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Histopathology and Bacterial Populations of Atypical Symptoms-Inducing *Xanthomonas axonopodis* pv. *citri* Strains in Leaves of Grapefruit and Mexican Lime

Hsin-Cheng Lin [1, 3], Shih-Tien Hsu [2], and Kuo-Ching Tzeng [2, 3]

[1.] Department of Biotechnology, Chung-Chou Institute of Technology, Changhua, Taiwan

[2.] Department of Plant Pathology, National Chung-Hsing University, Taichung, Taiwan

[3.] Corresponding author, E-mail: lin1368@dragon.ccut.edu.tw; Fax: +886-4-839-4070
kctzeng@nchu.edu.tw; Fax: +886-4-2285-4633

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ABSTRACT

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Histopathology of atypical symptoms-inducing *Xanthomonas axonopodis* pv. *citri* (*Xac*) strains XW47 (type *Xac*-A^{f}) and XW121 (type *Xac*-A^{r}) in citrus leaves was examined with light and electron microscopy, and compared with that of a typical Asiatic citrus canker strain XW19 (type *Xac*-A). Strain XW47 induces typical canker lesions on leaves of Mexican lime (*Citrus aurantifolia*) but causes flat necrotic lesions with water soaked margin on leaves of grapefruit (*C. paradisi*); whereas strain XW121 induces restricted and raised corky lesions without watersoaked margin on both citrus leaves. Light microscopic examinations showed that XW47 induced hyperplasia and hypertrophy of mesophyll cells and ruptures of the epidermis as those observed in Mexican lime leaves induced by XW19. However, neither hypertrophic cells nor eruption of the epidermis were observed in the brownish necrotic and hyperplastic mesophyll tissues induced by XW47 on grapefruit leaves. A distinct layer of lignified tissues around the brownish disintegrated erumpent tissues in which masses of fibrillar matrix agglutinated with bacterial cells was observed in the sections of a restricted and raised corky lesion induced by strain XW121. Bacterial populations of strain XW121 in mesophyll tissues were approximately 10^{3} folds lower than those of XW19 and XW47 under a lower inoculum concentration, but increased to those of XW19 and XW47 under a higher inoculum concentration. Although XW19 and XW47 induced distinct symptoms on grapefruit leaves, no significant difference of bacterial growth was observed.

Keywords: citrus canker, hypertrophy, hyperplasia, bacterial population, *Xanthomonas axonopodis* pv. *citri*

INTRODUCTION

Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) is one of the most important diseases in many citrus-producing countries [19]. *Xac* has a broad host range infecting members of the *Citrus* spp. and other species in Rutaceae [3]. The disease is characterized by erumpent lesions on leaves, stems and fruits of citrus species [3]. Under favorable conditions, blister-like lesions appear on leaves about 7-10 days after infection. Subsequently, the lesions become erumptently raised with watersoaked margins, often surrounded by chlorotic haloes. However, several strains of *Xac* producing atypical symptoms have been described. Strains from southwest Asia (*Xac*-A^w) and Florida (*Xac*-A^w) elicit typical canker lesions on Mexican lime but induce flat watersoaked lesions on grapefruit [20, 22]. Recently, we also reported atypical symptoms-inducing strains, *Xac*-A^f and *Xac*-A^r, in Taiwan [16]. A strain of *Xac*-A^f induces flat necrotic lesion with watersoaked margin and light chlorotic halo on leaves of grapefruit, lemon (*C. limon*) and Liucheng (*C. sinensis*), while induces typical canker lesions on Mexican lime. Strains of *Xac*-A^r induce restricted and raised corky lesions with no watersoaked margin and light chlorotic halo on leaves of citrus species. Histological changes in leaves or fruits of citrus caused by *Xac* infection have been examined in detail by Koizumi [9-13]. Histopathological comparisons on lesions produced by *Xac* and *Xanthomonas axonopodis* pv. *citrumelo* in *Citrus aurantifolia* using light and electron microscopy have also been reported [4, 15]. Information regarding atypical symptoms induced by *Xac* strains is not available.

In this study, we examined histopathology of Mexican lime and grapefruit leaves caused by atypical symptoms-inducing strains *Xac*-A^f strain XW47 and *Xac*-A^r strain XW121 and compared with that caused by a typical symptom-inducing strain XW19 using both light and transmission electron microscopes, and we also determined bacterial populations of the three strains in these leaves.

MATERIALS AND METHODS

Bacterial strains and culture media

Three *Xac* strains isolated from Taiwan were used in this study. They are one typical Asiatic type strain XW19 (*Xac*-A type) and two atypical symptoms-inducing strains XW47 (*Xac*-A^f type) and XW121 (*Xac*-A^r type) [16]. All strains were stored in YPD broth (yeast extract 7 g, bactopeptone 7 g, dextrose 7g, distilled water 1000 ml, pH 7.2) [21] containing 20% glycerol at -80℃. When required, each bacterial strain was streaked out from the glycerol stock and cultured on YPD agar plates at 30℃ for 3 days.

