J. Mycopathol. Res. 32 *(2)* : 155-159 (1994) *Printed in India*

Hypersensitive Reaction by Non-Specific Elicitors of *Colletotrichum lindemuthianum* in French Bean Protoplasts

M. J. LEINA

Department of Sericulture, University of Agricultural Sciences, G.K.V.K., Bangalore 560 065, Karnataka

and

RICHARD J. O'CONNELL

Department of Crop and Environmental Sciences, Long Ashton Research Station, Long Ashton, Bristol BS 189AF, U.K.

The culture filtrates of *Colletotrichnm lindemuthianum* race ♀ and intercellular fluids from leaves of *Phaseolus vulgaris* cv. Kievitsboon Koekock infected with the compatible race ♀ was investigated for tha presence of elicitor of necrosis. The test fluids were incubated with protoplasts of bean cultivars

The crude culture filtrate caused more death of protoplasts than intercellular fluid. Crude culture filtrate greatly reduced protoplast viability of bean eultivars than desalted culture filtrate indicating that, elicitors are of less than 6000 daltons in molecular weight. The elicitor molecules are non-specific.

Key words : *Colletorichum lindemuthianum*, protoplasts, elicitor, hypersensitive reaction, bean

INTRODUCTION

Resistance in plants involves more than one active defense mechanism which occur either simultaneously or consequtively following attack by pathogens. In resistant cultivars, the infected cell and one or two adjacent cells die restricting further development of the fungal hyphae, resulting in Hypersensitive reaction

(Health, 1980). The expression of resistance or susceptibility, first proven in the flax rust system (Flor, 1956), is explained by the Gene for Gene hypothesis. Several metabolites of *C. lindemuthianum* that elicit hypersensitive reaction have been isolated from culture filtrates and mycelial walls *in vitro*, which are both race-specific and non-specific elicitors. The plasma membrane is the site for reception and transduction of stimuli originating from the pathogens. The existence of surface membrane receptors of glucan elicitors is shown in the binding of β-glucosyl Yariv antigens by plant protoplasts, specific binding of β, I-3 linked storage glucan to soybean membrane fragments (Yoshikawa *et al.*, 1983), alteration of protoplast membrane potential by cell wall derived β-glucans (Kota and Stelzig, 1977) and its agglutination (Peters *et. al.*, 1978).

The study was conducted using protoplasts of different bean cultivars to determine the presence of race-specfic or non-specific elicitors of necrosis in the intercellular fluid from a compatible bean-*Colletotrichum* interaction and in culture filtrates of the fungus. The lack of a cell wall makes protoplast a good model system to study the recognition process between host and pathogen *in vitro*. The study was undertaken in the Department of Crop and Environmental Sciences, Long Ashton Research Station, Bristol, U. K.

MATERIALS AND METHODS

The French bean (*Phaseolus vulgaris* L) cultivar Kievitsboon Koekoek (KK) susceptible to race γ (LARS culture No. 129) of *C. lindemuthianum* (Sacc. & Mag.) Briosi. and Cav. was used. The primary leaves from 8 d old seedlings were inoculated with 5×10^6 spores ml^{-1} of *C. lindemuthiamum* race γ. The intercellular fluid was harvested 4 d after inoculation according to Crucifix and Mansfield (1984). Intercellular fluid from uninoculated leaves served as control.

C. lindemuthianum fungus was cultured on *Colletotrichum* broth medium (Mathur *et al*, 1950) at $17 \pm 1°C$ on an orbital shaker. The 3 d old liquid cultures were filtered through 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 using Sephadex 10 DG column (Econo-Pac, Bio-Rad) to remove molecules of less than 6000 daltons molecular weight. Sterile *Colletotrichum* broth served as control.

The protoplasts of french bean cultivars Imuna, Dark Red Kidney (DRK), Red Mexican (RM), Cornell 49-242, Processor R (resistant cultivars) and Kievitsboon Koekoek (KK) (susceptible cultivar) were used for the study. The seedlings of the cultivars were incubated in a Fison 600 growth cabinet at $12 \pm 1°C$, 80% RH, 40 M mol m^{-2} s^{-1} photon flux density and 16 h photoperiod. The primary leaves of 8 d, old seedlings were used to prepare protoplsts adopting the method of Nagata and Shigetaka (1979).

The sterile test fluids were diluted (9 : 1) with sterile 4 M sorbitol containing 100 mM MES (pH 6.5), 2 mM KH_2PO_4, 10 mM KNO_3, 40 mM $CaCl_3$ and 100 mM L-arginine. The test fluids were incubated with the protoplasts of each cultivar in 9 : 1 ratio to get a final concentration of 1×10^5 protoplasts ml^{-1}. The aliquots (10 ml) of each protoplast mixture was pipetted into the wells of a Terasaki micro-test plate (Sartedt Inc, U. S. A.) and incubated for 10 minutes.

At the end of 10 minutes, the protoplasts of each cultivar was stained with fluorescein diacctate (200 mg ml^{-1} FDA, Sigma Chemical Co., in acetone). Only viable protoplasts fluoresce green due to esterase enzyme activity resulting in the accumulation of fluorescein (Larkin, 1976). The fluorescense was observed under Phase contrast microscope using cxitation filter 450-490, dichronic mirror FT510 and barrier filter LP 520 (Fig. 1).

