**SHORT COMMUNICATION**

# PHENOTYPIC AND GENETIC VARIABILITY OF *PECTOBACTERIUM CAROTOVORUM* ISOLATED FROM ARTICHOKE IN THE SELE VALLEY

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**SUMMARY**

Phenotypic and genetic characteristics were investigated of 24 isolates of *Pectobacterium carotovorum*, recovered in summer 2001 from artichokes in the Sele valley (Campania, southern Italy). Based on biochemical tests, all isolates were identified as *P. carotovorum* subsp. *carotovorum*, except for four atypical ones. PCR-amplification of a 434 bp fragment of the pectate-lyase-encoding *pel* gene, gave the expected band from all isolates, whereas no amplicons were obtained using *P. atrosepticum*-specific primers. PCR-RFLP on *pel* gene, placed the four atypical isolates in the RFLP groups 8, 9 and 11 (ISPaVe 110A, ISPAVe 105A and ISPAVe 91B) of the subspecies *carotovorum* and in the RFLP group 3 of the subspecies *odoriferum* (ISPaVe CA). DNA-polymorphism investigated by rep-PCR and M13-PCR showed the isolates to be distributed in two main groups, totalling 14 haplotypes. Eight different haplotypes were obtained from samples collected in the same field and three different haplotypes were found in the same plant. The rep-PCR fingerprinting of ISPaVe CA was the same as *P. carotovorum* subsp. *odoriferum* NCPPB 3839. The majority of the isolates did not show a significative differential virulence response, with the exception of ISPaVe 114B, the most-virulent, and ISPaVe 105B, the least-virulent.

*Key words*: rep-PCR, M13-PCR, virulence, hypersensitive reaction.

*Erwinia carotovora* subsp. *carotovora*, recently proposed as *Pectobacterium carotovorum* subsp. c*arotovorum* (Hauben *et al*., 1998; Gardan *et al.,* 2003) (*Pcc*) is the causal agent of soft rot on a wide range of host plants (Toth *et al.,* 2003). In Italy, typical symptoms occur in artichoke mainly from July to September. Most of the external leaves are chlorotic and slightly wilted, show necrosis at the base of the petiole and soft rot of the pith. Severe wilt and collapse of the plants ensue. In summer 2001, a serious attack of soft rot of artichoke plants occurred in four production sites of the Sele valley (Campania, southern Italy). A preliminary molecular characterization showed a very large variability of the isolated bacterial population (Loreti *et al.*, 2001, 2006).

Twenty-four pectinolytic bacterial isolates were obtained on crystal violet pectate (CVP) medium by incubation at 27°C for 3-4 days. These isolates were characterized based on Lelliot and Stead (1987) tests for acidification of lactose, sorbitol, melibiose and α-methylglucoside, production of reducing substances from sucrose and growth at 36°C. Several *Pectobacterium* reference and type strains were included in the tests (Tab. 1). Experimental infection assays were performed on potato tubers of cvs Mona Lisa, Agria and Vivaldi, as described by Vitale *et al.* (2004), and the data obtained from four inoculation tests were analysed statistically using the Statgraphics Plus 5.1 Program. Hypersensitive reaction (HR) assay was performed on leaves of *Nicotiana tabacum* L. cv. Samsung using a bacterial suspension of $10^8$ CFU $ml^{-1}$.

Total genomic DNA was extracted from 1.5 ml broth cultures using the Puregene DNA isolation kit (Gentra System-Flowgen, UK). Polymerase chain reaction (PCR) was done with primers Y1 and Y2, specific for *P. carotovorum,* and primers Y45 and Y46, specific for *P. atrosepticum* (*Pa*) (Darrasse *et al.*, 1994; Frenchon *et al.*, 1995). PCR-RFLP was as described by Helias *et al.* (1998) using reference strains of *Pcc* (PD 1769 and CIP 009), *Pa* (NCPPB 549) and *P. carotovorum* subsp. *odoriferum* (*Pco*) (NCPPB 3839). All bacterial isolates were characterized by rep-PCR analyses, performed with REP and ERIC primers (Louws *et al.*, 1994) and by M13-PCR assay (Zaccardelli *et al*., 2000).

