ENVIRONMENT AND SPORULATION IN PHYTOPATHOGENIC FUNGI

I. MOISTURE IN RELATION TO THE PRODUCTION AND DISCHARGE OF CONIDIA OF PERONOSPORA TABACINA ADAM

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Summary

In an investigation into the effects of water relations on sporulation of *Peronospora tabacina* Adam in tobacco leaf disks the following results were obtained:

(i) Both diffusion pressure deficit (D.P.D.) and relative humidity (R.H.) were shown to be of critical importance to sporulation. The threshold values for maximum sporulation were 2·6 atm and 97 per cent. respectively. Minor deviations from these values towards either a higher D.P.D. or a lower R.H. very significantly reduced sporulation intensity.

(ii) Some aspects of the effect of R.H. level × time were analysed.

(iii) It was shown that in addition to the requirement of a minimum period of optimum conditions at a specific time of day for sporulation, there exists also a stage of conidiophore development which is completely dependent on favourable humidity conditions.

(iv) Subminimum periods of optimum humidity on consecutive days were shown to be non-cumulative, and to have no positive effect on sporulation.

(v) Both change in R.H. and mechanical shock were shown to be capable of causing conidial discharge.

These results are briefly discussed in relation to epidemiology of disease development.

I. INTRODUCTION

Yarwood (1956) has recently reviewed published work concerning the relationship between humidity and sporulation *in vivo* of plant pathogenic fungi. Little quantitative data on this subject has been published and qualitative results in most publications have been limited to field observations under uncontrolled conditions.

Studies of the sporulation of *Peronospora tabacina* Adam have been reported by Clayton and Gaines (1933), Armstrong and Sumner (1935), and Dixon, McLean, and Wolf (1936). These authors have shown that under field, seed-bed, and laboratory conditions sporulation was limited to the range of humidity from atmospheric saturation to slightly below dew-point and that it occurred most abundantly when these conditions occurred at night or over prolonged periods when the sky was overcast.

The mechanism of conidial discharge in *P. tabacina* has been previously studied by Pinkard (1942) who claimed that it was entirely a response to change in humidity.

In a preliminary note (Cruickshank and Müller 1957), a technique was described for the quantitative study of sporulation as a function of individual environmental factors under controlled conditions. This paper reports in greater detail the interaction between water relations and sporulation *in vivo*. An attempt is also made to evaluate the relative importance of humidity changes and mechanical shock in the discharge of conidia of this fungus.

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II. Materials and Methods

Physiologically similar leaves from uniform glass-house-grown tobacco (*Nicotiana tabacum* L. var. Virginia Gold) plants were used as the host plant material. Prior to the experiments described below the plants were inoculated with a spore suspension of *P. tabacina*. After incubation at high humidity (R.H.>97 per cent., temp. 20°C) for 48 hr the plants were transferred to a relatively low humidity in a glass-house until symptoms of vegetative development of the pathogen were visible as mild chlorosis of the leaves. At this pre-sporulation stage of symptom development, uniformly infected leaves were detached and disks (15 mm in diameter) were punched from them. These leaf disks were used as the basic leaf units in the quantitative experiments reported.

All experiments were carried out at constant temperature (20°C) and under a standard light intensity (600 lux, fluorescent light) and a 12-hr photoperiod (0600–1800 hr).

In the sporulation experiments, leaf disks were transferred, abaxial surface upwards, to micro-environment chambers (see Cruickshank and Müller 1957). The R.H. of the air above the leaf disks was controlled by glycerol–water mixtures (Carson 1931) in the base of the chambers. Refractive indices of the solutions were measured and the R.H. determined from a standard calibration curve. Where the effect of specific humidity levels was studied the chambers were sealed with glycerol. In all other experiments a high level of humidity (R.H. 98 per cent.) was readily maintained and sealing was unnecessary.

The diffusion pressure deficit (D.P.D.) of the leaf tissue was adjusted by floating leaf disks on mannitol and sucrose solutions contained in "Perspex" cells (15 mm in internal diameter, 5 mm deep) standing in the glycerol–water solutions.

