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Regulators of ethylene biosynthesis or activity as a tool for reducing susceptibility of host plant tissues to infection by Botrytis cinerea

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Abstract

Several compounds were tested for their ability to reduce development of grey mould on rose, tomato, pepper, eggplant, French bean and *Senecio* sp. Removal of ethylene from the atmosphere surrounding rose flowers, or leaves of tomato and pepper, by potassium permanganate, resulted in slower grey mould development. Inhibition of ethylene activity by 2,5-norbornadiene controlled disease on all crops but tomato. Carbon dioxide controlled grey mould on roses, but the potential for use of these agents is in doubt. Inhibitors of ethylene biosynthesis such as aminooxyacetic acid (AOA), cobalt ion, the uncoupler 2,4-dinitrophenol and the radical scavenger salicylic acid were differentially effective in controlling the disease in the various hosts. Fifty mM AOA reduced grey mould on rose flowers by up to 97% when flowers were partially aerated. AOA was not phytotoxic on the tested rose cvs Golden Times and Jaguar. Combinations of ethylene absorption, inhibition of ethylene activity and ethylene biosynthesis did not result in better control as compared with the disease reduction ability of the compounds alone, tested on the various hosts. Application of benzyladenine, which reduces the host responsiveness to ethylene, resulted in 39–99% grey mould reduction in rose flowers and in leaves of tomato and *Senecio* sp. but was not effective on pepper or eggplant. Manipulation of ethylene presence and of host plant susceptibility to grey mould is discussed.

Additional keywords: aminooxyacetic acid, benzyladenine, carbon dioxide, cobalt, 2,4-dinitrophenol, eggplant, French bean, 2,5-norbornadiene, potassium permanganate, pepper, (radical scavenger), rose, salicylic acid, *Senecio*, tomato, (uncoupler).

Introduction

Plant tissues react constantly to hormones and their physiological situation is controlled by these plant growth regulators (Goodman et al., 1986; Veen, 1987). Hormone biosynthesis, transport, metabolism and action as well as tissue sensitivity to the hormone are contributing factors to the hormonal homeostatic system of the tissue (Goodman et al., 1986; Veen, 1987).

Exogenous and endogenous ethylene plays a role in various physiological processes and is effective in shortening postharvest life and inducing senescence by triggering autocatalytic ethylene production. It is regulated in many plants by the induction of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the ethylene-forming enzyme (EFE) (Aharoni et al., 1979; Manning, 1985; Philosoph-Hadas et al., 1989).

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Blocking the binding sites of ethylene with various chemicals like silver ions or 2,5-norbornadiene (NBD) can prevent the autocatalytic production of ethylene and slow down senescence (Halevy and Kofranek, 1977; Staby et al., 1984; Sisler et al., 1985). Chemicals like benzyladenine can reduce the responsiveness of a tissue to ethylene (Veen, 1987). Carbon dioxide is a competitive inhibitor of ethylene activity (Burg and Burg, 1967; Sisler, 1979; Smith et al., 1985).

Ethylene production can be blocked by various compounds like aminooxyvinylglycine (AVG), cobalt ion (Yu and Yang, 1979), aminoxyacetic acid (AOA) (Yang and Hoffman, 1984; Elad, 1988a), salicylic acid (Leslie and Romani, 1986) and 2,4-dinitrophenol (DNP) (Yu et al., 1980; Apelbaum et al., 1981).

The removal of ethylene from air can also reduce its atmospheric concentration and thus minimize the effect of this plant hormone on susceptible tissue. Potassium permanganate was suggested as a potent ethylene remover from the atmosphere surrounding agricultural products (Saltveit, 1980; Granger and Rousselle, 1984).

Ethylene may have a promotive or an inhibitory effect on disease development. Promotion of disease development was demonstrated in systems like *Diplodia natalensis* on lemon (Barmore et al., 1976), *Helminthosporium sativum* on barley (Dehne et al., 1981), *Botrytis cinerea* on strawberry, tomato, cucumber, pepper, French bean and black currant (Dehne et al., 1981; Elad, 1988a; 1990; Barkai-Golan et al., 1989; McNicol et al., 1989) and *Colletotrichum lagenarium* on cucumber (Biles et al., 1990). On the other hand, a role of ethylene in disease resistance was demonstrated in various host–pathogen interactions (see reviews by Archer and Hislop, 1975; Boller, 1982).

