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THE ROLE OF EXTRACELLULAR PROTEIN ON THE PATHOGENICITY OF *XANTHOMONAS CAMPESTRIS* PV. *CITRI*

PERANAN PROTEIN EKSTRASEL TERHADAP PATOGENISITAS XANTHOMONAS CAMPESTRIS *PV.* CITRI

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INTISARI

Studi patogenisitas Xanthomonas campestris *pv.* citri, *penyebab penyakit kanker jeruk, telah dilakukan untuk mengevaluasi beberapa medium yang cocok untuk pertumbuhan patogen, sifat-sifat fisiologi, dan peranan protein ekstrasel dalam proses timbulnya gejala penyakit. Protein ekstrasel* X. campestris *pv.* citri *diekstraksi menggunakan metode presipitasi amonium sulfat. Sampel protein kemudian dielektroforesis pada 10% gel poliakrilamid (SDS-PAGE) 15mA/150V selama 1,5–2 jam. Pengujian patogenisitas dilakukan dengan menginokulasi suspensi bakteri dan protein ekstrasel pada jaringan daun jeruk. Hasil penelitian menunjukkan bahwa patogen mampu tumbuh baik pada semua media yang diuji. Protein spesifik yang dimiliki* X. campestris *pv.* citri *adalah protein dengan BM 25,71 KDa. Suspensi bakteri dan protein ekstrasel* X. campestris *pv.* citri *mampu menimbulkan gejala kanker, sedangkan patovar* Xanthomonas campestris *lain yang memiliki hubungan kekerabatan dekat hanya menyebabkan gejala hipersensitif.*

Kata kunci: Xanthomonas campestris *pv.* citri, *protein ekstrasel, patogenisitas*

ABSTRACT

A research on the pathogenicity of *Xanthomonas campestris* pv. *citri*, the causal agent of citrus canker, has been carried out to study the growth characteristics of the pathogen on some media, physiological characteristics, and the role of extracellular protein on the bacterial pathogenicity. Extracellular protein of *X. campestris* pv. *citri* was extracted using ammonium sulfate precipitation. The extracted protein samples were electrophoresed on 10% polyacrilamide gel in the presence of sodium dodecyl sulfate at 15mA/150V for 1.5–2 hrs. Pathogenicity assay was conducted by infiltration of bacterial cell and extracellular protein suspension into citrus leaf tissues. The results showed that *X. campestris* pv. *citri* was able to grow well on all media. It possess specific protein with molecular weight of 25.71 KDa. Bacterial cell and extracellular protein of *X. campestris* pv. *citri* were able to produce typical symptoms of canker, while other closely related *Xanthomonas campestris* pathovars were only able to produce hypersensitive reaction on citrus leaves.

Key words: *Xanthomonas campestris* pv. *citri*, extracellular protein, pathogenicity

INTRODUCTION

Citrus canker caused by *X. campestris* pv. *citri* is a common and endemic disease in many citrus growing countries throughout the world (Hartung & Civerolo, 1989). The disease was originated from India, Indonesia, and other countries in Asia (Semangun, 1994). Asiatic type, known as canker A, is the most widespread and the most severe form of the disease (Whiteside *et al.* 1989). In Indonesia and Malaysia, citrus canker is one of the most important diseases attacking many cultivars of citrus (Singh, 1980). Gail (1991) reported that *X.*

campestris pv. *citri* destroyed more than 20 million citrus trees in USA caused approximately 25 million dollars of losses.

The pathogen survives on infected leaves, stems, and fruits. When free moisture is present on the lesions, the bacterial ooze is existed and may function as secondary inoculum source of the disease (Whiteside *et al.* 1989). Primary infection occurs on the first flush in spring when the pathogen dispersed from the holdover cankers on angular shoots. Secondary infection of the disease occurs through the rain splash carrying the bacterial ooze from new lesions formed on young shoots (Goto, 1992).

