Kinetin Enhanced 1-Aminocyclopropane-1-Carboxylic Acid Utilization during Alleviation of High Temperatures Stress in Lettuce Seeds

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ABSTRACT

The thermoinhibition at 35 and 32°C of pregermination ethylene production and germination in lettuce (Lactuca sativa L. cv Mesa 659) seeds was synergistically or additively alleviated by 0.05 millimolar kinetin (KIN) and 10 millimolar 1-aminocyclopropane-1-carboxylic acid (ACC). The synergistic effect of KIN + ACC on ethylene production and germination at 35°C was inhibited by Co²⁺ (44-46%) but not by aminoethoxyvinyl glycine (AVG). The uptake of ACC by the seed was not influenced by KIN. Upon sitting of the seed coats (composed of pericarp, testa and endosperm), following the uptake of chemicals, ACC was readily converted into ethylene at all temperatures, and the synergistic effects of KIN + ACC at 35°C were lost. At 35°C, KIN acted synergistically with ACC or ethylene (ETH) in alleviating the osmotic restraint. At 25°C, ETH was more active than KIN or KIN + ACC in overcoming the osmotic restraint. Thus, the integrity of the seed coats, the KIN-enhanced ACC utilization, and an interaction of KIN with the ethylene produced may be the basis for the synergistic or additive effects of KIN + ACC at high temperature.

The first indication that cytokinins may mediate their effects via enhanced ethylene production came from studies with dormant peanut seeds in which the relief of dormancy by cytokinin was accompanied by enhanced ethylene production (6). The release of dormancy in cocklebur and Indian rice grass seeds was synergistically or additively enhanced by a combination of KIN + ETH (16).

Lettuce seeds subjected to high temperature and other stresses produce little ethylene (1, 8). In several nonseed tissues, the stress resulting from flooding, anaerobiosis, and high temperature appears to inhibit the ACC-to-ethylene conversion step in preference to S-adenosylmethionine (SAM)-to-ACC step in the ethylene biosynthetic pathway (2, 4, 9). In a preliminary report KIN, in combination with ACC, synergistically promoted the pregermination ethylene production and germination in lettuce seeds at supraoptimal temperatures (7).

The study reported here examines the interactive effects of KIN and ACC in ethylene production and germination of lettuce seeds at various temperatures and how these interactions are influenced by the seed coats and the osmotic restraint.

MATERIALS AND METHODS

‘Mesa 659’ lettuce (1987 harvest) seeds were obtained from the Harris-Moran Seed Company, Rochester, NY. They were kept at 7°C and 28% RH and small batches were removed as needed.

Intact Seed Treatments

Batches of 50 seeds (three replicates) were soaked in 5.0-cm Petri dishes lined with two layers of Whatman No. 1 filter paper and moistened with 3 mL of 0.05 mM KIN, 10 mM ACC, 1 mM AVG, 1 mM cobalt chloride, or combinations thereof. Germination (radicle protrusion) was recorded at various times at 25, 32, and 35°C in fluorescent light (9-16 μmol s⁻¹ m⁻²).

Slit Seed Treatments

Seeds in batches of 0.8 g were presoaked in 12 mL of water, 0.05 mM KIN, 10 mM ACC, or combinations thereof, at 25, 32, and 35°C for 1 and 4 h and dried for 2 h by forced air at 25°C. Of these seeds, 150 were slit by making a longitudinal

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2 Abbreviations: KIN, kinetin; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinyl glycine; EFE, ethylene forming enzyme; ETH, ethylene [(2-chloroethyl)phosphonic acid]; δ, water potential.
cut at the cotyledonary end, the cut extending one-third the length of the seed. Batches of 50 (three replicates) presoaked intact and slit seeds were then soaked in water for 16 h at temperatures initially used for presoaking. In slit seeds, germination occurred atypically with the cotyledon end protruding first (5). The various embryo coverings (pericarp, testa, and endosperm) will be referred to as seed coats in the present study.

**Water Potential Studies**

To determine the ability of chemicals to alter the germination potential of the seeds, seeds in batches of 0.2 g were presoaked for 2 h in 3 mL water, 0.05 mM KIN, 10 mM ACC, 10 mM ETH, or combinations thereof, and dried for 2 h in forced air at 25°C. Fifty treated seeds (three replicates) were soaked for 48 h in solutions of PEG of Ψ ranging from 0 to −0.6 MPa at 25, 32, and 35°C. The PEG solutions were prepared and adjusted for temperature changes as described before (11). The ability to germinate at any one Ψ was used to estimate the germination potential of the treated seeds.

