

## STUDIES ON PECTOLYTIC *ERWINIA* SPP. IN PORTUGAL REVEAL UNUSUAL STRAINS OF *E. CAROTOVORA* SUBSP. *ATROSEPTICA*

A.B. Costa<sup>1,2</sup>, M. Eloy<sup>2</sup>, L. Cruz<sup>2</sup>, J.D. Janse<sup>3</sup> and H. Oliveira<sup>1</sup>

<sup>1</sup> Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisboa, Portugal

<sup>2</sup> Direcção Geral de Protecção das Culturas, Tapada da Ajuda, Edifício 1, 1349-018 Lisboa, Portugal

<sup>3</sup> Plant Protection Service, 15 Geertjesweg 9102, 6700 HC, Wageningen, The Netherlands

### SUMMARY

The bacterial pathogens *Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* are responsible for soft rot diseases, affecting plants in the field and/or in storage. Thirty-one strains from Portugal and elsewhere were characterised by classical, fatty acid methyl ester (FAME) and molecular methods (MSP-PCR and BOX-PCR). Ten strains were identified as *E. carotovora* subsp. *atroseptica*, 10 as *E. carotovora* subsp. *carotovora*, and 11 as *E. chrysanthemi* by classical methods. Strains of *E.c.* subsp. *carotovora* and *E. chrysanthemi* were phenotypically, chemotaxonomically, and genetically highly variable. *E. c.* subsp. *atroseptica* strains isolated from Portuguese fields had low molecular variability, but FAME analysis revealed high phenotypic variability. Fatty acid profiles of the Portuguese strains were compared with 27 *E. c.* subsp. *atroseptica* profiles obtained from the Dutch Plant Protection Service (PD) collection. The Portuguese *E. c.* subsp. *atroseptica* strains clustered separately from PD *E. c.* subsp. *atroseptica* strains. However, the clusters are not distant enough for Portuguese strains to be included in separate subspecies. Therefore, these Portuguese *E. carotovora* subsp. *atroseptica* strains may constitute an ecotype occurring in Portugal.

*Key words:* *Erwinia*, FAME, MSP-PCR, BOX-PCR, soft rot, variability.

### INTRODUCTION

*Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* are pectolytic bacteria that cause soft rot diseases in many economically important crops (Pérombelon and Kelman, 1980). In temperate climates, such as in Portugal, *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* can all seriously affect the production of ornamental and/or horticultural crops.

The classification of soft rot bacteria has been controversial and several studies have been conducted in order to clarify their taxonomy (Kwon *et al.*, 1997; Hauben *et al.*, 1998; Avrova *et al.*, 2002). Gardan *et al.* (2003) proposed that pectolytic bacteria of the genus *Erwinia* should be included in a separate genus, *Pectobacterium*, and the subspecies *atroseptica* be elevated to species level. However, both classifications are still valid in the scientific literature. Although bacteria in these three taxons can cause soft rot in plants, their host range and optimum temperatures are distinct. *E. c.* subsp. *atroseptica* infects mainly potato plants and tubers at an optimum temperature of 20°C, whereas *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* have a wider host range and cause disease at higher temperatures, 20 to 35°C (Schober and Zadoks, 1999). Mixed infections by *E. c.* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* can occur within the same plant (Pérombelon, 1972). Since these pathogens have common hosts, a better understanding of the variability of these bacteria is needed in order to develop methods to effectively identify each pathogen.

Phenotypic studies using biochemical and physiological methods on soft rot erwinias have been and are still undertaken in parallel to other methods to identify and characterise the group (Dye, 1968, 1969; De Boer *et al.*, 1987; Toth *et al.*, 1999; Seo *et al.*, 2003; Yahiaoui *et al.*, 2003). Fatty acid analysis proved to be a reliable and accurate method to identify many organisms, including soft rot bacteria, and could distinguish sub-species (Persson and Sletten, 1995; Seo *et al.*, 2002). Among the pectolytic bacteria, *E. carotovora* subsp. *atroseptica* is the most serious in cool climates, causing severe losses to potato crops (Pérombelon and Kelman, 1980).

Most studies on the diversity of soft rot bacteria have therefore been on this organism. Molecular methods such as restriction fragment length polymorphism (RFLP) (Darrasse *et al.*, 1994; Nassar *et al.*, 1996; Van der Wolf *et al.*, 1996; Hélias *et al.*, 1998; Slédz *et al.*, 2000; Yahiaoui *et al.*, 2003), random amplified polymorphic DNA (RAPD) (Maki-Valkama and Karjalainen, 1994; Toth *et al.*, 1999) and enterobacterial repetitive intergenic consensus (ERIC) (Toth *et al.*, 1999) have been used.

