

FUNGAL SPORE GERMINATION INHIBITORS FROM WHEAT LEAVES

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ABSTRACT

When water droplets containing spores of *Botrytis cinerea* and *Cladosporium fulvum* are placed on wheat leaf segments, antifungal compounds diffuse into the droplets. The nature of these antifungal compounds is discussed.

INTRODUCTION

Most plants are resistant to pathogenic invasion, and the ways in which this resistance is manifest are varied. One of the most interesting methods involves an alteration of metabolism in the plant cells in response to infection. Compounds toxic to the pathogen are produced and halt the pathogen's progression. Müller (1956) used the term "phytoalexin" for such compounds and the accumulation of phytoalexins by a number of plants in response to both fungal and bacterial invasion is now well documented (Ingham 1972). The ready ability of plants to produce these novel antimicrobial agents might well be harnessed to provide resistant plants or novel means of controlling plant disease.

The investigation described here is an attempt to elucidate aspects of the resistance of wheat varieties to fungal pathogens by determining whether wheat plants produced phytoalexin-type compounds in response to challenge by fungal spores. Since wheat is vital to the world's food requirement, its resistance to disease is of major concern.

MATERIALS AND METHODS

PLANTS

Four commercial varieties of wheat (*Triticum aestivum*) supplied by Rothwell Plant Breeders, Lincolnshire, U.K. were used: Joss Cambier, Cappelle-Desprez, Maris Widgeon and Maris Ranger. Plants were grown under mercury vapour lamps with a 16 hr : 8 hr light : dark regime at temperatures of 27-32°C day and 15-18°C night. Nine-day old plants were used.

FUNGI

Two fungi from the culture collection, University of Hull, were used: *Botrytis cinerea* and *Cladosporium fulvum*. Both are non-pathogenic to wheat. They were maintained and propagated on potato-dextrose agar.

Spore suspensions of *B. cinerea* and *C. fulvum* were obtained by adding small quantities of sterile distilled water to sporulating cultures and gently agitating. Freshly collected spores were used immediately for the experiment.

PHYTOALEXIN PRODUCTION

Droplets (50 μ l) of spore suspensions (50 000 spores/ml) were applied to segments of the first leaf of plants of all four varieties of wheat. The leaves were removed from plants and 70-80 mm segments cut and floated on water contained in a 90 mm petri dish. Distilled water was used for the control droplets. The petri dishes were incubated at 18°C for 18 or 36 hours. Very little bacterial contamination was observed in any of the droplets. After incubation the droplets were collected and were centrifuged to render cell-free. The supernatant was concentrated by evaporation to 1 ml (x8 concentration). This concentrate was used for germination and characterisation studies.

The concentrate was assayed for fungitoxicity by the slide germination test and by a chromatographic plate method.

SLIDE GERMINATION (SG)

Droplets (0.01 ml and 0.025 ml) of the concentrated toxin or control fluid were placed on a microscope slide and equal volumes of a spore suspension (100 000 spores/ml) added. The slides were incubated in moist chambers at 15°C. The level of inhibition of germination by the toxin was estimated by counting the percentage germination of 200 spores for each droplet. All tests were made in triplicate.

CHROMATOGRAPHIC PLATE BIOASSAY

Silica gel plates were developed in ethanol/chloroform (5:100) to separate the fungitoxic component of the concentrate, dried in air and sprayed with a suspension of spores of *C. fulvum* in Czapek Dox solution (Oxoid CM95). The plates were then incubated in a moist chamber in the dark at 25°C for three days. In the absence of inhibitors the fungus grows extensively on the silica gel and dark hyphae and spores become visible. Inhibitory zones are revealed as white zones devoid of mycelia.

RESULTS

A germination inhibitor active against *B. cinerea* spores was detected in droplets containing *B. cinerea* spores from all varieties of wheat leaves (see Table 1).

TABLE 1. INHIBITION OF SPORE GERMINATION BY LEAF DIFFUSATES

WHEAT VARIETY	% INHIBITION OF GERMINATION (SG Test)
Joss	32
Cappelle	61
M. Widgeon	54
M. Ranger	86
Leaf Control	12
Distilled Water Control	4

The inhibitor was also active against *C. fulvum* spores.

Spores germinating in the presence of the toxin produced irregular walled, curled germ tubes with a marked granular appearance, which contrasted to the smoother walled, straighter germ tubes in the controls.

NATURE OF TOXIN

The toxin was thermostable and not adversely affected by visible light. It appeared stable with no detectable loss of activity when stored at -20°C for three months. It was not readily soluble in water but was more soluble in diethyl ether than in ethanol.

Spectrophotometric analysis of the toxin (as extracted in water) revealed absorption maxima at 280 nm and 297 nm (Fig. 1). Addition of alkali (NaOH to pH 11.5) caused bathochromic shifts to 310 nm and 340 nm respectively.

