Notas Científicas

Erwinia chrysanthemi: pectolytic bacterium causing soft rot outbreaks of arracacha in Brazil

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Abstract – The objetive of this work was to identify the pectolytic bacteria associated with soft rot of arracacha roots in Brazil. From 1998 to 2001, 227 isolates of *Erwinia* spp. were obtained from arracacha roots and identified by biochemical and physiological tests (pectolytic activity, lecithinase, α-methyl glucoside, phosphatase, erythromycin sensivity, growth at 37ºC). Of these isolates, 89.9% were identified as *E. chrysanthemi* (*Ech*), 9.7% as *E. carotovora* subsp. *carotovora* (*Ecc*) and 0.5% as *E. carotovora* subsp. *atroseptica*. The identity of seventeen out of twenty representative isolates of *Ech* and *Ecc* was confirmed by PCR (primers '149f', 'L1r', 'ADE1', 'ADE2').

Index terms: *Arracacia xanthorrhiza*, *Pectobacterium*, peruvian carrot, disease, etiology.

Erwinia chrysanthemi: bactéria pectolítica envolvida na "mela" da mandioquinha-salsa no Brasil

Resumo – O objetivo deste trabalho foi identificar as bactérias pectolíticas envolvidas na podridão-mole de raízes de mandioquinha-salsa no Brasil. De 1998 a 2001, 227 isolados de *Erwinia* spp. foram obtidos de raízes de mandioquinha-salsa e identificados por testes bioquímicos e fisiológicos (atividade pectolítica, lecitinase, α-methyl glucosídeo, fosfatase, sensibilidade à eritromicina, crescimento a 37ºC). Destes isolados, 89,9% foram identificados como *E. chrysanthemi* (*Ech*), 9,7% como *E. carotovora* subsp. *carotovora* (*Ecc*) e somente 0,5% como *E. carotovora* subsp. *atroseptica*. A identidade de 20 isolados representativos de *Ech* e *Ecc* foi confirmada por PCR (primers '149f', 'L1r', 'ADE1', 'ADE2'), com exceção de dois isolados de *Ech* e um de *Ecc*.

Termos para indexação: *Arracacia xanthorrhiza*, *Pectobacterium*, batata-baroa, podridão-mole, etiologia.

In tropical regions, postharvest losses of vegetable crops can easily reach 30% due to poor handling practices, diseases and inadequacy in packing and refrigeration. Deterioration caused by pectolyctic bacteria is one of the main causes of postharvest losses of perishable products worldwide.

Soft rot of fleshy plant organs of vegetables is typically caused by the *Erwinia carotovora* group, particularly, *E. carotovora* subsp. *carotovora* (*Ecc*), *E. carotovora* subsp. *atroseptica* (*Eca*) and *E. chrysanthemi* (*Ech*). Identification of pectolytic erwinias is traditionally based on biochemical and phenotypic characteristics (De Boer & Kelman, 2000), and more recently molecular techniques have also been applied. As a result, Hauben et al. (1998) suggested that the pectolytic bacteria should be placed in a separate genus (*Pectobacterium*) on the basis of the 16S rDNA sequences. Gardan et al. (2003) elevated three subspecies of *Pectobacterium carotovorum* to species level (*P. atrosepticum*, *P. betavascularum* and *P. wasabi*). Presently, the taxonomy of *Erwinia* is in a state of flux (Yap et al., 2004), and the proposed name *Pectobacterium* by Hauben et al. (1998) has not yet been accepted by many researchers working with this group of bacteria.

