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

II. OXYGEN AND TEMPERATURE DEPENDENCY

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**ABSTRACT**

Wound-induced ethylene synthesis by subapical stem sections of etiolated *Pisum sativum* L., cv. Alaska seedlings, as described by Saltveit and Dilley (Plant Physiol 1978 61: 447–450), was half-saturated at 3.6% (v/v) O₂ and saturated at about 10% O₂. Corresponding values for CO₂ production during the same period were 1.1% and 10% O₂, respectively. Anaerobiosis stopped all ethylene evolution and delayed the characteristic pattern of wound ethylene synthesis. Exposing tissue to 3.5% CO₂ in air in a flow-through system reduced wound ethylene synthesis by 30%. Enhancing gas diffusivity by reducing the total pressure to 130 mm Hg almost doubled the rate of wound ethylene synthesis and this effect was negated by exposure to 250 μl liter⁻¹ propylene. Applied ethylene or propylene stopped wound ethylene synthesis during the period of application, but unlike O₂, no lag period was observed upon flushing with air. It is concluded that the characteristic pattern of wound-induced ethylene synthesis resulted from negative feedback control by endogenous ethylene.

No wound ethylene was produced for 2 hours after excision at 10 or 38 C. Low temperatures prolonged the lag period, but did not prevent induction of the wound response, since tissue held for 2 hours at 10 C produced wound ethylene immediately when warmed to 30 C. In contrast, temperatures above 36 C prevented induction of wound ethylene synthesis, since tissue cooled to 30 C after 1 hour at 40 C required 2 hours before ethylene production returned to normal levels. The activation energy between 15 and 36 C was 12.1 mole kilocalories degree⁻¹.

A rapidly induced, transitory increase in the rate of ethylene synthesis has been observed in a variety of excised tissues (1, 24). The wound response was recently characterized in *Pisum sativum* L., cv. Alaska (24). In subapical stem tissue wound-induced ethylene production at 25 C increased linearly after a lag period of 26 min from 2.7 nl g⁻¹ hr⁻¹ to the first maximum of 11.3 nl g⁻¹ hr⁻¹ at 56 min. The rate of production then decreased to a minimum at 90 min, increased to a lower second maximum at 131 min, and then declined over a period of about 100 min to about 4 nl g⁻¹ hr⁻¹. The magnitude of the response varied slightly from experiment to experiment, but the time sequence was constant for tissue excised from a given region of the pea seedling. In this study we examined the O₂ and temperature dependency of wound-induced ethylene synthesis by etiolated 'Alaska' pea stem sections.

Vegetative, ripening, and senescent tissue require O₂ for ethylene synthesis (1, 3, 6, 13, 21, 29). Sfakiotakis and Dilley (25) found that induction of autotropical ethylene production by apples (*Malus domestica* Borkh.) occurred above 6.5% O₂ and that anaerobiosis prevented induced ethylene production. Lieberman and Spurr (17) showed that ethylene synthesis by broccoli (*Brassica oleracea* L.) was prevented by anaerobiosis and saturated at between 1 and 2.5% O₂. Mature green banana fruit (*Musa spp.*) maintained normal, though reduced, basal metabolism at O₂ tensions of 5%, but were unable to produce ethylene in atmospheres of less than 7.5% O₂ (18). Burg and Thimmann (10) showed that ethylene production by plums of climacteric apples decreased at around 10% O₂ and was half-maximal at around 2% O₂.

Yang (31) postulated that ethylene may be formed by an O₂-dependent reaction. He suggested that under anaerobic conditions the proposed intermediate accumulated and that a surge of ethylene production occurred upon exposure to O₂. Such a surge has been reported for apples (3, 10) and pears (14).

The rate of ethylene production increased in plants which had been partially frozen (13, 28, 32), cold-stressed at 5 C (11, 26, 30), or heat-stressed at 40 C (15). Plants continually exposed to 40 C produced very little ethylene, and required several hr at cooler temperatures to recover ethylene-synthesizing capacity (10, 14). Substrate availability was not limiting, since ethylene production by apple tissue fed methionine was also severely inhibited at 40 C (16). In general, ethylene production is reduced at temperatures above 35 C, and completely prevented at 40 C (1, 5).