Pathogenicity in the wide sense is a complex phenomenon. In addition to the obvious manifestation of damage to the plant, pathogenic bacteria have to get in contact and penetrate the plant tissues. In such process the pathogens also have to be able to resist the self defense of the host (Daniels, 1989). Bacteria, in contrast to many fungi, do not possess active mechanisms for penetrating intact plant surfaces and gaining entry into plants (Hugouvieux *et al.* 1998). Biochemical genetic studies begun in the mid 1980s revealed that some plant pathogenic Gram-negative bacteria secrete a surprising variety of plant cell wall degrading enzymes and these enzymes contribute a significant role to the virulence of the pathogen (He *et al.* 1993). Extracellular component secreted by the bacteria contains substances which are functioned as virulence factor to infect host-plant tissues. Nomura *et al.* (1998) stated that *Erwinia chrysanthemi*, the soft-rotting bacteria, secreted pectate lyase (PL) enzymes to degrade the pectate fraction of the plant cell wall. This research was conducted to study the role of extracellular protein on the pathogenicity of *X. campestris* pv. *citri*, the causal agent of citrus canker.

MATERIALS AND METHODS

Isolates and culture media. The bacterial pathogen, *X. campestris* pv. *citri* was isolated from citrus leaves showing specific symptoms of canker collected from Magelang. The leaves were cut into small pieces, rinsed in NaOCl 0.5% for two minutes and resuspended in two ml of sterilized distilled water. A loop of suspension containing bacteria was streaked onto Peptone Sucrose Agar (PSA) medium and incubated at 32°C in incubator (Memmert, West Germany) for 48 hrs. The isolates were then subcultured on Peptone Sucrose Agar (PSA), Potato Dextrose Agar (PDA), and Wakimoto's medium to observe the growth characteristics on each medium. The cultures were incubated for 48 hrs in incubator and stored in the refrigerator for further experiments. The isolates of *Ralstonia solanacearum*, *X. campestris* pv. *campestris*, *X. campestris* pv. *cassavae*, and *X. campestris* pv. *oryzae* used as control were taken from cultures collection at the Laboratory of Plant Bacteriology, Faculty of Agriculture, Gadjah Mada University.

Physiological tests. The 48 hrs-old bacterial isolates were tested for determining their physiological characteristics including Gram reaction, OF-test, Arginine dihydrolisis, Gelatin hydrolysis, Action in milk, and Starch hydrolysis (Fahy & Hayward, 1983).

a. **Gram reaction.** The colonies were picked out from the PSA medium with an inoculating loop and placed on a glass slide containing one or two drops of 3% potassium hydroxide (KOH). The materials were stirred in the KOH for 5–10 seconds and the inoculating loop was then raised up from the drop. A thread of slime following the loop for 0.5–2 cm was an indication of positive reaction and seen in Gram-negative bacteria.

b. **OF-test.** Isolates were grown in medium A (in g/l: $NH_4H_2PO_4$ 1.0, KCl 0.2, $MgSO_4.7H_2O$ 0.2, bromthymol blue powder 0.08, pH 7.2). The tubes were incubated at room temperature and examined daily for up to 14 days for evidence of acid production (a change to yellow). The cultures poured with mineral oil was used as control.

c. **Arginine dihydrolisis.** Isolates were grown in medium contained (g/l): Bactopeptone (Difco) 1, NaCl 5, K_2HPO_4 0.3, phenol red 0.01, L+arginine HCL 10, pH 7.2. The tubes were incubated at room temperature for 7 days. A positive reaction is indicated by a deep red colour change compared with orange-pink by controls.

d. **Gelatin hydrolysis.** Isolates were grown in nutrients gelatin containing (g/l) beef extract 3, peptone 5, and gelatin 120. The tubes were incubated at room temperature, then cooled at 4°C to determine liquefaction of gelatin, starting at 2 days after incubation during one week with the interval of one day.

e. **Action in milk.** Isolates were streaked onto nutrient agar added with 15% (w/v) sterile skim milk. Cultures were incubated at room temperature for 7 days and proteolysis was observed as a clear zone surrounding colonies.

f. **Starch hydrolysis.** Isolates were streaked onto medium containing starch 0.2%. Plates were incubated at room temperature for 2–7 days, then flooded with iodine solution. Starch stains blue-black, a clear zone was present where amylase activity has occurred.

Extracellular protein extraction. Extracellular protein was extracted using the method modification of De Boer & Schaad (1990). The 48 hrs-old bacterial cultures taken from PSA medium plate were grown in 50 ml Peptone Sucrose Broth containing 5% of citrus leaves extract and shaked for 48 hours on orbital shaker. The cultures were harvested and centrifuged at 4,000 rpm (Nr. 12159, Sigma 3K30) for 10 minutes. Thirty ml of supernatant was added with 39 ml of Ammonium Sulfate 85% and refrigerated overnight. The suspension was pelleted by centrifugation at 10,000 rpm (Nr. 12154, Sigma 3K30) for 15 minutes and resuspended in 5 ml Phosphate Buffer Saline 0.02 M pH 7.2. The suspension was then added with 5 ml of Ammonium Sulfate 85% and refrigerated for 30 minutes. The suspension was pelleted by centrifugation at 10,000 rpm for 10 minutes, resuspended in 500 µl Phosphate Buffer Saline 0.02 M pH 7.2 and dialyzed for 18 hours.