The amplification products (15 µl) were analysed on 1.6 % (w/v) agarose gel run in TAE buffer (0.04 M Tris, 0.001 M EDTA, 0.02 M acetic acid), stained with 0.5% ethidium bromide and photographed under UV light. Each experiment was repeated three times. Results of REP, ERIC and M13-PCR genomic fingerprinting were combined to obtain an single binary matrix. Cluster analysis was made with the NTSYSpc software (version 2.11j; Exeter Software, USA) using simple matching

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**Table 1.** List of *Pectobacterium carotovorum* isolates, collected from artichoke, used in this study. Their biochemical and physiological characteristics were compared with *P. carotovorum* subsp. *carotovorum* (*Pcc*), *P. atrosepticum* (*Pa*), *Pectobacterium wasabiae* (*Pw*) and *P. carotovorum* subsp. *odoriferum (Pco)* reference strains.

| Isolates [1] | Place of production, Location, Municipality | Reducing sugars from sucrose | Acid production from sorbitol | Acid production from melibiose | Acid production from lactose | α - methylglucoside | Grown 36°C | Pectolytic activity | HR Tobacco |
|---|---|---|---|---|---|---|---|---|---|
| ISPaVe CA | 1, Picciola, Pontecagnano | + | + | + | + | + | + | + | + |
| ISCI 89 b | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 89 B | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 89 L | " " " | – | – | + | + | – | + | + | – |
| ISCI 90 a | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 90 F | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 90 L | " " " | – | – | + | + | – | + | + | + |
| ISCI 91 a | " " " | – | – | + | + | – | + | + | + |
| ISCI 91 d | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 91 B | " " " | – | – | + | + | – | – | + | + |
| ISPaVe 91/2 | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 91 D | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 92 A | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 92 B | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 92 C | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 101 A | 2, Casella, Pontecagnano | – | – | + | + | – | + | + | + |
| ISPaVe 101 B | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 103 A | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 103 B | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 105 A | " " " | – | – | + | + | – | – | + | + |
| ISPaVe 105 B | " " " | – | – | + | + | – | + | + | + |
| ISPaVe 110 A | 3, Gramola, Capaccio | + | – | + | + | + | + | + | + |
| ISPaVe 114 B | 4, Gramola, Capaccio | – | – | + | + | – | + | + | + |
| Pcc PD 1769 | | – | – | + | + | – | + | + | + |
| Pcc CIP009 | | – | – | + | + | – | + | + | + |
| Pa NCPPB 549 | | + | – | + | + | + | – | + | + |
| Pco NCPPB 3839[T] | | + | + | + | + | + | + | + | + |
| Pw NCPPB 3701[T] | | – | – | – | NP | – | – | NP | NP |

+, Positive reaction; – negative reaction. NP: not performed

[1] Collections: ISPaVe - Culture Collection of Plant Pathology Research Center, Roma, Italy; ISCI - Culture Collection of Research Center for Industrial Crops, Salerno, Italy; PD - Culture Collection of Plant Protection Service, Wageningen, The Netherlands.; NCPPB - National Collection of Plant Pathogenic Bacteria, CSL, Sand Hutton, York, United Kingdom.

(SM) coefficient, according to the unweighed pair-group method with average linkages (UPGMA).