Inoculation

Citrus plants (grapefruit and Mexican lime) grown in pots under greenhouse conditions were used. Plants inoculated with *Xac* strains were kept in a growth chamber with 65-90% humidity and 12-hr light at 30℃ and 12-hr dark at 25℃.

To prepare inoculum, *Xac* strains were grown overnight in YPD broth, harvested by centrifugation, and resuspended in sterile distilled water to a concentration of approximately 10^8 colony-forming units (CFU)/ml. In needle-prick inoculation, six wounds in an 1 cm^2 area were made on young fully expanded citrus leaves with a standard 26-gauge needle. An aliquot (20 μl) of the bacterial suspension was dropped onto each wound and the drops were wiped off with sterile cotton right after inoculation. Symptoms were examined visually or with dissection microscope. For injection-infiltration inoculation, bacterial suspensions were adjusted to a concentration of approximately 10^5 or 10^8 CFU/ml. Young fully expanded citrus leaves with similar size and thickness were injection-infiltrated with the bacterial suspension by pressing the opening of a syringe without a needle against the leaf surface.

Bacterial population in citrus leaves

Leaves were inoculated by needle-prick inoculation and the injection-infiltration method as described above. For needle-prick inoculation assay, leaf disks containing 6 lesions were removed with a corkborer at a 7-day interval from 7 days to 56 days after inoculation. For injection-infiltration assay, leaf disks (9 mm in diameter) were removed with a corkborer by randomly punching within the inoculated area at various time intervals after inoculation. Four leaf disks per replicate and three replicates for each strain per host were assayed. Leaf disks were soaked in 1% sodium hypochlorite for 1 min followed by rinsing with sterile distilled water, and ground

with phosphate buffered saline (PBS)[22]. The appropriate dilutions of the ground suspension were plated with a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, England) on YPD agar plates containing cycloheximide (50 μg/ml), and incubated at 30℃. The number of colonies was counted 3 days after incubation. Bacterial populations were expressed as log CFU/disk.

Light and transmission electron microscopy

For light microscopy, the leaf tissue containing a lesion was excised with a dissection knife 26 days after inoculation by the needle-prick method as described above, and immediately fixed in a 20% gelatin solution, placed onto a frozen metal specimen holder, and sectioned with a Freezing Microtome (model FX-801, Yamato Kohki Industrial Co. Ltd, Japan). Sections were examined under a light microscope. For transmission electron microscopy, tissues excised from inoculated leaves were immediately fixed in 5% phosphate buffered glutaradehyde for 4 hr at room temperature and post-fixed in 1 % osmium tetraoxide solution for 4 hr. Fixed materials were dehydrated with a series of ethanol treatments followed by embedding in LR white resin (London Resin Company, Ltd, England). Embedded materials were sectioned with Leica Ultracut R (Leica, Ltd, Austria). The sections were stained with aqueous uranium acetate and followed by lead citrate, and examined under a JEM-1200EX II electron microscope (JEOL, Ltd, Japan).

RESULTS

Histopathology of *X. axonopodis* pv. *citri* in citrus leaves

No visible lesions on inoculated leaves were observed in the first six days after needle-prick inoculation. Ten days after inoculation, small erumpent or necrotic spots appeared at the inoculation sites. By 26 days after inoculation, *Xac*-A^f strain XW47 induced typical erumpent canker lesions with watersoaked margins surrounded by light chlorotic haloes similar to those induced by *Xac*-A strain XW19 on Mexican lime leaves (Fig. 1A and B). However, on grapefruit leaves, XW19 caused typical erumpent canker lesions, but XW47 caused the flat necrotic lesions with watersoaked margins and light chlorotic haloes (Fig. 1D and 1E). On both Mexican lime and grapefruit leaves, the *Xac*-A^r strain XW121 induced restricted and raised corky lesions with no watersoaked margins and light chlorotic haloes (Fig. 1C and F). The inoculation results confirmed our previous study[16]

Hyperplasia and hypertrophy of mesophyll cells, ruptures of the abaxial epidermis and protrusion of the adaxial epidermis were observed by light microscopy on sections of lesions induced by strain XW19 on leaves of Mexican lime and grapefruit 26 days post inoculation. (Fig. 2A and B). The similar histopathological symptoms to those induced by strain XW19 were found in Mexican lime leaves inoculated with strain XW47 (Fig. 2C). Strain XW47, however, caused brownish necrotic and hyperplastic mesophyll tissues without hypertrophic cells and eruption of the epidermis in grapefruit leaves (Fig. 2D). In the lesions induced by strain XW19 or XW47, masses of bacterial cells oozing out from infected tissue or growing around the disintegrated mesophyll cells were observed. The restricted and raised corky lesions induced by strain XW121 showed a distinct layer of lignified tissues surrounding the brownish disintegrated erumpent tissues in leaves of Mexican lime and grapefruit 26 days post inoculation (Fig. 2E and F). Comparing with lesions induced by XW19 or XW47, only a few bacterial cells oozed out from ruptured infected tissues and fewer bacterial cells were observed in the infected mesophyll tissues in lesions induced by XW121. In control leaves inoculated with sterile distilled water, mesophyll spaces appeared in spongy mesophyll (Fig. 2G and H).

