J. Crop Prot. 2016, 5 (1): 11-17

Short Paper

First detection of *Pantoea ananatis*, the causal agent of bacterial center rot of onion in Morocco

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Abstract: *Pantoea ananatis* is an important pathogen causing bacterial center rot in onion. Isolated strains from onion soft rot bulbs based on morphological characteristics were tested for hypersensitivity reaction on tobacco. Pathogenic strains underwent some biochemical tests which detected the presence of *P. ananatis*, based on RNA16S sequencing, this result was confirmed. Symptoms were reproduced by inoculating this species to onion leaves and bulbs. This study reports for the first time the presence of *P. ananatis* bacterium in Morocco causing the bacterial center rot in onion**.**

Keywords: *Allium cepa***,** bacterial center rot, Morocco, Onion, *Pantoea ananatis*

Introduction

In Morocco, the onion crop (*Allium cepa* L.) has a high economical importance, representing 11% of nationally produced vegetable crops (Anonymous, 2011). Therefore, detection and control of diseases of this plant is important. Thus onion crop is exposed to many physiological and phytosanitary constraints (Conn *et al.,* 2012), among which is the bacterium center rot caused by *Pantoea ananatis*.

The enterobacterium *P. ananatis* is an ecologically versatile species. It has been found in the environment, as plant epiphyte and endophyte, emerging phytopathogen, and as a presumptive, opportunistic human pathogen (De Maayer *et al*., 2012).

Pantoea ananatis causes disease symptoms in a wide range of economically important agricultural crops and forest trees all over the world. It has been reported to induce diseases in onion (Gitaitis and Gay, 1997; Schwartz and Otto, 2000), Eucalyptus (Coutinho *et al.,* 2002), corn (Paccola- Meirelles *et al.,* 2001), melons (Bruton *et al.,* 1986; Wells *et al.,* 1987), Sudan grass (Azad *et al.,* 2000), rice (Azegami *et al.,* 1983), maize (Pérez-y-Terrón *et al*., 2009) and pineapple (Serrano, 1928).

The onion seed lot associated with the first outbreak of center rot in Georgua, USA (Gitaitis and Gay, 1997), was reportedly produced in South Africa and Walcott *et al.* (2002)) suggested that the center rot pathogen was possibly introduced on infested seed lots. *P. ananatis* is both seed-borne and seed-transmitted in Sudan grass (Azad *et al*., 2000), rice (Tabei *et al*., 1988) and onions (Walcott *et al.,* 2002).

The disease affects the center leaves of onions, which become water-soaked, soft, and bleached white as the rot progresses. Advanced stages of the disease result in complete wilting and bleaching of all leaves (Mark *et al*., 2002; Walcott *et al*., 2002). Walcott *et al.,* 2002, reported natural infestation and transmission of *P. ananatis* in onion seed and implied that seed was the primary source of inoculum. The main objective of this work was to isolate and identify the causal agent of the bacterial soft rot on onion bulbs in Morocco using biochemical, molecular and pathogenicity tests.

Handling Editor: Naser Safaie

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Received: 24 November 2014, Accepted: 03 November 2015
Published online: 24 November 2015

Materials and Methods

Isolation

Onion bulbs showing soft rot symptoms (Fig. 1) were sampled on 27 March 2013 from vegetables storage areas at Elhajjeb (N33°41.45, W5°22.00) (Meknes-Tafilalt, Morocco).

Onion bulbs showing center rot were used for isolation. These were washed with tap water and cut longitudinally. The diseased scale tissues were cut into 5 mm cubes using sterilized surgical blade. Three pieces of onion scale were ground in 1 ml of distilled water using a mortar and pestle. The suspension was streaked onto YPGA medium (yeast extract, 5 g; peptone, 5 g; glucose, 10 g; of agar, 18 g; and distilled water, 1000 ml). Six bacterial isolates were identified by biochemical and physiological tests including Gram stain; Levan production, Oxidase reaction, Arginine dihydrolase [ADH] and Tobacco hypersensitivity test, Catalase, Gram test (KOH), and API 20 gallery. All tests were repeated at least twice (Schaad *et al*., 2001).

