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First Report on *Pseudomonas marginalis* Bacterium Causing Soft Rot of Onion in Morocco

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Abstract

***Pseudomonas marginalis* is an important postharvest pathogen causing soft rot in a wide variety of harvested fruits and vegetables. Isolated strains from rotten onion bulbs based on morphological characteristics, were tested for pathogenecity on tobacco. Pathogenic strains underwent a biochemical test which detected the presence of *P. marginalis*. Symptoms were reproduced by inoculating this species to onion leaves and bulbs. Based on ARN16S sequencing, reported associated species (*Pantoea agglomerans*, *Pseudomonas fluorescens*, *Klebsiella oxytoca*) were also confirmed. This study reports for the first time the presence of *P. marginalis* bacterium in Morocco causing the soft rot of onion bulb, in association with *Pantoea agglomerans*, *Pseudomonas fluorescens*, and *Klebsiella oxytoca*.**

Keywords: *Pseudomonas marginalis*, soft rot, onion, Morocco.

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Introduction

In Morocco, the onion crop (*Allium cepa* L.) has a high economical importance, representing 11% of nationally produced vegetable crops (Anonyme, 2011). However this crop is exposed to many physiological and phytosanitary constraints (Conn et al., 2012), among which, the onion bulb soft rot represents the most serious post harvest disease.

For onion storage two methods used in France, storage in a traditional dryer and temporary storage under plastic tunnels, also storage methods affect the soft rot incidence (Gourc et al., 2007).

The *Pseudomonas* and *Pectobacterium* bacterial genus are considered as the main bacteria that caused the damages in USA during storage (Agrios, 2005).

By means of pectinolytic enzyme products (pectin lyase and pectate lyase) (Hayashi et al., 1997, Liao et al., 1997), *P. marginalis* causes the post-harvest soft rot (Conn et al., 2012; Scortichini, 1994) of many harvested crops including: onion (Kim et al., 2002 ; Wright et al 1992 ; Dallaire, 2009), tomato (Ibe and Grogan, 1983), salad (Blancard et al., 2003), potato (Elumalai and Mahadevan, 1995), broccoli (Charron et al., 2002) and carrot (Godfrey and Marshall., 2002). *P. marginalis* is a foliar as well as a post-harvest disease (Conn et al., 2012; Scortichini, 1994). It's a bacterium present in Europe, India, South American, USA, Japan, New Zealand and Austria, etc.

Development of the soft rot disease caused by *P. marginalis* is optimal at low temperatures (between 5 and 25°C), the bacteria develop at 0°C and it can induce the soft rot at 5°C on onion crop (Kim et al., 2002). In Morocco, no evidence of such bacteria causing soft rot on onion or another crop.

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.

Materials and Methods

Isolation

Onion bulbs showing soft rot symptoms (Fig. 1) were sampled in March 2012 from vegetables storage areas at Elhajjeb (N 33°41.45, W 5°22 00) (Meknes-Tafilalt, Morocco).

Onion bulbs showing water-soaking or yellowish-brown rot were used for isolation. These were washed with tap water and cut longitudinally. The diseased scale tissues were cut into 5 mm cubes by 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 LPGA (yeast extract, 5g; peptone, 5g; glucose, 10g; agar, 18g; and distilled water, 1 l) and King B (proteose peptone, 20 g; $K_2HPO_4 \cdot 3H_2O$, 2.5 g ; $MgSO_4 \cdot 7H_2O$, 6 g ; glycerol, 15 ml ; agar, 15 g; and distilled water, 1 l) mediums. Six bacterial isolates were identified by biochemical and physiological tests including Gram strain; LOPAT (Levan production, Oxidase reaction, Potato soft rot, Arginine dishydrolase [ADH] and Tobacco hypersensitivity) test, Hydrolysis of Tween80, mobility test, Indole, Mannitol, Catalase, Hugh & Leifson (H.L), the bacteria development in low and high temperature (4 and 40°C), the salt tolerance (7 and 10% of NaCl) and API 20 gallery. All tests were repeated at last twice (Schaad et al., 2001).

