The Infection Site of *Sclerotinia sclerotiorum* Attacks on Sunflower terminal buds

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Abstract

A simple cold water spray apparatus was used to study the duration of leaf wetting necessary for infection of young leaves in the sunflower terminal bud by ascospores of *Sclerotinia sclerotiorum*. For temperatures between 15 and 18°C, 38h with continuously wet leaves were necessary for successful infection to occur. No infection was observed at temperatures above 20°C or if the 38h wetting cycle was interupted. Scanning electron microscope studies showed no evidence for any physical barrier to ascospore contamination of the young leaves. Fungal penetration of leaves was observed when the fungus had developed a sufficient mass of hyphae to penetrate the cuticle. The resistance of some sunflower genotypes was not related to any physical barrier but probably resulted from blockage of spore germination due to biochemical factors which remain to be identified.

Introduction

Sclerotinia sclerotiorum (Lib) de Bary attacks different parts of the sunflower plant : roots (Huang and Hoes, 1980), leaves (Cuk, 1978), capitula (Hoes, 1975) and terminal buds (Peres *et al.*, 1989). Ascospores produced by apothecia at the soil surface constitute the primary inoculum for all the aerial attacks. Different plants parts can be infected only if they remain wet for a sufficiently long period. Lamarque (1978) showed that 42h wetting were necessary, in the laboratory, for infection of the floral surface of sunflower capitula by *S.sclerotiorum* ascospores. Ascospores were observed on sunflower capitula by Says-Lesage and Tourvieille (1988) on the anthers, pistil, and internal suface of the corolla and extremities of the internal bracts. These authors observed that spore germination occured only on the anthers, pistil and internal surface of the corolla, and that development of hyphae from spore germination was favoured by the presence of pollen.
The aim of the present study was to evaluate the duration of wetting necessary for terminal bud infection by *S.sclerotiorum* ascospores and the penetration sites for this type of infection.

Materials and methods

Sunflower genotypes : Two inbred lines with extreme reactions to *Sclerotinia* terminal bud attack (Castaño *et al.*, 1993) were used : PAC1 (resistant) and CC40 (susceptible).

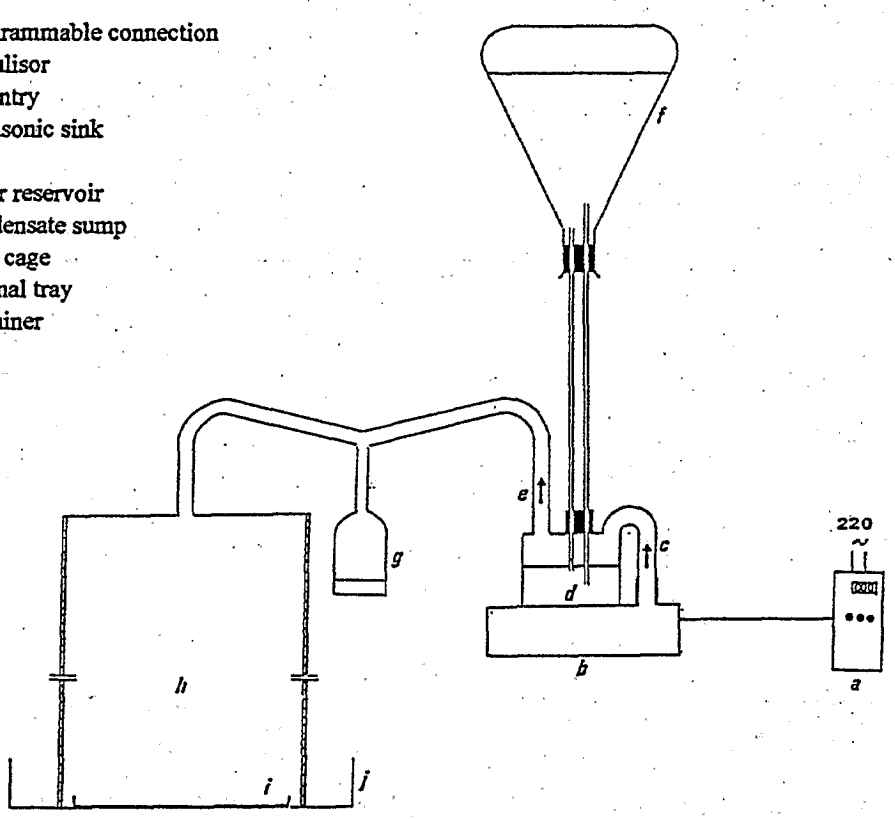
Fungal isolate : A mixture of ascospores produced from sclerotia collected from infected sunflower capitula was used for the artificial infections. The sclerotia were induced for ascospore production by placing them during 3 winter months in damp perlite, out of doors, then replacing them in damp soil-less compost in an unheated greenhouse at 12-18°C (Tourvieille *et al.*, 1978). Apothecia appeared after two weeks.