In Brazil, *Ecc* was considered the most important species of the pectolytic group, causing losses in more than fifty crop plants, such as lettuce, garlic, potato, sweet-potato, eggplant, zucchini, onion, carrot, cauliflower, arracacha, melon, cucumber, sweet-pepper, okra, cabbage, tomato, chicory, collard, among others

(Jabuonski et al., 1986; Michereff & Mariano, 1993). *Ech* affects many hosts and is considered more important in tropical and warm areas, regarded as highly aggressive in temperatures above 30ºC. *Eca* causes black-leg of potatoes and is less prevalent in tropical regions, being considered as a cool temperature variant of *Ecc*. Recently, atypical strains causing blackleg of potato in Southern Brazil were proposed as a new subspecies (*E. carotovora* subsp. *brasiliensis*) by Duarte et al. (2004).

During summer, the erwinia soft rot of arracacha roots can cause losses of up 100% in only three days after harvest (Henz, 2001). In Brazil, the problem was first described by Silva (1967) as "mela", the Portuguese word that better describes the sticky soft rot of the roots, but the author was unable to properly identify the causal agent. During the seventies, Camino & Díaz Polanco (1972) described *Erwinia amylovora* as the causal agent of soft rot in arracacha plants in Venezuela, and later on Zapata & Pardo (1974) reported the occurrence of *Erwinia* sp. in arracacha plants in Colombia. Romeiro et al. (1988) published the first paper identifying the causal agent of the disease at the subspecific level, associating *Erwinia carotovora* subsp. *carotovora* with soft rot of arracacha roots in Minas Gerais State, Brazil. The same group presented the report in an annual meeting, and later on a survey on bacterial diseases of vegetable crops (Gomide & Romeiro, 1992). In the Distrito Federal area, the three most important pectolytic erwinias (*Ecc*, *Eca* and *Ech*) were found associated to the soft rot of arracacha (Henz, 2001). Lopes & Quezado-Soares (1997) considered *Ecc* as the most important subspecies causing soft rot in arracacha roots in Brazil.

Although diseases caused by *Erwinia* spp. are very harmful and occur in a large number of agricultural crops in Brazil, there are few papers published on the pathogen recently. In a survey on potato plants showing blackleg symptoms in 22 fields of nine counties in Rio Grande do Sul State, 55% of the strains were identified as *Eca*, 44% as *Ecc* and only 1% as *Ech* (Oliveira et al., 2003). Arracacha soft rot continues to be a very important postharvest disease during summertime in Brazil, causing severe losses. Since some new methods to control the soft rot of arracacha roots are being tested, it is important to determine the prevailing bacterial species or subspecies.

The objective of this research was to identify the pectolytic erwinias associated to the outbreaks of the soft rot on arracacha in Brazil.

Arracacha roots with typical soft-rot symptoms were collected from wholesale and retail markets in São Paulo, Paraná, Minas Gerais and Rio de Janeiro states and also in Brasília, DF. Bacterial isolates were obtained both straight from the rotted host tissues or by inoculating sweet pepper fruits with toothpicks previously impregnated into rotted tissue in order to avoid secondary bacterial growth (Takatsu et al., 1980).

Bacteria were isolated in nutrient agar (NA) and Kado & Heskett '523' media and then incubated at 28ºC for two days (Klement et al., 1990; De Boer & Kelman, 2000). Single bacterial colonies resembling *Erwinia* were harvested and tested for pectolytic activity on arracacha roots and sweet pepper fruits kept on moist chambers. Isolates tested positive for pectolytic activity were submitted to the following physiological and biochemical tests: Gram test; growth at 37ºC; reducing substances from sucrose; phosphatase; lecithinase; α-methyl glucoside; erythromycin sensitivity; growth in NaCl 5% (Klement et al., 1990; De Boer & Kelman, 2000). Based on these test results, the isolates were categorized into species of *Erwinia* and subspecies of *Erwinia carotovora*. After that, all *Erwinia* isolates were kept in sterile distilled water for further identification.

