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Effects of leaf wetness duration, relative humidity, light and dark on infection and sporulation by *Didymella rabiei* on chickpea

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No infection occurred at less than 95% relative humidity (r.h.) when chickpea plants were dried after inoculation with conidia of *Didymella rabiei*. Infection was significant when the dry leaves were exposed to 98% r.h. for 48 h. When inoculated plants were subjected to different leaf wetness periods, some infection occurred with 4 h wetness, and disease severity increased with wetness duration according to an exponential asymptote, with a maximum value after about 18 h. Germination of conidia and germ tube penetration increased linearly with increasing wetness periods when recorded 42 h after inoculation. With a 24-h wetness period, germination of conidia was first observed 12 h after inoculation and increased linearly with time up to 52 h (end of the experiment). Dry periods immediately after inoculation, followed by 24-h leaf wetness, reduced disease severity; as the dry period increased the severity decreased. Disease severity increased with increasing periods of darkness after inoculation. The number of pycnidia and the production of conidia on infected leaves increased only slightly with high r.h. (either in the light or in the dark), but large increases occurred over an 8-day period when the leaves were kept wet.

Introduction

Chickpea (*Cicer arietinum*) is an important food legume grown in ecologically diverse environments, including India, the Mediterranean region, eastern Africa, the Americas and Europe (Saxena, 1990). The crop is subjected to a wide range of photoperiod and hydrothermal regimes that may affect crop yield, but large fluctuations in its productivity commonly coincide with the severity of attacks by Ascochyta blight (caused by *Ascochyta rabiei*: teleomorph *Didymella rabiei*) (Jhorar *et al*., 1997).

The pathogen and the disease are affected by the weather at all crop growth stages up to maturity (Singh & Sharma, 1998). However, in common with other foliar fungal pathogens, *D. rabiei* will respond to its immediate environment (the crop microclimate), which in turn is affected by the prevailing weather conditions. Precise information on the crop microclimate and the response of the pathogen therefore is a prerequisite to assess the importance of microclimate on disease (Butler, 1993).

Moisture in general is an important weather element for fungal pathogens infecting aerial plant parts. When the temperature is favourable and the moisture requirements of a pathogen on a susceptible host are fully met for a sufficient time, an epidemic is likely to occur. This kind of potential role of moisture has been exploited frequently as a basis for disease forecasting (Royle & Butler, 1986). The role of host tissue wetness in infection by pathogens has been demonstrated by a number of researchers and, for different pathogens, the wetness duration requirements range from 0·5 to more than 100 h (Huber & Gillespie, 1992). The effect of free water on the host surface on sporulation has been less widely investigated. This lack of information may limit the application of forecasting methods. For some pathogens, sporulation may require longer wetness periods than the infection process (Fitt *et al*., 1989).

In the case of Ascochyta blight on chickpea, conidia of *D. rabiei* are reported to be released only from wet pycnidia. Hassani (1981) showed that germination of *D. rabiei* conidia can be bipolar and reach 100% in free water. At least 6 h of wetness are reported to be necessary for infection of chickpea at the optimum temperature (Weltzien & Kaack, 1984) and longer wetness periods would be required for infection at suboptimal temperatures. As the leaf wetness period is increased beyond the minimum required for infection, the disease increases rapidly. However, Trapero-Casas & Kaiser (1992) reported that a short break in wetness (6 h) immediately after inoculation substantially increases the disease.

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Humidity directly affects sporulation by many fungi and has implications for the persistence of wetness. Relative humidity (r.h.) is often a good indicator of whether the plant surface is wet or dry, though r.h. may not always correlate with wetness (Royle & Butler, 1986). High r.h. in association with favourable temperature is reported to affect Ascochyta blight on chickpea, but different workers (Chauhan & Sinha, 1973; Hassani, 1981; Singh *et al*., 1982; Reddy & Singh, 1990) defined different humidity ranges for the disease. No systematic study has been reported with precisely controlled humidity values.

Light may affect spore germination, penetration, infection type, spore release and viability in fungal plant pathogens (Colhoun, 1973). Kaiser (1973) and Nene (1981) reported that continuous light increased mycelial growth and sporulation of *D. rabiei* on potato dextrose agar and chickpea seed meal agar, compared with continuous darkness. However, sporulation was always greater under alternate light and dark conditions. The effects of light and dark on disease development and sporulation of *D. rabiei* on chickpea plants have not been reported.