Toth *et al.* (2001) described use of the 16S-23rRNA intergenic transcribed spacer combined with PCR (ITS-PCR) followed by restriction length polymorphism (ITS-RFLP) analysis for identification and differentiation of the soft rot erwinias. Although several PCR-based methods have been used in genetic diversity studies, repetitive extragenic palindromic PCR genomic fingerprinting with the BOX primer set (BOX-PCR) and Mini-satellite primed (MSP)-PCR have not yet been tested on soft rot erwinias. While MSP-PCR has been used mostly for fungi (Longato and Bonfante, 1997; Meyer *et al.*, 2001; Gadanho and Sampaio, 2002), BOX-PCR fingerprinting has been used mainly for bacteria (Darrasse *et al.*, 1994; Rademaker and de Bruijn, 1999; Coenye *et al.*, 2002; Sahin *et al.*, 2003).

The aim of this study was to characterise Portuguese *E. c.* subsp. *atroseptica* and other soft rot *Erwinia* strains (*E. carotovora* subsp. *carotovora* and *E. chrysanthemi*) on the basis of biochemical, physiological and pathogenicity tests, by fatty acid analysis and molecular methods based on PCR (MSP-PCR and BOX-PCR).

## MATERIALS AND METHODS

### Bacterial strains, isolation and culture conditions.

The strains used are listed in Table 1. Twenty-two erwinia strains were isolated from potato plants showing soft rot symptoms from the Escaroupim (Marinhais, Portugal) experimental site of the Direcção Geral de Protecção das Culturas (DGPC) in 2001 and 2002. Eight strains of *E. carotovora* subsp. *carotovora* were obtained from the Dutch Plant Protection Service (PD) and one *E. chrysanthemi* strain was obtained from the National Collection of Plant Pathogenic Bacteria (NCPBB). All 31 strains were characterised by classical methods, fatty acid analysis, and molecular methods. Strains were stored in cryoprotector medium (Protect, Technical Service Consultants Limited, Lancashire, UK) at -80°C and included in the DGPC collection of plant pathogenic bacteria. Bacteria were grown at 27°C on King's medium B (KB) (King *et al.*, 1954) or Nutrient Agar (NA) as required.

**Cultural, biochemical and physiological tests.** All strains were tested for fermentative metabolism (Hugh and Leifson, 1953), oxidase and catalase activities, gelatin hydrolysis and the ability to cause soft rot in potato slices (Lelliott and Stead, 1987; Dickey and Kelman, 1988). The ability to degrade pectate was tested on crystal violet pectate (CVP) medium (Lelliott and Stead, 1987) and Stewart modified medium (Pérombelon, 1971). Strains showing characteristics of the genus *Erwinia* were then identified to the species (*E. chrysanthemi* and *E. carotovora*) or subspecies levels (*E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*)

on the basis of the following tests: erythromycin sensitivity (Dickey and Kelman, 1988), phosphatase activity (Lelliott and Stead, 1987), indole production (Dickey and Kelman, 1988), production of reducing substances from sucrose (Dye, 1969), acid production from malonate (Dye, 1969), ability to grow at 36-37°C and acid production from the following carbon sources: lactose, trehalose, maltose,  $\alpha$ -methyl-D-glucoside, melibiose and rhamnose (Dye, 1969).

**Pathogenicity tests.** All strains were tested for pathogenicity on tomato (*Lycopersicon esculentum* cv Money-maker), Chinese cabbage (*Brassica rapa* var. *chinensis*) and potato plants (*Solanum tuberosum* cv Kennebec). Bacterial cells grown for 24 h on Nutrient Agar (Difco, Detroit, USA) at 27°C were suspended in sterile distilled water (SDW) at a concentration of  $10^8$  CFU ml<sup>-1</sup> and 100 ml were inoculated by injection into the plant stem at the second and third axial leaf from the base. Each strain was inoculated in six plants per host. Ten control plants were injected with SDW. Inoculated plants were covered with plastic bags to maintain high humidity for 48 h in growth chambers at 22°C and 16 h photoperiod and evaluated for symptoms up to 21 days after inoculation.

**Fatty acid analysis.** Whole cell fatty acids were extracted and methylated as described by Sasser (1990). Fatty acid methyl esters (FAME) were separated by gas chromatography using the Microbial Identification System [MIS, Microbial ID, Inc. (MIDI), Newark, USA]. Fatty acid profiles of the 31 Portuguese strains were compared with profiles of *E. carotovora* subsp. *carotovora* strains present in the PD database (MIDI system including a standard library and a PPS Wageningen generated library) and submitted to statistical analysis.

