue through the newly exposed cut surfaces. Previously wounded apical sections did not exhibit a second response when rewounded. No significant correlation was found between wound-induced ethylene synthesis and either CO_2 or ethane production.

Ethylene production increases in a wide variety of plant tissues subjected to stress induced by noxious chemicals (2), pathogenic organisms (19), ionizing radiation (1), water imbalance (23), or mechanical injury (16). Abeles (3) has reviewed stress ethylene production. Stress-induced increases in ethylene production occur minutes (8, 10, 12, 17, 24), hours (6, 9, 11, 14, 18), or days (7, 16, 21, 22) after presentation of the stress. Appropriate controls are necessary to ascertain whether rapid increases in the rate of ethylene evolution result from facilitated diffusion of endogenous ethylene through the newly exposed cut surfaces, or from induced synthesis. Burg and Thimann (5) showed that in sliced apples the initial surge in ethylene and CO_2 evolution was the result of endogenous levels of these gases. Increased evolution of ethylene after a lag period suggested induced synthesis. This is supported by studies with inhibitors which established that protein synthesis is required during the long lag period preceding stress ethylene synthesis (2, 6).

Little work has been reported on the kinetics of rapidly induced ethylene production from stressed tissue (10, 11). This paper presents a detailed kinetic study of ethylene production from mechanically injured tissue during the first 4 hr after excision.

MATERIALS AND METHODS

Preparation of Plant Material. Seeds of *Pisum sativum* L., cv. Alaska were imbibed in aerated tap water for 6 hr at 23 ± 2 C and planted in moist vermiculite. All subsequent manipulations were performed in the dark or under dim green light. The growth cabinet was maintained at 24 ± 1 C and continuously flushed with humidified ethylene-free (<1 nl l^{-1}) air. Seven-day-old etiolated seedlings, with 3 to 8 cm long third internodes and with reflexed apical hooks were selected. Apical stem segments were cut 9 mm below the top of the apical hook and included the apical meristem, plumule, and apical hook. Subapical stem segments were excised from the region 9 to 18 mm below the apical hook and consisted primarily of elongating cells. Internodal stem segments were excised from the second internode and consisted primarily of mature differentiated cells. Root apical segments were cut 9 mm from the root apex and included the root cap, apical meristem, and adjacent regions. Except as noted, all experiments used 9-mm tissue segments. Tissue from other parts of the plant were used and are described in the appropriate text.

Other plant materials were used to investigate the ubiquitousness of the wound-induced phenomenon. These included 9-mm sections excised from the region 9 to 18 mm from the shoot apices of red bud (*Cercis canadensis* L.), honeysuckle (*Lonicera flexuosa* var *Halliana* Dipp.), and *Forsythia viridissima* Lindl. from mature plants growing under natural conditions. Tissue from etiolated plants prepared in the same manner as the peas were also used. They included excised cotyledons and hypocotyl segments from 7-day-old cucumber seedlings (*Cucumis sativus* L., cv. Pioneer), apical stem segments from 7-day-old tomato seedlings (*Lycopersicon esculentum* Mill., cv. Campbell 28), and apical coleoptile segments from 5-day-old seedlings of oats (*Avena sativa* L., cv. Victory), barley (*Hordeum vulgare* L., cv Maraini), winter wheat (*Triticum aestivum* L., cv. Ionia), and corn (*Zea mays* L., cv. D-5).

Experimental Procedure. The kinetic behavior of wound-induced ethylene synthesis was studied using a flow-through system and a static system in which evolved ethylene accumulated. The flow-through system used tissue segments enclosed in opaque glass tubes with volumes varying from 0.8 to 26 ml, depending on the experiment, and which were capped with serum stoppers. Humidified ethylene-free air (<1 nl l^{-1}), or other gases, were admitted at a positive pressure of 2 cm Hg through a hypodermic needle inserted in the bottom serum stopper. Samples were taken through the top serum stopper at regular intervals with gas-tight syringes to give flow rates of either 1 or 2 ml min^{-1} and immediately injected into a gas chromatograph which used N_2 at 60 C as the carrier gas, was equipped with a column (2 mm × 1 m) of

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activated alumina, and a flame ionization detector. The gas chromatograph readily detected ethylene at concentrations of 1 nl l^{-1} in a 1-ml sample with a retention time of about 25 sec.