This paper aims to investigate further the effects of leaf wetness, r.h., light and dark on the infection and sporulation of *D. rabiei* on chickpea. Germination of conidia, host penetration, infection and production of conidia are quantified in controlled environment experiments.

Materials and methods

Chickpea cv. L-550 was used in all experiments. Fifteen-day-old chickpea plants were grown in pots (two plants per pot) filled with steam-sterilized potting medium (50% loam, 25% sand and 25% compost) in a glasshouse at 20–28°C. Experiments were repeated four times unless otherwise stated.

Inoculum was multiplied from a culture of *D. rabiei* race 11 (obtained from Punjab Agricultural University, Ludhiana, Singh, 1990) on autoclaved (103·5 kPas, 20 min), water-soaked chickpea seeds at 20°C for 7 days. Chickpea seeds containing pycnidia with conidia were washed with distilled water by stirring for 10 min and the suspension was filtered through muslin cloth. A few drops of Tween 80 wetting agent (polyoxyethylene sorbitan mono-oleate) were added to each washing (about 250 mL water). The concentration of conidia in the suspension was determined with a haemocytometer and dilutions were made to obtain 10^6 conidia per mL. Plants were inoculated by spraying the suspension with an atomizer, ensuring that both sides of the leaves were completely wet. For experiments on infection, the treatments were imposed immediately after inoculation as described below.

A randomized block design (RBD) with four replicates was used in each experiment. After completing the treatments, plants were moved to a controlled environment growth cabinet (Conviron Model E15, Winnipeg, Canada), adjusted to a 12-h photoperiod with a photosynthetic photon flux density (PPFD) of 450 μmol m^{-2} s^{-1} and to constant temperature and r.h. of 20°C and 85%, respectively. In initial experiments, symptoms were assessed for each leaf on sequential days, using visual estimates of the percentage necrotic area. Since the disease did not increase beyond 7 days after inoculation, observations were recorded on the seventh day in subsequent experiments. Disease severity was recorded by making a visual assessment of the percentage necrotic area on each leaf (except the top leaf, which appeared after inoculation), and averaging the values to obtain an overall disease severity per plant.

An analysis of variance (ANOVA) was carried out for each experiment to obtain the critical difference (CD) between treatments and replications. A square-root transformation $(x + 0{\cdot}5)^{0{\cdot}5}$ was applied to the disease severity data for the ANOVA (Snedecor & Cochran, 1967). There were no significant differences between replicates within any experimental run, so these data were pooled and repeated experiments treated as replicates. Response curves were fitted to obtain relationships between environmental and pathogen/disease variables. For linear regressions, the coefficient of variation (r^2) was used to indicate the goodness of fit. Nonlinear responses were fitted by the Quasi-Newton method, where r^2 is calculated from the corrected sum-of-squares ($r^2 = 1 -$ residual sum-of-squares/corrected sum-of-squares (Wilkinson, 1990)).

Effect of leaf wetness duration on infection

Two sets of experiments (I and II) were carried out, one to observe germination of conidia and germ tube penetration, and another to observe Ascochyta blight symptoms. For set I, inoculated plants were exposed to wetness periods of 0, 2, 3, 4, 6, 12, 18 and 24 h at a constant temperature of 20°C in a dew chamber. The dew chamber design was modified from that of Clifford (1973), as described by Butler *et al*. (1994). At the end of the required wetness period, the plants were removed to the growth cabinet, where the leaves remained dry, and were kept there until symptoms appeared. For each treatment, four leaflets from each of the second, third, fourth and fifth leaves were detached for microscopic observations of spore germination and germ tube penetration. Samples were collected 0, 3, 6, 9, 12, 18, 24, 36, 42, 48 and 54 h after the beginning of the experiment. The detached leaflets were observed under a scanning electron microscope to aid recognition of nongerminated and germinated conidia and germ tube penetration by *D. rabiei* as these stages have not been clearly described in the available literature. Samples were also cleared by soaking for 4 h in a mixture of 3 parts glacial acetic acid and 1 part ethanol. The cleared leaflets were washed in running water to remove the acetic acid and ethanol, and then submerged in cotton blue dye to stain the fungus. The stained leaflets were examined under a light microscope with 400× magnification. Five

microscopic fields were selected, each with more than 100 conidia, and the numbers of nongerminated conidia, germinated conidia and host penetrations were recorded.

