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Pectobacterium carotovorum subsp. *brasiliense* and *Pectobacterium carotovorum* subsp. *carotovorum* as causal agents of potato soft rot in Algeria

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Abstract During surveys carried out in 2014 and 2015 in Algerian potato fields, severe bacterial tuber soft rot was observed. Twenty-one Gram-negative pectinolytic bacterial isolates were obtained from diseased potato tubers. These induced a hypersensitive reaction and soft rot symptoms when inoculated in tobacco leaves and potato tubers, respectively. PCR amplification using the Y1/Y2 primers demonstrated that all of the bacterial isolates belonged to the genus *Pectobacterium*. Phylogenetic analysis using partial malate dehydrogenase (*mdh*) gene sequences revealed that six and 15 isolates grouped with *Pectobacterium carotovorum* subsp. *brasiliense* and *Pectobacterium carotovorum* subsp. *carotovorum* reference strains, respectively. Multilocus sequence analysis of concatenated partial sequences of the *acnA*, *atpD*, *gyrB* and *infB* genes carried out on selected bacterial isolates confirmed the results obtained with the *mdh* gene sequences. PCR amplification with the BR1f/L1r primers that are specific for *P. carotovorum* subsp. *brasiliense* confirmed that six of the Algerian isolates belonged to this taxon.

Keywords Algeria · Soft-rotting bacteria · *Solanum tuberosum* · *Pectobacterium*

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Introduction

Each year, viral, bacterial, oomycete and fungal diseases and pests worldwide cause severe potato (*Solanum tuberosum* L.) yield losses that have been estimated at 22% of the total production, which corresponds to over 65 million tonnes (Czajkowski et al. 2011). In 2014, Algeria was in the 16th top potato producer worldwide, with the production of 4,673,516 t on 156,176 ha (FAOSTAT 2014). However, a number of potato diseases have been reported for Algeria, including potato leaf roll virus, common scab, late blight, dry rot and soft rot (Nassan and Fettouche 1991; Yahiaoui-Zaidi et al. 2003).

Bacteria belonging to the *Pectobacterium* and *Dickeya* genera are the main causal agents of potato tuber soft rot and/or blackleg (Pérombelon and Kelman 1980; Pérombelon 2002; Czajkowski et al. 2011). Pectolytic Pseudomonads can also sometimes cause potato soft-rot (Aremu and Babalola 2015). Five *taxa* of the genus *Pectobacterium* are involved in potato

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tuber soft rot and/or blackleg: *Pectobacterium atrosepticum* is mainly spread through temperate regions, and it almost exclusively infects potatoes; *Pectobacterium carotovorum* subsp. *carotovorum* has a broad host range worldwide; *Pectobacterium carotovorum* subsp. *brasiliense* was initially reported in Brazil and has now spread to several other countries; *Pectobacterium aroidearum* has been reported in Lebanon; and *Pectobacterium wasabiae* has been reported in a number of countries (Duarte et al. 2004; Czajkowski et al. 2011; Nabhan et al. 2012a, b; Nykyri et al. 2012; Moleleki et al. 2013; Waleron et al. 2013; Moretti et al. 2016; Zhang et al. 2016; Dees et al. 2017; Zoledowska et al. 2018). Recently, strains of *P. wasabie* were isolated from potato and were reclassified as *P. parmentieri* (Khayi et al. 2016). Consensus now indicates that *P. wasabie* isolated from horseradish is not a potato pathogen (Goto and Matsumoto 1987). *Dickeya dadantii*, *Dickeya dianthicola* and *Dickeya solani* are the main pathogens that cause potato blackleg and soft rot (van der Wolf et al. 2014; Potrykus et al. 2017).

During surveys carried out in 2014 and 2015 in potato fields in five provinces in north-west Algeria (i.e., Ain Defla, Chlef, Mostaganem, Relizane, Tiaret) severe and typical bacterial soft rot of tubers were observed. The aim of the present study was to define the aetiology of this disease.

