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Investigating the spread of *Pseudomonas savastanoi* pv. *savastanoi* the causal agent of Olive knot disease in Algeria

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Olive knot disease is one of the most important diseases affecting olive cultivation, and *Pseudomonas savastanoi pv. savastanoi* is recognised as its causal agent. The aim of our research is to explore the reasons behind the alarming spread of olive knot disease in Algeria for the regions of Bouira, Bejaia and Tizi-ouzou and to study the factors implicated on its infestation. Field surveys were carried out during the period 2019-2020 in several olive orchards in the Kabylie area on the northern tip of Algeria. The isolation of *P. savastanoi* was carried out on King-B medium from the sampled knots, the identification of the pathogen was done by biochemical, biological and molecular methods. The field survey revealed a heterogeneous distribution of the disease depending on the site. However, all varieties of *Olea europea* grown in the study area were affected regardless of the age of the tree. A total of 14 bacterial strains were isolated from knots on semi-selective medium (King B) and identified as *P. savastanoi* based on biochemical characteristics (LOPAT test), and phyto pathogenicity test on olive plants.The amplification of the iaaL gene confirmed that the selected strains concern*P. savastanoi*. The biochemical characterisation of the bacteria associated with olive knots found alone or in association with *P. savastanoi* revealed the identification of mainly *Pantoa*, to which were added *Stenotrophomonas, Rahnella*, *Seratia,Pseudomonasfluorescens*, *Enterobacter cloacae* and *Citrobacter freundi*. These associated bacteria would appear to contribute to the virulence of the causal agent and/or have antagonistic activity that may depreciate the disease.

Keywords: *Pseudomonas savastanoi pv. savastanoi*, olive knot, incidence, bacterial disease, Algeria.

INTRODUCTION

The olive tree (*Olea europea*), an ancient fruit tree cultivated throughout the world, particularly in the Mediterranean basin, is of great nutritional, economic and social importance (Zohary and Spiegel-Roy, 1975). Olive growing in Algeria dates back to ancient times and accounts for more than 50% of the national tree orchard. In 2020, it occupied the ninth place in the world with a harvested area of 438828 ha and an olive production of 1079 508 tonnes, distributed throughout the national territory (FAOSTAT, 2022). This culture is mainly concentrated in the central region, constituting a significant source of income for the rural population.

Among the bacterial diseases that can affect olive trees, olive knot caused by *Pseudomonas savastanoi pv. savastanoi* is the most common in Algeria. This bacterium can survive on olive trees as an epiphyte (Ercolani, 1978), and is an opportunistic wound phytopathogen (Wilson, 1935) responsible for a characteristic hypertrophy and hyperplasia called galls (tumours) on the aerial part of olive trees; twigs, trunk, and occasionally on leaves and fruits (Sisto et al. 2004). It is a Gram-negative rod, motile by peritrich ciliature, and producing a yellow-green fluorescent pigment soluble on king B medium. According to the LOPAT scheme, the bacterium is levan and oxidase-negative,arginine dihydrolasenegative, lacks pectinolytic activity and has a hypersensitivity reaction on tobacco leaves (Bradbury, 1986; (Ali Hassan et al. 2021, Nguyen PM. 2021, AL-Asmari et al. 2020).

Several factors are involved in triggering the infection process, such as wounds, which may be natural, such as those caused by leaf fall, or mechanical, such as those caused by pruning. The effect of insects is not negligible either, it has been demonstrated that a homoptera (*Ceresa sp*.) when laying its eggs causes wounds constituting a site of infection by the bacterium (Jardak et al. 2004). Indeed, the pathogen induces a DNA fragment (TADN), located on a specific plasmid designated by TI

(Tumor Inducing), its expression in the host cell induces the synthesis of hormones (indole-3-acetic acid and cytokinins) (Surico et al. 1985), and amino acids (opines) by the transformed cells. In addition to the production of phytohormones, *P. savastanoi* has a type III secretion system that plays a vital role as a virulence factor (Pérez-Martínez et al.2010;Sisto et al. 2004). The tumour thus formed can grow in the absence of the pathogen (Surico et al. 1975; Comai and Kosuge 1980; Iacobellis et al. 1994; Lepoivre, 2007).It seems that *P. savastanoi* needs signals released by the plant wound to activate tumour formation (Surico, 1993). to the Executive Decree No. 93-284 regulating phytosanitary control at borders (JORA, 1993). The intensification of production systems has increased the risk of contamination, but there are no official data on the current situation in Algeria.

This pathogen can also infect other hosts, in particular oleander (*Nerium oleander*), ash (*Fraxinus excelsior*), privet (*Ligustrum japonicum*), jasmine (*Jasminum officinale L*.) on which it causes diseases similar to olive knot (Marchi et al. 2005). More recently, myrtle (*Myrtus communis L*., Myrtaceae) and buckthorn (*Rhamnusalaternus* L., Rhamnaceae) have been reported as hosts of *P. savastanoi* (Saad and Hanna, 2002; Lamichhane and Varvaro, 2012).