Pathogenicity test

Cultures of bacterial isolates obtained from onion bulbs, labeled 2276-1, 2276-2, 2276-3, 2276-4, 2276-5 and 2276-6 were used in a greenhouse pathogenicity experiment. *In vitro,* onion bulbs were inoculated by a suspension (10^8 CFU / ml) of 24-h bacterial culture; the vegetable material was incubated in moist chamber at a temperature of 30 °C, six days after incubation symptoms were observed. All tests were repeated at last twice.

PCR amplification

To confirm the biochemical results, a molecular identification by sequencing was performed. The DNA extraction was made using GenElute Mammalian Genomic Kit (Anonymous, 2010). Quantification of DNA was performed using spectrophotometry. Amplification was carried with primers Fd1 (CAGAGTTTGATCCTGGCTCAG) and RP2 (AGAGTTTGATCCTGGCTCAG) (Klouche *et al.,* 2007; Parola *et al.,* 2002) at a PCR kit (Invitrogen). The PCR was accomplished in a total volume of 25 µl of the following reaction mixture: 2.5 µl 10X Buffer, 2 µl dNTP (10 mm), 0.125 µl of each primer (100 µM), 0.75 µl MgCl2 (50 mM), 0.2 µl Taq (5 units / µl) and 5 µl DNA. The PCR was performed using the following protocol: initial denaturation at 96 °C for 4 min, followed by 35 cycles of denaturation at 96 °C for 0.1 min, annealing at 52 °C for 0.4 min, and extension at 72 °C for 2 min, followed by an additional extension at 72 °C for 4 min.

Electrophoresis was performed in 1.5% agarose gel. Purification of PCR products was carried using the enzyme EXO-SAT according to the following schedule: 37 °C for 15 min followed by 80 °C for 15 min.

Figure 1 Onion bulbs showing soft rot.

16S DNA sequencing and sequence analysis

Selected PCR fragments, amplified from the isolates tested for pathogenicity, were sequenced in both strands, with the pA and pH' primers, using the BigDye terminator cycle sequencing ready reaction FS kit and an ABI PRISM 3700 DNA sequencer (both from Applied Biosystems Div., Foster City, Calif). So the sequencing of amplification product was carried out in a total volume of 10 µl of the following reaction mixture: 1 µl BigDye, 3 µl sequencing buffer x5, x µl primer (3.2-5 pmol), (0.75-1.5) µl DNA matrice, (2.5-3.25) µl H2O (MiliQ). The sequencing was performed using the

following protocol: initial denaturation at 96 °C for 1 min, followed by 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and an extension at 60 °C for 4 min, followed by an additional extension at 72 °C for 4 min.

Reading of sequencing results was done using NCBI-BLAST software (Altschul *et al*., 1997).

Results and Discussion

Bacterial colonies, which were consistently isolated from infected samples, were yellow and opaque on YPGA medium. All six representative strains isolated were Circular-shaped, Gram-negative, oxidase negative and catalase and arginine dihydrolase positive. The bacterial strains were able to induce hypersensitive reaction on tobacco leaves. All strains were capable of producing acid from sorbitol, sucrose, glucose, arabinose, melibiose but not able to utilize lysine (LDC), ornithine (ODC), thiosulfate (H_2S), Urea (URE), sodium pyruvate (VP), inositol (INO), gelatin (GEL), amygdaline (AMY). However, the strains showed ability to utilized, indole (IND), ONPG (beta-galactosidase enzyme), CIT (citrate), TDA (tryptophan deaminase), MAN (mannitol) and RHA (rhamnose) (Table 1).

Table 1 *Pantoea ananatis* strains (2276-1, 2276-2, 2276-3, 2276-4, 2276-5 and 2276-6) behavior on gallery API20 and classic biochemical tests.

