The Causal Organism of Bacterial Black Spot of Mangoes

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ABSTRACT

The pathogen of bacterial black spot of mangoes (*Mangifera indica*) in South Africa was isolated and subjected to a series of pathogenicity and diagnostic tests together with two isolates of *Pseudomonas mangiferaeindicae*, one of *Erwinia mangiferae* and a yellow-pigmented bacterium isolated from old fruit lesions. The pathogen was identified as *P. mangiferaeindicae* and found to be identical to the two isolates included for comparison. The yellow-pigmented organism was found to be identical to *E. mangiferae*, but neither of the two isolates was pathogenic to mangoes. It was concluded that *E. mangiferae* had never been responsible for bacterial black spot of mangoes and that *P. mangiferaeindicae* was the causal agent for all outbreaks of this disease in South Africa, India, and Pakistan. It is suggested that the official description of *P. mangiferaeindicae* be amended and extended, and that this species be removed from the fluorescent group in the key for the identification of species of the genus *Pseudomonas*.

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Bacterial black spot of mangoes was first observed in South Africa by Doidge (3) who isolated and described the pathogen as *Bacillus mangiferae* n.sp. The bacterium, a yellow-pigmented, gram-negative and peritrichously flagellated rod, has since been reclassified as *Erwinia mangiferae* Doidge (1). From black spot disease symptoms on mangoes in India, a polarly flagellated, gram-negative rod was isolated and described as *Pseudomonas mangiferaeindicae* by Patel, Moniz and Kulkarni (14). From apparently similar lesions on mangoes in Pakistan, Khan and Kamal (9) isolated a yellow-pigmented, peritrichously flagellated rod which, according to their description, resembled *E. mangiferae* more closely than *P. mangiferaeindicae*. During a recent outbreak of bacterial black spot of mangoes in South Africa, two bacterial types were isolated. From old lesions a yellow-pigmented, gram-negative, peritrichously flagellated rod was isolated, whereas polarly flagellated, short, gram-negative rods were isolated from young, watersoaked lesions (20). In subsequent experiments, the yellow rod proved to be nonpathogenic even under the most favorable conditions, whereas the short rod repeatedly produced typical symptoms on mango leaves and fruit. This bacterium was tentatively identified as a *Pseudomonas* species (21). This paper deals with the identification of the mango bacterial black spot pathogen, and the pathogenicity of *Erwinia mangiferae* and the yellow-pigmented organism isolated from old lesions.

MATERIALS AND METHODS.—*Cultures used and preparation of inocula.*—Five different isolates were used; namely, a pseudomonad isolated from young lesions on mango leaves (number 89), *E. mangiferae* (obtained from Dr. J. J. Joubert, Faculty of Agriculture, University of Natal, Pietermaritzburg), a yellow-pigmented organism (YPO) isolated from old lesions on mangoes (21), and two isolates of *P. mangiferaeindicae* (490 and 2387) kindly provided by the National Collection of Plant Pathogenic Bacteria, Hertfordshire, England. Each isolate was grown in Nutrient Broth (Difco) in shake culture on a Griffen shaker at 25 C for 24 h. Inocula were prepared by standardizing cell

TABLE 1. Diverse cultural characteristics of bacterial isolates from mango leaves [a yellow-pigmented organism (YPO) and isolate 89 of *Pseudomonas mangiferaeindicae*] and selected reference bacteria [*Erwinia mangiferae* (from J. J. Joubert) and isolates 490 and 2387 of *P. mangiferaeindicae* (from the National Collection of Plant Pathogenic Bacteria, Hertsfordshire, England)]