Sections of lesions formed 26 days after inoculation were also observed under a transmission electron microscope. In lesions induced by strain XW121, massive fibrillar matrixes agglutinated with bacterial cells in infected brownish disintegrated mesophyll tissues were observed (Fig. 3A). However, bacterial cells distributed freely and no fibrillar matrix agglutinated with bacterial cells in the infected mesophyll tissues was observed in lesions induced by strains XW47 and XW19 (Fig. 3B and C).

Bacterial growth of *X. axonopodis* pv. *citri* in leaf tissues

All three strains of the bacterium grew in leaves of grapefruit and Mexican lime. No significant differences

between the growth rate of XW19 and that of XW47 in either grapefruit or Mexican lime leaves inoculated with 10^5 CFU/ml of bacterial cells were observed (Fig. 4A and B). The bacterial populations of both XW19 and XW47 strains increased gradually from 10^3 CFU/disk to 10^6-10^7 CFU/disk 12 days post inoculation. Similar increases were observed for strain XW121 for the first 2 days after inoculation. Thereafter, the rates of growth differed. Four days after inoculation, populations of strain XW121 fell. It is approximately 10^3 folds lower than those of XW19 or XW47 12 days post inoculation (Fig. 4A and B). There were no significant differences in growth rates among XW19, XW47, and XW121 on citrus leaves inoculated with 10^8 CFU/ml. They all increased gradually from 10^6-10^7 CFU/disk to 10^{10}-10^{11} CFU/disk 12 days post inoculation (Fig. 5A and B). Difference in growth in citrus

