Phytopathol. Mediterr. (2008) 47, 87–91

RESEARCH PAPERS

Ralstonia solanacearum biovar 1 associated with a new outbreak of potato brown rot in Portugal

LEONOR CRUZ[1], MADALENA ELOY[1], FRANCISCO QUIRINO[1] and HERBERTO CARRINHO[2]

[1]Instituto Nacional de Recursos Biológicos, Ed. 1, Tapada da Ajuda, 1349-018 Lisbon, Portugal
[2]Direcção-Geral de Agricultura e Desenvolvimento Rural, Tapada da Ajuda, Ed. 1, 1349-018 Lisbon, Portugal

Summary. In May 2007, potato plants exhibiting symptoms possibly of brown rot were collected in some potato fields in the Baixo Mondego region (Center), Portugal, as a part of a nationwide programme to monitor *Ralstonia solanacearum*. All laboratory procedures laid down in Commission Directive 2006/63/EC, including dilution plating on semi-selective medium SMSA, indirect imunofluorescence (IIF), polymerase chain reaction (PCR) using specific primers and bioassays on tomato plants, were strictly followed and the causal agent of the disease was identified as *Ralstonia solanacearum*. The identity of the pure cultures of the isolated organism was confirmed by PCR, IIF and pathogenicity tests on several other plant species (eggplant, tobacco, pelargonium and eucalyptus). In biovar determination, the failure of the isolates to utilise/oxidise certain carbon sources indicated that the isolates were all biovar 1. This biovar has a broader host range than biovar 2 strains, and affects several crops of economic importance including ornamental plants and forest trees. Comparative analysis of 16S rRNA and endoglucanase (*egl*) gene sequences of these isolates with sequences that have been deposited at the GenBank revealed a similarity higher than 99% for several *Ralstonia solanacearum* isolates from biovar 1, including isolate DAR 64836 (Accession number DQ011551). This is the first report of *Ralstonia solanacearum* biovar 1 in Portugal. All control measures specified in the Commission Directive are being implemented.

Key words: brown rot, diagnosis, first record, gene sequencing.

Introduction

Ralstonia solanacearum (Smith 1896) Yabuuchi *et al.* (1995), the causal agent of potato brown rot and bacterial wilt disease on a variety of hosts, is a quarantine organism regulated by the European Union phytosanitary legislation. The organism is genomically very complex, thus exhibiting an extensive host range and a high plasticity in its adaptation to widely different climatic conditions and habitats. Brown rot was quite widespread in Portugal in the early 20th century (Maia and Oliveira, 1945). However, measures to eradicate the disease led to the disappearance of the disease caused by biovar 2, subphenotype A strains of the bacterium (Sousa-Santos, 1997). A recurrence of the disease was seen in the late 1990s, affecting potato and tomato fields as well as alternative host weeds, and the pathogen was also detected in surface irrigation water (Cruz *et al.*, 2001). This recurrence of brown rot led to the identification once more of biovar 2, subphenotype A strains, which are

Corresponding author: L. Cruz
Fax: +351 361 3277
E-mail: cruzleonor@gmail.com

characterized by a narrow host range, with solanaceous crops as their main natural hosts. In late May 2007, the detection of potato plants in an irrigated field in the Baixo Mondego region showing symptoms reminiscent of brown rot led to intensive monitoring of this and several other potato fields exhibiting the same symptoms nearby. The symptoms were wilting of the basal leaves, yellowing and reduced growth of the whole plant, associated with very high virulence patterns, which suggested that a different strain of *R. solanacearum* occurred in this area. Laboratory procedures laid down by the European Union phytosanitary legislation (Anonymous, 2006), including the isolation of the organism, the identification of pure cultures and pathogenicity tests on tomato plantlets, as well as on other hosts such as tobacco plants, *Pelargonium* sp. and *Eucalyptus globulus*, made it possible to identify the causal agent of the disease. Several other laboratory approaches, namely biovar determination (Hayward, 1964, 1994), 16S rRNA and the sequencing of partial endoglucanase (*egl*) (Fegan and Prior, 2005) genes enabled the new isolates obtained to be characterized at intra-specific level and the causal agent of this new outbreak to be identified.