RESULTS AND DISCUSSION

The result in Table 1 shows the highest proportion of protoplast (61.68%) of cv. KK remained alive after incubation with intercellular fluid. This is consistant with the fact that cv. KK is susceptible to *C. lindemuthianum race* γ. The receptors on the plasma membrane of susceptible bean protoplasts fail to recognise the elicitor molecules in the intercellular fluid. Thus the cells remain alive allowing the development of fungal hyphae and hence infection. The other cultivars tested showed higher cell death, indicating a hypersensitive reaction. The cvs. Processor R and Imuna showed maximum resistant reaction with 0.0% and 0.60% live protoplasts respectively in treatment with culture filtrate. The crude culture filtrate caused more cell death in all the cultivars tested compared to intercellular fiuid.

Cytological studies of cv. KK leaves on the 4th d after inoculation showed, long primary hyphae colonising 2-3 cells but none in the intercellular region. The low fungal biomass in the intercellular region attributes to the low level of elicitors in the intercellular fluid. O'Connell *et al.* (1985) also reported similar findings in the hypocotyl of bean cv. KK.

Incubation of protoplasts with crude culture filtrate caused maximum cell death (0.0% and 2.23%) than desalted culture filtrate (16.91% and 32.20%) in cv. DRK and RK respectively indicating a rcsistant reaction (Table 2). In desalted culture filtrate, Gel filtration removed molecules of less than 6000 daltons. Hence, elicitor molecules have molecular weight less than 6000 daltons. However, more protoplasts of cv. KK (31.11%) remained live after incubatiou with crude culture filtrate similar to the results of treatment with intercellular fluid.

Table 1. Effect of intercellular fluid and crude culture filtrate on viability of protoplasts of bean cultivars

Cultivars	Intercellular fluid		Crude culture filtrate		*Control	% Viable Proto-plasts
	Total No. Proto-Plasts	% Viable Proto-plasts	Total No. Proto-plasts	% Viable Proto-plasts	Total No. Proto-plasts	
Imuna	110	42.70	168	0.60	117	80.34
KK	167	61.68	160	14.40	248	68.46
DRK	62	40.30	72	25.00	226	46.10
KM	158	28.48	231	11.68	327	38.60
Cornell 49-242	108	30.55	125	15.20	184	31.19
Processor R	57	57.89	43	0.00	282	63.40

* Intercellular fluid from uninoculated leaf

Table 2. Effect of desalted and crude culture filtrates on viability of protoplasts of bean cultivars

Cultivars	Desalted culture filtrate		Crude culture filtrate		*Control	% Viable Proto-plasts
	Total No. Proto-plasts	% Viable Proto-plasts	Total No. Proto-plasts	% Viable Proto-plasts	Total No. Proto-plasts	
KK	135	2.74	135	31.11	135	63.70
Processor R	390	0.00	100	0.00	409	43.00
DRK	136	16.91	74	0.00	121	20.66
RM	118	32.20	134	2.23	91	21.09

* Sterile Colletotrichum broth media

These results further confirm the findings of O'Connell and Bailey (1988) that resistance in bean to the fungus is associated with premature cell death. The rapid cell death is attributed to recognition between the host receptor and the pathogen elicitor immediately after fungal attack of host cells (O'Connell *et al.*,

1985). The hypersensitive reaction observed in both resistant and susceptible cultivars to crude culture filtrate indicates the presence of non-specific elicitors.

ACKNOWLEDGEMENT

We thank Dr. John A. Bailey for his valued suggestions towards the research work and Dr. Richard Pring in helping with Microscopy. We sincerely thank Mr. K. P. Chinnaswamy, Associate Professor, for critical reading of the manuscript.

REFERENCES

Crucifix, D. N. and Mansfield, J. W (1984). Evidence that determinants of race specificity in lettuce downy mildew disease are highly localised. *Physiol. Plant. Pathol*, **24** : 93-106.

Flor, H. H. (1956). The complementary genic systems in Flax rusts. *Adv. Genet.* 8 : 29-54.

Heath, M. C. (1980). Effects of infection by compatible species or injection of tissue extracts on the susceptibility of non-host plants to rust fungi. *Phytopathol.* **70** : 357-360.

Kota, D. A. and Stelzig, D. A. (1977). Electrophysiology as a means of studying the role of elicitors in plant disease reactions. *Proc. American Phytopathol. Soc.* **4** : 216-217.

Larkin, P. J. (1976). Purification and viability determination of plant protoplasts. *Plants* (Beris). **128**, 213-216.

Mathur, R. S., Bennett, H. L. and Lilly, V. G. (1950). Sporulation of *Colletotrichum lindemuthianum* in culture. *Phytopathol.* **40** : 104-114.

Nagata, T. and Shigetaka, I. (1979). A rapid method for isolation of mesophyll protoplasts. *Canadian J. Bot.* **57** : 1820-1823.

O'Connell, R. J. and Bailey, J. A., (1988). Differences in the extent of fungal development, host cell necrosis and symptom expression during race-cultivar interactions, between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*. *Plaut Pathol.* **37** : 351-362.

O'Connell, R. J., Bailey, J. A. and Richmond, D. V. (1985). Cytology and Physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. *Physiol. Plant Pathol.* **27** : 75-78.

Peters. B. M , Cribbs, D. H. and Stelzig, D. A. (1978). Agglutination of plant protoplasts by fungal cell wall glucans. *Science*. **201**, 364-365.

Yoshikawa, M , Keen, N. T. and Wang, M. C. (1983). A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. *Plant Physiol.* **73** : 497-506.

(*Accepted for publication 18 July 1994*)