Requirement for Ethylene Synthesis and Action during Relief of
Thermoinhibition of Lettuce Seed Germination by Combinations
of Gibberellic Acid, Kinetin, and Carbon Dioxide

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ABSTRACT

Application of exogenous ethylene in combination with gibberellic acid (GA3), kinetin (KIN), and/or CO2 has been reported to induce germination of lettuce seeds at supraoptimal temperatures. However, it is not clear whether endogenous ethylene also plays a mediatory role when germination under these conditions is induced by treatment regimes that do not include ethylene. Therefore, possible involvement of endogenous ethylene during the relief of thermoinhibition of lettuce (Lactuca sativa L. cv Grand Rapids) seed germination at 32°C was investigated. Combinations of GA3 (0.5 millimolar), KIN (0.05 millimolar), and CO2 (10%) were used to induce germination. Little germination occurred in controls or upon treatment with ethylene, KIN, or CO2. Neither KIN nor CO2 affected the rate of ethylene production by seeds. Both germination and ethylene production were slightly promoted by GA3. Treatments with GA3 + CO2, GA3 + KIN, or GA3 + CO2 + KIN resulted in approximately 10- to 40-fold increases in ethylene production and 50 to 100% promotion of germination as compared to controls. Initial ethylene evolution from the treated seeds was greater than from the controls and a major surge in ethylene evolution occurred at the time of visible germination. Application of 1 millimolar 2-aminoethoxyvinyl glycerine (AVG), an inhibitor of ethylene synthesis, in combination with any of above three treatments inhibited the ethylene production to below control levels. This was accompanied by a marked decline in germination percentage. Germination was also inhibited by 2,5-norbornadiene (0.25–2 milliliters per liter), a competitive inhibitor of ethylene action. Application of exogenous ethylene (1–100 microliters per liter) overcame the inhibitory effects of AVG and 2,5-norbornadiene on germination. The results demonstrate that endogenous ethylene synthesis and action are essential for the alleviation of thermoinhibition of lettuce seeds by combinations of GA3, KIN, and CO2. It also appears that these treatment combinations do not act exclusively via promotion of ethylene evolution as the application of exogenous ethylene alone did not promote germination.

The optimum temperature for the germination of lettuce seeds is in the vicinity of 20°C, though differences are encountered among varieties and seed lots (12, 20, 23). Germination is inhibited at temperatures above the optimum (thermoinhibition), often falling sharply to reach zero within a narrow temperature range (12, 16). The seeds that fail to germinate upon imbibition at these supraoptimal temperatures, eventually enter a state of secondary dormancy termed as thermodormancy (2). Effects of a number of treatments have been studied in attempts to overcome thermoinhibition and thus prevent the induction of thermodormancy (2, 11, 13). Among these, GA3 has been generally found ineffective whereas KIN and ethylene have been reported to cause variable degrees of promotion (1, 11, 15, 18, 21). In addition to a variety of interactions among these plant hormones, their effects have also been reported to be markedly augmented by CO2 (10, 13, 14). However, owing largely to inadequate experimental techniques, the exact contribution of each of these factors to their collective effects in bringing about the relief of thermoinhibition had, until recently, remained rather unclear. Using techniques that allow a precise control of the gaseous environment, we have recently shown that GA3, KIN, ethylene, or CO2 applied singly have little effect on the germination of thermoinhibited (at 32°C) Grand Rapids lettuce seeds (17). However, recent studies have suggested that GA3 + KIN or GA3 + CO2 could induce approximately 50% germination. Addition of ethylene to either of these combinations or of CO2 to GA3 + KIN resulted in nearly 100% germination.

It has been suggested that GA3, KIN, or CO2 may induce germination in various species via the enhancement of endogenous ethylene production (2, 8, 11). However, little direct experimental evidence exists in support of these views, which are mainly based on comparisons of ethylene production by seeds with differing germination capabilities (2, 8, 11). Using inhibitors to manipulate ethylene synthesis and action, we have investigated whether the induction of germination at a supraoptimal (32°C) temperature by combinations of GA3, KIN, and CO2 is mediated by ethylene.

MATERIALS AND METHODS

Materials and Treatments. Seeds of Lactuca sativa L. cv Grand Rapids were purchased from Ferry Morse Seed Co., Mountain View, CA and were stored at 3°C in darkness until used. Seeds that were free from deformities and were uniform in size and color were used for experiments. Compressed air, CO2, and ethylene were obtained from Liquid Carbonic Canada and were purified as described below. Aldrich Chemical Co. was the source of 2,5-norbornadiene (bicyclo[2.2.1]hepta-2,5-diene) and other chemicals were supplied by Sigma Chemical Co. All chemicals were of the highest purity available.