SDS-polyacrilamide gel electrophoresis (SDS-PAGE). Extracellular protein samples were solubilized in SDS/buffer sample (4× Tris-Cl/SDS pH 6.8, Glycerol, SDS, 2-merchaptoethanol, Bromphenol blue) 5:1 (v/v) and boiled at 95°C for 3 minutes. Thirty µl of samples were loaded and electrophoresed on 10% polyacrilamide gel using vertical gel electrophoresis unit 11.3 × 10 cm (Sigma Chemical co.) at 15 mA/150 Volt for 1.5–2 hrs. The gel was removed from the electrophoresis apparatus, fixed in methanol:acetic acid:water (5:1:4) at room temperature, washed in distilled water, and stained for 2–3 hrs in 0.05% Coomassie Brilliant Blue (Sigma Chemical co.) (Ausubel *et al.*, 1989). The molecular weight of the protein bands were calculated based on the method of Weber & Osborn (Mulyadi, 1990).

Hypersensitivity test. A 100 µl of the bacterial cell and extracellular protein suspension of *X. campestris* pathovars were infiltrated using 1 ml disposable syringe into undersurface of tobacco leaf tissues (*Nicotiana tabacum* cv. White Burley).

Sterilized distilled water was infiltrated as control. Observation was done daily within one week starting at 2 days after inoculation for the symptoms formation.

Pathogenicity assay. Pathogenicity assay was conducted by inoculating bacterial cell and extracellular protein suspension on citrus leaves. The inoculations were done by infiltrating bacterial cell suspension of *X. campestris* pv. *citri*, extracellular protein of *X. campestris* pv. *citri*, other closely related species (*X. campestris* pv. *campestris*, *X. campestris* pv. *cassavae*, *X. campestris* pv. *oryzae*), *R. solanacearum* from tobacco isolate, and sterilized distilled water.

Inoculation was carried out by infiltration of inoculum suspension using 1 ml disposable syringe into citrus leaf tissues in plant house. The observation was done for one week starting at two days after inoculation with the interval of one day on the appearance of the symptom. Reisolation of *X. campestris* pv. *citri* was done from the inoculated citrus leaves showing citrus canker symptom.

RESULTS AND DISCUSSION

The pathogen isolates of citrus canker required approximately 48 hours to reach the colony's size of 1–2 mm in diameter on all media. They produced typical smooth mucoid yellow colonies and were able to grow on PSA, PDA, and Wakimoto's medium, however, they were different in producing the intensity of yellow pigmentation. The highest yellow intensity was shown by the colonies grown on Wakimoto's medium, whereas on the other media the colonies were lighter yellow.

The total of extracellular profile of *X. campestris* pv. *citri* contained approximately five distinct fragment banding pattern. The pattern produced were 72.02 KDa, 40.98 KDa, 25.71 KDa, 23.84 KDa, and 6.18 KDa. The protein with molecular weight of 72.02 KDa was general protein possessed by all *X. campestris* pathovars. However, 25.71 KDa protein pattern appeared to be present only in *X. campestris* pv. *citri*, while other protein patterns also belonged to other *X. campestris* pathovars. Such as the protein with molecular weight of 40.98 KDa was also possessed by *X. campestris* pv. *oryzae*, so was the protein with molecular weight of 23.84 KDa possessed by either *X. campestris* pv. *cassavae* or *X. campestris* pv. *oryzae*. The protein with molecular weight of 6.18 KDa was also possessed by *X. campestris* pv. *campestris*.

The physiological test showed that the pathogen was obligate-aerobe and Gram-negative. The reaction was positive in milk proteolysis that was shown by the evidence of clear zone surrounding colonies at three days incubation, whereas the reaction was negative in Arginine dihydrolisis. Pathogen was able to hydrolyze starch at two days incubations and hydrolyzed gelatin at three days incubations. The data indicated that pathogen produced either proteolytic enzyme to decompose gelatin and milk, or amylase to decompose starch.