Inhibitors of ethylene activity (silver thiosulfate) or production (AVG, AOA) and antagonism by CO_2 were reported in recent years to inhibit development of grey mould in flowers of carnation and rose and in leaves and fruits of vegetable crops (Elad, 1988a,b, 1990; Phillips et al., 1985).

The present study was carried out to evaluate physical and chemical methods to control production, accumulation or activity of ethylene as a tool to change the homeostatic equilibrium in the *B. cinerea* infected plant tissue in order to delay or prevent its susceptible reaction to the pathogen.

Materials and methods

Organisms and growth conditions. *Botrytis cinerea* Pers.: Fr. had been isolated from naturally infected flowers of rose (*Rosa hybrida* L.) (Elad, 1989). The fungus was maintained and grown on potato dextrose agar (PDA, Difco) and then tested on the following host plants: rose (cvs Mercedes, Golden Times, Jaguar, Ilseta, Diplomat, Florence and Lorena), bean (*Phaseolus vulgaris* L. cv. Brittle Wax), pepper (*Capsicum annuum* L. cv. Maor), tomato (*Lycopersicon esculentum* Mill. cv. VF 198), eggplant (*Solanum melongena* L. cv. Classic), and *Senecio* sp. Plants, leaves or flowers were (unless specified otherwise) inoculated by spraying conidial suspensions of *B. cinerea* (10^5/ml), supplemented with 0.1 M glucose and 0.07 M K_2HPO_4, except for rose inoculation.

Sclerotinia sclerotiorum (Lib.) de Bary was grown on PDA. In order to infest lettuce leaves (*Lactuca sativa* L. cv. Iceberg), a 1-week-old culture was chopped by an Ultraturax T25 homogenizer (Janke and Kunkel, IKA Labortechnik, Staufen, FRG) and suspended in water. Aliquots of 1.0 ml were spread on each leaf.

The inoculated plant material was incubated in boxes covered with polyethylene bags. Partial aeration was obtained by perforation of the polyethylene, thus reducing humidity in the incubation chamber and allowing escape of ethylene. Incubation was at 20 + 2 °C. A photoperiod of 12 h was maintained for the vegetable crops. Evaluation of disease was

carried out after 7–14 days of incubation. Disease severity was evaluated according to a scale of 0–5, where 0 = healthy plant and 5 = completely destroyed plant (Elad, 1988a, 1990).

Chemicals. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA): aminooxyacetic acid (AOA), 2,5-norbornadiene (NBD), 2,4-dinitrophenol (DNP), salicylic acid, $AgNO_3$, $Ca(NO_3)_2$ and $CoSO_4$. Silver thiosulfate (STS) was obtained from Asia Rizel, Ramat Gan, Israel. Different concentrations of these compounds were applied to plants by an atomizer operated on air pressure. Each flower or leaf received 0.2–0.1 ml of liquid, respectively, 1 h before inoculation.

Carbon dioxide from a pressure cylinder was introduced into an isolated container (20 × 40 × 60 cm) in which rose flowers were incubated.

Potassium permanganate, embedded on Al_2O_3 (Ethysorb, Stayfresh Ltd., UK), was put in cheese cloth (20 g/pack) for incubation in cartons (10 × 40 × 60 cm) into which naturally infected, but symptomless flower bunches were placed. The flowers were transferred after 4 days to humidity chambers to induce visible symptoms of grey mould.

Fungitoxicity tests. The compounds were added to PDA at rates of 0.1–10.0 mM in order to test any direct effect on the fungus. Mycelium disks from the growing margins of PDA cultures bearing mycelium of *B. cinerea* were inoculated on the amended media in order to test the effect of the chemicals on linear growth of the fungus. In addition, conidia of the fungus from 15-day-old cultures (10^6/ml) were inoculated in 20 µl drops onto plates amended with the chemicals, in order to test their effect on germination of the conidia. Germination was observed under a light microscope within 24 h after inoculation. All plates were incubated at 20 °C.

Ethylene determination. Five leaves (of 5–10 week old plants) were placed in a 32 ml glass test tube (25 mm diam. × 95 mm height) and the tube was closed with a rubber stopper. The leaves were incubated in the sealed containers for 2–5 h at 20 ± 2 °C. The ethylene present in gas samples removed from the containers was measured by a gas chromatograph (Gow-Mac Instrument Co., Series 750) fitted with a flame ionization detector and a 0.9 m glass column packed with alumina. The rate of ethylene production was expressed as nl g^{-1} fresh weight h^{-1}, using pure ethylene as a standard. There was no detectable ethylene in empty glass tubes.