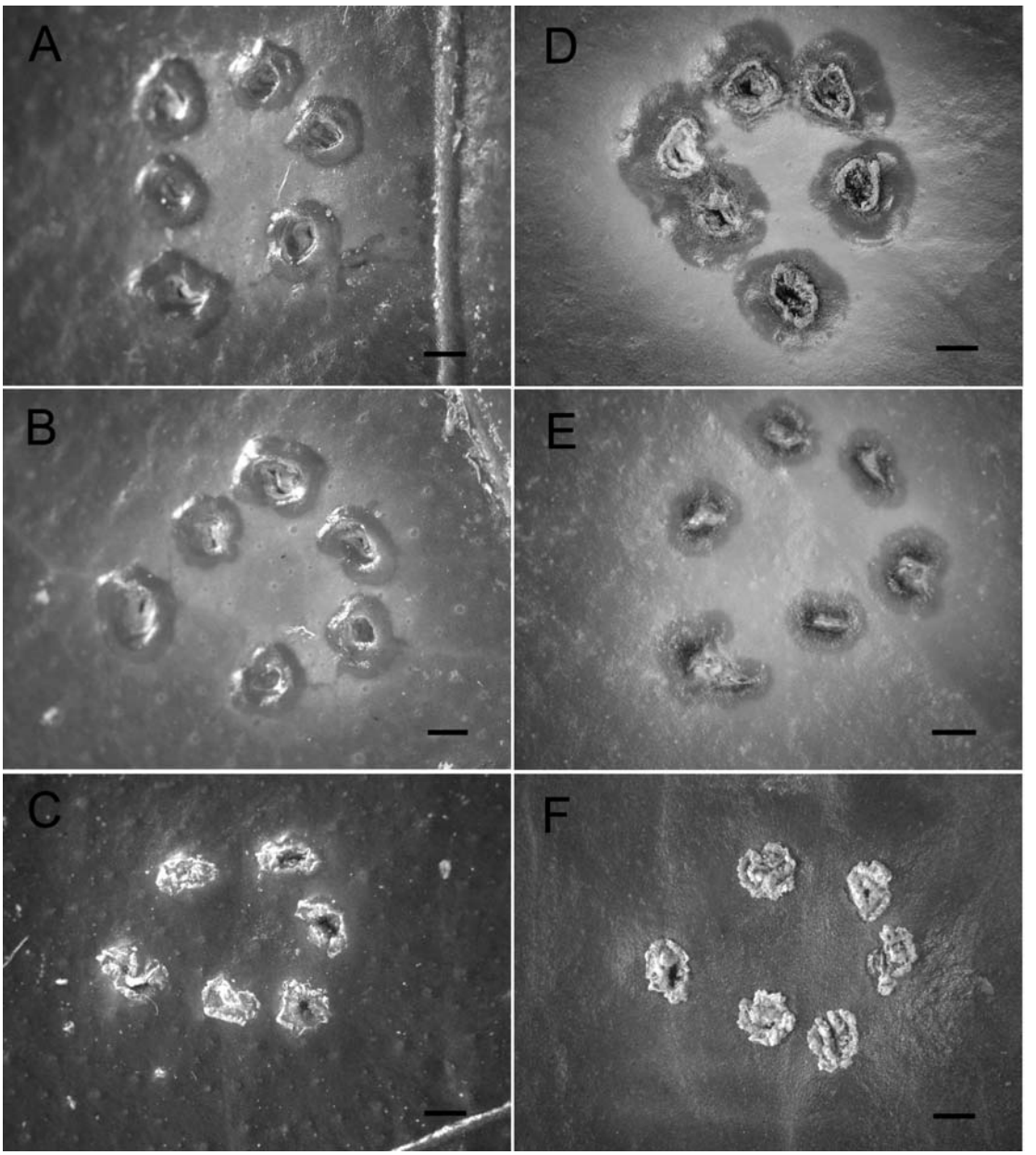

Fig. 1. Symptoms on leaves of Mexican lime (A, B, C) and grapefruit (D, E, F) induced by strains of *X. axonopodis* pv. *citri* 26 days after inoculation. A and D: Typical erumpent canker lesions with watersoaked margin and light chlorotic halo induced by *Xac*-A strain XW19; B: Typical erumpent canker lesions with watersoaked margin and light chlorotic halo induced by *Xac*-A^f strain XW47; E: The flat necrosis lesions with watersoaked margin and light chlorotic halo induced by *Xac*-A^f strain XW47; C and F: The restricted and raised corky lesions with no watersoaked margin and light chlorotic halo induced by *Xac*-A^r strain XW121. (Bars＝1mm)

