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# Epidemiology and Control of Plant Diseases Caused by Phytopathogenic Bacteria: The Case of Olive Knot Disease Caused by *Pseudomonas savastanoi* pv. *savastanoi*

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## 1. Introduction

*Pseudomonas savastanoi* pv. *savastanoi* (Gardan et al., 1992) (hereafter Psv, according to Vivian & Mansfield (1993)) is the causal agent of olive knot disease. It is considered one of the most serious diseases affecting olive trees (*Olea europaea* L.) in most olive growing regions worldwide and mainly in Mediterranean countries, where this crop has been growing for centuries. The disease can lead to severe damage in olive groves, causing serious losses in terms of production. This is probably the first disease clearly described in antiquity by Theophrastus (370-286 BC) (Iacobellis, 2001) and its bacterial etiology was known through the work of Savastano since 1887 (Smith & Rorer, 1904). However, there are currently many unknown facts about the epidemiology of this disease or its chemical control. Here we describe the most relevant studies performed on the epidemiology and chemical control of olive knot.

## 2. Biology of infection

Psv causes the formation of hyperplastic growth in olive trees, producing spherical knots on the trunk and branches, and less frequently on leaves and fruits (Sisto & Iacobellis, 1999; Smith, 1920). See details in figure 1. Psv infections in fresh wounds of olive trees start with a small cavity caused by the collapse of adjoining plant cells and are more frequent on trunks and branches, and rare on leaves and fruits. Subsequently, a proliferation of tissue follows the periphery of the cavity resulting in knot development (Smith, 1920; Surico, 1977). Tumor development is dependent on bacterial production of phytohormones indoleacetic acid and cytokinins (Comai & Kosuge, 1980; Iacobellis et al., 1994; Rodríguez-Moreno et al., 2008; Smidt & Kosuge, 1978; Surico et al., 1985). Besides, recent results have revealed that Psv strains contain two copies of all the genes involved in indoleacetic acid synthesis (Matas et al., 2009; Pérez-Martínez et al., 2008). It has been

reported that olive knots are also dependent on the *hrp/hrc* genes (Sisto et al., 2004), which encode the biosynthesis of a functional Type III Secretion System (TTSS). Recently, remarkable progress has been made in research into several aspects of the host-pathogen interaction of the causal agent of olive knot (Pérez-Martínez et al., 2008; Matas, 2010; Pérez-Martínez et al., 2010). Several putative virulence factors in Psv have been identified, including TTSS protein effectors and a variety of genes encoding known *P. syringae* virulence determinants (Pérez-Martínez et al., 2008). Analyses of TTSS protein effectors of Psv have recently shed light on the role of TTSS in pathogenicity and host range (Matas, 2010; Pérez-Martínez et al., 2010).

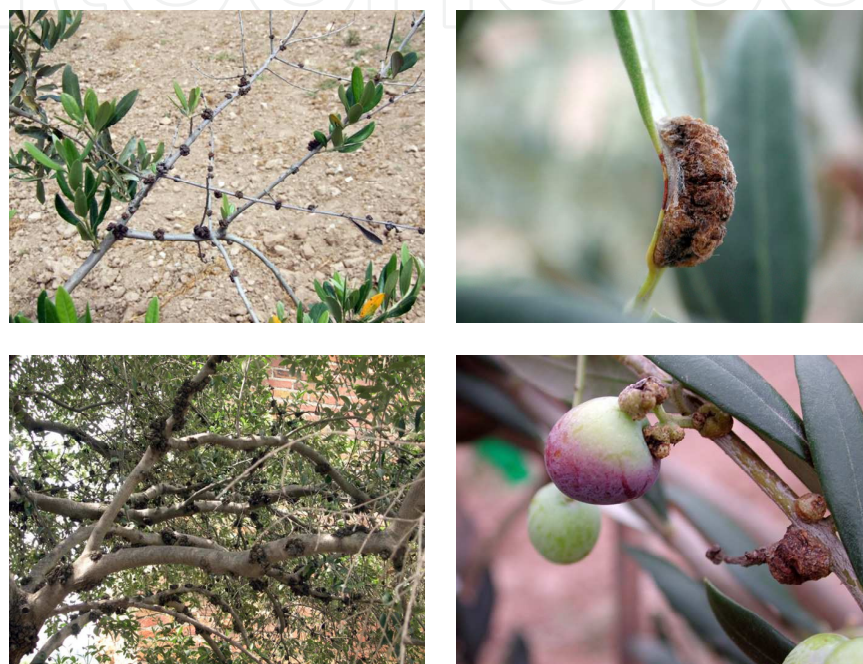


Fig. 1. Typical olive knot symptoms caused by *Pseudomonas savastanoi* pv. *savastanoi* on twigs (upper left), leaf (upper right), branches (lower left) and fruits (lower right).

Anatomical studies of knots have been performed in olive (Smith, 1920; Surico, 1977), oleander (Wilson & Magie, 1964; Wilson, 1965) and more recently, in buckthorn (Temsah et al., 2007a) and myrtle (Temsah et al., 2007b) by light microscopy. Rodríguez-Moreno et al. (2009) performed the first real-time monitoring of Psv disease development and the first illustrated description of the ultrastructure of Psv induced knots. They examined knot sections using a green fluorescent protein tagging a Psv strain, coupled with epifluorescence microscopy and scanning confocal electron microscopy. Additionally, scanning and TEM (transmission electron microscopy) were used for a detailed ultrastructural analysis within knot tissues (Rodríguez-Moreno et al., 2009).

Infection by Psv and subsequent knot formation in young twigs of oleander (*Nerium oleander*) requires vascular cambium activity (Wilson, 1965). The host invasion by the bacterium begins with the colonization of the infection site, followed by the disintegration and breakdown of adjacent plant cells that results in the formation of a large cavity around the area colonized by the bacteria. Curiously, this bacterium produces cell wall degrading enzymes *in vitro* such as cellulase, cellobiase, xylanase and peptinase (Magie, 1963). In a

second phase, intact cells surrounding the pathogen suffer the effect of the hormones that Psv produces and increase in size (hypertrophy) followed by an abnormal cell division (hyperplasia). Finally, there is a differentiation of certain cells of the hyperplastic area, elements of xylem and phloem.

