LOW-TEMPERATURE SCANNING ELECTRON MICROSCOPE STUDIES OF STOMATAL RESPONSES IN SNAP BEAN PLANTS TREATED WITH OZONE AND ETHYLENEDIUREA

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出版情報：BIOTRONICS. 28, pp.45-53, 1999-12. Biotron Institute, Kyushu University
バージョン：
権利関係：

BIOTRONICS **28**, 45–53, 1999

LOW-TEMPERATURE SCANNING ELECTRON MICROSCOPE STUDIES OF STOMATAL RESPONSES IN SNAP BEAN PLANTS TREATED WITH OZONE AND ETHYLENEDIUREA

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(Received; June 14, 1999; accepted July 21, 1999)

AGRAWAL, S. B. and AGRAWAL, M. *Low-temperature scanning electron microscopic studies of stomatal responses in snap bean plants treated with ozone and ethylenediurea.* BIOTRONICS **28**, 45–53. Pretreatment of plants with ethylenediurea (EDU, N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N′-phenylurea) protects plants from foliar injury caused by ozone (O_3), but the fundamental mechanisms underlying this protection are poorly understood. A low-temperature scanning electron microscope (LtSEM) study revealed that following O_3 treatment, plants treated with EDU as a soil drench 48 h prior to O_3 exposure exhibited greater numbers of stomata with closed apertures than those exposed to O_3 without EDU pretreatment. Stomatal resistance was increased significantly by O_3 in control and EDU-treated plants; however, this increase was maximal in plants pretreated with EDU. Photosynthetic rate was reduced significantly in EDU pretreated plants exposed to O_3 than treated with O_3 only. These results suggest that EDU increased stomatal closure during O_3 exposure, and thereby decreased O_3 uptake and toxicity.

Key words: LtSEM, Stomatal response, EDU, Ozone, Snap bean

INTRODUCTION

Ozone (O_3), and the oxyradicals production and its dissolution in the leaf apoplast, are potentially phytotoxic among the most damaging tissue toxicants (*17, 19, 28*). Ozone can cause leaf injury, stomatal damage, premature senescence, decreased photosynthetic activity, changes in enzyme activity and membrane permeability, reduced growth and lower yields in sensitive plant species (*1, 2, 13, 20*). The experimantal antiozonant, EDU (N-[2-(2-oxy-1-imidazolidinyl) ethyl]-N′-phenylurea) provides protection against visible O_3 injury in a number of plant sepcies when applied as an aqueous solution to the

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foliage or to roots (*6–8, 11, 18, 21, 27*). Lee and Bennett (*21*) correlated the protective effect of EDU with higher levels of superoxide dismutase (SOD) and catalase (CAT) in the trifoliate leaf of snap bean plants. However, other authors have detected no effects of EDU on SOD activity (*29*), on SOD, guiacol–peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) activities (*25*) despite EDU induced reduction of the injury due to O_3 (*9, 25*).

Stomata are recognized as important modifiers of plant responses to O_3 (*12, 35*), since the flux of the pollutant to the leaf interior is predominantly controlled by stomatal conductance (*36*). Engle and Gabelman (*10*) reported that certain cultivars of onion (*Allium cepa* L.) owe their O_3 resistance to rapid stomatal closure. Closure of stomata due to O_3 exposure was suggested as an important resistance mechanism in snap bean (*Phaseolus vulgaris* L.) (*5*), radish (*Raphanus sativus* L. cv. Baladey), turnip (*Brassica rapa* L. cv. Sultani) (*12*) and *Plantago major* (*31*). However, Bennett *et al.* (*3, 4*) found that EDU did not induce closure of stomata in snap bean plants; suggesting that the antiozonant activity of EDU is biochemical rather than biophysical in nature.

In the present investigation, our main objective was to determine the stomatal response of *Phaseolus vulgaris* L. plants (cv. BBL–290) to O_3 alone and in combination with EDU using a Low–temperature Scanning Electron Microscopy (LtSEM) and leaf gas exchange measurements.

MATERIALS AND MATHODS

Plant Propagation

Snapbean plants (*Phaseolus vulgaris* L., Bush Blue Lake–290) were grown in a glasshouse supplied with charcoal–filtered air (CFA) to remove any air contamination, in 13–cm diameter plastic pots containing Jiffy Mix potting mixture (Jiffy Products of America, Inc., W. Chicago, IL, USA). After the emergence of seedlings, plants were thinned to one per pot, and fertilized weekly with Peter's liquid fertilizer (20–20–20) according to the manufactures instructions (Peter's Fertilizer Products, W. R. Grace & Co., Fogelsville, PA, USA).