Materials and methods

Bacterial isolation

To isolate the causal agent(s) of this potato bacterial soft rot in Algeria, small amounts of tuber samples from the margins of diseased tissue were macerated in sterile water. Loopfuls of these homogenates were streaked onto nutrient agar and incubated at 27 ± 1 °C. The bacterial colonies that formed were purified in nutrient agar supplemented with 5% sucrose, and were stored in 15% glycerol solution at −80 °C.

Biochemical, physiological, pathogenicity and virulence tests

The ability of these purified bacterial isolates to degrade pectin was tested using crystal violet medium that was prepared according to Helias et al. (2012), and pectin (Dipecta, type AG366; Agdia Biofords, 91,030 Evry cedex, France).

The bacterial isolates were tested for induction of a hypersensitive reaction (HR) in tobacco leaves and for disease symptoms when inoculated into potato tubers. For the HR test, tobacco leaves (cv. Havana 425) were infiltrated with bacterial suspensions at 10^8 cfu mL^{-1}, using a syringe without a needle, while potato tubers of cv. Agata were inoculated as described by Laurila et al. (2008).

The bacterial isolates were also subjected to basic microbiological tests according to procedures described by Schaad et al. (2001) and Sands (1990), which included KOH solubility, oxidative/ fermentative metabolism, and growth at 37 °C.

DNA extraction and molecular characterisation

For DNA extraction, the bacterial isolates were cultivated in Luria–Bertani broth and incubated overnight at 27 ± 1 °C in an orbital shaker (130 rpm). The DNA was extracted using kits (GenElute Bacterial Genomic DNA kits; Sigma–Aldrich, St Louis, MO, USA), following the manufacturer instructions. The Y1/Y2 primers that are specific for *Pectobacterium* genus were used (Supplementary Table 1), as reported by Darrasse et al. (1994). The PCR amplicons were electrophoresed through 1.5% (*w*/*v*) agarose gels in 0.5× TAE buffer (20 min, 100 V), and visualised under UV light.

The bacterial isolates were characterised at the molecular level using sequencing of the malate dehydrogenase (*mdh*) gene. The PCR reactions were performed in a total volume of 50 μL, which consisted of PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer, 2 U Taq DNA polymerase and 50 ng bacterial DNA. The PCR temperature profile comprised an initial denaturation step at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were separated by gel electrophoresis using 1.5% (w/v) agarose in 0.5× TAE buffer (45 min, 100 V), and were visualised under UV light. The PCR products were sequenced by Macrogen Europe (The Netherlands). For phylogenetic comparisons, partial *mdh* sequences from enterobacteria that are related to soft rot bacteria were retrieved from the GenBank database. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 7 (Kumar et al. 2016) and the maximum parsimony

method. Clade stability was assessed by 1000 bootstrap replications. *Yersinia pestis* CO92 was used as outgroup. All of the sequences have been deposited in GenBank under the accession numbers reported in Supplementary Table 2.

PCR amplification with the BR1f/L1r primers that are specific for *P. carotovorum* subsp. *brasiliense* (Supplementary Table 1) were carried out as described by Duarte et al. (2004). The 16S rDNA was amplified using the P0 and P6 primers (Supplementary Table 1). PCR was performed in 20 μL reaction mixture that consisting of PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer, 2 U Taq DNA polymerase and 25 ng bacterial DNA. The PCR temperature profile (MyCycler Thermal Cycler; Bio-Rad, CA, USA) comprised an initial denaturation step at 95 °C for 1.5 min, 5 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 4.5 min, 5 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 4.5 min, 25 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 4.5 min, and final extension at 72 °C for 7 min. The PCR products were separated by gel electrophoresis using 1.2% (*w*/*v*) agarose in 0.5× TAE buffer (1 h, 100 V) and were visualised under UV light. The PCR products of 16S rDNA were sequenced by Macrogen Europe (The Netherlands), and were compared with those in GenBank using BlastN.