Pathogenicity Test

Cultures of bacterial isolates obtained from onion bulbs, labeled 2078-6-1, 2078-6-2, 2078-6-3, 2078-6-4, 2078-6-5, were used in a greenhouse pathogenicity experiment. *In vivo*, seven weeks old tomato plants grown in greenhouse and sixteen weeks old onion plants grow in nursery were inoculated. A suspension of tested bacterial isolate (1×10^8 CFU/ml) in sterile distilled water from 24-h cultures was used as the inoculums; plants in one experimental variant were inoculated by injection of approximately 0.2 ml of inoculums into leaves of onion and tomato plants. The plants were incubated in a greenhouse at 20°C to 25°C and 60–70% relative humidity. Sterile water was used as control (Kudela et al., 2010). *In vitro*, onion bulbs and leaves, and tomato leaves 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, after five days of incubation symptoms were observed.

Figure 1. Onion soft rot.

PCR Amplification

To complete the biochemical results, a molecular identification by sequencing was performed. The extraction was made using GenElute Mammalian Genomic Kit (Anonyme, 2010). Quantification of DNA was performed using spectrophotometry. Amplification was performed with primers Fd1 (CAGAGTTTGATCCTGGCTCAG) and RP2 (AGAGTTTGATCCTGGCTCAG) at a PCR kit (Invitrogen). The PCR was carried out in a total volume of 25 µl of the following reaction mixture: 2.5µl 10X Buffer, 2µl dNTP (10mm), 0.125µl of each primer (100 µM), 0.75 µl $MgCl_2$ (50 mM), 0.2µl Taq (5U/µl) and 5µl 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 0.1min, annealing at 52°C for 0.4min, and a extension at 72°C for 2min, 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 15min following by 80°C for 15min.

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. So the sequencing of amplification product was carried out in a total volume of 10µl of the following reaction mixture: 1µl BigDye, 3µl sequencing buffer x5, xµl primer (3.2-5pmol), (0.75-1.5) µl DNA matrice, (2.5-3.25) µl H_2O (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 10s, annealing at 50°C for 5s, and a extension at 60°C for 4min, 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 fluorescent on King's medium B. All six (2078-6-1, 2078-6-2, 2078-6-3, 2078-6-4, 2078-6-5 and 2078-6-6) strains caused hypersensitive reaction (HR) on tobacco leaves, indicating that they were pathogenic. They were Gram-negative. According to the LOPAT tests, the isolates were negative for Levan production and positive for Oxidase test, Pectinase test (Fig. 2) and Arginine dihydrolase. Based on the LOPAT and Gram tests (Schaad et al., 2001) and Kim et al.

(2002) results, representative isolates of the causal agent of soft rot of onion bulbs in Morocco was identified as *P. marginalis*, they also showed that strains can grow in a concentration of 7 and 10% of salt at 24°C with pH7. These results were different from those of Membre and Burlot (1994) who showed that, a lesser concentration of 2.5% of NaCl reduced *P. marginalis* growth and inhibited pectinolytic enzyme production. The conflicting results may be due to strains natures and isolation origins. All of our strains were Catalase positive and capable of hydrolyzing Esculin, but not Tween80 and Gelatin. None of the strains were able to produce Indole and reduce nitrate. However, all strains showed ability to utilize Arginine, Lysine (LDC), Ortnithine

Table 1. *P. marginalis* strains (2078-6-1, 2078-6-2, 2078-6-3, 2078-6-4, 2078-6-5,) behavior on gallery API20, classic biochemical tests and growth at different temperatures. (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 indolepyruvique 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; ND: No determinate).

Strains	2078-6-1	2078-6-2	2078-6-3	2078-6-4	2078-6-5	2078-6-6
Levan	(+)	(+)	(+)	(+)	(+)	(+)
Oxidase	(+)	(+)	(+)	(+)	(+)	(+)
Pectinase	(+)	(+)	(+)	(+)	(+)	(+)
ADH	(+)	(+)	(+)	(+)	(+)	(+)
H. Tabac	(+)	(+)	(+)	(+)	(+)	(+)
Growth at 41°C	(+)	(+)	(+)	(+)	(+)	(+)
Growth at4°C	(+)	(+)	(+)	(+)	(+)	(+)
H.L	(-)	(-)	(-)	(-)	(-)	(-)
Catalase	(+)	(+)	(+)	(+)	(+)	(+)
Hydrolysis of Tween 80	(-)	(-)	(-)	(-)	(-)	(-)
Mobility	(+)	(+)	(+)	(+)	(+)	(+)
Esculine	(+)	(+)	(+)	(+)	(+)	(+)
ONPG	(+)	(+)	(+)	(+)	(+)	(+)
LDC	(+)	(+)	(+)	(+)	(+)	(+)
ODC	(+)	(+)	(+)	(+)	(+)	(+)
CIT	(-)	(-)	(-)	(-)	(-)	(-)
H2S	(+)	(+)	(+)	(+)	(+)	(+)
URE	(+)	(+)	(+)	(+)	(+)	(+)
TDA	ND	ND	ND	ND	ND	ND
IND	(-)	(-)	(-)	(-)	(-)	(-)
VP	(-)	(-)	(-)	(-)	(-)	(-)
GEL	(-)	(-)	(-)	(-)	(-)	(-)
GLU	(+)	(+)	(+)	(+)	(+)	(+)
MAN	(+)	(+)	(+)	(+)	(+)	(+)
INO	(-)	(-)	(-)	(-)	(-)	(-)
SOR	(+)	(+)	(+)	(+)	(+)	(+)
RHA	(+)	(+)	(+)	(+)	(+)	(+)
SAC	(+)	(+)	(+)	(+)	(+)	(+)
MEL	(+)	(+)	(+)	(+)	(+)	(+)
AMY	(+)	(+)	(+)	(+)	(+)	(+)
ARA	(+)	(+)	(+)	(+)	(+)	(+)
NO2	(-)	(-)	(-)	(-)	(-)	(-)
N2	(-)	(-)	(-)	(-)	(-)	(-)

