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Response of 'Ousleti' Olive Pollen to *Pseudomonas syringae* pv. *savastanoi* Culture Filtrate

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ABSTRACT

Olive knot is the only bacterial disease of the olive tree, caused by *Pseudomonas syringae* pv. *savastanoi* L. The most effective measure for controlling this bacterial disease is the use of resistant cultivars because chemical control is not effective. 'Ousleti', an important local olive cultivar in Tunisia, is reported to be resistant to this bacterial disease although there is no scientific study to prove this. In this preliminary study, the effect of a toxic culture filtrate of *P. savastanoi* on pollen germination and pollen tube growth was evaluated *in vitro* on two Tunisian olive cultivars; one tolerant 'Ousleti' and one susceptible 'Chemlali'. The variety x treatment interaction was significant indicating that cultivars differed in their response to the treatment. Cultivar differences in the reaction of olive pollen to bacterial filtrate were noted. The addition of *P. savastanoi* culture filtrate to the germination medium had no significant effect on the percentage of pollen germination and the tube length in the tolerant olive cultivar 'Ousleti' in comparison to its control but they were highly reduced in the susceptible one, 'Chemlali'. In addition to inhibiting pollen germination and tube elongation in 'Chemlali', culture filtrate applied to culture medium also influenced tube morphology. Several abnormalities in pollen tube growth were observed when culture filtrate was added to germination medium of 'Chemlali' pollen. These results suggest that this tested method, based on pollen response, enabled rapid and effective evaluation of *P. savastanoi* on olive pollen grains then on olive resistance. Also, the percentage of pollen germination and tube growth in the presence of culture filtrate medium might be correlated with the plant's response to the pathogen.

Keywords: cultivar tolerance, *Olea europaea*, pollen germination and tube growth

INTRODUCTION

In the last few years, there has been an increase in interest in the olive crop, not only in traditional countries where it exists, but also in some American countries and in Australia, largely due to the benefits of olive oil for human health (Alarcon de la Lastra *et al*. 2001). In Tunisia, olive trees are widely cultivated and play an important role in the economy and culture. At present, about 1.68 million ha and 65.9 millions olive trees are grown, giving approximately 130.000 tons of olive oil in 2005 (DGEDA 2006).

Olive knot, caused by *Pseudomonas syringae* pv. *savastanoi* (ex Smith 1908) (hereafter *P. savastanoi*) (Gardan *et al.* 1992), is the only bacterial disease of the olive tree and is considered as one of the most important diseases affecting this crop because the bacterium produces tumorous outgrowths on different parts of affected plants (Wilson 1965; Nester and Kosuge 1981).

Affected trees consistently display distinct irregularly shaped proliferations (knots) mainly on primary and secondary branches. The virulence of *P. savastani*, which causes hyperplastic symptoms (knots) on olive plants, is associated with the ability of the bacterium to produce phytohormones (indole-3-acetic acid [IAA] and cytokinins) (Surico *et al*. 1985; Surico and Lacobellis 1992; Teviotdale and Krueger 1998) that alter the physiological hormone balance in infected tissues and cause the proliferation of plant cells surrounding the infection site, inducing cell proliferation and tumour formation. This causes serious problems in olive trees such as reducing plant growth. The severity of tumor formation depends on weather conditions, location and cultural practices (Baker 1992).

There is a general lack of knowledge on virulence and pathogenicity determinants specific to infection of woody plants (Perez-Martínez *et al.* 2007). Olive knot control measures are usually preventive; the disease is controlled by fall application of copper-containing bactericide after harvest. This practice protects against olive leaf spot but provides only minimal protection against olive knot (Teviotdale and Krueger 2004).

Because chemical treatments are not effective, disease control efforts should focus on the development of genetically resistant cultivars. Several research projects have been carried out on olive cultivars (Ben Jemaa *et al.* 1987; Sisto and Lacobellis 1999; Sisto *et al.* 2001), in which the authors suggested that the most effective measure for controlling *P. savastanoi* is through the use of resistant cultivars. In olive, various cultivars show different degrees of sensitivity to *P. savastanoi*, 'Manzanillo' and 'Picholine' cultivars are the most susceptible to infection; 'Koroneiki' is the least susceptible and 'Sevillano', 'Mission', and 'Ascolano' are more resistant to olive Knot.

