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Hypersensitive Reaction of Cowpea Leaf to Infiltration of *Xanthomonas campestris* pv. *oryzae*

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By infiltration with both wild type strains and virulent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-induced mutants of *Xanthomonas campestris pv. oryzae* cowpea leaf tissues produced necrosis within 24 hr, which could be designated as typical hypersensitive reaction (HR). Among fractions of bacterial culture tested, only living bacterial cells were effective for necrosis inductivity, while heat-killed cells, culture filtrate and extracellular polysaccharide were ineffective. HR was induced at a concentration level higher than ca. 10^7 cells/ml of virulent bacteria, while neither necrosis nor yellowish discoloration developed with the bacteria below 10^4 cells/ml. Avirulent mutant strains did not induce necrosis in cowpea leaf even at a concentration higher than 10^7 cells/ml, while the virulent revertant regained the necrosis inductivity associating with the restoration of virulence.

INTRODUCTION

The hypersensitive reaction (HR) is known to be one of the most important defense reaction of plants in incompatible host-pathogen combination. Since the first report of HR concerning with pseudomonads-tobacco combination (Klement et al., 1964). the phenomenon has been confirmed to occur with various host-bacteria combinations such as tobacco plant against ***Pseudomonas syringae*** or ***Erwinia amylovora*** (Klement and Goodman, 1967 b), apple tree against *E. amylovora* (Burkowicz and Goodman, 1969), pepper against ***Xanthomonas campestris*** pv. ***vesicatoria*** (Cook and Stall, 1971), bean plants against ***P. phaseolicola*** (Smith and Mansfield, 1981) and so on. Among them, some reports focused on the correlation between pathogenicity or virulence and HR (Averre and Kelman, 1964; Klement and Goodman, 1966, 1967 a; Lozano and Sequeira, 1970; O'Brien and Wood, 1973; Turner and Novacky, 1974).

In connection with X. *campestris* pv. *oryzae*, some host responses observed in resistant rice variety or uncongenial plant were referred to as HR (Cook, 1971; Horino, 1976; Kaku and Hori, 1977).

In the present study, the reaction of cowpea leaf tissues infiltrated with wild types and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-induced mutant strains of X. ***campestris*** pv. *oryzae* was investigated with particular attention to

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the relationship between intensity or characteristics of HR and virulence of the bacterium.

MATERIALS AND METHODS

Bacterial strains and test plant

Four wild type strains of X. *campestris* pv. *oryzae*, PXO 61, PXO 63-6, PX079 and PX071, which belong to different pathogenic groups in the Philippines, I, II, III and IV, respectively, were used. These bacterial cultures were supplied from the stock cultures maintained in the Department of Plant Pathology, the International Rice Research Institute (IRRI). Besides, two parent strains, PXO 61 PT and PXO 63-6 PT, and NTG-induced mutant strains with various phenotypes (Tsuchiya *et al.*, 1982) were also used.

Cowpea plant (Vigna *unguiculata* (L.) Wal. var. EG# 2) was grown for about 2 weeks under ordinary greenhouse conditions (temp. 25-30°C) and the fully expanded primary leaves were used for infiltration.

Inoculum preparation and infiltration to leaf tissue

Each bacterial strain was grown in peptone-sucrose broth (PSB) medium containing 10 g peptone, 10 g sucrose and 1 g sodium glutamate, in 1,000 ml distilled water, pH 7.0, at 30°C for 48 hr in shake culture. Cells were washed twice by centrifugation (10, 000 $\times g$, 20 min), then resuspended in sterile distilled water at an approximate concentration of 10^9 cells/ml. Leaf was pin-pricked at a site to be infiltrated, subsequently bacterial suspension was infiltrated into the intercellular spaces through wound with a hypodermic syringe (Fig. 1-A). To get informations concerning the facfors responsible for induction of necrosis bacterial culture of a virulent strain, PXO 61 PT, was fractionated and infiltrated. Forty eight-hr-old liquid culture of PXO 61PT was washed twice by centrifugation and resuspended bacteria was used as living whole cell fraction. Besides the living whole cells, heat-killed cells, culture filtrate, dialized culture filtrate and crude extracellular polysaccharide (EPS) were also served for infiltration. For preparing heat-killed cells, washed bacterial suspension in distilled water (ca. 10^9 cells/ml) was treated at 60 or 100°C for 10 min in a water bath. Crude EPS solution was prepared as follows. Three volumes of 95 % cold ethanol were slowly added to 500 ml of bacterial culture filtrate with stirring. The precipitate was recovered by centrifugation at 8, 000 $\times g$ for 30 min and twice with 95 % ethanol. After ethanol was removed by vacuum evaporation at 60°C EPS was finally dissolved in 25 ml of water and it was served as an inoculum. Infiltrated leaves were observed periodically up to 96 hr after infiltration under the greenhouse conditions.

