Molecular identification, *in vitro* copper resistance and antibiotics susceptibility of the causal agent of the olive knot disease in Morocco

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ABSTRACT

Aims: This study aimed to i) identify *Pseudomonas savastanoi* pv. *savastanoi* (Pss) as a causal agent of the olive knot on the basis of biochemical, pathogenicity and PCR technique ii) investigate *in vitro* bacterial resistance toward copper-based compounds and efficiency of some antibiotics on pathogen suppression.
Methodology and results: Biochemical, pathogenicity and molecular identification based on alkaline method for the DNA extraction were performed to identify possible causal agent of the olive knot. Copper resistance for Pss strains was evaluated by inoculation of bacterial suspensions into YPG medium, containing the cupric sulfate at 0, 100, 250 and 500 ppm. The efficiency of eight antibiotics on Pss strain was evaluated at different concentrations. Fifty-nine isolates caused typical knots at the site of inoculation with bacterial suspensions. All isolates have been identified as Pss using specific primers. No resistance to copper was detected with concentration of 500 ppm. In contrast, copper resistance was found during 48 h with lower concentration (100 or 250 ppm). The maximal inhibition of Pss 2102-4M was observed with the highest concentration (20 µg/mL) of the Aureomycin, Streptomycin and Novobiocin with inhibition diameters of 30, 24 and 10 mm, respectively. Whereas, Colchicine, Bacitracin, Cephalex, Ampicillin and Cycloserine have no inhibitory effect on the Pss 2102-4M strain.
Conclusion, significance and impact of study: The alkaline method for the DNA extraction from pure culture was reliable and rapid and can be recommended for molecular detection the causal agent of the olive knot. This is the first report determined copper resistance levels of Moroccan strains of Pss and *in vitro* evaluated for the susceptibility towards the antibiotics.

Keywords: Olive knot, pathogenicity test, PCR, copper resistance, antibiotics

INTRODUCTION

Olive knot caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Pss) is a limiting disease in many olive-producing areas (Young, 2004). The disease is manifested by the production of tumorous woody outgrowths, 2-10 cm of diameter, mostly formed on young stems as well as on branches and twigs. The leaves and fruits are rarely infected, but they usually harbour the bacteria on their surface (Sisto *et al.*, 2004). Olive knot disease is considered an important problem for olive crops because of its effect on vegetative growth (decline of branch and shoots, death of small branches and twigs), olive yield and even possibly on olive oil quality through inferior organoleptic characteristics such as unpleasant

smell and a bitter and rancid taste (Godena *et al.*, 2012). The characterized virulence factors in tumor-inducing isolates of *P. savastanoi* are the indol acetic acid (IAA) and cytokinins hormone (Surico *et al.*, 1985).

Natural isolates of Pss are phenotypically and genotypically heterogeneous (Ramos *et al.*, 2012), although they tend to generate clonal populations in colonized areas (Sisto *et al.*, 2007; Quesada *et al.*, 2008). There is an important variation in virulence, with strains showing low, intermediate or high virulence to diverse olive cultivars (Penyalver *et al.*, 2006), and also variation in the size and morphology of tumorous in artificial inoculations (Pérez-Martínez *et al.*, 2007). Certain isolates in central Italy are non-fluorescent and produce levan compared with most other isolates (Marchi *et al.*,

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2005). The identification of Pss is currently based on the isolation of the bacterium followed by pathogenicity tests, biochemical tests and PCR test (Penyalver *et al.*, 2000).

The occurrence of infection requires openings provided by leaf scars, pruning wounds or cracking due to freezing. For its control, it is necessary to make preventive pesticide sprays to reduce bacterial entry through the scars. Furthermore, pruning should be conducted carefully during the dry season, in order to remove knots from twigs.

After pruning it is necessary to do the disinfection by a copper-based solution and the required number of treatments for olive protection is three times. The spraying schedule is one in autumn, one before the winter rains and another in spring, following leaf abscission. No specific bactericides are available, so infected trees are treated with copper compounds or by application of preventive techniques that reduce the pathogen population (Lavermicocca *et al.*, 2003).

