ARE SUBSTITUTED BENZOTHIADIAZOLES SPECIFIC INHIBITORS OF ETHYLENE ACTION

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Introduction

Ethylene is known to promote seed germination. However, seeds of many plants do not respond to the gas and some of the promotive effects are small (1). The most thoroughly studied system is the lettuce "seed" (botanically an achene). Lettuce seed germination is due to the growth or cell expansion of the hypocotyl region which forces the radical through the micropyle. When sufficient phytochrome is present in the far red absorbing form (Pfr) and the temperature is less than 20°C, lettuce seeds germinate within 24 hours following imbibition. When the temperature is raised to 30°C germination of the seeds is inhibited. This effect is called thermal dormancy, but thermal inhibition is a better term since embryos germinate if the endosperm is removed or punctured. Lettuce seed germination is a simple and rapid way of measuring ethylene action. Other than the fact that ethylene promotes germination, little is known concerning the role, mechanism, or site of action of the gas. The experiments described here were designed to evaluate the role of inhibitors of ethylene action during germination. The inhibitors evaluated in this study included: DIHB (3,5-diiodo-4-hydroxybenzoic acid), STS (silver thiosulfate), CO_2, NBA (2,5-norbornadiene), and MCEB (5-methyl-7-chloro-4-ethoxy-carbonyl-methoxy-2,1,3-benzothiadiazole).

The first compound reported to be an inhibitor of ethylene action was CO_2 (4). Since then the following compounds were shown to have anti-ethylene effects: MCEB (5,6,9,19), NBA (21,25), DIHB (16), and STS (3). Inhibitors are useful tools in physiological experiments since they can indicate whether a hormone plays a role in a particular process. Inhibitors may also serve as probes as to the nature of the hormone attachment site and its location. Of the inhibitors described above, CO_2 and NBA are competitive inhibitors in the sense that they have a molecular structure similar to ethylene (carbon atom with a double bound). The action of silver is still unknown, but it may interact with ethylene by absorbing pi electrons. The action of DIHB and MCEB is unclear. It is not known whether these substances bind competitively to the site of ethylene action, or effect the action of the ethylene binding site complex.

Methods

Lettuce (Latuca sativa 'Grand Rapids') seeds were purchased from Burpee Seed Company, Warminster, Pennsylvania. Ethylene was measured by flame ionization gas chromatography. Germination studies were performed by placing 4 groups of 25 seeds in petri plates containing 9 cm diameter Whatman #3 filter paper imbibed with 4 ml of the appropriate test solution. Seeds were incubated for 2 hours in the dark at 30°C and then removed and exposed to fluorescent lights in the laboratory for at least 5 min to convert phytochrome to the Pfr form. They were then placed at the temperatures or in the gas mixtures indicated. Seeds were gassed by placing them in 4-L plastic paint cans or 10-L glass dessicators fitted with rubber vaccine stoppers. Germination was measured after 24 hours. In Fig. 4, germination was measured at 24 and 48 hours. Data are expressed as the mean of 4 samples and least significant difference (LSD) was calculated by the Duncan's multiple range test.

Results

Fig. 1 shows the result of treating lettuce seeds with various levels of DIHB in the presence of ethylene and air. DIHB did not inhibit germination at 25°C but had a slight affect at 30°C. Ethylene did not appear to reverse the small inhibition observed.

Fig. 2 shows that STS inhibited germination. The effect at 30°C was greater than that at 25°C. Ethylene blocked the inhibiting effect of silver in terms of the percent reduction in germination. In the absence of ethylene, 1 mM STS inhibited germination by 90%. In the presence of 1 ul/L ethylene, the inhibition by STS was reduced to 20%.

The effect of CO_2 on germination at 30°C is shown in Fig. 3. The action of this inhibitor is a paradox since it promotes rather than inhibits germination. This promotive effect has been reported earlier (2).

NBA was an effective inhibitor at both 25°C and 30°C (Fig. 4). In the presence of ethylene at 25°C (24 H, Fig. 4C), most of the NBA effect was lost. At 30°C, the higher NBA concentrations blocked germination (24 H, Fig. 4D). The seeds were then transferred to air or ethylene for an additional 24 hr treatment. In the absence of NBA (48 H AIR), germination increased. Germination was increased further if 10 ul/L ethylene was included in the gas phase (48 H ETH). Except for the experiment shown in Fig. 4C, 1000 ul/L of NBA appeared to be toxic or irreversibly bound to the seeds.