At the termination of each experiment the micro-environment chambers were opened and the leaf disks removed from the "Perspex" cells, drained on filter paper to remove surplus liquid from the adaxial surface, and dropped into 0·5 ml of 50 per cent. ethanol in McCartney bottles. The latter were agitated for $\frac{1}{2}$ min on a "Microid" shaker and the concentration of the spore suspension measured by counting standard fields in a haemocytometer chamber. Morphological examination showed that only mature spores were released by this method. Serial transfer and agitation tests of disks also showed that more than 90 per cent. of spores were released into solution during the first agitation. The spore concentration was taken as a measure of the sporulation intensity.

In the experiments on conidial discharge the following modifications to the basic technique were made. Water–agar blocks (15 mm in diameter, 2 mm thick) replaced the mannitol solutions. The leaf disks were placed with their adaxial surface against the agar blocks which were inverted and attached by surface tension to the inside of petri-dish lids. One disk was placed centrally within each lid. A standard microscope slide smeared with glycerol was supported between the leaf disk and the glycerol solution as a spore trap. The intensity of spores caught on the slides was assessed by microscopic examination on a 0–10 scale (0 = no spores, 10 = heavy spore deposit).

Leaf disks were randomized and six to nine replications were used in each experiment. All experiments were repeated in time and results from representative experiments are given. The means of the spore concentrations were calculated as percentages of the maximum spore concentration within experiments and were plotted against treatment in the figures presented.

III. Experimental

(*a*) *Sporulation*

(i) *Effect of Diffusion Pressure Deficit of Host Tissue*

To study the effect of D.P.D. of leaf tissue on sporulation intensity the D.P.D. of the leaf disks was adjusted from 2·6 to 20·7 atm by floating them on solutions of sucrose and mannitol ranging from 0·1 to 0·8m. Zero D.P.D. was obtained using glass-distilled water only. The R.H. was kept constant (98 per cent.) and experiments were run over a period of 24 hr.

The possibility of toxicity of either sucrose or mannitol solutions influencing the viability of the pathogen appeared unlikely as spores of *P. tabacina* were successfully germinated on 0·1–0·6m solutions of both compounds. The difference between response of disks floated on mannitol and sucrose was probably attributable to slight permeability of the host cells to sucrose.

The results of this experiment are presented in Figure 1. The shape and slope of the response curve shows that maximal sporulation occurs at pressure deficits below 2·6 atm and that with increasing D.P.D. sporulation intensity rapidly decreases until complete inhibition is reached at 20·6 atm. The first and most significant drop in sporulation intensity appeared to be independent of permanent wilting as it occurred at a D.P.D. value well below that of the osmotic concentration* of tobacco leaf tissues.

(ii) *Effect of Relative Humidity of the Atmosphere*

The relationship between sporulation and R.H. of the air immediately adjacent to the adaxial leaf surface was studied using micro-environment chambers. The proportion of glycerol in glycerol–water mixtures was varied to give vapour pressures equivalent to 100, 99, 98, 97, 96, 94, 92, and 90 per cent. R.H. The D.P.D. was kept constant (2·6 atm) during the 24-hr duration of the experiment. Results are presented in Figure 2. An analysis of the data using the transformation log $(x+10)$ showed that significant ($P<0{\cdot}001$) reduction in sporulation intensity occurred between the 97 and 96 per cent. R.H. levels. There was no significant difference in sporulation intensity between 97 and 100 per cent. R.H. The latter could possibly have been due to incomplete control of R.H. over this humidity range owing to slight temperature fluctuation ($\pm 0{\cdot}5$°C). As the R.H. levels dropped towards 90 per cent. the sporulation intensity rapidly approached zero.

(iii) *Sporulation in Relation to Time*

In these experiments an attempt was made to study the development of conidia with time under optimal conditions and to determine whether a relationship existed

*Osmotic concentration of tobacco leaf 8–8·5 atm (cryoscopic method).

between time of onset of optimal humidity conditions, time of initiation of conidia formation, and final intensity of sporulation.

Water relationships were kept constant during these experiments (R.H. 98 per cent., D.P.D. 2·6 atm).