For set II (to assess the effect of wetness duration on Ascochyta blight symptoms), inoculated plants exposed to different wetness periods (2, 3, 4, 6, 8, 10, 12, 18 and 24 h) at 20°C in the dew chamber were moved to the growth cabinet for symptom development, the same design being maintained as in the dew chamber.

Effect of drying leaves after inoculation

Immediately after inoculation, chickpea plants were exposed to different dry treatments (0, 0·5, 2, 4, 6 and 8 h dryness) in a plant growth cabinet, prior to 24 h wetness in a dew chamber. Those plants with 0 h dryness were placed in the dew chamber immediately after inoculation. Others were dried under a fan before being placed in the plant growth cabinet (at 20°C and 85% r.h.) for the designated dry period. In both the growth cabinet and the dew chamber, the pots were arranged in a random block design with four replicates. After 24 h wetness in the dew chamber, the plants were placed again in the growth cabinet to await symptom appearance.

Effect of relative humidity on infection

The effect of r.h. was studied in single-plant chambers housed in a basement room where the air temperature was maintained constant at 24°C with an evaporative cooling system. It was possible to maintain a constant r.h. in each chamber to ± 0·2%, by mixing moist and dry air streams with a proportional control system (Butler *et al*., 1995). After inoculation, the plants were dried under a fan and then exposed to 90, 92, 95, 98, 99 and 100% r.h. continuously for 48 h. They were then transferred to the growth cabinet to await symptom appearance. The first appearance of symptoms and disease severity per plant were recorded as described above.

Effect of light and dark on infection

This experiment was conducted in a growth cabinet adjusted to 20°C and 85% r.h.. The treatments were: (i) 12/12 h light and dark periods, (ii) 24 h dark followed by 12/12 h light and dark and (iii) 48 h dark followed by 12/12 h light and dark. The inoculated plants were kept in a 30 × 30 cm plastic tray containing free water and covered with polyethylene to maintain high r.h.. Transparent polyethylene was used to allow the plants to receive light and black polyethylene to keep the plants in the dark. After completion of the treatments, the plants were kept in the growth cabinet for symptom development. They were observed daily to note the first appearance of symptoms and, after 7 days, the disease severity per plant was recorded as described above.

Sporulation

Inoculated plants were placed in a dew chamber for 24 h and then moved to a growth cabinet set to 20°C and 85% r.h. Symptoms appeared after 5 days, by which time some pycnidia had already developed on the leaves. The diseased plants were divided into four sets. One was kept in the growth cabinet at 90% r.h.. The second set was moved to a dew chamber to provide continuous leaf wetness. The third set was covered with transparent polyethylene in a tray containing free water to provide high r.h. in the light. The fourth set, in a similar tray, was covered with black polyethylene in a tray containing free water to provide high r.h. in the dark. Although the r.h. was not measured in these containers, it was expected to be above 90%, but not to produce free water on the surface of leaves.

The two leaves with the highest disease severity were marked on each plant and pycnidia were counted daily on each leaflet using a stereo-microscope. Two other leaves from each treatment were detached and the conidia were dispersed in 40 mL of water by stirring for 20 min. The concentration of conidia in the resulting suspension was determined with a haemocytometer. The detached leaflets were washed a second time by stirring in a known volume of water for 20 min, but no conidia were detected in the second washing, confirming complete removal of detachable conidia in the first washing. This procedure was repeated for the first 3 days (while the number of conidia was increasing rapidly) and again after 8 days, when the number of pycnidia and conidia stopped increasing. The experiment was repeated twice.

Results

Effect of leaf wetness duration on infection

Germination of conidia, recorded 42 h after inoculation, was only 0·7% with 2 h of leaf wetness and increased linearly with increasing wetness duration up to 24 h (Fig. 1). When 24 h of leaf wetness was provided, germination was first observed 12 h after inoculation and increased linearly from 12 to 52 h after inoculation (Fig. 2).