CO_2 and O_2 were determined by injecting samples into a gas chromatograph which used helium at 70 C as the carrier gas, was equipped with a silica gel column (6 mm × 0.6 m) and a molecular sieve column (6 mm × 3 m) mounted in parallel, and a differential thermal conductivity detector. Ethylene, CO_2, and O_2 concentrations were determined by comparing peak heights with those produced by a standard gas mixture. Ethane production was analyzed in some experiments and in the absence of an ethane standard, was quantitatively related to ethylene by comparing their relative peak heights within an experiment.

In the first series of experiments, three sets of 15 excised 13-cm etiolated pea stems were held for 140 min in opaque glass tubes (1.5 × 15 cm) which contained 2 ml of water and were continuously flushed with humidified ethylene-free air. The stems were then wounded with five cuts halfway through the stem, perpendicular to the stem axis, and between 9 and 18 mm below the apical hook. Ethylene evolution was followed for 70 min after wounding by taking 2-ml samples every 2 min. This experiment was repeated three times.

Except as noted, all subsequent experiments were conducted with 9-mm segments of tissue excised from the region 9 to 18 mm below the top of the apical hook and enclosed in 0.8- to 2.2-ml glass sample containers. Small chambers were used to increase the system's responsiveness. The exchangeable gas volume was further reduced by the approximately 0.4-ml volume of the tissue. Decay of ethylene from tissue previously exposed to 10 μl l^{-1} ethylene showed that under actual conditions, approximately 90% of the gas was removed by every 2-ml sample. Smaller tissue samples also permitted study of specific regions of a plant and maintenance of an adequate flow rate by repeated sampling. The latter facilitated rapid gas exchange, yet allowed sufficient ethylene to accumulate for analysis. Three samples were run simultaneously with a sampling rate of 2 ml $(2\ min)^{-1}$ by injecting aliquots into the gas chromatograph on a staggered 40-sec schedule.

Ethylene accumulation experiments were done with tissue enclosed in 25-ml Erlenmeyer flasks, 21-ml test tubes, or 3- or 5-ml plastic gas-tight syringes. Internal humidity was maintained near saturation with moist filter paper, and CO_2 was absorbed by strips of filter paper moistened with a freshly prepared saturated solution of KOH.

The effect on the wound response of reducing endogenous ethylene was studied using five subapical pea sections in 3-ml syringes. Before wound ethylene production started, endogenous ethylene was removed by evacuating and flushing the tissue with ethylene-free air. The plunger was adjusted to 0.5 ml and the syringe was sealed with a serum stopper. The plunger was then withdrawn to the 3-ml mark which diluted the endogenous gases 6-fold. The syringe contents were returned to atmospheric pressure by admitting ethylene-free air. This procedure was repeated 10 times for each syringe. The controls were flushed 10 times at atmospheric pressure. All syringes were then set to 2 ml and capped with serum stoppers. After 30, 60, or 90 min, the plungers were reciprocated between the 1- and 3-ml marks about 10 times to equalize the internal and external concentrations of gases. Samples were analyzed for ethylene, CO_2, and O_2. The experiment was repeated three times with five replicates for each sample time.