During infection of young olive stems after inoculation, the bacteria multiply by a succession of phases which include a population increase, a stationary phase and a population decline. There is a clear parabolic trend whose maximum value depends on cultivar susceptibility (Varvaro & Surico, 1978). Pathogen multiplication inside tissues of micropropagated olive plants can reach densities of  $10^7$  to  $10^8$  cfu/ knot (Rodríguez-Moreno et al., 2008), values very similar to those previously described with 1-2 years old seedlings by Penyalver et al. (2006). The first reaction of tissue from the inoculated slit of a young stem is to renew or quickly increase cambium activity, although this depends on whether the inoculation takes place in winter, summer or spring (Surico, 1977). The increased activity of the cambium promotes the formation of two new tissue masses on both sides of the wound, which grow until their junction and form a knot. Differentiation of phloem and xylem elements, which are organized or not in vascular bundles, occurs within the new parenchyma tissue. Light microscopy shows the presence of vascular bundles of new formation in olive knots, connected with the stem vascular cylinder (Rodríguez-Moreno et al., 2009). Psv has been located in cavities formed after the collapse of intercellular plant cells, as well as in peripheral areas close to the epidermis or invading the newly formed xylem bundles (Rodríguez-Moreno et al., 2009). This could be related to the spread of the pathogen and its external output through plant exudates. Formation of bacterial aggregates, microcolonies and multilayer biofilms has been observed in knot sections by scanning electron microscopy. Besides, TEM analysis of knot sections shows the release of outer membrane vesicles from the pathogen surface (Rodríguez-Moreno et al., 2009).

Subsequently, in old knots, plant cells collapse and form cavities containing large numbers of bacteria. Fissures reaching the knot surface develop inside these cavities, allowing bacteria to escape to the external surface of intact knots (Surico, 1977). However, it remains unclear how knot formation in the host benefits Psv. Knots may represent a favorable environment for bacteria to multiply and also protect them against extreme environmental conditions, such as the usually dry and hot summers of the olive-growing areas (Comai & Kosuge, 1980).

The causative agent of olive knot disease is not the only organism living in knots, because there are also white or yellow saprophytic bacteria characterized as *Pantoea agglomerans* (García de los Ríos, 1989), other species of enterobacteria and even putative human pathogenic bacteria (Ouzari et al., 2008). The etiologic agent of olive knot disease has only been isolated from 5 to 10% of olive knots, similarly to that observed in other Psv hosts species, such as oleander and ash (García de los Ríos, 1989). Four new bacterial species belonging to the genus *Pantoea* were proposed in a study of endophytic bacteria from olive knots associated to Psv (Rojas, 1999) and one of them known as *Erwinia toletana* has been accepted as new species (Rojas et al., 2004). These bacteria would be incorporated to the knot subsequently to the infection caused by the etiologic agent, according to García de los Ríos (1989). Indeed, a symbiotic relationship may exist between Psv and *Pantoea* (or may be other bacteria) because they are found together not only in knots but also as epiphytic bacteria in infected plants (Ercolani, 1978, 1991; Quesada et al., 2007). Furthermore, preliminary tests have shown that strains of an uncharacterized *Erwinia*, isolated from Psv-related olive



knots, could have a synergistic effect with Psv in the development of typical symptoms of olive knot disease (Fernandes & Marcelo, 2002). Similar results have been reported in olive trees coinoculated with strains of Psv and *P. agglomerans*, which produced larger knots than inoculations with Psv strains alone and this effect could be due to auxin production by *P. agglomerans* (Marchi et al., 2006).

### 3. General characteristics of phyllosphere habitats

The surfaces of the aerial parts of plants, including stems, buds, flowers and mainly leaves, can be denominated phyllosphere (Lindow & Brandl, 2003). The leaf surface is a large microbial habitat and Morris & Kinkel (2002) estimated that in total terrestrial surface area there would be about  $4 \times 10^8$  km<sup>2</sup> of leaf surface area, which could be colonized by  $10^{26}$  bacteria.

Leaf-borne microbial communities are diverse and include many different genera of bacteria, filamentous fungi, yeasts, algae and, less frequently, protozoa and nematodes (Lindow & Brandl, 2003). The phyllosphere of field plants is a harsh environment for bacteria. This habitat is severe because they are exposed to extreme microclimatic changes in temperature, relative humidity, wind speed, radiation, etc. in time periods as short as hours. Plant nutrient resources are scarce and accordingly plants like olive trees have a thicker cuticle (Lindow & Andersen, 1996). The phyllosphere may also be exposed to long dry periods or, conversely, heavy rains could dramatically alter the microbiota by the “washing” effect (Hirano & Upper, 2000).

The phyllosphere colonizing microorganisms are called epiphytes and Psv is one of them. A bacterium is considered as epiphytic when it lives and multiplies in the phyllosphere and constitutes the main colonizing group of the leaf surface, with average numbers from  $10^6$  to  $10^7$  cfu/cm<sup>2</sup> of leaf (Hirano & Upper, 1983, 2000). Interestingly, not all epiphytic bacteria colonizing the phyllosphere have a strictly commensal relationship with their host plant. This has also been demonstrated in many plant pathogenic bacteria including Psv, which have also an epiphytic resident phase (Ercolani, 1971).

The size and composition of epiphytic bacterial populations vary according to plant characteristics (species, age) and factors related to nutritional and climatic conditions (Hirano & Upper, 2000; Lindow & Brandl, 2003). The size estimation of epiphytic bacterial populations in the laboratory depends on the sampling, bacteriological and statistical procedures used (Hirano & Upper, 2000; Jacques & Morris, 1995). Most studies about epiphytic bacteria have estimated population sizes by washing or sonication to release bacteria from the leaf, followed by plating of serial dilution of the washings (Hirano & Upper, 2000). The estimated population sizes with this technique correspond to a proportion of total microorganisms living in the phyllosphere (Wilson & Lindow, 1992). The error involved in the recovery of epiphytic bacteria on solid medium is not important compared to the high variability of populations in field samples, which could range from 5 to 6 orders of magnitude (Hirano & Upper, 2000).