The majority of the isolates showed the same biochemical properties as *Pcc* reference strains (Tab. 1). Only four of them, ISPaVe 91B, ISPaVe 105A and ISPaVe 110A and ISPaVe CA, showed atypical features, differing from *Pcc* and *Pa* for growth at 36°C, and a similarity with *Pco* (ISPaVe 110A and ISPaVe CA) or *P. wasabiae* (ISPaVe 91B and ISPaVe 105A) which also attack potato (Pitman *et al.*, 2008). However, ISPaVe 91B and ISPaVe 105A differed from *P. wasabiae* for their ability to produce acid from melibiose, and ISPaVe 110A from *Pco*, because it did not produce acid from sorbitol. Isolates with similar atypical biochemical features had been previously reported (Thomson *et al.*, 1981; Priou, 1992). Further analyses by PCR-RFLP included these atypical isolates in the groups 8 and 9 of the subsp. *carotovorum* (Priou, 1992; Darrasse *et al.*, 1994; Helias *et al.*, 1998).

The four atypical isolates did not respond to PCR assays performed with *Pa*-specific primers Y45 and Y46 whereas, following PCR-RFLP, they were assigned to group 8 (ISPaVe 110A), group 9 (ISPaVe 105A), group 11 (ISPaVe 91B) of *Pcc* and to group 3 of *Pco* (ISPaVe CA). Atypical aspects of some phenotypic traits could reflect the natural variability of this bacterial population. Such variability was also reported for *Pcc* isolates from tomato (Fiori *et al.*, 2005), potato (Yahiaoui-Zaidi *et al.,* 2003; Yap *et al.*, 2004), mulberry (Seo *et al.*, 2003) and other host plants (Smith and Bartz, 1990; Helias *et al.*, 1998). An exception was the isolate ISPaVe CA, that showed biochemical and nutritional properties similar to those of *Pco*, and displayed the same genotypic fingerprint pattern of the *Pco* pathotype strain NCPPB 3839 using ERIC and BOX primers (data not shown). These results suggest classification of isolates ISPaVe 110A**,** ISPaVe 105A and ISPaVe 91B as *Pcc,* and isolate ISPaVe CA as *Pco*.

A total of 23 and 94 bands were obtained by M13 and rep-PCR respectively, with amplicons ranging from 300 to 2500 bp in size. A total of 14 *Pcc* haplotypes were obtained by M13 and rep-PCR analyses; all isolates were contained in two clusters (á and â) with a similarity index of 0.7 (Fig. 1). The cluster á contained 18 *Pcc* isolates including 10 haplotypes, the majority (15 out of 16) collected in production site 1. The cluster â contained the remaining 5 *Pcc* isolates, divided in 4 different haplotypes, the majority (4 out of 5) coming from production site 2. The reference strains and isolate ISPaVe CA grouped all in cluster â. Moreover, up to 8 different haplotypes were isolated in the same production site and up to three different haplotypes were found in the same infected plant.

M13 and rep-PCR confirmed the high genetic diversity of this bacterial population, already reported by others (Avrova *et al.*, 2002; Yahiaoui-Zaidi *et al.*, 2003; Yap *et al.,* 2004). Previously, the extensive genetic variability of *Pcc* was hypothesized as being consequent to a non-monophyletic origin, in accordance with broad host-range and geographical distribution of this subspecies (Darrasse *et al.*, 1994; Mäki-Valkama and Karjalainen, 1994; Helias *et al.*, 1998). However, we now confirm this diversity among isolates collected from a homogeneous ecological 'niche'. A similar situation was reported for *Pcc* strains from potato by Yap *et al.* (2004) who showed that two strains with unusual genomes were less virulent than other strains collected in the same environmental niche. One of the two strains was unable to induce HR in tobacco and to oxidize cellobiose; moreover, eight *hrp-hrc* genes were missing. The presumed loss of the functional type three secretion system (TTSS) and of cellobiose degradation ability, were thought to partially account for the low virulence of the strain in question.