Analysis of Data. Whenever possible, actual data from a representative experiment were used to study the kinetics of wound-induced ethylene synthesis. When there was a large amount of variability, curves were either fitted manually or drawn using data subjected to a 5-point curve-smoothing equation. The first peak of ethylene production was reasonably symmetrical. A second degree polynomial was fitted to data from the first peak by the method of least squares. The quadratic equation was then used to calculate the time of maximum ethylene evolution (the first derivative) and the amount of ethylene evolved during a standard interval about the maximum (the area under the curve as calculated from the integral equation, and bounded by the time of maximum ethylene production ± 30 min). These parameters and similar data from accumulation studies were subjected to analysis of variance. Each experiment reported was repeated at least twice.

RESULTS AND DISCUSSION

Preliminary Observations. Ethylene production by 13-cm etiolated pea stems declined slightly after the apical region was cut, increased after about 25 min, and became significantly greater than the noninjured control after a lag period of about 30 min (Fig. 1). Production by the noninjured control remained virtually constant during the experiment. Similar patterns were observed in three experiments. Two-hr accumulation experiments showed that three cuts perpendicular to the stem axis and halfway through the stem were as effective in eliciting wound ethylene (5.3 nl g^{-1} hr^{-1}) as were three cuts parallel to the stem axis (5.5 nl g^{-1} hr^{-1}) or six perpendicular cuts (5.4 nl g^{-1} hr^{-1}). The rates of ethylene production were not significantly different from each other, but were significantly different from the noninjured controls (3.9 nl g^{-1} hr^{-1}).

Kinetics of Wound-induced Ethylene Synthesis from Subapical Sections. Subapical stem segments were used to further characterize the pattern of wound ethylene synthesis. The 9-mm sections were excised from the same region that was wounded in the preliminary series of experiments. The trauma of excising the segments was sufficient to elicit a characteristic pattern of wound ethylene production. Averaging the min-by-min rates of ethylene synthesis from 12 experiments resulted in a remarkably consistent pattern (Fig. 2). Wound-induced ethylene synthesis commenced after a definite lag period of 26 ± 1.4 min following excision of the subapical tissue segment. The rate of ethylene production increased linearly after this lag from 2.7 ± 0.3 nl g^{-1} hr^{-1} to the first maximum of 11.3 ± 1.8 nl g^{-1} hr^{-1} at 56 ± 2.1 min. The rate then decreased to a minimum of 4.9 ± 0.9 nl g^{-1} hr^{-1} at 90 ± 2.7 min, increased to a reduced second maximum of 8.4 ± 1.7 nl g^{-1} hr^{-1} at 1.31 ± 4.9 min, and then declined over a period of about 100 min to a production rate of about 4 nl g^{-1} hr^{-1}. Stem segments which had dissipated their initial wound response were not stimulated to produce a second response if rewounded.

Removal of endogenous ethylene by repeated evacuation and flushing of tissue sections with ethylene-free air did not signifi-

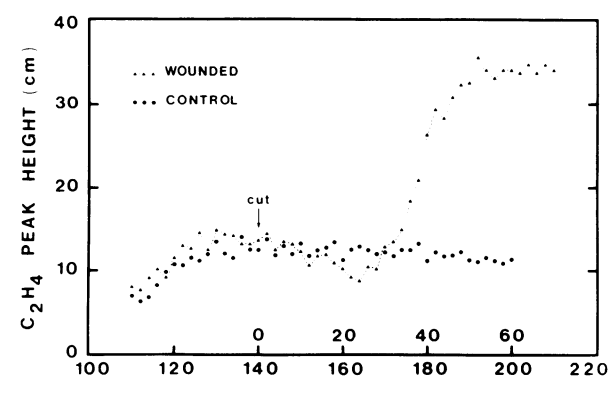

FIG. 1. Ethylene evolution with respect to time from 13-cm etiolated pea stems which were kept in 26-ml sample chambers continually flushed with humidified ethylene-free air. Replicates were either left uninjured or wounded with three cuts perpendicular to the stem axis in the region 9 and 18 mm below the top of the apical hook.