(1) *Development of conidia under optimal conditions.*—Conidial formation with time was studied by setting up 180 leaf disks under optimal conditions and removing at hourly intervals samples of nine disks for measurement of sporulation intensity. The experiment was initiated at 1300 hr and completed at 0900 hr. Sporulation was first detected at 0300 hr. The results presented in Figure 3 show that there is a very short interval of time between the first appearance of conidia and the attainment of the maximum level of sporulation intensity.

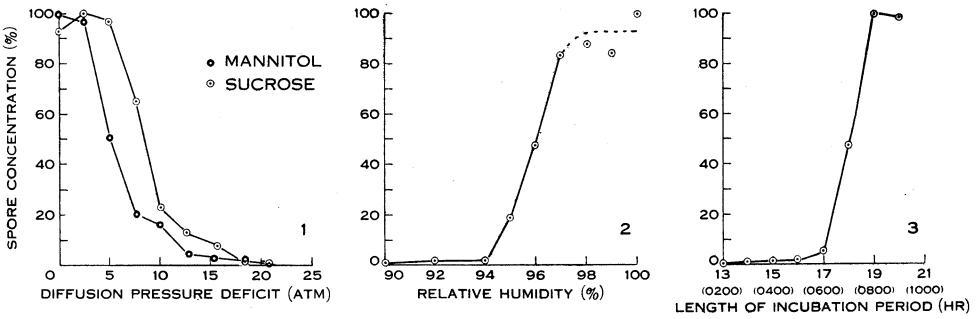

Fig. 1.—Relationship between diffusion pressure deficit of host tissue and sporulation.
Fig. 2.—Relationship between relative humidity of air and sporulation.
Fig. 3.—Sporulation response under optimal conditions. Time of day is given in parenthesis.

(2) *Relationship between humidity, time, and intensity of sporulation.*—Blocks of 30 leaf disks were set up in micro-environment chambers at 1400, 1800, 2200, 2300, 2400, 0100, 0200, and 0300 hr. Samples of six leaf disks were taken for measurement from each block at 0400, 0500, 0600, 0700, and 0800 hr. Later samples were omitted since preliminary tests had shown that normally no significant increase in sporulation intensity occurred after this time (see Fig. 3).

The results presented in Figure 4 illustrated three important points. Firstly, no simple relationship existed between onset of optimum humidity conditions and time of sporulation. Secondly, a minimum period of 3 hr of optimum humidity prior to 0500 hr was necessary for maximum sporulation to occur assuming that conditions remained optimal until 0800 hr. Finally, an analysis of the data using a suitable transformation ($Y = \log (x+10)$) indicated no significant difference in sporulation intensity at 0800 hr irrespective of the length of exposure of the leaf disks to optimum R.H. On the basis of the t-test, however, the sporulation resulting from the treatment initiated at 0300 hr appeared to be significantly lower ($P = 0{\cdot}05$) than all but one of the previous treatments. This latter test indicates that although conditions were satisfactory for sporulation to be initiated, there was barely adequate time for the maximum sporulation intensity to be reached during the duration of this experiment.

(iv) *Effect of Change in Humidity from Optimum to Suboptimum Level during Sporulation*

Since the above experiments had shown that humidity conditions must be satisfactory over a minimum period prior to sporulation for sporulation to occur, this

experiment was designed to test whether, given the above conditions, sporulation could be inhibited once it had begun by lowering the R.H. during and subsequent to the first appearance of conidia.

Leaf disks were placed under optimal conditions (R.H. 98 per cent., D.P.D. 2·6 atm) in micro-environment chambers at 1300 hr. From 2300 to 0600 hr inclusive nine leaf disk samples were transferred at hourly intervals to chambers at suboptimal humidity (R.H. 90 per cent., D.P.D. 2·6 atm). All disks were harvested at 0800 hr