Both bacterial cell and extracellular protein suspension caused hypersensitive reaction (chlorotic spot) on tobacco leaves at two days after inoculation. The spot turned yellow and dried down within one week (Figure 1). The necrotic lesions caused by bacterial cell were similar morphologically with those caused by the extracellular protein suspension. In this case, it seems that plant cell could recognize the presence of unexpected substances acted as pathogen. The interesting phenomenon is thought that extracellular protein contains a signal which was recognized by the plant genomic system. Moreover, extracellular

component is a product of bacterial genomic system playing an important role in the establishment of disease symptom. Goto (1992) concluded that extracellular component consisted of enzyme, EPS, and phytohormones involved in causing hypersensitive reaction. Hypersensitive reaction was also induced by the presence of elicitor. Mukherjee *et al.* (1997) reported that protein with molecular weight of 36 KDa encoded by *hrp*N_{Ecc} gene belonged to *Erwinia carotovora* subsp. *carotovora* acted as elicitor.

The symptoms of citrus canker appeared 2–3 days after inoculation on citrus leaves inoculated by bacterial cell and extracellular protein suspension of *X. campestris* pv. *citri* (Figure 2). This data suggested that protein secreted by the pathogen contains substances which caused hyperplasia in plant tissues. The two symptoms, either caused by bacterial cells or by extracellular protein were similar, however the symptom caused by bacterial cells then developed further to become infectious canker, whereas the extracellular protein produced limited lesion on the infiltrated spot and no pathogen was reisolated from the symptom produced by extracellular protein.

The other closely related species of *X. campestris* (*X. campestris* pv. *campestris*, *X. campestris* pv. *cassavae*, and *X. campestris* pv. *oryzae*) failed to produce canker on citrus leaves, however, they were able to induce hypersensitive responses on citrus leaves. Although they belong in the same species as well as morphologically and physiologically similar to one another, the bacteria were not able to infect the plant tissue and to cause the typical citrus canker.

