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## **Rapid Identification of *Erwinia chrysanthemi* Isolated from Soft Rotted Eggplant and *Phalaenopsis* sp. by Lipid and Fatty Acid Profiling**

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Rapid identification of *Erwinia chrysanthemi* isolated from an eggplant and a *Phalaenopsis* sp. was conducted by lipid and fatty acid profiling. In lipid profiles, the eggplant and phalaenopsis strains as well as *E. chrysanthemi* pv. *chrysanthemi* were differentiated from *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* on the basis of the presence or absence of a spot at Rf 0.66, which was specific for *E. carotovora*. Further, marked differences were also observed in fatty acid composition of the strains. The ratios of the amount of lauric acid (12:0) and myristic acid (14:0) clearly differentiated eggplant and phalaenopsis strains and *E. chrysanthemi* pv. *chrysanthemi* from *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*. Fatty acid profiles of the eggplant and phalaenopsis strains were identical with that of *E. chrysanthemi* pv. *chrysanthemi*. The results of lipid and fatty acid profiling indicated that the eggplant and phalaenopsis strains are *E. chrysanthemi*. Physiological and biochemical tests also confirmed the results. The lipid and fatty acid analysis will be a practical approach for differentiation and identification of *E. chrysanthemi* and *E. carotovora* at least presumptive level.

### INTRODUCTION

*Erwinia chrysanthemi*, originally isolated from chrysanthemum by Burkholder *et al.* (1953) is a member of the pathogenic enterobacteria causing soft rot, stunting and wilting on a wide range of plants in different parts of the world. Though the physiological and biochemical characteristics are the basis of differentiation and identification of the pathogen (Cothier and Powell, 1983; Ngwira and Samson, 1990), fatty acid profiling (Kori *et al.*, 1992), protein analysis (Moline, 1985; Uesugi *et al.*, 1990), DNA homology analysis (Brenner *et al.*, 1973) and PCR-RFLP analysis (Nassar *et al.*, 1996) have also been conducted to differentiate the bacterium from other enterobacteria.

Rapid identification of two strains of *E. chrysanthemi* isolated from an eggplant and a *Phalaenopsis* sp. by lipid and fatty acid profiling is described in this paper. Physiological and biochemical tests were also performed to confirm the results of the lipid and fatty acid profiling.

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## MATERIALS AND METHODS

### Bacterial strains

Two strains isolated from a soft rotted eggplant and a *Phalaenopsis* sp. were included in this experiment. Other three strains, *Erwinia carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup>, *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>r</sup> and *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377 were used as reference strains.

### Rapid extraction TLC for lipid profiles

The rapid extraction – TLC was conducted according to Khan and Matsuyama (1999b) for lipid profiling. One loopful of bacterial cells was gently suspended in 0.2 ml of chloroform – methanol – 0.3% NaCl solution (2:1:0.4, v/v/v) in a small glass vial and kept at least for 15 min at room temperature for lipid extraction. About 10  $\mu$ l of lipid extract was spotted on the origin of a silica gel TLC plate. The plate was developed with chloroform – methanol – 0.2%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (55:35:8, v/v/v) for 1 hr. Lipid spots were detected by spraying ninhydrin followed by heating at 100 °C for 10 min and chromatograms were recorded by photocopy/photograph/computer (Adobe photoshop 3.0J).

### GLC for fatty acid profiles

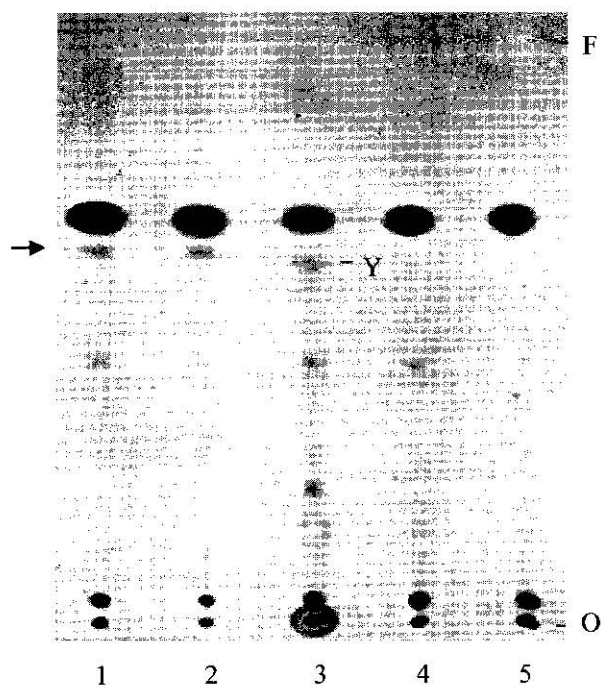
Whole cellular fatty acid analysis was conducted followed by Khan *et al.* (1999a). Bacterial cells were shake-cultured in 523 broth (Kado and Heskett, 1970) at 30 °C for 48 hr. Five milligrams of the lyophilized cells was methylated with 0.5 ml of 5% HCl-methanol at 100 °C for 3 hr in a sealed glass tube. The fatty acid methyl ester (FAME) derivatives were extracted with 0.5 ml petroleum ether. The solvent phase was washed with 0.5 ml distilled water and dehydrated by mixing with 0.5 g anhydrous sodium sulfate. The organic phase was concentrated by nitrogen gas blowing and subjected to gas liquid chromatography (GLC), Shimadzu GC-17A equipped with HR-SS-10 column. The column and injection-port temperatures were maintained at 180 °C and 250 °C, respectively, and the flow rate of nitrogen gas was 50 ml/min. Fatty acids were identified by the comparison of retention time with the standard. Relative similarities among the strains based on the fatty acid composition were assessed with average linkage cluster analysis procedure using the statistics package software SYSTAT.