Chemical and fumigation treatment

EDU solutions were prepared in deionized water and were applied 48 h prior to fumigation as a soil drench (500 ppm EDU, 100 ml/pot) to one set of 20 pots Another set of 20 plants received only 100 ml of deionized water. EDU treatments were administered when the first trifoliate leaf was fully expanded (at 3 weeks of age).

All EDU–treated and untreated plants were placed in controlled environment chamber at 25℃ for 2 h before O_3 fumigation. The relative humidity in the chamber was measured at 70–80% and the PPFD was 350 μmol $m^{-2}s^{-1}$. After a 2 h equilibration period, 10 plants of each group (EDU treated and untreated) were removed and exposed to 599 μg m^{-3} O_3 for 3 h. Ozone was generated by passing pure O_2 through a high voltage electric discharge ozonizer (O_3 generator Model 500M, Fischer Labor–und Verfahrenstechnik, Germany). The concentra-

tion of the gas was monitored with a chemiluminescent O_3 analyzer (Bendix Corp., Ronceverte, WV, USA) which was calibrated with a Dasibi Model 1003 PC O_3 calibrator (Dasibi Env. Corp., Glendale, CA, USA). The experiment was repeated after two days with similar conditions.

Low-temperature scanning electron microscope studies

Five samples from five plants in different treaments for cryofixation (thin strips of leaves) were rapidly cut from the central region of the first trifoliate leaf mounted on stubs using tissue tek (methyl cellulose), and placed directly in a container filled with liquid nitrogen. Samples were eventually transferred to the Scanning Electron Microscope (2000 A sublimation, staged freeze edge at -80℃, then re-cooled and sputter coated for 6 min, with Au 25 μA at CR #106 count) fitted with a cryostage (Model S-530 Hitachi, Japan). Ten photographs were taken (Polaroid) from each sample to determine stomatal numbers and appearance. This method (Lt SEM) minimizes the possibility of changes in stomatal response due to chemical action of 4% glutaraldehyde used as a fixative in conventional scanning electron microscopy.

Leaf gas exchange measurements

Stomatal resistance and net CO_2 uptake were measured before and after exposure to O_3 using a LI-6200 photosynthetic system (LI-COR Inc., Lincoln, NE, USA).

Statistical analysis

Data were subjected to analysis of variance. Duncan's Multiple Range Test was also used to test the significance of difference between means. All statistical analyses were performed using SPSS (*34*).

RESULTS

Scanning electron micrographs of stomata of control (CFA) snapbean plants show that epidermal cells are turgid and that exposure to 599 μg m^{-3} O_3 for 3 h resulted in stomatal closure, loss of turgor, and collapse of guard cells. The symptom of O_3 injury was water-logged flecks on the adaxial leaf surface, which became apparent 1 to 3 h after fumigation. Treatment with 500 ppm EDU and 599 μg m^{-3} O_3 for 3 h caused stomatal closure and loss of turgor but no collapse of guard and epidermal cells adjacent to the stomata. Photographs of stomata under lower and higher magnifications seen under LtSEM are depicted in Figs. 1 & 2.

In control, the percentage of stomata with open aperture were maximum i.e. 60.9 (Table 1). Snap bean plants pretreated with EDU showed no significant difference in stomatal resistance when compared with control plants (Table 2). However, pretreatment with EDU was shown to slightly increase the number of stomata with closed apertures (Table 1). EDU application 48 h prior to O_3 exposure caused greater closure of stomata than O_3 treatment without EDU-