Experimental design : For the microscopical observations following artificial infections, plants were grown in pots of soil-less compost.

Artificial infection methods : The teminal buds of plants at the susceptible stage (6-12 leaves) were either infected with dry ascospores discharged directly from ripe apothecia (Says-Lesage and Tourvieille, 1988) or sprayed with suspensions of ascospores in distilled water (Vear and Tourvieille, 1987). The plants were then transfered to a humid chamber. A saturated atmosphere was maintained using a medical "ultrasonic-nebuliser" apparatus (Europe Medical NU52), normally used to treat respiratory disorders. This apparatus produced a fine mist resulting in slow deposition of water on the leaves (fig.1). Thirty minutes misting followed by a two hours break , at 15 to 23°C, gave continuous wetting of leaves and saturated humidity in the cage.

Fig 1. Diagram of continuous misting apparatus used in the study of the duration of watting of sunflower terminal buds necessary for *Sclerotinia sclerotiorum* infection.

a = programmable connection
b = nebulisor
c = air entry
d = ultrasonic sink
e = mist
f = water reservoir
g = condensate sump
h = mist cage
i = internal tray
j = container



Microscopical techniques : Observations of ascospore germination were made by the method of Preece (1959) using a light microscope. Samples mounted in water showed excellent colour contrast between the ascospores and lesions which appeared red, and the rest of the leaf, which was green. Epifluorescence microscopy was used to determine ascospore viability. These observations were made using the fluorochromatin test (FCR) of Heslop-Harrison *et al.*(1983), developed to determine pollen viability. Live *S.sclerotiorum* ascospores appeared green under blue light (495nm), whereas

the dead ascospores were colourless. A scanning electron microscope, Philips SEM 505, with a Hexland cryogenic stage, was used to observe leaf structure and ascospore contamination using the technique of Says-Lesage and Tourvieille (1988). Ilford HP5 400ASA film was used for photography.

Observations : Under the microscope, a scale of ascospore concentration was defined for each leaf zone : 0 = no ascospores; 1 = 1 to 20 ascospores/mm²; 2 = 21 to 50 ascospores/mm²; 3 = 51 to 100 ascospores/mm²; 4 = more than 100 ascospores/mm².

Results

Duration of leaf wetness : Continuous wetting treatments varied in duration from 4 to 48h, with two temperatures, 15-18°C and 20-23°C. Observations of ascospore germination and leaf infection are presented in table 1. Germination of spores only occured when wetting lasted at least 24h without interuption, and infection required these conditions for at least 38h. Infections were obtained when the temperature was 15-18°C but not when it was above 20°C.

Table 1. Effect of duration of continuous wetting on infection of sunflower terminal buds by *Sclerotinia sclerotiorum* according to temperate (+ : active germination or infection; - : no germination or infection)

Wetting period (hour)	Number of sequences	spore germination at 15-18 °C	at 20-23 °c	infection at 15-18 °C	at 20-23 °C
4	1	-	-	-	-
8	1	-	-	-	-
12	1	-	-	-	-
16	1	-	-	-	-
18	1	-	-	-	-
24	1	+	-	-	-
	2 (12 + 12)	-	-	-	-
30	1	+	-	-	-
38	1	+	-	+	-
	2 (18 + 20)	-	-	-	-
42	1	+	-	+	-
	3 (14+14+14)	-	-	-	-
48	1	+	-	+	-
	2 (24 + 24)	+	-	-	-

Microscopical Observations :

Contamination sites : The morphological structure of the small leaves making up the terminal buds of the two sunflower genotypes, CC40 (susceptible) and PAC1 (resistant) appeared identical. The apical part of the leaves showed fewer epidermal hairs than the other zones, with leaf tips having almost none. There were no apparent differences in structure of epidermal hairs, cell architecture or stomata number between the two lines.

A magnification of at least x 312 enabled ascospores to be observed without confusion with other structures. On plants sprayed with ascospore suspensions, few ascospores were observed. In contrast, all leaves showed inoculum from the dry apothecia discharge, but there were considerable

differences in the ascospore concentrations on different leaf parts (table 2). The large leaves showed the greatest numbers of ascospores. On the small leaves (length < 0.8cm), which were very close together, with most of their surfaces covered by other leaves, ascospores were mainly limited to the extremities. In contrast, it was the central part of the larger leaves (length >1.5cm) which showed the greatest concentrations, few ascospores being observed on their points.

