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The effects of powdery mildew (*Blumeria graminis* f. sp. hordei) infection on stomatal resistance in cultivated and wild barley lines

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ABSTRACT

The objective of these experiments was to examine how stomatal resistance changed in infected leaves of Powdery Mildew (*Blumeria graminis*) tolerant and non-tolerant barley lines compared to uninfected leaves in both light and dark, and to determine how much changes in stomatal behaviour following infection could be involved in the reductions in photosynthesis and consequently in reduced dry matter production reported in Akhkha *et al*. (2003a, 2003b). It was found that infection by powdery mildew increased stomatal resistance in the light in all three lines indicating that mildew induced stomatal closure and consequently limiting CO_2 diffusion to the carboxylation sites and causing a decline in the rates of photosynthesis. In contrast, stomata failed to close completely in the dark in all three lines.

Key words: Powdery Mildew, (*Blumeria graminis*), stomatal resistance, wild barley lines.

INTRODUCTION

Water plays a very important role in all physiological processes in plants including photosynthesis, respiration, translocation, partitioning of metabolites, stomatal behaviour, protein synthesis, cell division, cell elongation and cell wall synthesis. Water stress will lead to the perturbation of all or some of these physiological processes and consequently will lead to reductions in plant growth and yield (Kramer, 1983).

Healthy plants can protect themselves against the development of water stress by regulating stomatal aperture. The stomata are sensitive structures that represent the greatest variable resistance in the pathway of water movement through the plant (Ayres, 1981) and any biotic or abiotic factor causing changes in the pattern of stomatal behaviour will affect plant water relations and consequently perturb growth and development. Many investigations have been carried out to determine the effects of obligate biotrophs on stomatal behaviour. These effects have been found to differ from one pathogen to another and from one host to another.

Infections of barley leaves by *Rhynchosporium secalis* (Ayres *et al*., 1975) and of potato by the blight fungus, *Phytophthora infestans* (Farrell *et al*., 1969) caused an increase in the rate of transpiration from the infected area of the leaf both in the light and in the dark. This increase was attributed to an increase in the mean stomatal aperture in the infected area in the light and the failure of the stomata to close in the dark. The downy mildew fungus *Peronospora tabacina* has also been found to affect stomatal opening in the leaves of its host tobacco in a similar manner (Cruickshank *et al*., 1961).

In contrast, stomatal opening in the light has been reported to be inhibited by rust and powdery mildew infections as well as by some viruses such as sugar beet yellows virus (Hall *et al*., 1972).

Transpiration in rust and powdery mildew infected tissues usually follows the pattern of stomatal behaviour, decreasing in the light and increasing in the dark (Walters, 1985). Rust fungi enter their hosts through stomatal pores, develop mainly in the intercellular spaces of the leaf and inhibit stomatal movements progressively until eventually the stomata became fixed in an almost closed position (Duniway *et al*., 1971). However, with rust fungi, once fungal sporulation has ruptured the cuticle, non-stomatal transpiration increases and becomes the significant factor (Johnson *et al*., 1934, 1940 and Murphy, 1935).

Paul *et al*. (1984) showed that after sporulation, groundsel (*Senecio vulgaris*) leaves infected with *Puccinia lagenophorae* transpired much more rapidly than did healthy controls. The same results were shown by Duniway *et al*. (1971a) in bean (*Phaseolus vulgaris*) leaves infected with *Uromyces phaseolus.*

Powdery mildew infections result generally in a failure of stomata to open fully in the light and to close fully in the dark (Majernick, 1971; Ayres, 1976). Wheat leaves infected with *B. graminis* f.sp. *tritici* were shown to have a significantly reduced stomatal opening within three to six hours after inoculation (Martin *et al*., 1975). Majernick (1965) working with barley leaves infected with *B. graminis* f.sp. *hordei* reported that stomatal transpiration had reduced within one day after inoculation. Ayres (1979) also, using barley leaves infected with *B. graminis* f.sp. *hordei*, observed that reduced stomatal opening was not apparent until three days after inoculation. Other plant species other than cereals showed similar responses to mildew infection. For example, although garden pea (*Pisum sativum*) leaves infected with *E. pisi* showed an initial increase in stomatal opening within the first 48 hours of inoculation, the stomatal opening became progressively reduced in the light and stomata failed to close completely in the dark (Ayres, 1976). Thomas *et al*. (1982) observed a 50% reduction in stomatal aperture five days after inoculation in sugar beet (*Beta vulgaris*) leaves infected with *E. polygoni*. Similar responses were observed in leaves of oak plants infected with *Microsphaera alphitoides*, but not until six days after inoculation, although, transpiration rates increased within two to three days after inoculation (Hewitt *et al*., 1975).