Treatment of olive trees with copper-based products has traditionally been used to prevent spread of the pathogen and to protect olive trees from disease. But the main disadvantage of chemicals copper compounds is their phytotoxicity on host plants. Furthermore, the classes of antibiotic that are more widely used in agriculture at the global level, which are of growing scientific concern with regards to their potential adverse effects and risk management steps, include the tetracyclines, aminoglycosides, β-lactams, lincosamides, macrolides, pleuromutilins, and sulphonamides (Finley *et al.*, 2013). Despite the beneficial consequences of antibiotics in agroecosystems, such as growth promotion and disease prevention (Williams-Nguyen *et al.*, 2016), the presence of these antibiotics in the environment may create selective pressure resulting in antibiotic resistance and in removal processes (Du and Liu, 2002).

The benefit of antibiotic products is that they are applied well in advance of harvest and result in zero residues present on olives at time of harvest. But these products can be used in rotation with copper to prevent the disease before becoming resistant to any one of these products. One research showed that orchards treated with the combination of these products resulted in excellent control of the olive knot disease (Adaskaveg, 2014).

The objective of this study was to i) identify the bacteria Pss on the basis of biochemical, pathogenicity and PCR technique and ii) to investigate *in vitro* bacterial resistance toward copper-based compounds and efficiency of some antibiotics on pathogen suppression.

MATERIAL AND METHODS

Pathogenic bacteria

Pathogenic bacteria were isolated from stem knots of infected olive trees from three different regions in Morocco (Meknes, Taounat and Ouazzane). Knots were surface disinfected with paper moistened with ethanol for 30 sec. Fragments of knots and leaves (1-2 mm) were cut aseptically and macerated in 200 µL of sterile distilled water (SDW). After 30 min, a spot (50 µL) of the resulting suspension was streaked on Petri plates containing King's medium B (King *et al.*, 1954) and incubated at 26 °C for 3 to 5 days. All colonies which discerned based on their morphology (colour, size and form) were purified in solid King's B medium and isolates were stored in 30% glycerol at –20 °C.

Identification of bacterial isolates

Biochemical analysis

Selected bacterial isolates were subjected to LOPAT tests as recommended by Lelliott and Stead (1987) including Levan production, oxidase, potato soft rot, arginine dihydrolase and hypersensitivity reaction to white burley tobacco leaves.

Pathogenicity test

Pathogenicity tests were performed on 1-year-old seedlings of olive (cv. Arbequina) to confirm the aggressively of different isolates. Wounds of 1 cm long were made in the bark with sterile scalpel dipped in bacterial suspensions (10^8 CFU/mL) (Surico *et al.*, 1984). Three wounds on plant were inoculated with each isolate. Control plants were inoculated with sterile distilled water. The inoculated wounds were covered with Parafilm for 96 h. The inoculated plants were maintained in the greenhouse at 26 ± 2 °C and then checked periodically for knots development.

Genomic DNA extraction from pure culture

Common extraction method of bacterial DNA is based on the protocol previously described by Llop *et al.* (1999). This method is laborious, expensive and time consuming. For this reason, many studies were conducted to develop other techniques of DNA extraction from pure culture.

The alkaline method described by Shams *et al.* (2013) was used to extract bacterial DNA. From 24h-old bacteria (at 28 °C) in King's B medium, each pathogenic isolate was mixed with 30 µL of NaOH (20 mM) and incubated at 37 °C for 10 min. The lyses bacterial cells were stored at 4 °C until usage.

PCR tests

Identification based on polymerase chain reaction was involved to identify and confirm the selected pathogenic bacteria using genomic DNA. A set of two specific primers of the bacterial gene *iaaL* described by Penyalver *et al.,* (2000) were used for DNA amplification; IAALF (5´-GGCACCAGCGGCAACATCAA-3´) and IAALR (5´-CGCCCTCGGAACTGCCATAC-3´). Each mixture of PCR reaction was prepared in final volume of 20 µL containing: 12.8 µL ultrapure water, 4 µL PCR buffer **x** 10 (Bioline), 1 µL of each primer, 0.2 µL *Taq* polymerase and 1 µL of DNA extract.

