J Plant Growth Regul (2005) 23:307–312
DOI: 10.1007/s00344-004-0011-9

J P G R
Journal of Plant Growth Regulation

The Effect of the Ethylene Action Inhibitor 1-Cyclopropenylmethyl Butyl Ether on Early Plant Growth

Saleema Saleh-Lakha,[1] Varvara P. Grichko,[2] Edward C. Sisler,[2] and Bernard R. Glick[1]

[1]*Department of Biology, University of Waterloo, N2L 3G1, Waterloo, Ontario, Canada;* [2]*Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695, USA*

Abstract

Increased levels of ethylene in plants are responsible for many deleterious effects such as early senescence, fruit deterioration and inhibition of root elongation. Several cyclopropene derivatives have previously been studied as inhibitors of ethylene action in plants. This study focuses on one such compound, 1-cyclopropenylmethyl butyl ether and its effect on the growth of roots and shoots of canola plants as well as rooting of mung bean seedlings. 1-cyclopropenylmethyl butyl ether increased root length in canola plants, but had no significant effect on shoot length. In rooting studies, mung bean seedlings treated with 1-cyclopropenylmethyl butyl ether prior to root excision had fewer numbers of roots than control plants that were not treated with the ethylene action inhibitor. The same rooting study, when repeated in the presence of 1-amino-cyclopropane-l-carboxylic acid (ACC), demonstrated an overall increase in the number of roots of inhibitor-treated and non-treated plants, however, the inhibitor was still effective in decreasing the number of roots, compared to its non-treated counterpart.

Key words: Ethylene; 1-MCP; Inhibitor; Mung bean; Rooting; Canola; Cuttings; ACC

Introduction

Ethylene modulates a wide range of plant responses and developmental steps. It is involved in seed germination, tissue differentiation, formation of root and shoot primordia, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, fruit ripening and degreening, production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission and the response of plants to biotic and abiotic stresses (Abeles and others 1992; Arshad and Frankenberger 2002; van Loon and Glick 2004).

Stress ethylene, an increased level of ethylene produced in response to temperature stress, pathogen attack, chemicals, insect damage, salt and water stress, can cause a variety of symptoms in plants such as epinastic curvature, leaf abscission, inhibition of root growth, formation of aerenchyma and the onset of senescence. High levels of ethylene are the primary cause of the rotting of fruits and

Received: 5 April 2004; accepted: 18 January 2005; Online publication: 7 April 2005
Corresponding author; e-mail: glick@sciborg.uwaterloo.ca

senescence of plants. At the same time lower levels of ethylene are required for cell wall strengthening, production of phytoalexins and the synthesis of defensive proteins (van Loon and Glick 2004).

Recently, cyclopropene and its derivatives have been found to be effective ethylene antagonists of ethylene action that often act at very low concentrations. When added simultaneously with ethylene, these cyclopropene compounds compete with ethylene for binding sites. However, when the cyclopropene compounds are allowed to bind before ethylene is applied, even a subsequent addition of very high concentrations of ethylene cannot remove the bound cyclopropene compound from the ethylene binding site (Sisler and others 2001, 2003; Feng and others 2004).

Specifically, 1-methylcyclopropene (1-MCP) is a very effective blocking agent for the ethylene receptor, inhibiting ethylene responses for long periods of time. 1-MCP has been shown to inhibit the effects of exogenous ethylene, such as bud abscission and flower wilting in potted plants of *Campanula carpatica* (Serek and Sisler 2001). In addition, pre-treatment of phlox flowers with 1-MCP and subsequent exposure to ethylene significantly inhibited ethylene-induced flower abscission and hence improved their shelf life (Porat and others 1995). An analog of 1-MCP, namely 3,3-dimethylcyclopropene is also active, however, requires about 1000 times the treatment concentration and protects for only 7 days (Sisler and others 1999). The compound 3-methylcyclopropene (3-MCP), another analog of 1-MCP, also binds to the ethylene receptor and blocks it for several days, however, concentration-wise, it is much less effective than 1-MCP (Sisler and others 1999). This suggests that both steric and/or electronic effects may be important for antagonist action; with 3-MCP, the methyl group is further away from the double bond, whereas in 1-MCP, the methyl group is directly adjacent to the double bond. An added chain just adjacent to the double bond may allow for more rapid binding of 1-MCP to the receptor than in the case of 3-MCP (Sisler and others 1999).