Ayres (1972 and 1975) investigated stomatal functioning in barley leaves infected with *Rhynchosporium secalis* and suggested that in the early stages of infection the increase in stomatal aperture was a result of the loss of osmotically active solutes from the epidermal cells of diseased leaves which consequently altered the turgor relations between guard cells and their surrounding epidermal cells. The increase in transpiration at later stages of infection was attributed to water loss through the ruptured cuticle (Ayres, 1975).

In the case of barley infected with *B. graminis* f.sp. *hordei*, Majernick (1965) suggested that a volatile product was involved in the inhibition of stomatal opening in the light. A similar suggestion was made by Martin *et al*. (1975) for wheat leaves infected with *B. graminis* f.sp. *tritici*.

The increased stomatal opening in the light that occurs in pea leaves infected with *E. pisi* 48 hours after inoculation contrasts with the reduced stomatal opening in wheat within 6 hours of inoculation (Martin *et al*., 1975) and in barley within 24 hours after inoculation (Majernick, 1965) with the cereal powdery mildew. The difference between peas and cereals (barley and wheat) was attributed to the lack of production of a volatile substances in peas infected with *Erysiphe pisi* or to the differences in the turgor pressures of guard cells and epidermal cells (Ayres, 1976). Furthermore, Ayres (1980) suggested that stomatal opening could be inhibited by substances synthesised by the host such as pisatin (a pterocarpan) which accumulates in pea leaves infected with *E. pisi*.

The increased rate of transpiration observed in barley leaves infected with *B. graminis* f.sp. *hordei* when 50% of the leaf was covered by mildew, was attributed to cuticular injuries caused by the infection (Paulech *et al*., 1970; Majernick, 1965). In contrast, the increase in the rate of

transpiration observed in oak leaves infected with *Microsphaera alphitoides* was attributed mainly to the fungal mycelium itself (Hewitt *et al*., 1975).

Many of the differences in host response to different pathogens are most likely to be mainly due to the different ways the pathogens grow and develop on or within their host's tissues. However, the experimental differences in host response reported for particular pathogens are also likely to be due to an extent to the experimental procedures used, but are also likely to be due to the fact that different cultivars were used.

One significant factor missing from most of the studies was any measure of the way in which or the rate at which parasite biomass accumulated during the course of the experiments. Even when parasite biomass accumulated to similar extents in the different cultivars used, reactions may be different due to different tolerances of the parasite in the tissues.

The effects of infection on stomatal resistance were determined in the two wild barley lines I-17-40 and B19909, and in the cultivated barley cv. Prisma. The experiment was carried out twice, with similar results being obtained on each occasion. Only the results of the second experiment are presented.

MATERIAL AND METHODS

Plant material & growth

The lines of wild barley (*Hordeum spontaneum)* and of cultivated barley (*Hordeum vulgare*) used in this study were obtained either from the John Innes Centre, Norwich Research Park or from the Scottish Crop Research Institute, Invergowrie, Dundee.

Seed germination was carried out following Akhkha *et al*. (2003a) procedure by treating wild lines seeds with 7 days of chilling temperature at 4°C. Seeds of both wild and cultivated lines were germinated in damp filter paper then sown in peat based potting compost (Levington Horticulture Ltd.). Before use, the wild lines were inbred as mentioned in Akhkha *et al*. (2003a).

Stomatal measurements

Stomatal resistance

An automatic diffusion porometer MK3 Delta-T Devices (128, Low Road, Burwell, Cambridge CB5 OEJ, UK) was used to measure stomatal diffusive resistance.

Principle of the measurements

The diffusion porometer measures the approximate rate of diffusion of water vapour through the stomata. Its operation assumes that water vapour diffusion out of a leaf into dry air is regulated by the degree of opening of the stomata (neglecting cuticular transpiration). A small chamber containing a relative humidity sensor is clamped to the leaf. Prior to reading, a small electric diaphragm pump blows a stream of air, dried by passing through silica gel, into the chamber. Water vapour emitted by the transpiring leaf surface causes the relative humidity (RH) within the chamber to rise and the sensor becomes moist. As the sensor becomes moist its conductivity increases and the rate of increase in conductivity to a set value is directly proportional to the rate of outward diffusion of water vapour through the stomata. The difference in temperature between the leaf and the cup is measured by two thermistors, which are built into the leaf clamp.