**Ethylene Analysis**

Batches of 0.5 g seeds (three replicates) were soaked in 9-cm Petri dishes with 6 mL of test solution at various temperatures. At indicated times, seeds were rapidly wiped dry on paper towels and transferred to 28-mL glass tube that was capped with a rubber septum and incubated on its side for 1 h at 25°C in light (9 μmol s⁻¹ m⁻²). In some cases, intact and slit seeds (in batches of 50, three replicates), prepared as described above, following a 1 and 4 h presoak treatment with KIN and/or ACC, were soaked for 6 h in water, wiped dry and transferred to 5.7 mL glass tubes, capped and incubated for 1 h at 25°C. Ethylene contents in the gas phase of the tube was determined by withdrawing a 1 mL sample with a gastight syringe and injecting into a Hewlett-Packard 5790 gas chromatograph equipped with a flame ionization detector and a 183 × 0.32 cm stainless steel column containing Poropak Q. A correction was made in calculating ethylene diluted due to vacuum created during removal of gas from 5.7 mL tubes.

**ACC Determination**

To determine ACC uptake, 0.5 g seeds (three replicates) were soaked in 10 mM ACC with or without 0.05 mM KIN for 1, 5.5, and 10 h. Seeds were extracted twice with 80% ethanol for 2 h at 70°C, the combined extract centrifuged at 10,000 g, and the supernatant evaporated in vacuo. After resuspending in 0.5 mL chloroform, ACC was extracted with 2 mL of water, and its content in the extract was determined by chemical conversion to ethylene as described by Lizada and Yang (10).

**RESULTS**

The germination response of seeds to KIN and/or ACC at various temperatures varied a great deal (Fig. 1). At supraoptimal temperatures of 32 and 35°C, little or no germination occurred in Mesa 659 lettuce seeds soaked in water. This thermoinhibition was relieved by KIN to a greater extent than by ACC. A synergistic or additive promotion of germination occurred by KIN + ACC at 32 and 35°C. At 25°C (Fig. 1) or lower temperatures of 15 and 20°C (data not shown), KIN +
ACC showed no synergistic effect during the course of germination.

The effects of KIN and/or ACC at various temperatures on ethylene production generally paralleled their effects on germination (Fig. 2). For example, after 12 h soak in ACC the amounts produced by the seeds at 25, 32, and 35°C were 336, 113, and 16 nL h⁻¹ g⁻¹, respectively. The combination, KIN + ACC, synergistically promoted ethylene production after a soak of 10 to 16 h at 35°C, and 10 to 12 h at 32°C. As in the case of germination, no synergism occurred at 25°C (Fig. 2) or at temperatures of 15 and 20°C (data not shown).

Although no ethylene was detected in seeds soaked in water or KIN for 10 h at 35°C, the KIN-induced germination was strongly inhibited (91–95%) by 1 mM AVG and 1 mM Co²⁺ (Table I). Ethylene derived from ACC or by KIN + ACC was, however, inhibited (46–49%) only by Co²⁺. Similarly, ACC- and KIN + ACC-promoted germination were inhibited (44–55%) by Co²⁺ but not by AVG. At 25°C, AVG had no effect on germination with or without KIN and/or ACC. The ethylene production in water- or KIN-soaked seeds was completely inhibited by AVG, whereas Co²⁺ was partially effective. In the presence of ACC or KIN + ACC, only Co²⁺ inhibited (about 40%) ethylene production.

To determine if KIN influenced ACC uptake by the seed at high temperature, ACC contents of the seeds soaked in 10 mM ACC with or without 0.05 mM KIN for 1, 5.5, and 10 h at 32°C were determined (Fig. 3). The amount of ACC taken up by the seed increased in a linear fashion with an increase in the duration of soaking from 1 to 10 h, irrespective of the presence or absence of KIN. The endogenous amounts of ACC recoverable from seeds soaked in water and KIN ranged from 0.3 ± 0.2 to 0.5 ± 0.2 nmol g⁻¹ seeds.