There was no germ tube penetration after 42 h with 2 h of wetness and it was negligible with 4 h (Fig. 1). Thereafter the number of conidia with a penetrating germ tube increased linearly, from 1·9% with 6 h of wetness to 11·0% with 24 h of wetness and followed a similar trend to that for germination of conidia, but with about half the magnitude. With 2 h of leaf wetness no symptoms developed and with 3 h of wetness, infection was slight, giving traces of disease (Fig. 3). When the wetness duration (WD) increased beyond 4 h, disease severity (DS) increased rapidly and approached a plateau by about 18 h. The fitted curve in Fig. 3 is an exponential asymptote:

$$DS = 45{\cdot}7\ (1 - \exp\ (-0{\cdot}181\ (WD - 3{\cdot}2))) \qquad r^2 = 0{\cdot}99 \quad (1)$$

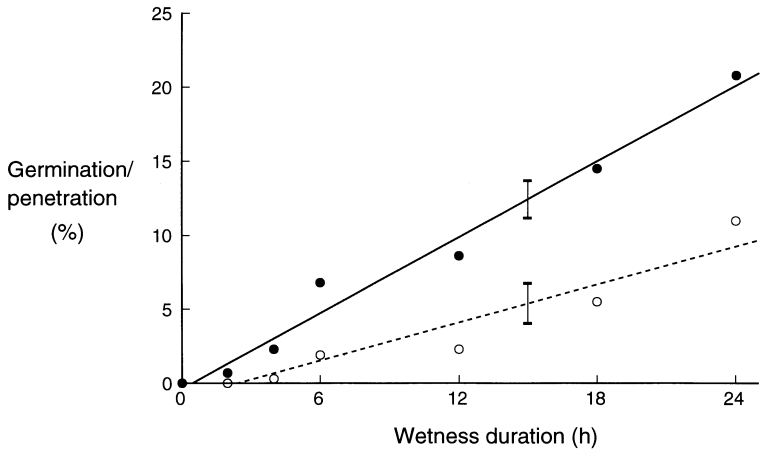

Figure 1 Effects of leaf wetness duration (WD) on germination of conidia and germ tube penetration. The regression line for germination (G, solid line) is $G = 0{\cdot}855 \times WD - 0{\cdot}391$ ($r^2 = 0{\cdot}98$) and that for penetration (P, dashed line) is $P = 0{\cdot}43 \times WD - 1{\cdot}07$ ($r^2 = 0{\cdot}91$). Key: ●, germination of conidia; ○, germ tube penetration. The vertical bar is the standard error.

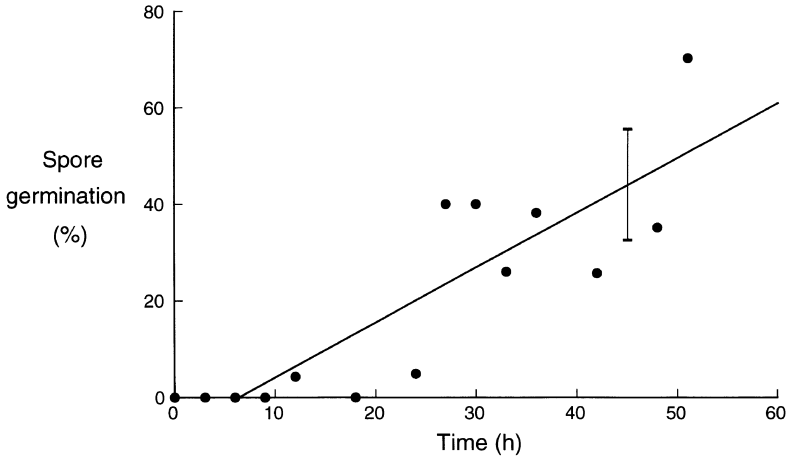

Figure 2 Germination of conidia with time (t), when 24 h wetness was provided. The regression line is $G = 1{\cdot}14 \times t - 7{\cdot}20$ ($r^2 = 0{\cdot}73$). The vertical bar is the standard error.

