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Production of Non-Specific Elicitors of Necrosis by *Collectotrichum lindemuthianum* Race ..

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ABSTRACT. *The presence of elicitors of necrosis in culture filtrates of Collectotrichum lindemuthianum race and intercellular fluids from leaves of Phaseolus vulgaris cv. Kievitsboon Koekoek infected with the compatible race was investigated. Culture filtrates and intercellular fluids were injected into leaves of bean cultivars resistant and susceptible to race. At 4 d after inoculation, during the bio-trophic phase of infection, the fungus was entirely intracellular. Culture filtrates produced necrotic lesions when injected into leaves of all cultivars, indicating the presence of non-specific elicitors. The crude culture filtrate produced more necrosis than dialyzed or desalted culture filtrate suggesting that, elicitors are of low molecular weight (less than 6 kioldaltons). Race-specific elicitors were not detected.*

INTRODUCTION

Many plant pathogenic fungi show specificity in causing disease, where only certain races of the pathogenic species can cause disease in certain cultivars of the crop species. Race-cultivar specificity was first xylose (Whitehead *et. al.*, 1982). Race-specific elicitors have also been reported in culture filtrates by Tepper and Anderson (1976) *C. lindemuthianum.*

Different race-cultivar combinations of *C. lindemuthianum* and bean show varied degrees of symptoms. Resistance in bean is associated with premature death and browning of infected cells (O' Connell and Bailey, 1988).

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The present investigation was conducted to determine whether diffusible race-specific or non-specific elicitor molecules are secreted by the fungus *C. lindemuthianum* in the course of infection in bean tissue and in culture filtrates *in vitro*. The study was conducted in the Department of Crop and Environmental Sciences, Long Ashton Research Station, Bristol (U.K.).

MATERIALS AND METHODS

Elicitors of necrosis in culture filtrates of *C. lindemuthianum*

The race (LARS culture No. 129) of *C. lindemuthianum* (Sacc. and Mag.) Briosi and Cav. was used in the experiment. The fungus was cultured on collectotrichum medium broth (Mathur *et. al.*, 1950) at 17±1°C on an orbital shaker.

The 3 d old liquid cultures were filtered using Whatman No. 1 filter paper and sterilized by passage through a 0.2 μm membrane filter (Acrodisc, Gelman Sciences). Some of the culture filtrate was desalted by gel filtration and some by dialysis using a dialysis membrane. For gel filtration, 3 ml of culture filtrate was applied to a Sephadex 10 DG column (Econo-Pac, Bio-Rad) and eluted with 4 ml of 10 mM sodium phosphate buffer (pH 7.0). The un-inoculated nutrient broth served as control discovered in bean, and *Collectotrichum lindemuthianum* fungus interaction (Barrus, 1918) that causes Anthracnose disease of French bean, *Phaseolus vulgaris* L.

The elicitor molecules are either bound to the fungal cell wall surface or secreted as freely diffusible molecules. Race-specific elicitors have been detected from compatible race-cultivar interaction of tomato and *Cladosporium fulvum*, (De Wit, *et. al.*; 1985), soybean and *Phytopthora megasperma* f sp. *glycinea* (Keen and Legrand, 1980); and a non-diffusible elicitor has been detected in the lettuce and *Bremia lactucae* system (Crucefix and Mansfield, 1984).

Albersheim and Anderson - Prouty (1975) and Keen and Legrand (1980) explained the molecular basis of specificity through the specific elicitor specific receptor model. A resistance gene in the host encodes a receptor protein on the plasma membrane, while an avirulence gene in the fungus encodes an enzyme responsible for synthesizing diffusible or surface bound elicitors. Recognition between the elicitor and receptor leads to a sequence of events (hypersensitive reaction, phytoalexin accumulation, lignification

etc.) culminating in resistance. Virulent races of the pathogen escape recognition and successfully cause infection.

Both race-specific and non-specific elicitors have been isolated from culture filtrates and mycelial walls of *Collectotrichum lindemuthianum* growing *in vitro*. The non-specific elicitors released from autoclaved mycelial cell walls were glucans with ß-1, 3 and ß-1, 4 linkages (Anderson, 1976), and those isolated from culture filtrates were polysaccharides.

Elicitors of necrosis in intercellular fluids of infected bean leaves

The French bean (*Phaseolus vulgaris* L.) cv. Kievitsboon Koekoek (KK) susceptible to *C. lindemuthianum* race, was used; as it would be possible to detect fungal products in the intercellular fluid of a susceptible host-pathogen interaction. The primary leaves from 8 day old seedlings were inoculated with $5x10^6$ spores ml^{-1} of race. Intercellular fluid was harvested 4 d after inoculation, as the fungus was in its biotrophic phase and the host cells were intact.

The infected leaves were first rinsed with water and then vacuum infiltrated with deionized water for 10 min using a water pump. The leaves were blotted dry and placed in a 50 ml centrifuge tube containing an aluminium mesh held 2 cm above its base to support the leaves. The leaves were then centrifuged for 20 min at 1000xg. The fluid that collected at the base of the tube was sterilized using a 0.2 μm membrane filter fitted to a syringe. Some of the intercellular fluid was concentrated four fold by lyophilization and redissolving in a known volume of deionized water. The fluid was concentrated, because infiltration of leaves with water would dilute the small amounts of elicitor molecules, if present in the apoplast. Intercellular fluid from un-inoculated leaves served as control.