The role of susceptible cultivars in the spread of olive knot underlines the importance of screening for resistance to the disease. This requires a screening method that can be carried out with many cultivars or selections simultaneously without being too laborious, expensive and time consuming. A simple method of screening olive cultivars for resistance to *P. savastanoi* has been developed. Small sectors of the interior knot tissue were excised and crushed in water to release the bacteria. The use of King's B medium favoured bacterial growth and gave distinctive colony types (Perez-Martínez *et al.* 2007). The confirmation of pathogenicity is achieved by inoculation heavy suspension of bacteria into the stems of actively growing plants of susceptible and resistant olive plants kept under controlled conditions and

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this resembles more closely what occurs during natural infection of *P. savastanoi* in olive trees (Sisto *et al.* 2001). However, results from those experiments were often inconclusive because the pathogenicity tests are best done between spring and mid summer and in autumn when plants are most actively growing, but they can be done at all times of the year.

In several species, the use of selective agents has been found to increase the efficiency of selection. We used in this study, pollen grains as explants but little information about pollen response to selective agents in olive trees exists (Mehri *et al.* 2006). Application of three insecticides and a bio-insecticide *Bacillus thuringiensis* (Bt) on olive, inhibited pollen germination and tube growth both *in vitro* (added to the germination media) and *in vivo* assays (sprayed on shoots during green-cluster stage and incubated on insecticide-free medium).

In order to study the resistance of two Tunisian olive cultivars to *P. savastanoi*, selection pressure, using bacterial filtrate, was applied to germination medium of pollen grains. In this preliminary study, the effects of a toxic culture filtrate of *P. savastanoi* on pollen germination and tube growth was evaluated *in vitro* on two olive cultivars; one tolerant 'Ousleti' and one susceptible 'Chemlali' (based on our observations in olive orchards).

MATERIALS AND METHODS

Olive cultivars

'Ousleti' and 'Chemlali' are both important local olive cultivars in Tunisia. 'Ousleti', was reported to be tolerant and 'Chemlali' to be susceptible to *P. savastanoi* disease without any scientific study.

'Chemlali' is the most frequently planted olive cultivar in the center and southern parts of Tunisia; it covers 60% of olive orchards and contributes to 70% of olive oil production in Tunisia (Fourati *et al.* 2003). The tree is vigorous with a drooping growth habit. It has a very small fruit size with high oil content (26-28%), high amount of saturated fatty acids (palmitic and stearic acids), high polyphenol content and a strongly aromatic fruitiness.

'Ousleti' is a local olive cultivar cultivated in the central part of Tunisia (Djebel Ouslet and Ousletia). It is appreciated and well adapted in its home localities because it develops a compact canopy, more efficient for mechanical harvest and valuable for high density plantings. It performs very well in Djebel Ouslet for dual purposes: oil and table production (Mehri and Hellali 1995).

Pollen collection and germination

Pollen of each cultivar was collected from flowers having the same physiological stage from various inflorescences and shoot locations. The germination solid medium, composed of distilled water supplemented with 0.7% agar, 20% sucrose, 100 ppm H_3BO_3 at pH 5, was sterilised by autoclaving during 20 min at a temperature of 120°C and a pressure of 1bar (Mehri *et al.* 2003). Pollen cultures were maintained at 25°C for 72 h.

To evaluate the germination of pollen grains, 5 ml of pure bacterial culture filtrate was added to100 ml sterilized germination media at 60°C containing pollen grains, and then poured equally into five Petri plates (80 mm of diameter). In control plates, the quantity of bacterial culture filtrate was replaced by the same quantity of sterile distilled water. Germination rate and tube growth of pollen grains were scored during incubation. For each olive cultivar tested and during 72 h under selection pressure, the treated and the control pollen grains were microscopically observed to determine the germination rate and the percentage of grains that developed tubes longer than their diameters (Stanley and Linkens 1974). Pollen germination and tube growth were determined after 3, 6, 12, 24, 48 and 72 h on 200 pollen grains chosen at random from various locations in the pollen sample. Germination rate was determined using three replicates of approximately 100 grains (Pinney and Polito 1990).

