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The influence of pollen on the development of Cladosporium herbarum in the phyllosphere of rye

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Abstract

The natural occurrence of actively sporing colonies of *Cladosporium herbarum* near pollen clusters on the surfaces of rye leaves, suggested that pollen might be an important source of nutrients in the phyllosphere. One month after flowering, concentrations of ca. 100 pollen grains/cm^2 were not uncommon.
When artificially inoculated to rye, the development of *C. herbarum* was enhanced by simultaneous inoculation with 100 pollen grains/cm^2. Numbers of *C. herbarum* colonies developing on agar media from leaf washings taken 14 days after inoculation, were increased by a factor 2 to 3. Numbers of spores counted in situ increased by factors ranging from 4 to 16.
The naturally occurring sudden increases in numbers of colonies of *Cladosporium* spp. and other micro-organisms, isolated from rye leaves after flowering, might be attributed, directly or indirectly, to an effect of pollen.

Introduction

Populations of *Cladosporium* spp., *Alternaria tenuis* and other micro-organisms on rye leaves increase rapidly shortly before these leaves yellow (Kerling, 1964). The mechanism of this sudden change in the phyllosphere is unknown but may be attributable to the increasing deposition of airborne spores originating from older senescent leaves where these fungi readily sporulate. On the other hand metabolites released by the host at this stage may favour a gradual increase in population density, though it is difficult to explain the sudden increase by this factor.
When studying the phyllosphere microflora of green leaves of rye, it was noted that heavily sporulating colonies of *Cladosporium herbarum* Link ex Fr. occurred in clusters around pollen deposits (Fig. 1). Large numbers of pollen grains, suddenly released at flowering, are commonly found on leaves, and may affect nutrient concentrations in the phylloplane so favouring increased microbial activity.
The natural deposition of pollen was studied microscopically on leaves of field-grown crops of rye. It was later simulated in glasshouse experiments where the effect of pollen on the development of *C. herbarum* was tested.

Fig. 1. Natural deposits of pollen and *C. herbarum* on the flag (leaf 1) of field-grown rye. A: magnification × 75; B: magnification × 290.

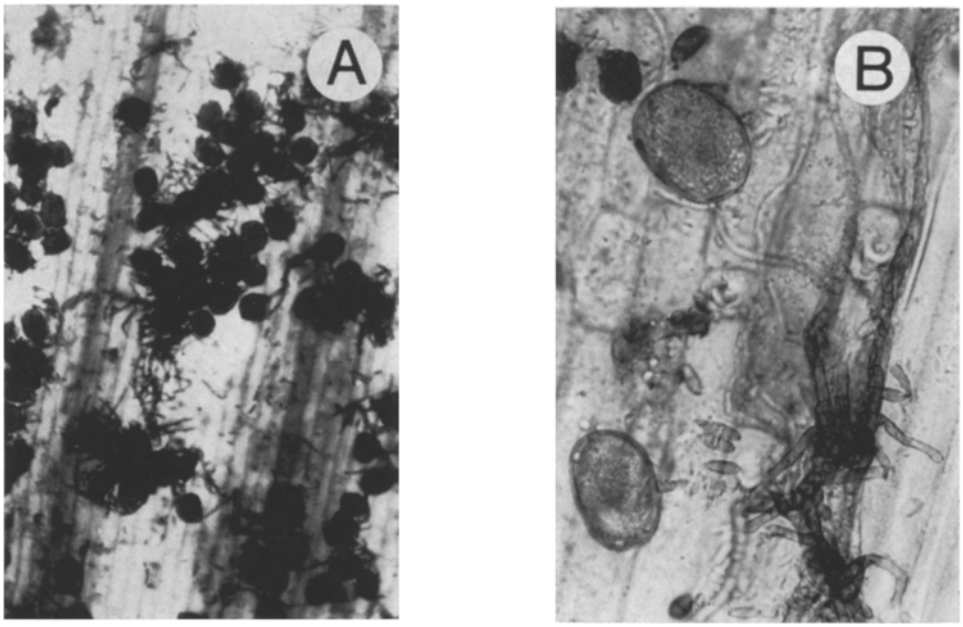

Fig. 1. Stuifmeel en C. herbarum op de vlag (blad 1) van rogge uit het veld. A: vergroting 75 ×; B: vergroting 290 ×

Materials and methods

The host
Summer rye, *Secale cereale* L. cv. 'Petkuser', was grown in the laboratory garden on humous sandy soil. Pollen deposition was studied on foliage sampled one month after flowering, and subsequently stored at –30 °C until examined.
Experiments were done with glasshouse plants, grown at temperatures between 18 ° and 22 °C, in which the lamina and ligule of the flag leaf were just visible. In this paper the flag leaf is denoted as leaf 1, the penultimate as leaf 2, and so on, leaves 2 and 3 being used experimentally.