Characteristics	YPO	*E. mangiferae*	*P. mangiferaeindicae* isolates		
			2387	490	89
Accumulation of poly-β-hydroxy-butyrate	–[a]	–	–	–	–
Capsule	–	–	–	–[b]	–
Gelatinase	–	–	+	+	+
Oxidase	–	–	±	–	–
Catalase	+	+	+	+	+
Arginine dihydrolase	–	–	–	–[c]	–
Cellulase	+	+	+	+	+
Pectinase	–	–	–	–	–
Urease	–	–	+	+	+
Amylase	–	–	+	+	+
Lipase	–	–	+	+	+
Indole	–	–	–	–	–
H_2S	–	–	–	–	–
Levan	±	±	++	++	++
Litmus milk	–	–	–	–	–
Fluorescent pigment	–	–	–	–	–
NO_3-reduction	+	+	–	–	–
Growth in vitamin-free medium on:					
Lactate	++	++	+	+	+
Tartrate	++	++	–	–	±
Asparagine	++	++	–	+	+
β-alanine	–	–	–	±	–
Arabinose	++	++	±	–	–
Sucrose	++	++	++	++	++
Erythritol	±	+	–	+	±
Mannitol	++	±	±	±	±
Sorbitol	++	++	–	+	+
Salicin	++	±	±	±	±
Inulin	+	+	+	+	+
Tobacco hypersensitivity	–	–	+[d]	+[d]	+[d]
Potato soft rot	–	–	–	–	–
Gluconate	–	–	–	–	–

[a] + = positive reaction; – = negative reaction; ± = weak positive reaction.
[b] Slime produced.
[c] A strong acid reaction was observed, indicating that arginine was utilized as a carbon and energy source.
[d] Only a slight yellow discoloration of the induced water-soaked lesions developed.

suspensions spectrocolorometrically to a density of 10^8 cells per ml.

Leaf infections under greenhouse conditions.—Young mango trees of Kent, Sabre, and Peach cultivars were used. Prior to infection, the trees were predisposed by a spray of water for 24 h. The inoculum was applied in a water suspension with an Aimer atomizer and each tree was then enclosed in a transparent polyethylene sleeve for another 24 h. Greenhouse temp ranged from 20 to 30 C, and relative humidity from 50-60%.

Identification of the cultures.—The two isolates of *P. mangiferaeindicae* were included in most of the tests for comparison with the pathogen. *Erwinia mangiferae* was likewise included for comparison with the YPO, although the diagnostic tests were not meant to be exhaustive in this case.

Unless otherwise stated, all tests were carried out according to the methods of the Society of American Bacteriologists (18) on Bacto Agar, or other Difco media. Known positive- and negative-reacting bacteria were included in all tests except those for substrate utilization and the fermentation tests.

Cellular and cultural characteristics.—Cell morphology and size were determined on cultures grown in nutrient broth at 30 C for 24 h. Gram and flagellar stains were formulated according to Salle (15) and Leifson (11), respectively. Tests for intracellular accumulation of poly-β-hydroxybutyrate were conducted according to Burdon's method as described by Cruickshank (2). Colony morphology was investigated by streaking the organisms on the following agar media in petri dishes: nutrient, King's B (10), potato-dextrose, tetrazolium chloride (8), MacConkey, Endo, and D4 (7).

Physiological, metabolical, and enzymological studies.—Growth in stationary and shake culture was studied in nutrient broth. Salt tolerance was tested in nutrient broth with 2% (mass/volume) NaCl. Cardinal temp were determined on nutrient agar slants in thermostatically controlled water baths. Optimum pH was determined in nutrient broth according to Salle (15). Formation of fluorescent pigment at 25 and 30 C respectively was determined by irradiation of streaks on King's B agar plates (10) with an Hanovia ultraviolet lamp after 7 and 14 days. The production of hydrogen sulphide and indole was determined by stabbing in SIM medium, using Kovacs' reagent for detection of indole (Salle, 15). The utilization of carbohydrates was investigated according to the method of Hugh and Leifson as described by Cruickshank (2). The production of acid from carbohydrates was determined on Medium C and peptone water (Dye, 4). The utilization of several organic compounds as sole source of carbon and energy was carried out according to a modification of the method of Stanier, Palleroni, and Doudoroff (19). The vitamin-

TABLE 2. Acid production from several carbohydrates by bacterial isolates from diseased mango leaves [a yellow-pigmented organism (YPO) and isolate 89 of *Pseudomonas mangiferaeindicae*] and selected reference bacteria [*Erwinia mangiferae* (from J. J. Joubert) and isolates 490 and 2387 of *P. mangiferaeindicae* (from the National Collection of Plant Pathogenic Bacteria, Hertsfordshire, England)]