Experimental design. Experiments were repeated three to six times, and included at least 10 replicates of each treatment. Results were statistically analysed by Duncan's Multiple Range Test ($P \leq 0.05$).

Percent disease reduction was calculated according to the formula (a-b) × 100/a, where a = disease level in the control and b = level of disease in a certain treatment.

Results

Effect of ethylene absorption. Naturally infected symptomless flowers of rose cv. Lorena were stored in boxes incubated at 4 °C in order to simulate transport conditions. Ethysorb was placed in the boxes before incubation; boxes containing flowers without ethysorb served as control. After 5 days in grey mould-conducive conditions, disease severity (index 0–5) on flowers and leaves was significantly reduced from 1.47 in untreated control to 0.57 and from 1.82 to 0.61, respectively. Similar results were obtained with rose cvs Golden Times, Diplomat, Ilseta, Mercedes and Florence. However, less

effectivity was obtained with other cultivars. Elongation of vase life of flowers by 1–3 days was observed with the respective cvs Mercedes, Golden Times, Lorena and Ilseta, whereas in other tested cultivars ethysorb did not affect vase life.

Leaves of tomato, pepper and *Senecio* sp. which were inoculated and then incubated with or without ethysorb in a humidity chamber composed of a box placed in a polyethylene bag (containing five leaves per litre volume). Levels of disease severity of control tomato, pepper and *Senecio* sp. leaves were 4.4, 3.45 and 3.15 (out of maximum index 5.0), respectively. The percent of grey mould reduction in ethysorb treatments was 38 and 62.5 (both significantly different, $P \leq 0.05$) and 24 (not significant) on tomato, pepper and *Senecio* sp., respectively.

Effect of inhibitors of ethylene production or activity on rose grey mould. AOA was sprayed on rose flowers (cvs Golden Times and Jaguar) at concentrations of 0, 5 and 50 mM and flowers were incubated under humid conditions with partial aeration or no aeration (Fig. 1). Under partial aeration, the disease developed at a lower rate (50–65% reduction) than under no aeration. Grey mould developed sometimes better at 15 °C than at 10 °C. The rate of reduction of severity of grey mould by AOA varied with the cultivar, temperature of incubation, practice of aeration and concentration. More than 97% reduction in severity of disease was observed in both cultivars at both incubation temperatures when partial aeration and 50 mM AOA were examined. Only partial control was obtained by 5 mM AOA at both incubation conditions (Fig. 1). The highest concentration of AOA was not phytotoxic to the flowers.

Further experiments were conducted to test whether the combinations of the above-mentioned treatments might improve the control of grey mould of rose flowers. A combination of ethysorb with AOA, DNP, $AgNO_3$ or STS and a combination of AOA with NBD did not result in improvement of grey mould control as compared with either treatment alone.

Carbon dioxide at 4% significantly reduced grey mould severity on rose flowers cv. Jaguar from index 1.4 (untreated control) down to index 0.37 and on cv. Mercedes from index 1.52 to 0.59.

Effect of inhibitory compounds on grey mould of various crops. NBD, CoSO4, DNP and salicylic acid were sprayed at rates of 0.1–10.0 mM on leaves of pepper, tomato, eggplant, bean and *Senecio* sp. and on flowers of rose (cv. Mercedes). The leaves were inoculated later by *B. cinerea* whereas the flowers were naturally infected (Table 1). The percent reduction of disease severity by each compound on each host varied considerably. The highest concentration of NBD and $CoSO_4$ was effective on rose and of NBD also on bean. All concentrations of NBD and $CoSO_4$ (0.1, 1.0 and 10.0 mM) had a significant effect on pepper and eggplant. DNP significantly inhibited the disease on pepper at both tested doses (0.1 and 1.0 mM), on tomato at the lower dose, and on bean and *Senecio* sp. at the higher concentration. Salicylic acid had a significant effect on pepper (0.1 mM) and tomato (1.0 mM). Benzyladenine significantly reduced disease on rose (1.0 mM) and on tomato and *Senecio* sp. (0.1 and 1.0 mM) (Table 1).

