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Determination of Genomovar Status of *Burkholderia cepacia* Causing Banana Finger-tip Rot and Development of a Simple Semi-selective Medium for Isolation of the Pathogen

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ABSTRACT

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Banana finger-tip rot disease was observed in commercial fields in Pingtung County, Taiwan. Symptoms included small and externally distorted shape of diseased fingers, and brown discoloration of pulps. The pathogen was isolated and identified as *Burkholderia cepacia* in a previous study. The genomovar status of banana finger-tip rot pathogen in *B. cepacia* complex was further determined by PCR-RFLP pattern analysis of 16S rDNA and *recA*. The results suggested that the banana finger-tip rot pathogen was closely related to *B. cepacia* genomovar III. In addition, the *recA* nucleotide sequence-based phylogenetic tree showed that banana finger-tip rot pathogen aligned more closely with the *recA* group IIIA of genomovar III. For epidemiological studies, a simple semi-selective medium (QY-TP) was developed for the isolation of banana finger-tip rot pathogen from diseased and symptomless banana fingers. All *B. cepacia* strains tested grew well on QY-TP medium, but other plant pathogenic bacteria such as *Erwinia carotovora*, *E. chrysanthemi*, *Ralstonia solanacearum*, and *Xathomonas* spp. failed to grow on the medium. The medium allowed recovery of 92 to 98% of the *B. cepacia* colonies grown on Luria-Bertani agar medium, and inhibited growth of most background bacteria. Use of QY-TP medium made it possible to determine the infestation rate of banana fingers in the field.

Key words : Banana finger-tip rot, *Burkholderia cepacia*, semi-selective medium

INTRODUCTION

anana finger-tip rot disease affects banana fruit fingers of cultivars in the triploid (AAA) genomic group. Diseased fingers are recognized externally by their distorted shape, often being narrow at the tip, smaller, and curved out of line with the other fingers [(13)]. Parts of the pulp within the finger appear slightly gelatinous and yellow. Often, a brown discoloration is evident at the tip of the flower end. Losses from the disease are not severe and only a few fingers from the youngest hands are affected.

Finger-tip rot, which was first described from Honduras in 1962, was called "Mokillo" because of its superficial resemblance to fruit infected with *Ralstonia solanacearum* (syn. *Pseudomonas solanacearum*) race 2[(2)]. It occurs throughout Central America and in Taiwan [(2)]. This may be part of the reason why Taiwan was once considered an infestation area of Moko disease by European and Mediterranean Plant Protection Organizations.

Although *Pseudomonas* sp. was reported to be a causal pathogen of finger-tip rot, the identity of the species was not determined [(2)]. In the previous study, strains were isolated from diseased samples collected by Taiwan Banana Research Institute at Pingtung, and were identified as *Burkholderia cepacia* using physiological and biochemical features and the sequences of the16S ribosomal RNA gene (16S rDNA) [(13)]. However, the *B. cepacia* complex is a very diverse group of bacteria [(21)]. Current taxonomic classification divides strains

previously classified as *B. cepacia* into six genomovars [4, 21]. This study was undertaken to determine the genomovar status of banana finger-tip rot pathogen in *B. cepacia* complex and further to develop a semi-selective medium specifically for the recovery of the pathogen causing banana finger-tip rot from diseased and symptomless banana fingers. Such a medium could be used to determine the survival of *B. cepacia* on banana fingers, and to assess the potential for banana fingers to serve as an inoculum source for the pathogen.

MATERIALS AND METHODS

Isolation method, bacterial strains, and culture conditions

Banana fruit fingers showing tip rot symptoms were collected in Pingtung County. Part of the discolored pulp within fingers was removed and immersed in sterile water for 10 min. One loopfu1 of the suspension was streaked onto Luria-Bertani agar (LA) medium [18]. Plates were incubated at 28℃ for two days. Colonies were further streaked on the same medium to purify single colonies. The purified colonies were cultured on LA medium and stored at 4℃, or frozen at -80℃ in 25% glycerol. Where required, an *Escherichia coli* host, strain DH5α (Gibco-BRL Life Technologies, Inc., Gaithersburg, MD), was used and cultured in LA at 37℃. Ampicillin (50 μg/ml) was added as necessary to maintain selection of the resistance marker in pGEM®-T Easy vectors (Promega Corp. Madison, WI).