Medium[a]	Isolate	Ara[b]	Fru	Gal	Glu	Manno	Lac	Mal	Suc	Raf	Tre	Gly	Manni
H&L (aerobic):	YPO	++[c]	++	++	++	++	*k*	*k*[d]	++	*k*	++	++	++
	E. mangiferae	++	++	++	++	++	*k*	*k*	++	*k*	+	++	++
	P. mangiferaeindicae												
	isolate 2387	±	±	+	±	+	*k*	*k*	±	*k*	++	±	−
	isolate 490	−	±	+	±	−	*k*	*k*	±	*k*	+	±	−
	isolate 89	+	+	+	+	+	*k*	*k*	±	*k*	+	±	−
H&L (anaerobic):	*YPO*	−	++	−	++	−	+	+++	++	+	−	+	++
	E. mangiferae	−	++	−	++	−	+	+	++	+	−	+	++
	P. mangiferaeindicae												
	isolate 2387	−	±	−	−	−	+	−	±	+	−	+	±
	isolate 490	−	±	−	−	−	+	−	±	+	−	+	±
	isolate 89	−	±	−	−	−	+	−	±	+	−	+	±
Dye (4) (peptone-water):	*YPO*		++				−			−		±	++
	E. mangiferae		++				−			−		±	++
	P. mangiferaeindicae												
	isolate 2387		−				−			−		−	*k*
	isolate 490		−				−			−		−	*k*
	isolate 89		−				−			−		−	*k*
C	YPO		++				−			−		++	++
	E. mangiferae		++				−			−		++	++
	P. mangiferaeindicae												
	isolate 2387		++				+			−		+	±
	isolate 490		++				+			−		+	±
	isolate 89		++				+			−		+	±

[a] H&L = Hugh and Leifson medium, as described by Cruickshank (2); Dye (4) peptone-water medium; and C.

[b] Ara = Arabinose; Manno = Mannose; Raf = Raffinose; Fru = Fructose; Lac = Lactose; Tre = Trehalose; Gal = Galactose; Mal = Maltose; Gly = Glycerol; Glu = Glucose; Suc = Sucrose; and Manni = Mannitol.

[c] + = acid production; ++ = abundant acid production; − = no acid production; ± = doubtful acid production.

[d] *k* = weak alkaline reaction.

free medium was prepared from the following stock solutions: Solution A: distilled water, 200 ml; KH_2PO_4, 1.67 g; $Na_2HPO_4 \cdot 12H_2O$, 6.34 g; $(NH_4)_2SO_4$, 1.0 g; pH 6.8. Solution B: distilled water, 300 ml; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.05 g; $CaCl_2$, 0.02 g; $MnCl_2 \cdot 4H_2O$, 0.002 g; $NaMoO_4 \cdot 2H_2O$, 0.001 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; $Co(NO_3)_2 \cdot 6H_2O$, 0.002 g; $CuSO_4 \cdot 5H_2O$, 0.02 g; Solution C: distilled water, 500 ml; Ion agar, 20 g. The organic compounds were membrane filter sterilized as 10% solutions (m/v, pH7) of the sugars or organic ions and 0.2 ml of the sugars and 0.1 ml in other cases added to a petri dish. Solutions A, B, and C were separately autoclaved, cooled to 50 C, and 4 ml of A, 6 ml of B, and 10 ml of C added to each petri dish and thoroughly mixed. Observations were made 7 days after inoculation. Reaction on litmus milk was determined in reconstituted litmus milk. Cellulose production was investigated in carboxymethylcellulose medium (13). Tests for inducible and constitutive production of pectinase were carried out according to the method of Smith (17) on citrus pectin (Nutritional Biochemical Co.). The method of Smith as published by the Society of American Bacteriologists (18) was used to test for gelatinase. Urease production on Christensen's medium and lipase production were determined according to Cruickshank (2). Alpha-amylase production was determined on certified starch agar plates (15). The LOPAT properties (production of levan and oxidase, induction of potato soft rot, arginine dihydrolase production and tobacco hypersensitivity) and the utilization of gluconate were determined by the methods as described by Lelliott, Billing, and Hayward (12). Unless otherwise stated, all liquid cultures were incubated on a Griffen shaker at 25 C, whereas all other cultures were incubated at 30 C.