The combination of 1.0 mM of NBD and 1.0 mM of $CoSO_4$ was 30% and 25% better in reducing grey mould of pepper and eggplant, respectively, as compared with either treatment alone (not significant, $P \leq 0.05$). The combination of compounds did not result in an additive effect on other crops.

Ethylene production. Production of ethylene by leaves of tomato and pepper was tested after application of 1.0 mM of the compounds NBD, $CoSO_4$, DNP benzyladenine and

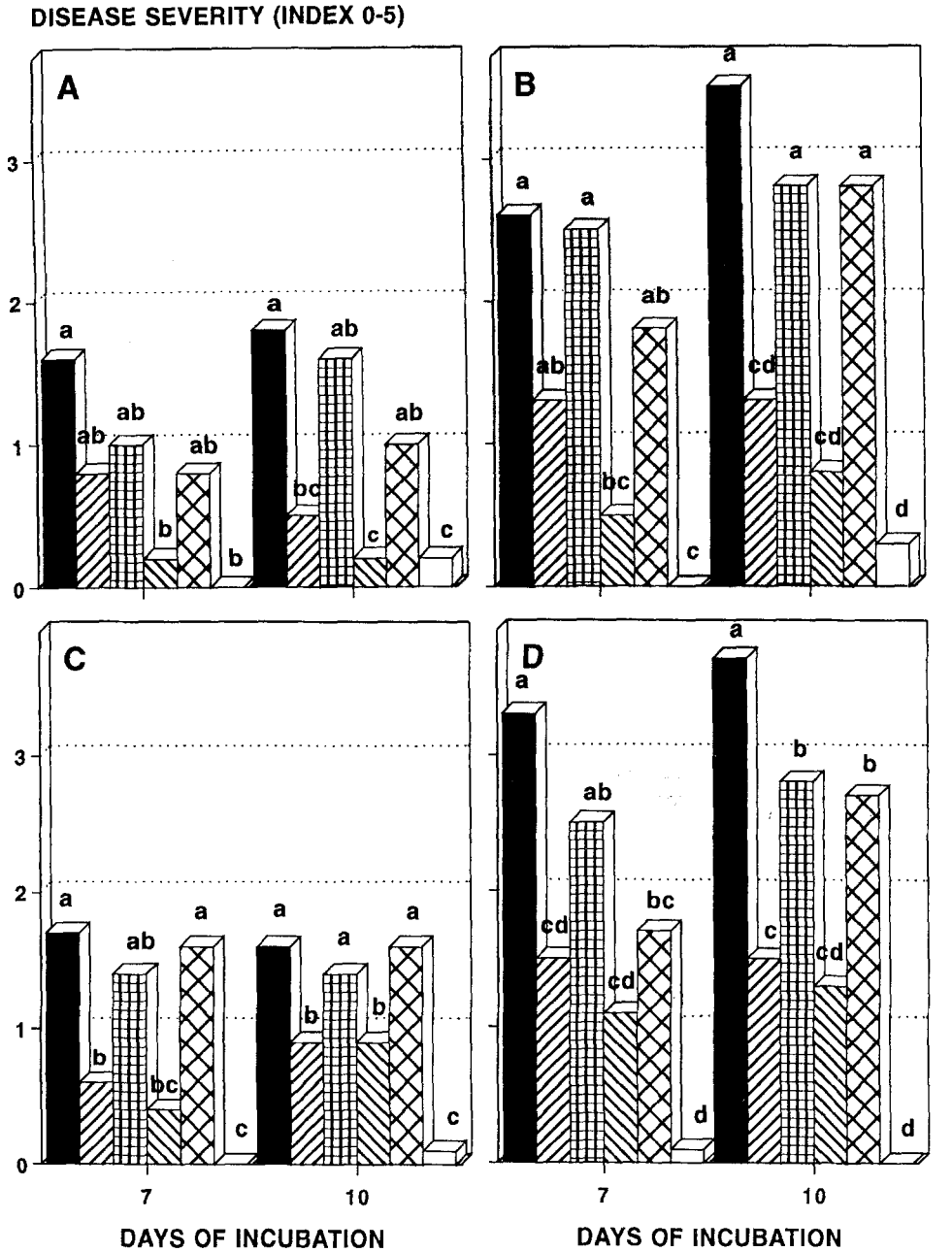

Fig. 1. Effect of aninooxyacetic acid (AOA) at rates of 5.0 (■, ▨, ▦, ▧) and 50 mM (▩, □) on rose flowers of cvs Golden Times (A,B) and Jaguar (C,D) incubated at 10 °C (A,C) and 15 °C (B,D) under conditions of no aeration (■, ▦, ▩) or partial aeration (▨, ▧, □). Disease severity was evaluated after 7 and 10 days of incubation according to an index of 0–5 where 0 = healthy flowers and 5 = completely rotted flowers.
Within each cultivar and sampling day, columns with a common letter do not differ significantly according to Duncan's Multiple Range Test ($P \leq 0.05$).

Table 1. Effect of 2,5-norbornadiene (NBD), cobalt sulfate, 2,4-dinitrophenol (DNP), salicylic acid and benzyladenine on the incidence of grey mould on rose flowers and leaves of various hosts.

Host plant	Compound and concentration (mM)												
	NBD			$CoSO_4$			DNP		Salicylic acid			Benzyladenine	
	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	0.1	1.0	10.0	0.1	1.0
Rose	15[1]	10	62*	45	50	85*	0	10	15	16	4	16	39*
Pepper	61*	69*	89*	74*	63*	91*	61*	70*	44*	20	12	15	10
Tomato	43	48	20	38	45	25	71*	37	28*	60*	7	42*	37*
Eggplant	85*	71*	65*	62*	60*	52*	11	38	0	8	34	0	–5
Bean	17	37	100*	0	5	0	42	56*	—	—	—	—	—
Senecio sp.	65*	45	62*	94*	47	85*	0	56*	28	4	5	82*	99*

[1] Disease reduction was calculated according to the formula (a-b) x 100/a, where a= disease severity of nontreated control and b=disease severity of leaves or flowers treated with certain dose of a compound.
* = Significant reduction of disease severity according to Duncan's Multiple Range Test ($P \leq 0.05$); — = not determined.

salicylic acid. Leaves of the two plants produced 34 and 7.8 ethylene nl g^{-1} h^{-1}, respectively. NBD and benzyladenine did not reduce ethylene production by the leaves. $CoSO_4$ reduced the production in tomato and pepper to 13.4 and 2.1 nl g^{-1} h^{-1}, respectively. DNP completely inhibited ethylene production on tomato and decreased it to 6.2 nl g^{-1} h^{-1} on pepper. Salicylic acid reduced ethylene production by tomato and pepper leaves to 21.4 and 3.4 nl g^{-1} h^{-1}, respectively. All reductions of ethylene production were to levels significantly lower than those of untreated noninoculated controls ($P \leq 0.05$).