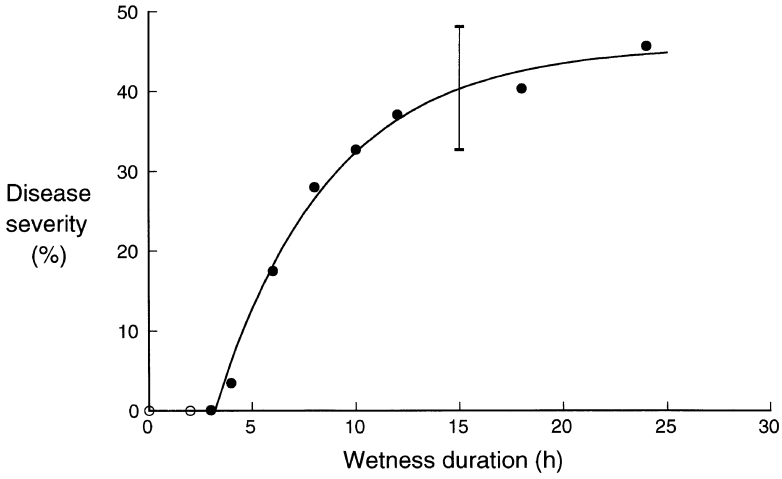

Figure 3 Effect of leaf wetness duration on disease severity for Ascochyta blight. The curve, given by eqn 1, was fitted by the Quasi-Newton method (Wilkinson, 1990). The open circles (for which the disease intensity was 0) were omitted in the fitting procedure. The vertical bar is the standard error.

where the asymptote is 45·7% and the minimum wetness period for infection to occur is 3·2 h.

Effect of drying leaves after inoculation

The maximum disease severity (44·2%) was observed with no dryness (24 h wetness) and disease severity decreased as the dry period increased from 0·5 to 8 h (Fig. 4). Differences were significant ($P < 0{\cdot}05$) between each specific dry period.

Effect of relative humidity on infection

In the single-plant controlled humidity chambers, the leaf surface always remained dry up to 98% r.h.; however, some transient free moisture may have

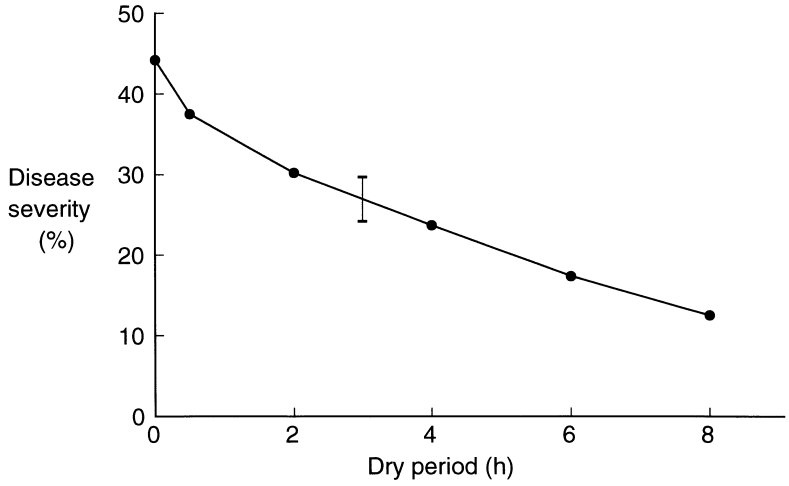

Figure 4 Effect of dry periods after inoculation, followed by 24 h leaf wetness, on disease severity. The vertical bar is the standard error.

condensed at 99 and 100% r.h. The results (Fig. 5) indicate that no infection occurred with less then 95% r.h. There was a large increase in disease severity between 95% r.h. (0·8% disease) and 98% r.h. (24·8% disease), and an even steeper rise between 98 and 100% r.h.

Effect of light and dark on infection

Disease severity increased with the total period of darkness provided before the 12-h photoperiod (Fig. 6). There was significantly less disease with no preceding dark period than when a dark period was provided, but the difference between 24 and 48 h darkness was not significant ($P > 0{\cdot}05$).

Sporulation

The number of pycnidia that developed with continuous leaf wetness was more than double that in all the other treatments (90% r.h. in the light, high r.h. in the light and high r.h. in the dark, Fig. 7). The wetness treatment showed a steep increase in the density of pycnidia for the first 4 days, after which there was little further increase. An exponential asymptote fitted to the data gave the equation:

$$Py = 1293\ (1 - \exp\ (-0{\cdot}349 \times t)) \qquad r^2 = 0{\cdot}96 \quad (2)$$

where Py is the number of pycnidia per leaf and t is time (days). There were no significant differences between the other treatments, where only a slight increase in the density of pycnidia was detected 7 days after starting the treatments.