### 4. Epiphytic populations of Psv

There are few studies on the epidemiology of olive knot disease and most data of epiphytic Psv populations on the aerial surface of the tree come from studies done in southern Italy

(Ercolani, 1971, 1978, 1979, 1983, 1985, 1991, 1993) and southeastern Spain (Quesada et al., 2007; Quesada et al., 2010a; 2010b). In the aforementioned studies, epiphytic Psv populations in the phyllosphere of olive trees were estimated by washing, followed by plating serial dilution of the washings.

Microbial communities of the olive tree phylloplane can grow embedded in a matrix of exopolysaccharides and form biofilms adhered to the leaf surface (Morris et al., 1997). Furthermore, a great diversity of pigmented bacteria colonizing the surface of olive tree was observed by washing olive leaves and plating the washings. In Italy, Ercolani collected bacteria from the leaf surfaces of olive trees for two sampling periods over several years in the 70s and 80s (Ercolani, 1978, 1991). Phenotypic characterization of these isolates allowed Ercolani to record over 20 bacterial species colonizing the leaf surface. The three highest frequency values of occurrence corresponded to Psv, *Xanthomonas campestris* and *Pantoea agglomerans* with 51, 6.7 and 6%, respectively (Ercolani, 1991).

Spanish studies found that averages of the total bacterial population from leaves and stems were generally significantly higher in Psv-inoculated than in non-inoculated olive trees, suggesting that Psv might have a positive effect on the growth of other epiphytic bacteria or on their ability to colonize olive organs (Quesada et al., 2010a). Populations of *P. agglomerans* could accompany Psv and contribute to the significant differences in total bacterial populations between inoculated and non-inoculated olive trees. Besides, there was a positive correlation between Psv and yellow *P. agglomerans* either on stems or leaf surfaces of naturally infected olive trees (Quesada et al., 2007), and a similar fluctuation of both bacterial populations on the same host. This is of interest because both bacteria produce indoleacetic acid and this can contribute to the epiphytic fitness of Psv in olive trees (Varvaro & Martella, 1993).

The bacterial community composition on the surface of olive leaves is more strongly influenced by the sampling season than by leaf age (Ercolani, 1991). The diversity and size of the total bacterial populations within the olive phyllosphere were lower during the hot dry months and higher during the cold rainy months (Ercolani, 1991). Our observations on one olive orchard showed that seasonal fluctuations of Psv populations fitted into the pattern of seasonal shifts described above (Quesada et al., 2007). Interestingly, Psv population sizes in stems and leaf surfaces were correlated (with  $r^2$  values of 0.7 and 0.43, respectively) with rainfall, temperature and relative humidity (Quesada et al., 2007). Therefore, these climatic parameters may exert a more or less strong influence on the Psv population values. Epiphytic bacterial communities on olive leaves were more uniform in mature leaves than in young leaves (Ercolani, 1991). In addition, the olive phyllosphere apparently selects specific genotypes of the bacterial community (Lindow & Brandl, 2003). This gives us an idea of the great variability, in terms of epiphytic populations, existing among leaves of the same tree.

Over 50% of bacterial isolates collected from olive leaves by Ercolani (1978, 1991) were identified as Psv and this bacterium survived and multiplied on the leaf surfaces of olive trees (Varvaro & Ferrulli, 1983). Abu-Ghorrah (1988) observed maximum Psv population levels of about  $10^7$  cfu/cm<sup>2</sup> in olive trees and the Psv generation time in this host was 24 to 36 hours. In studies of leaves inoculated by spraying a suspension of Psv, the bacteria

colonized the lower leaf surface better than the upper surface (Surico, 1993). Basically, they stucked to the vein depressions and to specific structures such as the shields of pectate hairs (Surico, 1993).

The seasonal fluctuation of Psv populations on olive leaves in Italy, recorded over three consecutive years by Ercolani (1971, 1978), showed that Psv populations were higher in spring and fall (about  $10^4$  cfu/cm<sup>2</sup> of leaf) than in winter and summer (about  $10^2$ - $10^3$  cfu/cm<sup>2</sup>) (Ercolani, 1971, 1978; Varvaro & Surico, 1978). Psv populations on olive leaves in Spain, also recorded over three consecutive years, reached the highest (ca.  $10^3$ - $10^4$  cfu/cm<sup>2</sup> of leaf) densities mainly in warm and rainy months (mainly spring season) and the lowest (ca. 0-10 cfu/cm<sup>2</sup> of leaf) in hot and dry months (summer season) (Quesada et al., 2007). Significant differences were observed between Psv populations in summer and in the other seasons over the three-year study (Quesada et al., 2007).

Lavermicocca & Surico (1987) simultaneously analyzed Psv populations on olive tree leaves and stems for the first time and during one year, reporting higher frequencies of Psv isolation in stems than in leaves with relatively high epiphytic Psv populations in July (about  $10^5$  cfu/cm<sup>2</sup>) and only 10 cfu/cm<sup>2</sup> in September and March. However, in Spain no significant differences were found between either leaves or stems with respect to the number of analyzed samples where Psv was isolated, detected by PCR, or regarding the average Psv populations over several years (Bertolini et al., 2003a; Quesada et al., 2007; Quesada et al., 2010a, 2010b). Given that in such studies the Psv number were evaluated on stems after they were cut into pieces, some endophytic Psv could be also counted (Penyalver et al., 2006). Furthermore, both types of plant material (stems and leaves) should be analyzed from symptomless shoots to make the evaluation of Psv populations in the phyllosphere more accurate (Bertolini et al., 2003a; Quesada et al., 2007). Psv was also isolated from the surface of olive fruits, but at lower frequency than from leaves, reaching a high Psv population size in September ( $10^6$  bacteria / g fresh weight) (Lavermicocca & Surico, 1987).

Between 70 and 95% of the maximum variance of some microbiological parameters, such as Psv density in the olive tree phyllosphere, was explained by the influence of seven factors, four of which were related with the weather: summer, summer rainfall, winter rainfall and warm fronts (Ercolani, 1985) and the three remaining factors were cambium activity, leaf age and time of flowering. As described for other epiphytic bacteria and hosts (Kinkel, 1997), Psv population densities varied over several orders of magnitude among leaves sampled concurrently from the same shoot, as assessed by the comparison of leaf printing and isolation experiments (Quesada et al., 2007). Due to the low detection level associated with leaf printing (Jacques & Morris, 1995), such results also suggested that Psv probably colonizes low numbers of leaves with high populations, in bulked samples.