Biochemical and physiological tests

Diagnostic tests for biochemical and physiological characterizations were conducted according to Hildebrand *et al*.[9] The API 20E system test (BioMerieux Vitek Inc., Hazelwood, MO) was used for some biochemical tests. The ability to catabolize different carbon compounds was also determined by using the Biolog GN system according to the manufacturer's directions (Biolog Inc., Hayward, CA). Utilization of some other carbon sources was evaluated on solid minimal medium (K_2HPO_4 1.5g/L, $(NH_4)_2SO_4$ 1.0g/L, $MgSO_4.7H_2O$ 1.5g/L, agar 15g/L) [12]. The carbon sources selected were benzoic acid, p-hydroxybenzoic acid, polygalacturoniac acid (sodium salt), quinic acid, and starch (soluble potato). The carbon sources were added to this minimal medium at a final concentration of 0.5%. The medium was adjusted to pH 7.2-7.5 and autoclaved for 10 minutes at 121℃. Resistance to antibiotics was tested on LA medium with gentamycin, kanamycin, polymyxin, and tetracycline (Sigma, St. Louis, MO), respectively.

PCR-RFLP analysis of 16S rDNA and *recA*

16S rDNA and *recA* gene fragments were amplified by PCR with UN12/UN15 and BCR1/BCR2 primer pairs as described by Mahenthiralingam *et al*.[16], respectively. PCR amplifications were performed with a GeneAmp® PCR system 2400 (Perkin-Elmer Corporation, Norway, CT) in a 30-μl reaction mixture containing 2 mM $MgCl_2$, 0.5 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate and 1.25 U of *Taq* DNA polymerase (DyNAzyme II, Finnzymes Oy, Finland) by using the following program: 1 cycle of denaturation for 1 min at 94℃; 35 cycles consisting of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 1 min. For restriction fragment length polymorphism (RFLP) analysis, PCR products were digested with *Dde*I, *Hae*III, or *Mnl*I (New England Biolabs, Inc., Beverly, MA.) and analyzed by 2.5% agarose gel electrophoresis as described by Mahenthiralingam *et al*.[16]. The amplified PCR fragments were also cloned into pGEM®-T Easy vectors (Promega Corp. Madison, WI) and then sequenced.

16S rDNA and *recA* sequence analysis

16S rDNA and *recA* gene fragments were amplified by PCR with primer pairs as described by LiPuma *et al*. [15] and Mahenthiralingam *et al*. [16], respectively. The amplified fragments were cloned into pGEM®-T Easy vectors (Promega Corp. Madison, WI), transferred into *E. coli* DH5α, and then sequenced. Double-stranded sequencing was performed using a 373A automated DNA sequencer and an ABI Prism DNA Sequencing kit (Applied Biosystems, Foster City, CA), based on the dideoxy chain termination method [19]. Sequence data were compiled and analyzed using the computer programs of GCG (Genetics Computer Group, Madison, WI). Sequences and selected GenBank 16S rDNA sequences of some representative strains of *Proteobacteria* were aligned. Distance analyses were performed using the phylip package in the dnadist program [5]. Divergence between two sequences was estimated using the Kimura two-parameter method [11]. A phylogenetic tree was inferred using the neighbor-joining method [17].

Semi-selective medium and evaluation of the medium

A quinate-yeast extract-tetracycline-polymyxin (QY-TP) semi-selective medium was developed for isolation of the finger-tip rot pathogen, *B. cepacia*, based on carbon source utilization and antibiotic resistance. One litre of QY-TP medium contained 5 g quinic acid, 5 g yeast extract, 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 1.0 g $(NH_4)_2SO_4$, and 15 g agar. The medium was adjusted to pH 7.2-7.5 with 10 N NaOH and autoclaved for 10 minutes at 121℃. Tetracycline (Sigma, St. Louis, MO) and polymyxin (Sigma) stock solutions were prepared separately and added to the medium after autoclaving to give final concentrations of 50 mg/L and 600,000 U/L, respectively.

