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Changes in susceptibility of bean leaves (Phaseolus vulgaris) to Sclerotinia sclerotiorum and Botrytis cinerea by pre-inoculative ozone exposures

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Abstract

The effects of ozone on the susceptibility of leaves of *Phaseolus vulgaris* to *Sclerotinia sclerotiorum* and *Botrytis cinerea* have been investigated. Seedlings of one ozone-sensitive ('Pros') and five relatively ozone-insensitive cultivars ('Gamin', 'Precores', 'Groffy', 'Narda', 'Berna') were exposed to different ozone concentrations (0, 120, 180 and 270 $\mu g\ m^{-3}$) for 8 h. One day after the exposures, primary leaves were detached and immediately inoculated with spores of either pathogen suspended in water or in a 62.5 mM KH_2PO_4 (Pi) solution. Visible ozone injury differed between the cultivars and increased with increasing ozone concentration. On the leaves of non-exposed plants, spores of the pathogens suspended in water caused very few lesions, whereas fungal pathogenicity was stimulated by addition of Pi to the inoculum. Ozone-injured leaves of all cultivars exhibited lesions after inoculation of the leaves with the pathogens suspended in water, and the number of lesions was positively correlated with the level of ozone injury for either pathogen and cultivar. The increase in susceptibility of bean leaves in response to increasing ozone concentrations was greater for *B. cinerea* than for *S. sclerotiorum* when spores were suspended in water, but was similar when the spores were suspended in Pi.

In general, the number of lesions following inoculation with spores in Pi increased with increasing ozone concentration. However, the number of lesions in the ozone-insensitive 'Groffy' was reduced by an exposure to 120 $\mu g\ m^{-3}$ but increased with higher concentrations. This pattern of susceptibility response to the pathogens was not found in the other ozone-insensitive cultivars and, thus, did not appear to be related to the inherent ozone-insensitivity in bean.

Additional keywords: air pollution, white mould, grey mould, pollutant–disease interaction, predisposition, necrotrophic pathogens.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary, the causal agent of white mould, is a common plant pathogen which can infect over 360 species of plants (Purdy, 1979). Field-grown beans (*Phaseolus vulgaris* L.) are among the more susceptible crops (Lohuis, 1991) and serious losses may be suffered from infection by this fungus (Steadman, 1983; Hall, 1991). White mould epidemics of beans are initiated by ascospores produced by sclerotia and all aerial parts of the plants can be affected (Abawi and Grogan, 1979). The asco-

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spores require external nutrients to infect healthy plant tissues, the most common source being senescent bean flowers. Successful infections by ascospores can also be readily obtained in bean tissues that are injured by various causes (Abawi et al., 1975).

Grey mould, caused by *Botrytis cinerea* Pers.: Fr., is also a common disease in beans (Hall, 1991). As for ascospores of white mould, conidia of grey mould are generally unable to infect healthy leaves in the absence of an external nutrient source or dead plant material (Blakeman, 1980). Recent experiments have shown that conidia of *B. cinerea*, suspended in water without any added nutrients, were able to infect the leaves of bean plants exposed to realistic concentrations of ozone for 8 h prior to inoculation (Leone and Tonneijck, 1990a). The ozone-induced increase in susceptibility was significantly correlated with the level of visible ozone injury. Ozone symptoms have been recorded on beans in the Netherlands (Tonneijck, 1983) and, thus, ambient ozone could play a role in increasing the susceptibility of field-grown beans to *B. cinerea*. Recent reports from the United Kingdom (Archer et al., 1992) and the Netherlands (Lohuis, 1991) have indicated that the infection of susceptible crops by white mould has increased in the field during the last years. Thus, the question arose whether ozone could be involved in this increased susceptibility of field-grown crops to *S. sclerotiorum*.