Multilocus sequence analysis (MLSA) was used for identification of *Pectobacterium* species, using partial sequences of the aconitase hydrase 1 (*acnA*), ATP synthase F1, β subunit (*atpD*), DNA gyrase B subunit (*gyrB*), and initiation translation factor 2 (*infB*) genes. Partial fragments of the *acnA*, *atpD*, *gyrB* and *infB* genes were amplified using the protocol of Brady et al. (2008). The PCR products were sequenced by Macrogen Europe (The Netherlands). Phylogenetic and molecular evolutionary analyses were conducted as reported above.

Table 1 Soft-rot bacterial strains isolated from potato tubers in different Algerian locations and selected tests for their identification

Bacterial species	Strain	Location	Cultivar	Virulence on potato[a]	BR1f/L1r amplification
Pectobacterium carotovorum subsp. *carotovorum*	HNA 1	Ain Defla	Spunta	14.1 abc	−
	HNA 11	Ain Defla	Désirée	2.7 a	−
	HNA 12	Ain Defla	Spunta	4.7 abc	−
	HNA 13	Ain Defla	Spunta	12.2 abc	−
	HNA 20	Ain Defla	Condor	15.2 abc	−
	HNA 21	Ain Defla	Spunta	12.1 abc	−
	HNA 22	Ain Defla	Spunta	29.3 c	−
	HNA 6	Ain Defla	Spunta	24.9 c	−
	HNA 17	Chlef	Spunta	16.7 abc	−
	HNA 3	Chlef	Spunta	24.4 c	−
	HNA 4	Chlef	Condor	11.6 abc	−
	HNA 18	Mostaganem (Debdaba)	Désirée	5.7 abc	−
	HNA 24	Relizane	Spunta	9.1 abc	−
	HNA 5	Relizane (Hmadna)	Spunta	13.2 abc	−
	HNA 19	Tyaret	Désirée	10.8 abc	−
Pectobacterium carotovorum subsp. *brasiliensis*	HNA 8	Ain Defla	Spunta	15.2 abc	+
	HNA 15	Ain Defla	Spunta	23.9 c	+
	HNA 9	Chlef	Condor	12.9 abc	+
	HNA 26	Chlef (Mejaja)	Désirée	18.3 bc	+
	HNA 2	Tyaret	Désirée	3.8 ab	+
	HNA 27	Tyaret	Désirée	16.3 abc	+

[a] Virulence on potato tubers is expressed as g of rotted tissue (3 days after the inoculation). Each value is the mean of three replicates. Values followed by the same letter are not significantly different according to Tukey HSD test (p= 0.05)

Results and discussion

Twenty-one Algerian bacterial isolates that degraded pectin were investigated in the present study (Table 1). All of them induced HR in tobacco leaves that were evident from 24 h after inoculation, and were also pathogenic on potato tubers. The isolates, that cause the most severe disease symptoms were isolated from potato tubers of cv. Spunta sampled in the Algerian provinces of Ain Defla (isolates HNA 6, 15, 22) and Chlef (isolate HNA 3) (Table 1). Koch's postulates were fulfilled, as the isolates from the inoculated tubers had the same culture characteristics as the inoculated isolates.

All of the isolates were soluble in KOH, and were therefore Gram negative; they had oxidative/ fermentative metabolism, and grew at 37 °C.

All 21 of these isolates generated a 434 bp PCR product with the Y1/Y2 primers; therefore they belong to the *Pectobacterium* genus (Table 1).