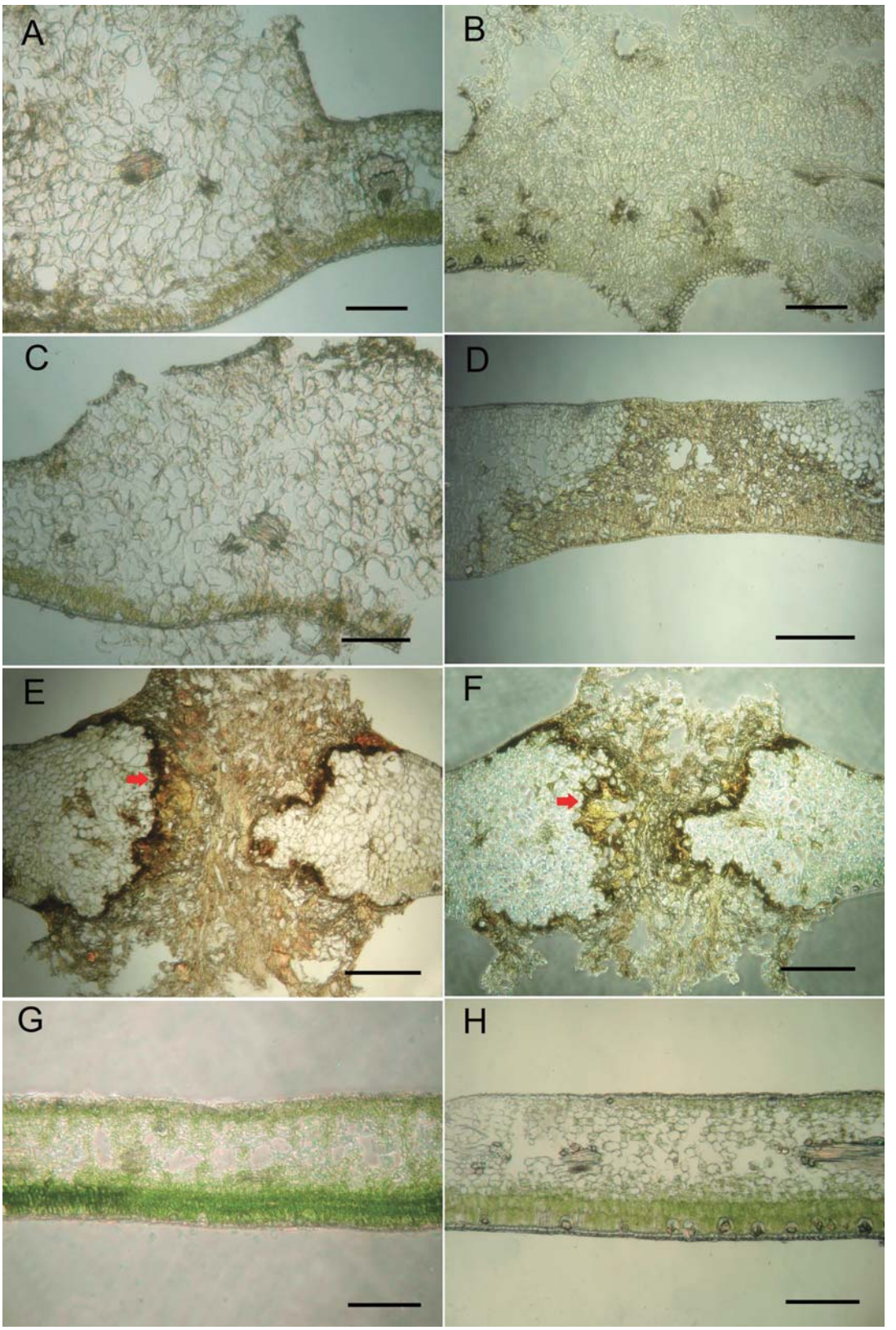

Fig. 2. Histopathology of lesions induced by *Xanthomonas axonopodis* pv. *citri* strains on leaves of Mexican lime (A, C, E, G) and grapefruit (B, D, F, H) 26 days after needle-prick inoculation. A and B: The sections of typical erumpent canker lesions induced by strain XW19; C: A section of typical erumpent canker lesion induced by strain XW47; D: A section of flat necrotic lesion induced by strain XW47; E and F: The sections of restricted and raised corky lesions showing a distinct layer of lignified tissues formed around the brownish disintegrated erumpent tissues (red arrows) induced by strain XW121; G and H: The sections of tissues inoculated with sterile distilled water served as the controls. (Bars = 125 μm).

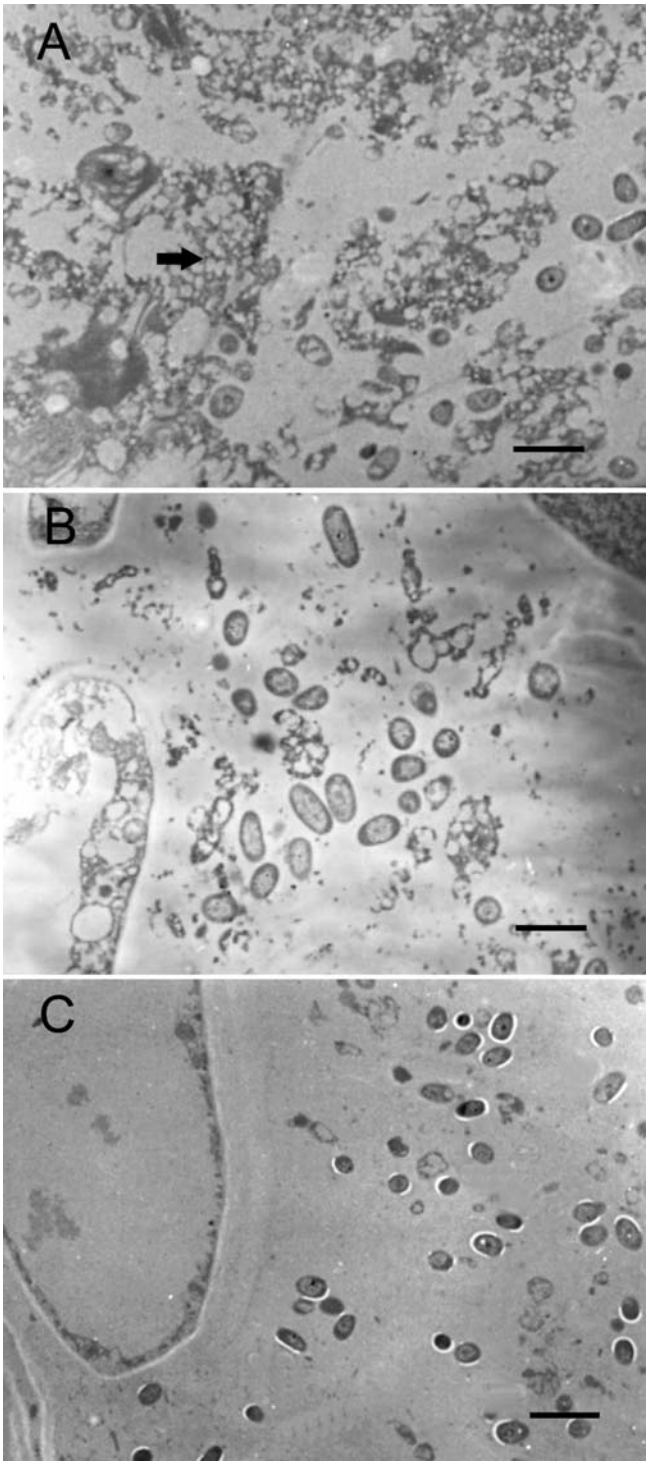

Fig. 3. Transmission electron microscopy of lesions induced by *Xanthomonas axonopodis* pv. *citri* strains XW121, XW47 and XW19 on grapefruit leaves 26 days after inoculation. A: Masses of fibrillar matrix (red arrow) agglutinated with bacterial cells of strain XW121 in infected brownish disintegrated mesophyll tissues; B and C: Bacterial cells of strains XW47 and XW19, respectively, distributed freely in the infected mesophyll tissues. (Bars = 1 μm).