Olive knots are ideal niches for bacterial growth, not only of the causal agent of the disease, but also of a number of endophytic species of the class Gamma proteo bacteria such as *Erwinia toletana*, *Pantoeaagglomerans*. As well as other bacteria of the genus *Burkholderia*, *Hafnia*, *Pseudomonas* and *Stenotrophomonas* (Marchi et al. 2006; Ouzari et al. 2008; Moretti et al. 2011).

This disease is considered as an important problem threatening olive cultivation, as it affects the vegetative growth and olive yield and oil quality (Penyalver et al. 2006; Bouaichi et al. 2019).

The objective of our research is to investigate the spread of olive knot disease in olive orchard sin Algeria for the regions of Bouira, Bejaia and Tizi-ouzou, to study the factors implicated on its infestation, to isolate, identify and characterise its causal agent *P.savastanoi*, as well as other possible associated germs.

MATERIALS AND METHODS

Distribution survey of olive knotin the study area

Field surveys were conducted during the period from March 2019 to October 2020 in all communes of the wilayas of Bouira, TiziOuzou and Béjaia. These cover a total area of 1.07 million hectares, including more than 110 thousand hectares of olive orchards. In each commune, five sites were randomly surveyed in an attempt to map the infected orchards.

Assessment of the incidence and intensity of attack

The evaluation of the incidence of the disease as well as the intensity of attack was carried out on one infected orchard randomly selected among the five surveyed in each commune of the study area. The difficulty of systematically counting the number of tumours on all the trees in an infected olive orchard or in a given region led us to work only on a part of the total branching of the tree; this part represents randomly selected twigs whose number is between 1/6 and 1/4 of the total number of twigs of the tree (Benjama, 2003; Bachouche et al. 2018). For an overall assessment of the infection intensity of the tree, the average number of knots/cm of twig was calculated. Trees defined as heavily attacked have an average equal to or higher than 0.15 knots cm^{-1} of twig, others are weakly attacked with an average lower than or equal to 0.06 cm^{-1}, and finally moderately attacked trees with values between the two (Benjama, 2003; Krid et al. 2011). In addition to the infection intensity of the tree, the percentage of affected trees per orchard (incidence) was also calculated by dividing the number of affected trees in an orchard by the total number of trees.

Sampling procedure

In order to be able to isolate the pathogen, sampling was carried out in spring and autumn 2019-2020 in 28 sites in the study region (10 communes in Bouira, 9 in TiziOuzou and 9 in Béjaia).Sampling concerned only olive trees with tumours. Young green tumours with regular outlines usually located on the twigs were taken from each orchard from infected olive trees: 3 trees per orchard (Krid et al. 2011) and 3 twigs per tree were chosen randomly (Quesada et al. 2007). The samples were placed in sterile plastic bags, sealed and transported directly to the laboratory.

Isolation from knots

Knots were disinfected with sterile filter paper soaked in 95% ethanol (Marchi et al. 2005). The internal tissue was finely cut with a sterile scalpel. The resulting fragments were crushed using pellet plungers into sterile Eppendorfs tubes containing 200 µl of sterile distilled water. After 30 min, 10 µl of the supernatant was inoculated onto Petri dishes containing King B semi-selective medium (King et al. 1954) and incubated at 26°C for 3-5 days.

Phenotypic characterisation of isolates

The isolated colonies were purified and then characterised by morphological and biochemical tests. Isolates were tested for levan production on nutrient agar medium combined with 5% sucrose (NAS), for polymerisation of fructose to poly fructose, oxidase, catalase, arginine dihydrolase, and pectinolytic activity, fluorescent pigment production on King B medium; and gelatin hydrolysis (Lelliott et al. 1966; Hildebrand, 1971; Schaad et al. 2001). For the hypersensitivity test on tobacco (*Nicotiana tabacum*) leaves, a bacterial suspension of 10^8 CFU/ml obtained on King B medium was injected into the secondary leaf veins. Negative

control plants were injected with sterile distilled water (Klement, 1964).

Other biochemical tests present on the Api 20E gallery of biomérieux were carried out, allowing the rapid evaluation of several physiological and biochemical properties (Lacroix et al. 1995).

Pathogenicity tests

One year old certified healthy olive plants (*Olea europaea*) from the Institut National de Protection des Végétaux (INPV, Algiers) were artificially inoculated with 10 µl of a bacterial suspension of 10^8 CFU ml^{-1}, at the main stems (on the bark of the branch) by means of a wound made with a sterile scalpel (2 mm deep and 2 mm wide). The incision is then covered with cotton wool and removed after 36 hours. The olive plants are then placed in the growing room at a temperature between 25 and 30 °C and inspected weekly for knotsformation until 90-120 days (Alvarez et al. 1998; Lamichhane and Varvaro, 2013).

Another alternative test proposed by FilizDoksöz and Bozkurt in 2020 was performed. This phytopathogenicity test performed on fresh carrot (*Daucus carota*) slices is considered simple, sensitive and relatively fast giving a reliable positive result after 14 days. The roots were washed under tap water, disinfected by soaking in 70% ethanol for 5 min, rinsed 3 times with sterile distilled water, and dried on sterile filter paper. After surface sterilisation, the carrot roots were peeled and sliced. From a 48-hour culture on King B medium, a bacterial suspension of 10^8CFU ml^{-1} was prepared. The slices were inoculated with 50 µl of a previously prepared suspension, then placed on moistened sterile filter paper in Petri dishes. Petri dishes sealed with Parafilm were incubated at 25 ± 2 ° C, and inspected twice a week for knot formation (FilizDoksöz and Bozkurt, 2020). Negative controls were inoculated with sterile distilled water and installed under the same conditions.