**MSP-PCR and BOX-PCR fingerprinting.** All the thirty-one strains were fingerprinted using the mini-satellite csM13 primer (5'-GAGGGTGGCGTTCT-3') and BOX-A1R primer (5'-CTACGGCAAGGC-GACGCTGACG-3') from the BOX subunit (Versalovic *et al.*, 1994). DNA template was prepared by boiling bacterial suspensions in 500  $\mu$ l SDW for 6 min.

The PCR conditions and amplification programs were as described by Gadanho and Sampaio (2002) and Louws *et al.* (1995), respectively. Amplifications were performed in a Programmable Thermal Cycler 100 (MJ Research, Incline Village, USA). PCR products (10  $\mu$ l) were separated by gel electrophoresis in 2% agarose gels in Tris-acetate-EDTA (TAE) buffer (0.04 M Tris-HCl, 20 mM sodium acetate, 2 mM EDTA). At least two lanes with 1Kb plus ladder (Invitrogen Life Technologies, California, USA) were included on each gel.

Following staining with ethidium bromide, the gels were viewed and photographed under UV illumination.

**Data analysis.** Statistical analysis was performed using Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) software package version 2.1 (Rolph, 2000).

Twenty-seven fatty acid profiles of *E. carotovora* subsp. *atroseptica* from the PD collection were included in the analysis. The fatty acid profiles were submitted to both cluster analysis and principal component analysis (PCA).

Cluster analysis was done using UPGMA on dissimilarity matrices calculated with the Euclidian coefficient. Genetic relationships among and within strains (*E. c.* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*) were determined by cluster analysis performed by UPGMA on distance matrices calculated with the Dice coefficient. The robustness of clusters was evaluated by calculating cophenetic correlations.

## RESULTS

**Identification by biochemical and physiological methods.** Bacteria were identified on the basis of biochemical and physiological profiles according to Dye (1969) and Lelliott and Stead (1987). From the 22 strains isolated from Portugal, 10 were identified as *E. c.* subsp. *atroseptica*, two as *E. carotovora* subsp. *carotovora* and 10 strains as *E. chrysanthemi*. The identification of eight strains received as *E. carotovora* subsp. *carotovora* from the Dutch Plant Protection Service (PD) collection and one as *E. chrysanthemi* from NCPPB was confirmed (Table 2). No deviating results were found regarding Portuguese *E. carotovora* subsp. *atroseptica* strains. However, *E. chrysanthemi* strains 982, 984 and

**Table 1.** Source and origin of the 31 *Erwinia* spp. strains used in this study isolated from Portugal and obtained from PD collection.

Received/Obtained as	DGPC Strain Code	Host	Origin	Date
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	974	<i>Solanum tuberosum</i> cv. Spunta	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	976	<i>Solanum tuberosum</i> cv. Spunta	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	977	<i>Solanum tuberosum</i> cv. Spunta	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	981	<i>Solanum tuberosum</i> cv. Pentland Dell	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	985	<i>Solanum tuberosum</i> cv. Rooster	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	995	<i>Solanum tuberosum</i> cv. Kennebec	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	999	<i>Solanum tuberosum</i> cv. Kennebec	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	1000	<i>Solanum tuberosum</i> cv. Maris Piper	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	1074	<i>Solanum tuberosum</i>	Portugal	2002
<i>E. carotovora</i> subsp. <i>atroseptica</i> <sup>1</sup>	1105	<i>Solanum tuberosum</i> cv. Spunta	Portugal	2002
<i>E. carotovora</i> subsp. <i>carotovora</i> <sup>1</sup>	1012	<i>Solanum tuberosum</i> cv. Kennebec	Portugal	2002
<i>E. carotovora</i> subsp. <i>carotovora</i> <sup>1</sup>	1132	<i>Apium vulgare</i>	Portugal	2002
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 932	1115	<i>Aglaonema</i> spp.	Jamaica	1987
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 934	1116	<i>Lactuca sativa</i>	Netherlands	1987
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 1060	1117	<i>Dracaena</i> spp.	Puerto Rico	1988
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 1070	1118	<i>Solanum tuberosum</i> cv. Baraka	Brazil	1988
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 1278	1119	<i>Kalanchoe</i> spp.	Israel	1989
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 1679	1120	<i>Ornithogalum arabicum</i>	Kenya	1990
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 2412	1122	<i>Solanum tuberosum</i> cv. Spunta	Sudan	1994
<i>E. carotovora</i> subsp. <i>carotovora</i> PD <sup>3</sup> 3882	1125	<i>Zamioculcas</i>	Costa Rica	2000
<i>E. chrysanthemi</i> <sup>1</sup>	982	<i>Solanum tuberosum</i> cv. Kondor	Portugal	2002
<i>E. chrysanthemi</i> <sup>1</sup>	984	<i>Solanum tuberosum</i> cv. Kondor	Portugal	2002
<i>E. chrysanthemi</i> <sup>1</sup>	989	<i>Solanum tuberosum</i> cv. Désirée	Portugal	2002
<i>E. chrysanthemi</i> <sup>1</sup>	996	<i>Solanum tuberosum</i> cv. Kennebec	Portugal	2001
<i>E. chrysanthemi</i> <sup>1</sup>	998	<i>Solanum tuberosum</i> cv. Kennebec	Portugal	2001
<i>E. chrysanthemi</i> <sup>1</sup>	1004	<i>Solanum tuberosum</i> cv. Baraka	Portugal	2001
<i>E. chrysanthemi</i> <sup>1</sup>	1005	<i>Solanum tuberosum</i> cv. Baraka	Portugal	2001
<i>E. chrysanthemi</i> <sup>1</sup>	1028	<i>Solanum tuberosum</i> cv. Baraka	Portugal	2002
<i>E. chrysanthemi</i> <sup>1</sup>	1041	<i>Zea mays</i>	Portugal	2001
<i>E. chrysanthemi</i> <sup>1</sup>	1084	<i>Solanum tuberosum</i>	Portugal	2002
<i>E. chrysanthemi</i> bv. 7 NCPPB <sup>2</sup> 3710	934	<i>Solanum tuberosum</i> cv. Sante	United Kingdom	1990