Materials and methods

Sampling and isolate characterization

Samples of potato plants and tubers collected as part of the National Monitoring Programme on *Ralstonia solanacearum* in the Baixo Mondego irrigated area were routinely analyzed at the Bacteriology Laboratory of the Portuguese Plant Protection Services (ex-DGPC), following the procedures laid down in the EU Directive 2006/63/CE (Anonymous, 2006), which include a range of steps such as screening, identification, characterization of the pathogen and confirmation of its identity.

Pieces of plant tissue collected from the basal part of the stems and heel end cores from potato tubers were processed and analysed by dilution plating on the semiselective medium SMSA (Elphinstone *et al.*, 1996), the enrichment of plant extracts in a semiselective broth for indirect imunofluorescence with a validated polyclonal antiserum (Plant Research International, The Netherlands) (Janse, 1988) and PCR detection (Opina *et al.*, 1997), supplemented with bioassays on susceptible tomato plantlets (Janse, 1988). Presumptive bacterial colonies were re-isolated on general sucrose peptone agar medium (SPA) (Lelliott and Stead, 1987), and their identity was confirmed using PCR and imunofluorescence. Pathogenicity tests on susceptible tomato plantlets were also performed to fulfil Koch's postulates.

In addition, samples from potato plants and tubers and from irrigation water were taken from visibly affected fields and from areas near those fields. Samples from surface irrigation water were always checked for temperature at the time of sampling in order to assess the possibility of isolating *R. solanacearum*. These samples were also analyzed according to the stringent procedures laid down in the EU legislation mentioned above (Anonymous, 2006). Intra-specific characterization of isolates was done by determining the biovar profile according to the utilization of six carbon sources: maltose, lactose, cellobiose, mannitol, sorbitol and dulcitol (Hayward, 1964, 1994). Subphenotypes from biovar 2 isolates were characterized on the basis of their utilization of threalose, innositol and D-ribose (Hayward, 1994). Bacterial water suspensions from fresh pure cultures grown on SPA and containing about 10^8 cfu ml^{-1} (OD 0.1 at 630 nm) were used to stem-inoculate tomato, tobacco, eggplant and *Pelargonium* sp. with two to three true leaves, and *E. globulus* plantlets, in order to evaluate their host range, following Janse (1988).

DNA extraction and semplificaton

Minisatellite primed PCR (MSP-PCR) genomic fingerprinting with primer csM13, obtained from the core sequence of the capsid protein from phage M13 using bacterial water suspension lysates, was performed for several Portuguese isolates and for the foreign type strain K 60, obtained by Kelman from tomato and belonging to biovar 1, to assess the similarity of the genomic fingerprinting profiles (Gadanho *et al.*, 2003).

The housekeeping gene 16S rRNA and the *egl* megaplasmid virulence gene were partially sequenced on two selected isolates. The DNA was purified using the Invitrogen easy DNA Kit (Invitrogen, Paisley, UK). The 16S rRNA sequence was amplified using the pA and pH primers. The *egl* 750 bp partial sequence was determined using the Endo-F, Endo-R and Endo 837r primers, following Fegan and Prior (2005) and Castillo and Greenberg (2007). Raw sequences from both strands were corrected and assembled using BioEdit Version 7.0.5.3 software (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Nucleotide BLAST was done by comparing the obtained sequences with the accessions deposited at the 'National Center for Biotechnology Information' (NCBI) database (www.ncbi.nlm.nih.gov).

Nucleotide sequence accession numbers

Nucleotide sequences were deposited in the nucleotide sequence database NCBI under accession numbers EU574930 and EU574931 for gene 16S rRNA, and EU334565 and EU334564 for the *egl* gene.