The ozone-induced increase in the susceptibility of bean to *B. cinerea* has been found to be dependent on the selected cultivar, the rate of enhancement being greater in ozone-sensitive cultivars than in the relatively insensitive cultivar Groffy (Leone and Tonneijck, 1990a). However, recent experiments revealed that an exposure to a low concentration of ozone that did not result in visible injury, reduced the susceptibility of 'Groffy' to this fungus when its leaves were subsequently inoculated with conidia suspended in an inorganic phosphate solution (Tonneijck, 1993).

The aim of the present investigation was (1) to compare the effects of pre-inoculative ozone exposures on the susceptibility of bean leaves to *S. sclerotiorum* and to *B. cinerea* and (2) to compare the susceptibility of several ozone-insensitive cultivars with that of 'Groffy' when the leaves were inoculated with the spores of one of the two fungi suspended in a Pi solution after the plants had been exposed to incremental ozone concentrations.

Materials and methods

Cultivation and fumigation. Seeds of the ozone-sensitive cultivar Pros and of the relatively ozone-insensitive cultivars Gamin, Precores, Groffy, Narda and Berna (Tonneijck, 1983) were planted in plastic pots (diameter 12 cm) filled with a high fertility peat-clay potting mixture (Triomf no. 17, modified, Trio BV, Westerhaar, the Netherlands). Plants were grown in a glasshouse with charcoal-filtered air at a 20/18 °C day/night temperature regime and 75% relative air humidity. In the winter experiment, plants received additional light from high pressure mercury vapour lamps (HPL 400 W, Philips, the Netherlands) during 12 h a day.

In each fumigation experiment, 3-week-old plants were exposed to 0, 120, 180 and 270 μg ozone m^{-3} for 8 h. Fumigations were conducted in 3.3 m^3 environmentally controlled exposure chambers (Mooi and Jolink, 1989). Plants were placed in the chambers c. 16 h before the start of the fumigation and maintained at 75% relative air humidity, 20/18 °C day/night temperature and at 22 000 lux (c. 75 W m^{-2}) at plant height from 5.00 till 21.00 hours. Charcoal-filtered air flowed through the chambers at a rate of 4000 m^3 h^{-1}, resulting in a wind velocity of at least 50 cm sec^{-1}. Ozone was generated by UV irradiation of pure oxygen and added to the air stream of three chambers. Plants in the fourth chamber received filtered air only. Ozone concentrations were monitored con-

stantly with a Dasibi 1003 AH analyzer calibrated with air of known ozone content from a UV generator.

Inoculum production, inoculation and incubation procedures. *B. cinerea* and *S. sclerotiorum* had been isolated from naturally infected bean plants and maintained on potato dextrose agar (PDA) at 4 °C. Spores were obtained as previously described (Leone and Tonneijck, 1990b). For each ozone level and fungus, four primary leaves per cultivar were detached 24 h after the start of the exposure period, placed in plastic trays and inoculated immediately with 20-µl drops (ten drops per leaf) of a spore suspension in water (2×10^6 spores ml^{-1}). Similarly, four other primary leaves were detached and inoculated with 20-µl drops of a 62.5 mM KH_2PO_4 (Pi) solution with the same concentration of spores (Leone and Tonneijck, 1990a,b). After inoculation, the trays were enclosed in transparent plastic bags and incubated in an environmentally controlled chamber under a 16-h photoperiod (fluorescent light, c. 1 200 lux) and a 20/18 °C day/night regime.

Statistics. The number of lesions and the proportion of leaf area affected by ozone were determined 48 h after inoculation of the leaves. Ozone-induced visible injury was assessed by using a modified Horsfall-Barratt scale (Hofstra and Ormrod, 1977). Each fumigation experiment was performed three times, both in summer and in winter. Thus, the responses of 24 primary leaves were observed for each combination of cultivar, ozone level, pathogen and inoculum suspension (water and Pi), totalling 96 combinations. Data for all responses were subjected to regression analysis with a generalized linear model by using the statistical package Genstat V. With respect to the number of lesions, data were analyzed separately for the two inoculum suspensions. The methods of maximum likelihood and of maximum quasi-likelihood were applied to the number of lesions and to the percentage of ozone injury, respectively (Mc Cullagh and Nelder, 1983). The logit function was used as link function which implies a sigmoidal dependence of fungal lesions and injury on ozone concentration. Treatment effects were assessed eliminating the effects of the factors 'season' and within season the factor 'experiment' and effects were considered statistically significant at $P \leq 0.05$.