To evaluate plating efficiency (number of colonies per plate on test medium/number of colonies per plate on LA medium, X 100), serially diluted bacterial suspensions of

B. cepacia were plated onto media. Cultures were incubated at 37℃ for 2-3 days. Colony characteristics were observed and colonies were counted. Selectivity of the medium was evaluated by testing the growth of other plant pathogenic bacteria on QY-TP medium, and by recovery of *B. cepacia* from the soil. Growth was tested by streaking four cultures of plant pathogenic bacteria onto each plate and incubating for 4 to 6 days at 37℃. To evaluate the efficiency in recovering *B. cepacia* from soil, a soil sample (0.1 g) from a farm in the banana cultivation areas at Pingtung was infested by adding 0.1 ml of a suspension of *B. cepacia* (1.0×10^5 cfu/ml) from a 24-h LA culture. The infested soil was then suspended in 1 ml of sterile distilled water. The suspension was serially diluted and plated onto QY-TP medium.

Recovery of *B. cepacia* from banana fruit fingers

Naturally diseased and symptomless banana fruit fingers were collected from three banana cultivation areas in Pingtung County. The stigma, which is considered an infection court for *B. cepacia*, was cut from healthy and diseased fingers and placed into 2 ml of sterile water and ground in mortar. The extract was serially diluted and plated onto QY-TP medium. In addition, parts of the discolored pulp within the diseased finger were removed and immersed in sterile water for 5 min. One loopfu1 of the extract was streaked onto QY-TP medium. Bacterial colonies growing on the medium were further assayed by injecting 20-μl aliquots into onion bulbs to test pathogenicity [13].

Nucleotide sequence accession number

The 16S rDNA and *recA* partial sequences of *B. cepacia* strain B9 have been assigned the GenBank accession number AY207313 and AY598028, respectively.

RESULTS

Characterizations of the banana finger-tip rot pathogen

Three *B. cepacia* strains were selected for further characterization to determine the genomovar status and to develop a semi-selective medium. All strains grew at 42℃, were positive for oxidase, lysine decarboxylase, ornithine decarboxylase, and β-galactosidase, but negative for arginine dihydrolase and tryptophanase (indole production). The strains produced acids by utilizing lactose, sucrose, maltose, D-mannitol, D-sorbitol, and dulcitol, but not adonito, melibiose or rhamnose. Current taxonomic classification divides *B. cepacia* strains into six genomovars [4, 21]. The most important biochemical characteristics for differentiation of genomovars are listed in Table 1 according to Coenye *et al.*[4]. Biochemically, *B. cepacia* B9 strain was closely related to *B. cepacia* genomovar III. In addition, all strains could utilize benzoic acid, p-hydroxybenzoic acid, polygalacturoniac acid, or quinic acid as a sole carbon source for growth. The strains were resistant to gentamycin (10 μg/ml), kanamycin (50 μg/ml), polymyxin B (600 U/ml), and tetracycline (200 μg/ml). These characterizations were used to develop a semi-selective medium.

Determination of genomovar status of banana finger-tip rot pathogen by PCR-RFLP analysis

The genomovar status of isolated *B. cepacia* strains was determined by PCR-RFLP analysis of 16S rDNA and *recA* as described in Mahenthiralingam *et al.* [16] The PCR amplification using UN12/UN15 and BCR1/BCR2 primers amplified a 1,020-bp 16S rDNA fragment and a 1,041-bp *recA* gene fragment, respectively, from three isolated *B. cepacia* strains tested. Restriction analysis of the amplified fragments was then performed using DdeI for 16S rDNA fragment, and *Hae*III and *Mnl*I for *recA* gene fragment. Only one 16S rDNA RFLP pattern was found for all *B. cepacia* strains tested (Fig. 1A). The pattern is identical to those of *B.*

Table 1. Phenotypic characteristics of *B. cepacia* genomovar I-VI and a representative strain (B9) of the causal agent of banana finger-tip rot.

Characteristics [1]	Genomovar						
	I	II [2]	III	IV [2]	V [2]	VI	strain B9
Growth at 42℃	− [3]	+	+	−	−	+	+
β-Galactosidase	+	+	+	−	+	+	+
Decarboxylation of:							
Lysine	+	v	+	+	+	−	+
Ornithine	+	−	+	+	+	−	+
Oxidation of							
Sucrose	+	−	+	−	+	−	+
Lactose	+	+	v	+	+	+	+
Adonitol	+	+	v	+	+	+	−
Maltose	+	+	v	+	+	+	+

[1] Phenetic characteristics of *B. cepacia genomovar* I-VI were based on Coenye *et al.*[4]
[2] The genomovar II, IV, and V were proposed as new species *B. multivorans* [21], *B. stabilis* [22], and *B. vietnamiensis* [6], respectively.
[3] +: positive reaction, -: negative reaction, v: variable (strain dependent)