Results

Potato plant and tuber samples from ware potato fields in the 'Baixo Mondego' region were infected with a strain of the *R. solanacearum* species complex pathogenic to tomato. The species-specific primers 759f and 760r produced the expected 288 bp amplicons after PCR amplification of DNA from the plant and tuber tissue extracts. Colonies from those extracts exhibited the characteristic morphology on a specific isolation medium. The identity of the isolated organism was confirmed by indirect imunofluorescence with a commercially available validated polyclonal antiserum, the bacterial cells showing a characteristic morphology and fluorescence intensity. Water samples at a temperature of about 20ºC consistently produced negative results, indicating that these samples were not contaminated with this organism.

Further characterization showed that the isolates did not utilize or oxidise the three disaccharides or the three hexose alcohols, revealing that they belonged to biovar 1 phenotype.

Stem inoculation as described in the EU legislation performed under a plant quarantine confinement facility produced symptoms on all the tomato, eggplant, tobacco and *Pelargonium* sp. test plantlets a few days after inoculation. On *E. globulus* the first symptoms arose one month after inoculation.

MSP-PCR fingerprinting profiles of the Portuguese isolates from biovar 1, including CPBF 674 and CPBF 1192, were very similar among each other, but clear differences were observed between these and other isolates from the same biovar, namely the type strain K 60 isolated from tomato and the isolate NCPPB 1029 obtained from *Pelargonium capitatum*. The Portuguese isolate CPBF 1212, also from potato tubers but belonging to biovar 2, produced a clearly distinct genotypic profile (Fig. 1).

Sequences from the 16S rRNA gene of isolates CPBF 674 and CPBF 1192 were identical. The same happened for *egl* partial gene sequences from both isolates. Blast of the 16S rRNA gene sequences from isolates CPBF 674 (EU574930) and CPBF 1192 (EU574931) revealed 100% nucleotide identity with several *R. solanacearum* accessions deposited at the GenBank, such as AF207891 (CFBP 1036). In addition, blast of the *egl* gene partial sequences from those isolates (accession numbers EU334565 and EU334564, respectively) had an identity of 99% with

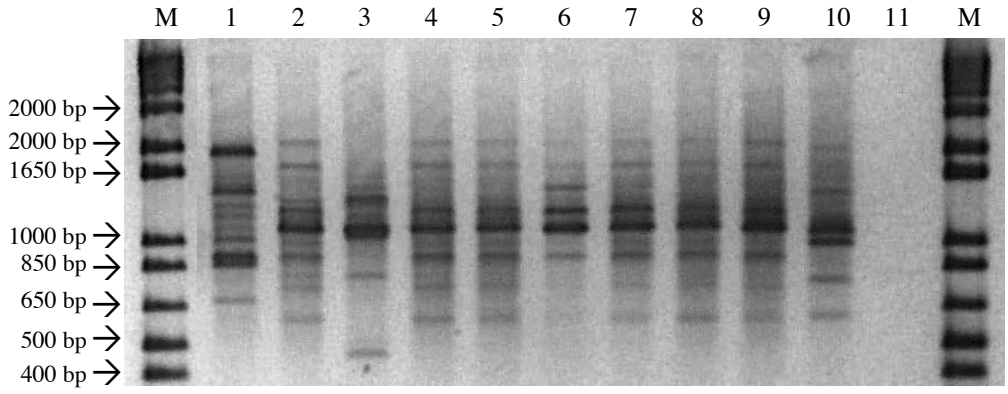

Fig. 1. MSP-PCR of Ralstonia solanacearum with primer CsM13. M, DNA ladder 1Kb plus (Invitrogen); Biovar 1 isolates - Lanes 1 to 10: 1, NCPPB 1029; 2, CPBF 674; 3, K60; 4, CPBF 1189; 5, CPBF 1192; 6, CPBF 1199; 7, CPBF 1200; 8, 1201; 9, CPBF 1211. Biovar 2 isolate- lane 10, CPBF 1212. Lane 11, negative control.

the corresponding sequences from other isolates of biovar 1, such as isolate DAR 64836 (DQ011551).