(-): Negative; (+): Positive; ND: Indeterminate

Table 2. *Table 2. The sequence identity/similarity. (The sequences of the isolates strain amplified by RNA 16S, present a 99% similarity with the LMG 2214 Pseudomonas marginalis several strain (1204b/1208b with a score equal to 2165 bits (2400)).*

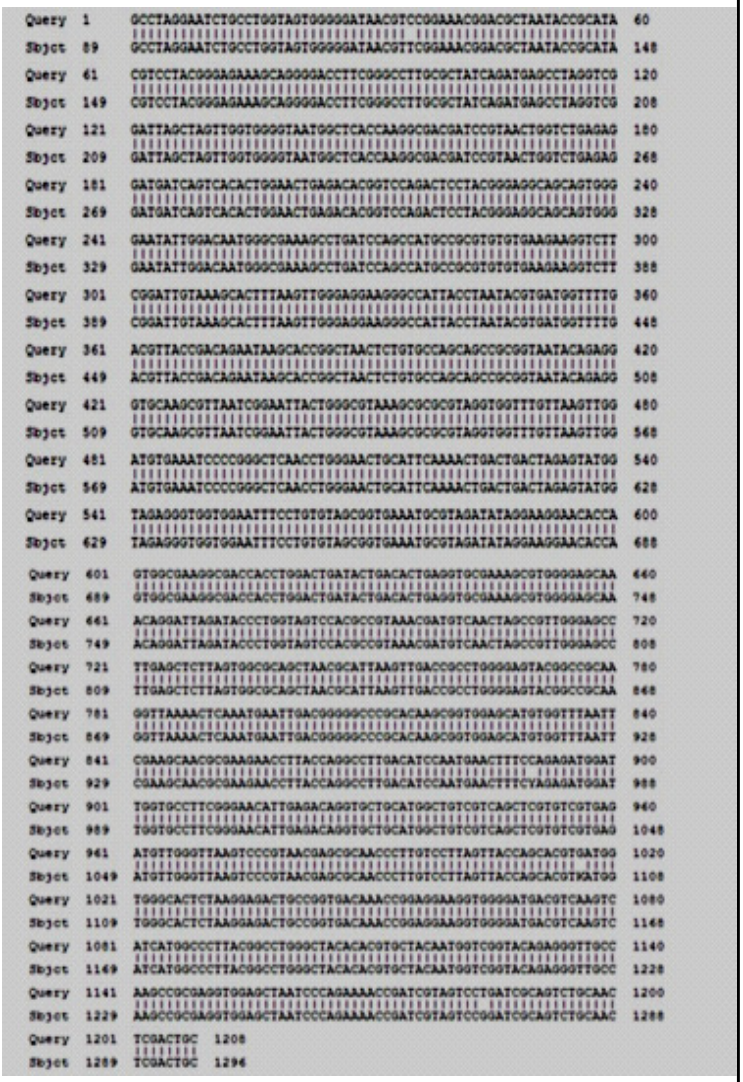
The Sequence of Isolates strains (2078-6-1, 2078-6-2, 2078-6-3, 2078-6-4, 2078-6-5, 2078-6-6)	Sequence Identity/Similarity	GenBank Accession Numbers
GCCTAGGAATCTGCCTGGTAGTGGGGGA TAACGTCCGGAAACGGACGCTAATACCGC ATACGTCCTACGGGAGAAAGCAGGGGAC CTTCGGGCCTTGCGCTATCAGATGAGCCTA GGTCGGATTAGCTAGTTGGTGGGGTAATG GCTCACCAAGGCGACGATCCGTAACTGGT CTGAGAGGATGATCAGTCACACTGGAACT GAGACACGGTCCAGACTCCTACGGGAGG CAGCAGTGGGGAATATTGGACAATGGGC GAAAGCCTGATCCAGCCATGCCGCGTGTG TGAAGAAGGTCTTCGGATTGTAAAGCACTT TAAGTTGGGAGGAAGGGCCATTACCTAAT ACGTGATGGTTTTGACGTTACCGACAGAAT AAGCACCGGCTAACTCTGTGCCAGCAGCC GCGGTAATACAGAGGGTGCAAGCGTTAAT CGGAATTACTGGGCGTAAAGCGCGCGTA GGTGGTTTGTTAAGTTGGATGTGAAATCCC CGGGCTCAACCTGGGAACTGCATTCAAAA CTGACTGACTAGAGTATGGTAGAGGGTG GTGGAATTTCCTGTGTAGCGGTGAAATGC GTAGATATAGGAAGGAACACCAGTGGCG AAGGCGACCACCTGGACTGATACTGACAC TGAGGTGCGAAAGCGTGGGGAGCAAAC AGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGTCAACTAGCCGTTGGGAGCC TTGAGCTCTTAGTGGCGCAGCTAACGCATT AAGTTGACCGCCTGGGGAGTACGGCCGC AAGGTTAAAACTCAAATGAATTGACGGGG GCCCGCACAAGCGGTGGAGCATGTGGTT TAATTCGAAGCAACGCGAAGAACCTTACCA GGCCTTGACATCCAATGAACTTTCCAGAGA TGGATTGGTGCCTTCGGGAACATTGAGAC AGGTGCTGCATGGCTGTCGTCAGCTCGTG TCGTGAGATGTTGGGTTAAGTCCCGTAAC GAGCGCAACCCTTGTCCTTAGTTACCAGCA CGTGATGGTGGGCACTCTAAGGAGACTG CCGGTGACAAACCGGAGGAAGGTGGGG ATGACGTCAAGTCATCATGGCCCTTACGGC CTGGGCTACACACGTGCTACAATGGTCGG TACAGAGGGTTGCCAAGCCGCGAGGTG GAGCTAATCCCAGAAAACCGATCGTAGTC CTGATCGCAGTCTGCAACTCGACTGC	 Query 1 GCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGACGCTAATACCGCATA 60 Sbjct 89 GCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATA 148 Query 61 CGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCG 120 Sbjct 149 CGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCG 208 Query 121 GATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG 180 Sbjct 209 GATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG 268 Query 181 GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG 240 Sbjct 269 GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG 328 Query 241 GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT 300 Sbjct 329 GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT 388 Query 301 CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCCATTACCTAATACGTGATGGTTTTG 360 Sbjct 389 CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCCATTACCTAATACGTGATGGTTTTG 448 Query 361 ACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGG 420 Sbjct 449 ACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGG 508 Query 421 GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGG 480 Sbjct 509 GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGG 568 Query 481 ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGG 540 Sbjct 569 ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGG 628 Query 541 TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA 600 Sbjct 629 TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA 688 Query 601 GTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA 660 Sbjct 689 GTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA 748 Query 661 ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCC 720 Sbjct 749 ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCC 808 Query 721 TTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAA 780 Sbjct 809 TTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAA 868 Query 781 GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT 840 Sbjct 869 GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT 928 Query 841 CGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGAT 900 Sbjct 929 CGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCYAGAGATGGAT 988 Query 901 TGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG 960 Sbjct 989 TGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG 1048 Query 961 ATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTGATGG 1020 Sbjct 1049 ATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTKATGG 1108 Query 1021 TGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1080 Sbjct 1109 TGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1168 Query 1081 ATCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCC 1140 Sbjct 1169 ATCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCC 1228 Query 1141 AAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCTGATCGCAGTCTGCAAC 1200 Sbjct 1229 AAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC 1288 Query 1201 TCGACTGC 1208 Sbjct 1289 TCGACTGC 1296	HE586394.1

Figure 2. Pectinase test. Symptoms induced by artificial inoculation of isolates of *P. marginalis*.