Identification moléculaire par test PCR

For bacterial DNA preparation, a single colony of fresh pure culture was suspended and homogenized in 1ml of molecular-grade water; bacterial cells lysis was performed at 95°C for 15 min using block heater (Stuart);and followed by immediate cooling with ice for 5 min. For purification, the bacterial extract was centrifuged for 3 min at 5 000 g (Centrifuge: eppendorf), the supernatant was used for PCR.

The primers designed for *iaaL* gene amplification were as follows: primer iaaL Forward, 5'-GGCACCACGGGCAACATCAA-3'; primer iaaL Reverse, 5'-CGCCCTCGGAACTGCCATAC-3' (Penyalver et al. 2000). PCR reactions were performed by combining the following reagents in a reaction mix: 5 ul *Taq*buffer (SOLIS BIODYNE), 3µL of $MgCl_2$25 mM (SOLIS BIODYNE), 2µl of dNTPs10mM(mixture of the four deoxynucleoside triphosphates: Sigma), 1 µL of each iaaLFprimer 10 µ Man diaaLR primer 10 µM (PRIMM Technology), 0.4µL of TaqDNA polymerase 5U $µL^{-1}$ (FIREPol DNA polymerase from SOLIS BIODYNE) and 35.6 µl of molecular-grade water per reaction (final volume 48µl. Then, 2µl of the DNA extraction from bacterial cultures was added. Samples were amplified through 1 cycle of 94°C (5 min), followed by 35 cycles of 94°C (30 s), 60°C (45 s), and 72°C (45 s) and then 1 cycle of 72°C for 5 min in a 9600 SimpliAmp thermocycler (applied bio-systems). After amplification, 3µL of Gel loading buffer (Sigma Aldrich) were added for each sample.

For 1% agarose gel preparation, 1.4 g of Agarose molecular Biology-grade (Fischer Scientific) were diluted in 140mL of Tris Acetate EDTA buffer (Sigma Aldrich) , after molting, 14 µl of SUBR safe DNA Gel Stain (invitrogen by ThermoFicher Scientific) were added. For separation; horizontal agarose Electrophoresis was used in Tris Acetate EDTA buffer, it was performed for one hour at 100 V. Molecular weight marker of 100 bp (PCR 100 bp low Ladder, Sigma-Aldrich) was used DNA fingerprints were visualized with a UV transilluminator (Penyalver et al. 2000).

RESULTS AND DISCUSSION

Field surveys conducted in several regions of central Algeria revealed that the incidence of the disease varies from one region to another and even from one commune to another (Table 1).

Olive orchards are ubiquitous throughout northern Algeria and more particularly in the surveyed area and are mainly concentrated in mountainous areas, which means that the olive tree has a preference for a rather mild climate and high rainfall, which are both favourable conditions for its development but also for the development of diseases, such as knot olive caused by *P. savastanoi*. Olive knots are easily recognisable when the symptoms are well developed (figure 1a).

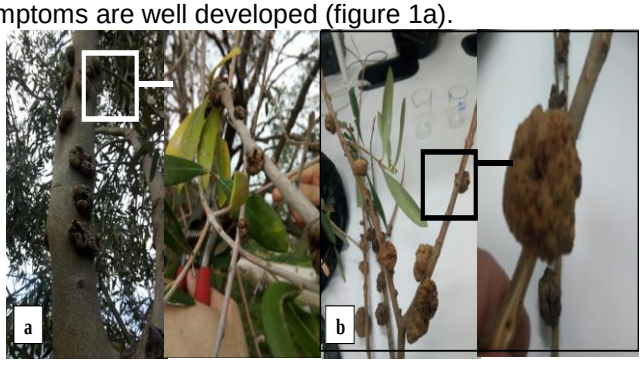

Figure 1: Knots observed during the survey of an orchard.

a- Characteristic symptoms observed on trunks and branches during the surveys.

b- Aspect of the nodes used for the analysis and research of the causal agent.

Surveys carried out in the various olive orchards have revealed knots growths of 0.5 to 12 cm in diameter, occurring on the twigs, branches and trunks of the tree,

but never on the leaves, contrary to several authors (Ercolani, 1978). These parenchymal tumours are regular to irregular in shape, green to blackish in colour, and have smooth to rough surfaces (figure 1b).

Based on literature data, this disease has been reported in 21 countries on five continents, the majority of which exist in the Mediterranean basin, and Algeria is among the most affected (Chliyeh et al. 2017).

The present study demonstrated that the disease is widespread throughout the study region. Our results can be explained by the high dissemination power of the pathogen. Indeed, populations of *P. savastanoi* on olive trees can spread over long distances (Ercolani, 1978). If the knot is observed in the trees of an orchard block, it can be assumed that the whole block is at risk, and that transmission of the pathogen occurs within a radius of about 500 m (Young, 2004).