Kinetin was dissolved in 1 N HCl and then diluted with double distilled H2O so that the final HCl concentration was 0.001 N. The pH of kinetin solution was then adjusted with 0.25 N NaOH to 6.0 at 25°C. The solution of GA3 was prepared in double distilled H2O. Germination was induced by the application of

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3 Abbreviations: KIN, kinetin; AVG, 2-aminoethoxyvinyl glycerine.
GA₃ + KIN, GA₃ + CO₂, or GA₃ + KIN + CO₂, since these treatments have been previously reported to overcome thermoinhibition (17).

All manipulations of seeds were done in a darkroom fitted with a green safelight obtained by filtering the light from one 15-W cool-white fluorescent tube through a green plexiglass No. 2092 filter (24). The seed level quantum flux density from this source was 4 × 10¹⁸ Q m⁻² s⁻¹.

**Seed Germination. Experiments with Endogenous Ethylene Measurement.** Seeds were surface sterilized with 2 ml of 1% NaOCl for 2 min, washed for 15 min in flowing sterile distilled H₂O, and were imbibed on two layers of Whatman No. 1 filter paper, soaked with 5 ml of the relevant solution, in modified 250 ml glass Erlenmeyer flasks (200 seeds/flask). Each flask was fitted with a light-tight glass stopper and an inlet and outlet to allow continuous gaseous flow. The flasks were wrapped in two layers of black polyethylene sheet and one layer of foil and were placed inside an incubator maintained at 32 ± 0.2°C. Each flask was connected to a supply of the relevant gas of known concentration flowing at the rate of 20 ml min⁻¹. When ethylene or CO₂ were not a part of the treatment, any contamination by these gases was eliminated prior to the entry of a gaseous stream into the flask. CO₂ was removed by bubbling the inflow through two successive traps, each containing 30 ml of 20% (w/v) KOH whereas ethylene was eliminated through oxidation using the procedure described earlier (5). Gaseous effluents from each flask were passed through 30 ml of 75% (w/v) KOH contained in a glass tube immersed in an ice-bath to remove CO₂ and moisture, following which ethylene was collected for 30 min in a U-tube containing 0.5 g silica gel (60–80 mesh) kept at −86°C in a dry ice-acetone bath. Ethylene collections were done at frequent intervals over a 24 h period and measured on a Hewlett-Packard 5880A series gas chromatograph. The details of the method of ethylene collection and gas chromatography were as described earlier (5). Ethylene evolution from seeds was not measured when these were treated with exogenous ethylene.

All experiments were repeated at least twice with similar results. Each set of ethylene evolution data is based on one representative replicate whereas seed germination data are composites of all replicates (at least three) of an experiment. Germination (radicle protrusion) was recorded after 24 h, a period that allows maximum germination in all treatments. When germination was recorded at different times during the 24 h period, counts were taken from separate flasks on each occasion to avoid any possible effect of seed movement on germination, and the flasks were then discarded.

**Experiments with 2,5-Norbornadiene.** Since it was difficult to control the concentration of norbornadiene vapor in a flow-through system, the experiments were done in a closed system but large volume containers were chosen so as to minimize the effects of gases released by the seeds on the gaseous composition within the containers. Seeds were imbibed in 6 × 1.5 cm Petri dishes on three layers of Whatman No. 1 filter paper wetted with 3 ml of the appropriate solution. Three Petri dishes, containing 50 seeds each, were placed inside darkened 3.8 L airtight glass bottles. Each bottle was flushed for 5 min with a 5 L min⁻¹ flow of 10% CO₂ through a small hole in the lid which was then sealed with a rubber septum. Concentration of CO₂ in the bottles was measured and was found to be 10%. Since these manipulations were done at 23°C, 113 ml of the gas was removed from each bottle to compensate for the pressure change upon subsequent incubation at 32°C. Liquid norbornadiene was injected in microliter quantities through a rubber septum on to a watch-glass suspended below the septum and the compound completely evaporated within 10 min. When appropriate, ethylene was injected into the bottles to obtain the desired concentration. The bottles were incubated at 32 ± 0.1°C for 24 h, following which the germination was recorded.