Table 2. Ascospore concentration indices on leaves around the sunflower terminal bud

Inbred line	CC40	PAC1
Leaves of lengths :		
<0.8cm	0 - 1	0
0.8 - 1.5cm	1 - 2	1 - 2
1.5 - 2.0cm	2	4
2.0 - 3.0cm	4	4
3.0 - 4.0cm	4	4

0 = 0 ascospores 1 = 1--20 ascospores/mm²
2 = 21--50 ascospores/mm² 4 = >100 ascospores/mm²

Ascospore germination and mycelial development : No infections were obtained on plants sprayed with ascospore suspensions. When direct apothecia discharge was used, on the susceptible genotype, CC40, the first sign of infection was a discoloration of the zone around the ascospores, especially when they were in high concentrations. This effect was visible when there was a water film on the leaf. Some leaf regions appeared more favorable than others, in particular, the tips and bases of young leaves. At these sites, there was considerable development of *S.sclerotiorum* mycelium. Nevertheless, germination did occur on other parts of the leaf. A mucilaginous substance was observed around the ascospores. Hyphae were not attracted to the stomata and no penetration was observed through these openings. In contrast with CC40, observations made on the resistant genotype PAC1 showed an ascospore exudate but only very rare ascospore germination, and no further development of mycelium.

Discussion

Epidemiologists consider that two physical factors, temperature and relative humidity, are important in fungal infection processes. For *S.sclerotiorum* on sunflower, the duration of wetting required depends on the tissue invovled. On adult leaves, Sedun and Brown (1987) found that 72h was necessary for infection. On capitula at flowering , Saharan (1993), found that 30h were necessary for infection whereas Lamarque (1978) reported a need for 42h wetting. Our observations showed that 24h wetting were necessary for germination of spores on terminal buds, but that infection occurd only if continuous wetting remained 38h.

Microscopical studies on a susceptible sunflower genotype have provided some information about the beginning of the infection process of *S.sclerotiorum* ascospores on young sunflower leaves. The dry ascospores used in this study, freshly emitted by apothecia, adhered solidly to the leaf epidermis. In contrast, when ascospore suspensions in water were applied, they did not adhere. Such a suspension in water may dilute substances necessary for adhesion, as reported by Azmeh (1976) for *Puccinia* spp. spores on barley.

The presence of an exudate secreted by spores was observed, a phenomenon which may be necessary for recognition between the fungus and its host (Achbani, 1993). Various enzymes and soluble carbohydrates compose these exudates. For example, Fournet (1971) reported the presence, for *Phoma destructiva* spores, of pectinolytic enzymes which may stimulate penetration of the fungus in tomato leaf tissues.

Germination of *S.sclerotiorum* ascospores requires the presence of senescent tissues or some other nutritive support (Mellinger and Hooker, 1969) The present observations showed that infections of the susceptible genotypes always occured at the tip of young leaves around the terminal bud. On older leaves, infection generally starts at the base, near the main vein (Sedun and Brown, 1987), where a leaf exudate is produced. A comparison can also be made with *S.sclerotiorum* attacks on rapeseed where senescent petals provide the nutritive base (Brun *et al.*, 1983) and on sunflower capitula, where infection only occurs through the florets at anthesis (Says-Lesage and Tourvieille, 1988) when pollen and nectar provide the sources of energy necessary for ascospore germination. No leaf exudate was observed on the very young sunflower leaves, but the cuticle may not have formed, or may be very thin at the tip, permitting direct absorption of nutrients by *S.sclerotiorum* mycelium.

According to Purdy (1958), *S.sclerotiorum* ascospore germination will be followed by colonisation only if a sufficient mass of mycelium is produced. Rapilly (1991) reported that spores of *S.sclerotiorum* and *Botrytis cinerea* will not infect sunflower if they are present in very small numbers. Here, it was observed that direct discharge of an apothecium generally gave high concentrations of ascospores on the young sunflower leaves, and this was visible by the discoloration of the contaminated tissues. If the concentration of ascospores was low, no discoloration was visible, and in this case no infection was observed. .

Thus the resistant and susceptible genotypes showed no difference in their leaf morphology and no differences were observed concerning the presence of a mucilaginous substance produced by the ascospores. It was ascospore germination that differentiated the two lines, for this occured only very rarely on the resistant inbred. A hypothesis to explain this would be that the leaves of this genotype do not produce sufficient nutritive exudate to stimulate ascospore germination. As a result, only a few ascospores germinate and do not provide the mass of mycelium necessary for infection. Evidence for this comes from the observations by Achbani (1993) that ascospores could infect leaves of PAC1 if these were covered with a nutritive suspension such as a vegetable extract, or were necrosed in humid conditions.

Similarly, mycelial explants always infect sunflower leaves, whatever the genotype, although the mycelium extends more slowly on resistant genotypes than on susceptible ones (Achbani *et al.*, 1994). This suggests that there could also be production of some fungistatic compound by the leaves of resistant genotypes. Analyses of leaf surface exudates, not only for carbohydrates, but also for phenolic compounds which could hinder ascospore germination, are necessary to determine the validity of these hypotheses.

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