### Physiological and biochemical tests

Gram stain, oxidase, catalase, potato soft rot, glucose fermentation, acetoin production, nitrate reduction, gelatin liquefaction, urease production, indole test, sensitivity to erythromycin, gas from glucose, methyl red test, lecithinase, reducing substance from sucrose and phosphatase tests were conducted followed by the standard methods (Mc Clung and Toabe, 1947; Hugh and Leifson, 1953; Kovacs, 1956; Lelliot *et al.*, 1966; Dye, 1968; Suslow *et al.*, 1982; Schaad, 1988). Acid production from various organic substrates was tested by Dye's method (Dye, 1968). Utilization of organic acids was determined by streaking one loopful bacterial suspension on the medium of Ayer's *et al.* (1919) supplemented with 0.1% of the substrates. The growth of bacteria was observed after 7, 14 and 21 days of incubation at 30 °C.

## RESULTS AND DISCUSSION

As can be seen in Fig. 1, chromatograms of the eggplant and phalaenopsis strains as well as *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377 were different from those of *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup> and *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>T</sup>. A benchmark spot at Rf 0.66 (arrow in the figure) was observed in *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup> and *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>T</sup>, while absent in the eggplant and phalaenopsis strains as well as *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377. Matsuyama and Furuya (1993) reported that distinct differences were observed between chromatographic profiles of *E. carotovora* subsp.



**Fig. 1.** TLC chromatograms of lipids from eggplant and phalaenopsis strains as well as reference strains by the rapid extraction-TLC.

1. *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup>
2. *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>T</sup>
3. *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377
4. Eggplant strain
5. Phalaenopsis strain

O: origin, F: solvent front, arrow indicates the benchmark spot for *E. carotovora* and Y: indicates a yellow spot for *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377.

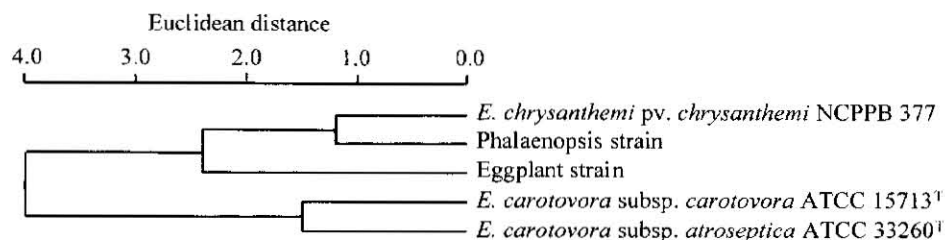
*carotovora* and *E. chrysanthemi* by the direct colony TLC, and a characteristic spot at Rf 0.57 was always detected on the chromatograph of *E. carotovora* subsp. *carotovora*. The results of the present experiment agreed well with the previous findings. A yellow spot (Y in the figure) was observed in *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377, while absent in the other strains (Fig. 1). This spot at Rf 0.62 was pigment produced by this strain. Therefore, this spot was not considered. These results suggested that the eggplant and phalaenopsis strains would be *E. chrysanthemi* but not *E. carotovora*.

Ten kinds of fatty acids were detected in GLC analysis of the FAMES of the strains. Among them, lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1 *cis* 9), stearic acid (18:0), oleic acid (18:1 *cis* 9) and *cis* vaccenic acid (18:1 *cis* 11) were common in all strains. The eggplant and phalaenopsis strains showed identical fatty acid profiles with those of *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377, and these were distinctly different from those of *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup> and *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>T</sup>. Higher amount of lauric acid (12:0) and lower amount of myristic acid (14:0) were characteristically detected in *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup> and *E. carotovora* subsp. *atroseptica* ATCC

**Table 1.** Percentage composition of whole cellular fatty acids in eggplant and phalaenopsis strains as well as reference strains.