Discussion

The diagnostic procedures used in the present study identified the isolates obtained from samples of potato tubers and plants. Pathogenicity tests showed that the isolates were highly virulent on tomato, eggplant, *Nicotiana* sp. and *Perlargonium* sp. plants, leading to their death in a few days. *E. globulus* plants were also susceptible to these isolates when inoculated on the stems and potential hosts under natural conditions. Other hosts, including *Musa acuminata*, will soon be inoculated with these isolates in order to establish the race of the isolates as defined by Janse (1991). Strains of *R. solanacearum* biovars 1 and 3 both produce disease on *Eucalyptus* sp. (Alfenas *et al.*, 2006; Fouché-Weich *et al.*, 2006), but the high reproducibility of the biovar determination tests allowed these isolates to be typed as biovar 1. Only one report on the interception of *R. solanacearum* biovar 1 infecting *Begonia eliator* plants in Europe was found (Janse, 2006), and the corresponding eradication measures were carried out successfully.

MSP-PCR produced highly reproducible fingerprints and characterized the Portuguese biovar 1 isolates as belonging to a type clearly different from the resident strains of biovar 2, subphenotype A. *R. solanacearum* biovar 1 has a broad host range and its genomic diversity is higher (Gillings and Fahy, 1994; Fegan and Prior, 2005). MSP-PCR indicated that the putative biovar 1 strains tested had some degree of diversity, and suggested that these Portuguese strains may belong to a group distinct from the type strain K 60, isolated from tomato and belonging to race 1.

The 16S rRNA gene sequence from the *R. solanacearum* species complex is extremely conserved, revealing a high reliability in identifying specimens from this group. Variability was less than 1% (Taghavi *et al.*, 1996; Fegan *et al.*, 1998). The *egl* gene sequence was more variable, correlating well with the virulence profile and adaptative performance of the strains and usefully confirming the identification and the intra-specific characterization of these Portuguese isolates as belonging to biovar 1 of the *R. solanacearum* species complex.

So far, it has not been possible to trace the origin of the current outbreak affecting several potato fields in the Baixo Mondego region. However, the most likely origin is a single potato seed lot of foreign origin, with a latent infection, distributed and marketed in this area. In fact, the same agricultural machinery used by different farmers may have played an important role in the dissemination of this organism to neighbouring potato fields. The potato fields are located near an area where there are several ornamental and forest nurseries which may also represent a possible alternative source of contamination or alternative host reservoir that should not be neglected.

The National Monitoring Programme, implemented several years ago, made it possible to detect and identify this new pathogen population with a broader host range, which is confined to this irrigated area. Since the irrigation water is not contaminated the pathogen has probably been recently introduced, suggesting a positive prospect towards the implementation of a new eradication program. Specific phytosanitary measures, including quarantine, to control and eradicate this new outbreak, are now being undertaken by the competent national authorities.

Further pathogenicity assays and biomolecular characterization are ongoing to assess the epidemiologic profile of these new isolates and to determine whether there are any new variants of *R. solanacearum* in Portugal that are pathogenic on unexpected host plants.

Acknowledgements

The authors would like to express their gratitude to the Laboratório de Sequenciação e Análise de Fragmentos (ICAT, Lisbon) for collaboration in the sequencing analysis. Authors also would like to acknowledge the phytosanitary inspectors at "Direcção Regional de Agricultura e Pescas do Centro".

Literature cited

Alfenas A.C., R.G. Mafia, R.C. Sartório, D.H.B. Binoti, R.R. Silva, D. Lau and C.A. Vanetti, 2006. *Ralstonia solanacearum* em viveiros clonais de eucalipto no Brasil. *Fitopatologia Brasileira* 31, 357–366.

Anonymous, 2006. Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. *Official Journal of the European Union* L 206 from 27/07/2006, 36–106.

Castillo J. and J.T. Greenberg, 2007. Evolutionary dynamics

of *Ralstonia solanacearum*. *Applied and Environmental Microbiology* 74, 1225–1238.

Cruz L., M. Sousa-Santos, A. Costa and H. Carrinho, 2001. Present status of *Ralstonia solanacearum* in Portugal. In: *Proceedings of the 11th Congress of the Mediterranean Phytopathological Union*, Évora, Portugal, 252–254.