The PCR was performed in thermal cycler (PTC-Techne) with the following program: 1 cycle of 95 °C (5 min), followed by 25 cycles of denaturation at 95 °C (30 sec), annealing at 62 °C (30 sec), and extension at 72 °C (30 sec) and then a final extension cycle of 72 °C for 5 min. PCR-products were separated on electrophoresis gel (1.5% agarose). After that, the gel was stained with ethidium bromide and DNA sequences were visualized under ultraviolet (UV) transilluminator (Penyalver *et al.*, 2000).

Assessment of copper resistance

Three Pss strains (Pss 2102-4M; Pss 2064-8T and Pss 2112-8O) selected in this study were isolated from Meknes, Taounat and Ouazzane regions respectively.

Copper resistance was assessed by spotting 400 µL aliquots of bacterial suspension into YPG medium, containing different concentrations of cupric sulfate ($CuSO_4$): 0, 100, 250 and 500 ppm. Bacterial concentration was evaluated after 0, 48, 72 and 96 h at 26 °C by measuring the optical density at 600 nm using the spectrophotometer (UV-mini 1240, Shimadzu) (Oufdou *et al.*, 2016).

Antibiotics and cupric sulfate susceptibility test

For this test, 250 µL of microbial inoculum (10^8 CFU/mL) of the aggressive strain Pss 2102-4M from cultured nutrient broth was spread on the surface of King B agar. Then, eight commercial antimicrobial compounds; Aureomycin, Colchicine, Bacitracin, Cephalex, Ampicillin, D-cycloserine at 5, 10, 15, 20 µg/mL and Streptomycin, Novobiocin at 1, 5, 10, 20 µg/mL were investigated. Cupric sulfate at 1, 2, 4, 8 mg/mL was also used to assess the susceptibility of selected strains. These compounds were spotted (5 µL) on the surface of Petri dishes according to Islam et *al.* (2014). After incubation (48 h at 26 °C), the inhibitory effect was assessed, and the diameters of inhibition zone were measured.

Statistical analysis

Data on the effect of the copper concentration factor on growth *in vitro* Pss are analyzed with the Duncan test with a P = 0.05. These statistical analyzes are conducted with SPSS 24 software.

RESULTS

Biochemical and pathogenicity identification

Based on biochemical and physiological characterization, the reactions of these bacterial isolates to Levan production, oxidase, pectinase and arginine dihydrolase

tests were all negative, but hypersensitive reaction on tobacco leaves was positive (necrosis of leaf tissue) and the obtained results were in accordance with Lelliott and Stead, (1987). Fifty-nine isolates induced typical knot symptoms when they have been inoculated on olive seedlings (cv. Arbequina) (Figure 1). No symptoms were observed on plants inoculated with sterile distilled water (control plants).

Figure 1: Typical knots formation at the site of inoculation on olive (cv. Arbequina) plants artificially inoculated with isolate Pss 2102-4M.
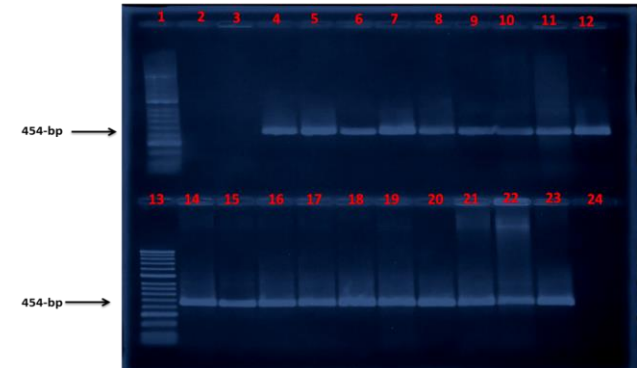