**MATERIALS AND METHODS**

**Plant Material.** Seven-day-old etiolated seedlings of 'Alaska' pea were grown and prepared as previously described (24). Subapical stem sections (9-mm) excised 9 mm from the top of the apical hook were used in all kinetic studies. Some experiments employed tissue which had dissipated their initial wound response (aged sections). The procedures for identifying and quantifying ethylene, CO₂, and O₂ were the same as previously described (24). Data points are not shown in Figures 2 through 7 because the short sampling interval of 45 sec produced data which generated a smooth curve.

**O₂ Dependency.** The O₂ requirement for wound-induced ethylene synthesis was studied using 20 subapical sections enclosed in 25-ml Erlenmeyer flasks with moist filter paper and a CO₂ absorber. The flasks were immediately purged with N₂ and an appropriate volume of air or pure O₂ was injected by syringe to give a specified O₂ concentration. After 2 hr the gaseous contents of the flasks were sampled and analyzed for ethylene, O₂, and CO₂.

**Effect of Endogenous Gases.** Equilibration of endogenous and ambient gas concentrations in aged subapical sections was accomplished in a flow-through system by inserting the needle of a 50-ml syringe through the top serum stopper of an 0.8-ml opaque glass sample chamber. A partial vacuum was created by closing the inlet and outlet ports of the sample chamber and pulling the plunger of the syringe to the 50-ml mark. After a few sec the inlet tube was opened and ethylene-free air entered the evacuated
Table I. Ethylene and carbon dioxide production by excised subapical 'Alaska' pea stem sections held at 760 or 130 mm Hg for 2 hr.

<table>
<thead>
<tr>
<th>Partial pressure of gases (mm Hg)</th>
<th>Total Pressure</th>
<th>130 mm Hg</th>
<th>760 mm Hg</th>
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<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>CO₂</td>
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<td>O₂</td>
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<td>760</td>
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<td>130</td>
<td></td>
<td>351</td>
<td>1.13</td>
</tr>
<tr>
<td>130</td>
<td></td>
<td>336</td>
<td>1.11</td>
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<tr>
<td>130 (250 μl/1)</td>
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<td>149</td>
<td>1.16</td>
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1C₂H₄ as pmol g⁻¹ hr⁻¹
2CO₂ as ml g⁻¹ hr⁻¹

chamber. The syringe was withdrawn when the flow into the chamber stopped, and routine C₂H₄ sampling was resumed.

Endogenous concentrations of gases were varied by changing gas diffusivity by adjusting the total pressure. Ten subapical sections were subjected to 130 mm Hg O₂ partial pressure at absolute pressures of 760 or 130 mm Hg and to O₂ at 760 mm Hg in 30-ml gas-tight syringes. The syringes were flushed with ethylene-free air or O₂, set to 4 ml, capped with rubber serum stoppers, and 1 ml of air or O₂ was introduced. Propylene was injected into some of the syringes to yield a final concentration of 250 μl liter⁻¹ at absolute pressures of 760 or 130 mm Hg (Table I). The plungers were set at 5 ml (760 mm Hg) or held at 30 ml (130 mm Hg). After 2 hr the contents of all syringes were analyzed for ethylene and CO₂.

Temperature Dependency. The effect of temperature was studied using a flow-through system in which seven subapical sections were enclosed in 0.8-ml opaque glass sample chambers immersed in water baths. Three chambers were sampled in series by withdrawing 2-ml samples on a staggered 40-sec schedule. Measurements were also made using 30 subapical sections enclosed in a 1.7-ml opaque glass sample chamber with a thermometer (0.01 C divisions) inserted through the top serum stopper and in contact with the tissue. This permitted direct reading of the tissue temperature, rather than inferring the temperature from that of the water bath. Gas samples were analyzed for ethylene every 5 min and at the flow rate indicated the tissue produced quantities of ethylene easily detectable by GC. The rate of ethylene synthesis was calculated as picomol (g fresh wt)⁻¹ hr⁻¹.