Tests	2276-1	2276-2	2276-3	2276-4	2276-5	2276-6	*P. ananatis* reference strain
IND	(+)	(+)	(+)	(+)	(+)	(+)	(+)
VP	(-)	(-)	(-)	(-)	(-)	(-)	(-)
GEL	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Glucose	(+)	(+)	(+)	(+)	(+)	(+)	(+)
MAN	(+)	(+)	(+)	(+)	(+)	(+)	(+)
INO	(-)	(-)	(-)	(-)	(-)	(-)	(+)
Sorbitole	(+)	(+)	(+)	(+)	(+)	(+)	(+)
RHA	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Sucrose	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Melobiose	(+)	(+)	(+)	(+)	(+)	(+)	(+)
AMY	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Arabinose	(+)	(+)	(+)	(+)	(+)	(+)	(+)
H. Sensibilité au tabac	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Oxydase	(-)	(-)	(-)	(-)	(-)	(-)	(-)
ADH	(+)	(+)	(+)	(+)	(+)	(+)	(+)
H.L	(+)	(+)	(+)	(+)	(+)	(+)	(+)
YDC	(+)	(+)	(+)	(+)	(+)	(+)	(+)
NA	(+)	(+)	(+)	(+)	(+)	(+)	(+)
ONPG	(+)	(+)	(+)	(+)	(+)	(+)	(+)
LDC	(-)	(-)	(-)	(-)	(-)	(-)	(-)
ODC	(-)	(-)	(-)	(-)	(-)	(-)	(-)
CIT	(+)	(+)	(+)	(+)	(+)	(+)	(+)
H2S	(-)	(-)	(-)	(-)	(-)	(-)	(-)
URE	(-)	(-)	(-)	(-)	(-)	(-)	(-)
TDA	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Catalase	(+)	(+)	(+)	(+)	(+)	(+)	(+)

ONPG: determination of enzyme beta-galactosidase presence, ADH: transformation of arginine by dishydrolase argnine, LDC: transformation of lysine by decarboxylase lysine, ODC: transformation of ornithine by decarboxylase ornithine, CIT: utilization of citrate as alone source of Carbone, H2S: production of hydrogen sulfate from thiosulfate, URE: liberation of ammoniac from urea by urease, TDA: formation of indole pyruvique acid from tryptophan by desaminase tryptophan, IND: formation of indole from tryptophan, VP: formation of acetone from sodium piruvate, GEL: liquefaction of gelatin, GlU: glucose, MAN: mannitol, INO: inositol, SOR: sorbitol,, RHA: rhamnose, SAC: sucrose, MEL: melibiose, AMY: amygdaline, ARA: arabinose (formation of acid by utilization of carbon hydrate tests) (-): Negative; (+): Positive.

As a result, representative isolates of the causal agent of soft rot of onion bulbs in Morocco were identified as *P. ananitis* (Kido *et al*., 2008; Goszczynska *et al.,* 2006).

The 2276-1, 2276-2, 2276-3, 2276-4, 2276-5 and 2276-6 strains gave a positive reaction on pathogenicity test *in vitro*. This test showed that there was development of centre soft rot after six days of incubation on onion bulbs by *P. ananatis* (Carr *et al*., 2008; Conn *et al.,* 2012). Result of DNA quantification showed that the quantity of DNA was sufficient for PCR (25 ng / µl). A band of 1550 bp size was observed in agarose gel (Fig. 2). BLAST-NCBI analyses of sequences proved that 2276-1, 2276-2, 2276-3, 2276-4, 2276-5 and 2276-6 were strains of *P. ananatis* (Table 2).

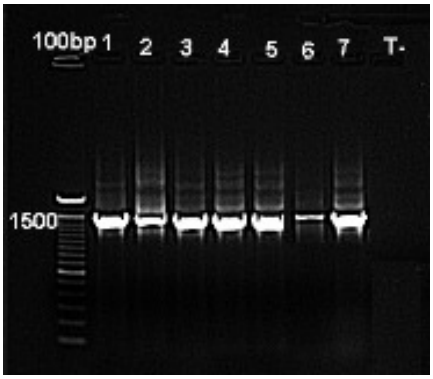

Figure 2 Electrophoritic profile of *P. ananatis* strains: (1) 2276-1. (2) 2276-2. (3) 2276-3. (4) 2276-4. (5) 2276-5. (6) 2276-6. (7) *P. ananatis* reference strain. (T-) Negative control (H2O).