The effects of different treatments on the number of conidia per leaf are presented in Fig. 8. With wetness there was a steep rate of increase in the number of conidia for the first 3 days, followed by a decline. Again, the trend followed that of an exponential asymptote, for which the equation is:

$$S = 6{\cdot}51 \times 10^8\ \ (1 - \exp\ \ (-0{\cdot}304 \times t)) \qquad r^2 = 0{\cdot}97 \quad (3)$$

where S is the number of conidia per leaf. The other treatments were similar to each other, with little or no increase in the number of conidia with time.

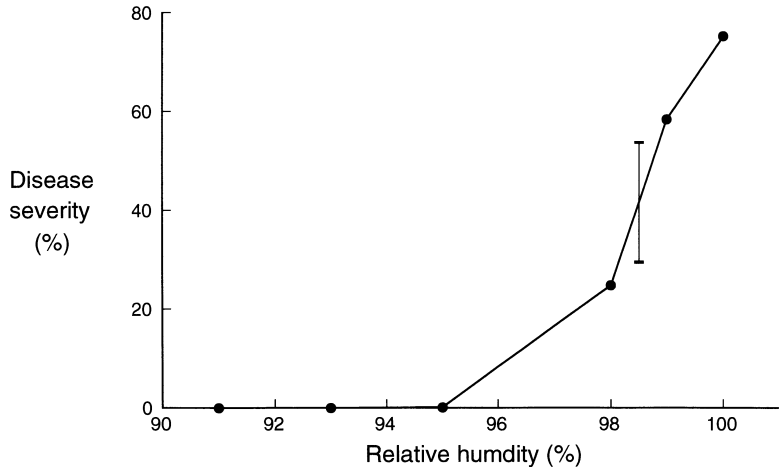

Figure 5 Effect of relative humidity on the severity of Ascochyta blight infection. After inoculation, the leaves were dried prior to exposure in the controlled humidity chambers for 48 h. The vertical bar is the standard error.

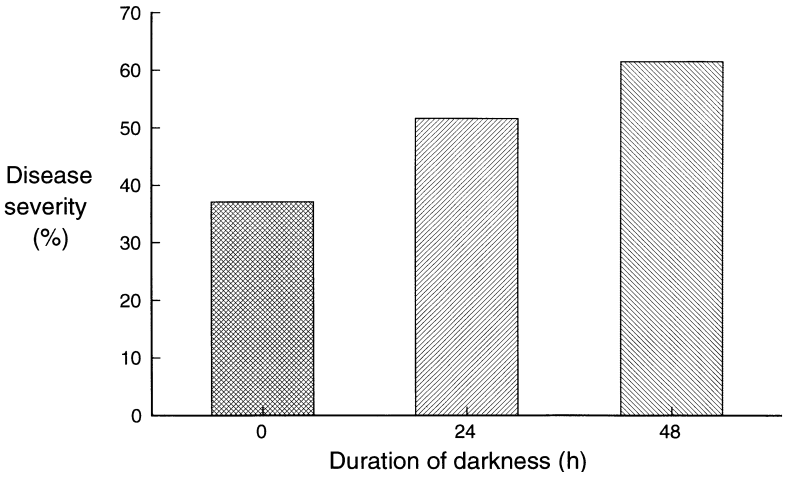

Figure 6 Effect of light and dark on Ascochyta blight severity. The critical difference (CD) between the treatments is 11·6.