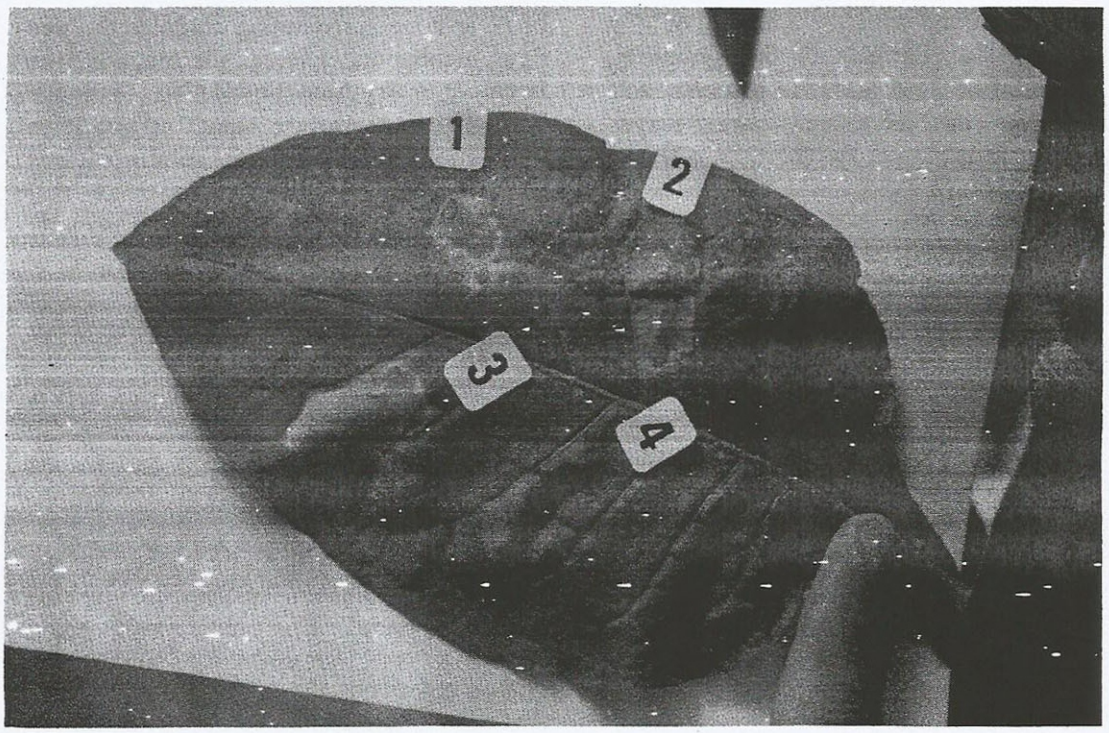

Figure 1. Symptom showing hypersensitive reactions on tobacco leaf infiltrated with extracellular protein of *X. campestris* pv. *campestris* (1), *X. campestris* pv. *cassavae* (2), *X. campestris* pv. *citri* (3), and *X. campestris* pv. *oryzae* (4) at 3 days after inoculation.

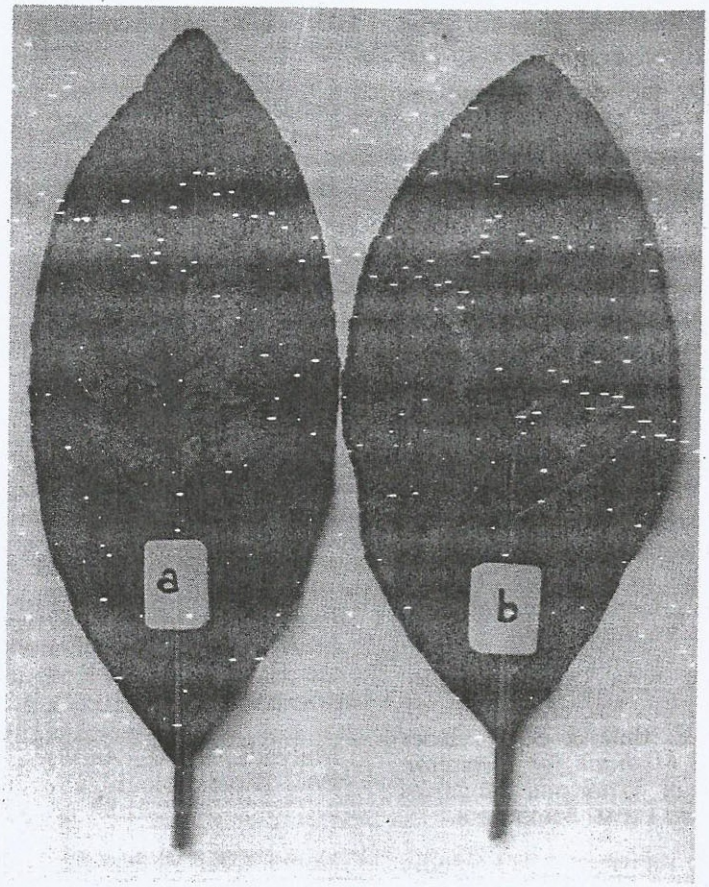

Figure 2. Typical symptoms of citrus canker on citrus leaves produced by extracellular protein of *X. campestris* pv. *citri* (a) and bacterial cell of *X. campestris* pv. *citri* (b).

Plant cell produced hypersensitive reaction as a response of the existence of these bacteria. The result strengthened the **gene for gene** and **protein for protein hypothesis** proposed by Flor (1942) and Vanderplank (1978). Disease symptoms will develop when virulence gene of the pathogen is more dominant than the resistance gene of the host. The pathogen will produce non-pathogenic protein when there is a compatibility interrelationship between avirulence (avr) genes of the pathogen and the resistance genes of the host on the contrary. It is called elicitor protein, which induced hypersensitive reaction. Elicitors are molecules that stimulate any number of defense responses in plants. Hahn (1996), showed that elicitor proteins behave as products of avirulence (avr) genes in the pathogens which are thought to interact with the products of plant resistance genes.

R. solanacearum and sterilized distilled water did not produce any hypersensitive reaction in the form of canker symptoms on the infiltrated citrus leaves, presumably because *R. solanacearum* has far genetic relationship (different genus) from *X. campestris* pv. *citri*, thus the protein secreted does not affect plant cell. The mechanism of disease infection and plant resistance of both pathogens (*R. solanecearum* and *X. campestris*) are also different. Vanderplank

(1982) revealed that in gene-for-gene disease, the mutual recognition of host and pathogen is not by the genes themselves but by their coded proteins. The protein of the pathogen polymerize with its partner host protein, failure of any to polymerize means resistance to disease.

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