Abstract – The objective 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 sensitivity, growth at 37°C). 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.

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 pectolytic 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.
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 28°C 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 37°C; reducing substances from sucrose; phosphatase; lecinthinase; α-methyl glucoside; erythromycin sensitivity; growth in NaCl 15% (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: 94°C/2 min, (94°C/45 sec, 62°C/45 sec, 72°C/90 min) 25X, 72°C/10 min, 4°C/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 \textit{E. carotovora} isolates, and seven showed two more separated bands, typical of \textit{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 \textit{pel} genes, which code for the yield of pectolytic enzymes specific for \textit{Ech} (Nassar et al., 1996), an important trait for separating this species from \textit{E. carotovora}.

Only six isolates (‘46’, ‘53’, ‘P5’, ‘P6’, ‘Q5’, ‘Q20’) showed the same band as the check strain (\textit{Ech} 571), while other isolates did not show this RNA sequence (Figure 2). Isolate ‘Q2’, identified as \textit{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 \textit{Ech} and isolate ‘46’ as \textit{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 \textit{Ech} and isolate ‘46’ as \textit{Ecc}. Isolate ‘P14’ was weakly positive for phosphatase, negative for lecithinase and α-methyl glucoside, and apparently belonged to \textit{E. carotovora}, as confirmed by the primers. The identity of a second group of 22 \textit{Ech} isolates from arracacha was confirmed by primers ‘ADE1’ and ‘ADE2’.

Finally, the two isolates of \textit{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 \textit{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 $^{(3)}$ |
|---------|-----------------------------------|---------------------------------|--------------------------|---------------------|
| 41      | Ecc                               | -                              | -                        | Ec                  |
| 42      | Ecc                               | -                              | -                        | Ec                  |
| 46$^{(4)}$ | Ecc                          | +                              | +                        | \textit{Ech}        |
| 47      | Ecc                               | -                              | -                        | Ec                  |
| 48      | Ecc                               | -                              | -                        | Ec                  |
| 49      | Ecc                               | -                              | -                        | Ec                  |
| 50      | Ecc                               | -                              | -                        | Ec                  |
| 53      | \textit{Ech}                      | +                              | +                        | \textit{Ech}        |
| 54      | Ecc                               | -                              | -                        | Ec                  |
| 56      | Ecc                               | -                              | -                        | Ec                  |
| P1      | Ecc                               | -                              | -                        | Ec                  |
| P2      | Ecc                               | -                              | -                        | Ec                  |
| P5      | Ecc                               | +                              | +                        | \textit{Ech}        |
| P6      | Ecc                               | +                              | +                        | \textit{Ech}        |
| P9      | Ecc                               | -                              | -                        | Ec                  |
| P10     | Ecc                               | -                              | -                        | Ec                  |
| P14     | Ecc                               | -                              | -                        | Ec                  |
| Q1      | \textit{Ech}                      | -                              | -                        | Ec                  |
| Q2$^{(4)}$ | \textit{Ech}                     | +                              | +                        | \textit{Ech}        |
| Q4      | \textit{Ech}                      | +                              | +                        | \textit{Ech}        |
| Q20     | Ecc                               | +                              | +                        | \textit{Ech}        |

$^{(1)}$Ecc: \textit{Erwinia carotovora} subsp. \textit{carotovora}; Ecc: \textit{E. carotovora} subsp. \textit{atrorectica}; \textit{Ech}: \textit{E. chrysanthemi}. $^{(2)}$: positive of 380 bp and 480 bp. $^{(3)}$Ec: \textit{E. carotovora}; \textit{Ech}: \textit{E. chrysanthemi}. $^{(4)}$Isolates with discordant results: strain ‘46’ confirmed as \textit{Ech} by PCR and biochemical tests; strain ‘Q2’: confirmed as \textit{Ech} but negative with primers “ADE1” and “ADE2”.

### Figure 1. Result of PCR amplification of IGS rDNA 16S-23S regions of \textit{Erwinia} with primers “149LF” and “L1ra”. Lines: 1: strain ‘571’ (\textit{Ech}); 2: strain ‘31’ (\textit{Eca}); 3: strain ‘71’ (\textit{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’ (\textit{Eca}); 27: strain ‘P1’ (\textit{Ecc}); and 28: ‘tomato’ strain (\textit{Ec}). IGS 16S-23S; \textit{E. chrysanthemi} (\textit{Ech}): 354–356 (smaller) and 480 pb (larger); \textit{E. carotovora} (\textit{Eca}): 440–453 (smaller) and 475–490 pb (larger).
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), corroborating the results of these papers.

References

CAMINO, J.M.; DÍAZ POLANCO, C. Identificación de una bacteriosis en apio (Arracacia xanthorrhiza). Agronomía Tropical, v.22, p.563-567, 1972.

DE BOER, S.H.; KELMAN, A. Gram-negative bacteria: Erwinia soft rot group. In: SCHAAD, N.W.; JONES, J.B.; CHUN, W. (Ed.). Laboratory guide for identification of plant pathogenic bacteria. St. Paul: APS, 2000. p.56-72.