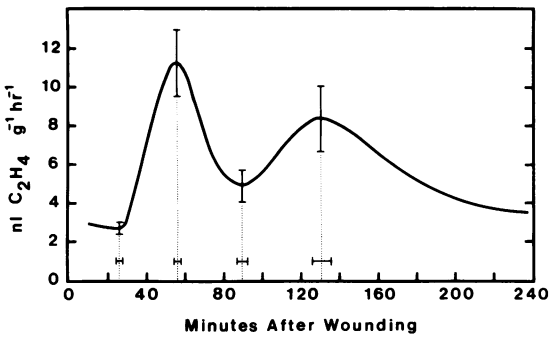

FIG. 2. Kinetics of wound-induced ethylene synthesis from subapical pea stem sections. Data for this graph were taken from 12 representative experiments. Vertical lines denote SD of the rates of ethylene synthesis; horizontal lines denote SD of the times at which the inflection points occurred.

cantly affect the timing or rate of wound ethylene synthesis. The characteristic pattern must therefore be the result of increased ethylene synthesis and not merely the result of facilitated diffusion of ethylene already present in the tissue through the newly exposed cut surfaces. This conclusion is supported by the presence of a definite 26-min lag period. If the increase in ethylene evolution was due to enhanced diffusion a much more rapid increase would occur following cutting. This was tested by exposing subapical pea sections, in which the wound response had dissipated, to 10 $\mu l\ l^{-1}$ ethylene for 15 min, then quickly flushing the sample chamber with ethylene-free air and following the subsequent evolution of ethylene from the tissue. A sampling rate of 1 ml min^{-1} caused an immediate exponential decrease in the rate of ethylene evolution which reached barely detectable levels after 7 min. This indicates that dissolved ethylene diffuses out of the tissue much too rapidly to be the source of the observed increase.

Effect of Internode Length. The length of the internode from which the subapical sections were excised significantly affected both the time and the rate of maximum wound ethylene synthesis. As the internode length increased from 2 to 8 cm, the time of maximum synthesis shifted from 52 to 57 min. An increase in internode length from 6 to 8 cm caused a greater increase (1.8 min) than did an increase from 2 to 4 cm (1.1 min). The rate of maximum synthesis increased slowly from 5.5 nl g^{-1} hr^{-1} for sections excised from 2-cm internodes to 6.9 g^{-1} hr^{-1} for sections excised from 6-cm internodes, and then markedly increased to 8.2 nl g^{-1} hr^{-1} for sections excised from 8-cm internodes.

It was observed that etiolated peas form the fourth node when the third internode was about 8 cm long. The great increase in wound ethylene synthesis from subapical sections excised from 8-cm internodes may reflect some morphological or physiological change in the stem. Tissue excised closer to the meristematic regions of the hook produced more wound-induced ethylene than tissue from differentiated regions. Extension of the meristematic region farther from the apical hook could account for the increased wound response, as could accumulation of substrates near the apex.

Effect of Gravity Orientation. Changing the gravity orientation of excised subapical sections by rotating the sample chamber through 90 or 180 degrees after the onset of wound ethylene synthesis did not significantly affect the rate of ethylene production during a subsequent 0.5 hr. However, apical sections oriented in their normal vertical position had significantly higher rates of ethylene synthesis (12.3 ± 1.8 nl g^{-1} hr^{-1}) than did sections oriented at random (9 ± 1.3 nl g^{-1} hr^{-1}). The lag time and the rate of increase were identical for both treatments.

Effect of Light. Exposure to fluorescent light had a slightly inhibitory effect on wound-induced ethylene. The timing was not changed, but exposure to light did reduce the maximum rate of ethylene synthesis by about 18%, from 10.3 ± 1.7 nl g^{-1} hr^{-1} in the dark, to 8.4 ± 1.5 nl g^{-1} hr^{-1} in the light.