MCEB was an effective inhibitor of germination at both 25°C and 30°C. At 25°C, 10 ul/L ethylene reversed the effect of 10 mM MCEB (Fig. 5). However, higher concentrations of MCEB blocked germination. As shown in Fig. 6, 10 ul/L ethylene reversed the effect of 10 mM MCEB but had only a small effect on seeds treated with 50 mM MCEB. Germination at 30°C can also be stimulated by cytokinins, such as

N6-benzyladenine (N6-BA). The specificity of MCEB in terms of blocking ethylene action was tested by examining the effect of MCEB on seeds whose germination was enhanced by N6-BA. In the experiment shown in Fig. 7, seeds were placed in water (control), 25 mM MCEB for 3 hrs, or 25 mM MCEB for 24 hrs. The 3-hr treatment consisted of transferring seeds after 3 hrs to filter paper saturated with water or N6-BA. As shown in Fig. 7, both ethylene and N6-BA increased germination. Both hormones promoted germination in the presence of MCEB. Seeds exposed to MCEB for 3 hrs had a higher rate of germination than those treated for 24 hrs, indicating that the seeds either detoxified MCEB or the chemical diffused into the surrounding medium.

Discussion

DIHB appeared to have little or no effect on germination at 25°C or 30°C. Either the action of this compound is not as specific as originally proposed by Larque-Saaredra (16) or the compound failed to reach the embryo under the conditions of these experiments. The surrounding endosperm is a significant barrier to solutes and this problem has been discussed earlier by others (10). However, triiodobenzoic acid, a compound similar to DIHB, was shown to prevent lettuce seed germination and its effect was reversed by Ethrel (20).

Lack of penetration may also explain the results obtained with STS. At 30°C some inhibition with STS was observed, and depending upon the concentration used, this inhibition could be overcome by 0.1 ul/L or 1 ul/L ethylene.

The observation that CO_2 enhanced instead of inhibited germination is opposite to that one would expect for a competitive inhibitor of ethylene action. This effect has been reported earlier (2,11,15,17,18,24). However, CO_2 reduced the ability of ethylene to promote witchweed germination (8). CO_2 also blocked the ability of ethylene to promote lettuce seed germination after seeds were imbibed in 0.2 M NaCl (18). Under these conditions, germination was observed only when ethylene was added to the gas phase. The role of the Na^+-K^+/ATPase proton pump in cell expansion may explain the paradoxical effect of CO_2 (7). In this system, carbonic acid may supply the hydrogen ions needed for proton extrusion and the bicarbonate ions for sodium or potassium ion uptake. The importance of K^+ uptake for hypocotyl growth and germination may also explain the observation that KNO_3 promotes germination in lettuce (13).

NBA was reported to be an effective inhibitor of ethylene action by Sisler and Pian (21). They reported that NBA prevented senescence and seed germination. NBA has been used to prevent abscission (22) and germination of Amaranthus seeds (14). NBA was an effective inhibitor of lettuce seed germination. At both 25°C and 30°C, it blocked germination at concentrations of 100 ul/L and higher. The inhibitory effect of NBA was lost when seeds were treated simultaneously with ethylene. The NBA effect was reversible. Placing seeds treated with NBA in air or

ethylene resulted in the resumption of germination. Concentrations of 500 ul/L appeared to be either toxic or irreversibly bound since some seeds did not germinate after treatment with these levels of the gas.

MCEB also proved to be an effective and partially reversible inhibitor of germination. At a concentration of 10 uM, germination was inhibited 50%. Full germination was observed when 10 ul/L ethylene was added to the gas phase. The effects of higher concentrations of MCEB were not fully reversible by ethylene. As shown in Fig. 7, some of the MCEB effect was lost if seeds were incubated in water. It is not clear if MCEB lost its effectiveness by diffusion or metabolism. Unlike other inhibitors of ethylene action, the structure of MCEB does not suggest a mode of action dependent upon competitive binding. MCEB may work through an as yet unknown mechanism. The observation that MCEB also inhibited N6-BA action suggests that MCEB either blocks the action of other hormones or that germination can be promoted through alternative routes. Thus, even though the effect of ethylene is lost, an alternative cytokinin mediated pathway can promote germination.