The compound 1-MCP is an effective ethylene action blocker and has been used commercially as a means of delaying senescence of ornamental and horticultural crops (Watkins and Miller 2003); however, it is limited in its use in agriculture. Hence, new compounds that are analogs of 1-MCP, and also function in blocking ethylene action, are being developed. The work reported here focuses on the effects of 1-cyclopropenylmethyl butyl ether, a derivative of 1-MCP, on growth promotion of canola seedlings as well as on the rooting of mung bean cuttings.

Materials and Methods

Synthesis of 1-Cyclopropenylmethyl Butyl Ether

Propargyl butyl ether was first prepared by the direct alkylation of propargyl alcohol with 1-bromobutane in the presence of powdered potassium hydroxide in cyclohexanone (Vartanian and others 1974). Following this reaction, 2-bromo-2-propenyl butyl ether was synthesized by direct hydrobromination of propargyl butyl ether with tetraethylammonium hydrogen dibromide in dichloromethane (Cousseau 1980). Finally, dibromocarbene was added to 2-bromo-2-propenyl butyl ether under phase transfer conditions to produce 1-(1,2,2-tribromocyclopropyl)methyl butyl ether. Subsequent reaction with an excess amount of methyl lithium under nitrogen yielded 1-lithiocyclopropyl methyl butyl ether (Al Dulayymi and others 1996). The solvent, 1-lithiocyclopropyl methyl butyl ether was then removed under vacuum and stored at −20°C. Prior to treatment of plant material, 5 mL of diethyl ether were added with a syringe through the rubber stopper of a closed tube containing 1 g of 1-lithiocyclopropyl methyl butyl ether. At the same time, a 60 mL syringe was inserted into the tube to relieve the pressure that built up during the reaction. Upon addition of diethyl ether, the tube was mixed on ice. Five mL of water were added drop by drop to the tube on ice to quantitatively convert 1-lithiocyclopropenylmethyl butyl ether to 1-cyclopropenylmethyl butyl ether. The extremely rapid and exothermic reaction was allowed to proceed to completion for approximately 5 min after which an aqueous as well as an organic layer formed. The tube was placed in a dry ice/acetone bath to freeze the aqueous layer, and the organic layer was extracted with a syringe and placed in a glass bottle. The volume was made up to 5 mL with diethyl ether and the concentration of the 1-cyclopropenylmethyl butyl ether determined by GC analysis.

GC Analysis

A Varian 2700 gas chromatograph with an FID and a 5 foot, 2-mm packed glass column was used for the determination of the 1-cyclopropenylmethyl butyl ether concentration in ether solution by comparison of the 1-cyclopropenylmethyl butyl ether peak area with the peak area of the standard. The column was packed with 10% SE-30, 80/100 packing material (Supelco, Park Bellefonte, PA). The flow rate of nitrogen carrier gas was 2 mL/min during the run and increased to 10 mL/min

between the runs to clean the column. To ascertain a separation of the obtained products, the oven was operated in a programmed temperature range, in order to elute the low boilers in a well-spaced pattern and the high boilers in a reasonable period of time. The temperature settings for the oven were as follows: 30°C (5 min), 4°C/min to 80°C, and 10°C/min to 200°C.

Growth Pouch Assay

The protocol for assessing the effect of 1-cyclopropenylmethyl butyl ether on the elongation of canola seedling roots followed the method described by Lifshitz and others (1987).