Effect of the compounds on B. cinerea. Linear growth of *B. cinerea* was tested on PDA amended with the test compounds. NBD inhibited the growth of the fungus by 23% only at the rate of 10.0 mM. Ten mM $CoSO_4$ reduced the growth of the fungus by 33% and DNP reduced growth of *B. cinerea* by 22% and 47% when added to PDA at 1.0 and 10.0 mM, respectively. Salicylic acid significantly reduced growth rate of *B. cinerea* by 42% when applied at 10.0 mM. Germination of conidia of *B. cinerea* on media amended with 10.0 mM of any of the compounds was similar to that on non-amended PDA plates except for salicylic acid, which was inhibitory to conidial germination (52% reduction) at this high rate.

Control of Sclerotinia sclerotiorum. One mM of the compounds DNP, AOA, STS, $CoSO_4$, salicylic acid and benzyladenine was sprayed on lettuce leaves in order to control Sclerotinia white rot. The only compound which reduced white rot of lettuce was salicylic acid, which resulted in a disease index of 3.5, while that in the untreated control was 5.0.

Discussion

Results of this study indicate that the different measures tested in order to confront the grey mould-enhancing effect of ethylene, were effective, in spite of the fact that some of the methods did not always result in a statistically significant reduction of disease severity. Less pronounced results were obtained against white rot.

Absorption of ethylene from the atmosphere surrounding susceptible organs resulted in significant control of grey mould severity in several rose cultivars. Potassium permanganate was also effective in prolonging vase life of flowers of various rose cultivars even in the absence of visible *B. cinerea* infection. The combination of ethylene oxidation by potassium permanganate with sprays with silver ions (nitrate or thiosulfate) did not improve the control of grey mould, probably due to the large amount of ethylene in air which were released by plant tissues treated with the silver treatment. There are indications that such a treatment induces a greater production of the growth hormone in treated tissue (Elad, 1988a).