Porometer calibration

The porometer is supplied with a moulded polypropylene calibration plate with six groups of holes each of known diffusion resistance. A source of water vapour is provided by backing the plate with damp filter paper, which is sealed to the plate with waterproof tape. The sensor head is clipped onto the calibration plate and readings are taken from each set of holes. A calibration graph of plate resistances is plotted against the corresponding counts (Automatic porometer MK3 operating manual,) and this graph is used to convert the counts obtained from the leaf measurements into diffusion resistance values.

Experimental procedure

Fifty seedlings were raised. When two weeks old, the fully expanded third leaves on 25 plants of each line were inoculated in the middle region of the adaxial surface, using a camel hair

brush. The tip and the base of the leaf blades were kept free of mildew. The other 25 plants of each line were kept free of mildew by adding 0.05% Benlate solution to the pots at weekly intervals. The inoculated and uninoculated plants were then placed randomly in the growth cabinet. The stomatal resistance measurements were taken from four plants per treatment per line. The first measurements in the light were made 24 hours after inoculation, and then the same plants were placed in a dark room for 24 hours after which porometer measurements were made under green light. Subsequent measurements were made at two-day intervals until 5 sets of measurements had been made.

Stomatal resistances were measured in the middle and tip of both adaxial and abaxial surfaces of infected and uninfected third leaves on each plant line.

Statistical analysis

The means and standard errors (shown in graphs) were calculated using Excel (Microsoft Office 2000). Analysis of variance was performed using Minitab's ANOVA and General Linear Model (version 13).

RESULTS

Stomatal resistance was measured in the middle and tip regions of infected and uninfected leaves of the three lines. The results are plotted graphically in Figs 1A and 1B.

Ontogenetic changes in stomatal function in the uninfected third leaf

Changes in the light

Stomatal resistance in the light in both the middle and tip regions of uninfected leaves remained relatively constant throughout the period of measurement, in all three lines (Figs. 1A and 1B).

Changes in the dark

In contrast, in the dark, marked changes occurred as the leaves aged in line I-17-40 and cv. Prisma at both the middle and the tip regions (Figs. 1A and 1B). For cv. Prisma and line I-17-40, at the beginning of the experiment, the stomatal resistances increased reaching a maximum two days later, but from then on, resistances declined and then an approximately steady state was reached and maintained to the end of the experiment.

In line B19909, stomatal resistance increased rapidly in the middle region from the second to the seventh day after inoculation and then stayed at a relatively constant state. In contrast to the other two lines, stomatal resistance in the tips of the leaves of line B19909 remained at an almost constant level during the whole period of the experiment.

Effects of infection on stomatal function in the light

Stomatal resistance in the middle, inoculated region, of the leaf

Within 24 hours of inoculation, stomatal resistance had significantly ($p < 0.01$) increased in infected leaves when compared to uninfected leaves in all three lines, but then as infection progressed it began to decrease. By the eighth day after inoculation it was similar to that of uninfected leaves in both cv. Prisma and line B19909, but in line I-17-40, it was still slightly higher ten days after inoculation. From the eighth day, the stomatal resistance of cv. Prisma continued to decrease to levels significantly ($p < 0.05$) below uninfected leaves. In contrast, stomatal resistance in line B19909 remained at control levels.

Stomatal resistance at the leaf tip

Infection in the centre of the leaf increased stomatal resistance in the uninfected tip of that leaf in all three lines (Fig. 1B) although the increase did not become significant, until around 10 days after inoculation ($p < 0.05$).

Effects of infection on stomatal function in the dark

Stomatal resistance in the middle inoculated region of the leaf

The high stomatal resistances of uninfected leaves in the dark indicate that the stomata were probably closed (Fig. 1A). Infection decreased the stomatal resistance of infected leaves significantly ($p < 0.02$) in both the wild line I-17-40 and cv. Prisma. As infection progressed the differences became

smaller but still remained significant. In contrast, by two days after inoculation, stomatal resistance in leaves of line B19909 had increased significantly above control levels ($p < 0.001$), but as infection progressed, it decreased again and followed the same pattern as in the other two lines.