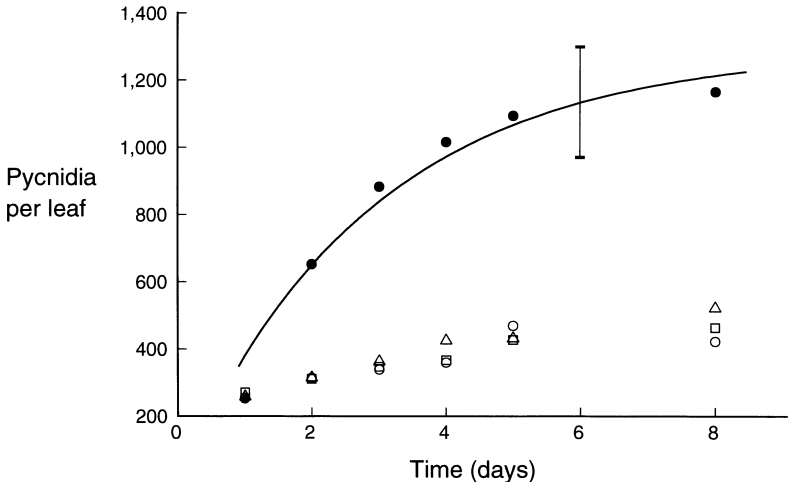

Figure 7 Effects of wetness, humidity and darkness on the production of pycnidia. The curve for the effect of wetness, given by eqn 2, was fitted by the Quasi-Newton method (Wilkinson, 1990). Key: ●, continuous leaf wetness; △, high r.h. in the dark; □, high r.h. in the light; ○, 90% r.h. in the light. The vertical bar is the standard error.

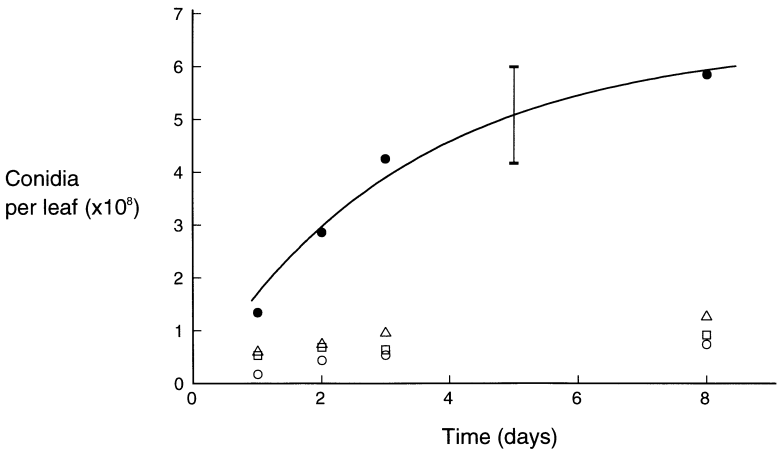

Figure 8 Effects of wetness, humidity and darkness on the production of conidia. The curve for the effect of wetness, given by eqn 3, was fitted by the Quasi-Newton method (Wilkinson, 1990). Key: ●, continuous leaf wetness; △, high r.h. in the dark; □, high r.h. in the light; ○, 90% r.h. in the light. The vertical bar is the standard error.

Discussion

Experiments were conducted in controlled environments at a constant temperature to investigate the effects of leaf wetness duration, dryness duration, r.h., and light and dark on various components of infection and on the establishment of Ascochyta blight on chickpea. Effects of temperature on the disease were not considered, but relevant information is available in the literature (Bedi & Aujla, 1970; Singh *et al*., 1982; Weltzien & Kaack, 1984; Trapero-Casas & Kaiser, 1992).

Although some conidia (0·7%) germinated on the leaf surface with a wetness period of only 2 h, significant germ tube penetration did not occur until the wetness period was 4 h or more. Results from this microscope study provide support to those of the experiments where disease symptoms were assessed. Disease appeared in traces with 3 h of wetness, but the value did not become significantly different from zero until 4 h of wetness was provided. The evidence indicates that the minimum requirement of leaf wetness for significant infection at 20°C is 4 h, somewhat less than the earlier reported value of 6 h (Weltzien & Kaack, 1984; Trapero-Casas & Kaiser, 1992). In these previous reports, disease occurred with 6 h leaf wetness or less, but the levels were not significantly different from zero. In the present work, wetness treatments less than or equal to 6 h were examined closely to identify the minimum wetness period for infection.

Germination of conidia and germ tube penetration increased linearly with increasing wetness duration; however, disease severity approached an asymptote after about 18 h of wetness. These results support the suggestion of Pandey *et al*. (1985) that longer wetness periods, with a favourable temperature, would favour disease development by maintaining the viability of germ tubes longer, thereby increasing the chances of penetration. However, disease severity will only be limited by wetness periods shorter than those needed to approach the disease asymptote. The difference in disease severity between 18 and 24 h wetness was not significant so it appears that, for a single disease cycle, a wetness period of 18 h would allow almost maximum infection. Additional experiments to assess infection with wetness periods longer than 24 h are needed to confirm this.