The size of Psv populations on each leaf correlated with leaf age, the time when it formed and the time of the year when the sample was taken (Ercolani, 1991). In addition, phenotypically distinct Psv isolates from the phyllosphere, succeed each other in time in the olive tree phyllosphere (Ercolani, 1983). This was discovered because Psv isolates obtained by washing leaves of a certain age at a particular time of the year (over eight years) showed more phenotypic similarity with each other, than with isolates obtained by washing leaves

of different ages at different times of the year. Most of the Psv isolates obtained by washing leaves of different ages taken at random in April, were less similar to each other in 60 phenotypic characters (they formed a single group at 65% similarity) than Psv isolates obtained from six-month-old knots in October (one group formed 85% similarity). A similar result was obtained when Psv isolates obtained by washing leaves in October were compared with Psv isolates obtained from six-month-old knots in April (Ercolani, 1993). Psv isolation from these knots was performed six months after washing leaves in April and October as above indicated. Almost all isolates from knots reflected the dominant phenotype of the isolates obtained from the phyllosphere six months earlier. Most Psv isolated by washing leaves in April and October were phenotypically classified near to isolates obtained by washing 13-month-old leaves. According to these authors, senescent leaves (13 months old) could be the main source of bacteria for knot formation in April and October (Ercolani, 1993).

## 5. Endophytic populations of Psv

Endophytic bacteria are defined as bacteria living in plant tissues without doing substantive harm or gaining benefit other than residency (Kado, 1992). The endophytic colonization of plants is probably fundamental for plant-associated bacteria to develop sustainable epiphytic populations (Manceau & Kasempour, 2002). Although the information about endophytic populations of plant pathogenic bacteria is still scarce, it is very likely that most of the pathogens can undergo an endophytic step during the disease cycle. However, as their numbers are low inside the asymptomatic plants and their distribution is not homogeneous, laborious studies are required to detect them. Consequently, little is known about the real importance of the endophytic phase for most bacterial pathogens.

Psv could also present an endophyte phase spanning a considerable part of its life cycle due to its multiplication in the intercellular spaces, substomatal cavities or in vascular tissues of the plant, without any visible symptoms (Schiff-Giorgini, 1906; Smith 1908, 1920; Wilson & Magie, 1964). Regarding Psv, the bacteria that survive inside and outside the knots could have a greater impact than the bacteria colonizing the olive as a symptomless endophyte. In fact, some studies described the endophytic phase of Psv in olive plants as rare (Wilson & Magie, 1964). According to other authors, Psv could also present an endophytic phase, moving through the intercellular spaces and even in the xylem vessels and infecting areas close to the first infected zone (Penyalver et al., 2006; Schiff-Giorgini, 1906; Smith 1908, 1920; Wilson & Magie, 1964; Wilson & Ogawa, 1979). Further studies are needed to reliably assess the importance of this phase of Psv in olive knot epidemiology. Nowadays, this is relatively easier to address, thanks to an established model system for the study of olive knot disease covering a wide range of aspects. It is formed by a micropropagated olive plant, coming from an *in vitro* germinated seed and a Psv strain (NCPPB 3335) producing the characteristic symptoms of olive knot disease in both woody and micropropagated olive plants (Pérez-Martínez et al., 2007; Rodríguez-Moreno et al., 2008). Besides, Psv strain NCPPB 3335 has been studied in depth and many genetic resources are available because its genome has been sequenced and analyzed using appropriate bioinformatic tools (Rodríguez-Palenzuela et al., 2010; Matas, 2010).



Endophytic Psv populations could contribute to a dramatic increase in Psv numbers and in olive knots in infected olive groves, when the copper-based control treatments are not applied (Quesada et al., 2010b).

## 6. Epidemiology and disease cycle

Disease caused by Psv populations has an epiphytic-pathogen type cycle. The bacteria have an epiphytic phase in which they multiply on the surface of olive tree stems and leaves without developing symptoms (Ercolani, 1978, 1991; Varvaro & Ferrulli, 1983). Interestingly, Psv populations were recovered from symptomless shoots from non-inoculated control trees prior to the appearance of symptoms, suggesting that these epiphytic bacteria were the potential source of inoculum for infection of healthy plants (Quesada et al., 2010a).

The temperature range in which Psv can initiate infection is between 5 and 37 °C and this would allow the bacteria to cause infections throughout the year. However, optimal conditions for disease development are about 22-25 °C and the subsequent time periods with high infection probability are fall and spring (Protta, 1995). Psv can infect olive trees at any time of the year and trigger knot formation only when conditions are favorable. Thus, when the bacteria infect an olive tree in the fall, knots will begin to develop several months later, but if the infection occurs during the spring, the time required for knot formation may be only two weeks (Wilson, 1935). Field trials performed in California showed that Psv inoculations of olive trees carried out in April caused higher levels of olive knot disease than Psv inoculations carried out in December (Teviotdale & Krueger, 2004).

Dissemination of Psv bacteria from infected (or inoculated) to non-infected (or non-inoculated) trees was suggested (Quesada et al., 2010a). Bacteria could spread over long distances due to the introduction and planting of infected material, or over short distances transported by splashing rain, windblown aerosols, insects and cultural practices (Horne et al., 1912; Wilson, 1935). Currently, bacterial dissemination is facilitated by cultural practices, new plantations with high tree density, frequent severe pruning and with small distance between plants (Tous et al., 2007). Wounds caused by harvesting and pruning, as well as by hail, frost and leaf scars, create niches where infection occurs (Wilson, 1935; Janse, 1982) and olive tree infection by Psv is directly related to the degree of wounding of the trees (Smith et al., 1991). In an assay to evaluate natural dissemination of the bacteria on young plants, knots were not observed in inoculated and non-inoculated trees until 3 and 10 months after inoculations, respectively (Quesada et al., 2010a). The compatible Psv-olive tree interaction facilitates the invasion, infection and multiplication, triggering hypertrophy and hyperplasia of the plant tissues with subsequent knot formation. Bacteria can survive inside the knots from one season to another and when humidity is high enough, exudates containing large amounts of bacteria are emitted in which they can survive as epiphytes (Wilson, 1935). However, the bacteria can only survive in soil for a few days (Wilson & Ogawa, 1979). The disease cycle is summarized in Figure 2.