The potato tubers assay performed in our study showed a variable response of different isolates. In particular, isolate ISPaVe 114B was significantly the most virulent and isolate ISPaVe 105B the least virulent (Tab. 2). The hypersensitive response assay showed that another

**Table 2.** Results of experimental inoculation, on potato tubers, of *Pectobacterium carotovorum* isolates obtained from artichoke. Reference strains were *P. carotovorum* subsp. *carotovorum* CIP 009 and *P. atrosepticum* NCPPB 549. The data were subjected to analysis of variance and Duncan's test (p=0,01).

| Isolate | Mean* area of rotten tissue (mm) | |
|---|---|---|
| ISPaVe 105 B | 0.03 | a |
| *Pa* NCPPB 549 | 0.07 | ab |
| ISPaVe 91 B | 0.18 | abc |
| ISCI 91 d | 0.26 | abcd |
| ISPaVe 101 A | 0.34 | abcde |
| ISCI 89 a | 0.38 | abcde |
| ISPaVe 91 -2 | 0.48 | abcde |
| ISCI 89 b | 0.52 | abcde |
| ISPaVe 92 B | 0.54 | abcde |
| ISPaVe 89 L | 0.56 | abcdef |
| ISPaVe 89 B | 0.60 | abcdef |
| ISPaVe 92 C | 0.60 | abcde f |
| ISCI 90 a | 0.60 | abcdef |
| ISPaVe 90 L | 0.62 | abcdef |
| *Pcc* CIP 009 | 0.63 | abcdef |
| ISPaVe 105 A | 0.63 | abcdef |
| ISPaVe 91 D | 0.70 | bcdef |
| ISPaVe 90 F | 0.74 | cdef |
| ISPaVe 103 A | 0.75 | cdef |
| ISPaVe 103 B | 0.82 | cdef |
| ISPaVe CA | 0.83 | cdef |
| ISPaVe 110 A | 0.86 | def |
| ISCI 91 a | 0.91 | def |
| ISPaVe 101 B | 0.94 | ef |
| ISPaVe 114 B | 1.2 | f |