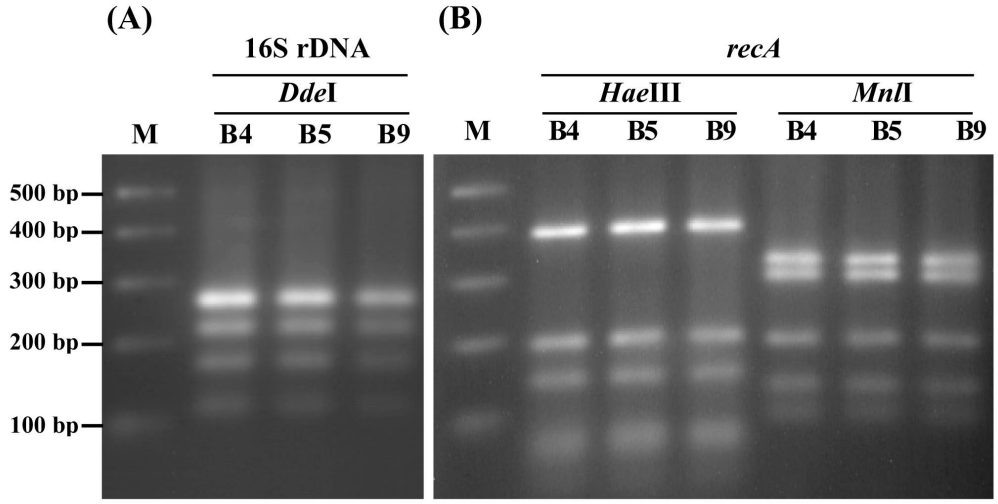

Fig. 1. PCR-RFLP analysis of the PCR-amplified 16S rDNA and *recA* fragments of three strains (B4, B5, B9) of banana finger-tip rot pathogen, *B. cepacia*. (A) *Dde*I digestion of 16S rDNA fragment; (B) *Hae*III or *Mnl*I digestion of *recA* DNA fragment. Molecular size standards (100-bp ladder) are in lane M.

cepacia genomovar I, III and *B. stabilis* as described in Mahenthiralingam *et al.* [16].

Strains of *B. cepacia* genomovar I, III and *B. stabilis* could be discriminated by analysis of PCR-RFLP patterns of *recA* gene fragment. The HaeIII and MnlI digestions of *recA* gene fragments obtained one RFLP pattern, respectively (Fig. 1B). Each pattern is identical to that of *B. cepacia* genomovar III as described in Mahenthiralingam *et al.* [16].

16S rDNA and *recA* nucleotide sequence analysis of *B. cepacia* genomovars

To confirm the results of PCR-RFLP analyses of the amplified 16S rDNA and *recA*, nucleotide sequences 16S rDNA and *recA* gene of a strain of banana finger-tip rot pathogen (*B. cepacia* B9 strain) were determined and compared with those of *B. cepacia* genomovars. Computational analysis of the *Dde*I, *Hae*III, and *Mnl*I restriction sites within 16S rDNA and *recA* nucleotide sequences matched those sites determined by RFLP analysis of 16S rDNA and *recA* amplicon of *B. cepacia* B9 strain. The 16S rDNA sequence of strain B9 was also compared with available 16S rDNA sequences of members of *B. cepacia* complex, *B. cepacia* genomovar I ATCC25416 (GenBank accession no. AF097530), genomovar II LMG14293 (AF097531), genomovar III LMG12614 (AF097532), genomovar IV LMG14294 (AF148554), genomovar V LMG10929 (AF097534), and genomovar VI LMG 18941 (AF175314). The sequence was highly similar (99.87%) to that of *B. cepacia* genomovar III LMG 12614.

In addition to 16S rDNA, phylogenetic analysis of *recA* has demonstrated that the gene is very useful for the separation of *B. cepacia* genomovars [16]. The *recA* nucleotide sequence of *B. cepacia* B9 strain was aligned with those of *B. cepacia* genomovars for the construction of phylogenetic tree. The resulting nucleotide sequence-based phylogenetic tree is shown in Fig. 2. All previously determined genomovars formed distinct arms within the tree, and the sequence of B9 strain aligned closely with *recA* group IIIA of genomovar III.