Figure 2: Pattern of electrophoresis gel showing DNA sequences from different strains using IAALF/ IAALR primers. Lane 1 and 13, a marker of molecular weights 2 kb Ladder. Lanes 2-3, negative PCR product. Lanes 4–12 and Lanes14-23, PCR product from different isolates. Lane 24, negative control (PCR mix without DNA). Arrow shows amplification of a 454-bp DNA fragment.
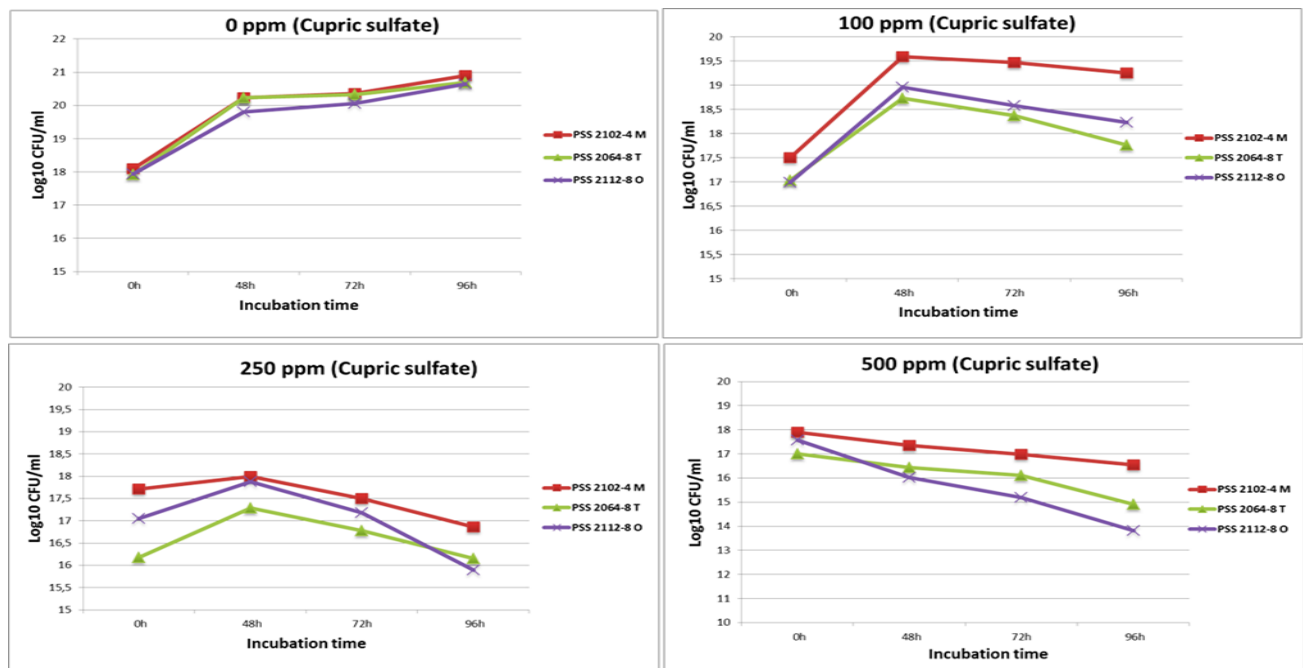
Figure 3: *In vitro* effect of cupric sulfate at different concentrations on the multiplication of Pss (Pss 2102- 4M, Pss 2064-8T and Pss 2112-8O).

Table 1: Inhibition zone (mm) of different antibiotics applied *in vitro* against Pss 2102-4M.

	Antibiotics	Concentrations				
		1 µg/mL	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL
Inhibition zone (mm)	Aureomycin	*	26	27	29	30
	Streptomycin	1	12	20	*	24
	Novobiocin	0	0	08	*	10
	Colchicine	*	0	0	0	0
	Bacitracin	*	0	0	0	0
	Cephalex	*	0	0	0	0
	Ampicillin	*	0	0	0	0
	D-cycloserine	*	0	0	0	0

(*) not tested

Molecular identification

Extraction technique with alkaline condition used in this work was reliable and successful. The alkalinity and high temperature cause the lysis of cell and therefore the liberation of the DNA in the solution. The sensitivity of alkaline protocol can be explained by the additional NaOH property of the hydrogen bond perturbation between the DNA base pairs, which denatures genomic and plasmid DNA and allow their amplification.