Pathogen isolation

The culture filtrate was provided by the phytopathology laboratory of the High Institute of Agronomy in Chott-Meriem (Tunisia).

'Ousleti' and 'Chemlali' trees were grown in an experimental field in Chott-Meriem situated in the central part of Tunisia. Fresh green-grayish olive knots were collected during spring and early summer. They were taken from symptomless olive plants carefully detached from the shoots, and were surface disinfected with absolute ethanol. Then, they were crushed in mortar and soaked in physiological water (0.85%) for 30 min to release the bacteria. The bacterial suspension was then spread on a surface-dry solid King's medium B (KB) (King *et al.* 1954) that favoured both bacterial growth and gave distinctive colony types (Schaad *et al.* 2001). After an incubation period of about 2-3 days at 27°C, colonies of bacteria were re-streaked to obtain pure cultures of representative strains. Biochemical tests were applied for all the isolates. They were performed according to the method reported by Schaad *et al.* (2001). Tests included utilization of levane, oxydase, pectinase and arginine deshydrolase activity.

Statistical analysis

Experimental design used to determine the effect of bacterial culture filtrate on pollen germination is a complete plan with one factors corresponding to pollen of each cultivar. The number of repetitions is 5 plates by elementary treatment. Statistical analyses were conduced using the general linear models procedures (GLM) of the Statistical Package for the Social Science (SPSS 10.0). Experiments were analysed using the standard analysis of variance (ANOVA) with factorial treatment structure. Significance was evaluated at $P<0.05$ for all tests. Means separation was accomplished using Student-Newman-Keuls (SNK) test.

RESULTS

The results of the *in vitro* study (germination and the growth patterns of pollen grains) of tolerant ('Ousleti') and susceptible olive cultivar ('Chemlali'), in the presence or absence of bacterial filtrate of *P. savastanoi* are presented in **Fig. 1A**, **1B**. 'Chemlali' and 'Ousleti' pollen germination was achieved on media containing 20% sucrose, 0.7% agar and 100 ppm boric acid.

Control

The control was made of pollen grains which were cultured on germination medium without culture filtrate of *P. savastanoï*. For both 'Ousleti' and 'Chemlali' cultivars, pollen germination followed a typical sigmoidal curve. The mean germination percentage in the control, after an incubation period of about 3 h, was 12 and 3.8% for 'Ousleti' and 'Chemlali', respectively. The germination rate increased to 61 and 56.8% after an incubation period of 72 h (**Fig. 1A**). In 'Chemlali' grains, pollen tube elongation did not follow the pattern of pollen germination. Pollen tube growth in the control medium was poorer in the susceptible cultivar than in the tolerant 'Ousleti'. **Fig. 1B** indicates that pollen tube length reached after 72 h of incubation was significantly higher for 'Ousleti' pollen (642 µm) than for 'Chemlali' (496 µm).

Treatments

Germination and the pollen tube growth analysis distinguished distinct response patterns according to the cultivar and to the treatment. Mean comparisons between media (culture media with and without culture filtrate of *P. savastanoi*) were carried out for each of the 2 cultivars separately and showed cultivar x medium interactions for both percentage of pollen germination and tube growth.

Both 'Ousleti' and 'Chemlali' pollen grains exhibited slower germination in presence of culture filtrate of *P. savastanoi* in comparison to the control (**Fig. 1A, 1B**). Pollen germination in the medium containing the culture