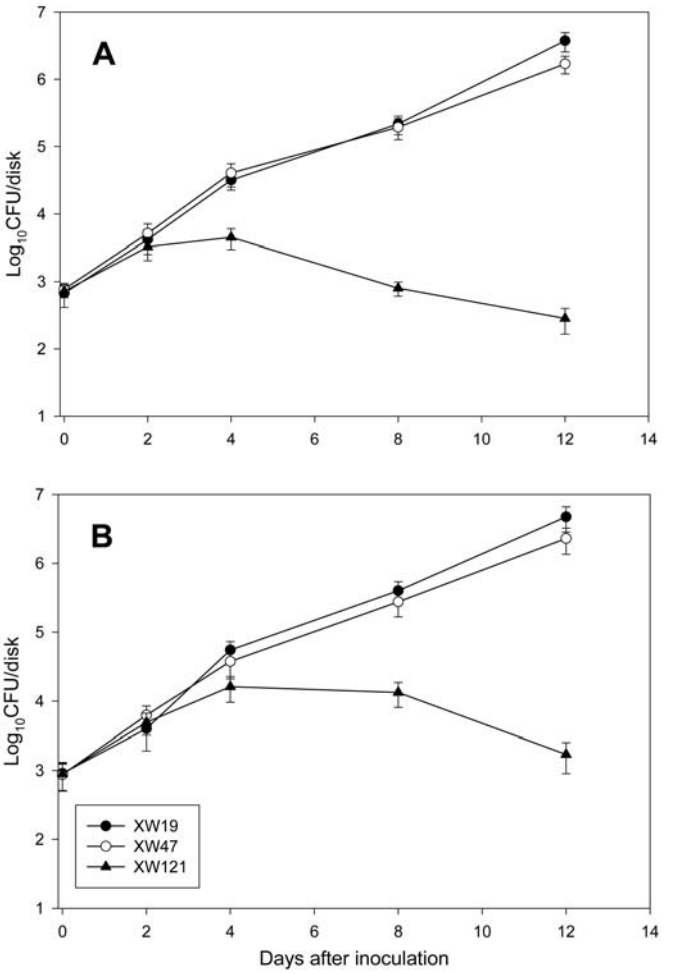

Fig. 4. Bacterial populations of *X. axonopodis* pv. *citri* strains XW19, XW47, and XW121 in leaves of grapefruit (A) and Mexican lime (B). Each bacterial strain was inoculated into citrus leaves by injection-infiltration at a concentration of 1×10^5 CFU/ml. Each value is mean of three replicates and vertical bar represents the standard error.

leaves among XW19, XW47 and XW121 were also confirmed after inoculation by needle-prick inoculation (Fig. 6A and B). Populations of strains XW19 and XW47 increased similarly up to approximately 10^7-10^8 CFU/lesion 28 days post inoculation, and slowly declined thereafter to about 10^6-10^7 CFU/lesion 56 days post inoculation. However, populations of strain XW121 fell gradually from 10^5 CFU/lesion to 10^2-10^3 CFU/lesion 56 days post inoculation. It is approximately 10^3-10^4 folds lower than those of XW19 or XW47 56 days post inoculation (Fig. 6A and B).

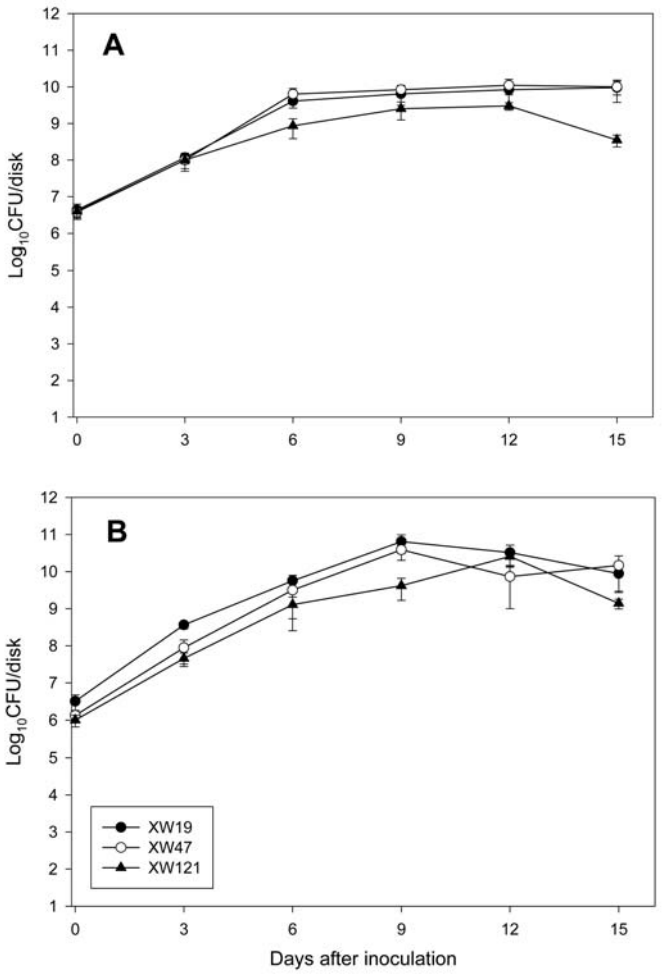

Fig. 5. Bacterial populations of *X. axonopodis* pv. *citri* strains XW19, XW47, and XW121 in leaves of grapefruit (A) and Mexican lime (B). Each bacterial strain was inoculated into citrus leaves by injection-infiltration at a concentration of 1×10^8 CFU/ml. Each value is mean of three replicates and vertical bar represents the standard error.