The PCR reaction produced sequence of 454 bp such as described in Figure 2. The PCR amplification carried out in this study confirmed the identity of our selected isolate. Indeed, 59 isolates was detected by PCR technique. These results showed clearly the reproducibility of involved technique and higher sensibility of primers (IAALF, IAALR) yielding the expected 454 bp

DNA sequence from all the 59 pathogenic isolates. The alkaline method was the most sensitive, specific and rapid method for detection of Pss from pure culture.

Assessment of copper resistance

The *in vitro* study of resistance to copper of three strains from 59 was performed into different concentration (0, 100, 250 and 500 ppm). The monitoring of the bacterial growth over time (0, 48, 72 and 96 h) showed that the three bacteria (Pss 2102-4M, Pss 2064-8T and Pss 2112-8O) are able to grow during the first 48 hours in 100 ppm of copper to reach 19.58, 18.73 and 18.96 log10 CFU/mL respectively. A weak decrease in bacterial concentration was detected after 48 h, thus, the values 19.25, 17.76 and 18.23 log10 CFU/mL were recorded for the three bacteria after 96 h respectively. At 250 ppm, all these

strains were able to grow during the first 48 h, whereas, after 96h, the bacterial growth remained very low compared to the control (0 ppm) and to 100 ppm. Moreover, the concentration of 500 ppm showed the most significant inhibition of the growth of the three bacteria from the first hours to reach 16.55, 14.91 and 13.82 log10 CFU/mL respectively after 96 hours of incubation (Figure 3).

Copper has shown its ability to inhibit the growth of all three bacteria. This inhibition depends not only on the copper concentration, but also on the incubation period. We conclude that Pss 2102-4M strain is the most resistant to copper compared with Pss 2064-8T and Pss 2112-8O strains for the three concentrations and during all incubation periods. The statistical analyzes of the resistance test of copper strains in liquid medium at 48 and 72h showed that the bacterial growth of the Pss 2102-4M strain isolated from the Meknes region is high compared to the growth of the two strains Pss 2112-8O and Pss 2064-8T isolated from the region of Ouazzane and Taounate respectively. This difference has been reported for concentrations of 100 and 500 ppm of copper. However, no significant difference between the different strains was recorded in the case of the 250 ppm copper concentration. Moreover, the multiple comparisons of the different concentrations and including the control showed a highly significant difference between these concentrations. Indeed, the bacterial growth of the three strains decreases with the increase of the copper concentration. This inhibition depends not only on the copper concentration, but also on the incubation period.

Antibiotic and copper sulphate susceptibility

The antibiogram of the antibiotics (Aureomycin, Streptomycin, Novobiocin, Colchicine, Bacitracin, Cephalex, Ampicillin and D-Cycloserine) and copper sulphate used to control Pss 2102-4M strain showed different inhibition diameters depending on the antibiotic nature and concentration (Table 1).

Low concentration of Aureomycin (5 µg/mL) showed an inhibition diameter which exceeded the inhibition diameter observed in the case of high concentrations of Streptomycin and novobiocin (20 µg/mL). In fact, Aureomycin inhibited bacterial growth at 5 µg/mL with an inhibition diameter of 26 mm, followed by Streptomycin (12 mm). However, Novobiocin had no effect on the bacterium at this concentration (0 mm). Furthermore, maximal inhibition was observed with the highest concentration (20 µg/mL) in the case of the three antibiotics, Aureomycin, Streptomycin and Novobiocin, with inhibition diameters of 30, 24 and 10 mm respectively (Table 1 and Figure 4). Finally, other antibiotics tested *in vitro* (Colchicine, Bacitracin, Cephalex, Ampicillin, D-cycloserine) have no inhibitory effect on the strain Pss 2102-4M.

Copper sulphate was used with the concentrations (1, 2, 4 and 8 mg/mL) and it showed a maximum inhibition diameter (18 mm) with the concentration of 8 mg/mL.