<sup>1</sup> DGPC: Portuguese Plant Protection Service; <sup>2</sup> NCPPB: National Collection of Pathogenic Plant Bacteria; <sup>3</sup> PD: Dutch Plant Protection Service

**Table 2.** Biochemical, physiological and pathogenicity characteristics of ten strains of *E. carotovora* subsp. *atroseptica* from Portugal, ten *E. carotovora* subsp. *carotovora* (two from Portugal and eight worldwide) and eleven *E. chrysanthemi* (ten from Portugal and one from UK). Characteristics of the strains were evaluated by comparison with a typical strain reaction according to Dye (1968) and Lelliott and Stead (1987).

Biochemical, physiological and pathogenicity characteristics	DGPC Strain Code													
	<i>E. carotovora</i> subsp. <i>atroseptica</i>		<i>E. carotovora</i> subsp. <i>carotovora</i>					<i>E. chrysanthemi</i>						
	Typical reaction	974, 976*, 977, 981, 985, 995, 999, 1000, 1074, 1105	Typical reaction	1132	1012, 1115*, 1117, 1120, 1122, 1125	1116	1118, 1119	Typical reaction	934	982, 984	998	989	996	1004, 1005, 1028, 1041, 1084
Erythromycin sensitivity	-	-	-	+	-	-	-	+	+	-	+	+	+	+
Phosphatase activity	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	+	V	+	+	+	+	-	+
Reducing subst. auces from sucrose	+	+	-	-	-	+	-	V	-	-	-	+	-	-
Malonate utilisation	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Growth at 36-37°C	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Acid production from:														
Lactose	+	+	+	+	+	+	+	-	-	+	+	-	-	-
Trehalose	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Maltose	+	+	V	-	-	-	-	-	-	-	-	-	-	-
α-methyl-D-glucoside	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	+	+	+	V	-	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pathogenicity on:														
Potato	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tomato	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cabbage	+	+	+	+	+	+	+	+	+	+	+	+	+	+