**RESULTS AND DISCUSSION**

No germination occurred at 32°C in H₂O controls (CO₂ and ethylene free atmosphere) and ethylene evolution remained at a constant level of less than 0.25 nl/200 seeds/30 min during the 24 h period of incubation (Fig. 1). Similar levels of ethylene emanation and germination occurred in seeds treated with KIN or CO₂ (data not shown). Treatment with GA₃ resulted in 8.0 ± 2.5% germination and an increase in ethylene evolution from approximately 0.1 to 0.8 nl/200 seeds/30 min during 24 h of incubation. Germination (Table I) and ethylene evolution (Fig. 2) were dramatically enhanced by the application of GA₃ + CO₂, GA₃ + KIN, or GA₃ + KIN + CO₂. The temporal relationship between germination and ethylene evolution from seeds is illustrated in Figure 1 by the example of seeds treated with GA₃ + KIN + CO₂. Initial ethylene evolution by treated seeds was slightly greater than in controls and increased slowly during the first 10 h of imbibition followed by an accelerated increase up to 24 h. Visible germination commenced approximately 12 h after imbibition, concomitantly with the surge in ethylene evolution. Similar relationships between germination and ethylene evolution were observed in seeds treated with GA₃ + KIN or GA₃ + CO₂ (data not presented). Thus, of the two phases of ethylene evolution from the seeds subjected to these treatments, only the initial phase of a slower rate of ethylene production could be a potential cause of germination. The progressive increase in ethylene evolution may be partially attributable to an increase in the number of metabolically active seeds that produce ethylene within a treated population.

Application of AVG along with GA₃ + CO₂, GA₃ + KIN, or GA₃ + KIN + CO₂ inhibited the ethylene evolution throughout the incubation period (Fig. 2) to levels even lower than those arising from untreated control seeds (Fig. 1). This inhibition of ethylene evolution was accompanied by a marked reduction in the percent germination in each case, although complete inhibition of germination was not observed (Table I). The inhibitory effects of AVG on germination were completely reversed by the exogenous application of ethylene to seeds subjected to all three germination-promoting treatments in the presence of AVG (Ta-
Table 1. Effects of GA₃ + KIN, GA₃ + CO₂, or GA₃ + KIN + CO₂ Applied in the Presence or Absence of AVG and in Combination with Various Concentrations of Ethylene on the Germination of L. sativa Seeds

Concentrations of GA₃, KIN, AVG, and CO₂ were 0.5 mM, 0.05 mM, 1 mM, and 10%, respectively. Germination was recorded following incubation for 24 h at 32°C in the dark. Each value denotes mean ± se.

<table>
<thead>
<tr>
<th>Medium</th>
<th>0 Ethylene (10 μL L⁻¹)</th>
<th>10 Ethylene (μL L⁻¹)</th>
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<tbody>
<tr>
<td>H₂O</td>
<td>0.5 ± 0.5</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>GA₃ + KIN</td>
<td>52.0 ± 2.5</td>
<td>70.5 ± 4.5</td>
</tr>
<tr>
<td>GA₃ + KIN + AVG</td>
<td>22.7 ± 2.7</td>
<td>72.3 ± 0.8</td>
</tr>
<tr>
<td>GA₃ + CO₂</td>
<td>55.6 ± 0.7</td>
<td>71.0 ± 3.5</td>
</tr>
<tr>
<td>GA₃ + CO₂ + AVG</td>
<td>9.6 ± 0.4</td>
<td>58.0 ± 0.5</td>
</tr>
<tr>
<td>GA₃ + KIN + CO₂</td>
<td>93.8 ± 2.3</td>
<td>94.0 ± 0.0</td>
</tr>
<tr>
<td>GA₃ + KIN + CO₂ + AVG</td>
<td>40.3 ± 2.1</td>
<td>89.0 ± 1.0</td>
</tr>
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In fact, ethylene at 10 μL L⁻¹ not only reversed the AVG-induced inhibition, it was also able to accentuate the effects of GA₃ + CO₂ and GA₃ + KIN to the same extent as observed in the absence of AVG (Table 1).

Since reduction in ethylene synthesis did not completely eliminate germination, inhibition of ethylene action was attempted using 2,5-norbornadiene (19). Only one of the treatments, GA₃ + KIN + CO₂, was employed for the induction of germination since this combination included all the factors being studied. Over 90% of the seeds germinated in response to this treatment. The germination was inhibited by norbornadiene at all the concentrations between 0.25 and 2 ml L⁻¹ with only 7% germination at the highest concentration (Fig. 3A). Ethylene, at concentrations of 1 to 100 μL L⁻¹, affected a concentration-dependent reversal of the norbornadiene-induced inhibition of germination (Fig. 3A). Germination induced by GA₃ + KIN + CO₂ was reduced to nearly 60% by the addition of AVG and the residual germination was further inhibited by norbornadiene to 7% at the highest concentration of 2 ml L⁻¹ (Fig. 3B). Application of 10 μL L⁻¹ ethylene alleviated the inhibition caused by AVG + norbornadiene (Fig. 3B).