Results

Effect of ozone on the susceptibility of six bean cultivars to S. sclerotiorum and B. cinerea. Immediately after the exposures with high concentrations of ozone, leaves of the ozone-sensitive cultivar showed a grey/green flecking between the veins, which developed in white/tan bifacial necrosis. Similar symptoms were also observed on the relatively insensitive cultivars after 2 days. All cultivars exhibited coloured, mostly dark brown lesions, while some leaves became chlorotic. The level of ozone-induced injury significantly depended on the ozone concentration and the cultivar used and differences in foliar injury between cultivars were significantly dependent on the concentration. Injury increased most rapidly with increasing concentration of ozone in 'Pros' (Fig. 1). Symptoms in this cultivar were clearly visible after the exposure to 120 µg ozone m^{-3} while the other cultivars were hardly injured by this exposure. Among the insensitive cultivars, ozone injury in 'Gamin' and 'Precores' was generally greater than that in 'Groffy' whereas it was slightly smaller in 'Berna' and 'Narda'.

Hardly any infection was observed when the primary leaves of non-exposed plants were inoculated with conidia of *B. cinerea* or with ascospores of *S. sclerotiorum* suspended in water (Fig. 2). Only on leaves of non-exposed 'Groffy' plants, was the mean number of lesions higher than 10%. Statistical analyses indicated that the effect of ozone on suscep-

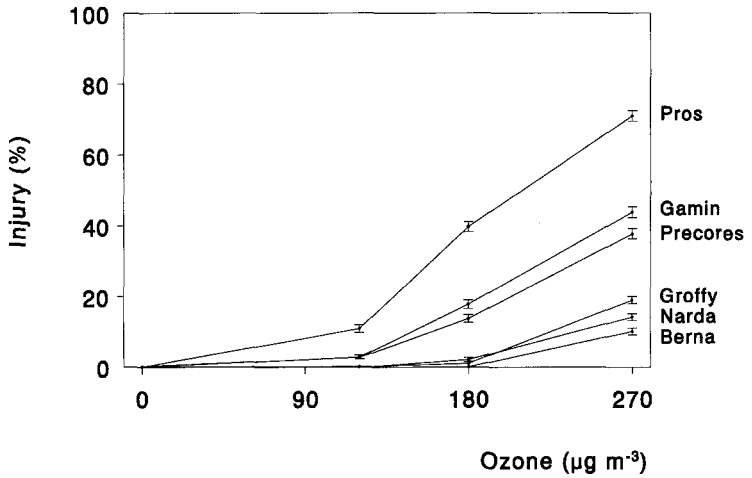

Fig. 1. Injury (% leaf area affected) on primary leaves of six bean cultivars after 8 h exposures to incremental ozone concentrations. The vertical bars represent ± SE.

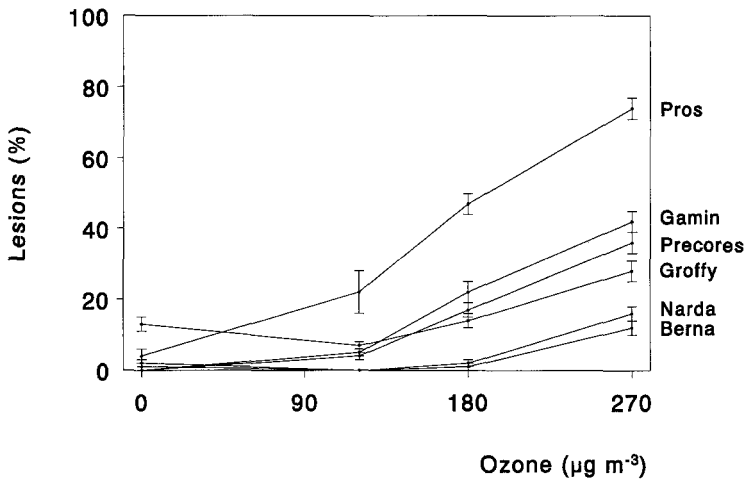

Fig. 2. Proportion of inoculation sites with lesions (%) after inoculation with spores of *Botrytis cinerea* and *Sclerotinia sclerotiorum* (data are combined for the pathogens) suspended in water, on primary leaves of six bean cultivars, exposed for 8 h to incremental ozone concentrations. The vertical bars represent ± SE.