Table 2 The sequence identity/similarity.

The sequence of isolates strains (2276-1, 2276-2, 2276-3, 2276-4, 2276-5 and 2276-6)	Sequence identity / Similarity	GenBank Accession Numbers
GCGTGTATGAGAAGGCCTTCGGG TTGTAAAGTACTTTCAGCGGGGA GGAAGGCGATGTGGTTAATAACC GCATTGATTGACGTTACCCGCAG AAGAAGCACCGGCTAACTCCGTG CCAGCAGCCGCGGTAATACGGAG GGTGCAAGCGTTAATCGGAATTA CTGGGCGTAAAGCGCACGCAGGC GGTCTGTTAAGTCAGATGTGAAA TCCCCGGGCTTAACCTGGGAACT GCATTTGAAACTGGCAGGCTTGA GTCTCGTAGAGGGGGGTAGAATT CCAGGTGTAGCGGTGAAATGCGT AGAGATCTGGAGGAATACCGGTG GCGAAGGCGGCCCCCTGGACGAA GACTGACGCTCAGGTGCGAAAGC GTGGGGAGCAAACAGGATTAGAT ACCCTGGTAGTCCACGCCGTAAA CGATGTCGACTTGGAGGTTGTTCC CTTGAGGAGTGGCTTCCGGAGCT AACGCGTTAAGTCGACCGCCTGG GGAGTACGGCCGCAAGGTTAAAA CTCAAATGAATTGACGGGGGCCC GCACAAGCGGTGGAGCATGTGGT TTAATTCGATGCAACGCGAAGAA CCTTACCTACTCTTGACATCCACA GAACTTGGCAGAGATGCTTTGGT GCCTTCGGGAGCCCTGAGACAGG TGCTGCATGGGCTGTCGTCAGCTC GTGTTGTGAAATGTTGGGTTAAG TCCCGCA	1 tctcgggtga cgagtggcgg acgggtgagt aatgtctggg gatctgcccg atagaggggg 61 ataaccactg gaaacggtgg ctaataccgc ataacgtcgc aagaccaaag agggggacct 121 tcgggcctct cactatcgga tgaacccaga tgggattagc tagtaggcgg ggtaacggcc 181 cacctaggcg acgatcccta gctggtctga gaggatgacc agccacactg gaactgagac 241 acggtccaga ctcctacggg aggcagcagt ggggaatatt gcacaatggg cgcaagcctg 301 atgcagccat gccgcgtgta tgaagaaggc cttcgggttg taaagtactt tcagcgggga 361 ggaaggcgat gtggttaata accgcattga ttgacgttac ccgcagaaga agcaccggct 421 aactccgtgc cagcagccgc ggtaatacgg agggtgcaag cgttaatcgg aattactggg 481 cgtaaagcgc acgcaggcgg tctgttaagt cagatgtgaa atccccgggc ttaacctggg 541 aactgcattt gaaactggca ggcttgagtc tcgtagaggg gggtagaatt ccaggtgtag 601 cggtgaaatg cgtagagatc tggaggaata ccggtggcga aggcggcccc ctggacgaag 661 actgacgctc aggtgcgaaa gcgtggggag caaacaggat tagataccct ggtagtccac 721 gccgtaaacg atgtcgactt ggaggttgtt cccttgagga gtggcttccg gagctaacgc 781 gttaagtcga ccgcctgggg agtacggccg caaggttaaa actcaaatga attgacgggg 841 gcccgcacaa gcggtggagc atgtggttta attcgatgca acgcgaagaa ccttacctac 901 tcttgacatc cacagaactt agcagagatg ctttggtgcc ttcgggaact ctgagacagg 961 tgctgcatgg ctgtcgtcag ctcgtgttgt gaaatgttgg gttaagtccc gcaacgagcg 1021 caacccttat cctttgttgc cagcgattcg gtcgggaact caaaggagac tgccggtgat 1081 aaaccggagg aaggtgggga tgacgtcaag tcatcatggc ccttacgagt agggctacac 1141 acgtgctaca atggcgcata caaagagaag cgacctcgcg agagcaagcg gacctcataa 1201 agtgcgtcgt agtccggatc ggagtctgca actcgactcc gtgaagtcgg aatcgctagt 1261 aatcgtggat cagaatgcca cggtgaatac gttcccgggc cttgtacaca ccgcccgtca 1321 caccatggga gtgggttgca aaagaagtag gtagcttaac cttcgggagg gcgcttacca 1381 ctttgtgatt catgactggg gtgaagtcgt aacaaggtag cc	AY7411 61.1