Evaluation of a semi-selective medium and recovery of *B. cepacia* from banana fingers

All *B. cepacia* strains tested grew on QY-TP medium and colonies became visible after incubation for 1-2 days at 37℃ as white, shiny, mucoid colonies. Other plant pathogenic bacteria tested, such as *Erwinia carotovora*, *E. chrysanthemi*, *R. solanacearum*, *Xanthomonas campestris* pv. campestris, and *X. campestris* pv. citri failed to grow on the medium. Plating efficiencies (number of colonies per plate on test medium/number of colonies per plate on LA medium) of 4 strains of *B. cepacia* on QY-TP medium ranged from 92 to 98%. QY-TP medium significantly reduced the number of saprophytes from infested soil compared with LA. Although the soil was infested with 1.0×10^5 viable cells, no *B. cepacia* colonies were detected on LA due to the presence of soil bacteria. Fewer than 5 soil bacterial colonies were present on QY-TP agar, and *B. cepacia* colonies could be easily recognized and differentiated from other bacterial colonies. Bacteria from the stigma of banana fruit fingers were significantly reduced on QY-TP medium. *B. cepacia* was detected on QY-TP medium in 100% of stigmata and pulps from banana fingers showing tip rot symptoms, and 8% from stigmata of fingers showing no symptoms. All isolated *B. cepacia* strains caused rotting symptom on onion.