To confirm the identity, forty representative isolates were chosen by hazard and sent for additional identification to the Centre of Expertise for Potato Diseases, Canadian Food Inspection Agency, in Charlottetown, Canada. The IGS region of the DNA of *Erwinia* isolates were amplified with primers IGS '1491f' and 'L1r' (Fessehaie et al., 2002) and by primers 'ADE1' and 'ADE2' (Nassar et al., 1996). After 25 cycles, the results of PCR were separated by agarose gel (1%) electroforese for one hour. The PCR-IGS were performed at the following conditions: 94ºC/2 min, (94ºC/45 sec, 62ºC/45 sec, 72ºC/90 min) 25X, 72ºC/10 min, 4ºC/5 min (Duarte et al., 2004). Two representative isolates of *Ech* from arracacha roots were also submitted to the Biolog system, based on the utilization of 95 sources of carbon.

More than 400 bacterial isolates were obtained from arracacha roots with typical soft-rot symptoms during a four-year period (1998–2001). According to the identification with the traditional biochemical tests (Klement et al., 1990; De Boer & Kelman, 2000), 204 isolates were characterized as *Ech*, 22 as *Ecc* and one as *Eca*. Twenty isolates of *Erwinia* from arracacha roots were also submitted to PCR. Identification of six isolates of *Ech* was confirmed by specific primers

'ADE1' and 'ADE2' (Table 1). For primers IGS '1491f' and 'L1r', 13 isolates showed two close bands (600 pb), typical of *E. carotovora* isolates, and seven showed two more separated bands, typical of *E. chrysanthemi* (Figure 1). Primers IGS '1491f' and 'L1r' developed by Fessehaie et al. (2002) are universal for enterobacteria, including the pectolytic erwinias. Primers 'ADE1' and 'ADE2' recognize *pel* genes, which code for the yield of pectolytic enzymes specific for *Ech* (Nassar et al., 1996), an important trait for separating this species from *E. carotovora*.

Only six isolates ('46', '53', 'P5', 'P6', 'Q5', 'Q20') showed the same band as the check strain (*Ech* 571), while other isolates did not show this RNA sequence (Figure 2). Isolate 'Q2", identified as *Ech* by biochemical tests and primers IGS, did not have the characteristic band for the species with primers 'ADE1' and 'ADE2', probably due to a deficiency in this specific sequence.

Isolates 'P14' and 'Q1' were identified as *Ech* and isolate '46' as *Ecc* by biochemical tests, which was not confirmed by the primers used (Table 1). Additional biochemical tests were performed for these isolates (phosphatase, α-methyl glucoside, lecithinase), and the identity of isolate 'Q1' was confirmed as *Ech* and isolate '46' as *Ecc*. Isolate 'P14' was weakly positive for phosphatase, negative for lecitinase and α-methyl glucoside, and apparently belonged to *E. carotovora*, as confirmed by the primers. The identity of a second group of 22 *Ech* isolates from arracacha was confirmed by primers ADE1' and 'ADE2'.

Finally, the two isolates of *Ech*, 'B2' and '154', submitted to the Biolog system showed, respectively, 96.7 and 89.1% of similarity, higher than the two check isolates, confirming their identity. The combination of traditional biochemical and physiological tests with molecular tools seems to be the best way to confirm the identity of the pectolytic erwinias, since the taxonomy of this group is in a flux (Yap et al., 2004). Traditional identification of pectolytic erwinias is still useful, when molecular tools are not available.

Table 1. Identification of isolates of pectolytic *Erwinia* by PCR with primers IGS '1491f' and 'L1r' and "ADE1" and "ADE2".