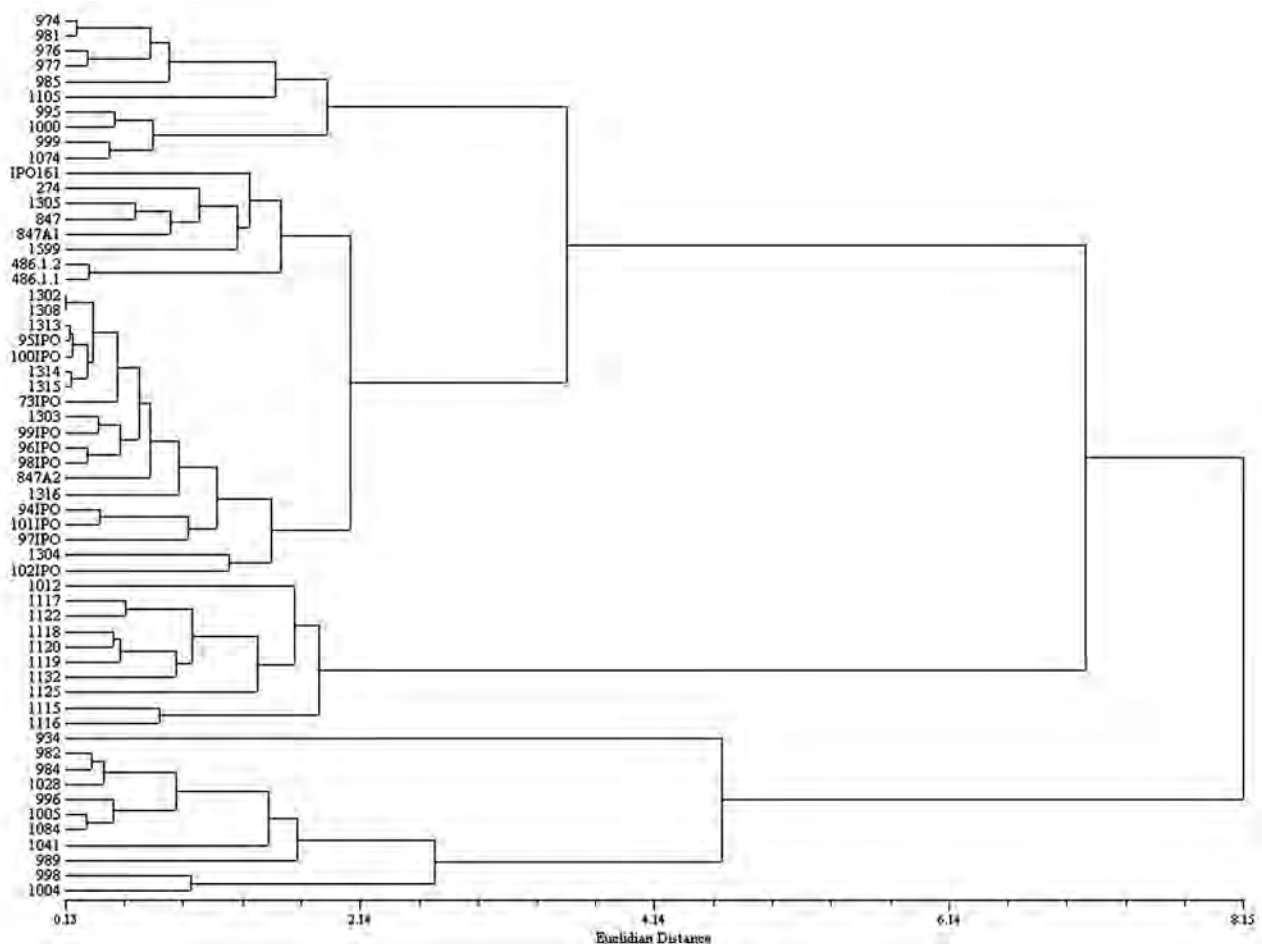
Positive result: +; Negative result: -; Variable result: V.

998 from Portugal were able to produce acid from lactose and strains 982 and 984 also did not show sensitivity to erythromycin. *E. c.* subsp. *carotovora* strain 1132 from Portugal showed sensitivity to erythromycin. *E. carotovora* subsp. *carotovora* 1118 and 1119, from the PD collection, showed the ability to produce indole and strain 1116 reduced compounds from sucrose.

**Pathogenicity tests.** All *E. c.* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains inoculated to tomato, Chinese cabbage and potato plants were pathogenic, with the exception *E. carotovora* subsp. *atroseptica* 976 (negative on cabbage) and *E. carotovora* subsp. *carotovora* 1115 (negative on potato) (Table 2). *E. carotovora* subsp. *atroseptica* strains were more virulent on potato plants, although they also induced symptoms in the other hosts, such as wilt, leaf chlorosis and stem soft rot (not shown). The majority of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains were more virulent on tomato and Chinese cabbage than *E. carotovora* subsp. *atroseptica* strains, leading to plant death.

**FAME analysis.** The identification of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains performed with biochemical and physiological tests was confirmed by FAME. However, FAME analysis was not able to identify the 10 Portuguese strains previously identified as by classical methods as *E. carotovora* subsp. *atroseptica*. No match was obtained between these bacteria and the PD database. In order to clarify these results, 27 fatty acid profiles of *E. carotovora* subsp. *atroseptica* from the PD collection were compared with profiles from Portuguese strains. Fatty acid profiles of the 31 strains and 27 from the PD database were analysed together. The dendrogram obtained (cophenetic coefficient of 0.93) confirmed that *E. c.* subsp. *atroseptica* strains form two main clusters, corresponding to Portuguese and PD, separated by 3.5 Euclidian distances (Fig. 1).

