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Characterization of *Pantoea agglomerans* Strains Isolated from Olive Knot disease in Morocco

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ABSTRACT

On the base of prospecting conducted during the agriculture season 2012-2013, the results revealed that the olive knot disease caused by *Pseudomonas savastanoi* pv.*savastanoi* was observed in different olive groves in Morocco. The disease incidence varies with location and cultivar. However, high disease incidence was reported in Taounate, Ouazzane and Meknes. The biochemical and the molecular tests were revealed the presence of saprophytic bacteria; *Pantoea agglomerans*, *Enterobacter* sp., *Pseudomonas* sp., associated with *Pseudomonas savastanoi* pv.*savastanoi.* Moreover, a hypersensitive reaction on tobacco leaves caused by *Pantoea agglomerans* strains (2074-1 and 2074-4) was noted. Nonetheless, the strain of *Pantoea agglomerans* (2066-7) did not affect the tobacco leaves.

Keywords: Olive knots / *Pseudomonas savastanoi* pv. *savastanoi* / *Pantoea agglomerans* / molecular tests.

INTRODUCTION

P*seudomonas savastanoi* is a plant pathogen of the *Oleaceae* family (Gardan *et al.* 1992).Thus, *Pseudomonas savastanoi* pv. *savastanoi* (hereafter termed *PSV*) reported specific to olive trees. However, the disease is widespread in olive producing Mediterranean countries and has recently been detected in Australia (Hall *et al.* 2004). Besides, its symptoms are tumourous overgrowths occurring on young twigs, branches and stems but occasionally on the leaves and fruits as well (Surico 1986). The knots develop in reaction to factors produced by the bacterium; including indole-3-acetic acid (IAA) and cytokinins (Comai and Kosuge1980; Comai *et al*. 1982; Iacobellis et *al.* 1994; Surico and Iacobellis 1992).The length of the pathogen's incubation period and the size of the knot are regulated by the amounts of IAA and cytokinin, respectively. For many years, the phytohormones produced by *PSV* were considered to be its main virulence factors (Surico and Iacobellis 1992). More recently, however, products of the type III secretion system (TTSS), encoded by the *hrp/hrc* gene clusters and a variety of genes encoding *P. syringae* virulence determinants, have been discovered to play key roles in *PSV* pathogenicity (Rodríguez- Moreno *et al*. 2008; Sisto *et al*. 2004). In addition, other auxin-producing bacteria, such as *Pantoea agglomerans*, seem to interact with *PSV* and contribute to its

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pathogenesis (Cimmino *et al*. 2006; Marchi *et al*. 2006; Ouzari *et al*. 2008). *16S rDNA* sequence analysis revealed the presence of many saprophytic bacteria associated with *PSV*, such as *Pantoea agglomerans*, *Enterobacter sp.*, *Pseudomonassp.*, *P. fluorescens*, *Stenotrophomonas maltophilia* and *Burkolderia cepacia*. However, in the leaves, *PSV* was often associated with *Bacillus subtilis* and *Acinetobacter* species (*Krid et al*. 2010).

Pantoea agglomerans which is often associated with olive and oleander knots (Azad and Cooksey 1995; Fernandes and Marcelo 2002). It is also used as a biocontrol agent (Nunes *et al.* 2002; Trotel- Aziz *et al*. 2007), but apparently has a positive role in knot formation by producing IAA which contributes to increasing the size of knots (Marchi *et al*. 2006).

In this study we report (i) disease cartography of olive knot in different regions prospects in Morocco (ii) the isolation and characterization of some bacteria associated with *PSV* in knots of olive trees

MATERIALS AND METHODS

Olive knot disease cartography

Field prospections were conducted in 2012-2013 in several Morocco regions to make cartography of the different locations attacked. Prospections results are detailed in table 1.

Sampling procedures and isolation from knots

Sampling was released in the spring and autumn during 2012-2013. The knots were placed in sterile plastic bags, transported to the laboratory and processed immediately. The knots were surface-disinfected with a paper moistened with ethanol (Marchi *et al*. 2005). Small fragments (1–2 mm) were cut aseptically with a sterile scalpel then placed in 200 µl of sterile distilled water. After 30 min, a loopful of the resulting suspension was streaked with a sterile loop onto the surface of *Kings B* (KB) medium containing gL^{-1}, 10 g protease peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 15 g agar and 15 ml glycerol, and the pH was adjusted to 7.2-7.4 (King *et al*.1954). Plates were incubated at 25 ± 2ºC for 72 hours and subjected to long wave (367 nm) dark blue ultra violet light for the detection of fluorescent colonies. The single fluorescent colonies were restreaked onto new KB plates and incubated at the same conditions. Fluorescent colonies were grown on KB test tubes and kept in refrigerator for further identification (Schaad, *et al.* 2001).