Isolate	Preliminary Identification[1]	Primers IGS '1491f' and 'L1r'[2]	Primers 'ADE1' and' ADE2'	Identification with primers[3]
41	*Ecc*	-	-	*Ec*
42	*Ecc*	-	-	*Ec*
46[4]	*Ecc*	+	+	*Ech*
47	*Ecc*	-	-	*Ec*
48	*Ecc*	-	-	*Ec*
49	*Eca*	-	-	*Ec*
50	*Ecc*	-	-	*Ec*
53	*Ech*	+	+	*Ech*
54	*Ecc*	-	-	*Ec*
56	*Eca*	-	-	*Ec*
P1	*Ecc*	-	-	*Ec*
P2	*Ecc*	-	-	*Ec*
P5	*Ech*	+	+	*Ech*
P6	*Ech*	+	+	*Ech*
P9	*Ecc*	-	-	*Ec*
P10	*Eca*	-	-	*Ec*
P14	*Ech*	-	-	*Ec*
Q1	*Ech*	-	-	*Ec*
Q2[4]	*Ech*	+	-	*Ech*
Q5	*Ech*	+	+	*Ech*
Q20	*Ech*	+	+	*Ech*

[1]*Ecc*: *Erwinia carotovora* subsp. *carotovora*; *Eca*: *E. carotovora* subsp. *atroseptica*; *Ech*: *E. chrysanthemi*. [2]+: positive of 380 bp and 480 bp. [3]*Ec*: *E. carotovora*; *Ech*: *E. chrysanthemi*. [4]Isolates with discordant results: strain '46': confirmed as *Ech* by PCR and biochemical tests; strain 'Q2': confirmed as *Ech* but negative with primers "ADE1" and "ADE2".

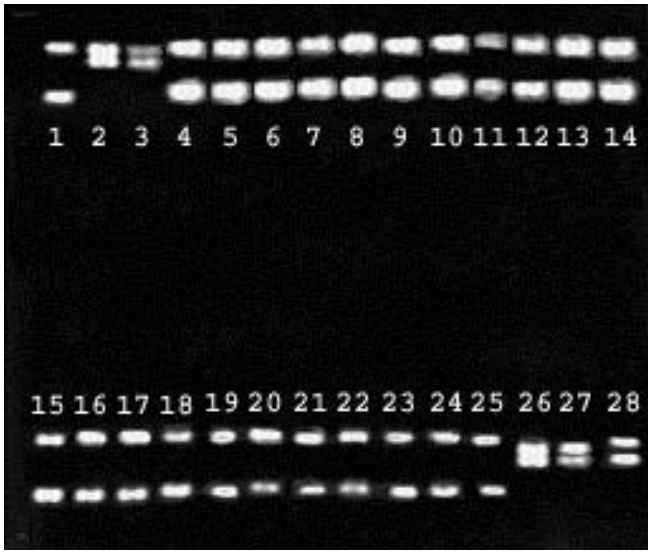

Figure 1. Result of PCR amplification of IGS rDNA 16S-23S regions of *Erwinia* with primers "149LF" and "L1ra". Lines: 1: strain '571' (*Ech*); 2: strain '31' (*Eca*); 3: strain '71' (*Ecc*); 4: strain 'a'; 5: strain 'B2'; 6: strain 'B8'; 7: strain 'B11'; 8: strain 'B12'; 9: strain 'C1'; 10: strain 'C3'; 11: strain 'C6'; 12: strain 'C7'; 13: strain 'C9'; 14: strain 'C16'; 15: strain 'D5'; 16: strain 'D7'; 17: strain 'D9'; 18: strain 'I'; 19: strain '154'; 20: strain '155'; 21: strain '161'; 22: strain '163'; 23: strain '164'; 24: strain '166'; 25: strain '171'; 26: strain 'F5' (*Eca*); 27: strain 'P1' (*Ecc*); and 28: 'tomato' strain (*Ec*). IGS 16S-23S; *E. chrysanthemi* (*Ech*): 354–356 (smaller) and 480 pb (larger); *E. carotovora* (*Ec*): 440–453 (smaller) and 475–490 pb (larger).