The sequences of the isolates strain amplified by RNA 16S, present a 99% similarity with accession number of AY741161.1.

To our knowledge, this is the first report of bacterial bulbs soft rot of onion in Morocco caused by *P. ananatis*. Because the soft rot of onion is the most destructive disease during storage, this study is considered as a basis for works that will target the *P. ananatis* ecologic and behaviors, to solve the soft rot problem by applying a biological control by using antagonistic bacteria. A development of a molecular method is very important for a rapid detection of *P. ananatis* in soft rot bulbs.

Acknowledgments

This study was carried out in Laboratory of Plant Protection URPP-INRA-Meknes Morocco. Authors acknowledge the support of INRA regional center of Meknes.

References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, 25: 3389-3402.

Anonymous. 2010. GenElute™ Mammalian Genomic DNA Miniprep Kit, User Guide, 16 p.

Anonymous. 2011. Ministère de l'agriculture et de la pêche maritime. Available on: http://www.marocagriculture.com

Azad, H. R., Holmes, G. J., and Cooksey, D. A. 2000. A new leaf blotch disease of Sudan grass caused by *Pantoea ananatis* and *Pantoea stewartii*. Plant Disease, 84: 973-979.

Azegamì, K., Ozaki, K. and Matsuda, A. 1983. Bacterial palea browning. A new disease of nice caused by *Erwinia herbicola*. Bulletin of the National Institute of Agricultural Science Series: C, 39: I-12.

Bruton, B. D., Wells, J. M. and Ester, G. E. 1986. Pathogenicity of *Erwinia ananas* to muskmelons in Texas. Phytopathology, 76: 1136.

Carr, E. A., Lorbeer J. W. and Beer S. V. 2008. Detection of *Pantoea ananatis* in New York-Grown Onions and Development of Pathogenicity Tests for *Pantoea ananatis*. Department of Plant Pathology and Plant-Microbe Biology, Cornell University. 42 pp.

Conn, E. K., Lutton, J. S. and Rosenberger, S. A. 2012. Onion Disease Guide. Plant Health, 72 pp.

Coutinho, T. A., Preisig, O., Mergaert, J., Cnockaert, M. C., Riedel, K. H., Swings, J. and Wingfield, M. J. 2002. Bacterial blight and dieback of Eucalyptus species, hybrids and clones in South Africa. Plant Disease, 86: 20-25.

De Maayer, P., Rezzonico, F., Bühlmann, A., Venter S. N., Blom, J., Goesmann, A., Frey J. E., Smits T. H. M., Duffy, B. and Coutinho T. A. 2012. Complete Genome Sequence of Clinical Isolate *Pantoea ananatis* LMG 5342. Journal of Bacteriology, 194 (6): 1615-1616.

Gitaitis, R. D. and Gay, J. D. 1997. First report of a leaf blight seed stalk rot, and bulb decay of onion by *Pantoea ananas* in Georgia, Plant Disease, 81: 1096.

Goszczynska, T., Moloto, V. M., Venter, S. N. and Coutinho, T. A. 2006. Isolation and identification of *Pantoea ananatis* from onion seed in South Africa. Seed Science and Technology, 34: 677-690.

Kido, K., Adachi, R., Hasegawa, M., Yano, K., Hikichi, Y., Takeuchi, S., Atsuschi, T. and Takikawa, Y. 2008. Internal fruit rot of netted melon caused by *Pantoea ananatis* (= *Erwinia ananas*) in Japan. Journal of General Plant Pathology, 74: 302-312.