tibility significantly depended on cultivars and on fungi. All three factor interactions were not significant. Thus, differences in the susceptibility responses between the cultivars significantly depended on ozone concentration when the leaves were inoculated with the spores suspended in water (Fig. 2). The number of lesions increased with increasing ozone concentrations in all cases except one. Compared to non-exposed controls, a slight but insignificant decrease in number of lesions was observed in 'Groffy' after exposure to the

lowest ozone concentration. The concentration-dependent increase in susceptibility to the pathogens was the highest in 'Pros' and the number of lesions remained relatively low in 'Berna' and 'Narda' even after the exposure to 270 μg ozone m^{-3}.

Ozone predisposed the plants to either fungus. The increase in the number of lesions in response to the increasing ozone concentrations was significantly different for the two fungi and was more pronounced for *B. cinerea* than for *S. sclerotiorum* (Fig. 3). As compared to the non-exposed controls, an increase in number of lesions by *B. cinerea* could already be observed on plants exposed to 120 μg ozone m^{-3} whereas an exposure to 180 μg ozone m^{-3} was required to enhance the infection by *S. sclerotiorum*. With respect to inoculations with spores of either fungus in water, a positive and highly significant correlation between the number of lesions and the level of ozone injury was always found for all cultivars (Table 1).

Compared with inoculation in water, addition of Pi to the inoculum stimulated fungal infection on primary leaves of all cultivars. Statistical analyses indicated that the main effects of ozone concentration, cultivar and fungus were significant and that the two and three factor interactions were not significant. Thus, the effect of the ozone exposures on the susceptibility of the leaves can be presented by combining the data for all cultivars and the two fungi. As shown in Table 2 (right column), the number of lesions increased with increasing ozone concentration. Conidia of *B. cinerea* caused significantly more lesions than ascospores of *S. sclerotiorum*, but the increase in number of lesions in response to increasing ozone concentrations did not differ significantly for the two fungi (Fig. 4). A positive and significant correlation between the number of lesions and the level of ozone injury was found on leaves of exposed plants of four cultivars when inoculated with the Pi suspensions of either fungus (Table 1). The number of lesions was not significantly correlated with injury in 'Groffy' for both fungi and in 'Berna' for *B. cinerea*.

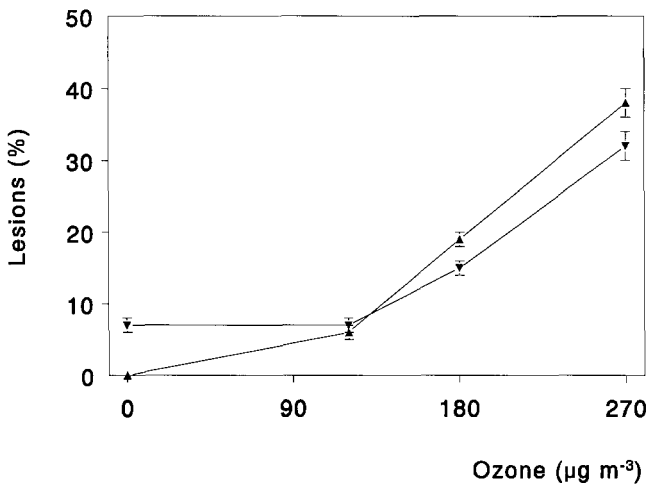

Fig. 3. Proportion of inoculation sites with lesions (%) after inoculation with conidia of *Botrytis cinerea* (▲) and ascospores of *Sclerotinia sclerotiorum* (▼) suspended in water, on primary leaves of bean plants (data are combined for the cultivars) exposed for 8 h to incremental ozone concentrations. The vertical bars represent ± SE.