Wound-induced Ethylene Synthesis from Various Tissues. Different regions of etiolated pea plants were tested for their ability to produce wound-induced ethylene. Sections excised from meristematic regions of the stem or root exhibited high rates and more than two peaks of wound ethylene (Fig. 3). Tissue excised from different regions near the stem apex showed similar lag times, rates of increase, and maximum ethylene synthesis. As sections were excised progressively farther from the stem apex, the second peak diminished and disappeared before the magnitude of the first peak was significantly affected. Segments excised still farther from the stem apex had lower rates of maximum ethylene synthesis during the first peak, but neither the lag time nor the time of maximum synthesis was affected. Fully elongated stem or root tissue did not produce measurable amounts of wound-induced ethylene.

A wound-induced increase in the rate of ethylene production occurred in tissue excised from many different species of plants (Table I). Segments from etiolated herbaceous plants had similar kinetics of wound ethylene synthesis, while tissues from green woody plants were more variable in the timing and rates of ethylene production. Tissue from etiolated plants had similar lag times (20 ± 3 min) and times of ethylene synthesis (53 ± 5 min). The lag time for green tissue segments was variable, ranging from 22 min for *Cercis canadensis* to 55 min for *Lonicera flexuosa*. *Taxus canadensis* did not exhibit wound ethylene synthesis. The time to the maximum was over twice as long for green tissue as for etiolated tissue; ranging from 104 min for *C. canadensis* to 133 min for *Forsythia viridissima*. These differences may reflect specie differences, since etiolated *versus* nonetiolated tissues from one specie were not compared.

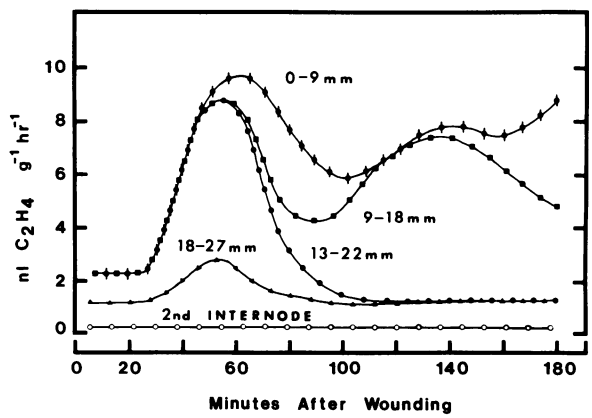

FIG. 3. Ethylene synthesis with respect to time for various 9-mm segments of tissue from 7-day-old etiolated pea seedlings. Stem segments were excised from the region 0 to 9 mm (●——●), 9 to 18 mm (■——■), 13 to 22 mm (●——●), and 18 to 27 mm (▲——▲) from the top of the apical hook. Sections were also excised from the center of the second internode (○——○).

Table I. Wound-induced ethylene synthesis from various species of plants

Species	Tissue	Etiolated	Time lag	Time peak	Maximum rate	Basal rate
			min		nl g^{-1} hr^{-1}	nl g^{-1} hr^{-1}
Pisum sativum	sub-apical	yes	26	55	10.6	2.6
Cucumis sativus	cotyledons	yes	24	57	3.9	0.5
Cucumis sativus	hypocotyl	yes	19	55	6.3	0.8
Lycopersicon esculentum	apical	yes	18	39	5.3	2.4
Hordeum vulgare	coleoptile	yes	24	47	22.4	15.8
Zea mays	coleoptile	yes	20	58	29.7	1.2
Zea mays	leaves	yes	20	58	60.3	2.6
Triticum aestivum	coleoptile	yes	18	53	30.9	18.8
Avena sativa	coleoptile	yes	16	50	42.0	7.0
Cercis canadensis	sub-apical	no	22	104	6.9	1.4
Forsythia viridissima	sub-apical	no	30	133	17.0	1.1
Lonicera flexuosa	sub-apical	no	55	129	1.3	0.5
Taxus canadensis	sub-apical	no			no wound-induced ethylene	