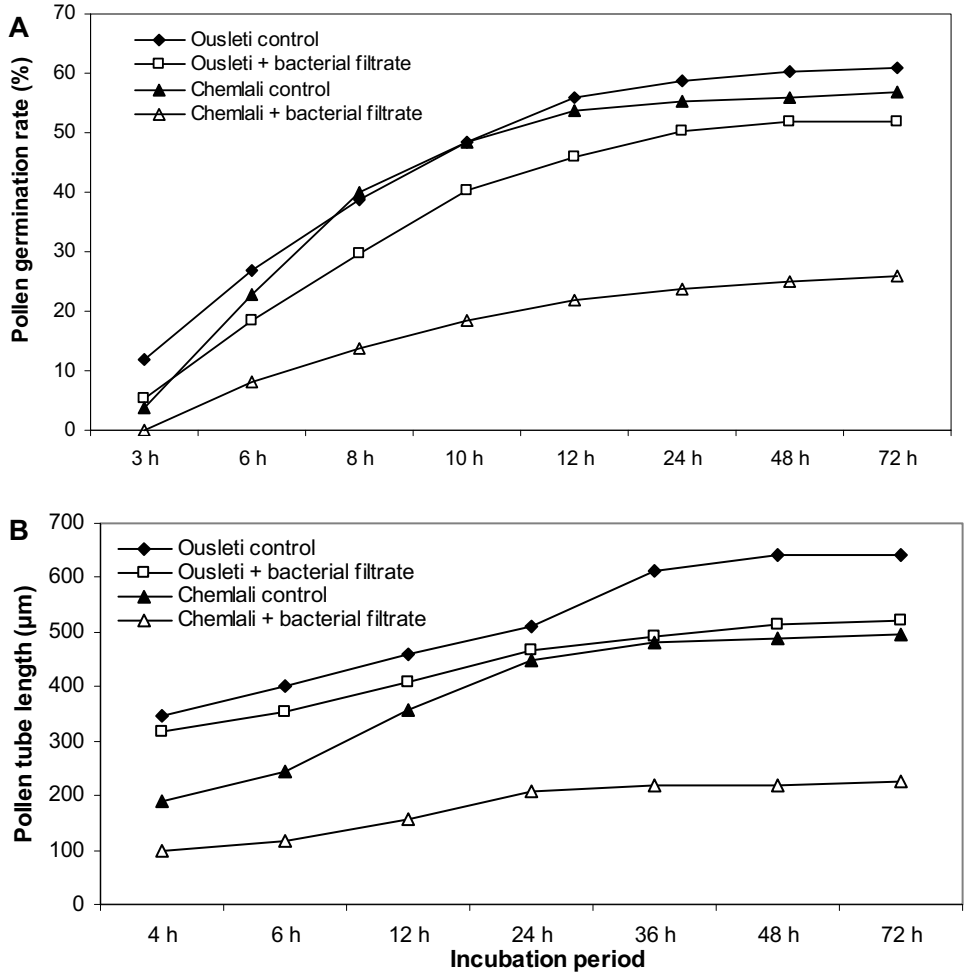

Fig. 1 ***In vitro*** **pollen germination (A) and tube growth (B) of 2 olive cultivars 'Ousleti' and 'Chemlali'.** Pollen grains were cultured on solid medium containing 0.7% agar, 20% sucrose, 100 ppm H_3BO_3 at pH 5 and held at 25°C for 72 h (control) in the presence of *P. savastanoi* culture filtrate.

filtrate was not significantly affected in the tolerant cultivar 'Ousleti' but was highly reduced in the susceptible one 'Chemlali'.

The toxic culture filtrate did not affect significantly either pollen germination or tube growth of the tolerant cultivar 'Ousleti' but negatively affected these two parameters by diminishing significantly the percentage of pollen germination and tube length of the susceptible cultivar 'Chemlali'.

In 'Ousleti' (**Fig. 1A**), there were no significant differences in mean per cent pollen germination among control (without filtrate) and treatment (in presence of culture filtrate of *P. savastanoi*). The similarity of germination of pollen grains in presence or not of *P. savastanoi* was revealed that there are no significant differences between treatment (52.03%) and control (60.9%) after 72 h incubation. Also, a similar rate of pollen tube growth was observed for 'Ousleti' pollen with or without culture filtrate, but there was slower and irregular growth in presence of *P. savastanoi* (**Fig. 1B**).

For 'Chemlali', the shape of the graph was similar for pollen grains cultured with or without culture filtrate of *P. savastanoi* but the maximum germination rates were significantly different (**Fig. 1A**). In presence of the bacteria, the maximum germination percentage reached, after 72 h of incubation, 26% compared to the control (when pollen grains were cultured on bacteria-free medium) with 56.8% after 72 h. This was probably due to the degradation of pollen grain capacity in the presence of bacteria. The tube growth was also affected by the culture filtrate on germination medium. It reached 227.4 µm after 72 h incubation while 496 µm in the control (**Fig. 1B**).