*Means followed by the same letter do not differ significantly at p=0.01

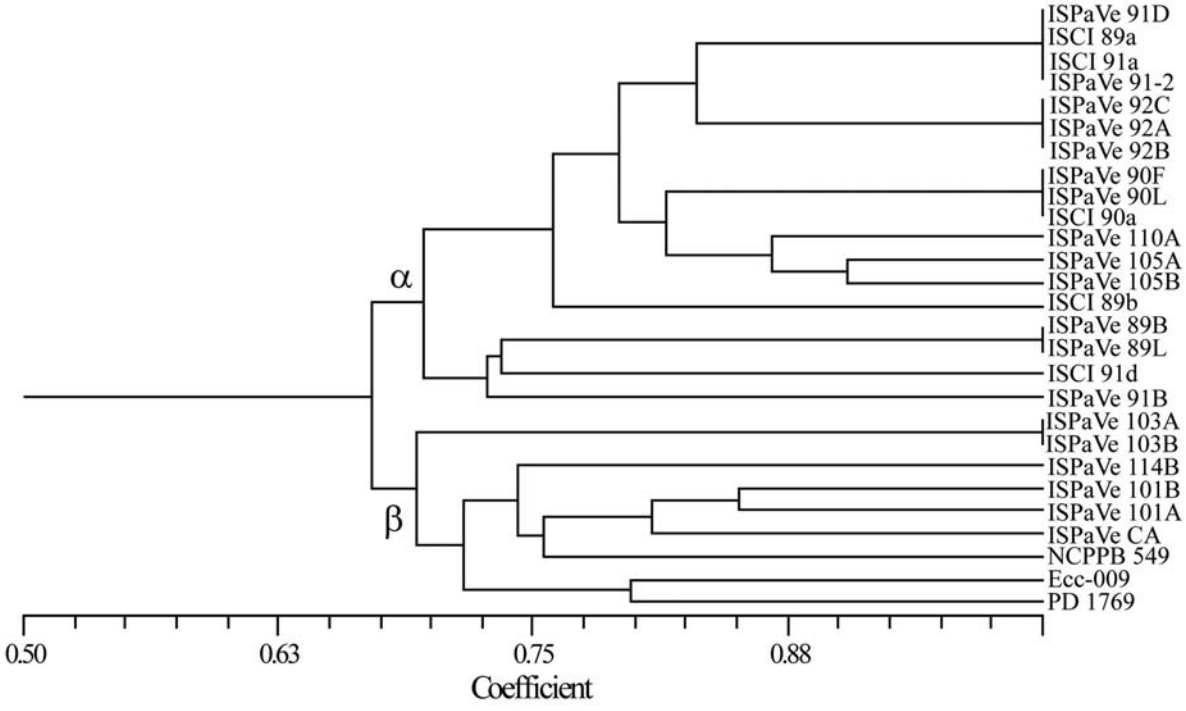


**Fig. 1.** Dendrogram of genetic similarity of *Pectobacterium carotovorum* subsp. *carotovorum* isolates from artichoke in comparison with references strains *P. carotovorum* subsp. *carotovorum* Ecc 009 (= CIP 009), PD 1769 and *P. atrosepticum* NCPPB 549. The similarity is the result of combined data sets of ERIC, BOX and M13-primer sets using UPGMA and simple matching (SM) coefficient. The scale at the bottom indicates the degree of genetic similarity among isolates.

isolate (ISPaVe 89L) was unable to induce the typical necrosis on tobacco leaves.

In conclusion, quite a high variability emerged among *Pcc* isolates recovered from artichoke in southern Italy by assessing genomic variability and testing some phenotypic traits. However, no correlation was observed among genotyping, virulence and atypical biochemical responses of some isolates. Finally, one isolate of *P. carotovorum* subsp. *odoriferum* (the ISPaVe CA) was isolated from a diseased artichoke plant. *Pco* is responsible for soft rot of witloof chicory (*Cichorium intybus* L.), celery (*Apium graveolens*), leek *(Apium porrum)* and hyacinth (Gallois *et al*., 1992), but was never isolated from artichokes. Since only one isolate of this subspecies was isolated during this study, it is difficult to define its role. Further study will investigate *hrp* and housekeeping genes, for characterizing other important genetic traits of this variable *Pcc* bacterial population. As observed by Yap *et al.* (2004), the highly genomic variability among strains obtained from this homogeneous ecological niche suggests that factors other than host plant, geographic origin or year of isolation, drive the evolution of this pathogen. This aspect may be important also for breeding programs, to avoid the overcoming of resistance by taking into consideration isolates with peculiar characteristics.

## REFERENCES

Avrova A.O., Hyman L.J., Toth R.L., Toth I.K., 2002. Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. *Applied and Environmental Microbiology* **68**: 1499-1508.

Darrasse A., Priou S., Kotoujansky A., Bertheau Y., 1994. PCR and restriction fragment length polymorphism of a pel gene as a tool to identify *Erwinia carotovora* in relation to potato disease. *Applied and Environmental Microbiology* **60**: 1437-1443.

Fiori M., Virdis S., Schiaffino A., 2005. Phenotypic and genetic characterization of *Erwinia carotovora* ssp. *carotovora* (Jones) Bergey et al. isolates from grafted tomato in Sardinia, Italy. *Phytopathologia Mediterranea* **44**: 50-57.

Frenchon D., Exbrayat P., Gallet O., Guillot E., LeClerc V., Payet N., Bertheau Y., 1995. Sequences nucleotidiques pour la detection des *Erwinia carotovora* subsp. *atroseptica*. Brevet 95. 12-803 INRA INA-PG, SDP.

Gallois A., Samson R., Ageron E., Grimont P.A.D., 1992. *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory (*Chicorium intybus* L.). *International Journal of Systematic Bacteriology* **42**: 582-588.

Gardan L., Gouy C., Christen R., Samson R., 2003. Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium*

*wasabiae* sp. nov. *International Journal of Sistematic and Evolutionary Microbiology* **53**: 381-391.

Hauben L., Moore E.R.B., Vauterin L., Steenackers M., Mergaert J., Verdonk L., Swings J., 1998. Phylogenetic position of phytopathogens within the Enterobacteriaceae. *Systematic and Applied Microbiology* **21**: 384-397.