(ODC), Thiosulfate (H2S), Urea (URE), Glucose (GLU), Mannitol (MAN), Sorbitol (SOR), Rhamnose (RHA), Sucrose (SAC), Melibiose (MEL), Amygdaline (AMY), Arabinose (ARA), but they did not use Citrate (CIT), Tryptophan (IND), Sodium Piruvate (VP), Gelatin (GEL), Inositol (INO) (Table 1).

The strains can grow at 4°C; low temperatures during this season (2011-2012) may have favored soft rot *P. marginalis* bacteria development. These results correlate with those of Kim et al. (2002), who showed that the optimal temperature development is 0°C. Also we have shown the development strains at 40°C.

Pathogenicity Test

The 2078-6-1, 2078-6-2, 2078-6-3, 2078-6-4, 2078-6-5 and 2078-6-6 strains gave a positive reaction *in vitro* (Fig. 3) and in planta. *In vitro* tests showed that there was development of soft rot after five days of incubation on onion bulbs by *P. marginalis* as indicated by chlorosis of infected area and necrosis development at onion and tomato leaves. In planta tests necrosis development was observed in onion and tomato leaves, after five days of incubation and after 14 days onion leaves died. Dallaire (2009) and (Ibe and Grogan, 1983) show that *P. marginalis* is a cause of onion and tomato decay.

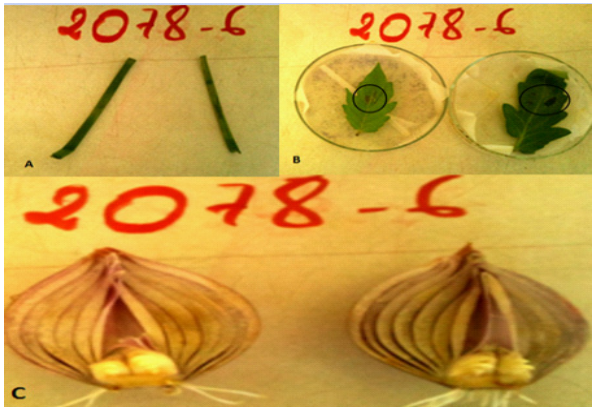


Figure 3. *In vitro* symptoms induced by artificial incubation. (a) Yellowing of onion leaves. (b) Necrosis in tomato leaves. (c) Soft rot in onion bulbs, after five days of inoculation.

Molecular Results

Result of DNA quantification showed that the quantity of DNA was sufficient for PCR (20.18 ng/µl). A band with 1550 bp size was observed in agarose gel (Fig. 4). Analysis of the 16SrDNA sequence, by BLAST-NCBI, revealed that, the strains isolates (2078-6-1, 078-6-2, 2078-6-3, 2078-6-4, 2078-6-5and 2078-6-6) were identical (100% identity over 1208 nucleotides) and that they were most closely related to 16S rDNA sequences from several strains of *P. marginalis* (LMG 2214) (Table 2). We also found the presence of associated bacteria namely: *Pantoea agglomerans*, *Pseudomonas fluorescens*, and *Klebsiella oxytoca*. El-hendawy (2004) showed that *Serratia marcescens* and *Klebsiella oxytoca* are associated bacteria with *P. marginalis* in onion crop.

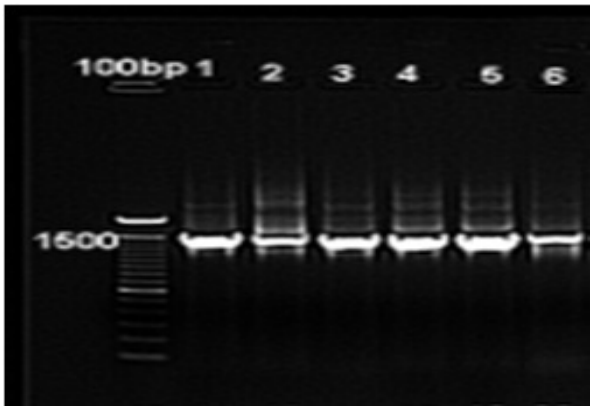


Figure 4. Electrophoritic profile of *P. marginalis* stains: (1) 2078-6-1. (2) 2078-6-2. (3) 2078-6-3. (4) 2078-6-4. (5) 2078-6-5. (6) 2078-6-6. (T+) P. marginalis reference strain. (T-) Negative control (H_2O).

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

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