Elphinstone J.G., J. Hennessy, J.K. Wilson and D.E. Stead, 1996. Sensitivity of detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO Bulletin* 26, 663–678.

Fegan M. and P. Prior, 2005. How complex is the "*Ralstonia solanacearum* species complex"?. In: *Bacterial Wilt Disease and the Ralstonia solanacearum Species Complex* (C. Allen, P. Prior, A.C. Hayward, ed.), The American Phytopathological Society, St. Paul, MN, USA, 449–462.

Fegan M., M. Taghavi, L. Sly and A.C. Hayward, 1998. Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum*. In: *Bacterial Wilt Disease – Molecular and Ecological Aspects* (P. Prior, C. Allen, J. Elphinstone, ed.), Springer-Verlag, INRA, France, 19–33.

Fouché-Weich J., S. Poussier, D. Trigalet-Demery, D. Berger and T. Coutinho, 2006. Molecular identification of some African strains of *Ralstonia solanacearum* from eucalypt and potato. *Journal of Plant Pathology* 72, 369–373.

Gadanho M., J.M. Almeida and J.P. Sampaio, 2003. Assessment of yeast diversity in a marine environment in the south of Portugal by microsatellite-primed PCR. *Antonie Van Leeuwenhoek* 84, 217–227.

Gillings M.R. and P. Fahy, 1994. Genomic fingerprinting towards a unified view of the *Pseudomonas solanacearum* species complex. In: *Bacterial Wilt: The Disease and its Causative Agent,* Pseudomonas solanacearum (A.C. Hayward, G.L. Hartman, ed.), CAB International, Wallingford, UK, 95–112.

Hayward A.C., 1964. Characteristics of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology* 27, 265–277.

Hayward A.C., 1994. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. In: *Bacterial Wilt: The Disease and its Causative Agent,* Pseudomonas solanacearum (A.C. Hayward, G.L. Hartman, ed.), CAB International, Wallingford, UK, 123–136.

Janse J.D., 1988. A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. *EPPO Bulletin* 18, 343–351.

Janse J.D., 1991. Infra-intraspecific classification of *Pseudomonas solanacearum* strains, using whole cell fatty acid analysis. *Systematic and Applied Microbiology* 14, 335–345.

Janse J.D., E. Goossens, E.A. van Beuningen, K. Gaisch and N.N.A. Tjou Tam Sin, 2006. *Begonia elatior*, a new host for *Ralstonia solanacearum* race 1, biovar 1. In: *The 4th International Bacterial Wilt Symposium. Programme and Abstracts Book*. York, UK, 32.

Lelliott R.A. and D.E. Stead, 1987. *Methods for the Diagnosis of Bacterial Diseases of Plants*. Blackwell Scientific Publicatons Ltd., Oxford, 216 pp.

Maia R. and O. Oliveira, 1945. Doença da batateira causada por uma variedade do *Bacterium solanacearum* E.F. Smith. *Agronomia Lusitana* 7, 195–205.

Opina N.F., F. Tavner, G. Holloway, J.F. Wang, T.H. Li, R. Maghirang, M. Fegan, A.C. Hayward, V. Krishnapillai, W.F. Hong, B.W. Holloway and J.N. Timmis, 1997. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asian Pacific Journal of Molecular Biology and Biotechnology* 5, 19–33.

Sousa Santos M., 1997. The recurrence of *Burkholderia solanacearum* in southern European countries. In: *Seed Health Testing – Progress Toward the 21st Century*. (J.D. Hutchins, J.C. Reeves, ed.), CAB International, Wallingford, 21–34.

Taghavi M., C. Hayward, I.S. Lindsay and M. Fegan, 1996. Analysis of the Phylogenetic relationships of strains of *Burkholderia solanacearum*, *Pseudomonas syzygii* and the blood disease bacterium of banana based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* 46, 10–15.

Yabuuchi E., Y. Kosako, I. Yano, H. Hotta and Y. Nishiuchi, 1995. Transfer of two Burkholderia and an Alcaligenes species to *Ralstonia* gen. nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiology and Immunology* 39, 897–904.

Accepted for publication: April 3, 2008