Out of a total of 140 orchards surveyed, 65 were infected (46%), which is almost half of the rate reported in Morocco (13%) by Barguigua et al. (2020) working on 9 communes. However, we noted a variability in the distribution, the orchards of the wilayas of Bouira and Tizi-Ouzou are very infected, in most of the surveyed communes more than 3 orchards out of 5 are affected by the disease, and with a higher incidence going up to 69% in the sampled orchard of the region of Tighzirt, while those of the wilaya of Béjaia turn out to be the least infected in which the incidence of the disease varied from 8 to 30%.

Similar surveys in several Moroccan regions show that the incidence of the disease varies from one region to another and even from one commune to another, ranging from 1% to 100% (Barguigua et al. 2020).

The progression of the disease is favoured by climatic conditions such as humidity and temperature (Bouaichi et al. 2021). High temperatures and heavy rainfall increase the effect of bacterial pathogens in general. Olive knots seem to be more severe in spring and autumn, which would be due to the climatic influence, the physiological state of the trees (Ercolani, 1978; Surico,1977, Lavermicocca and Surico, 1987).

Humidity influences the development of kontsin olive trees, and seems to contribute to the heterogeneity of the distribution; we have noticed that the orientation of the plots influences the development of tuberculosis, so that north and west exposure favours infection and increases the incidence of the disease in an orchard, as well as the intensity of attack, being a humid area that receives more rainfall and less exposure to the sun. As noted by (Rhimini et al. 2018); the incidence was 68% and 28% for the North and West orientation respectively, moreover, the condensation resulting from some unpruned olive orchards creates shade thus humidity.

We surveyed areas of altitude ranging from less than 100m (Melbou region at the seaside) to more than 1200m (the heightsof Ain El Hammam). Altitude seems to be a limiting factor of attack intensity, as demonstrated by Rhimini et al. (2018) who observed that in high and medium altitudes, olive trees present a higher number of knots, as noticed in California (Fayard et al. 2011). In our study, olive trees located by the sea were heavily infected, which would be due to the relative humidity in this area (Young, 2004). As well as the high altitude areas, the olive trees were moderately to heavily infected, probably due to the air currents, the currents at these levels causing injury to the olive branches and therefore easy penetration of the bacteria.

Furthermore, infection of the plant requires the presence of openings such as leaf scars, pruning wounds, frost and hail cracks or harvesting operations (Benjama, 1990; Bouaichi et al. 2019). Abscission points on leaves, flowers and fruits are also entry points for the bacterium (Benjama, 1990).

The bacterium is spread by insect bites, bird bites and human activity (pruning, pruning and harvesting). In addition, many conditions can help in its spread such as variation in climatic factors (continentality, altitude and exposure to slopes). Moretti et al. (2008; 2017) and Krid et al. (2011) found an association with geography, but not for Scortichini et al (2004). Another determining factor for the spread of the disease seems to be the very active circulation of plants and the origin of the olive trees (Bnjama, 2003).

All olive varieties can be affected, but at very variable levels of susceptibility. The virulence of the disease and its spread within the orchard seem to be more related to the bacterial strain. As demonstrated in several previous studies, especially in Italy, and all these studies agree that there is no resistant genotype so far (Panagopoulos, 1993; Hassani et al. 2003; Penyalver et al. 2006; Quesada et al. 2010; Valverde et al. 2020). Algerian strains isolated by Moretti et al. (2017) were classified as the most virulent among many strains from 15 different countries.

The unaffected orchards in the surveys were typically extensive and family-scale olive groves with almost no cultivation practices, the olive trees were never pruned according to their owners, which would have strongly contributed to their sanitary state as explained above that the pruning operation and tools present a triggering factor for the disease.

Algeria, thanks to its geographical situation and its pedoclimatic diversity, has a fairly rich assortment of varieties, each marking the edaphic and climatic features that characterize its area of implantation (Douzane et al. 2010). The main varieties cultivated are Chemlal, which occupies 45% of the national olive orchard, located mainly in the Kabylie region, and Azeraj, which accounts for 10% and is located in the Béjaia region (Mendil and Sebai, 2006).

The Chemlal variety, which is mainly planted, is self-sterile and is always associated with other varieties that ensure its pollination, such as Azeradj. Most trees are grafted onto Oleaster seedlings. Despite its adaptability, the Chemlal variety has the disadvantage of being

susceptible to diseases, and to attacks by the *P. savastanoï* (Monji, 2002).

The Azeradj variety was found to be more resistant, and in most of the orchards visited, only 1/5 were contaminated and with low levels of attack.

We also observed infection of oleaster in some orchards visited from different sites in Bouira and TiziOuzou (and not in Béjaia), which has already been reported in the literature, and which has been identified as a host plant of the pathogen (Bradbury, 1986).

This susceptibility is favoured by the presence of certain plant species that are very sensitive to infection by *P savastanoi*, such as oleander on the banks of rivers, ash trees, privet, forsythia, jasmine, etc., which grow within oleaster populations and can constitute a source of inoculum, and this infection of oleaster would be responsible for transmission of the pathogen to the olive trees to which they are grafted (Rhimini et al. 2014). And the severity of the disease increases with the number of scars and wounds (Teviotlade and William, 2004).