The results have shown that endogenous ethylene synthesis and action are essential for the relief of thermoinhibition of lettuce seed germination by combinations of GA₃, KIN, and CO₂. Since only the pregermination ethylene synthesis could be implicated in the induction of germination, it is significant that ethylene production during this phase from seeds that eventually germinated was greater than that by nongerminating controls (Fig. 1). Application of AVG depressed the ethylene production by treated seeds to levels lower than those emanating from seeds imbibed in H₂O (cf. Figs. 1 and 2). Further, the promotive effects of the GA₃, KIN, and CO₂ treatment on germination were markedly diminished by AVG. Though germination declined considerably in the presence of AVG, it was not completely inhibited despite a substantial decline in ethylene evolution. However, application of 2,5-norbornadiene, a competitive inhibitor of ethylene action (7, 19) to seeds treated with GA₃ + KIN + CO₂ or GA₃ + KIN + CO₂ + AVG caused nearly complete inhibition of germination. The inhibition caused by AVG, norbornadiene, or a combination of the two compounds was reversed by the exogenous application of ethylene. These results suggest that the seeds that germinated even after the ethylene synthesis was inhibited by AVG, probably had a very low ethylene requirement, which was fulfilled by the residual ethylene synthesis occurring in the presence of AVG. A low ethylene requirement by these seeds is also indicated by the fact that AVG was less inhibitory in the closed bottles than in the 'flow-through' system (cf. Fig. 3 and Table I); some ethylene accumulation could have occurred in the closed system. Ethylene accumulation to levels above the threshold for activity could also be a possible

![Fig. 2. The effects of various combinations of 0.5 mM GA₃, 0.05 mM KIN, and 10% CO₂ applied in the presence or absence of AVG (1 mM) on ethylene evolution from L. sativa seeds during incubation for 24 h at 32°C in darkness. A, GA₃ + KIN; B, GA₃ + CO₂; C, GA₃ + KIN + CO₂. (C), Treated with AVG; ( ), no AVG.](attachment://image-url)
ETHYLENE SYNTHESIS AND LETTUCE SEED GERMINATION

Fig. 3. The effects of various concentrations of 2,5-norbornadiene and ethylene on the germination of L. sativa seeds. A. Seeds treated with GA3 + KIN + CO2; B, seeds treated with GA3 + KIN + CO2 + AVG. Germination was recorded following incubation for 24 h at 32°C in the dark. The concentrations of GA3, KIN, CO2, and AVG were 0.5 mM, 0.05 mM, 10%, and 1 mM, respectively. (C), (A), (B), and (D), ethylene concentrations of 0, 1, 10, and 100 μL L⁻¹, respectively. Vertical bars, SE, where no bar exists, the SE value was smaller than the symbol.

reason that Kępczyński and Karssen (7) found no effect of AVG but a marked effect of norbornadiene on the germination of Amaranthus caudatus L. in a closed system.

Previously published evidence indicates that the inhibition of germination at supraoptimal temperatures may not result from a decline in the ethylene producing ability of the seeds (3, 4). Dunlap and Morgan (4) have suggested that supraoptimal temperatures raise the threshold concentration of ethylene required for germination. Regardless of whether or not a curtailed ability to synthesize ethylene is the cause of thermoinhibition, our results show that continued ethylene synthesis and action are required for the relief of thermoinhibition.

Though ethylene synthesis and action were found to be essential for the relief of thermoinhibition of lettuce seeds, germination was not induced as a result of ethylene action only since the exogenous ethylene applied alone in darkness was without any effect (17) (Table I). It appears that combinations of GA3, KIN, and CO2, apart from enhancing ethylene production, induce other metabolic changes that act in concert with ethylene to bring about germination. This possibility was also suggested by Dunlap and Morgan (4).

Although exogenous ethylene has been shown to affect the germination of many species (8, 14, 22), a positive requirement for the action of endogenous ethylene has been previously demonstrated only for the germination of nondormant seeds of A. caudatus L. (7). Most other reports that implicate endogenous ethylene in germination have been based on indirect evidence such as the observation of an increase in ethylene production prior to germination, correlations between dormancy levels and ethylene production, or a depression of germination upon removal of ethylene from the air surrounding the seeds (6, 8, 9).

Since the present results demonstrate that endogenously produced ethylene is an indispensable requirement for lettuce seed germination even though exogenous ethylene alone (in darkness) induces little germination, the regulatory function of ethylene in seed germination merits reevaluation. One should be cautious about dismissing a seed as having no ethylene requirement for germination simply because it does not respond to exogenous ethylene.

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LITERATURE CITED