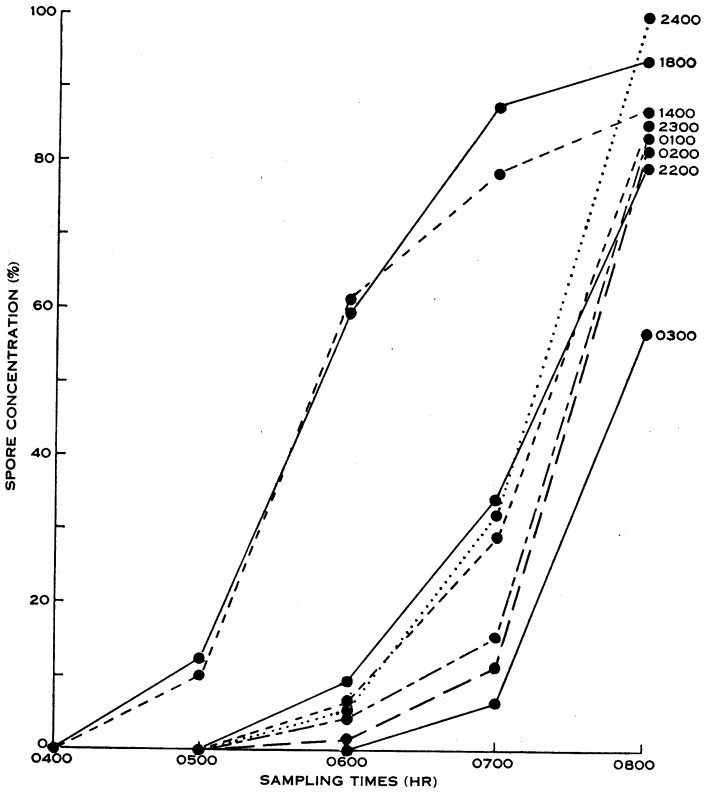

Fig. 4.—Relationship between onset of optimum relative humidity, first appearance of mature conidia, and final sporulation intensity. Treatment initiating times are indicated for each graph.

and sporulation intensity measured. The results presented in Figure 5 show that a change from optimal to suboptimal R.H. during the early stages of sporulation inhibits its further development but that, when the growth of the reproductive structures had reached a certain stage in their development, maturation occurs irrespective of subsequent changes in the R.H. Intermediate conditions also occur where sporulation, although not completely inhibited, is significantly reduced in intensity. The latter is probably due to unequal development within the conidiophore population.

(v) *Effect of Subminimal Periods of Optimal Humidity on Consecutive Days*

Preceding experiments showed that the most important time of day in relation to humidity and sporulation was between 2400 and 0600 hr. It was known from these

experiments that single subminimal periods of optimal humidity prior to 2400 hr were not inducive to sporulation but no data existed on the cumulative effect of subminimal periods of high humidity.

Optimum and suboptimum levels of humidity were similar to those given in Section III(*a*)(iv). Leaf disks were maintained under optimal conditions for periods of 3, 6, 9, 12, and 24 hr from 1200 hr on each of 4, 4, 3, 2, and 1 days respectively. Each day the disks were transferred for the balance of the 24 hr to the suboptimal humidity level. Other conditions remained unchanged. From Figure 6 it is clear that intermittent high humidity during the afternoon and evening hours is non-cumulative in its effect. Potential sporulation was not, however, affected by the treatments as continuation of the incubation period for a further 24 hr at 98 per cent. R.H. resulted in each case in normal sporulation.

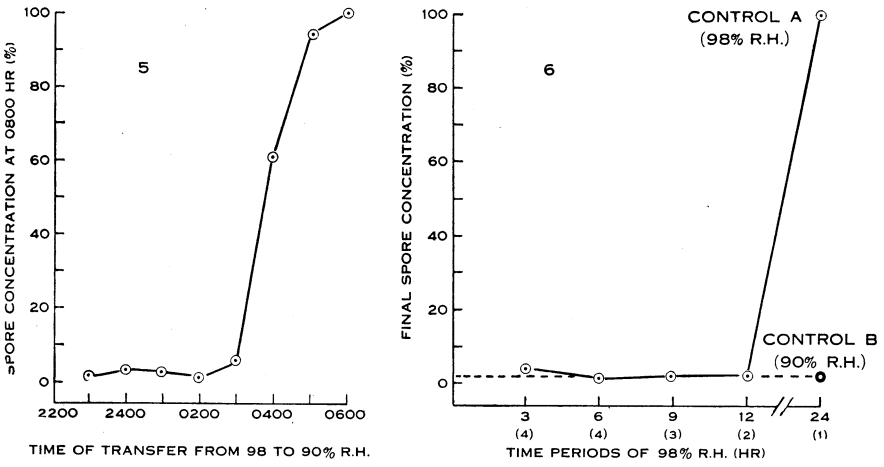

Fig. 5.—Effect on final sporulation intensity of change in relative humidity during sporulation.