Klouche, N., Fardeau, M. L., Lascourreges, J. F., Cayol, J. L., Hacene, H., Thomas, P. and Magot, M. 2007. *Geosporobacter subterraneus gen. nov*., sp. nov., a spore-forming bacterium isolated from a deep subsurface aquifer. International Journal of Systematic and Evolutionary Microbiology, 57: 1757-1761.

Mark, G. L., Gitaitis, R. D. and Lorbeer, J. W. 2002. Bacterial diseases of onion. In: Rabinowitch, H. D. and Currah, L. (Eds.), Allium Crop Science: Recent Advances. CAB International, Wallington, UK. pp. 267-292.

Paccola-Meirelles, L. D., Ferreira, A. S., Meirelles, W. F., Marriel, I. E. and Casela, C.

R. 2001. Detection of a bacterium associated with a leaf spot disease of maize in Brazil. Journal of Phytopathology, 149: 275-279.

Parola, P., Roux, V., Camicas, J. L., Baradji, I., Brouqui, P. and Raoult, D. 2000. Detection of ehrlichiae in African ticks by PCR. Transactions of the Royal Society of Tropical Medicine and Hygiene, 94: 707-708.

Pérez-y-Terrón, R., Villegas, M. C., Cuellar, B. A., Muñoz-Rojas, J., Castañeda-Lucio, M., Hernández-Lucas, I., Bustilos, C. R., Bautista-Sosa, L., Munive J. A., Caicedo-Rivas, R. and Fuentes-Ramírez, L. E. 2009. Detection of *Pantoea ananatis*, causal agent of leaf spot disease of maize, in Mexico. Australasian Plant Disease Notes, 4: 96-99.

Schaad, N. W., Jones, J. B. and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd Ed. APS Press, St. Paul, 373 pp.

Schwartz, H. F. and Otto, K. 2000. First report of a leaf blight and bulb decay of onion by *Pantoea ananatis* in Colorado. Plant Disease, 84: 808-814.

Serrano, F. B. 1928. Bacterial fruitlet brown rot of pineapples in the Philippines. The Philippine Journal of Science, 36: 271-305.

Tabei, H., Azegami, K. and Fukuda, T. 1988. Infection site of rice grain with *Erwinia herbicola*, the causal agent of bacterial palea browning of rice. Annals of the Phytopathological Society of Japan, 54: 637-639.

Walcott, R. R., Gitaitis, R. D., Castro, A. C., Sanders, F. H. and Diaz-Perez, J. C. 2002. Natural infestation of onion seed by *Pantoea ananatis*, causal agent of center rot. Plant Disease, 86: 106-111.

Wells, J. M., Sheug, W. S., Ceponis, M. J. and Chen, T. A. 1987. Isolation and characterization of strains of *Erwinia ananas* from honeydew melons. Phytopathology, 77: 511-514.

اولین گزارش از ردیابی *Pantoea ananatis*، عامل پوسیدگی باکتریایی مغز پیاز در مراکش

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دریافت: ۳ آذر ۱۳۹۳؛ پذیرش: ۱۲ آبان ۱۳۹۴

چکیده: گونه *Pantoea ananatis* یک بیمارگر مهم عامل پوسیدگی مغز پیاز می‌باشد. استرین‌های جدا شده از غده‌های پیاز با علایم پوسیدگی نرم براساس ویژگی‌های مورفولوژیک برای واکنش فوق حساسیت روی توتون آزمایش شدند. براساس آزمون‌های بیوشیمیایی استرین‌های بیماریزا *P. ananatis* تشخیص داده شد و با تعیین توالی ناحیه RNA 16S ریبوزومی نتایج تأیید شد. با مایه‌زنی این گونه روی برگ‌ها و غده پیاز علایم مشابه مشاهده شد. این بیماری اولین گزارش از وقوع باکتری *P. ananatis* به‌عنوان عامل پوسیدگی مغز پیاز در مراکش می‌باشد.

واژگان کلیدی: *Allium cepa* ، پوسیدگی باکتریایی مغز، مراکش، پیاز، *Pantoea ananatis*