The effects of various temperatures (25, 32, and 35°C) on

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**Table I. Effect of AVG and Co²⁺ on Ethylene Production and Germination Promoted by KIN, ACC, and KIN + ACC at 25 and 35°C**

Ethylene production and germination were determined following 10 and 24 h soak, respectively. Concentrations of chemicals: KIN, 0.05 mM; ACC, 10 mM; AVG, 1 mM; Co²⁺, 1 mM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethylene</th>
<th>Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVG</td>
<td>Co²⁺</td>
</tr>
<tr>
<td>Water</td>
<td>0 0 0</td>
<td>7 0 0</td>
</tr>
<tr>
<td>KIN</td>
<td>0 0 0</td>
<td>35 0 20</td>
</tr>
<tr>
<td>ACC</td>
<td>29 28 15</td>
<td>15 155 165</td>
</tr>
<tr>
<td>KIN + ACC</td>
<td>48 59 26</td>
<td>148 145 89</td>
</tr>
<tr>
<td>LSD</td>
<td>4 4 2</td>
<td>10 9 7</td>
</tr>
</tbody>
</table>

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**Table II. A Comparison of Ethylene Producing and Germination Capacities of Intact and Slit Seeds Incorporated with the Same Amount of ACC, KIN, or KIN + ACC**

Following incorporation of chemicals into seeds during 4 h soak at 35, 32, and 25°C, a portion of the seeds were slit. Treated intact and slit seeds were soaked in water at temperatures used for presoaking with chemicals, and ethylene production and germination were determined after 6 and 16 h, respectively. Concentrations of chemicals: KIN, 0.05 mM; ACC, 10 mM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethylene</th>
<th>Germination</th>
<th>Ethylene</th>
<th>Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nL h⁻¹ g⁻¹</td>
<td>%</td>
<td>nL h⁻¹ g⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>Water</td>
<td>0 0 0</td>
<td>96 95 98</td>
<td>0 0 0</td>
<td>96 95 98</td>
</tr>
<tr>
<td>KIN</td>
<td>0 0 0</td>
<td>9 75 93</td>
<td>0 5 15</td>
<td>35 45 90</td>
</tr>
<tr>
<td>ACC</td>
<td>9 16 52</td>
<td>95 142 152</td>
<td>37 48 92</td>
<td></td>
</tr>
<tr>
<td>KIN + ACC</td>
<td>22 33 55</td>
<td>98 147 152</td>
<td>35 43 92</td>
<td></td>
</tr>
<tr>
<td>LSD 5%</td>
<td>3 4 5</td>
<td>6 11 7</td>
<td>4 5 5</td>
<td></td>
</tr>
</tbody>
</table>

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the ethylene-producing capacity and germination of slit and intact seeds incorporated during a 4 h presoak with the same amounts of KIN and/or ACC are shown in Table II. Unlike intact seeds, slit seeds produced much greater amounts of ACC-derived ethylene and showed no synergistic or additive effects with KIN + ACC on ethylene production or germination at high temperatures. Intact seeds treated for 1 h with KIN and/or ACC and subsequently soaked for 6 h in water (total soak period of 7 h) at 32°C showed a KIN-ACC synergistic stimulation of ethylene production (water or KIN, 0 nL h⁻¹ g⁻¹; ACC, 2 nL h⁻¹ g⁻¹; KIN + ACC, 10 nL h⁻¹ g⁻¹). Slit seeds 1 h after incorporation of chemicals and soaked additionally for 6 h in water showed no synergistic improvement with KIN + ACC in ethylene production. In no case did intact or slit seeds germinate before 10 h of soaking in water following a period of presoak and drying.

The germination potential of seeds with KIN, ACC, ETH, or combinations thereof, and exposed to different temperatures and Ψs varied a great deal (Table III). At 35°C, little or no germination occurred within 48 h after treatment with 10 mM ACC or 10 mM ETH. Treatment with 0.05 mM KIN resulted in 36 and 9% germination at 0 and −0.2 MPa, respectively. A synergistic improvement in germination potential occurred from a treatment with KIN + ACC (74 and 13% germination at 0 and −0.2 MPa, respectively) or KIN + ETH (77 and 42% germination at 0 and −0.2 MPa, respectively). At 32°C, the effectiveness of KIN + ACC or KIN + ETH to synergistically enhance germination remained the same up to −0.4 MPa, but KIN + ETH generated a greater potential than KIN + ACC at −0.6 MPa (77% versus 16% germination). At 25°C, the seeds germinated readily in up to −0.4 MPa Ψ in all treatments. At −0.6 MPa, the germination potential generated by ETH (90% germination) was greater than that by KIN + ACC (63% germination), even though KIN + ACC additively improved the germination.