In 1909, Petri isolated Psv from the intestinal tract and eggs of the olive fly (*Bactrocera oleae*), but there is no other scientific evidence that this, or other insects, can be efficient vectors of olive knot disease. Additionally, there is no conclusive evidence about the role that birds may play as vectors of this disease (Wilson, 1935), although they can transport living bacteria from plant to plant.

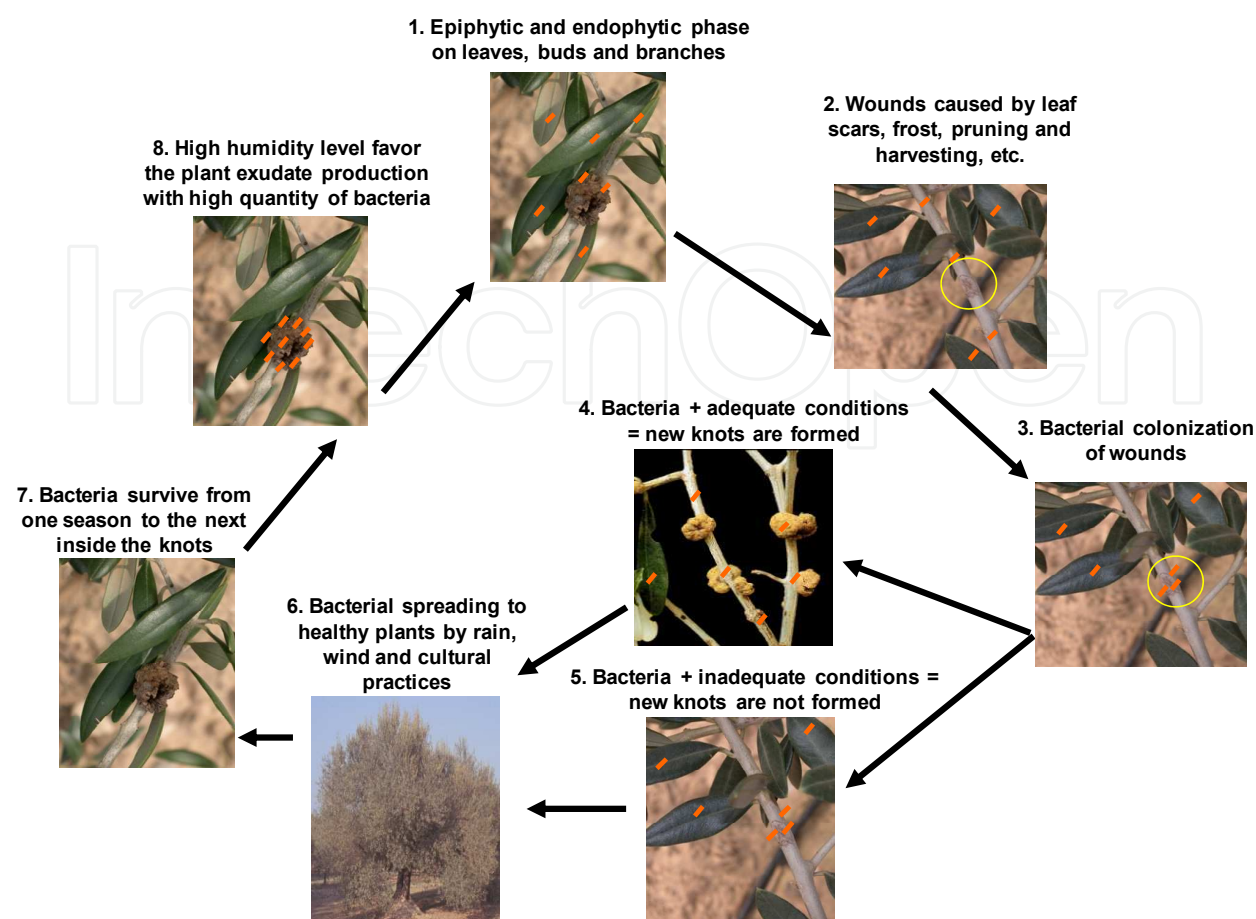


Fig. 2. Disease cycle of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi* simulated as red bacilli (kindly provided by E. Bertolini, 2003).

## 7. Effect of olive knot disease on the vigor and yield of olive trees

Although the olive knot disease is widespread throughout most olive-growing areas, there is no accurate estimation of the losses it causes. This is very difficult to measure because many factors can influence the severity of the symptoms. Severe infections can cause death of branches and a progressive weakening, resulting in a loss of tree vigor (Tjamos et al., 1993) and thus of harvest. De Andrés (1991) estimated that Psv-related losses were around 1.3% of national olive production in Spain. Occasionally, this disease has caused the loss of almost the olive local harvest due to the combination of optimal weather conditions for bacterial entry and multiplication, as observed in two Spanish localities in 1987 and 2001 (B. Celada, personal communication) after severe hail storms. Furthermore, this disease is present with variable incidence in many nursery plants, as it limits their commercialization due to the visible symptoms. This is especially important in plants for export because several countries that import plants from the European Union (EU), like Chile, consider Psv as a quarantine organism.

The quantitative effects of olive knot disease on vigor and olive fruit yields are not yet well established because there is only information available from one study in California and another in Spain. In a commercial orchard in California (USA) significant differences were

not found in vigor between 40-year-old olive trees lightly (0.10-0.30 knots in 0.3 m of fruit wood) and mildly (0.31-0.50 knots) infected with the disease (Schroth et al., 1973). In contrast, in Spain in a study on non-inoculated and inoculated trees 7-year-old of cv. Arbequina in a high-density grove, vigor was significantly higher in non-inoculated trees (Quesada et al., 2010a). Therefore, vigor was higher in trees of cv. Arbequina where olive knot disease was lower during the study, suggesting a negative effect over time of the disease on plant development. Schroth et al. (1973) in California showed that there was a clear relationship between crop losses and the number of tumors caused by Psv in branches. They observed significant differences in the weight of olive fruits per tree in only one year between lightly and mildly infected olive trees with 121.3 and 94.6 kg, respectively. In the Spanish assays, the different levels of the disease did not significantly affect cumulative olive yield (Quesada et al., 2010a) in young trees.