Pathogenicity test

A pathogenicity test was carried out on a limited number of samples, representing one isolate per locality. Wounds measuring around 1 cm were made in the bark of 1-year-old olive stems (Picholine marocaine) with a scalpel dipped in a bacterial suspension (10^8 $CFUml^{-1}$) of pure culture grown for 48 h on *King's B* medium. Each isolate was inoculated at five wound sites. Wounds were protected with parafilm for 3 days. The inoculated trees were kept in a greenhouse at 24 ± 2°C and inspected for knot formation after 2 months. Negative control trees were inoculated with sterile distilled water.

Biochemical studies of endophytic bacteria associated with *PSV*

The following biochemical tests were performed: levan, oxidase, pectinolytic activity, and presence of arginine dehydrolase and tobacco hypersensitivity (LOPAT) according to Lelliott *et al*. (1966).

Hypersensitivity on Nicotiana tabacum (Klement *et al*. 1964) was tested with bacteria from 24 h *Kings B* medium suspended in sterile distilled water to a concentration of about 10^8 CFU /ml. Control plants were injected with sterile distilled water. Reactions were considered positive if necrosis of infiltrated areas appeared within 48 h after injection.

Table-1. Different locations prospected and their incidences of Olive knot in Morocco

Isolate reference	region	Location	cultivars	Disease incidence
2088	Taounate	bouhouda	Picholine marocaine	10%
2064		bouaidal	Picholine marocaine	100%
2075		Mrouz	Picholine marocaine	1%
2076		askar	Picholine marocaine	50%
2089		zrizar	Picholine marocaine	1%
2090		zrizar	Picholine marocaine	60%
2066		Mkabra	Menara	100%
2065		Mkabra	Picholine marocaine	100%
2074	Meknes	Sidi ali	Picholine marocaine	10%
2073		dkhissa	Picholine marocaine	100%
2102		lamhaya	Picholine marocaine	100%
2107		taoujtate	Picholine marocaine	50%
2108	Ouezzane	Bni kla	Picholine marocaine	40%
2112		Bni kla	Picholine marocaine	30%
2113		ouezzane	Menara	1%

Three isolates were presumptively identified to species level using the multitube API 20E system (Biomerieux) following manufacturer's recommendations.

DNA Extraction and 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 MgCl2 (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. The PCR products (almost 1500 bp) were separated by gel electrophoresis on 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.

Sequencing and Phylogenetic 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 Big Dye, 3µl sequencing buffer x5, xµl primer (3.2-5pmol), (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 10s, annealing at 50°C for 5s, and an 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

Olive Knots cartography

The conducted prospections in field of different regions in Morocco have shown that the disease is mainly located in the north of the country (Taounate, Ouezzane), it present again in the center (Meknes) (Figure-1). The progression of the disease in areas of central and northern of the country is favorites by climatic conditions (humidity and temperature).

Pathogenicity test

Sixty days after inoculation of 15 isolates on 1-year-old olive stems, five isolates (2064-8, 2065-1, 2065-2,2066-1,2066-2) caused the of olive knot symptoms (Figure.2)

Biochemical identification of endophytic bacteria associated with *PSV*

Bacterial colonies which were consistently isolated from the olive knots (2074-1, 2074-4) caused hypersensitive reaction (HR) on tobacco leaves (Figure-3) indicating that they are phytopathogenic bacteria.

Figure-1. Disease cartography of olive Knots of Moroccan prospected regions in 2012-2013

Figure-2. Symptoms on 1-year-old olive stems (Picholine marocaine) 60 days after inoculation with isolates 2064-8(a) and 2065-1, 2065-2 (b)

According to the OPAT tests, the 2074-1 and 2074-4 isolates were able to product Levan and positives for Oxidase, Pectinase and Arginine dihydrolase tests, but not for Tobacco test. However, the 2066-7 isolate were the same characteristic with a difference response concerned the Tobacco and Arginine dihydrolase tests.

Figure-3. Hypersensitivity reaction in tobacco caused by the isolates (2074-1, 2074-4)

Based on the results of biochemical analysis (gallery API20) ,Tow strains (2074-1,2074-4) showed ability to utilize Citrate (CIT), Sodium Piruvate (VP), Glucose (GLU), Man¬nitol (MAN), Rhamnose (RHA), Sucrose (SAC), Melibiose (MEL), Ortho-Nitro-Phényl Galactoside (ONPG), Arabinose (ARA), and formation of indolepyruvic acid from tryp¬tophan by desaminase tryptophan (TDA) however they aren't able to form the indole from tryptophan but they did not use Lysine (LDC), Ortni¬thine (ODC), Gelatin (GEL), Inositol (INO), Thiosulfate (H2S) , Urea (URE), Sorbitol (SOR). However, the 2066-7 isolate were the same characteristic with a difference response concerned the Ortho-Nitro-Phényl Galactoside (ONPG),Tryptophane désaminase (TDA), Glucose (GLU), Mannitol (MAN), Rhamnose (RHA), Sucrose (SAC), Melibiose (MEL), Arabinose (ARA) and Sodium Piruvate (VP) (Table 2).