Canola seeds (*Brassica rapa* cv. Reward, kindly provided by Dr. Gerry Brown, Agrium Inc., Saskatoon, Saskatchewan, Canada) stored at 4°C, were surface sterilized by soaking them in a 1.5% sodium hypochlorite solution (bleach) for 10 min in polystyrene Petri dishes (100 × 15 mm; Falcon). The seeds were subsequently rinsed with sterile distilled water five times to remove any residual traces of bleach. The seeds were then incubated in 15 mL of sterile distilled water for 1 h at room temperature, to initiate seed imbibition. Seeds were placed in sealed jars containing 1-cyclopropenylmethyl butyl ether, doused onto a filter paper to yield varying gas phase concentrations of 106 μL/L, 212 μL/L, 425 μL/L and 637 μL/L. These jars were then incubated at room temperature for 18 hours. Six seeds with the same treatment were placed in each sterile growth pouch (Mega International, Minneapolis, MN, USA), which had previously been filled with 10 mL distilled water and autoclaved. For each treatment, ten pouches were prepared and placed upright in racks, with two empty pouches at each end of a row. The plastic box containing the growth pouches was filled with 3 cm of distilled water and loosely covered with Saran Wrap™. Pouches were incubated in a growth chamber at 20°C with a light/dark cycle beginning with 12 h of dark, followed by 12 h of light (18 μmol photons/m^2/sec). After five days, primary root lengths, as well as shoot lengths were measured and analyzed with a one-way analysis of variance (ANOVA) using Instat™ version 2.01 for Macintosh. Seeds that failed to germinate in two days after sowing were marked and roots that subsequently developed from these seeds were not measured.

Rooting Assay

The effect of 1-cyclopropenylmethyl butyl ether on the development of adventitious roots on mung bean (*Vigna radiata*, purchased from Ontario Seed Company, Waterloo, Ontario, Canada) cuttings was measured as described by Mayak and others (1999). Mung bean seeds were washed (first in bleach, then in water, similar to the canola seeds in the growth pouch assay described above), imbibed in water for one hour and then planted in sterile vermiculite. Incubation was in a growth chamber at 20°C with a light/dark cycle beginning with 12 h of dark, followed by 12 h of light (18 μmol photons/m^2/sec) for seven days. Twenty seedlings were then excised at the vermiculite surface and incubated at 20°C in the growth chamber with the same light/dark cycle conditions as above, in a closed jar containing 1-cyclopropenylmethyl butyl ether, doused onto a filter paper to yield a gas phase concentration of 425 μL/L. Twenty additional mung bean seedlings with the root system still attached were treated in a similar manner with the same compound. The seedlings were left at room temperature for 18 hours. The following day, the roots were excised from the second batch of seedlings. Both sets of explants were immersed in either 3 mL of distilled water, or 3 mL of 5×10^{-5} M ACC solution, in a 5 mL glass culture tube. The tubes were covered with Saran Wrap™, placed in a tray containing 3 cm of distilled water and incubated in a growth chamber at 20°C with a light/dark cycle beginning with 12 h of dark, followed by 12 h of light (18 μmol photons/m^2/sec). On the eighth day following the end of the chemical treatment, root lengths and root numbers were measured for each treatment, and analyzed using a one-way analysis of variance (ANOVA) using Instat™ version 2.01 for Macintosh. The mean difference of the treatments was considered to be significant at the level of $P = 0.05$

Results and Discussion

Effect of 1-Cyclopropenylmethyl Butyl Ether on Canola Seedlings

Canola seeds treated with 1-cyclopropenylmethyl butyl ether demonstrated a small (15%–26%), but statistically significant increase in root length compared to untreated plants (Figure 1A). Often, when seeds are imbibed, the breaking of dormancy and subsequent initiation of germination requires a burst of ethylene, however, a continued high level of ethylene actually impedes root growth (Glick and others 1999). Hence, a chemical such as 1-cyclopropenylmethyl butyl ether that prevents ethylene from binding to its receptor can offset the inhibitory effects of ethylene on root growth. Therefore, an