Furthermore, low oil quality was reported when olive fruits were harvested from olive trees moderately affected with olive knot presenting odors and flavors such as bitter, stale or salty, but the data were lacking of statistical support (Schroth et al., 1968; Tjamos et al., 1993). In another study, olive knot disease incidence did not modify either the chemical or organoleptic characteristics of virgin olive oil extracted from young olive tree fruits in a high-density grove (Quesada et al., unpublished data).

## 8. Control methods

The methods used to control plant pathogenic bacteria are based on preventive and curative measures and the combination of the two should be used in the context of an integrated control. The five main goals of an integrated plant disease control program are to eliminate or reduce the initial inoculum, reduce the effectiveness of the initial inoculum, increase host resistance, delay disease onset and slow the secondary cycles (Agrios, 2005). The key of any integrated control program is a question of sustainability at different levels (Caballero & Murillo, 2003). In economic terms, it must ensure farmers' profits and at the environmental level, select control methods that minimize environmental impact. And finally, control methods by themselves should ensure sustainability and they must remain effective over time. The monitoring of a strategy of this type is essential to ensure safe and sustainable agriculture.

Disease management of bacterial pathogens in the field is mainly based on preventive procedures, because it is difficult to eradicate pathogens once established. Due to the economic impact of the olive knot disease, growers require adequate control methods to overcome its negative repercussions on yield and even on olive fruit quality (Quesada et al., 2010a; Schroth et al., 1973). Olive knot control should be based on an integrated control strategy, giving priority to the most effective measures that are of preventive type. These are very diverse and can be grouped into regulatory measures, preventing introduction of the pathogen in protected areas and prophylactic measures to reduce or eliminate the pathogen or hinder its establishment in nurseries or orchards (Montesinos & López, 1996).

### 8.1 Regulatory measures

The production, maintenance and use of certified plant material which is pathogen free, is one of the main preventive measures used to control plant pathogens. In this regard, it is

very important that Psv appears in the EU list of pests and diseases that significantly affect plant quality standards, drawn up by the European Commission (Directive N° 92/34/EU). As advised by the European and Mediterranean Plant Protection Organization (EPPO-OEPP), new olive groves should be established using Psv-free certified plant material (EPPO, 2006).

As an example, fifteen years ago, there was scarce reliable information available on the sanitary status of Spanish olive plants with respect to pathogenic bacteria (Bertolini et al., 1998; Padilla, 1997). Although there is now more information and analyses have been performed, there is still a lack of scientific published data available on the status of olive plants in the field or in nurseries, either in Spain or in other olive-growing countries. Government agencies have shown an interest to control the planting material given the increase number of plantations, the notable changes in production technologies and the frequent commercial exchange of olive plants. All these facts emphasize the convenience of providing plant material with certain quality standards and the implementation of certification programs (Cambra et al., 1998).

With respect to this issue the EU has, so far, required the minimum conditions for the nursery plants of type Agricultural Conformitas Comunitatis (or CAC). The implementation of certification systems is the responsibility of each member state, but the European and Mediterranean Plant Protection Organization (EPPO) has developed specifications for certification of olive plants. These referred specifically to health although they were based on studies conducted in Italy and Portugal and thus should be contrasted with the situation in Spain (Chomé, 1998).

Italy was the first country to publish standards for certification of plant material from olive trees in 1993. Certified plants should be free from Psv, *Verticillium dahliae* and six virus (*Olive latent virus 1* (OLV-1), *Olive latent virus 2* (OLV-2), *Cucumber mosaic virus* (CMV), *Arabidopsis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV) and *Strawberry latent ring spot virus* (SLRSV)) (Martelli, 1998). Besides Italy, other countries like Portugal, Israel, Argentina and Spain, have also established certification programs for olive plant material. In the Argentinan Certification program, the mother plants are annually tested and must be free of *P. syringae*, Psv, *Agrobacterium tumefaciens*, *V. dahliae* and *Phytophthora cinnamomi* but viruses are not considered.

There are more than 260 nurseries registered in Spain, mainly located in Andalusia and Valencia, which produced about 5.5 million olive plants in 1999-2000 for new plantations and also for international trade (Chomé, 1998). Given this significant production, the *Real Decreto 1678/1999* (Anonymous, 1999) established quality control and certification requirements for olive seedlings in the certification program of olive plant material in Spain. Currently, the qualification of certified plant material is the responsibility of the competent institutions in each region. To qualify for certification, plant material must meet certain conditions such as having a known origin and having been submitted to cultivar analyses and sanitary tests. Mother plants of the starting material and base material should be officially inspected to verify that they are free of *V. dahliae*, Psv and viruses OLV-1, OLV-2, CMV, ArMV, CLRV and SLRSV. Each year the plants for certification should be sampled and tested for Psv by the responsible official body using approved techniques, which include isolation, serology and nested multiplex RT-PCR. With this technique, Psv and four



olive viruses (CMV, CLRV, SLRSV and ArMV) can be detected simultaneously in a sensitive single reaction (Bertolini et al., 2003b). Finally, parent plants of certified nursery stock must be at least free of symptoms of diseases caused by fungi, bacteria and the viruses previously cited.

## 8.2 Prophylactic measures

Prophylactic measures are designed to reduce or eliminate the pathogen levels or impede its establishment in a crop and these measures can also be of eradication nature, based on cultivar susceptibility or by direct protection (Montesinos & López, 1996). In the case of Psv, they include all those performed for disinfecting plants, agricultural machinery, or anything in contact with plants.

### 8.2.1 Eradication

The presence of knots in a tree is related to a high level of disease after several years, and this highlights the need of using preventive control methods or eradication methods to maintain olive trees without knots (Quesada et al., 2010a). In affected plantations the main olive knot disease eradication method would be the uprooting of the affected trees or the use of cultural practices to reduce the inoculum source, performing copper treatments, pruning of infected branches and reduction of number of wounds during the growing season and especially at harvest (Beltrá, 1956; Penyalver et al., 1998; Trapero & Blanco, 1998; Wilson, 1935). This is especially relevant in new plantations with high tree density and frequent severe pruning, where control measures should be accurately monitored (Tous et al., 2007).

The removal of knots is very laborious and may not be entirely effective because new wounds are usually done when knots are removed and new knots can develop in these wounds in the following years, even when treated with preventive chemicals (Wilson, 1935). Pruning of infected branches is more effective than knot removal as fewer wounds are caused to the olive tree and the bacterial inoculum load is minimised (Quesada et al., 2010a; Teviotdale & Krueger, 2004; Wilson, 1935). All cut branches should be burned in the same field to prevent the spread of the disease (Trapero & Blanco, 1998).