Silver nitrate, STS, AOA and calcium nitrate treatments were found previously to be effective in controlling grey mould on cut rose flowers (Elad, 1988a; Elad and Volpin, 1988). These results were confirmed in the present work. AOA is an inhibitor of pyridoxal enzymes. ACC synthase is a pyridoxal enzyme (Yu et al., 1979). The conditions under which the flowers were incubated dictated the effectiveness of AOA. Partial aeration of the flowers, which probably reduced the concentration of ethylene in the surrounding atmosphere, reduced grey mould severity by itself, and when combined with AOA. With partial aeration AOA was more effective than under conditions of no aeration. AOA was more effective at the high rate tested than at the low rate. Although toxicity to the flowers was not observed, it may occur with flowers of cultivars not tested in the present study. There it should be applied at the lower rate. Temperature of incubation also influences the development of grey mould (Elad, 1989) as well as the production of ethylene by the host flower (Elad, 1988a). However, since picked flowers are handled at low temperatures, it may be concluded that AOA is a potent agent for controlling the disease even at the lower rate tested in the present study.

Carbon dioxide was effective in controlling the disease on rose flowers, as was reported also by Phillips et al. (1985). However, the danger of a change of colour of petals in some cultivars of rose may interfere with the use of this gas in controlled atmosphere transportation or storage.

2,5-Norbornadiene is a cyclic olefin and a very powerful agent which competitively blocks the physiological ethylene-binding sites (Sisler et al., 1985). It was also an effective agent for the control of grey mould on some of the hosts tested in this study. However, practical application of this compound is in doubt because it is highly volatile, and has an unpleasant smell and carcinogenic properties (Smith and Hall, 1985). Hence, there is a need to develop analogues of NBD which possess its useful biological properties and lack its negative nature.

Cobalt ions which are inhibitors of ethylene production were effective in disease control similarly to NBD. Co^{2+} has been shown to inhibit the conversion of ACC to ethylene, which is the last step in the biosynthetic sequence in fruit or vegetative tissues (Yu and Yang, 1979). Co^{2+} controlled grey mould on most of the tested crops (Table 1).

2,4-Dinitrophenol is an uncoupler of oxidative phosphorylation. It also exerts its effect on the conversion of ACC to ethylene; however, at high concentrations it inhibits the conversion of methionine to ACC by affecting S-adenosylmethionine conversion to ACC (Yu et al., 1980).

Salicylic acid also affects ethylene production by blocking the conversion of ACC to ethylene (Leslie and Romani, 1986). It is an antioxidant and as such may affect disease also by influencing membranes of the host or by other mechanisms. Antioxidants and uncouplers are potent inhibitors of ethylene (Yang and Hoffman, 1984). Salicylic acid reduced grey mould in tomato and pepper only at one of the concentrations tested (0.1 and 1.0 mM, respectively) and white rot on lettuce.

Benzyladenine reduced grey mold on rose, tomato and *Senecio* sp. This compound was

found to lower the responsiveness of the host tissues to ethylene (Veen, 1987). The fact that benzyladenine and NBD did not reduce ethylene production by leaves of tomato and pepper but still reduced grey mould on these hosts may hint that another mechanism is involved in their activity rather than ethylene inhibition. Anyhow the possible toxic direct effect of the tested compounds on *B. cinerea* should be ruled out as possible mechanism since the only inhibitory effect on fungal gemination and growth was observed in rates higher than those which achieved significant disease control on plants.

Since ethylene promotes development of diseases of various hosts (Barkai-Golan et al., 1989; Barmore et al., 1976; Biles et al., 1990; Dehne et al., 1981; Elad, 1988a, 1990; McNicol et al., 1989), there is a possibility to control them by the methods described here. The various inhibitors of ethylene biosynthesis or activity, tested in the present study, can change the sensitivity of host plants to development of infection. Some of the tested compounds have a good potential for practical application, especially in cut flowers like roses. Further research is needed to understand the mechanisms involved in reduced sensitivity of the host plants to *B. cinerea* which is achieved by ethylene inhibition.

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