To refine the identification of these Algerian isolates of *Pectobacterium*, partial *mdh* sequences were obtained, as these have been reported to be phylogenetically informative for species of the genus *Pectobacterium*

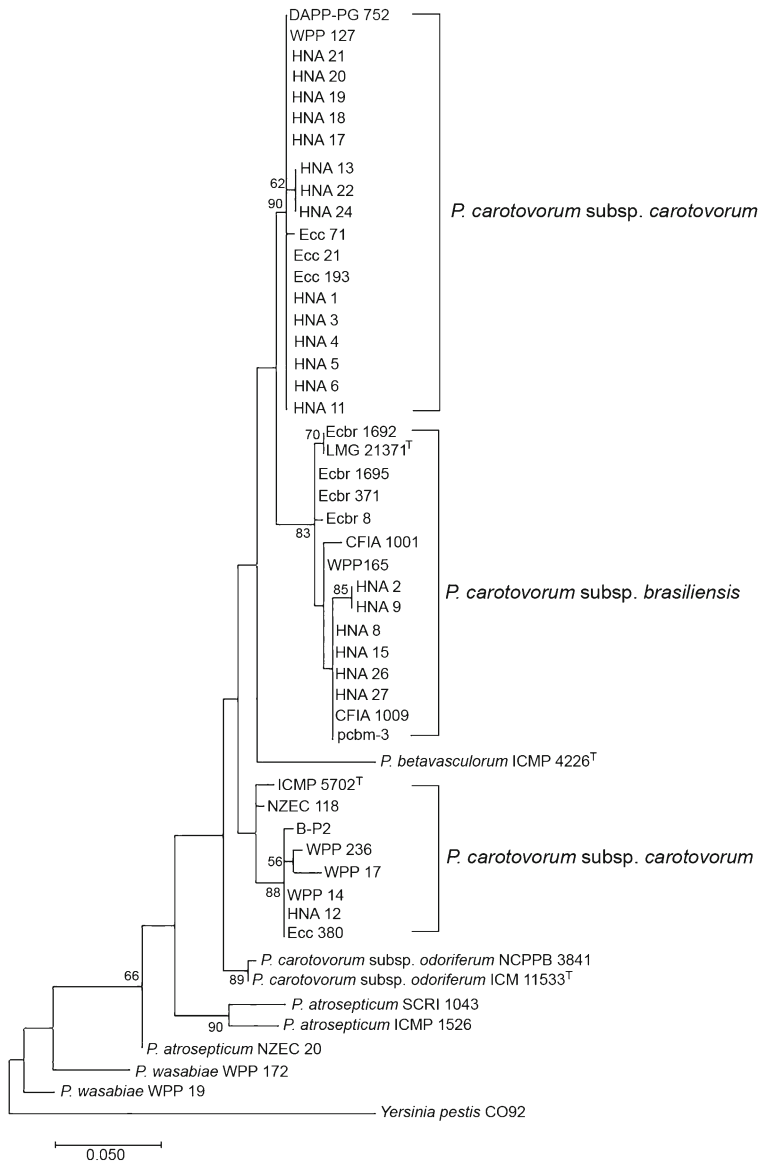

Fig. 1 Maximum likelihood tree based on partial *mdh* gene sequences (229 nt) showing the phylogenetic position of the 21 Algerian isolates (HNA) from potato tubers with soft rot symptoms within the genus *Pectobacterium*. Bar, 5% nucleotide substitutions. Numbers at branching points are bootstrap percentages based on 1000 replications. Only values ≥50% are shown. Strains marked with a superscript "T" represent the type strains of the species or subspecies

(Pitman et al. 2010; Yap et al. 2004; Moretti et al. 2016). These were compared with sequences of *Pectobacterium* spp. deposited in GenBank. The phylogenetic tree for *mdh* sequences is shown in Fig. 1, and this reveals that six of these Algerian isolates (HNA 2, 8, 9, 15, 26, 27) formed a cluster with all of the strains of *P. carotovorum* subsp. *brasiliense* considered in the present study, including the LMG 21371^T type strain. In particular, isolates HNA 8, 15, 26 and 27 have an *mdh* sequence identical to the Canadian CFIA 1009 strain, the genome of which has been sequenced recently (Li et al. 2015), and the Japanese pcbm-3 strain (Fujimoto et al. 2017).