Fig. 6.—Effect of subminimal periods of high humidity (R.H. = 98 per cent.) on sporulation. Number of days of each period is given in parenthesis.

(*b*) *Conidial Discharge*

Using the agar-block technique described above, leaf disks were set up under optimum conditions for sporulation for a period of 24 hr. The following treatments were then carried out and the intensity of spore discharge estimated.

(i) *Change of Relative Humidity.*—A second set of petri-dish bases were partly filled with glycerol–water mixtures designed to produce vapour pressures equivalent to 98, 90, 80, 70, 60, and 50 per cent. R.H. Glycerol slides were placed in position as described above and petri-dish lids carrying the sporulating leaf disks were rapidly transferred from the optimum humidity conditions to the lower R.H. series. The lids were left in the new positions for 1 hr. Six leaf disk replications were used.

(ii) *Mechanical Shock.*—The R.H. was maintained constant at 98 per cent. Glycerol slides were set up in the petri dishes prior to the initial 24-hr incubation period. The dishes were then transferred, with adequate protection against disturb-

ance in transit, to an apparatus in which a weight (1 g) was mounted centrally over the petri dish directly above the leaf disk. The weight was dropped on to the lid from heights of 5, 10, 20, 40, and 60 mm. Six leaf disk replications were used. Results of both experiments in Section III(*b*) are presented in Figures 7(*a*) and 7(*b*). From a comparison of the slopes of the curves it appeared that both changes in R.H. of the air adjacent to the sporulating surface and the magnitude of the mechanical shock suffered by the infected leaf were important contributing factors in the discharge of spores. This response increased with increasing magnitude of humidity change and size of mechanical shock.

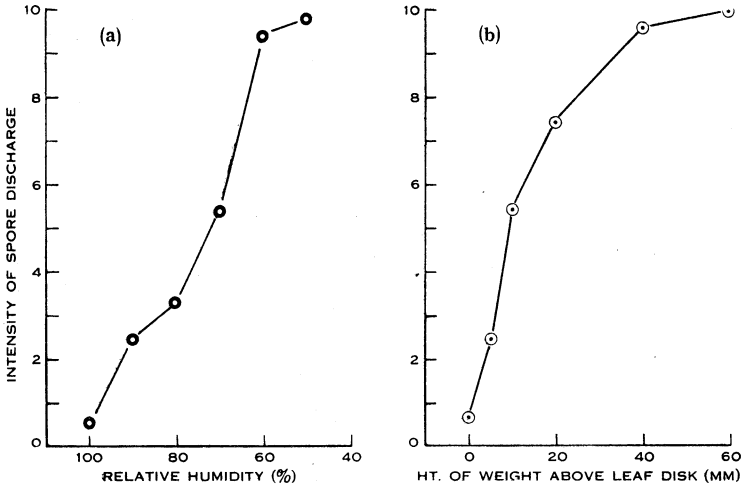

Fig. 7.—Intensity of spore discharge in relation to: (*a*) change in relative humidity; (*b*) mechanical shock (R.H. constant, 98 per cent.; magnitude of weight, 1 g).

IV. Discussion and Conclusions

Water relations, as shown by earlier workers, proved to be an important factor in the environment in relation to the phenomenon of sporulation in *P. tabacina*. While the general relationship between atmospheric humidity and sporulation has long been observed, the critical relationship between sporulation intensity and D.P.D. of the host tissue and R.H. of the air in the immediate vicinity of the abaxial leaf surface have not been previously reported. The leaf-disk technique has enabled the effect of hydrostatic pressures inside and vapour pressures outside the leaf tissues to be studied independently of each other while maintaining other conditions constant.

Threshold values of the D.P.D. and R.H. for maximum sporulation were 3–4 atm, and 97 per cent. respectively. Small increases in D.P.D. (Fig. 1) or decreases in R.H. (Fig. 2) very significantly decreased sporulation intensity. These analyses have demonstrated the critical sensitivity of the sporulation mechanism in *P. tabacina* to both internal and external water relations.