Regarding the severity of knot olive, the results of a study conducted by Rhimini et al. (2018) in Morocco showed that Oleaster trees are more susceptible to knot olive than olive trees, thus more infected.

Moreover, the disease wasfound in all olive trees, both in adult and young plants; given that our sample showed age heterogeneity ranging from 1 to 100 years, this was also observed by the work of Perez-Martínez et al. (2010).

Intensity of attack

Olive trees are unevenly affected by the disease, so three levels of attack were defined for each plot studied. Some trees were defined as weakly attacked, others as moderately attacked and finally trees that were strongly attacked (figure 2).

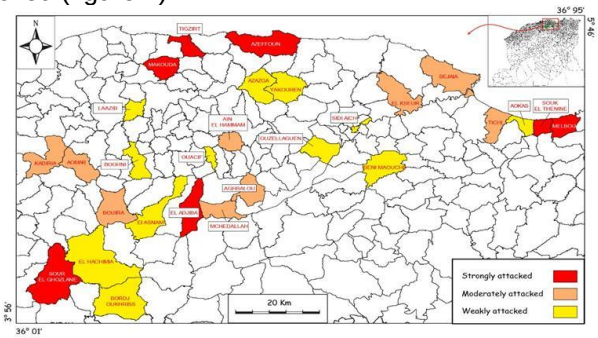

Figure 2: Mapping of olive knot in the surveyed sites.

In our study, the orchards used to assess the intensity of attack were mostly moderately to heavily attacked by the disease, which is not different from the observations of Oulebsir-Mohandkaci et al. (2017) who noted a high infestation rate exceeding 50% in the majority of the olive orchards surveyed (9 communes surveyed, including sites visited during our surveys in the wilayas of Bouira and TiziOuzou).

The work of Krid et al. (2011) allowed the identification of four degrees of attack in the northern region of Tunisia, namely strong, medium, weak and very weak infestation. This study revealed that the intensity of attack is positively correlated with the size and number of knots.

Olive knots, whose pathogen has a very heterogeneous population and a chemical pathogenic process, has an impact on annual autumn and spring shoot growth and on fruit yield. A close correlation was found between the number of tumours per linear metre of shoot and shoot growth. As the number of tumours increases, shoot growth is retarded and the number of fruit clusters is affected. The disease also causes leaf dryness. For the Meslala variety, desiccation and defoliation starts as soon as the number of tumours per tree reaches or exceeds a threshold of 600 tumours (Benjama, 2003).

The incidence of the disease differs between olive varieties although knots develop under all conditions evaluated (Nguyen et al. 2017).

Isolation and phenotypic characterisation

White to pale yellow colonies of 1-3 mm diameter resembling fried eggs, mostly expressing a characteristic fluorescence typical of *P. savastanoi*, were observed on King B medium after 2-4 days of incubation. However, some isolates did not show fluorescence, according to Surico and Marchi (2003), these populations are increasingly frequent, and show a fractal growth capacity under limiting environmental conditions (adaptation to unfavourable conditions).

All isolates were negative for the levan, oxidase, pectinolytic activity, arginine dihydrolase and gelatin liquefaction tests, except for the isolate from the orchard of the commune of Asnam which showed a positive result for the levan test. Several authors have detected levane-positive strains from isolates from several countries such as Spain and Italy (Janse, 1981; Alvarez, et al. 1998; Scortichini et al. 2004; Marchi et al. 2005).

However, the isolates were positive for catalase, tobacco hypersensitivity with reactions varying from slow (more than 48h) to very fast (less than 24h).

According to the Api 20 E biochemical gallery, sugar utilisation was variable between isolates, 93% were able to degrade glucose, and 64% degraded mannose and sucrose, only one isolate degraded melibiose (Table 2).

Several authors have reported variable responses towards sugar utilisation within *P. savastanoi* strains (Taghavi and Hasani, 2012, Khezri et al. 2019).

There is clear phenotypic heterogeneity in *P. savastanoi* strains, and this heterogeneity in biochemical traits may be due to environmental factors, resource availability in the host plant, or the fact that they are different pathovars (Alvarez, 1998).

Results of pathogenicity test

The inoculated olive plants were periodically inspected for the appearance of symptoms of the pathology. 14 isolates allowed the development of

characteristic knots, symptoms appeared after an interval of 25 to 70 days (5-10 weeks) for *O. europaea* (Figure 3), this reaction is relatively longer compared to that noticed by other authors; citing Lamichhane and Varvaro (2013), having observed the production of knots after 4 to 5 weeks of inoculation, and after a maximum of 6 weeks in another study (Campos et al. 2009). On the other hand, Saadi (2009) reported duration of 90 days after infection for the appearance of knots on healthy olive plants.

Figure3: Positive plant pathogenicity test on olive plant.