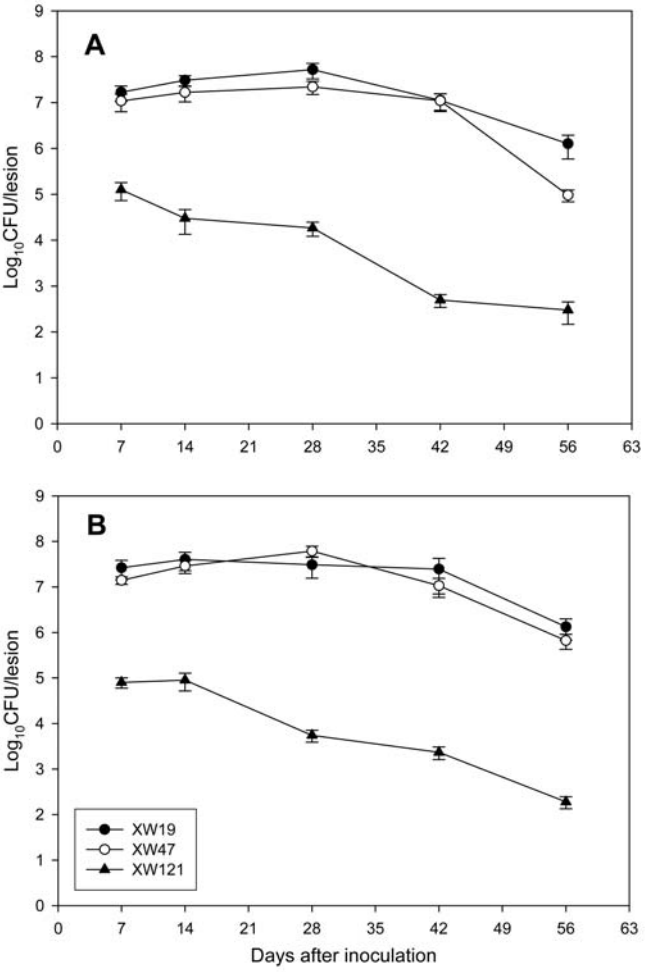

Fig. 6. Bacterial populations of *X. axonopodis* pv. *citri* strains XW19, XW47 and XW121 in the leaves of grapefruit (A) and Mexican lime (B). Each bacterial strain was inoculated into citrus leaves by needle-prick inoculation at a concentration of 1×10^8 CFU/ml. Each value is mean of three replicates and vertical bar represents the standard error.

DISCUSSION

Examination by light microscope revealed hypertrophy and hyperplasia of mesophyll cells in typical canker lesions induced by XW47 on Mexican lime leaves [5]. Only hyperplasia of mesophyll cells was observed in the flat necrotic lesions induced by XW47 on grapefruit leaves. (Fig. 2D). A pathogenicity gene, *pthA*, is required for *Xac* to cause typical canker lesions on citrus. Expression of the *pthA* gene in citrus cells is also sufficient to induced hypertrophy, hyperplasia, and death of citrus cells [1, 5, 6]. Strain XW47 induced typical canker lesions on Mexican lime indicating that it may possess functional PthA to interact with cells of Mexican lime. The reasons why XW47 fails to induce typical canker lesions on grapefruit leaves are not known. Since XW47 induces only cell hyperplasia which is not sufficient to cause erumpent canker lesion, the phenotypes of hypertrophy and hyperplasia in grapefruit mesophyll cells associated with the *pthA* gene might be differentially regulated or independently expressed. Although XW19 and XW47 induced different symptom types on grapefruit leaves, there was no significant difference in bacterial growth between the two strains in the grapefruit leaves. Thus, symptom alone does not affect the growth of bacteria in grapefruit leaves induced by either strain.