RESULTS AND DISCUSSION

O₂ Dependency. Wound-induced ethylene synthesis was reduced at O₂ concentrations below 10% (v/v) (Fig. 1). The 1-e⁻ⁿᵗ curve was fitted to the data by the method of least squares and demonstrates that the rate of wound ethylene synthesis is proportional to O₂ at low O₂ concentrations and independent of the O₂ concentration above 10%. The half-maximal rate of synthesis occurred at around 3.6% O₂. This is higher than the half-maximal concentration reported for broccoli (17), but similar to those for apples and bananas (10, 19). Wound ethylene synthesis was not detectable (less than 1 μl liter⁻¹) in flow-through systems in which the O₂ concentration was maintained below 0.01%. However, the lowest concentration attained in the 2-hr accumulation experiments was around 0.5% O₂. The intercept at 0.7% O₂ suggests that either an O₂ threshold exists or that wound ethylene synthesis is limited by O₂ diffusion into the tissue.

CO₂ production was half-saturated at 1.1% O₂ and saturated at around 10% O₂ (data not shown). In a variety of excised tissues, respiration is generally saturated at 10% O₂ and half-saturated at 2 to 4% O₂ (2, 4). The difference between the half-maximal O₂ concentration for respiration (1.1%) and for wound ethylene synthesis (3.6%) could result from competition for O₂ by various other metabolic reactions.

The characteristic pattern of wound-induced ethylene synthesis was delayed by the length of time freshly excised tissue was deprived of O₂ (Fig. 2). Both the lag period and the time to maximum ethylene production were increased in tissue held under N₂ for 25 or 50 min. However, the maximum rate of synthesis was not affected by the period of anaerobiosis. Interrupting the normal response with a 30-min period of anaerobiosis stopped ethylene synthesis during the time O₂ was withheld (Fig. 3). Admission of O₂ reinstated the characteristic pattern at the point at which it was interrupted when the stoppage occurred at 20 min. Oscillations in

FIG. 1. Effect of O₂ concentration on wound-induced ethylene synthesis by 9-mm subapical stem sections of etiolated 'Alaska' pea seedlings. Dashed lines indicate point of half-maximal synthesis at 3.6% O₂.

FIG. 2. Effect of anaerobiosis on wound-induced ethylene synthesis by freshly excised subapical sections of etiolated pea stems.

FIG. 3. Effect of a 30-min period of anaerobiosis, given before or after induction of increased synthesis, on wound-induced ethylene synthesis by etiolated pea stems.
the rate of wound ethylene synthesis were not observed if the 30-min period of anaerobiosis was given at 40 min, after the rise in wound ethylene synthesis had started.

Subapical sections in which the wound response had dissipated (aged sections) and which had been deprived of \( \text{O}_2 \) for 1 to 3 hr, showed a rapid increase in ethylene production as soon as air was readmitted, rising to between 30 to 50% of the rate before anaerobiosis (Fig. 4). A slight decline then occurred before a second increase started about 35 min after exposure to air. The second rise continued to a peak at around 70 min which was about twice the original rate of ethylene production. Ethylene production then declined and stabilized near the original rate in about 1 hr. Although the exact timing differed, the general pattern (i.e. a 30-min lag preceding a rapid increase) was similar to that of wound-induced ethylene synthesis.

Within 2 hr, anaerobiosis gradually reduced the rate of \( \text{CO}_2 \) production by freshly excised sections from 585 to 287 \( \mu \text{l g}^{-1} \text{ hr}^{-1} \). No surge in \( \text{CO}_2 \) production was observed following the change from \( \text{N}_2 \) to air. A burst of ethane sometimes accompanied the change from air to \( \text{N}_2 \) or from \( \text{N}_2 \) to air, but the results were not consistent. Palmer and Loughman (22) reported that the rate of respiration of etiolated pea epicotyls decreased following wounding. However, in this study wounding increased respiration (as measured by \( \text{CO}_2 \) production) from 509 \( \mu \text{l g}^{-1} \text{ hr}^{-1} \) before wounding to 597 \( \mu \text{l g}^{-1} \text{ hr}^{-1} \) 50 min after wounding. During the same interval, ethylene synthesis increased from 2.6 to 10.4 \( \text{nl g}^{-1} \text{ hr}^{-1} \).