The 14 isolates that allowed the development of symptoms on olive plants belonged to 14 sites among the sampled sites located in several communes of three wilayas; the wilaya of Bouira (Bireghbalou, Hachimia, Sour El Ghozlane, Bouira, Mechdallah, El Asnam), the wilaya of TiziOuzou (Ouacif, Azazga, Yakouren, Azefoun, Ain El Hammam), the wilaya of Béjaia (Beni Maouche, Souk Thnin, El Kseur).

For the carrot test, white growths appeared for only 4 isolates after about 25 days of inoculation, the 4 positive isolates came from 4 sites; Ain El Hammam, Beni Maouche, Souk Thnin, El Kseur, these sites are included among those positive to the phyto pathogenicity test on olive plants. The other slices remained negative whatever the extension of the incubation period (figure 4).

According to Doksöz and Bozkurt (2020), the first knot formation on carrot slices was observed one week after inoculation and typical knots were fully developed within two weeks after inoculation, the authors attest that this technique could be successfully used for the pathogenicity of *P. savastanoi*. This is quite different from our results, as the growths only appeared completely after 4 weeks of inoculation and not forall isolates.

The biochemical and biological characteristics, as well as the sugar assimilation tests of the 14 strains identified and confirmed by a positive reaction of phytopathogenicity test on olive plant are listed in table 2.

Figure 4: Positive plant pathogenicity test on a slice of carrot

Molecular confirmation by PCR test

Only the 14 isolates positive for the phytopathogenicity test on olive plants were positive in the PCR test, inducing an amplification of the iaaL gene (454 bp) confirming the presence of *P. savastanoi* (Figure 5).

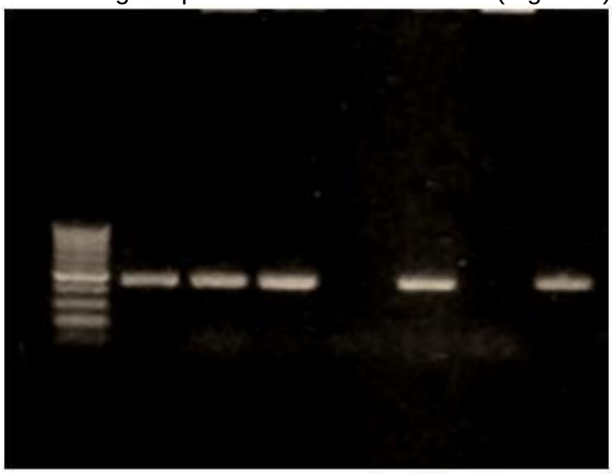

Figure 5: Visualisation of PCR amplification products of the iaaL gene from some *P. savastavoi* strains isolated in the wilaya of Bouira.

1 :*Bou*, 2 : *EAs*, 3 : *AEl*, 4 : Negative isolate of the commune of Tighzirt, 5 : *STh*, T+ : Positive control, T- : Negative control

The Associated bacteria

Bacterial colonies isolated from olive knots that caused hypersensitivity reactions on tobacco leaves indicating that they are phytopathogenic (a characteristic shared by many phytopathogenic bacteria) (Marchi et al. 2006; Hosni, 2010; Buonaurio, 2008), but without causing the formation of characteristic symptoms on olive plants were identified by means of biochemical tests on Api 20 E gallery. The study of the different parameters made it possible to say that the bacteria associated with olive knots seem to be Pantoaspp alone or associated with others such as *Stenotrophomonas*, *Rahnella*, *Seratia*, *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Citrobacter freundi*.

Table 1: Different localities surveyed and the incidence of tuberculosis of olive trees in the central region of Algeria.

Province	Region	Number of fields attacked / fields visited	Sampledfield		
			Variety	Number of affected trees/total trees (Incidence%)	Tree attack intensity
Bouira	Semach	4/5	Chemlal	21/115 (18%)	Heavilyattacked
	Sour el Ghozlane	2/5	Chemlal	4/46 (8%)	Heavilyattacked
	Kadiria	3/5	Chemlal	33/90 (37%)	Moderatelyattacked
	El Hachimia	3/5	Chemlal	4/70 (5%)	Weaklyattacked
	Mechdallah	4/5	Chemlal	17/142 (12%)	Moderately attacked
	Bireghbalou	2/5	Chemlal	6/45 (13%)	Moderatelyattacked
	El Asnam	3/5	Azeradj	150/300 (50%)	Weaklyattacked
	Borj Khris	2/5	Chemlal	15/92 (16%)	Weaklyattacked
	Bouira	3/5	Chemlal	7/60 (12%)	Moderatelyattacked
	Aomar	3/5	Chemlal	17/50 (34%)	Moderatelyattacked
Tizi Ouzou	Ouacif	3/5	Chemlal	24/100 (24%)	Weaklyattacked
	Azazga	2/5	Chemlal	14/98 (14%)	Weaklyattacked
	Yakouran	2/5	Azeradj	3/31 (10%)	Weaklyattacked
	Laazib	3/5	Chemlal	17/83 (20%)	Weaklyattacked
	Azefoun	4/5	Chemlal	44/102 (43%)	Heavilyattacked
	Tighzirt	3/5	Chemlal	62/90 (69%)	Heavilyattacked
	Makouda	2/5	Azeradj	17/55 (31%)	Heavilyattacked
	Ain El Hammam	3/5	Chemlal	40/120 (33%)	Moderatelyattacked
	Boghni	1/5	Azeradj	7/75 (9%)	Weaklyattacked
Béjaia	Béjaia	1/5	Limli	12/85 (14%)	Moderatelyattacked
	Tichy	2/5	Chemlal	6/48 (12%)	Moderatelyattacked
	Aoukas	1/5	Azeradj	9/80 (11%)	Weaklyattacked
	Melbou	1/5	Chemlal	4/38 (10%)	Heavilyattacked
	Ouzelaguen	2/5	Azeradj	10/78 (13%)	Weaklyattacked
	Sidi Aich	1/5	Chemlal	6/70 (9%)	Weaklyattacked
	Souk Thenin	2/5	Chemlal	25/83 (30%)	Heavilyattacked
	Beni Maouch	1/5	Azeradj	5/60 (8%)	Weaklyattacked
	El Kseur	2/5	Taksrit	7/60 (11%)	Moderatelyattacked