Although the Portuguese *E. carotovora* subsp. *atroseptica* strains displayed high variability, the Euclidian distance (3.5) indicated that Portuguese strains belong to the subspecies *atroseptica*. In addition, FAME analysis revealed a higher content of palmitic acid associated with *E. carotovora* subsp. *atroseptica* Portuguese



**Fig. 1.** Dendrogram derived from FAME analysis of 31 Portuguese *E. carotovora* subsp. *atroseptica* strains compared with *E. carotovora* subsp. *atroseptica* strains from the Dutch Plant Protection Service collection. Analysis was performed with Average linkage clustering (UPGMA) of correlation coefficients and using Euclidian distance dissimilarity coefficient.  $R=0.93$ .

strains than with PD strains. The subspecies *carotovora* and *atroseptica* were separated by 7 Euclidian distances. As expected, *E. chrysanthemi* strains formed a cluster distant from *E. carotovora* strains (8.15 Euclidian distances), confirming the identity of these strains (Fig. 1).

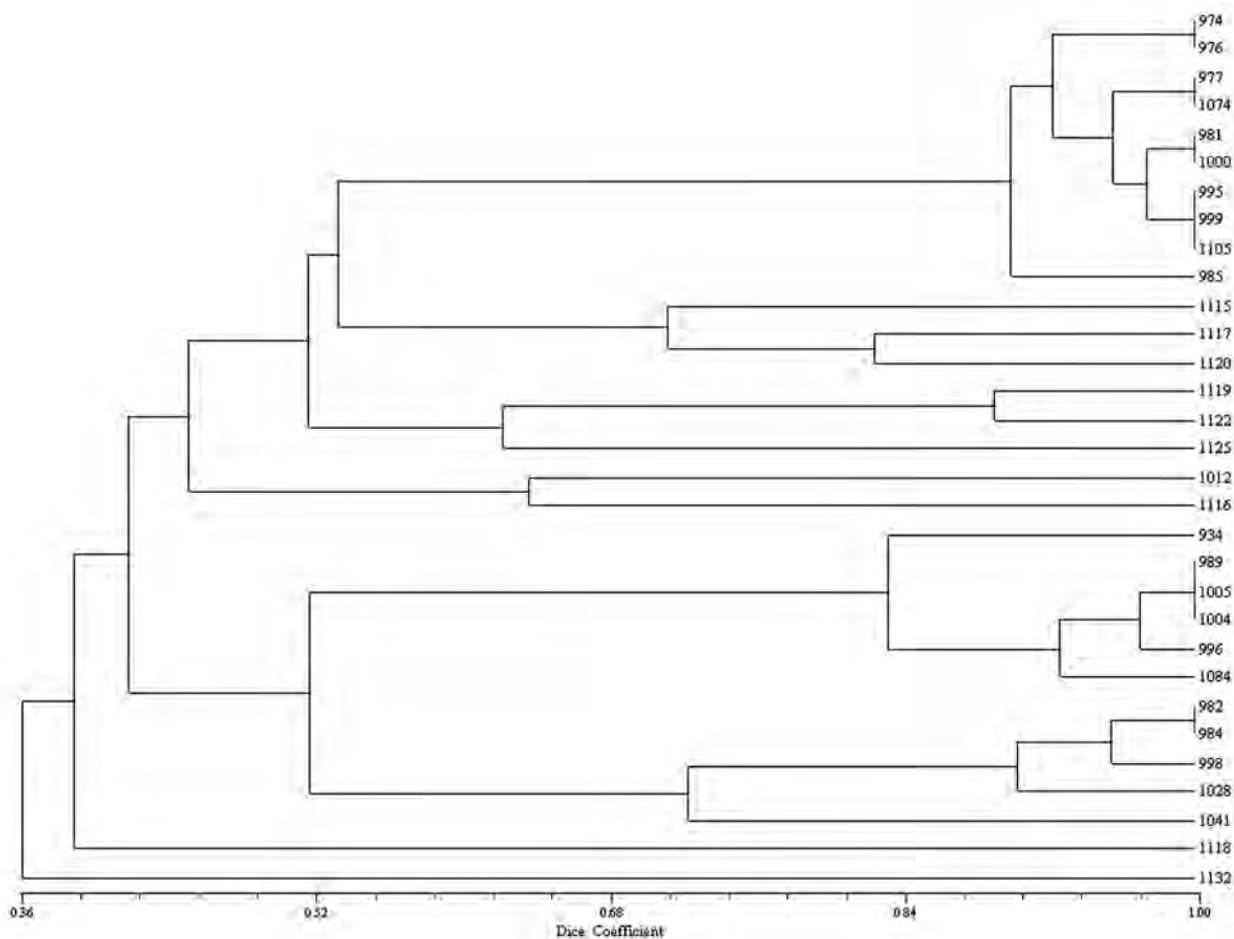
**MSP and BOX-PCR fingerprinting.** The dendrogram generated with BOX-PCR and MSP-PCR fingerprints (cophenetic coefficient of 0.92) showed three main clusters formed by *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains (Fig. 2). While *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* strains were grouped into individual clusters, clearly delimited, *E. carotovora* subsp. *carotovora* strains were divided into one main cluster and a minor cluster. This minor cluster is distant from the major cluster by 0.64 and includes Portuguese strain 1132 and one strain from the PD collection (1118) (Fig. 2).