Bacterial strain	Fatty acid composition									
	12:0	14:0	16:0 3-OH	16:1 <i>cis</i> 9	18:0	18:1 <i>cis</i> 9	Un-1	18:1 <i>cis</i> 11	18:3 <i>cis</i> 6, 9, 12	
<i>E. carotovora</i> subsp. <i>carotovora</i> ATCC 15713 <sup>T</sup>	14.12	4.96	0	17.20	30.39	1.16	7.53	0	24.61	0
<i>E. carotovora</i> subsp. <i>atroseptica</i> ATCC 33260 <sup>T</sup>	13.74	4.52	0	18.02	27.16	0.68	5.73	0	30.13	0
<i>E. chrysanthemi</i> pv. <i>chrysanthemi</i> NCPPB 377	4.60	6.02	4.99	16.97	20.88	1.15	5.08	2.67	35.6	1.99
Eggplant strain	5.34	8.83	7.19	10.37	22.46	0.82	2.49	1.28	39.72	1.46
Phalaenopsis strain	3.30	9.61	3.50	20.26	19.97	1.08	4.42	2.53	34.19	1.09

Un-, unidentified



**Fig. 2.** Dendrogram of cluster analysis on eggplant and phalaenopsis strains as well as reference strains.

**Table 2.** Physiological and biochemical properties of eggplant and phalaenopsis strains as well as reference strains.

Property	Present strain		Reference strain		
	Eggplant strain	Phalaenopsis strain	<i>E. chrysanthemi</i> pv. <i>chrysanthemi</i> NCPPB 377	<i>E. carotovora</i> subsp. <i>carotovora</i> ATCC 15713 <sup>†</sup>	<i>E. carotovora</i> subsp. <i>atroseptica</i> ATCC 33260 <sup>†</sup>
Gram stain	-	-	-	-	-
Oxidase	-	-	-	-	-
Catalase	+	+	+	+	+
Potato soft rot	+	+	+	+	+
Glucose fermentation	+	+	+	+	+
Acetoin production	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+
Urease production	-	-	-	-	-
Indole test	+	+	+	-	-
Sensitivity to erythromycin	+	+	+	-	-
Gas from glucose	+	+	+	-	-
Methyl red test	-	-	-	+	+
Lecithinase	+	+	+	-	-
Phosphatase	+	+	+	-	-
Reducing substances from sucrose	-	-	-	-	+
Acid production from:					
Mannitol	+	+	+	+	+
Dulcitol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Adonitol	-	-	-	-	-
Inositol	-	-	-	+	-
Inulin	-	-	-	+	+
Salicin	+	+	+	+	+
Maltose	-	-	-	-	-
Cellobiose	+	+	+	+	+
Lactose	-	-	-	+	+
Xylose	+	+	+	+	+
L-Arabinose	+	+	+	+	+
Ribose	+	+	+	+	+
Raffinose	+	+	+	+	+
Mellibiose	+	+	+	+	+
Mannose	+	+	+	+	+
Trehalose	-	-	-	+	+
L-Rhamnose	+	+	+	+	+
Melezitose	-	-	-	-	-
$\alpha$ -Methyl glucoside	-	-	-	-	+
Utilization of:					
Malonate	+	+	+	-	-
D-Tartrate	-	-	-	-	-
Galacturonate	+	+	+	+	+
Na-Citrate	+	+	+	+	+

+, positive; -, negative

33260<sup>T</sup>. However, this trend was *vice versa* in the case of the eggplant and phalaenopsis strains as well as *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377 (Table 1). These results showed that *E. carotovora* will be differentiated clearly from *E. chrysanthemi* by the fatty acid profiles. In particular, the ratio of lauric acid (12:0) and myristic acid (14:0) will be useful marker. Similar observation was reported earlier by Kori *et al.* (1992). Furthermore, 3-hydroxy capric acid (10:0 3-OH), linolenic acid (18:3 *cis* 6, 9, 12) and one unidentified fatty acid (unidentified - 1) were not recorded in *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup> and *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>T</sup> but observed in the eggplant and phalaenopsis strains as well as *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377 (Table 1).

Dendrogram based on the fatty acid composition formed two clusters. The eggplant and phalaenopsis strains were grouped with *E. chrysanthemi* pv. *chrysanthemi* NCPPB 377. On the other hand, *E. carotovora* subsp. *carotovora* ATCC 15713<sup>T</sup> and *E. carotovora* subsp. *atroseptica* ATCC 33260<sup>T</sup> formed another cluster (Fig. 2). These results indicated that the eggplant and phalaenopsis strains are *E. chrysanthemi*.

Physiological and biochemical properties of the eggplant and phalaenopsis strains showed that they were positive in catalase, potato soft rot, glucose fermentation, acetoin production, nitrate reduction, gelatin liquefaction, indole production, sensitivity to erythromycin, gas from glucose, lecithinase and phosphatase tests. They were negative in Gram staining, oxidase, urease production, methyl red test and production of reducing substances from sucrose. The results of the tests for acid production and utilization of carbon source summarized in Table 2 confirmed that the eggplant and phalaenopsis strains are *E. chrysanthemi*. This experiment proved the effectiveness of lipid and fatty acid profiling for identification of phytopathogenic bacteria at least presumptive level.

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