DISCUSSION

Olive knot disease caused by Pss has long been noticed to afflict olives in Morocco (Rieuf, 1960; Benjama, 1988). Today, many different regions in Morocco are noticed to be affected by this disease as evident by the presence of knot symptoms (Bouaichi *et al.*, 2015). Based on symptoms on olive plants, the results of biochemical, pathogenicity tests and PCR test (Figures 1 and 2), it can be concluded that the causal agent of hyperplastic outgrowths on olive trees in Morocco is Pss. PCR methods have provided an advantage because they are fast, specific, and sensitive (Goussous and Al-Gharaibeh, 2010). The PCR was designed to amplify the *iaaL* gene of Pss (Penyalver *et al.*, 2000; Roberto *et al.*, 1990). The designed primers were highly specific, yielding the expected 454 bp DNA fragment from all isolates. These results indicate that the *iaaL* gene is present among the Moroccan isolates of Pss.
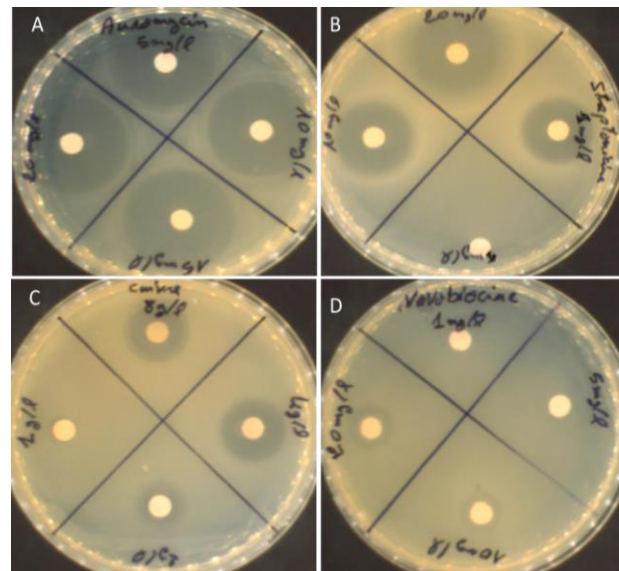

Figure 4: Antibiogram of applied antibiotics on Pss 2102-4M: A, Aureomycin; B, Streptomicin, C; cupric sulfate; D, Novobiocin.

Copper-based chemical treatments showed an important reduction of epiphytic Pss populations as well as a prevention of Pss infection through the injuries. In addition, cases of resistance to copper were not found in surviving Pss bacteria and copper-based bactericides significantly decreased pathogen population after inoculation (Quesada *et al.*, 2010). Adaskaveg (2014) demonstrated that antibiotics such as Kasugamycin and oxytetracycline have different action modes which are very active on Pss. Also, copper sensitivity levels of bacterial pathogens depend on years of copper usage, while Pss was sensitive of copper at concentration 50 ppm (Adaskaveg, 2014). Khlaif (2010) tested many antibiotics *in vitro* such as cuprosan, trimiltox, copper

sulfate, streptomycin sulfate and misaram extra blue. He showed these antibiotics were able to inhibit Pss growth with an inhibition zone that exceeds 30 mm. Also, *in vivo* experiment showed that cuprosan and trimiltox were highly efficient in reducing disease incidence followed by misaram extra blue, copper sulfate and copper hydroxide respectively. Mechanisms of resistance include exclusion of copper ions from the cell by the production of copious quantities of extracellular polysaccharides and detoxification of copper ions (Loper *et al.*, 1991). Finally, other management tools can be integrated with antibiotics to provide disease control and minimise the development of resistance. Managing bacterial diseases depends mostly on host plant resistance, sanitation (e.g. preventing the introduction of pathogens and removing symptomatic plants) and cultural practices (e.g. avoiding overhead irrigation and limiting nitrogen fertilisation). In some cases, chemical bactericides (e.g. copper compounds) and biological control agents can be integrated into the disease management programme (Stockwell and Duffy, 2012).

CONCLUSION

This work described the isolation of causal agent of olive knot from different regions in Morocco and the biochemical, pathogenic and molecular identification (based on PCR) of Pss. The molecular identification was carried out by using PCR technique with the *iaaL*-derived primers. The PCR was 100% specific and extremely rapid and the alkaline method for the DNA extraction from pure culture was reliable and rapid and can be recommended for molecular characterization and detection of olive knot disease. This is the first report determined copper resistance levels of Pss strains isolated from Morocco and *in vitro* evaluation of the susceptibility towards numerous antibiotics.

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