RESULTS AND DISCUSSION.—*Pathogenicity tests*.—Only the isolated pseudomonad and isolates 490 and 2387 of *P. mangiferaeindicae* caused typical black spot symptoms. It was impossible to distinguish between symptoms caused by the three different isolates.

Identification.—The pathogen was a gram-negative, monotrichous, polarly flagellated rod, 0.3 to 0.5 μm by 1.0 to 1.5 μm. No poly-β-hydroxybutyrate was accumulated intracellularly. Colonies on nutrient agar were round, raised, and glistening with an entire margin; originally smoke-gray, but creamy colored after 3 days. Older colonies had irregular margins. Colonies on King B agar were round, raised, and white to creamy after 24 h. On potato-dextrose agar, colonies were white-to-creamy, round, raised, and glistening with an entire margin. Streak cultures changed from smoke-gray to white with time. Colonies on tetrazolium chloride agar were glistening with a purple-to-pink center after 2 days. On MacConkey agar, colonies were glistening, white, and raised. No growth occurred on Endo agar and medium D4. A nutrient broth shake culture became turbid within 24 h. In stationary culture, a ring (but no pellicle) was formed within 3-4 days while only a slight turbidity of the medium was observed. The organism was salt-tolerant with an optimal growth temp of between 25 and 30 C, and minimum and maximum temp of 7 C and 36 C, respectively. No growth occurred at 37 C. The optimum pH for growth was between 6.6 and 7.4. Other characteristics of the pathogen as well as the other four isolates are presented in Tables 1 and 2.

Five keys were consulted for the identification of the pathogen. It was impossible to identify the pathogen by using the key to the species of the genus *Pseudomonas* in Bergey's Manual (1). However, with the aid of the host-plant key of the same authors, the pathogen was identified as *Pseudomonas mangiferaeindicae* Patel et al. (14). Some deviations from the description in Bergey's Manual were observed, but the identity of the pathogen was confirmed by the high degree of similarity between the pathogen and the two isolates of *P. mangiferaeindicae* which were included in most of the tests. It should be noted that neither the pathogen nor the two isolates produced blue-green fluorescent pigments, and that Patel et al. (14) did not mention the production of such a pigment. Therefore the inclusion of the production of a fluorescent pigment as a determinative property for *P. mangiferaeindicae* in Bergey's Manual (1) is subject to criticism. The official description and key for the identification of this species should therefore be amended and extended. The remaining three keys for plant-pathogenic pseudomonads led to a different identification of the mango pathogen. The key of Schroth and Hildebrand (16) does not assign prime importance to the production of a blue-green fluorescent pigment as a determinative characteristic and accordingly the pathogen was previously tentatively identified as *P. syringae* van Hall (20). The keys of Hildebrand and Schroth (6) and Lelliott et al. (12) were explicitly compiled for fluorescent phytopathogenic pseudomonads, thus excluding the mango pathogen. If this characteristic was ignored, the pathogen could be identified as *P. syringae*. However, the pathogen clearly differed from *P. syringae* as described in Bergey's Manual (1) with respect to size and number of flagella, as well as a number of physiological and biochemical characteristics. *Pseudomonas mangiferaeindicae* should therefore not be regarded as a nonfluorescent subspecies of *P. syringae*, but rather it should be recognized as a separate species.

In comparative tests, the YPO and *Erwinia mangiferae* were found to be identical, and the differences observed were only quantitative. This was in agreement with the opinion of Dye (5) that *E. mangiferae* is synonomous with *E. herbicola*, a saprophyte common on plants throughout the world. *Pseudomonas mangiferaeindicae* grows slowly and sometimes occurs as a contaminant with the YPO in isolations from old lesions. This possibly explains the results obtained by Doidge (3) and Khan and Kamal (9). It is doubtful whether *E. mangiferae* could ever have been responsible for bacterial black spot on mangoes in South Africa as reported by Doidge (3), or in Pakistan as reported by Khan and Kamal (9).

It is concluded that *P. mangiferaeindicae* was responsible for all cases of bacterial black spot on mangoes, and that *E. mangiferae* was erroneously regarded as the pathogen.

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