Vacuum-flushing aged subapical sections to equilibrate the internal and ambient gases resulted in an increase in ethylene production before the equilibrium was reestablished (Fig. 5). This suggested that the rate of ethylene synthesis was limited by the internal concentration of \( \text{O}_2 \), \( \text{CO}_2 \), ethylene, or other gases. Enhanced diffusion of gases across the cut surface of subapical sections was not the cause of wound ethylene synthesis, inasmuch as (a) coating the cut ends of freshly excised subapical sections with silicon stopcock grease did not prevent the characteristic pattern of wound ethylene evolution; and (b) holding 13-cm apical stem segments in 100% \( \text{O}_2 \) did not induce a wound response or increase the rate of ethylene production. If the concentration of \( \text{O}_2 \), \( \text{CO}_2 \), or ethylene limited wound ethylene synthesis, then increasing gas diffusivity by lowering the total pressure should increase the rate of wound ethylene synthesis. An 80% \( \text{He}-20\% \text{O}_2 \) (v/v) gas mixture increased gas diffusivity, but did not significantly affect the kinetics of wound ethylene synthesis. Hypobaric ventilation at a total pressure of 150 mm Hg \( \text{O}_2 \) is sometimes used to lower the internal concentration of ethylene below the threshold level needed to ripen fruits or vegetables (8). Since a flow-through system was not available which would permit sampling ethylene evolution at subatmospheric pressures, a series of experiments was performed using a static system.

When sections were held at 130 mm Hg \( \text{O}_2 \) at 760 or 130 mm

**FIG. 4.** Effect of anaerobiosis on ethylene production by aged subapical sections of etiolated pea stems. The atmosphere was changed from air to nitrogen, and from nitrogen to air at the designated times. Aged tissue left in air beyond 4 hr maintained a constant rate of ethylene production during the experiment.

Hg total pressure, almost twice as much wound ethylene was synthesized at the reduced pressure (Table I). Addition of \( \text{CO}_2 \) to give the normal partial pressure of 0.23 mm Hg (i.e. 0.03% at 760 mm Hg) had no significant effect of wound ethylene synthesis. However, \( \text{CO}_2 \) was shown to be an inhibitor of wound ethylene synthesis, since gassing subapical sections with 3.5% \( \text{CO}_2 \) in air in the flow-through system at 760 mm Hg significantly reduced the maximum rate of ethylene synthesis by 30%, from 12.3 \( \pm 1.3 \text{ nl g}^{-1} \text{ hr}^{-1} \) in air, to 8.2 \( \pm 1.5 \text{ nl g}^{-1} \text{ hr}^{-1} \) in 3.5% \( \text{CO}_2 \). The time of maximum wound ethylene synthesis was not significantly affected by \( \text{CO}_2 \). If \( \text{CO}_2 \) controlled wound ethylene synthesis by competing with endogenous ethylene for positive feedback sites, as it does in climacteric fruit (1, 7), then fluctuations in the internal concentration of \( \text{CO}_2 \) could produce the characteristic pattern of wound-induced ethylene evolution. However, previous work has shown that during the first 2 hr, \( \text{CO}_2 \) production remained almost constant as wound ethylene production fluctuated over 400% (24).

Addition of the ethylene analog propylene (9, 20) significantly reduced wound ethylene production at 760 or 130 mm Hg (Table I). This is not totally unexpected since ethylene reportedly inhibits ethylene synthesis in green tomato fruits (\textit{Lycopersicon esculentum Mill.}), immature fig fruit (\textit{Ficus sycomorus L.}, cv. Balami) (33), and in slices of green banana fruit (27). Propylene has a similar effect in these systems, but competes with ethylene in other systems. Propylene competes with ethylene in its ability to increase stem thickness, reduce stem growth, and cause diageotropism in etiolated ‘Alaska’ pea seedlings (12). Propylene may therefore not be an analog of ethylene, but like \( \text{CO}_2 \), a competitive inhibitor of ethylene action in wound ethylene synthesis. Reduction of wound-induced ethylene synthesis may result from competition between ethylene and propylene for positive feedback sites, rather than propylene augmenting activation by ethylene of a negative feedback site. This question was investigated by gassing freshly excised subapical sections with ethylene or propylene.