Table-2. Biochemical characteristics of isolates (2074-1, 2074-4, and 2066-7) on gallery API20

Tests	desired character	2074-1	2074-4	2066-7
Levan	Levan production	(-)	(-)	(-)
Oxidase	Cytochrome-oxydase	(-)	(-)	(-)
Pectinase	Pectinase	(-)	(-)	(-)
ADH	Arginine dihydrolase	(-)	(-)	(+)
H. Tabac	hypersensitivity reaction in tobacco	(+)	(+)	(-)
ONPG	Beta-galactosidase	(+)	(+)	(-)
LDC	Lysine décarboxylase	(-)	(-)	(-)
ODC	Ornithine décarboxylase	(-)	(-)	(-)
CIT	Citrate utilization	(+)	(+)	(+)
H2S	H2S production	(-)	(-)	(-)
URE	Uréase	(-)	(-)	(-)
TDA	Tryptophane désaminase	(+)	(+)	(-)
IND	Indole production	(-)	(-)	(-)
VP	Sodium Piruvate	(+)	(+)	(-)
GEL	Gélatinase	(-)	(-)	(-)
GLU	Glucose	(+)	(+)	(-)
MAN	Mannitol	(+)	(+)	(-)
INO	inositol	(-)	(-)	(-)
SOR	Sorbitol	(-)	(-)	(-)

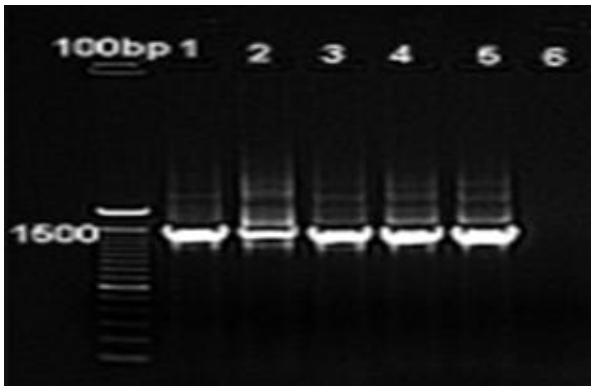
RHA	Rhamnose	(+)	(+)	(-)
SAC	Sucrose	(+)	(+)	(-)
MEL	Melibiose	(+)	(+)	(-)
AMY	Amygdaline	(-)	(-)	(-)
ARA	Arabinose	(+)	(+)	(-)
NO2	NO2 production	(-)	(-)	(-)
N2	N2 reduction	(-)	(-)	(-)

(-): Negative; (+): Positive.

Molecular identification

Result of DNA quantification showed that the quantity of DNA was sufficient for PCR. A band with 1500 bp size was observed in agarose gel (Figure. 4). Sequences of strains 2066-3, 2066-4 and (2066-7, 2074-1, 2074-4) were submitted to GenBank and were assigned respectively for accession numbers KF843716.1, KF782800.1 and HG799974.1.

Figure-4. Electrophoritic profile of (1), (2), (3) *Pantoea agglomerans* stains (4) *Pseudomonas sp*. (5) *enterobacter sp*. (6) Negative control (H_2O).



Based on the results of biochemical and sequence analysis of the *16S rDNA* gene of strains 2066-3, 2066-4 and (2066-7, 2074-1, 2074-4), these isolates isolated was identified as *Enterobacter sp*, *Pseudomonas sp* and *P. agglomerans* respectively (Table 3).

DISCUSSION

The present study demonstrated that the disease is widespread in Taounate, Ouezzane and Meknes regions. Thereby, we showed that "Picholine Marocain" is the most attacked variety by Knot disease than other varieties in Taounate and Meknes regions (table.1). In 1994 Benjama showed that this variety was moderately sensitive of the disease, so our results can be explained by a genetically heterogeneous population of the variety (Kartas *et al* 2015) and/or increased aggressiveness of the pathogen because of genetic changes.

60 bacteria were isolated from stem knots (Table. 1).Among the 36 fluorescent bacteria, were oxidase-negative, levan-negative, pectinolytic activity-negative, arginine dehydrolase-negative and tobacco hypersensitivity-positive (data not shown). Only 2066-7, 2074-1 and 2074-4 were not fluorescent. According to the analysis of the *16S rDNA* sequence and biochemical tests (API20 gallery), 5 were identified as bacteria associated with the Pathogen. These associated bacteria are more abundant in knots than in leaves (Marchi *et al*. 2006).