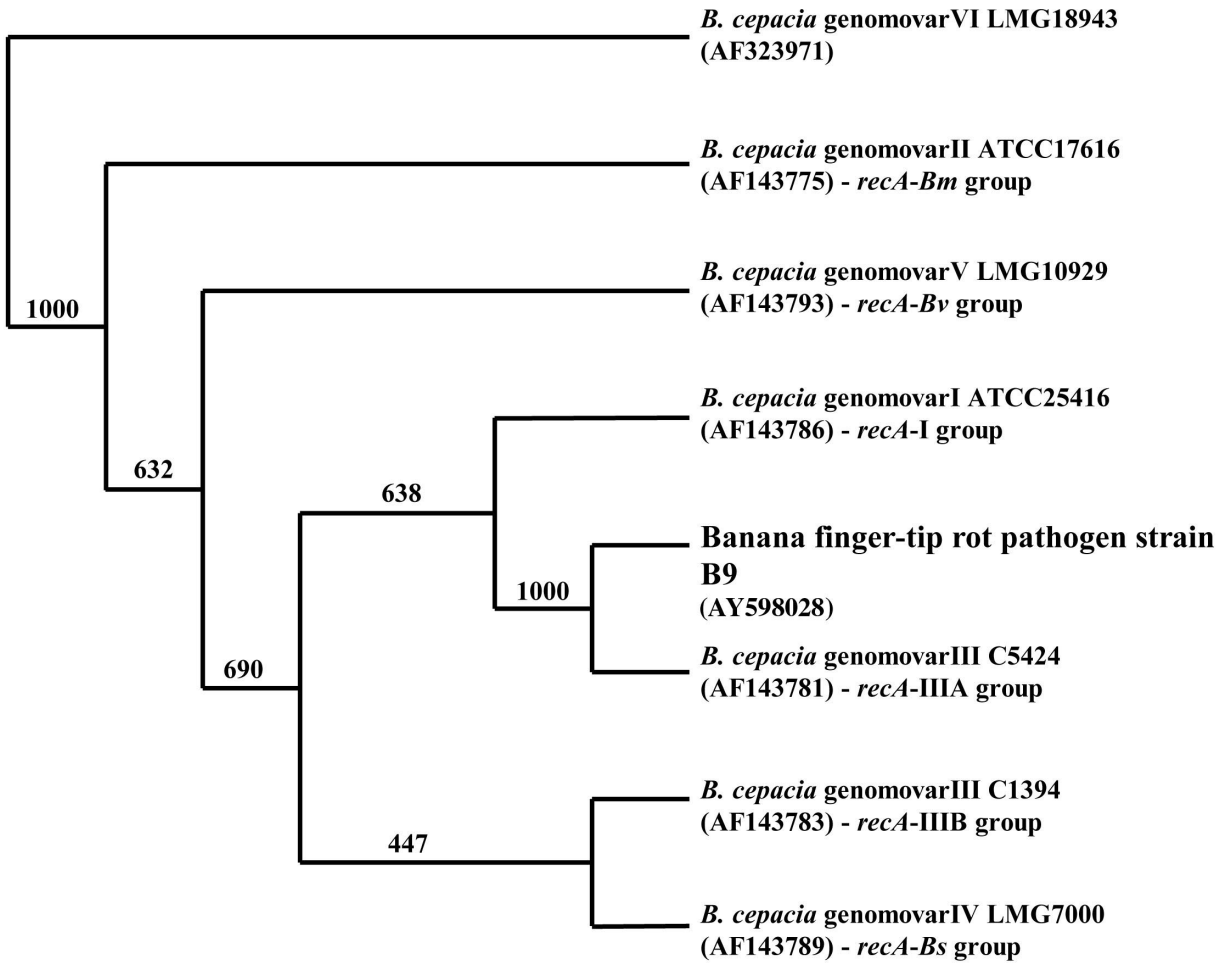

Fig. 2. Unrooted phylogenetic tree derived from neighbor-joining analysis of the *recA* nucleotide sequences of banana finger-tip rot pathogen strain B9 and strains of *B. cepacia* genomovars. Bootstrapping resampling statistics were applied to the tree (1,000 data sets), and bootstrap values are shown on each horizontal branch of the tree. GenBank accession numbers are given in parenthesis after the bacterial names. The genomovar II, IV, and V were proposed as new species *B. multivorans* [21], *B. stabilis* [22], and *B. vietnamiensis* [6], respectively.

DISCUSSION

B. cepacia was originally reported as the causal agent of onion decay [3], which also occurred in Taiwan. In the previous study, the causal agent of banana finger-tip rot was identified as *B. cepacia*, and the isolated *B. cepacia* strains caused rotting symptoms on banana fruit fingers and onion bulbs [13]. In Taiwan, major cultivation areas of banana and onion are located in the southern counties, such as Kaohsiung and Pingtung. *B. cepacia* can be spread between these areas by infested bananas and onions, transportation vehicles, or animals, such as insects and birds. As a result, banana finger-tip rot and onion decay often occur in these areas and become endemic, although the diseases cause only minor losses.

Symptoms of banana finger-tip rot caused by *B. cepacia* were superficially similar to symptoms of fruit infected with *R. solanacearum* race 2. Although both *B. cepacia* and *R. solanacearum* race 2 can infect banana fingers and cause internal fruit discoloration, infection pathways of the two pathogens are quite different. *B. cepacia* invades the edible pulp through the stigma of growing fingers and causes pulp discoloration which never spreads further into the vascular system within the floral stem. In comparison, the Moko disease pathogen, *R. solanacearum* race 2, infects banana cultivars typically via the roots or rhizome [20]. The first symptoms of Moko disease are yellowing and wilting of the oldest leaves. The Moko disease pathogen can spread further into fruits through vascular tissues, causing fruit discoloration and eventually rot. Alternatively, the Moko pathogen can be transmitted by insects, and symptoms are first seen in the flower buds and peduncles [20]. In this study, the identity of the causal pathogen of banana finger-tip rot was diagnosed as *B. cepacia*, not *R. solanacearum*. The symptom caused by *B. cepacia* is limited to fruit fingers and is quite different from that caused by *R. solanacearum* race 2.

The *Burkholderia cepacia* complex is a very diverse

group of bacteria [21]. The ability of *B. cepacia* to cause disease is not limited to plant hosts, as these bacteria are also important opportunistic pathogens of humans causing devastating infections in patients with cystic fibrosis [7, 14]. Current taxonomic classification divides *B. cepacia* strains into six genomovars [4, 16]. Among these genomovars, *B. cepacia* genomovar III is a common plant-associated bacterium. The bacterium can survive on the rhizosphere and in inner tissues of wheat, lupine, and maize [1]. In addition, most strains associated with the cepacia syndrome in patients with cystic fibrosis belong to genomovar III. This syndrome is characterized by a dramatic necrotizing pneumonia that results in rapid death of the patient [8, 10]. PCR-RFLP and nucleotide sequence analyses of 16S rDNA and *recA* indicated that strains of banana finger-tip rot pathogen belong to *recA* group IIIA of *B. cepacia* genomovar III. Strains of *recA* group IIIA included epidemic cystic fibrosis strains from cable pilus-encoding lineage [16]. Because there is considerable genetic variation among strains within genomovar III [1], *B. cepacia* strain B9 may not be associated with clinical stains causing cepacia syndrome. Nevertheless, results from this study suggest that banana may be another source of *B. cepacia*, and therefore, its epidemiological distribution and impacts on public health should be further considered.