The other 15 Algerian isolates grouped in a cluster that included strains of *P. carotovorum* subsp. *carotovorum*. In particular, 11 of these isolates (HNA 1, 3, 4, 5, 6, 11, 17, 18, 19, 20, 21) have an *mdh* sequence identical to the Lebanese DAPP-PG 752 strain (Moretti et al. 2016), the US WPP 127, Ecc21 strains, and the Ecc 193 from The Netherlands (Ma et al. 2007).

To confirm that the six Algerian isolates belong to *P. carotovorum* subsp. *brasiliense*, PCR amplification was carried out with the subspecies-specific BR1f/L1r primers. All six of these isolates generated a 690-bp product, which confirmed that they belong to *P. carotovorum* subsp. *brasiliense* (Table 1); no such PCR product was observed when the other 15 isolates were analysed.

To confirm the identity of the tested isolates as *P. carotovorum* subsp. *carotovorum* or *P. carotovorum* subsp. *brasiliense*, the 16S rRNA sequences of one representative isolate of each subspecies (HNA 18, 26, respectively) were obtained. The 16S rRNA sequences of isolates HNA 18 and HNA 26 show similarity to the RY29 and RY13 strains and the FM3 and FM4 strains of *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense*, respectively.

Identification was also carried out by MLSA by sequencing of representative Algerian isolates that belonged to the subspecies *brasiliense* (HNA 9, 27) and *carotovorum* (HNA 5, 12, 18, 22), which were selected on the basis of the *mdh* phylogram, using the housekeeping genes *acnA*, *atpD*, *gyrB* and *infB* (Yap et al. 2004; Ma et al. 2007; Kim et al. 2009; Pitman et al. 2010; Baghaee-Ravari et al. 2011; Moretti et al. 2016). The phylogenetic tree obtained with the concatenated