Leaf injection assay

A range of bean cultivars having differential resistance or susceptibility to race were used to test the presence of race cultivar specific elicitors in the test liquids. The cultivars were Immuna, Widusa, Cornell 49-242, Red Mexican (RM), Dark Red Kidney (DRK), Processor R (resistant) and Kievitsboon Koekoek (KK) (susceptible).. Seeds of the cultivars were obtained from Long Ashton Research Station, Bristol.

Approximately, 200-300 μl of the test fluids were injected directly into the lateral veins, on the lower surface of primary leaves (8d old seedlings), at 5 to 6 different points per leaf, using a sterile hypodermic syringe. The injected leaves were tagged with labels, and the seedlings were incubated in a Fison 600 growth cabinet at 17 ± 1°C, 80% RH, 40 M mol m^{-2} s^{-1} photon flux density and a 16 h photoperiod. The injected leaves were assessed daily for the appearance of symptoms. There were 5 replications (5 plants/ replication) per treatment. The experiment was repeated twice.

RESULTS AND DISCUSSION

Intercellular fluids

The leaves of cultivars injected with dilute and concentrated intercellular fluids did not show any response even after 7 d. This could be due to low fungal biomass when the fluids were collected at 4d, leading to a low level of elicitors.

Race-specific elicitors would be expected to induce chlorosis or necrosis in resistant but not in susceptible cultivars; as reported by De Wit *et. al.*, (1985), where tomato leaves were infected by *Cladosporium fulvum.*

The cytological studies showed globose infection vesicles in the epidermal cells, beneath the appressoria, at 2d after inoculation and short, thick primary hyphae within single spidermal cells at 3d after inoculation. At 4d, long primary hyphae had colonized 2-3 cells, and very few hyphae were found in the intercellular spaces. It is thus clear that on 4d after infection the primary hyphae remain intracellular. This could be another reason for a low concentration of elicitors in the intercellular fluids. The intracellular primary hyphae had also grown below the epidermis into the cortical cells. O' Connell *et. al.*, (1988) reported similar findings in the hypocotyls of bean *cv.* KK.

Comparison of dialyzed, desalted and crude culture filtrates

The early symptoms of brown spots at the injection site began to appear 5d after injection, and by 9d, fully developed large necrotic lesions were seen. The results in Table 1 show that severe symptoms were produced in all cultivars injected with crude culture filtrates. Irregularly shaped, large

dry brown lesions extending beyond the injected area were visible on both sides of the leaves. This confirms the findings of O' Connell and Bailey (1988), that resistance in bean to the fungus is associated with premature cell death, appearing as brown spreading lesions (Hypersensitive reaction). The rapid cell death (hypersensitive reaction) is attributed to recognition between the host receptor and the pathogen elicitor immediately after penetration (O' Connell *et. al.*, 1985). In *cv.* KK, symptoms were observed only on the lower leaf surface.

Table 1. Symptom expression in leaves of French bean cultivars 9d after injection with dialyzed, desalted and crude culture filtrate of C. lidemuthianum race ...

Cultivars	Dialyzed	Desalted	Crude	Control
Imuna (R)*	+	+	+++	-
Widusa (R)*	++	+++	+++	-
KK (S)**	++	+	+++	-
Cornell 49-242 (R)*	-	++	+++	-
RM (R)*	-	+	+++	-
DRK (R)*	+	++	+++	-
Processor R (R)*	++	+++	+++	-

\- = No symptoms
\+ = brown spots at injection site
++ = browning along the veins with small dry lesions
+++ = browning along veins with large irregularly shaped dry brown lesions
*R = Resistant
**S = Susceptible
Three replicates per treatment.

Necrosis was observed in the leaves of both resistant and susceptible cultivars in response to crude culture filtrate. This indicates that non-specific elicitor(s) on necrosis are present in the culture filtrates. Whitehead *et. al.*, (1982) also reported the presence of non-specific elicitors of necrosis in culture filtrates of *C. lindemuthianum,* which were identified as polysaccharides.

The extent of symptoms produced in response to dialyzed culture filtrate was less than crude culture filtrate such as small brown spots at the site of injection as in *cv.* imuna and DRK and browning along the veins with small dry lesions as in *cv.* KK, Processor R and Widusa. This could be because dialysis removed molecules having molecular weight less than 10-12 kilodaltons. This indicates that, the elicitor molecules released by the fungus in the culture filtrate are less than 10-12 kilodaltons molecular weight.

The desalted culture filtrate produced severe symptoms only in *cvs.* Widusa and Processor R. The pale brown lesions in *cv.* Cornell 49-242 were more pronounced on the lower leaf surface. Crude culture filtrate produced more severe necrotic symptoms than desalted filtrate. Gel filtration removed molecules less than 6 kilodaltons, suggesting that most of the elicitor molecules have molecular weight less than 6 kilodaltons.

CONCLUSIONS

The intercellular fluids injected into leaves of a range of differential cultivars did not elicite any response. The cytology of infection revealed that 4d after inoculation, when intercellular fluid was harvested, the fungus was almost entirely intracellular; which may explain the absence of elicitors in intercellular fluids.

The leaves of cultivars injected with culture filtrate produced necrotic symptoms. The symptoms were more severe with crude culture filtrate than with dialyzed or desalted culture filtrate; suggesting that, most of the elicitor molecules have molecular weight, less than 6 kilodaltons.

Severity of symptoms varied among cultivars injected with dialyzed and desalted culture filtrates. Both the resistant and susceptible cultivars produced symptoms in response to injection with culture filtrates. The study revealed that culture filtrates contain a low molecular weight and non-specific elicitor molecules (toxins) that induce necrosis.

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