RESULTS

Leaf reactions to bacterial infiltration

Leaf areas infiltrated with ca. 10^9 cells/ml suspensions of virulent wild

type strains were water-soaked, showing vellowish discoloration and slight chlorosis by 8 to 12 hr after infiltration. Within 24 hr brown necrosis resembled to HR was induced and then those areas were withered and dried (Fig. 1-B and Table 1). Such reaction was induced similarly regardless of pathogenic group of the bacteria.

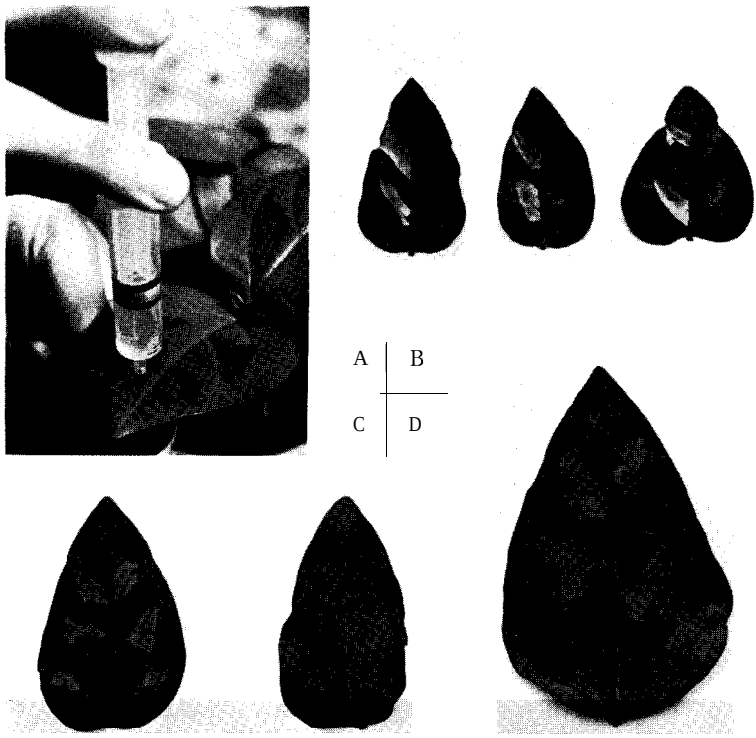

Fig. 1. (A) Infiltration method. (B) Reactions of cowpea leaf tissue to virulent strain (left halves) and avirulent mutant strain (right halves), from the left 24, 72 and 96 hr after infiltration, respectively. (C) Reactions of cowpea leaf tissue to living whole cell (left) and heat-killed bacteria (right) 24 hr after infiltration. (D) Reaction of cowpea leaf tissue to parent strain (left half) and white colony mutant strain (right half) 48 hr after infiltration.

On the other hand, infiltration with avirulent strains such as PX061 NT 3 and PX061 NT 3 RV-1 usually induced no response or yellowing within 48 hr at the infiltrated areas, but necrosis was not induced. These areas occasionally became brownish yellow later on but did not develop to the same symptom as shown by virulent strains (Fig. 1-B). The virulent revertant, PXO 61 NT 3 RV-2, which was induced from an avirulent mutant, regained necrosis inductivity similar to the parent strain (Table 1).

PXO 63-6 NT 5 (weakly virulent mutant) and PXO 63-6 NT 8 (virulent mu-

tant with white colony) also induced necrosis at the infiltrated areas, though the host reaction was somewhat slow and weak with the former (Table 1 and Fig. I-D).

Table 1. Reactions of cowpea leaf tissue to infiltration with different strains of X. *campestris* pv. ***oryzae.***

Bacterial strain	Phenotype[2]	Period after infiltration (hr)			
		12	24	48	96
Wild type					
PXO61 (I)[1]	V	(Y)[3]	++	++	++
PXO63-6 (II)	V	(Y)	++	++	++
PXO79 (III)	V	(Y)	+	++	++
PXO71 (IV)	V	(Y)	++	++	++
Parent					
PXO61PT	V	(Y)	++	++	++
PXO63-6PT	V	(Y)	++	++	++
NTG-induced mutant					
PXO61NT3	AV, w	(Y)	Y	Y	Y or BY
PXO61NT3RV-1	AV	(Y)	Y	Y	Y or BY
PXO61NT3RV-2	V	(Y)	++	++	++
PXO63-6NT5	WV	(Y)	+	++	++
PXO63-6NT8	V, W	(Y)	++	++	++

[1] Pathogenic group designated at IRRI.
[2] V: virulent; WV: weakly virulent; AV: avirulent ; W: white colony.
[3] (Y): yellowish discoloration ; Y: yellowing; BY: brownish yellowing; +: weak necrosis; ++: typical HR with both withering and drying.

Effect of bacterial concentration on HR induction

To determine the critical concentration of bacteria for necrosis inductivity, bacterial suspension of both virulent and avirulent strains were serially diluted

Table 2. Reactions of cowpea leaf tissue to infiltration with different concentration of bacteria.