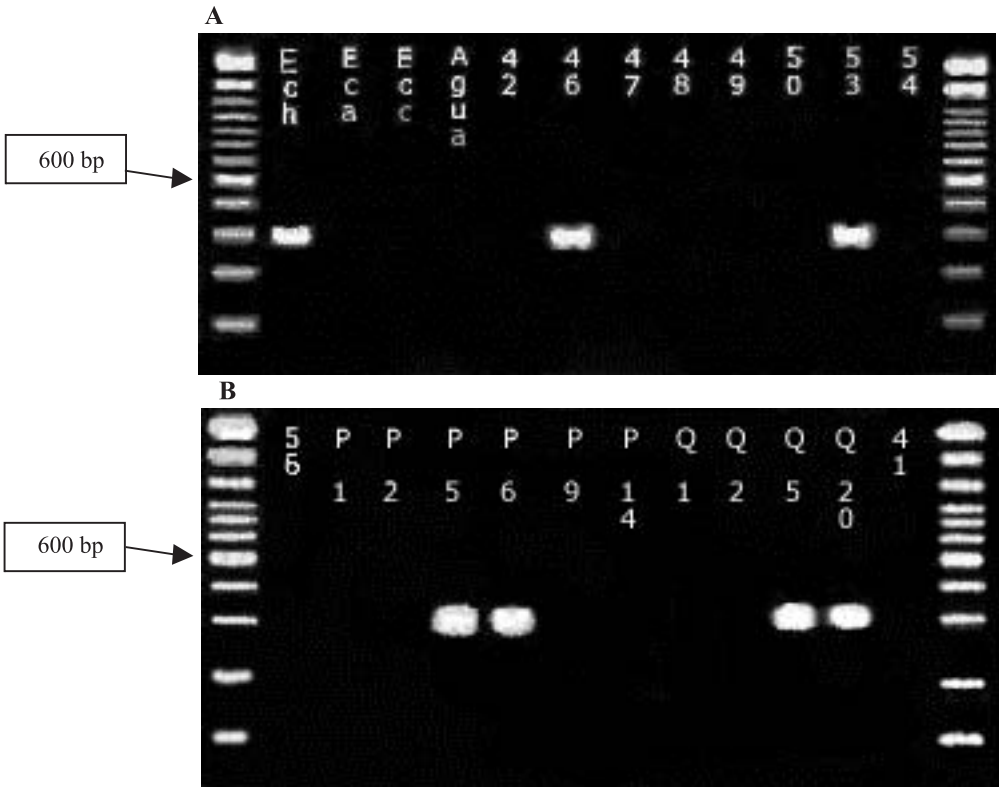

Figure 2. Result of PCR amplification of genes *pel* fragments of *Erwinia chrysanthemi* with primers "ADE1" e "ADE2". (*Ech* isolates had one band: '46', '53', 'P5', 'P6', 'Q5', 'Q20'; *Eca*, *Ecc* and check ('agua') show no band).

The first papers on soft rot of arracacha in Venezuela (Camino & Díaz Polanco, 1972) and Colombia (Zapata & Pardo, 1974) only identified correctly the genus (*Erwinia*). The predominance of *Ech* (89.8%) causing soft rot in arracacha roots in Brazil is a novel finding, since previous papers reporting the disease considered *Ecc* as the predominant one (Romeiro et al., 1988; Lopes & Quezado-Soares, 1997), although based on the identification of few isolates. Lately, Oliveira et al. (2003) described *Eca* (55%) and *Ecc* (44%) as the predominant subspecies in potato fields in Southern Brazil, which was unexpected, and more recently, Duarte et al. (2004) reported a new, atypical potato blackleg strain called *E. carotovora* subsp. *brasiliensis*. These recent findings justify the survey and identification of a large amount of pectolytic erwinias isolates to set control measures and better understand disease epidemiology.

Soft rot in arracacha roots usually occurs during summertime (December to March), which coincides with warmer temperatures in Southern Brazil (Henz, 2001). In environmental conditions such as these, *Ech* is usually considered more aggressive than *Ecc* and *Eca* (Pérombelon et al., 1995), corrobating the results of these papers.

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