Helias V., Le Roux A.C., Bertheau Y., Andrivon D., Gauthier J.P., Jouan B., 1998. Characterisation of *Erwinia carotovora* subspecies and detection of *Erwinia carotovora* subsp. *atroseptica* in potato plants, soil and water extracts with PCR-based methods. *European Journal of Plant Pathology* **104**: 685-699.

Lelliot R.A., Stead D.E., 1987. Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publication for the British Society of Plant Pathology, Oxford, UK.

Loreti S., Gallelli A., Zaccardelli M., Parisi M., 2001. Characterization of isolates of *Erwinia carotovora* from artichoke. *Journal of Plant Pathology* **83**: 236.

Loreti S., Gallelli A., Galli M., Zaccardelli M., 2006. Marciume radicale del carciofo da *Erwinia carotovora*: variabilita' genomica e fenotipica di una popolazione omogenea. *Atti del Convegno Il carciofo dal Laboratorio al Mercato, Roma, 2006*: 99-101.

Louws F.J., Fullbright D.W., Stephens C.T., De Bruijn F.J., 1994. Specific genomic fingerprinting of phytopathogenic Xanthomonas and Pseudomonas pathovars and strains generated with repetitive sequence and PCR. *Applied and Environmental Microbiology* **60**: 2286-2295.

Maki-Valkama T., Karjalainen R., 1994. Differentiation of *Erwinia carotovora* subsp. *atroseptica* and *carotovora* by RAPD-PCR. *Annals of Applied Biology* **125**: 301-309.

Pitman A.R., Wright P.J., Galbraith M.D., Harrow S.A., 2008. Biochemical and genetic diversity of pectolytic enterobacteria causing soft rot disease of potatoes in New Zealand. *Australasian Plant Pathology* **37**: 559-568.

Priou S., 1992. Variabilitè phènotypique et gènètique et carachterisation des sous-espèce dè *Erwinia carotovora* en relation avec le pouvoir pathogiene sur pomme de terre. Ph. D. thesis. Ecole National Superieure Agronomieque de Rennes, Rennes, France.

Smith C., Bartz J.A., 1990. Variation in the pathogenicity and aggressiveness of strains of *Erwinia carotovora* subsp. *atroseptica* isolated from different host. *Plant Disease* **74**: 505-509.

Seo S.T., Furuya N., Lim C.K., Takanami Y., Tschiya K., 2002. Phenotipic and genetic diversity of *Erwinia carotovora* ssp. *carotovora* strains. *Journal of Phytopathology* **150**: 120-127.

Thomson S.V., Hildebrand D.C., Schroth M.N., 1981. Identification and nutritional differentiation of the erwinia sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi*. *Phytopathology* **71**: 1037-42.

Toth I.K., Bell K.S., Holeva M.C., Birch P.R.J., 2003. Soft rot erwiniae: from genes to genomes. *Molecular Plant Pathology* **4**: 17-30.

Vitale S., Alberino S., Zoina A., Parisi B., Corazza L., 2004. Evaluation of resistance to dry and soft rot of potato clones adapted to Mediterranean regions. *Phytopathologia Polonica* **34**: 13-20.

Yahiaoui-Zaidi R., Jouan B., Andrivon D., 2003. Biochemical and molecular diversity among *Erwinia* isolates from potato in Algeria. *Plant Pathology* **52**: 28-40.

Yap M.N., Barak J., Charkowski A.O., 2004. Genomic diversity of *Erwinia carotovora* subsp. *carotovora* and its correlation with virulence. *Applied and Environmental Microbiology* **70**: 3013-3023.

Zaccardelli M., Vitale S., Luongo L., Corazza L., 2000. Molecular characterization of *Fusarium solani* var. *coeruleum* on potato. *Proceedings 5th Congress of the European Foundation for Plant Pathology, Taormina-G. Naxos 2000*: 29-33.

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