The initial peak of wound-induced ethylene synthesis was delayed by holding tissue for 60 or 120 min in either 11 \( \mu \text{l liter}^{-1} \) ethylene or a physiologically equivalent concentration (500 \( \mu \text{l liter}^{-1} \)) or propylene (Fig. 6). The rate of ethylene synthesis under propylene was only slightly less than the rate during the normal lag period or from the stable rate of production from nonwounded apical regions of pea stems (24). Exposure to either ethylene or propylene reduced the rate, and broadened the first peak when the tissue was returned to air. Normal events of the lag period occurred while the tissue was being gassed with ethylene or propylene for 1 hr since wound ethylene production proceeded immediately when the exogenous olefins were removed. Neither ethylene nor propylene stimulated wound ethylene synthesis, since no increase in ethylene production was observed while the tissue was gassed with propylene, and the initial rate of ethylene production was low when these sections were returned to air. Both
gases apparently inhibited wound-induced ethylene synthesis, but did not inhibit induction of the wound response. If propylene, like CO₂, was a competitive inhibitor of ethylene synthesis, then the rate, but not the timing of the response, should have been altered. However, the timing was actually more perturbed than the maximum rate of synthesis. These data indicate that propylene is an analog of ethylene in this system, and that endogenous ethylene exerts negative feedback control on the rate of wound-induced ethylene synthesis.

Negative feedback control by endogenous concentrations of ethylene may cause the oscillations in wound-induced ethylene synthesis as the tissue recovers from the initial effect of wounding. High levels of endogenous ethylene, resulting from the rapid rise in production during the first maximum, may deactivate part of the wound ethylene-synthesizing system and thereby decrease rates of production. As the internal concentration falls, synthesis may be reactivated. The decline following the second maximum may be the result, not of further negative feedback, but of depletion of a limiting substrate. This is supported by the observation that tissues in which the initial wound response had dissipated, and which were producing ethylene at a stable basal rate, were not stimulated to produce a second wound response when rewounded. Apical sections appear to contain more of this substrate since they produce higher rates of wound ethylene and oscillate through more cycles (24). If the characteristic pattern were the result of negative feedback control, then a period of anaerobiosis before induction of wound ethylene should not perturb the pattern, while a similar period of anaerobiosis after induction should reduce the internal concentration of ethylene, modify the feedback control, and cause the observed radical perturbation of the response (Fig. 3). The ability of ethylene and CO₂ to inhibit wound ethylene synthesis is a paradox since CO₂ is a competitive inhibitor of other ethylene-induced responses in this tissue (9).

The reported surge in ethylene production following removal of tissue from anaerobiosis (10, 14) may be the result, not of the accumulation of an intermediate (31), but of the lowering of the internal concentration of ethylene. Since anaerobic tissue does not produce ethylene, its internal concentration will equilibrate with the ambient atmosphere. The observed overshoot above the equilibrium rate of ethylene synthesis could therefore result from a slow response time of negative feedback control on the rate of ethylene synthesis. Examination of the kinetics of wound-induced ethylene synthesis from 'Alaska' pea sections supports this hypothesis. The time between the inflection points of wound ethylene synthesis was around 35 min (start of increase to first peak = 30 min, first peak to minimum = 34 min, minimum to second peak = 41 min). The midpoint of each of these times, i.e., 15, 17, and 20 min before or after the inflection point, occurred at a production rate of about 290 pmol g⁻¹ hr⁻¹. If the internal concentration is directly related to the rate of production, then the concentration associated with a production rate of 290 pmol g⁻¹ hr⁻¹ may be the threshold for modulating ethylene synthesis, and around 17 min is required to turn the synthesizing system on or off. A similar time lag of 17 min occurred in the surge of ethylene from aged subapical sections in which the endogenous and ambient concentrations of gases were equilibrated (Fig. 5). The surge in ethylene synthesis was probably the result of reduced internal concentrations of ethylene rather than of accumulated precursors.