**DISCUSSION**

The results of this study show that a combination of KIN and ACC synergistically enhances pregermination ethylene production and germination at high temperatures of 35 and 32°C but not at 25°C (Figs. 1 and 2) or lower temperatures of 20 and 15°C (data not shown). When seeds are slit, ACC taken up by the seed is readily converted to ethylene and the synergistic effects of KIN + ACC are lost (Table II). These results indicate that cytokinins may play an important role in regulating ethylene biosynthesis and germination in intact seeds at high temperatures, and the seed coats may be essential for such a regulation.

At 25°C, KIN does not enhance the pregermination ethylene production in the presence of ACC (Fig. 2). Further, inhibition of ethylene production at this temperature in the presence of KIN or KIN + ACC by inhibitors of ethylene production does not affect germination (Table I). Several lines of evidence, however, indicate that KIN may promote ACC utilization and germination at high temperatures: (a) At 35°C the synergistic promotion of ethylene production and germination by KIN + ACC was inhibited 44 to 49% by CO₂, an inhibitor of ACC-to-ethylene step, but not by AVG, an inhibitor of ACC production (17) (Table I); (b) the ACC-derivated ethylene production and germination decreased with an increase in temperature and the addition of KIN partially reversed the inhibition, particularly at 32 and 35°C (Figs. 1 and 2); (c) the amount of ACC taken up by the seed was not affected by the presence of KIN; however, ACC taken up interacted synergistically with KIN but only under conditions that prevented germination (Fig. 3; Table II); and (d) little or no germination or increase in germination potential occurred with ACC or ETH at 35°C; the addition of KIN to ACC or ETH, however, synergistically enhanced the germination potential (Table III).

A comparison of the effectiveness of KIN, ACC, ETH, or their combinations to induce germination at various temperatures and Ψs indicates that the KIN + ACC alleviation of high temperature stress is somewhat different from its alleviation of osmotic restraint (Table III). At 35°C, KIN was more active than ETH in promoting germination and interacted with ACC or ETH in the alleviation of temperature and osmotic stress. At 32°C, KIN was as active as ETH up to a Ψ of −0.4 MPa (but not at −0.6 MPa) and continued to interact with ACC or ETH in the alleviation of stress. At 25°C, KIN

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**Table III. Effect of Temperatures and Osmotic Restraint (0, −0.2, −0.4, −0.6 MPa PEG Solution) on Germination of Lettuce Seeds Pretreated with 0.05 mM KIN, 10 mM ACC, 10 mM ETH, or Combinations Thereof**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PEG Solution (MPa)</th>
<th>35°C</th>
<th>32°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ACC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ETH</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KIN</td>
<td>36</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>KIN + ACC</td>
<td>74</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>KIN + ETH</td>
<td>77</td>
<td>42</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>LSD 5%</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Seeds were presoaked for 2 h in test solutions, dried, and germinated for 48 h in PEG solution of varying Ψ.
was less effective than ETH but was still able to interact with ACC in alleviating osmotic restraint.

Thus, the synergistic or the promotive effect of KIN + ACC at 35 and 32°C, with or without the osmotic restraint, might involve both enhanced ACC utilization and an interaction of the ACC-derived ethylene with KIN in the alleviation of thermoinhibition. In seeds experiencing only osmotic restraint at 25°C, the KIN + ACC promotion may depend solely on the enhanced conversion of ACC to ethylene, the ethylene produced acting independently of KIN (ETH was as effective as KIN + ETH) in the alleviation of osmotic restraint. The KIN + ACC alleviation of osmotic restraint at 25°C may be similar to its alleviation of salt stress reported previously (8). In that study, as in the present, ethylene by itself was highly effective in alleviating the stress. These results are consistent with the reports in the literature that cytokinin is more active than ethylene in alleviating the high temperature stress on germination of lettuce seed (1, 13, 14; see also Table III) and is able to generate a growth potential at 35°C which is strong enough to override the restraining force of the seed coats (15). The presence of a cytokinin during thermoinhibition, with or without an osmotic restraint, might permit oxidative reaction promoting EFE activity and stress alleviation.

ACKNOWLEDGMENTS

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LITERATURE CITED