Fingerprints obtained with BOX-PCR and MSP-PCR (Fig. 3 and 4) revealed low genetic variability within *E. c.* subsp. *atroseptica* strains. Polymorphisms generated with BOX-PCR were mainly observed in strains 974, 976 and 985, characterised by the presence of an 850 bp frag-

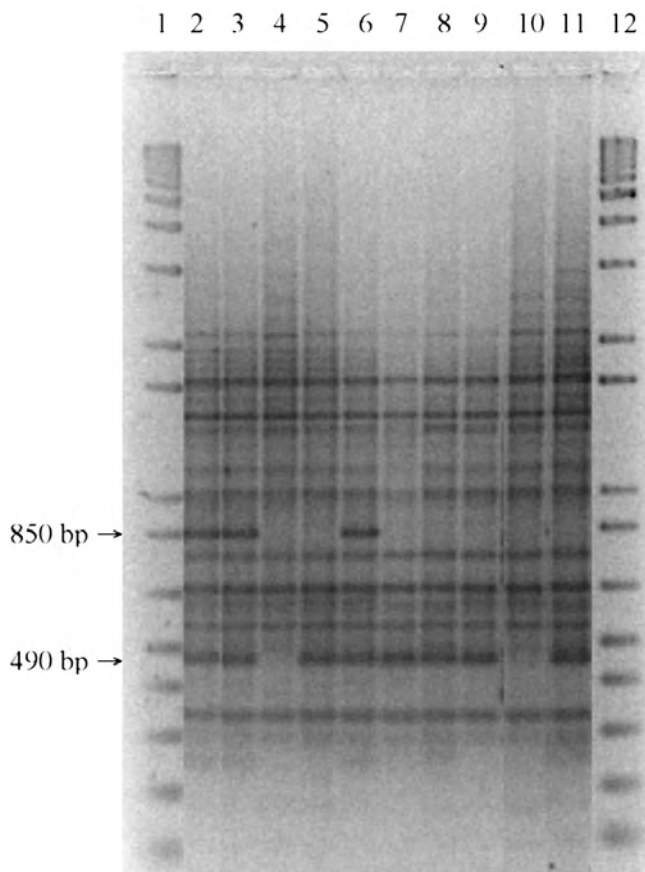
ment whereas strains 977 and 1074 lacked a 490 bp fragment (Fig. 3). Strain 985 also showed polymorphisms when analysed by MSP-PCR such as the presence of 900 bp and 1500 bp fragments (Fig. 4). *E. c.* subsp. *carotovora* and *E. chrysanthemi* fingerprints showed high genetic variability (not shown).

## DISCUSSION

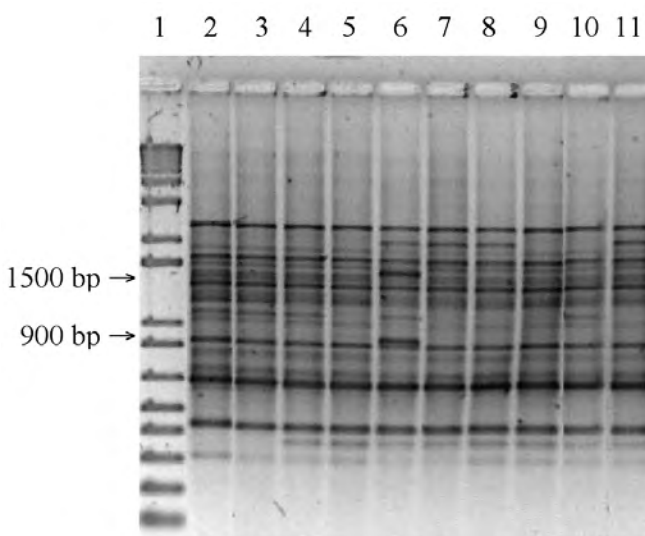
Characterisation of soft rot bacteria *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* is necessary to understand variability between these bacteria and thus enable the development of rapid diagnostic methods. In Portugal all three soft rot bacteria can cause severe damage. The current characterisation of Portuguese and worldwide soft rot bacteria by different methods represents a major contribution to understanding the Portuguese population of these pathogens. FAME analysis of 10 *E. c.* subsp. *atroseptica* isolates from Portugal showed high phenotypic variability not detectable by classical methods. Comparison of 27 fatty acid profiles from the PD database confirmed that



**Fig. 2.** Dendrogram derived by cluster analysis of similarities between *E. chrysanthemi*, *E. carotovora* subsp. *carotovora*, and *E. carotovora* subsp. *atroseptica*, based on BOX and MSP-PCR profiles. R=0.92.