The fungus
Field isolates of *C. herbarum* were cultured from 'Petkuser' rye. Spore suspensions were made from 8–10 days-old monospore cultures, grown on potato dextrose agar.

The pollen
Pollen grains were collected from ears of 'Petkuser' rye, picked at the beginning of anthesis, which were inverted for 24 h over petri dishes in a moist chamber. Because experiments were done when rye was no longer shedding pollen in the field some pollen was stored at –30 °C so that it, like fresh pollen, remained viable. Attemps to induce pollen germination were unsuccessful. Therefore staining of pollen with Nitro

Blue Tetrazolium (Nitro-BT) according to Hauser and Morrison (1964) was used as an indication of viability. Fresh and deep-frozen pollen stained on an average of 84% and 74%, respectively, suggesting a loss of viability during storage at −30 °C which was, however, relatively small compared with that on leaves. Pollen freshly deposited on leaves rapidly lost viability, the proportion of grains staining decreasing from 58 to 34 and 2% one and two days later. The deep-frozen and fresh pollen used in the experiments to be described, were uncontaminated.

Inoculation technique

Leaves were inoculated with freshly prepared suspensions containing ca. 5×10^6 *C. herbarum* spores/ml and 5 mg rye pollen/ml, plus 0.5% Tween 80. The mixture was shaken for 10 min, prolonged periods caused clumping, before 0.1 ml was spread over the upper surface of each leaf, leaves being drawn between the moistened thumb and index finger to ensure uniform distribution. Plants after inoculation and with ca. 100 pollen grains/cm² of leaf surface, were incubated in a moist chamber at 18 °–22 °C, and the development of *Cladosporium* was observed 14 days later. A control series of leaves was inoculated only with spores.

Methods of recording

Direct microscopic examination. Two drops of lactophenolcottonblue were carefully spread over upper leaf surfaces so as to stain pollen and fungi. When dry, the adaxial surface of a section of leaf was pressed against the adhesive of a strip of sellotape. The lower epidermis and mesophyll were then gently removed with a scalpel so that only the undamaged upper epidermis remained adhering to the sellotape. Lactophenol was added to the epidermis to expel air and to prevent drying. The sellotape with the epidermis was then inverted and placed on a slide. Numbers of *C. herbarum* spores per 6–10 mm² were readily counted on leaves prepared in this manner.

Indirect determination of Cladosporium populations. Sections of leaf of known area shaken for 3 h in volumes of sterile water. Loosely held spores and mycelial propagules of *Cladosporium* and other micro-organisms, became suspended (Kerling, 1964) and aliquots were plated on cherry agar. Most developing colonies, whose numbers were counted, were identified as *C. herbarum.*

Numbers of *C. herbarum* colonies on leaves not inoculated with this fungus, and attributable to natural contamination, rarely exceeded 5% of those developing after inoculation.

Results

Pollen deposits on leaves of field-grown rye

Numbers of pollen grains were counted on epidermal strips made from leaves 1–3 of ten plants stored at −30 °C, each leaf being divided into sections extending 3 cm and numbered from the base to the tip (Table 1).

When sampled 1 month after anthesis numerous pollen grains were still adhering to leaves. Although pollen was generally scattered on leaves 1, 2 and 3 greater numbers per cm² tended to occur on basal sections. Populations of ca. 100 grains/cm² were not uncommon – the density used in artificial inoculations.

The influence of pollen on the development of C. herbarum

Leaves inoculated with suspensions of *C. herbarum* spores, with or without pollen, were harvested 14 days later, when epidermal strips were prepared of a central 2 cm length of leaf. The remaining sections of leaf were rinsed, the resulting suspension being plated. Numbers of colonies developing from propagules washed from leaves treated with deep-frozen pollen were two to three times greater than those from leaves

Table 1. Numbers of pollen grains/cm² naturally occurring on leaves of field-grown rye, 1 month after flowering. Leaves were divided into 3 cm lengths, numbered 1 to 6 from the tip backwards.