The effect of humidity on infection was assessed by drying chickpea plants immediately after inoculation and then exposing them to different humidity levels. The effect of continuously high r.h. for 48 h on these plants in the humidity chambers indicated that no infection occurred when the r.h. was less then 95%. At 95% r.h., the disease appeared in traces (0·8%) and at 98% it reached a severity of 24·8%. This was significantly different from the other treatments ($P < 0{\cdot}05$). Greater values of disease severity at 99 and 100% r.h. could have been affected by free water condensing on the leaf surface, but at 98% r.h. the temperature and vapour pressure in the chambers were sufficiently stable to avoid condensation (Butler *et al*., 1995). The experiments provide evidence that infection is possible with a dry surface when the r.h. is greater than 95%. Such conditions may occur on cloudy nights. This result supports the studies conducted under controlled conditions by Hassani (1981), which revealed that germination of conidia occurred at 98% r.h. and showed a large increase at 100% r.h. Arauz & Sutton (1989) also suggested that conidia may germinate under high humidity even without wetness. Singh *et al*. (1982) reported that wet inoculated plants (without drying the plant surface) became highly infected when exposed to r.h. > 85%. This would have been possible if wetness on the plant surface persisted for sufficient time to allow infection, an argument supported by our evidence that significant disease can develop with as little as 4 h wetness.

When 24 h leaf wetness was provided, no germination of conidia was observed in the first 9 h after inoculation (Fig. 2), yet germination can occur with only 2 h of leaf wetness when assessed 42 h after inoculation (Fig. 1). It is likely therefore that germination of conidia (and host penetration) may have occurred after the plants were removed from the dew chamber. This hypothesis is supported by evidence, from experiments in the controlled humidity chambers, that pathogen growth is possible on dry leaves with high r.h. (Fig. 5).

When the plants were dried for different periods immediately after inoculation, the reduction in disease severity was greatest initially (dry periods from 0 to 2 h), with a gradually decreasing slope as the dry period increased (Fig. 4). It is likely that germination and penetration of the pathogen was restricted by the break in wetness, and the conidia may have lost viability because of desiccation. This result contradicts the findings of Trapero-Casas & Kaiser (1992), who reported a substantial increase in disease when a 6-h dry period immediately after inoculation was followed by 24 h wetness. The reason for this difference in results is unclear, but may be related to the methods used to dry the leaves after inoculation.

The study on effects of light and dark indicated that continuous darkness resulted in significantly more disease. This may be because the plants became predisposed in the dark, the pathogen penetrating more aggressively through the cuticle. This hypothesis is supported by studies conducted by Benedict (1971) on *Puccinia graminis* on barberry leaves, which revealed that low light conditions induced penetration through the cuticle, thereby increasing the disease. Pandey *et al*. (1985) reported direct penetration by *D. rabiei* through the cuticle after appressorial formation instead of penetration through open stomata.

Results of the sporulation study showed a clear effect of surface wetness on both pycnidia and production of conidia. A similar effect was reported by Hassani (1981), where the effects of wetness, high r.h. and

darkness on sporulation were studied. In the present study, the plants exposed to continuous leaf wetness were also in the dark. In the concurrent treatments where light and dark were compared under high r.h., there was no significant effect on the production of pycnidia and only a slight increase in production of conidia in the dark (Fig. 8). This result agrees with the findings of Chauhan & Sinha (1973), that darkness favours sporulation, but differs from the reports of Nene (1981) and Kaiser (1973) from laboratory studies on artificial media. They found that continuous light favoured sporulation more than continuous dark. The reaction to the pathogen on artificial media is clearly different from that on live chickpea plants.

Results of the present study highlight the importance of both leaf wetness duration and r.h. to the epidemiology of Ascochyta blight. Daily periods of leaf wetness are commonly related to the diurnal pattern of r.h. (Butler, 1992), so a relationship might be expected between r.h. and Ascochyta blight in nature. Such a relationship was found in an analysis of historical weather data and the disease in the Punjab, India (Jhorar *et al*., 1997). For this region, disease was empirically related to both temperature and r.h., and a combined factor, the humid/thermal ratio, was suggested for predicting infection. The results of the present study provide a sound basis for better understanding of the mechanisms behind such empirical relationships.

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