**Fig. 3.** BOX-PCR done with BOXA1R primer, generating fingerprint patterns of ten *E. carotovora* subsp. *atroseptica* strains (Lanes 2-10): 2-974, 3-976, 4-977, 5-981, 6-985, 7-995, 8-999, 9-1000, 10-1074, 11-1105. Lane 1 and 12, 1Kb plus ladder.



**Fig. 4.** MSP-PCR done with csM13 primer, generating fingerprint patterns of ten *E. carotovora* subsp. *atroseptica* strains: (Lanes 2-10): 2-974, 3-976, 4-977, 5-981, 6-985, 7-995, 8-999, 9-1000, 10-1074, 11-1105. Lane 1, 1Kb plus ladder.

the Portuguese strains differ from other *E. c.* subsp. *atroseptica* due to a higher content of palmitic acid associated with Portuguese strains. However, the Euclidian distance of 3.5 between Portuguese and PD *E. c.* subsp. *atroseptica* strains shows that despite the variability, Portuguese *E. c.* subsp. *atroseptica* strains belong to the *atroseptica* subspecies (Fig. 1). The subspecies *carotovora* and *atroseptica* (including both Portuguese and PD collection strains) were separated by 7 Euclidian distances, a result strongly supported by Persson and Sletten (1995), who showed *E. c.* subsp. *atroseptica* is separated from *E. carotovora* subsp. *carotovora* by 7 Euclidian distances.

Molecular fingerprints showed low genetic variability within *E. carotovora* subsp. *atroseptica* strains. Low variability using PCR was observed in *E. c.* subsp. *atroseptica* and other organisms (Darrasse *et al.*, 1994; Hélias *et al.*, 1998; Yahiaoui-Zaidi *et al.*, 2003) and the variability detected could reflect the adaptation of this pathogen to local environmental conditions. Atypical strains of *E. carotovora* subsp. *atroseptica* were found in Brazil. Features of these strains such as the ability to grow at 37 °C and failure of DNA amplification with specific primers, however, are not characteristic for strains found in Portugal (Duarte *et al.*, 2004). Although the host specificity of *E. carotovora* subsp. *atroseptica* strains to potato is well known, the Portuguese strains were found pathogenic to potato and also to tomato and Chinese cabbage. This was not unexpected as *E. carotovora* subsp. *atroseptica* strains have been previously shown to infect tomato and Chinese cabbage under natural conditions (De Boer *et al.*, 1987). As expected, due to their wide range of hosts and geographic origins, *E. c.* subsp. *carotovora* strains were characterised by high phenotypic and genetic variability (Smith and Bartz, 1990; Avrova *et al.*, 2002; Seo *et al.*, 2002; Seo *et al.*, 2003; Yahiaoui *et al.*, 2003).

Strains 1118 and 1132 were distant from the remaining strains included in this study when analysed by molecular methods, although no evident polymorphisms were observed in their molecular profiles (not shown). When analysed by FAME, these strains clustered with the remaining *E. carotovora* subsp. *atroseptica*. However two deviating features associated with these strains were observed by classical methods and could explain in part this variability: strain 1132 was sensitive to erythromycin and strain 1118 was able to produce indole. *E. chrysanthemi* strains were phenotypically and genetically diverse. These results tally with those of previous studies (Janse and Ruissen, 1988; Nassar *et al.*, 1996; Toth *et al.*, 2001; Avrova *et al.*, 2002).

Variability revealed by biochemical and physiological tests was expected and may reflect the subdivisions within *E. chrysanthemi* species. Of the methods used in this study, FAME analysis was found to sensitively detect the variability among strains. Classical methods are laborious and time-consuming and, although allowing identification of species and subspecies, they were limited for

detecting variability within subspecies. By contrast, molecular methods based on BOX-PCR and MSP-PCR were valuable for studying diversity.

Molecular methods included the 31 strains used in this study, therefore only *E. c.* subsp. *atroseptica* strains isolated in Portugal were analyzed. Further work including strains from other collections will be needed to support results obtained by FAME analysis. The unusual group of *E. carotovora* subsp. *atroseptica* strains from Portugal may constitute an ecotype of *E. carotovora* subsp. *atroseptica*. Factors affecting the variability of *E. carotovora* subsp. *atroseptica* strains and consequent effects at the genomic, protein, and lipid levels have yet to be investigated. The reason for this variability is unknown, but may be related to environmental conditions present in Portugal, a temperate climate with relatively high temperatures during the vegetation period. In order to clarify these questions it would be interesting to survey the distribution of this possible new ecotype throughout the country.

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