Plant No.	*First leaf Section No.*						*Second leaf Section No.*						*Third leaf Section No.*					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1	223	167	82				71	151	63	34	53		94	96	137	155	23	63
2	116	43	20				118	50	57	15	37		142	74	51	17	26	
3	29	10	3	6	3		40	8	26	9	2	2						
4	13	9	14				90	40	10	13			200					
5	46	30					123	45	20	6								
6	180	7					577	434	235	84	19	16	602	628	18			
7	357							184	239	20			198	59	85			
8	300	11	16				185	75	22				215	88	19	117	28	26
9	366	298	131	66	20	17	282	261	103	40	11	8	135	125	102	33		
10	350	15	19				297	104	49	37			312	214	123	88		

Tabel 1. Aantal stuifmeelkorrels/cm² uit het veld op roggebladeren 1 maand na de bloei. De bladeren zijn verdeeld in secties van 3 cm lengte, genummerd 1 tot 6 van basis naar top.

Table 2. Effects of deep-frozen pollen on the development of *C. herbarum* on rye leaves, assessed 14 days after inoculation

Leaves with pollen (a)				*Leaves without pollen (b)*			
Plant	*leaf No.*	*numbers per cm²*		*plant*	*leaf No.*	*number per cm²*	
		*colonies**	*spores***			*colonies**	*spores***
A	2	23,000	16,000	D	2	8,000	760
	3	17,000	4,400		3	7,000	1,930
B	2	18,000	4,200	E	2	5,000	680
	3	21,000	–		3	6,000	–
C	2	48,000	6,200	F	2	8,000	490
	3	15,000	–			11,000	–
Mean		24,000	7,700		3	7,500	960

* Colonies developing on cherry agar from propagules in leaf washings

** Spores counted in situ on leaf surfaces

Tabel 2. Invloed van diepvriesstuifmeel op de ontwikkeling van C. herbarum op roggebladeren, 14 dagen na inoculatie

without pollen (significant, Wilcoxon's two sample test; $\alpha = 0.02$), mean numbers being increased from 7,500 to 24,000/cm^2 (Table 2). Similarly, numbers of *Cladosporium* spores, counted in situ on epidermal strips, were increased when rye leaves were also inoculated with pollen. Numbers of *Cladosporium* spores counted on leaves with pollen averaged 7,700/cm^2, and without pollen only 960/cm^2 (Table 2). Most *C. herbarum* spores appeared, when epidermal strips were examined, to have germinated irrespectively of the presence or absence of pollen. Subsequent growth of mycelium and sporulation, however, was more intense near pollen grains (Fig. 2), an observation that could still be made although many spores were displaced during the preparation of epidermal strips.

Similar results were obtained with pollen collected in November from glasshouse-grown rye. The plants were poorly developed because of a shortage of light, but nevertheless the influence of this pollen on the development of *C. herbarum* on leaves was evident (Table 3). Numbers of *C. herbarum* colonies developing from leaf washings collected 2 weeks after inoculating rye with this fungus plus pollen were 50% greater than those washed from leaves inoculated with *C. herbarum* alone (significant, Wilcoxon's two sample test; $\alpha = 0.1$).

Discussion

It is clear that pollen grains of rye increased the development of *C. herbarum*, when pollen and *C. herbarum* were simultaneously inoculated to leaves. Irrespective of recording methods, numbers of *C. herbarum* colonies isolated from leaf washings exceeded numbers of spores counted by direct microscopy. This observation suggests

Fig. 2. Pollen and developing colonies of *C. herbarum* on leaf 2 of glasshouse-grown rye. Photographed 18 days after inoculation. Magnification × 370.