The results suggest that infection by powdery mildew prevented the stomata from closing in the dark as fully as those in uninfected leaves.

Stomatal resistance at the leaf tip

Infection had little effect on stomatal resistance in the uninfected tip regions of inoculated leaves of the wild line I-17-40. In contrast, infection decreased stomatal resistance, in uninfected tips of infected leaves, both of cv. Prisma and line B19909. These decreases became significant ($p <$

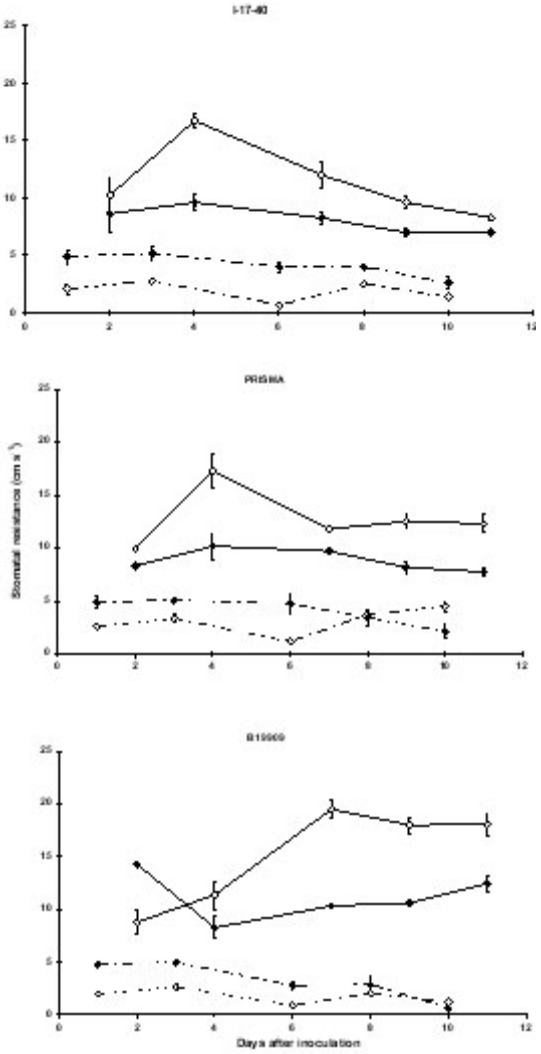

Fig. 1(A): Stomatal resistance in the third leaves (middle region) of the three barley lines in light (.....) or in darkness (_____) following inoculation with *B. graminis* f.sp hordei. Each darum is the mean of four replicates, with standard error. (◊) uninfected, (♦) infected

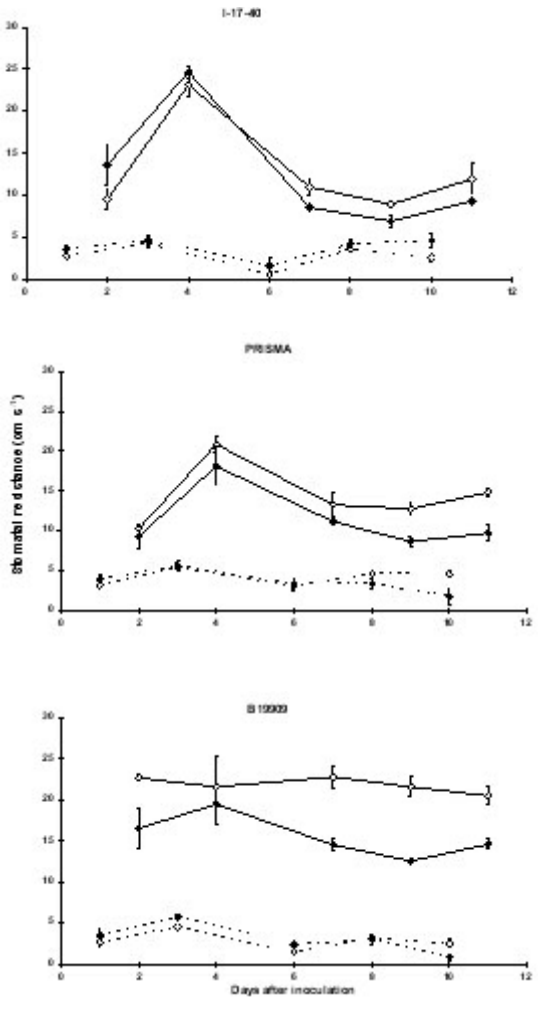

Fig. 1(B): Stomatal resistance in the third leaves (tip region) of the three barley lines in light (.....) or in darkness (_____) following inoculation with *B. graminis* f.sp hordei. Each darum is the mean of four replicates, with standard error. (◊) uninfected, (♦) infected

0.05) between seven and nine days after inoculation.