In summary, even though DIHB and CO_2 act as inhibitors of ethylene action in other systems, they failed to function in the lettuce seed germination system. Problems with penetration, or secondary effects of CO_2, may explain the results observed. The other three compounds tested, STS, NBA, and MCEB inhibited germination and their effect was reversed in part by ethylene. Lack of total reversibility may indicate that these compounds have toxic side effects or irreversibly bind to their site of action. The results reported here indicate that ethylene may play an endogenous role in the germination of lettuce seeds. The action of the gas is unknown but may be associated with its ability to promote radial cell expansion (12,23).

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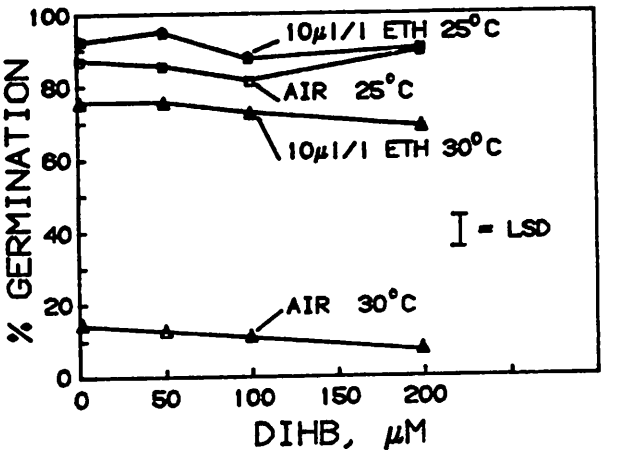

Fig. 1. Effect of a combination of DIHB and 10 ul/L ethylene on lettuce seed germination.

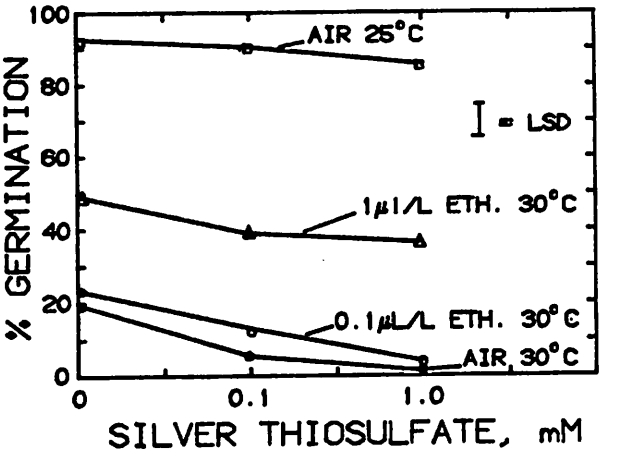

Fig. 2. Inhibition of lettuce seed germination by STS and a partial reversal of this inhibition by ethylene.

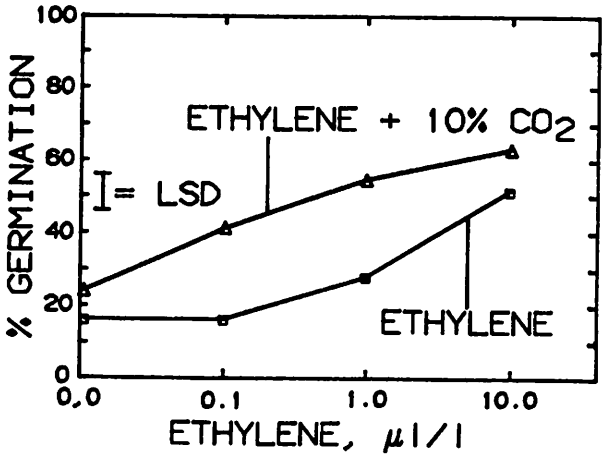

Fig. 3. The enhancement of ethylene action on lettuce seed germination by CO_2.