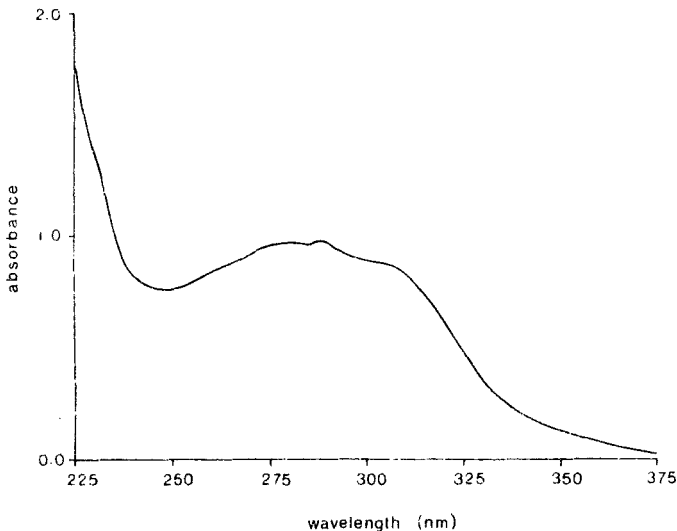


Fig. 1. Spectrum of the toxin produced by *Botrytis cinerea* on Joss Cambier leaf segments.

These data suggest the toxin may be phenolic in nature. However, two dimensional thin layer chromatography of the toxin on microgranular cellulose, using benzene-acetic acid-water (6:7:3 upper phase) for the first solvent and 2% acetic acid for the second direction revealed no fluorescent spots when examined under ultraviolet light and showed no reaction when sprayed with the phenolic indicator, diazotized paranitroaniline. This tends to militate against the phenolic nature of the toxin. Acidification of the toxin (with HCl) to pH 3.0

and extraction with redistilled diethyl ether followed by spore germination tests of the ether extract made aqueous, revealed that the ether fraction contained the spore germination inhibitory principle as shown in Table 2.

TABLE 2. INHIBITION OF SPORE GERMINATION BY
ETHER EXTRACTS OF DIFFUSATES

EXTRACTS	% INHIBITION OF GERMINATION
Spore droplet on leaf	73
Water droplet on leaf	14
Distilled water control	9

The ether fraction gave an inhibitory zone at Rf 0.48 in the chromatographic plate bioassay.

Many fungal spores are known to contain germination inhibitors and it is possible that these were responsible for some of the inhibition recorded. To investigate this possibility, *B. cinerea* spores were placed in droplets of distilled water for 18 h and the "washings" tested for inhibitory activity. Similarly, an ether extract of the "washings" was made and again tested by the slide germination technique.

The absorption spectrum obtained from the "spore washings" was markedly different from that of the inhibitor droplets (Fig. 2).

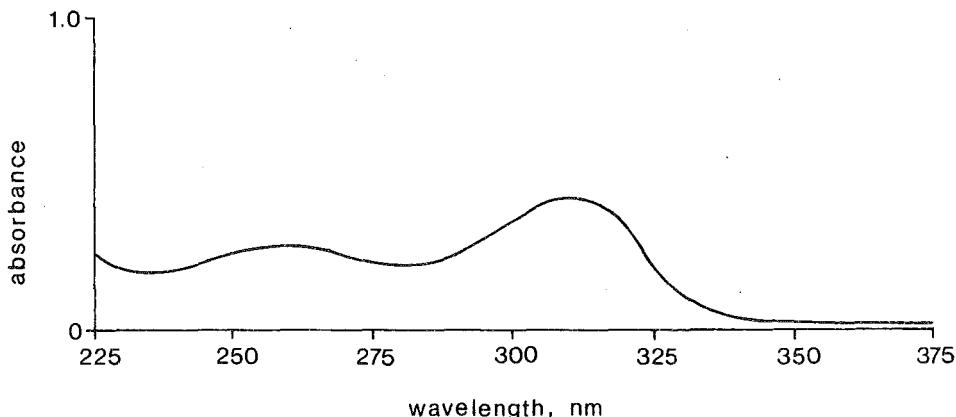


Fig. 2. Spectrum of washings from *Botrytis cinerea* spores.

A trough was obtained in the 280-285 nm range. The inhibitor spectrum shows peaks in this region (cf. Fig. 1).

DISCUSSION

The accumulation of toxic substances in tissues after infection is thought to be an important form of resistance of many plants to potential pathogens. The production of these substances is closely linked with a phenomenon known as hypersensitivity, where active responses of resistant plants cells to fungi can be seen. After contact or penetration by fungal germ tubes, the host plant cells, and often neighbouring cells, react to cause disorganization, browning and death of these cells. When *B. cinerea* spores are placed on wheat leaves, the germ tubes penetrate the epidermis and cause such a hypersensitive response; a brown flecking of the leaf at the site of spore droplet application is seen. Accompanying this hypersensitive response is the production of phytoalexins by the plant around the penetration area which assist to control the growth of the pathogen. Such a situation has been found in wheat leaves: infection by *B. cinerea* leads to a hypersensitive response, a production of fungitoxic compounds and the limitation of the fungal invasion.

Knowledge of the potential production of these antimicrobial compounds suggests the possibility of new disease control measures. Since phytoalexin formation can be stimulated by a variety of chemical agents (Perrin and Cruickshank 1965, Schwochau and Hadwiger 1968), chemicals could be used to induce the formation of phytoalexins prior to fungal spore alightment on the plant and thus spore germination and infection prevented.

Trace amounts of phytoalexins have been detected in many healthy plants and it may be possible to enhance the production of phytoalexins by suitable chemical treatments. Finally, genetic manipulations may enable susceptible plants to be converted to resistant plants if the appropriate genes for phytoalexin production can be incorporated into susceptible hosts.

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