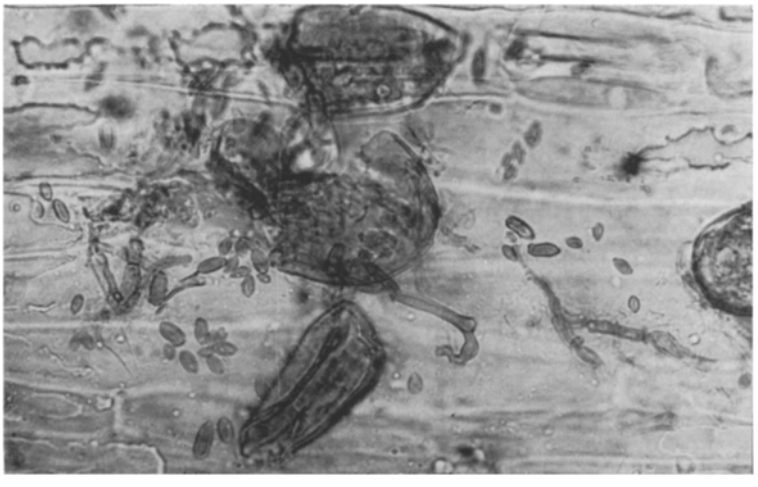

Fig. 2. Stuifmeel en C. herbarum op het 2e blad van een roggeplant, gekweekt in de kas, 18 dagen na inoculatie. Vergroting 370 ×.

Table 3. Effects of fresh pollen on the development of *C. herbarum* on rye (leaf 2) assessed 14 days after inoculation

Leaves with pollen		*Leaves without pollen*	
plant	*colonies* per cm²*	*plant*	*colonies per cm²*
G	12,900	M	8,300
H	6,100	N	8,400
I	8,600	O	3,300
J	9,000	P	5,400
K	10,000		
L	11,400		
Mean	9,700		6,300

* Colonies developing on cherry agar from propagules in leaf washings

Tabel 3. Invloed van vers stuifmeel op de ontwikkeling van C. herbarum op rogge (blad 2), 14 dagen na inoculatie

that most colonies from leaf washings were derived from mycelial propugales. Other tests suggested that mycelium could be readily removed from leaves by washing and was therefore mainly superficial.

Although an attempt was made to standardize inoculation and incubation conditions, there were nevertheless considerable differences between replicates. However, these conformed with the predicted trend, for large numbers of *Cladosporium* colonies from leaf 2 of plant C viz. 48,000/cm², were associated with 105 pollen grains/cm²; 18,000 *C. herbarum* colonies on leaf 2 of plant B were associated with 12.5 pollen grains/cm².

In favour of pollen *C. herbarum*, of which generally an active growth on green leaves was considered to be improbable (Last and Deighton, 1965), is able to develop on green leaves.

Sudden increases in populations of *Cladosporium* spp. and other micro-organisms on rye leaves, reported by Kerling (1964), might be attributed to the seasonal occurrence of pollen. The precise nature of the stimulant still needs investigation, also its effects on the activities of plant pathogens.

Acknowledgments

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Samenvatting

De invloed van stuifmeel op de ontwikkeling van Cladosporium herbarum in de fyllosfeer van rogge

Het voorkomen van sporulerende kolonies van *Cladosporium herbarum* in de nabijheid van stuifmeelconcentraties op roggebladeren wekte de indruk dat stuifmeel een

belangrijke bron van voedingsstoffen in de fyllosfeer zou kunnen zijn (Fig. 1 en 2). Een maand na de bloei bleek het stuifmeel nog in concentraties van ongeveer 100 korrels per cm^2 bladoppervlak voor te komen (Tabel 1). Deze concentratie werd in de proeven aangehouden. De ontwikkeling van *C. herbarum* op bladeren, geïnoculeerd met een sporensuspensie, werd vergeleken met die opbladeren behandeld met een mengsel van sporen en stuifmeelkorrels. Deze ontwikkeling werd 14 dagen na inoculatie bepaald door tellen van het aantal kolonies van *C. herbarum*, verkregen uit het spoelwater, en van het aantal sporen aanwezig op epidermis-strips (Tabel 2 en 3). Het aantal kolonies van bladeren met stuifmeel was twee tot drie malen hoger dan het aantal kolonies van bladeren zonder stuifmeel Op epidermis-strips bleek de aanwezigheid van stuifmeel de sporulatie 4 tot 16 malen verhoogd te hebben. De door Kerling (1964) vermelde plotselinge toename van *Cladosporium* spp. en andere microörganismen op bladeren van rogge, in het veld na de bloei, kan verklaard worden door aanwezigheid van stuifmeel.

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