**Temperature Dependency.** Temperature had a pronounced effect on the rate and timing of wound-induced ethylene synthesis (Fig. 7). The rate of production during the lag period was similar at temperatures of 10 and 38°C. Between these temperatures, the rate of ethylene synthesis increased from 50 pmol g⁻¹ hr⁻¹ at 10°C to a maximum of 120 pmol g⁻¹ hr⁻¹ at 30°C, and then declined to 60 pmol g⁻¹ hr⁻¹ at 38°C. The length of the lag period decreased from over 120 min at 10°C to a relatively constant 25 min at 30°C to 36°C. At above 38°C wound ethylene synthesis was completely inhibited, and only the basal rate was observed. The time to the first maximum decreased from over 120 min at 15°C to around 45 min at 30°C and then increased slightly to 50 min at 36°C. The rate of maximum wound ethylene synthesis increased from around 400 pmol g⁻¹ hr⁻¹ at 10°C to 880 pmol g⁻¹ hr⁻¹ at 36°C.

An Arrhenius plot of the maximum rate of wound ethylene synthesis showed that an abrupt change from linearity occurred at 36°C (Fig. 8). A linear equation fitted to the points between 15 and 36°C had an r² of 0.95 (N = 10). An activation energy of 12.1 kcal mol⁻¹ degree⁻¹ was calculated from this equation. This activation energy corresponds to a temperature coefficient (Q₁₀) of approximately 2 for temperatures between 15 and 36°C.

Although a change in the activation energy for respiration by peas does not occur between 9 and 12°C (18, 23), a phase change in the components of the ethylene-synthesizing system in this temperature range could account for the observed deviation in activation energy. However, the phase change was not drastic.
since changing the temperature to 30 C, after 2 hr at 10 C, immediately initiated the characteristic pattern of wound ethylene synthesis. It appears that 10 C allows induction of the wound response, while slowing down the reactions necessary for wound ethylene synthesis.

Temperatures above 36 C must have reduced wound ethylene synthesis by more drastic means, since about 2 hr at 30 C was required for recovery from a 40-min exposure to 40 C. Exposure to 40 C for a min produced the lag period by 7 min, from 26 to 33 min. Exposure to 40 C for 5 min shifted the time of maximum wound ethylene production from 50 min to 80 min, broadened the peak, and reduced the maximum rate from 300 pmol g⁻¹ hr⁻¹ to 140 pmol g⁻¹ hr⁻¹. Tissue sections held above 37 C did not produce wound-induced ethylene. However, if the subapical sections were held at 36 C until the wound response was induced (i.e., for the 26-min lag period), then the temperature had to exceed 38 C before wound ethylene synthesis was reduced. If the wound ethylene-synthesizing system is associated with membranes, comparison of the phase change temperatures with specific membrane fractions (e.g., plasmalemma, endoplasmic reticulum, tonoplast, mitochondria) could assist in its localization.

CONCLUSION

Similarities in the O₂ and temperature dependency of wound-induced ethylene synthesis by 9-mm subapical sections of etiolated 'Alaska' pea seedlings and of ethylene synthesis by other plant tissues suggest that ethylene is formed by the same enzymatic system in both cases. Other data suggest that wound ethylene is either produced in a different compartment of the cell, or is controlled by a different regulator. Wound ethylene synthesis had a higher apparent half-maximal O₂ saturation (3.6%) than that reported for ethylene synthesis by other tissues (around 2.0%) and O₂ was required for its induction during the 26-min lag period. Propylene, an active ethylene analog, prevented wound ethylene synthesis, but did not affect the basal rate of ethylene production. The rate of synthesis was apparently regulated by the endogenous concentration of ethylene through negative feedback control. Temperature had a profound effect on the kinetics of synthesis, but had little effect on the almost constant basal rate of ethylene production during the lag period.

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