The developed QY-TP semi-selective medium was easy to prepare and was highly selective and effective for isolation of *B. cepacia*. The medium will be useful for undertaking epidemiological studies of banana finger-tip rot pathogen in the field. Quinate was selected as the primary carbon source because some plant pathogenic bacteria, such as *Xanthomonas* spp., are reported to grow poorly or do not utilize it at all [12]. Addition of selective antibiotics, tetracycline (50 mg/L) and polymyxin (600,000 U/L), inhibited growth of most saprophytes. *B. cepacia* strains tested could tolerate tetracycline and polymyxin at the levels exceeding 200 mg/L and 600,000 U/L, respectively, and grew on QY-TP medium without any effects. Yeast extract (0.5%) was added to accelerate the growth and increase the recovery rate of *B. cepacia*. Plating efficiency was significantly higher on the medium with yeast extract than without it. Although QY-TP medium does not eliminate growth of all saprophytes, they can be differentiated from *B. cepacia* by colony morphology. All *B. cepacia* strains tested grew on QY-TP medium as white, shiny, mucoid colonies. To enable further distinction, reference strains could be included in the test to ensure proper identification of the bacteria.

QY-TP medium was useful for isolation and detection of *B. cepacia* from banana fingers. *B. cepacia* could be easily detected on QY-TP medium in stigmata and pulps of banana fingers showing tip rot symptoms. The results indicated that the stigma was an infection court for *B. cepacia*. Eight percent of stigmata from symptomless fingers were infested with *B. cepacia*. Stigma infestation could explain why small amount of controls plants also showed symptoms in inoculation tests [13]. *B. cepacia* could be disseminated in water drops splashed by rain, moving from the surfaces of infected tissues to those of healthy ones. The relatively high humidity in stigmata could favor the pathogen for multiplication and then penetration through natural openings of stigmata into banana fingers. In Taiwan, most farmers protect flower buds with a heavy paper bag during fruit development. This cultural practice can prevent contact of pathogens and insects with banana fruits, and also reduce the incidence of finger-tip rot. The disease is a minor disease and causes little loss in Taiwan.

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摘 要

李永安[1,3]、詹志文[1]、蕭伃佑[1]、趙治平[2]. 2004. 香蕉果指尖腐病病原之 genomovar 鑑定及 *Burkholderia cepacia* 之半選擇性培養基之研發. 植病會刊 13：267-274. ([1.] 輔仁大學生命科學系；[2.] 台灣香蕉研究所；[3.] 聯絡作者，電子郵件：bio1007@mails.fju.edu.tw；傳眞：+886-2-29021124)

在台灣屏東縣種植的香蕉，發現有香蕉果指尖腐病的病徵，此病會造成香蕉果實短小及變形，內部果肉有褐變現象。先前的研究結果，已以生理生化的特性測試及 16S rDNA 基因的核苷酸序列分析結果，鑑定該病原菌爲 *Burkholderia cepacia*。本研究進一步利用 16S rDNA 及 *recA* 基因的 PCR-RFLP 的分析，鑑定此病原細菌屬 *B. cepacia* complex 內之 genomovar III。另以 *recA* 基因之核苷酸序列進行親緣關係分析，進一步確認引起香蕉果指尖腐病之病原細菌屬 genomovar III 內之 *recA* group IIIA。爲進行生態研究，研發出可自罹病及沒有病徵的香蕉，分離出 *B. cepacia* 的半選擇性培養基-QY-TP。在 QY-TP 培養基，所測試 *B. cepacia* 菌株均能生長，而其他病原細菌，如 *Erwinia carotovora*、*E. chrysanthemi*、*Ralstonia solanacearum*、及 *Xathomonas* spp. 則無法生長。此選擇性培養基與 Luria-Bertani 培養基比較，可回收 92 至 98 % 的 *B. cepacia* 菌落，並抑制多數其他細菌的生長。利用此培養基將可用以研究田間的香蕉含有 *B. cepacia* 的比率。

關鍵詞：香蕉果指尖腐病、伯克氏菌、半選擇性培養基