DUARTE, V.; DE BOER, S.H.; WARD, L.I.; OLIVEIRA, A.M.R. Characterization of atypical Erwinia carotovora strains causing blackleg of potato in Brazil. Journal of Applied Microbiology, v.96, p.535-545, 2004.

FESSEHAIE, A.; DE BOER, S.H.; LÉVESQUE, C.A. Molecular characterization of DNA encoding 16S-23S rRNA intergenic spacer regions and 16S rRNA of pectolytic Erwinia species. Canadian Journal of Microbiology, v.48, p.387-398, 2002.

GARDAN, L.; GOUY, C.; CHRISTEN, R.; SAMSON, R. Elevation of three subspecies of Pectobacterium carotovorum to species level.
Erwinia chrysanthemi and soft rot outbreaks of arracacha

Pectobacterium atrosepticum sp. nov., Pectobacterium betavasculorum sp. nov. and Pectobacterium wasabi sp. nov.

International Journal of Systematic and Evolutionary Microbiology, v.53, p.381-391, 2003.

GOMIDE, A.F.; ROMEIRO, R.S. Levantamento de doenças bacterianas em hortaliças na região do cinturão verde de Belo Horizonte. Fitopatologia Brasileira, v.17, p.47-52, 1992.

HAUBEN, L.; MOORE, E.R.B.; VAUTERIN, L.; STEENACKERS, M.; MERGAERT, J.; VERDONCK, L.; SWINGS, J. Phylogenetic position of phytopathogens within the Enterobacteriaceae. Systematic Applied Microbiology, v.21, p.384-397, 1998.

HENZ, G.P. Perdas pós-colheita e métodos de manejo da podridão-mole causada por Erwinia chrysanthemi e Erwinia carotovora subsp. em mandioquinha-salsa (Arracacia xanthorrhiza Bancroft). 2001. 256p. Tese (Doutorado) - Universidade de Brasília, Brasília.

JABUSONS, R.E.; TAKATSU, A.; REIFSCHEIDER, F.J.B. Levantamento e identificação de espécies de Erwinia de diferentes plantas hospedeiras e regiões do Brasil. Fitopatologia Brasileira, v.11, p.185-195, 1986.

KLEMENT, Z.; RUDOLPH, K.; SANDS, D.C. (Ed.). Methods in phytopathobiology. Budapest: Akademiai Kiado, 1990. 657p.

LOPES, C.A.; QUEZADO-SOARES, A.M. Doenças bacterianas das hortaliças: diagnose e controle. Brasília: Embrapa-CNPH/Embrapa-SPI, 1997. 70p.

MICHEREFF, S.J.; MARIANO, R.L.R. Gênero Erwinia no Brasil. Summa Phytopathologica, v.19, p.137-144, 1993.

NASSAR, A.; DARRASSE, A.; LEMATTRE, M.; KOTOUJANSKY, A.; DERVIN, C.; VEDEL, R.; BERTHEAU, Y. Characterization of Erwinia chrysanthemi by pectolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR-amplified fragments of pel genes. Applied and Environmental Microbiology, v.62, p.2228-2235, 1996.

OLIVEIRA, A.M.R.; DUARTE, V.; SILVEIRA, J.R.P.; MORAES, M.G. Incidence of pectolytic erwinias associated with blackleg of potato in Rio Grande do Sul. Fitopatologia Brasileira, v.28, p.49-53, 2003.

PÉRÔMBELON, M.C.M.; SALMOND, G.P.C. Bacterial soft rots. In: SINGH, U.S.; SINGH, R.P.; KOSHMOTO, K. Pathogenesis and host specificity in plant diseases: histopathological, biochemical, genetic and molecular bases. Oxford: Pergamon/Elsevier Science, 1995. p.1-20.

ROMEIRO, R.S.; SOUSA, R.M.; MUCHOVEJ, J.J.; KIMURA, O. Soft rot of Peruvian carrot due to Erwinia carotovora in Brazil. Plant Pathology, v.37, p.300-302, 1988.

SILVA, J.R. Principais aspectos da cultura da mandioquinha-salsa ou batata-baroa. FIR, v.10, p.32-37, 1967.

TAKATSU, A.; MELO, S.; GARCIA, E.J. Fruto do pimentão como meio parcialmente seletivo para o isolamento de Erwinia carotovora. Fitopatologia Brasileira, v.6, p.550-551, 1980.

YAP, M.N.; BARAK, J.D.; CHARKOWOWSKI, A.O. Genomic diversity of Erwinia carotovora subsp. carotovora and its correlation with virulence. Applied and Environmental Microbiology, v.70, p.3013-3023, 2004.

ZAPATA, M.A.; PARDO, V.M. Estudios sobre el marchitamiento de la arracacha (Arracacia xanthorrhiza) causado por Erwinia sp. Revista Facultad Nacional de Agronomía, v.29, p.39-42, 1974.