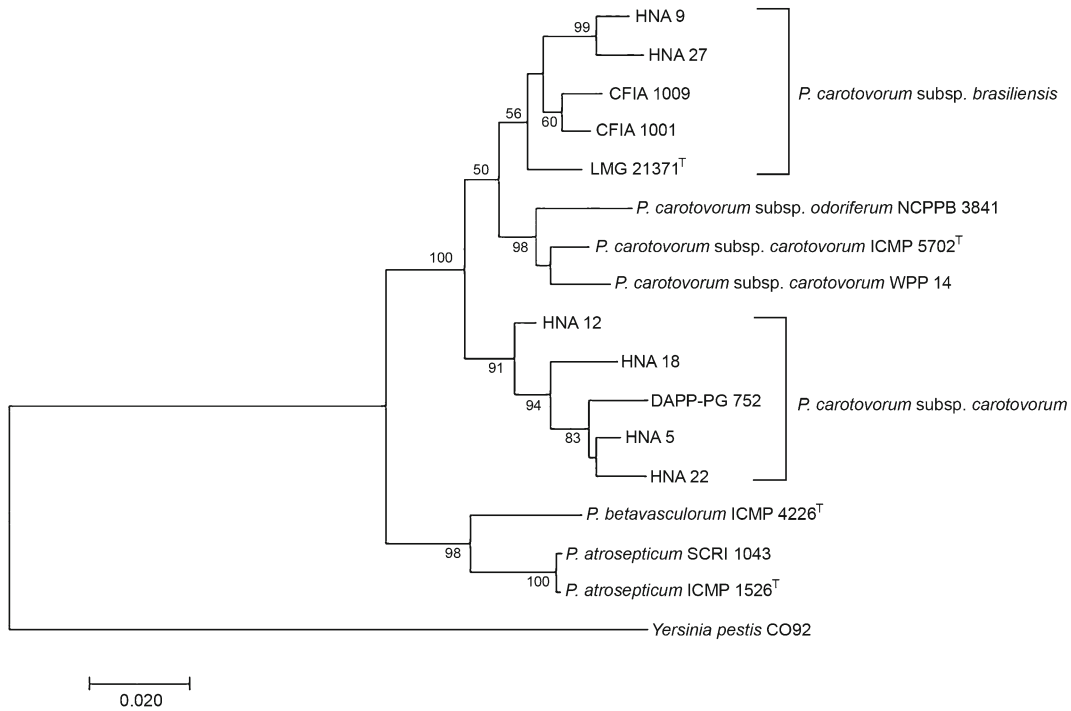

Fig. 2 Maximum likelihood tree based on concatenated partial *atpD*, *gyrB*, *infB* and *rpoB* gene sequences (*atpD*, 642 nt; *gyrB*, 747 nt; *infB*, 615 nt; *rpoB*, 605–637 nt) showing the phylogenetic position of the Algerian isolates (HNA) from potato tubers with soft rot symptoms within the genus *Pectobacterium*. Bar, 2% nucleotide substitutions. Numbers at branching points are bootstrap percentages based on 1000 replications. Only values >50% are shown

acnA, *atpD*, *gyrB* and *infB* partial nucleotide sequences were examined implementing the maximum likelihood method (Fig. 2), which confirmed that the Algerian isolates HNA 9 and 27 grouped with *P. carotovorum* subsp. *brasiliense*, and that isolates HNA 5, 12, 18 and 22 grouped with the Lebanese DAPP-PG 752 strain that belongs to *P. carotovorum* subsp. *carotovorum*. Of note, the ICMP 5702^{T} subspecies type strain of *P. carotovorum* subsp. *carotovorum* clustered in a separate clade (Fig. 2).

The present study thus characterises at the molecular level a number of bacterial isolates that cause potato tuber soft rot in Algeria, and demonstrates that they belong to both *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *carotovorum*. To the best of our knowledge, the present study represents the first occurrence of *P. carotovorum* subsp. *brasiliense* in Algeria as the causal agent of potato tuber soft rot, while the presence of *P. carotovorum* subsp. *carotovorum* on potato has been reported previously (Yahiaoui-Zaidi et al. 2003, 2010).

After the first description of *P. carotovorum* subsp. *brasiliense* (Duarte et al. 2004), it was later reported on potato in Canada (De Boer et al. 2012), Kenya (Onkendi and Moleleki 2014), Japan (Fujimoto et al. 2017), New Zealand (Panda et al. 2012), Peru (Nabhan et al. 2012a, b), Poland (Waleron et al. 2015), South Africa (van der Merwe et al. 2010), Switzerland (de Werra et al. 2015), Syria (Nabhan et al. 2012b), Turkey (Ozturk and Aksoy 2016) and the USA (McNally et al. 2017). *P. carotovorum* subsp. *brasiliense* can also cause mainly soft rot symptoms on artichoke (Cariddi and Bubici 2016), cucumber (Meng et al. 2017), pepper (Gillis et al. 2017; Choi and Kim 2013), tobacco (Wang et al. 2017), tomato (Caruso et al. 2016; Rosskopf and Hong 2016; Jaramillo et al. 2017), sugar beet (Secor et al. 2016) and courgettes (Moraes et al. 2017).

Among the four *P. carotovorum* subspecies *carotovorum*, *odoriferum*, *brasiliense* and *actinidiae*, only the first two have valid publication. However, a plethora of different approaches strongly suggests that subsp. *brasiliense* should be allocated to a separate species from *P. carotovorum*, which have included sequencing of 16S rRNA and intergenic spacer regions and biochemical analysis (Duarte et al. 2004), MLSA (Ma et al. 2007), a pan-genomic approach (Glasner et al. 2008), average nucleotide identity (ANI), and *in-silico* DNA-DNA hybridisation analysis (Zhang et al. 2016). Using whole-genome sequencing data from 83 *Pectobacterium* strains, Zhang et al. (2016) also demonstrated that the clade that contained all of the *P. carotovorum* subsp. *brasiliense* strains showed significant genetic diversity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Human and animal rights This study does not include any studies with human participants or animals (vertebrates) performed by any of the authors.

Informed consent Informed consent was obtained from all of the individual participants included in the study.

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