In the case of partially contaminated olive groves, healthy trees should be harvested and pruned first (Wilson, 1935). Besides, growers should harvest olives in dry weather only and avoid the use of techniques like knocking the olive tree branches with wooden poles (Krueger et al., 1999). Manual harvesting methods, like the “milking” method or the use of mechanical vibration are more suitable. It is important to assess the index of tree damage in terms of broken branches and compare this to the olive fruit harvested. It has been reported that knocking the olive tree branches with wood poles can break from 13 to 18% of branches while for mechanical vibration this is only 6 to 9%, on complete harvesting (Civantos et al., 2008).

### 8.2.2 Cultivar susceptibility

The use of resistant cultivars, or low susceptibility cultivars to bacterial plant diseases would be one of the most appropriate disease control methods (Montesinos & López, 1996).

However, in woody crops, such as olive trees, breeders and plant pathologists are hindered by the slow improvement in breeding processes as a result of delayed entry into fruition.

Another drawback is that the information available about cultivar susceptibility to olive knot disease is scarce and mainly comes from field observations, such as those reported in the USA and Spain (Barranco, 1998; Trapero & Blanco, 1998; Wilson, 1935). Very few data are available from comparative inoculation experiments and is limited to several cultivars (five to eight) from Italy, Greece, Morocco, and Portugal (Benjama, 1994; Catara et al., 2005; Hassani et al., 2003; Marcelo et al., 1999; Panagopoulos, 1993; Varvaro & Surico 1978), with the exception of Spain where 29 cultivars were evaluated (Penyalver et al., 2006). Field observations do not always give universally valid information on the intrinsic susceptibility of each cultivar because the initial quantity of bacterial inoculum differs between plants and factors favoring infection can vary in different areas.

Varvaro & Surico (1978) compared the behavior of six Italian olive cultivars inoculated with Psv and found no difference, because more than 95% of the inoculated wounds developed tumors. This was probably due to the high inoculum dose applied (more than  $10^6$  bacteria per wound) and because the inoculated plants were only one year old. Different doses of eight Psv isolates were inoculated in six olive cultivars in comparative inoculation experiments from Morocco (Benjama, 1994). The results showed that the cultivar Frantoio was the most susceptible among those tested, followed by Ascolana dura, Manzanilla, Picholine marocaine, Dahbia and Gordal Sevillana, which was the least susceptible, although a statistical analysis of the data was not performed. Marcelo et al. (1999) evaluated six Portuguese cultivars and found they differed in the percentage of knots formed at inoculation points, ranging from 36 to 66%. These authors considered that the cultivars Blanqueta, Cobrancosa, Cordovil de Serpa, Galega Vulgar, Redondil and Santulhana were moderately susceptible to olive knot disease, but their data were not statistically analysed. Hassani et al. (2003) evaluated the Italian cultivars Frantoio, Leccino, Moraiolo and Nostrale di Rigali by inoculation of five Psv strains with an inoculum dose of  $5 \times 10^7$  bacteria per wound and subsequently knot weights were compared. Although they did not indicate the percentage of inoculation sites that developed knots, it is likely that this parameter exceeded 90% in the four cultivars because an excessively high inoculum dose was used.

Penyalver et al. (2006) developed a methodology for evaluation of cultivar susceptibility to Psv and reported that most of that 21 Psv strains evaluated in virulence tests showed a high degree of aggressiveness but also, in some combinations, cultivar-strain interactions were observed. Consequently, strain selection for inoculation is a pre-requisite to obtaining useful data, and at least two strains should be used for accurate evaluations. The methodology was optimized for the first time with 29 olive cultivars. It was concluded that plant material should be genetically homogeneous, at least two or three years old, inoculated in spring or early summer by wounds made with a sterile scalpel. The use of at least two Psv strains with high degree of virulence was also recommended. They should be inoculated at low inoculum doses ( $10^2$  bacteria per wound) to differentiate among different cultivars, as well as at a high dose ( $10^6$  bacteria per wound) to identify the less susceptible cultivars. Ten olive plants should be used per bacterial strain and dose. Five wounds should be performed thus per plant and several measurements of symptoms taken for each combination, although the measurement taken at 90 days was the data included in the analysis of 29 cultivars from the World Olive Germplasm Bank of Spain, located in Cordoba.

Disease severity of a particular cultivar was found to be highly dependent on the pathogen dose applied at the inoculation point. In addition, secondary knot formation in non-inoculated wounds in previously inoculated plants, would suggest pathogen migration in the plant tissues. They also observed a correlation between the number of inoculation sites in which knots developed and the number of secondary knots formed when the initial wounds were inoculated with low bacterial doses. All cultivars developed knots at inoculation points, at least when high inoculum doses were applied. According to the results, six cultivars were classified as highly susceptible (Arbequina, Arróniz, Nevadillo Blanco de Jaén, Pajarero, Picudo and Vallesa). Some cultivars were classified as slightly susceptible (Azapa, Cerezuela, Chemlali, Dulzal de Carmona, Frantoio, FS-17, Gordal de Archidona, Gordal de Hellín, Lechín de Granada, Manzanilla Cacereña, Manzanilla de Sevilla, Nevadillo negro and Villalonga). The remaining cultivars (Changlot Real, Morisca, Gordal sevillana, Lechín de Sevilla, Oblonga, Picual, Ascolana tenera, Royal de Cazorla, Mollar de Cieza and Koroneiki) were classified as moderately susceptible.

So far studies on cultivar susceptibility to olive knot disease suggest that true resistance to this disease is uncommon among cultivated olive cultivars. In contrast, significant differences were observed in the degree of susceptibility among the cultivars tested. In addition, *in vitro* studies of Psv interaction with cell cultures of the cultivar Galega vulgar showed the typical events of a hypersensitive response in inoculated plant cells, such as an increase in reactive oxygen species, the activation of programmed cell death and decreased cell viability (Cruz & Tavares, 2005). High resolution liquid chromatography and mass spectrometry analysis of Psv-related knot extracts from outbreaks in olive trees of cultivar Koroneiki revealed high amounts of phenolic compounds, o-diphenols (oleopurina) and polyamines (spermidine, spermine, putrescine), in addition to auxins (Roussos et al., 2002). These authors postulated that the production of indole-3-acetonitrile and phenolic compounds could be related to the olive tree's defense mechanisms in knots. Cayuela et al. (2006) identified verbascoside as the main phenolic compound produced at significant levels in Psv-related knot extracts in olive trees of the cultivar Picual.