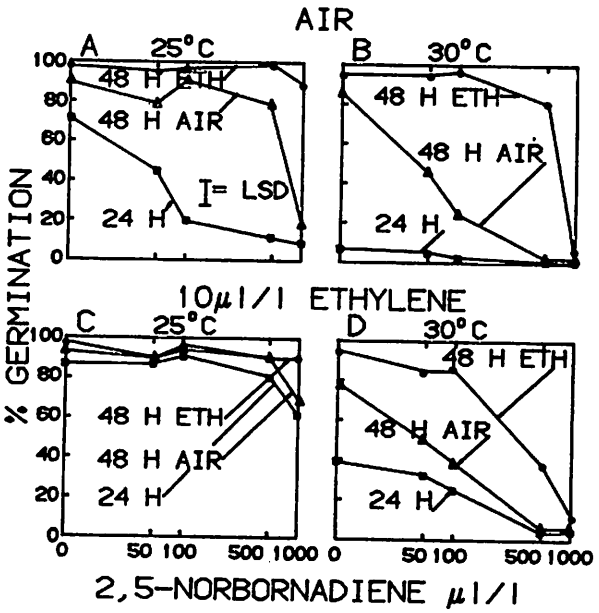

Fig. 4. Reversal of the inhibitory effect of NBA on lettuce seed germination by ethylene. For the first 24 hr, (24 H), seeds were treated with NBA at 25°C or 30°C in air (A, B) or with 10 ul/L ethylene (C, D). After 24 hr, NBA was flushed out. Germination was then measured again after an additional 24 hr incubation in either air (48 H AIR) or 10 ul/L ethylene (48 H ETH).

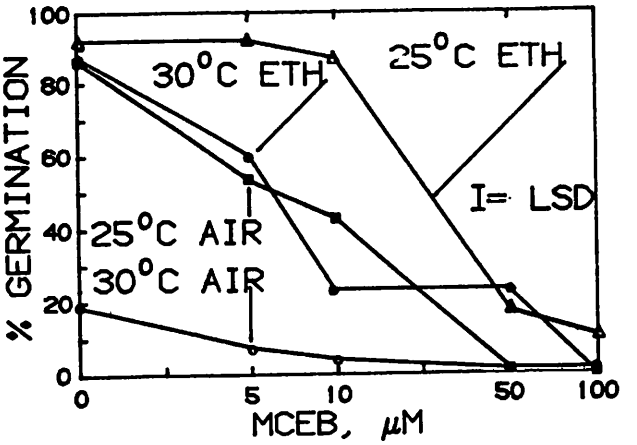

Fig. 5. Effect of a combination of various concentrations of MCEB and 10 ul/L ethylene on lettuce seed germination.

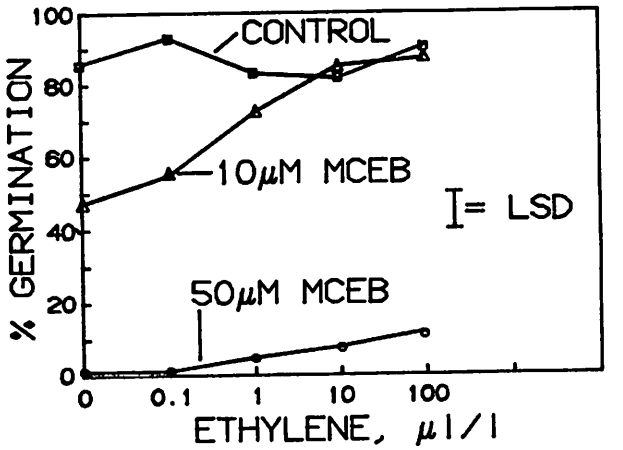

Fig. 6. Reversal of the inhibitory effect of MCEB on lettuce seed germination by various concentrations of ethylene.

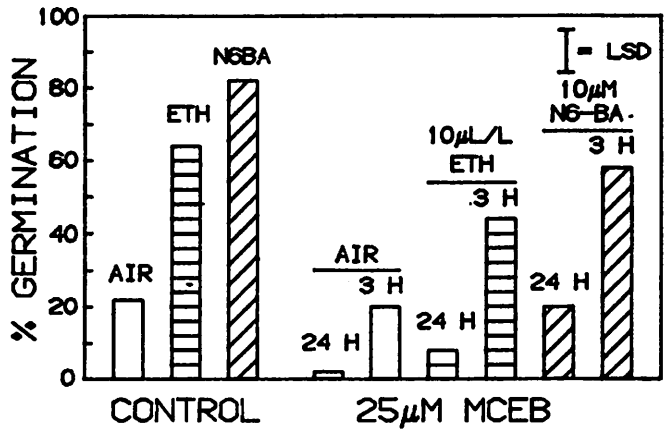

Fig. 7. Effect of 10 ul/L ethylene and 10 uM N6-BA on seeds exposed to a pretreatment with 25 uM MCEB. Seeds were exposed to 30°C during the course of this experiment. The three treatments on the left hand side of the figure represent control seeds not treated with MCEB. In the remaining treatments, MCEB was present for either 3 hr or 24 hr. In the case of 3 hr treatments, the seeds were transferred to filter paper saturated with water or N6-BA after 3 hr.