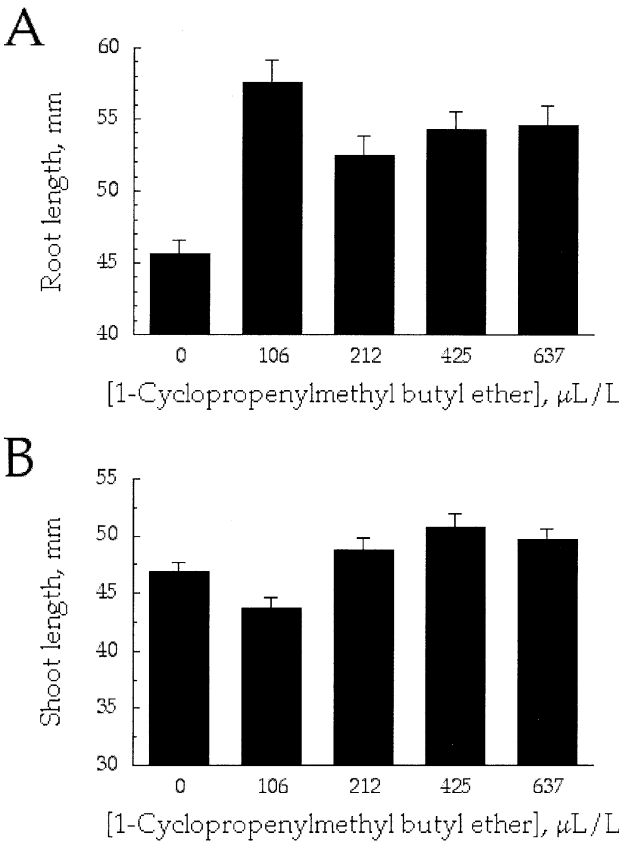

Figure 1. Effect of 1-cyclopropenylmethyl butyl ether on canola root (A) and shoot (B) length. The data shown is the mean root or shoot length ± SE. A typical experiment is shown where 60 seedlings were examined. The entire experiment was repeated three times.

increase in root growth is observed when seeds are treated with the 1-MCP analog, 1-cyclopropenylmethyl butyl ether. Because an increase in the concentration of 1-cyclopropenylmethyl butyl ether above 106 μL/L did not yield any additional growth stimulation, most probably the existing ethylene binding sites had already been saturated by 1-cyclopropenyl butyl ether. Moreover, the higher inhibitor concentrations that were employed did not cause any growth inhibition. This is consistent with a previous observation in which it was found that 0.7 nL/L of 1-MCP inhibited ethylene catalyzed senescence in banana plants (Sisler and others 2001). No significant difference was observed in shoot lengths of treated canola seedlings upon application of 1-cyclopropenylmethyl butyl ether (Figure 1B). This is consistent with what is observed when ethylene production is inhibited either chemically with aminoethoxyvinylglycine (AVG) (Penrose and others 2001), or biologically with plant growth promoting bacteria that contain the enzyme ACC deaminase (Saleh and others 2001).

Effect of 1-Cyclopropenylmethyl Butyl Ether on Rooting of Mung Bean Cuttings

In addition to inhibiting the length of plant roots, ethylene is thought to be involved in the formation of root primordia (Glick and others 1999). Thus, it might be expected that treatment of cuttings with 1-cyclopropenylmethyl butyl ether would result in a decrease in the number of adventitious roots formed. Figure 2A summarizes the effect of treating mung bean seedlings with 1-cyclopropenylmethyl butyl ether prior to root excision (at which point they become cuttings) on the number and length of adventitious roots that formed eight days after the treatment. The most notable result is that upon application of 1-cyclopropenylmethyl butyl ether prior to cutting the mung bean seedling, a significantly smaller number of very short roots (<1 mm) are observed, compared to the control. Consistent with previous experiments in which mung bean cuttings were treated with various plant growth-promoting bacteria, all of which synthesized indoleacetic acid and some of which lowered ethylene levels through the action of the enzyme ACC deaminase (Mayak and others 1999), cuttings treated with 1-cycloprepenylmethyl butyl ether had fewer roots than cuttings not treated with this ethylene action inhibitor. When 1-cyclopropenylmethyl butyl ether was present, it presumably bound to the ethylene receptor sites, and prevented ethylene from binding. Hence, ethylene activated physiological responses such as root initiation and inhibition of root elongation, are decreased in magnitude. Thus, with plants treated with 1-cyclopropenylmethyl butyl ether, a smaller number of roots were observed as compared to the control, when no inhibitor was added. However, when 1-cyclopropenylmethyl butyl ether was added after the roots of mung bean seedlings were excised, no significant inhibitory effect on the number of adventitious roots was observed (data not shown). This could reflect the fact that the root excision produces a wound, which initiates the production of stress ethylene. This ethylene could then bind to the receptors immediately, before 1-cyclopropenylmethyl butyl ether is added. When this inhibitor is added after excision, it cannot overcome the effect of ethylene, and hence does not alter the number of adventitious roots presumably initiated by ethylene. When 1-cyclopropenylmethyl butyl ether is added prior to root excision, the compound saturates the ethylene binding sites before stress ethylene is produced. Once bound, the inhibitor does not compete with ethylene any longer. A similar mode of action has also been observed in the case of the inhibitor 1-