HR reaction induced on tobacco leaves by P. agglomerans (2074-1, 2074-4) was put into evidence by other research (Moore 1979; Prinsen *et al*.1993).they showed that HR reaction on tobacco leaves is due to indolepyruvic acid formation from tryptophan by desaminase tryptophan. The indole-3-pyruvate (IPA) pathway is a major auxin pathway in plants. The IPA pathway could be shown in many bacteria such as phytopathogens (*P. agglomerans*), it consists of three steps. In a first step the precursor tryptophan is transaminated to IPA by an aminotransferase. In the rate-limiting step IPA is decarboxylated to indole-3-acetaldehyde (IAAld), which is then oxidized to IAA by a dehydrogenase. The decarboxylation step is catalyzed by the key enzyme indole-3-pyruvate decarboxylase. Inactivation of these genes results in impaired IAA production (sometimes >90%), indicating the importance of the encoded enzymes and consequently the IPA pathway in the IAA biosynthesis (Moore 1979; Prinsen *et al*. 1993).

In addition, the HR induced by the strains (2074-1, 2074-4) can be explained by the presence of a complete *hrp/hrc* gene clustern (Moretti *et al*. 2014). *hrp* gene clusters encode components of a type III protein secretion system that is believed to be used by phytopathogenic bacteria to transport, directly into the host cells, virulence proteins which are ultimately responsible for leakage of plant nutrients to the extracellular space of infected tissues and suppression of host defenses (Sisto *et al*. 2004 ; Greenberg and Vinatzer 2003).

Table-3. Endophytic bacteria associated with *PSV revealed 16S rDNA sequence analysis*

Isolate reference	Closest species using 16S rDNA sequences	GenBank Accession Numbers	Similarity (%)
2066-3	*Enterobacter sp.*	KF843716.1	99
2066-4	*Pseudomonas sp.*	KF782800.1	100
2074-1,2074-4,2066-7	*Pantoea agglomerans*	HG799974.1	100

Competition of *P. agglomerans* with *PSV* was probably for nutrients and space and perhaps more intensively, by means of the production of antibiotic substances (Marchi *et al.* 2006). The positive role of *P. agglomerans* in knot formation in certain cases was mostly probably caused by an increased accumulation of IAA at the sites where it multipled together with *PSV*. In culture, *P. agglomerans* accumulated not only indole-3-aldehyde, an alternate end-product of the oxidation of IAA (Moore, 1979), and indole-3-ethanol, a shunt product of the biosynthesis of IAA from tryptophan via indolpyruvic acid (Moore, 1979), but also IAA in amounts similar to those reported for some virulent *PSV* strains (Surico *et al.* 1985). *Pantoea agglomerans* has often been reported to be associated with olive and oleander knots (Azad and Cooksey 1995; Fernandes and Marcelo 2002; Surico and Lavermicocca 1989). The bacterium is considered an epiphyte and is very common on the aerial parts of plants (Starr and Chatterjee, 1972). Three pathovars of *P. agglomerans* have been described, pv. *Gypsophilae* (Brown 1932), pv.*Betae* (Burr *et al*. 1991) and pv.*Milletiae* (Opgenorth *et al*. 1994); all these pathovars cause diseases characterized by galls similar to the knots caused by *PSV*.

Moreover, the strain 2066-7 of *P. agglomerans* showed an antagonist effect against phytopatogenic bacteria. Thus, *P. agglomerans* 2066-7 was revealed its potential biocontrol against many phytopathogenic bacteria *in vitro* and *in planta*. Its utilization allowed a reduction of Soft Rot Disease in Potato Caused by *Pectobacterium carotovorum* (Faquihi *et al*.2014) and Bacterial Onion Diseases Caused by *Pseudomonas marginalis*, *Pseudomonas viridiflava, Xanthomonas retroflexus* and *Pantoea ananatis* (Sadik *et al*.2015).

The strain 2066-7 also revealed its contribution to improving the growth and yield of some crops such as Potato culture (Faquihi *et al*. 2014). This strain 2066-7 was revealed an antagonist effect against other phytopathogenic bacteria (data not shown).

CONCLUSION

In conclusion, our study showed that tumors of the knot disease are rich in olive endophytic bacteria associated with *PSV*. Then, future studies would focus on the study the Effects of co-inoculation of *PSV* and the *P. agglomerans* on the growth dynamics of these bacteria Strains of *PSV* and of the *P. agglomerans* were inoculated either alone or in combination in the stems of 1-year-old self-rooted olive cuttings “Picholine Marocain” in early spring. Also, the 2066-7 strain can be used like a base of a biopesticid product.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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