We noted also that pollen grains in presence of bacterial filtrate start germination later than control indicating that pollen need a long period to germinate. The filtrate could be of great significance for keeping pollen integrity and direct exposure of pollen grains to bacterial filtrate can be detrimental to viability and germination because of chemical toxicity. The duration of coculture (pollen-bacteria) may be an important factor for successful germination.

The mixture of resistance level of olive cultivar and the culture filtrate may be critical, the concentration of bacterial filtrate used in this study was perhaps high and increase its toxicity for pollen grains and thus resulted in poor germination.

Tube growth and morphology

There were highly significant differences in the mean elongation of pollen tubes among treatments. In control, the mean length of pollen tube in 'Ousleti' cultivar (642 µm) was significantly greater than in 'Chemlali' (496 µm).

Tested culture filtrate reduced tube growth of pollen and was more effective on 'Chemlali' than on 'Ousleti'. Pollen tube elongation in the presence of culture filtrate was lower in the susceptible cultivar (227.4 µm) than in the tolerant one (523 µm). The progression of pollen tube of 'Chemlali' was normal until 24 h incubation and stopped thereafter (**Fig. 1B**). The pollen grains turned rapidly brown within 24 h incubation and died just after. On the contrary, 'Ousleti' pollen had survived the action the action of bacterial filtrate until 72 h and continued growing very well. On the control medium, no symptoms of degradation were apparent before 72 h incubation.

The addition of culture filtrate to the germination medium altered pollen tube growth in the susceptible cultivar with respect to the control but had no effect on tube

growth in the tolerant one 'Ousleti' (**Fig. 1B**). For the latest, the tube growth was also affected by the culture filtrate on germination medium. It reached 523 µm after 72 h incubation while 642 µm in the control. There was no significant difference in tube growth of 'Ousleti' pollen cultured on germination medium with culture filtrate but there was less tube growth of 'Chemlali' pollen. However, the factors responsible for a reduction of activity of pollen grains have not been identified.

In 'Chemlali' grains, pollen tube elongation did not follow the pattern of pollen germination. Pollen tube growth in the control medium was poorer in the susceptible cultivar than in the tolerant one 'Ousleti'. The addition of culture filtrate to the germination medium had no effect on pollen tube growth in the tolerant cultivar but significantly increased it in the susceptible one in comparison with the control.

It is possible that some factors required for pollen germination and tube length of the susceptible cultivar are absent in the culture medium or the diminution of pollen germination in presence of bacteria is a defense response of the pollen against the pathogen attack. Also, *Pseudomonas* can produce plant growth inhibitors or analogous compounds in the susceptible cultivar 'Chemlali' that supplied by the culture filtrate and altered pollen germination and tube growth. It is in contradiction with the findings of Roussos *et al*. (2002). High level of auxins (indole-3-acetic acid, IAA and indole-3-acetonitrile, IAN) has been detected in olive knot crude extract which promote rooting *in vitro* of 'Koroneiki' olive explants.

In addition to inhibiting pollen germination and tube elongation in 'Chemlali' cultivar, culture filtrate applied to culture medium, also influenced tube morphology. This work shows that culture filtrate was associated with the germination capacity of pollen grains and the degree of tolerance of the olive cultivar. However, several abnormalities in pollen tube growth were observed mainly when pollen grains of 'Chemlali' cultivar were incubated in presence of *P. savastanoi* culture filtrate. These abnormalities consist of variable tube diameter, sigmoid growth and protuberant tube extremes. In control, 'Chemlali' and 'Ousleti' pollen had straight, long and smooth tubes. Pollen tubes grown in the presence of culture filtrate were characterized by swelling and rupture in the apical region, resulted in pollen tube smaller and with more abnormal morphology. Pollen tubes exhibited abnormal growth, with a sinuous and wavy configuration. These abnormalities in tube morphology, not reported in the literature on pollen reaction to pathotoxins, are identical to those described by Mehri *et al*. (2007) when insecticides at different concentrations were added to pollen germination medium of 'Coratina' olive cultivar.