DISCUSSION

Measurements of stomatal function in whole leaves of the three lines cv. Prisma and the two wild lines, B19909 and I-17-40 following inoculation showed significant alterations as the result of infection. Stomatal resistance in the light in the inoculated middle region of infected leaves was initially increased by infection in all three lines, but as the leaves became more heavily infected, it began to decrease to a level similar to that of uninfected plants in line B19909 but lower than the uninfected plants in cv. Prisma. In contrast, stomatal resistance in the dark in the inoculated middle regions of infected leaves began to fall in both line I-17-40 and cv. Prisma from two and four days after inoculation respectively, but not until seven days after inoculation in line B19909. However, stomatal resistance eventually fell to lower levels in line B19909 than in the other two lines.

The initial increase in stomatal resistance in infected leaves in the light is likely to reduce the diffusion of CO_2 to the mesophyll cells and could thus be partly responsible for the decline in photosynthesis that occurred following inoculation. However, the subsequent reductions in stomatal resistance should allow increased CO_2 uptake. Altered stomatal behaviour following infection could also be expected to alter the rate of transpiration and the leaf water content, and reductions in leaf water content could affect rates of photosynthesis. The transpiration rate from leaves usually follows the pattern of stomatal behaviour. Thus in mildew infected barley leaves transpiration would be expected to initially decrease in the light because infection causes the stomata to close. It would also increase in the dark, when the stomata failed to close completely. Changes in transpiration in infected leaves could also result partly from the increase in the boundary layer resistance caused by the presence of the fungal mycelium over the leaf surface and partly from the mycelium itself which also provides an increased route for cuticular transpiration. In the light, stomatal resistance increased significantly from 24 hours after inoculation when mildew development was very limited in all three lines, suggesting that the stomata provided the main control over water loss during the early stages of infection. However, in the dark, stomatal resistance decreased from 48 hours after inoculation in cv. Prisma, but not until 72 hours after inoculation in line I-17-40 and 168 hours after inoculation in line B19909. Thus stomatal function was impaired at a much earlier stage in cv. Prisma than in leaves of line B19909, the latter being able to control water loss up to quite a late stage of infection when 25% or more of the leaf area was colonised by mildew.

The increased stomatal closure in the light from 24 hours after inoculation of barley leaves is in agreement with the observations of Majernik (1965) who found that *B. graminis* f.sp. *hordei* decreased stomatal opening of barley leaves from 24 hours after inoculation. Martin *et al*. (1975) also observed that mildew infection decreased stomatal opening but slightly earlier than Majernik (1965), from around six hours after inoculation. However, neither of these studies related changes in stomatal function to the amount of mildew present at the time of stomatal resistance change and also they used different cultivars to the ones used here.

Infection also decreased stomatal opening in the light in mildewed pea leaves from three days after inoculation (Ayres, 1976) and from five days after inoculation in mildewed oat plants (Sabri, 1993). However, an initial increase in stomatal aperture was observed in pea leaves 48 hours after inoculation with *Erysiphe pisi* (Ayres, 1976) and in oat plants 72 hours after inoculation (Sabri, 1993). Martin *et al*. (1975) and Majernik (1965) in studies of wheat infected with *B. graminis* f.sp. *tritici* suggested that a volatile product of the fungus could be involved in the alteration of stomatal behaviour. If this is so, it may be that *B. graminis* f.sp. *hordei* produces a similar substance. However, other causes have been suggested, such as infection induced changes in the turgor pressures of the guard cells and of other epidermal cells (Ayres, 1976). Future work should concentrate on investigating the involvement of any metabolic substances in the alteration of stomatal behaviour in infected leaves as this could lead to an explanation of plant tolerance or intolerance of diseases.

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