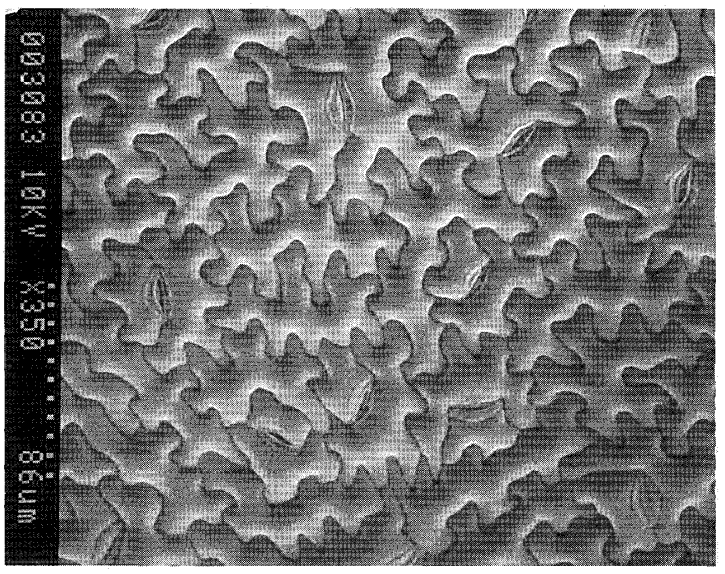

Fig. 1. Stomata at lower magnification×250

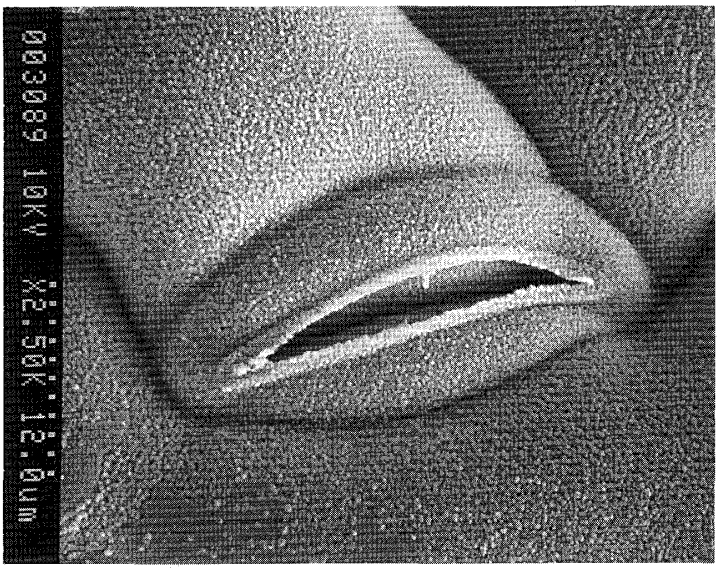

Fig. 2. Enlarged view of a stomata×2500

pretreatment. The percentage closed stomata were 39.1, 45.7, 55.3 and 66.7, respectively in control, EDU-treated, O_3-exposed and EDU+O_3-exposed snap bean plants (Table 1). Thus, EDU pretreatment, in combination with O_3 reduced stomatal opening to a greater extent than all other treatments.

Photosynthetic rate was reduced significantly in plants pretreated with EDU and then fumigated with O_3 and O_3-treated plants as compared to untreated controlled plants. The percentages of decrease in comparison to control values for photosynthetic rate were 20.2 and 4.2 for O_3-exposed and EDU-treated+O_3 exposed plants, respectively (Table 2). Photosynthetic rate, however, increased by 13.9% in EDU treated plants as compared to control.

Table 1. Effects of EDU and O_3 alone and in combination on the number of open and closed stomata in snap bean plants (Mean±S. E.) observed with low-temperature Scanning Electron Microscopy.

Treatment	Total No. of Stomata ($0.08\ mm^{-2}$)	No. of Stomata with open aperture	No. of Stomata with closed aperture
Control	14.6 ± 0.77^b	8.9 ± 0.95^b (60.9)	5.7 ± 1.05^a (39.1)
EDU only	12.9 ± 0.33^a	7.0 ± 0.50^b (54.3)	5.9 ± 0.64^a (45.7)
O_3 only	13.2 ± 0.72^c	5.8 ± 0.74^c (44.7)	7.4 ± 0.82^c (55.3)
EDU+O_3	12.6 ± 0.74^a	4.2 ± 0.44^a (33.3)	8.4 ± 0.76^b (66.7)

Data in parentheses indicate % values of open and closed apertures.
Values within each column followed by the same letter are not significantly different at 0.05 of level significance using Duncan's Multiple Range Test.

Table 2. Effects of EDU and O_3 alone and in combination on the rate of photosynthesis and stomatal resistance (Rs) in snap bean plants (Mean of 3 replicates±S. E.)

Treatment	Photosynthesis (m mol $m^{-2}s^{-1}$)	Rs (scm^{-1})
Control	7.74 ± 0.06^b	1.53 ± 0.03^a
EDU only	8.82 ± 0.21^c (+13.95)	1.80 ± 0.19^a (17.64)
O_3 only	4.17 ± 0.08^a (−20.28)	4.13 ± 0.13^b (169.9)
EDU+O_3	7.41 ± 0.18^b (−4.26)	6.52 ± 0.12^c (326.14)

Data in parentheses indicate % change relative to control.
Means in the same column bearing the same subscript are not significantly different at 5% level.