Table 2 : Phenotypic identification of PCR confirmed strains.

Regions	Code	Tests LOPAT					Cat	Use of sugars								
		Lev	Oxy	Pec	Adh	Ht		Glu	Man	Ino	Sor	Rha	Sac	Mel	Amy	Ara
Bireghbalou	*Bir*	_	_	_	_	+	+	+	+	_	_	_	+	_	_	_
Hachimia,	*Hac*	_	_	_	_	+	+	+	_	_	_	_	_	_	_	+
Sour El Ghozlane	*SEG*	_	_	_	_	+	+	+	+	_	_	+	+	_	+	+
Bouira	*Bou*	_	_	_	_	+	+	+	_	_	+	_	+	_	_	_
Mechdallah	*Mec*	_	_	_	_	+	+	+	+	_	_	_	+		_	_
El Asnam	*EAs*	+	_	_	_	+	+	_	_	_	_	_	+	+	_	_
Ouacif	*Oua*	_	_	_	_	+	+	+	+	+	+	_	_	_	+	_
Azazga	*Aza*	_	_	_	_	+	+	+	+	+	+	_	_	_	+	_
Yakouren	*Yak*	_	_	_	_	+	+	+	_	_	+	_	_	_	_	_
Azefoun	*Aze*	_	_	_	_	+	+	+	+	_	_	+	+	_	_	_
Ain E l Hammam	*AEl*	_	_	_	_	+	+	+	_	_	_	+	+	_	+	+
Beni Maouche	*BMa*	_	_	_	_	+	+	+	+	+	+	_	_	_	+	_
Souk Thnin	*STh*	_	_	_	_	+	+	+	+	_	_	_	+	_	_	_
El Kseur	*EKs*	_	_	_	_	+	+	+	+	+	_	+	+	_	_	_

***Lev** :levane, **Oxy** : oxydase, **Pec** : pectinase, **Adh** : arginine dihydrolase,**Ht** : hypersensibilité sur feuilles de tabac, **Cat** : catalase, **Glu** : glucose, **Man** : mannose, **Ino** : inositol, **Sor** : sorbitol, **Rha** : rhamnose, **Sac** : sucrose, **Mel** : melibiose, **Amy** : amygdalin **Ara** : arabinose.*

Co-inoculations of some of these species with *P. savastanoi* have shown that they result in larger nodes and better bacterial colonisation compared to single inoculations of P. savastanoi. These bacteria cohabit with the pathogen within the knots, leading to the formation of stable bacterial clusters that can facilitate the exchange of group sensing signals and metabolites. There is a growing interest in olive knot as a molecular model system for the study of the role of bacterial communities in plant disease development, indeed, the knot is considered a microbiome (Buonaurio et al. 2015: Moretti et al. 2021).

It is worth noting that *Pantoaagglomerans* was identified by Savastano as the causal agent of olive tuberculosis, until the work of Smith and Petri who rejected the identity of the isolated bacterium by calling it *P. savastanoi* and announcing its association with Pantoa (Surico and Marchi, 2003).

Pantoeaagglomerans has often been reported to be associated with olive knots, it is considered epiphytic and very common on aerial parts of plants (Surico and Lavermicocca, 1989; Fernandes and Marcelo, 2002; Bouaichi et al. 2015).

It turns out that *P. agglomerans*, although it would be involved in the increase in knotsize induced by *P. savastanoi*, competes with the latter probably for nutrients and occupied space, and can even produce antibiotics (Cimmino et al, 2006; Marchi et al. 2006); Studies by Fernandez and Marcelo (2002) noted a 15-fold higher number of *P. agglomerans* colonies than *P. savastanoi* isolated from the same knot; this could explain the absence of P. savastanoi on the knotafter it is formed. Furthermore,Buonaurio et al. (2015) state that co-isolation of bacterial species in a given niche does not automatically imply cooperation or synergy between them; it indicates that some type of reaction is taking place such as competition in our case.

The hypersensitivity reaction on tobacco leaves produced by *P. agglomerans*has been demonstrated in several studies to be due to the formation of indole pyruvic acid from tryptophan by tryptophan deaminase. The indole-3pyruvate pathway is a major auxin pathway in plants and is induced by many phytopathogenic bacteria (Moore and Warren, 1979; Prinsen et al. 1993).