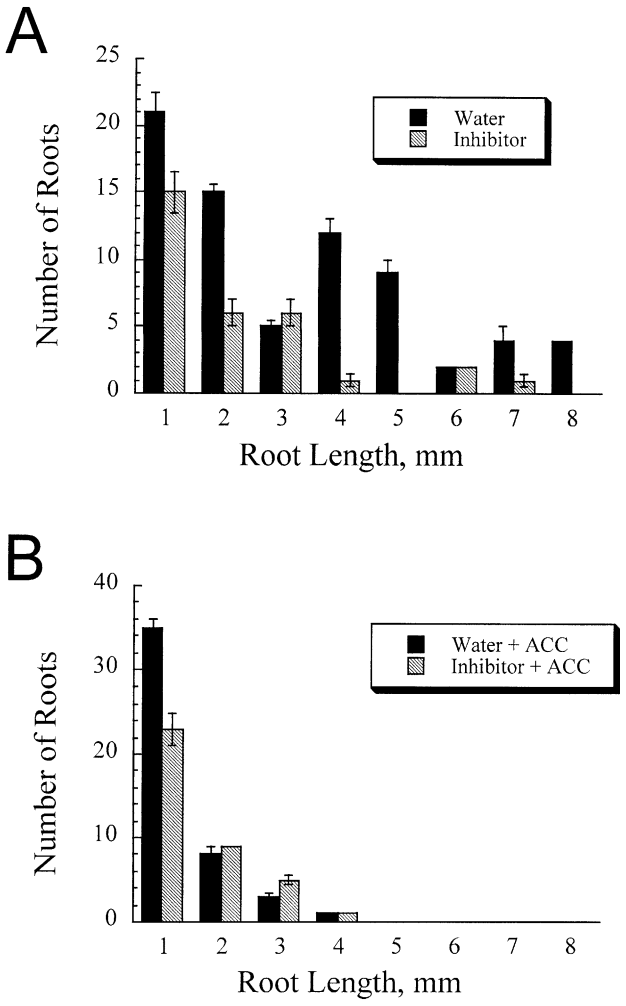

Figure 2:. Effect of the ethylene inhibitor, 1-cyclopropenylmethyl butyl ether on the number of adventitious roots that developed on ten mung bean cuttings after eight days of incubation . (A) The plants were either not treated with inhibitor (Water) or treated with Inhibitor (425 μL/L) before the root system was detached (Inhibitor). (B) Plants were immersed in 5×10^{-5} M ACC after being treated with Water or Inhibitor, as above. The data shown is the mean number of roots per ten cuttings ± SE. A typical experiment is shown here where twenty cuttings were examined. The entire experiment was repeated twice.

MCP (Sisler and others 1999, 2001). Interestingly, despite the fact that a relatively high concentration of inhibitor was employed in the present study, and unlike what was observed with plant growth-promoting bacteria, the decrease in the number of small adventitious roots was not accompanied by an increase in the number of larger roots. This probably reflects the fact that in this case, ethylene action is inhibited and plant auxin levels remain low. While with plant growth-promoting bacteria, ethylene is decreased while growth-stimulatory auxin is provided by the bacteria.

The experiment whose results are summarized in Figure 2A was repeated in the presence of 5×10^{-5} M ACC (Figure 2B). As a consequence of the addition of ACC, which is an immediate precursor of ethylene, a significant increase in the number of smaller roots was seen, both in inhibitor treated and non-treated plants. This presumably reflects an increase in the ethylene level, and its effect on both the initiation of adventitious roots and the elongation of these roots. Notwithstanding the higher level of ACC and hence ethylene, the inhibitor remained effective at decreasing the number of adventitious roots. Although it is unclear whether the compound 1-cyclopropenylmethyl butyl ether will ultimately be used commercially, this inhibitor should find use in experiments designed to understand and elaborate the mechanism(s) of ethylene action, including the formation of nodules by *Rhizobia* spp. (Ma and others 2001) and the role of ethylene in induced systemic resistance (van Loon and Glick 2004).

ACKNOWLEDGEMENTS

This work was supported by grants awarded to B.R.Glick by the Natural Science and Engineering Research Council of Canada (NSERC) as well as support from the United States - Israel Binational Agricultural Research and Development Fund to E.C.Sisler

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