Stomatal resistance increased in plants exposed to O_3. The increase was maximal for plants pretreated with EDU and then exposed to O_3. Percentages of increase over control values were 169.9 and 326.1 for plants exposed to O_3 and for plants pretreated with EDU and then exposed to O_3, respectively (Table 2).

DISCUSSION

Ethylenediurea has been shown to be an effective method of protecting plants against O_3 and oxidative stress (*4, 6–9, 16, 21, 24*). However, the mechanism of EDU–induced O_3 resistance in plants is still not clearly defined. Bennett *et al.* (*3*), Lee and Bennett (*21*) reported that the mechanism of EDU action is biochemical rather than biophysical in nature. No change in stomatal conductance was observed when EDU treated snapbean plants were exposed to O_3 (*3, 11*) indicating that EDU application did not cause changes in the doses of O_3 observed by individual leaves.

In the present study, stomatal resistance increased and photosynthetic rate reduced in terminal first trifoliate leaves of snap bean plants in response to O_3 exposure, and stomatal resistance was greater in the EDU pretreated plants while photosynthetic rate was slightly reduced. The data in Tables 1 and 2 are consistent with respect to % stomata with open apertures and stomata with closed apertures and stomatal resistance. Resistance to O_3 injury has often been corrected with stomatal closure (*13*). Depression of photosynthesis and increase in stomatal closure have occurred simultaneously in O_3 treated oats (*15*). However, in our study also, O_3 enhanced stomatal resistance with significant affect on photosynthesis. Interestingly, increase in stomatal resistance was more apparent in EDU pretreated plants and might have resulted in reduced injury to cellular processes. This suggests that biophysical processes are also involved and the protective effect of EDU against O_3 injury may be associated with stomatal closure during O_3 stress. This finding contradicts the statement that the action of EDU is only biochemical (*21*).

Miller *et al.* (*27*) found greater non–carbon exchange rate (NCER) in plants pretreated with EDU and exposed to O_3, whereas EDU pretreatment reduced NCER in carbon filter treatments. However, stomatal conductance increased in plants due to EDU treatment and O_3 exposure of snap bean plants (*27*). Our results slightly contradict this report but the former is a field study and the latter is a glass house study having different microenvironmental conditions. Raschke (*30*) also reported that decreased photosynthesis in the mesophyll probably leads to an accumulation of CO_2 in intercellular spaces, and this could increase stomatal closure. The aperture of the stomate is governed by a variety of environmental signals, including light level and quality, concentration of CO_2, air humidity, and leaf/soil water status (*14*). In addition, the activity of growth substances such as abscisic acid and cytokinin affects stomatal function (*14, 26*). Runeckles and Resh (*32*) reported that acute and chronic O_3 injury can be suppressed by the application of cytokinin to bean plants. They suggested that cytokinin retarded senescence and thereby modified the O_3 response of treated tissues. Lee and Chen (*23*) reported that EDU has cytokinin–like activity in a tobacco callus bioassay, and that EDU also retarded senescence in plants (*22*). Similarities of chemical structure between cytokinin and EDU help to rationalize the similarities of their effects in controlling stomatal behavior. Our observation of SEM studies suggested that control of aperture of stomata by EDU could lead

to protection of plants against O_3 injury.

Our current paper provides new evidence that the protective effects of EDU against O_3 leaf injury are associated with stomatal closure. Hence, such protection probably involves biophysical as well as biochemical effects. More work is required to reveal the exact mechanisms involved for the protective action of EDU.

ACKNOWLEDGEMENTS

Senior author (SBA) is thankful to the University Grants Commission, New Delhi for financial assistance to work with Drs. E. H. Lee at the Climate Stress Lab, and W. P. Wergin at Electron Microscopy Lab., USDA, Beltsville, MD 20705. We are particularly grateful to Eric Erbe (E. M. laboratory, USDA/ARS, Beltsville, MD, U.S.A) for help provided in LtSEM work. This work was supported in part by a Fulbright Foundation for Visiting Scholars to Dr. M. Agrawal. We would also like to thank Profs. J. N. B. Bell (U.K.), J. Barnes (U.K.) and Prof. R. B. Lal, Director, Allahabad Agricultual Institute, Allahabad (India) for helpful suggestions and encouragements.

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