Microscopic examination of lesions induced by XW121 on leaves of Mexican lime and grapefruit showed a distinct layer of lignified tissues formed surrounding the brownish disintegrated erumpent tissues (Fig. 2E and 2F). Masses of fibril materials agglutinated with bacterial cells in the disintegrated tissues were observed (Fig. 3A). In our previous study, the lesion size induced by XW121 is within a range from 1.0 to 1.6 mm in diameter 26 days post inoculation, and the lesion size does not expand further. The lesion size caused by XW121 is significantly smaller than those by XW19 and XW47 strains on citrus leaves [16]. The formation of lignified layer may serve as a physical barrier to prevent further expansion of the lesion. Immobilization or agglutination of bacterial cells by fibril materials in host cells is normally associated with incompatible reaction between plant pathogen and its host [2, 7, 17, 18]. Whether the fibrillar matrix agglutination with XW121 bacterial cells observed in the present study was due to incompatible reaction or hypersensitivity reaction, a higher concentration (10^8 CFU/ml) of XW121 was syringe-infiltrated into leaves of Mexican lime and grapefruit. The result showed that XW121 induced confluent lesions in which bacterial cells multiplied and maintained at a high level for a period of time (Fig. 5), and no rapid necrosis was observed in the infiltrated tissues. It has been shown that the gene expression of cinnamoyl-coA reductase (CCR), a key enzyme in the phenylpropanoid pathway, is induced during the hypersensitivity reaction [14]. We used RT-PCR [8] to determine whether CCR gene was expressed in citrus leaves inoculated with strains XW121, XW47 and XW19. No amplification products were detected from RNA extraction of XW121-infiltracted leaf tissues post inoculation (a constitutively expressed control gene, *HistonH4*, was used as an internal positive control). All these results indicated that the restriction and raised corky lesions induced by XW121 is unlikely due to incompatible reaction or hypersensitivity reaction.

Assays of bacterial populations in leaves of Mexican lime and grapefruit after needle-prick inoculation showed that populations of XW121 were approximately 10^3 folds lower than those of XW19 or XW47. Similar results were also observed with injection-infiltration at a lower inoculum concentration (10^5 CFU/ml). However, inoculation with injection-infiltration at a higher concentration (10^8 CFU/ml) of XW121 seemed to overcome the host defense and resulted in higher multiplication of bacteria inside the leaf tissues. These results suggest that XW121 is a less virulent or weakly aggressive strain. Pectolytic activity was detected only from *Xac*-A strains and *Xac*-A^f strain, but not *Xac*-A^r strains [16]. Further studies are needed to examine the role of pectolytic enzymes in pathogenesis of citrus canker disease or in bacterial growth in citrus leaves.

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摘 要

林信成[1, 3]、徐世典[2]、曾國欽[2, 3]. 2009. 引起非典型病徵之柑橘潰瘍病菌 *Xanthomonas axonopodis* pv. *citri* 菌株在葡萄柚及墨西哥萊姆葉片上之組織病理學及菌量變化. 植病會刊 18: 125-134. ([1] 中州技術學院生物技術系；[2] 國立中興大學植物病理學系；[3] 聯絡作者，電子郵件：lin1368@dragon.ccut.edu.tw；傳眞：+886-4-839-4070；電子郵件：kctzeng@nchu.edu.tw；傳眞：+886-4-2285-4633)

應用光學與電子顯微鏡技術，比較柑橘潰瘍病菌 (*Xanthomonas axonopodis* pv. *citri*) *Xac*-A^{f} 類型菌株 XW47 及 *Xac*-A^{r} 類型菌株 XW121，在柑橘葉片上所引起之非典型病斑與 Xac-A 類型菌株 XW19 所引起之典型潰瘍病斑病組織之差異。XW47 菌株在墨西哥萊姆葉片上可引起典型潰瘍病斑，但在葡萄柚葉片上則引起扁平壞疽病斑；而 XW121 菌株在此兩種柑橘葉片上皆引起木栓化突起不具水浸狀邊緣的小病斑。以光學顯微鏡觀察由 XW47 菌株在墨西哥萊姆葉片上所引起之典型潰瘍病斑之病組織切片，顯示在葉肉組織裡可見許多增生及增大的細胞，並造成葉表面之突起破裂，與 XW19 菌株所引起之病組織相似。然而在葡萄柚葉片上，由 XW47 菌株所引起之扁平壞疽病斑之葉肉組織，則呈現細胞增生與組織褐色壞疽現象，然未見葉肉細胞增大及葉表面突起破裂情形。於葡萄柚及墨西哥萊姆葉片上，由 XW121 菌株所引起之木栓化突起小病斑的病組織切片中，在褐色崩解的突起葉肉組織周圍，可見明顯木質化組織形成，在崩解的葉肉組織，則見細菌菌體爲大量纖維狀物質所纏聚。利用低濃度細菌懸浮液進行注射滲透接種於葡萄柚及墨西哥萊姆葉片，在病斑裡 XW121 菌株之菌量比 XW19 或 XW47 菌株少約 10^3 倍，若以較高濃度細菌懸浮液進行注射滲透接種時，在葉肉組織裡 XW121 菌株的菌量增加則與 XW19 或 XW47 菌株相近。雖然 XW19 與 XW47 菌株在葡萄柚葉片上引起不同的病徵，但是在病組織裡的菌量變化並無明顯差異。

關鍵詞：柑橘潰瘍病、細胞增大、細胞增生、菌量、柑橘潰瘍病菌