Wolf and McLean (1940) stated that under natural conditions conidia were produced at daybreak each morning. An analysis of the progressive development of

fructification in terms of conidia production with time under optimum conditions (Fig. 3) confirmed this claim. Similar experiments to the one reported showed that the position of the curve may move a little to the left or right but that the shape and slope are constant. On the basis of morphological observations at the time of sampling (Cruickshank, unpublished data) it was apparent that only fully developed spores were released into the alcohol solution by agitation. Conidiophores were macroscopically visible with immature conidia attached in samples taken up to 3 hr prior to the general detection (50 per cent. level of intensity) of fully developed spores. These observations along with the spore measurement data presented suggest that the incubation period required for sporulation may be divided into a relatively long one of vegetative growth of conidiophores and a short period of conidia formation and maturation.

Yarwood (1937) has suggested that light may exert a controlling influence on sporulation in certain downy mildews. The coincidence of conidia formation and maturation with the onset of light suggested it may play a role in determining the time of sporulation. Other results obtained confirmed that light was involved but that its effect was more indirect than previously reported.

The minimum period of optimum conditions required for sporulation of infected leaf has not previously been reported. Studies of sporulation in relation to varying periods of optimum conditions showed that 3 hr prior to 0500 hr were necessary (Fig. 4). However, when the results of Figures 4 and 5 are considered together it becomes apparent that, as the 0300 hr treatment would have matured irrespective of humidity change, if the experiment had been continued beyond 0800 hr, the minimum period of optimum conditions does not need to occur prior to 0500 hr. Infected leaf disks cut from plants grown under natural day length conditions could not be induced to sporulate at any other time of the day. A 12-hr difference (1400–0200 hr) in length of optimum conditions caused only a 2-hr difference in time of onset of conidia formation (10 per cent. level of intensity) and no significant difference in the final intensity of sporulation.

Change in R.H. during the hours of 2300 hr and 0600 hr from 98 to 90 per cent. (Fig. 5) showed that in addition to the requirement of a minimum period of optimum conditions at a specific time of day for sporulation, there exists also a stage in conidiophore development that is completely dependent on optimum humidity conditions. A drop in R.H. to 90 per cent. before this stage resulted in inhibition of conidia formation. If conidiophore development had progressed beyond this stage when the humidity was altered conidia formation and maturation continued to occur irrespective of the change in R.H.

The effect of high levels of humidity for periods of varying length from 1200 to 2400 hr on consecutive days was shown (Fig. 6) to be non-cumulative and to have no positive significance to the phenomenon of sporulation. The potential sporulation capacity of the infected tissues was not, however, affected as normal sporulation occurred when the necessary conditions were provided.

Pinkard (1942) has described the mechanism of conidial discharge in *P. tabacina* and attributed this phenomenon to R.H. changes. Yarwood (1943) in the course of studies on *P. destructor* concluded that conidia were released by changes in humidity and mechanical effects. In the present analysis it is shown that either change in

humidity or mechanical shock (Figs. 7(*a*) and 7(*b*)) may be involved in conidial discharge. Although under field conditions it would be difficult to separate these two factors, it was shown in the experiments described that each may act independently of the other and that their effect increases with the magnitude of the change in R.H. or the size of the shock. Spore dispersal was progressive and not simultaneous as reported by Wolf and McLean (1940).

Müller and Haigh (1953) and Waggoner (1956) have recently shown mathematically the relationship between sporulation intensity and the epidemiology of plant disease. The studies reported in this paper emphasize the great importance of favourable water relations to the sporulation *in vivo* of *P. tabacina* and show that minor deviations from the optimum humidity–time balance described will either extend the length of the incubation period of the development cycle or significantly decrease the intensity of sporulation. Either or both of these results would greatly affect the epidemiology of blue mould (*P. tabacina*) as it would result in a decrease in the speed of build-up of the spore population. The results presented may also explain much of the variation in blue mould that occurs both within and between tobacco crops (Angell and Wark 1955).

V. Acknowledgments

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