Table 1. Correlation coefficients for the relationship between the proportion of inoculation sites with lesions (%) by spores of *Botrytis cinerea* and *Sclerotinia sclerotiorum* suspended in water and in a Pi solution, and foliar injury (% leaf area affected) on primary leaves of six bean cultivars after 8 h exposures to ozone.

Cultivar	*B. cinerea*		*S. sclerotiorum*	
	Water	Pi	Water	Pi
Pros	0.92**	0.60**	0.92**	0.74**
Gamin	0.92**	0.64**	0.93**	0.77**
Precores	0.96**	0.50*	0.94**	0.49*
Groffy	0.89**	0.30	0.68**	0.39
Narda	0.91**	0.44*	0.96**	0.56**
Berna	0.99**	0.28	0.98**	0.42*

* and ** indicate significance at $\alpha = 0.05$ and 0.01, respectively.

Table 2. Proportion of inoculation sites with lesions (%) after inoculation with spores of *Botrytis cinerea* and *Sclerotinia sclerotiorum* suspended in a Pi solution, on primary leaves of six bean cultivars, exposed for 8 h to incremental ozone concentrations. Data for the two fungi are combined and represent means ± SE.

Ozone ($\mu g\ m^{-3}$)	Cultivar						Average
	Pros	Gamin	Precores	Groffy	Narda	Berna	
0	55 ± 6	26 ± 6	42 ± 6	66 ± 6	28 ± 6	39 ± 6	43 ± 3
120	66 ± 5	31 ± 5	56 ± 5	51 ± 5	28 ± 5	34 ± 5	44 ± 2
180	76 ± 4	47 ± 5	63 ± 5	59 ± 5	33 ± 5	43 ± 5	53 ± 2
270	88 ± 4	77 ± 5	70 ± 6	82 ± 5	44 ± 6	64 ± 7	71 ± 2

Ozone-induced changes in the susceptibility of 'Groffy' and four other ozone-insensitive cultivars. As can be seen in Table 2, the number of lesions on leaves of 'Groffy' that were inoculated with the fungal spores suspended in Pi, was reduced significantly by the pre-inoculative exposure to the lowest ozone concentration as compared to the non-exposed controls. This reduction was not observed in the other cultivars except in 'Berna' where a very slight but insignificant reduction was found after exposure to 120 $\mu g\ m^{-3}$ ozone. When the data of the ozone-insensitive cultivars were analyzed only, results showed that the susceptibility response of 'Groffy' to increasing ozone concentrations was significantly different from the mean response of the four other ozone-insensitive cultivars since the number of lesions in the latter cultivars steadily increased with increasing ozone concentrations (Fig. 5).

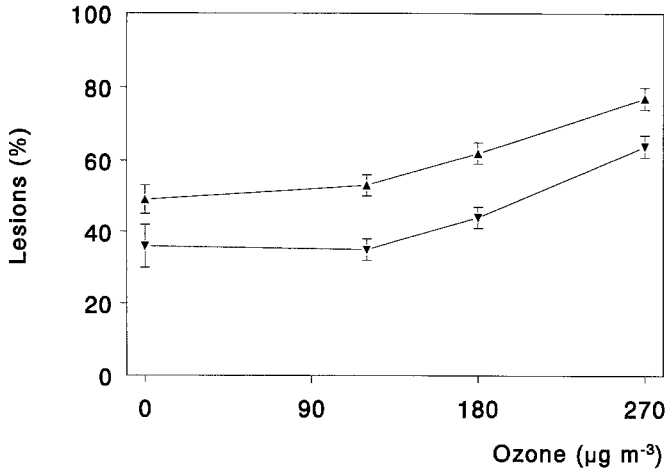

Fig. 4. Proportion of inoculation sites with lesions (%) after inoculation with conidia of *Botrytis cinerea* (▲) and ascospores of *Sclerotinia sclerotiorum* (▼) suspended in a Pi solution, on primary leaves of bean plants (data are combined for the cultivars) exposed for 8 h to incremental ozone concentrations. The vertical bars represent ± SE.