All these results suggest that the percentage of pollen germination and tube length in presence of the culture filtrate of *P. savastanoi* might be correlated with the olive cultivar response to the pathogen. The reaction of pollen to culture filtrate was unknown but a significative correlation between plant reaction to the pathogen (tolerance or susceptibility) and the pollen reaction to the culture filtrate exists. These results are similar to those reported for *Asparagus officinalis* pollen with *Fusarium oxysporum* f. sp. *asparagi* toxins (Pontarolli *et al*. 2000).

DISCUSSION

The variety x treatment interaction was significant indicating that olive cultivars 'Ousleti' and 'Chemlali' differed in the reaction of their pollen to bacterial filtrate of *P. savastanoi.*

Evaluation of *P. savastanoi* resistance of olive trees has been achieved after artificial germination of pollen grains, but little information about pollen response to selective agents to control resistance cultivars to bacteria in olive trees. Pollen germination and tube growth *in vitro* have been used to assess self-compatibility in many species olive trees (Mehri and Kamoun-Mehri 1995; Mehri *et al*. 2003), and almond (Bernad and Socias I Company, 1995), to examine the effectiveness of three insecticides and a bio-insecticide (*Bacillus thuringiensis* (Bt) sprayed during olive bloom period of 'Coratina' olive cultivar (Mehri *et al.* 2006) and to control resistance cultivars to fungus (Pontarolli *et al*. 2000).

Our work shows that using pollen is a rapid and reliable technique for testing the resistance to *P. savastanoi* on olive trees. It also overcomes problems of the large amount of labour needed to inoculate artificially trees in the field. On the other hand, results with pollen were generated in few days instead of several weeks needed with previous methods. But evaluation of *Pseudomonas* resistance by using pollen grains is not enough.

This technique is valuable for an initial screening of material. Pollen can be very useful also for the early detection of *P. savastanoi* resistance in olive plants but a selection program for olive plants must include *P. savastanoi* in the certification schemes which required sensitive and specific methods for the detection of this bacterium.

Our tests showed that pollen grains of 'Ousleti' were the least susceptible to bacterial filtrate and survived to the selection pressure, on the contrary 'Chemlali' pollen was the most susceptible. This can suggest that the later was unsuitable for orchards in which the conditions are favourable for *Pseudonomas* disease. Therefore, an integrated approach including chemical measures and cultural control practices is recommended to manage diseases caused by *P. savastanoi* in olive orchards, mainly in the northern part of Tunisia which is the higher rainfall area. Sisto and Lacobellis (1999) note the importance of identifying resistant cultivars of olive in order to contain the disease and to improve both the quantity and the quality of olive fruit and oil yields. But many precautionary measures should be considered to eliminate possible sources of pathogenic bacteria, until the identification of resistant cultivars of olive is fully understood. Some practices must be avoided mainly the traditional method of harvesting fruit by beating branches with sticks can lead to severe infections (Panagopoulos, 1993). Little is known about the sensitivity of different olive cultivars to infection by *P. savastanoi* (Penyalver 2006) and until now, no olive cultivars are known that are completely resistant to olive knot and resistance alone will not control this disease (Young 2004).

It is clear that the reaction of pollen germination *in vitro* by the addition of olive knot extract to the medium is a complicated procedure and screening for resistance against *P. savastanoi* is not easy. Several factors such as climate conditions and time of exposure to bacteria have important effects.

In vitro pollen germination and bacterial filtrate are rapid and can be used to distinguish between cultivars in susceptibility to *P. savastanoi.* Wounding of plants with heavy suspension of bacteria is particularly time consuming and results not always consistent. Susceptibility of olive trees to *P. savastanoi* is known to fluctuate between years and during the year. Olive knot appears to be most severe in spring and autumn (Krueger *et al.* 1999).

The results of this study have important implications for the management of *P. savastanoi* under Tunisian orchard conditions. Generally, it was concluded that none of the olive cultivars was completely resistant to *P. savastanoi*. Therefore, chemical and cultural control measures must be integrated into an overall strategy for managing olive knot in olive orchards, mainly in the northern part of Tunisia which is the higher rainfall area.

The positive response of pollen grains to bacterial filtrate as well as its role in the initial screening of olive material in the evaluation of *P. savastanoi* and its possible reaction to the pathogen should be the topics of some further research. It would be interesting to investigate the possible role of pollen grains in the plant defence mechanism.

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