Furthermore, the hypersensitivity reaction induced by the strains can be explained by the presence of the hrp/hrc gene; which encodes components of a type III secretion protein system believed to be used by plant pathogenic bacteria, and transported directly into host cells, these virulence proteins are thought to be responsible for the leakage of plant nutrients into the extracellular space of infected tissues and suppression of host defences (Moretti et al. 2014).

Ercolani (1978);Krid et al. (2010);da Silva et al. (2014) when isolating endophytic bacteria associated with olive tumours reported the presence of other species besides *P. savastanoi* belonging to the family of enterobacteria or others, among others *Pantoa*, *Stenotrophomonas*, *Enterobacter*, and *P. fluorescens*.

P. fluorescens may have antagonistic activity against *P. savastanoi* (Krueger et al. 1999), possibly due to the production of siderophores. Several strains of *P. fluorescens* produce different antibiotics such as 2,4-diacetylphloroglucinol (DAPG) with antibacterial, antifungal, antiviral and antihelminthic properties (Velusamy et al. 2006). In the study conducted by Benyoub et al. (2019) in western Algeria, isolated *P. fluorescens* expressed inhibitory activity towards *P. savastanoi*.

The presence of *Citrobacter freundi* in the orchard located in Sour El Ghozlane would be due to its location near a water source (Puits).

For many years, phytohormones produced by *P. savastanoi* were considered as main virulence factors (Caponero et al. 1995). More recently, other bacteria, such as *Pantoeaagglomerans*, *Enterobacter sp*., *Pseudomonas sp*., *P. fluorescens*, *Stenotrophomonasmaltophilia* and *Burkolderiacepacia* appear to interact with *P. savastanoi*. and contribute to its pathogenicity based on 16S rDNA analysis (Cimmino et al. 2006; Marchi et al. 2006; Ouzari et al. 2008; Krid et al. 2010).

CONCLUSION

The mild climate and high rainfall of our mainly mountainous study area would have contributed to the dissemination of the pathogen *P. savastanoi*, the symptoms of olive knot were observed in 65 orchards among the 140 surveyed and spread over the whole study area, but mainly in the wilayas of Bouira and TiziOuzou (at least 3 infected orchards out of 5), and an incidence reaching 69%, and for most of them moderately to strongly attacked by the disease

North and West exposure favours infection and increases the incidence of the disease in an orchard as well as the intensity of attack, being a humid area that receives more rainfall and less exposure to the sun. The location of olive trees along roadsides and tracks as well as near water points (wells, stagnant water on heavy soils) would increase the risk of infection.

Whatever the olive tree varieties (chemlal, azzeradj, limli, taksrit), and the heterogeneity of ages (from 1 to 100 years) of our sampling, the strains proved to be virulent for all olive trees

In our study, the olive trees located at the seaside were highly infected from (less than 100 m altitude), which would be due to the relative humidity level. As well as the high-altitude areas (above 1200 m), the olive trees were moderately to heavily infected probably due to air currents, the currents at these levels causing injury to the olive branches and therefore easy penetration of the bacteria.

We also observed oleaster infection in some orchards visited from different sites in Bouira and TiziOuzou, which

are known to be resistant to biotic and abiotic factors.

According to our field surveys, what really seems to be a limiting factor is the pruning of the trees, as healthy olive trees were never pruned

Phenotypic characterisation of the strains confirmed by phyto pathogenicity test on healthy olive plants and by PCR revealed a Levane positive strain, and variability in sugar utilisation. However, the carrot slice phyto pathogenicity test was not a good indicator in terms of speed of results and sensitivity (positive reaction after about 4 weeks of inoculation, and for only 4 of the 14 confirmed strains).

From morphological, biochemical and biological tests, endophytic bacteria seem to be associated with olive knots, such as Pantoaspp alone or associated with others such as *Stenotrophomonas*, *Rahnella*, *Seratia*, *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Citrobacter freundi*. These bacteria could contribute to the depreciation of *P. savastanoi* populations and their non-detection in some samples.

Management of this disease is difficult, and only a limited number of treatments are currently available for its control. On the agronomic level, preventive measures based on good tillage in orchards with heavy soils allowing good water run-off and the installation of natural windbreaks on the edges of orchards on the one hand, and on the other hand the control of neighbouring vegetation which may harbour the pathogen, could reduce the risk of contamination.

Epidemiologically, it is necessary to study the role of endophytes in the formation and evolution of the disease, such as *Pantoa*, which seems to compete with *P. savastanoi* probably for nutrients and space, and can even produce antibiotics. The selection of bacteria from the olive phyllosphere, especially *P. fluorescens*, capable of antagonism to *P. savastanoi* would be useful to reduce symptom expression in olive trees infected with *P. savastanoi.*

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

MAH, SM, KM, SM designed and performed the experiments and also wrote the manuscript. MAH, SM, KM, SM and SL carried out formal treatments, resources, data curation and data analysis. SM, KM, SM and SL supervised and edited the manuscript. All authors read and approved the final version.

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