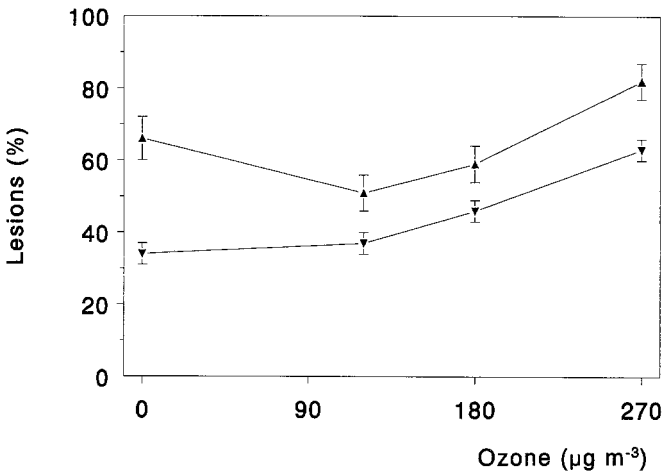

Fig. 5. Proportion of inoculation sites with lesions (%) after inoculation with spores of *Botrytis cinerea* and *Sclerotinia sclerotiorum* suspended in a Pi solution, on primary leaves of bean cultivar Groffy (▲) and of four other ozone-insensitive cultivars (▼, mean response for 'Precores', 'Gamin', 'Narda' and 'Berna'), exposed for 8 h to incremental ozone concentrations. The vertical bars represent ± SE.

Discussion

To our knowledge, this is the first report showing that ozone exposures can increase the susceptibility of plants to *S. sclerotiorum*. Leaves of bean plants exposed to acute ozone concentrations prior to inoculation, were generally more susceptible than the non-exposed controls. This predisposition effect corresponded with the ozone-induced increase in

susceptibility of bean leaves to *B. cinerea* found in this and earlier studies (Leone and Tonneijck, 1990a; Tonneijck, 1993). Leaves of non-exposed bean plants were barely infected by spores of *S. sclerotiorum* or *B. cinerea* suspended in water without any added nutrient. Under these conditions, ascospores of *S. sclerotiorum* were slightly more infectious than conidia of *B. cinerea*, as was already found by Leone and Tonneijck (1990b). Ozone-injured bean leaves became readily susceptible to either fungus, irrespective of the inoculum composition. However, the rates of leaf infection in response to the increasing ozone concentrations significantly differed between the two pathogens when inoculations were performed with spores in water. The number of lesions increased more rapidly with increasing ozone concentrations for *B. cinerea* than for *S. sclerotiorum*. Thus, the conidia of *B. cinerea* profited more by the changes in bean leaves due to ozone exposures than did the ascospores of *S. sclerotiorum*. The increase in number of lesions in response to increasing ozone concentrations was not significantly different for both fungi when inoculations were performed with the spores suspended in Pi.