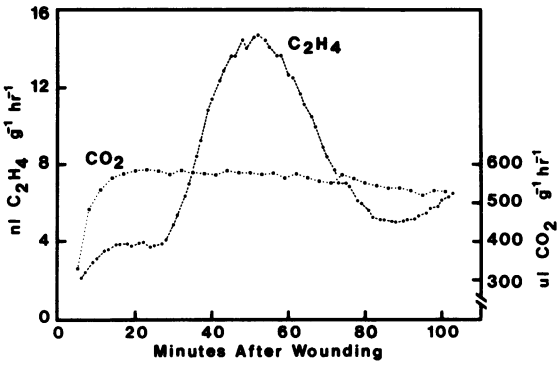

FIG. 4. Ethylene and CO_2 evolution with respect to time from 9-mm subapical pea stem sections.

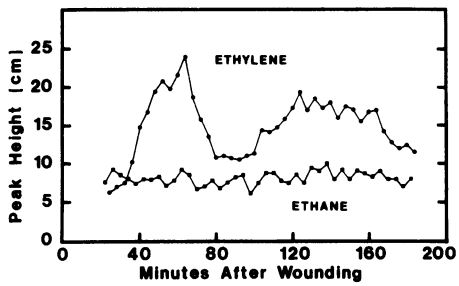

FIG. 5. Ethylene and ethane evolution with respect to time from 9-mm subapical pea stem sections.

Apical coleoptile segments from etiolated monocots had much higher rates of wound ethylene synthesis (28 ± 3.7 nl g^{-1} hr^{-1}) than did stem tissue segments from etiolated dicots (6 ± 3.0 nl g^{-1} hr^{-1}). Excised leaves from 5-day-old etiolated *Zea mays* seedlings had an astonishingly high rate of ethylene synthesis (60 nl g^{-1} hr^{-1}).

Effect of Wounding on Ethylene, CO_2, and Ethane Production. Since cutting fruits and vegetables stimulates the rate of respiration, as measured by O_2 uptake (20), wound ethylene might merely reflect fluctuations in the rate of respiration. The rate of ethylene production by subapical stem segments increased almost four times from 4 nl g^{-1} hr^{-1} during the lag phase to a maximum of around 15 nl g^{-1} hr^{-1}. During this same period the rate of CO_2 production, after an initial increase, remained constant at around 580 μl g^{-1} hr^{-1} (Fig. 4). Ethylene production fluctuated greatly during the first 100 min while CO_2 production showed a slight and almost linear decrease from 580 μl g^{-1} hr^{-1} to around 530 μl g^{-1} hr^{-1}. It should be noted that the rate of CO_2 production was over four orders of magnitude greater than the rate of ethylene production. Analysis of data from nine experiments showed no consistently significant correlation between ethylene and CO_2 production during the first peak of wound-induced ethylene synthesis.

Ethylene and ethane production were followed for 3 hr following excision of the sections. In three experiments the rate of ethane production did not change significantly as ethylene production went through its characteristic wound-induced pattern (Fig. 5). Lieberman and Kunishi (13) reported that in two model systems for ethylene synthesis from peroxidized linolenic acid, the ratio of ethylene to ethane production was about 15:1 and 4:1. The absence of a constant ratio between wound ethylene production and ethane evolution argues against a similar *in vivo* biosynthetic system for the production of wound ethylene from linolenic acid. Experiments with labeled linolenic acid (15), and its peroxidized product propanal (4), have shown that neither is converted to ethylene in living cells.

CONCLUSION

Mechanically wounded etiolated pea stem sections produce a remarkably consistent pattern of wound-induced ethylene synthesis. Variations in tissue preparation and execution of the experiment slightly perturb the magnitude, but do not markedly alter the timing of the response. A definite lag period of 26 min precedes the induction of one or two well defined surges of ethylene synthesis which occur at 56 and 131 min. This is a rapid, stable, and predictable system with which to study the physiology of induced ethylene synthesis.

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