Bacterial strain (original conc.)	Serial dilution	Period after infiltration (hr)		
		24	48	96
PXO61PT (V) (2.0 X 10^9cells/ml)	10^0	++[1]	++	++
	10^{-2}	+	++	++
	10^{-3}	–	(Y)	(Y)
	10^{-4}	–	(Y)	(Y)
	10^{-5}	–	–	–
PXO61NT3 (AV) (5.1 x 109 cells/ml)	10^0	Y	Y	Y or BY
	10^{-2}	(Y)	Y	Y or BY
	10^{-3}	–	(Y)	(Y)
	10^{-4}			
	10^{-5}			
PXO61NT3RV-2 (V) (3.8 x 10^9 cells /ml)	10^0	++	++	++
	10^{-2}	+	++	++
	10^{-3}	–	(Y)	Y
	10^{-4}	–	–	(Y)
	10^{-5}			

[1] See Table 1.

and infiltrated into cowpea leaf tissue, respectively. The distinct necrosis was induced at the infiltrated areas with virulent strain at a concentration higher than ca. 10^7 cells/ml by 24 hr after infiltration. At a concentration of ca. 10^6 cells/ml, however, the infiltrated areas showed discoloration but not necrosis. No visible response occurred when bacterial concentration was below 10^4 cells/ml (Table 2). With avirulent strain, PXO 61 NT3 or PXO61 NT3 RV-1, infiltration did not induce necrosis until *48* hr after infiltration and the affected areas remained yellow even at a concentration higher than 10^9 cells/ml (Table 2).

Reaction of cowpea leaf tissue to infiltration with various inoculum preparation

Among preparations infiltrated in this experiment, only the fractions containing living cells induced necrosis and none of heat-killed cells, culture filtrate and EPS had critical effect for necrosis inductivity (Fig. 1-C and Table 3).

Table 3. Factors necessary for inciting hypersensitive reaction in cowpea leaf tissue.

Inoculum infiltrated	Reaction of cowpea leaf tissue (3 days after infiltration)
48-hr-old broth culture (ca. 10^9 cells/ml)	++[1)]
Washed bacterial cell (ca. 10^9 cells/ml) in distilled water	it
Washed bacterial cell (2.5×10^5 cells/ml) in distilled water	–
48-hr-old broth culture heated at 60 or 100°C for 10 min	–
48-hr-old cell-free culture filtrate	–
Crude extracellular polysaccharide solution	

1) See Table 1.

DISCUSSION

Since Klement et al. (1964) reported HR caused by phytopathogenic bacteria, many workers have studied on this phenomenon in connection with its mechanisms from the viewpoints of physiological and ultrastructural changes (Klement and Goodman, 1967 a, b; Huang and Goodman, 1970; Novacky, 1972 ; O'Brien and Wood, 1973; Sigee and Epton, 1976; Roebuck et *al.*, 1978). Furthermore, several workers have studied HR in connection with virulence of bacteria by using virulent strains and avirulent mutants (Averre and Kelman, 1964 ; Klement and Goodman, 1966; Burkowicz and Goodman, 1969; Loaano and Sequeira, 1970 ; Stall et *al.*, 1974). Regarding the relationship between virulence and HR inductivity in incompatible host-bacteria combination, some workers reported that avirulent mutant also induced HR as well as virulent strains (Averre and Kelman, 1964; Klement and Goodman, 1966). Lozano and Sequeira (1970), on the other hand, reported that HR inductivity of avirulent mutants was either positive or negative depending upon the strains.

In the present study, when cowpea, an uncongenial test plant, was infiltrated with high concentration (more than 10^7 cells/ml) of virulent strains of X.

campestris pv. *oryzae*, necrosis was induced at the infiltrated leaf area by 24 hr after infiltration. This necrosis could be considered as the results of HR which was reported by other combinations of bacteria and incompatible plants. Because, the host response against X. *campestris* pv. *oryzae* satisfies the primary conceptions of HR, that is, leaf reactions were generally developed rapidly to induce necrosis within 24 hr, and the necrosis associating with withering or drying were induced by only living bacterial cells at a concentration higher than 10^7 cells/ml (Klement ***et al., 1964***; Klement and Goodman, 1967 b; Roebuck et al., 1978).

Inductivity of HR in cowpea leaf tissues was quite different depending upon virulence of infiltrated bacteria, namely, rapid necrosis was induced with virulent strain within 24 hr after infiltration and withered later on, while no response or sometimes yellowish discoloration was induced with avirulent strain. In the latter case, brownish yellowing was occasionally developed later but its appearance was quite different from the response shown in the former. Virulent with white colony mutant, PXO 63-6 NT 8, also induced HR as well as parent strain. All of the virulent wild type strains and some virulent mutants with different phenotypes induced by NTG were positive in HR inductivity so far as tested, suggesting close correlation between virulence of this bacterium to rice and HR inductivity in cowpea.

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