It is a well-known characteristic of both fungi that the spores are generally unable to invade healthy green tissues in the absence of a nutrient source (Abawi et al., 1975; Clark and Lorbeer, 1977). However, the presence of Pi in the spore suspensions stimulated the infection by either fungus on leaves of bean plants not exposed to ozone. The stimulative effect of Pi on the infectivity may be similar to that produced by external nutrients such as present in senescent or dead plant material (Leone, 1992). *B. cinerea* and related pathogens like *S. sclerotiorum* are common necrotrophic plant pathogens and it is logical to expect that these fungi would invade air pollution-weakened or damaged tissues (Manning, 1976). Interactions between visible ozone injury and increased infections of *B. cinerea* have been reported for potato (Manning et al., 1969), geranium (Manning et al., 1970) and bean (Leone and Tonneijck, 1990a). Wukasch and Hofstra (1977b) exposed onion plants to filtered and unfiltered ambient air in open-top chambers and significantly more lesions were observed on ozone-injured plants in the unfiltered chamber than on uninjured plants in the filtered chamber after inoculations with *B. squamosa*. Field-grown onions that were partly protected from ozone injury by an antioxidant chemical, were less infected by *Botrytis* spp. than injured onions (Wukasch and Hofstra, 1977a). However, Rist and Lorbeer (1984a) found that the ozone-induced predisposition of onion leaves to enhanced infection by *B. cinerea* occurred in both the presence and absence of visible injury. Ozone can damage the plasmalemma through the action of free radicals that may eventually lead to premature senescence (Thompson et al., 1987) and to leakage of nutrients toward the leaf surface. These nutrients can then stimulate infection by necrotrophic pathogens. Thus, Rist and Lorbeer (1984b) showed that ozone exposures caused nutrients to leak from exposed onion leaves and that these nutrients increased the infection by *B. cinerea* on leaves of onion plants that were not exposed to ozone. Our investigation showed that the rate of infection on bean leaves by both *B. cinerea* and *S. sclerotiorum* increased with increasing ozone injury. If the level of ozone injury as such is directly related to an increase in the susceptibility of bean leaves to the pathogens, then it is difficult to explain why the rate of infection caused by *B. cinerea* was more enhanced by ozone than that caused by *S. sclerotiorum* when the leaves were inoculated with spores suspended in water but not in Pi. The results suggest that other processes such as the ozone-induced leakage of nutrients are also involved in the ozone-induced increase in susceptibility of bean, the more so since *B. cinerea* appeared to be more infectious in the presence of Pi than *S. sclerotiorum*. Also, McKersie et al. (1982) have shown that a short-term exposure to ozone induced the leakage of nutrients such as sugars and amino acids toward the leaf surface of two bean cultivars.

The level of ozone-induced foliar injury differed among the cultivars, 'Pros' being the

most sensitive and 'Berna' the most insensitive cultivar. Among the five cultivars that had been classified earlier as relatively insensitive to ozone (Tonneijck, 1983), 'Groffy' showed an intermediate foliar injury response to ozone exposure. When leaves of 'Groffy' plants were inoculated with the fungal spores suspended in Pi, the number of lesions was reduced initially by an exposure to 120 μg ozone m^{-3} and increased again when the concentrations further increased. Leaves of plants of this cultivar exposed to this low ozone concentration and inoculated with spores suspended in water, also tended to be less susceptible to the fungi than the non-exposed controls. The fumigation with 120 μg ozone m^{-3} did not cause any visible injury in this cultivar. The described results corroborated the findings of previous studies (Leone and Tonneijck, 1990a; Tonneijck, 1993) in which pre-inoculative exposures to low concentrations of ozone induced resistance to *B. cinerea* in the ozone-insensitive 'Groffy'. Defense mechanisms in plants can be triggered by free radicals in an aspecific manner in response to biotic or abiotic challenges (Thompson et al., 1987; Sutherland, 1991). Whether or not these or other mechanisms are involved in the ozone-induced resistance of 'Groffy' to necrotrophic pathogens is as yet unknown. The susceptibility of 'Groffy' in response to increasing ozone concentrations may be cultivar-specific and does not appear to be related to the inherent ozone-insensitivity in bean since this response pattern was not observed in other ozone-insensitive bean cultivars.

The ozone levels used in the experiments are within the range of maximal 8 h concentrations that have been recorded in ambient air in the Netherlands (Slooff et al., 1989). Thus, laboratory exposures for 8 h to concentrations that occur in ambient air, can enhance, reduce or not alter the susceptibility of bean leaves to subsequent inoculations with spores of *B. cinerea* and *S. sclerotiorum*. An increase in susceptibility is related to the level of ozone-induced injury which is dependent on the ozone sensitivity of the cultivar and the applied concentration of ozone. Ozone exposures that do not result in visible injury, do not alter the susceptibility of bean leaves to the necrotrophic pathogens or can even decrease it in a cultivar-specific way.

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