Balanced soil fertilization, avoiding excess nitrogen, may increase plant resistance to infection (Paoletti, 1993). However, in modern olivicultural practices such a balance is hard to maintain because the rapid development of young plants is valued, with early production onset and increased yields from one year to the next. A common mistake made to meet the demands of modern oliviculture is to apply an excess of nitrogen fertilizer, as this increases susceptibility to olive knot disease (Balestra & Varvaro, 1997). It is advisable to perform main fertilization of olive trees in January-February (Baratta & Di Marco, 1981) with low winter temperatures.

### 8.2.3 Direct control

Direct protection measures are mainly based on chemical or biological principles and are used by the growers when prophylactic measures have failed to stop disease progression in one zone (Montesinos & López, 1996), or are combined with all the other measures in an integrated control strategy.

### 8.2.3.1 Chemical control

Chemical control of bacterial plant diseases is only effective when they are used in preventive strategies before the onset of infection or very early in the bacterial infection process (Montesinos & López, 1996). Specifically, chemical control of olive knot disease has given inconsistent results in field experiments and may also have low efficacy and even show phytotoxicity to some tissues. This variability is due to several factors, such as the amount of inoculum, timing of treatments, climatic conditions, cultivar susceptibility, treatment application method, or physiological state of the host plant.

Copper compounds are the main preventive chemical treatment recommended against olive knot disease and their use is recommended every year when there is a risk of infection, in spring and fall before the rains, after the leaf fall and especially after hail and frost or other events causing olive injuries (Penyalver et al., 1998; Protta, 1995; Smith et al., 1991; Wilson, 1935). A positive correlation has been found between disease incidence and spring rains (Teviotdale & Krueger, 2004) and it was observed that moist winds in coastal areas promote infection (Smith et al., 1991).

The copper-based compounds used in olive groves in Spain include various salts and formulations (hydroxides, oxychlorides, oxides or sulfates) as well as their mixture with organic compounds obtained by chemical synthesis. An interesting example is the combination of cuprocalcic sulfate plus mancozeb because it has a synergistic effect against several bacterial diseases (Hausbeck et al., 2000; Jones et al., 1991; Marco & Stall, 1983). Currently copper oxychloride is the copper compound most commonly recommended against olive knot disease by the Spanish extension services. The active ingredient in these products is the divalent copper ion solubilized and both, bacteria and plant exudates, contain compounds which are capable of solubilizing copper. Generally, these products have a toxic or bacteriostatic effect, only preventing the multiplication of bacteria and most bacteria may die due to the toxic effects of  $\text{Cu}^{++}$  ions, or enter in the VBNC (Viable But Non Culturable) state in which they are unable to grow on solid medium. This state could be induced by copper ions, as previously reported for several plant pathogenic bacteria (Alexander et al., 1999; Grey & Steck, 2001; Ordax et al., 2005). These preventive chemical treatments are recommended for both to reduce epiphytic Psv populations and prevent their penetration through the plant wounds.

The effectiveness of chemical control of olive knot has been poorly evaluated, both in the field and in experimental assays under controlled conditions. The effect of the treatments against epiphytic inoculum of Psv or the optimal time of application are not well known. Several studies suggest that the management of epiphytic Psv populations probably reduces the incidence of olive knot disease (Ercolani, 1978, 1991; Lavermicocca & Surico, 1987; Quesada et al., 2007, 2010a). In this context, a chemical control program using copper compounds was proposed, based on field observations in California (Horne et al., 1912; Wilson, 1935). The first field experiments described in the literature were conducted in California where several Bordeaux mixture formulations controlled olive knot disease with minimal phytotoxicity symptoms in commercial olive groves with prevalent Psv infections (Krueger et al., 1999; Teviotdale & Krueger, 2004; Wilson, 1935). Assays performed with copper hydroxide showed that a single post-harvest copper application provided only minimal protection against the disease and subsequently, additional sprays in spring were needed to substantially improve its control (Teviotdale & Krueger, 2004). The efficacy of



copper hydroxide to control the incidence of knots was higher after three sprays than after two or one single spray. New information has been gathered about the effect of copper compounds on the population dynamics of epiphytic Psv, the possible appearance of copper resistance, or its role in decreasing olive knot incidence under Mediterranean conditions in high-density groves (Quesada et al., 2010b).

The effect of copper oxychloride or cuprocalcic sulfate plus mancozeb treatments on Psv populations and subsequent disease development were evaluated in an olive grove planted with two susceptible cultivars, Arbequina and Picudo, over a four-year period. Unlike the previous studies, to homogenize the knot number per tree before beginning treatments, olive trees were inoculated. The effect of copper on Psv populations was observed after the first application, but the greatest differences between copper-treated and untreated plants were observed in the third year, after five copper applications. Two applications of copper compounds per year, reduced Psv populations effectively. We also found that treatment with copper compounds had a drastic effect on reducing disease incidence (Quesada et al., 2010b). These results for both cultivars, in this high-density grove, supported previous observations by Teviotdale and Krueger (2004) in California, in standard groves. Unlike other plant pathogenic bacteria that develop copper resistance after extensive exposure to copper compounds (Cazorla et al., 2002; Cooksey, 1990; Garret & Schwartz, 1998; Marco & Stall, 1983; Scheck et al., 1996; Sundin et al., 1989, 1994), copper resistance was not detected in the remaining Psv bacteria in copper-treated olives trees.

Chemical treatments based on antibiotics and oil-water emulsion containing hydrocarbons have also been recommended but without encouraging results (Scrivani & Bugiani, 1955; Schroth & Hildebrand, 1968). The use of antibiotics such as streptomycin and terramycin has been successful under experimental conditions (Trapero & Blanco, 1998) but their application against plant pathogenic bacteria is currently forbidden by the EU legislation, although it is permitted in some other countries.

Systemic acquired response, or SAR, is plants' ability to generate defense reactions against external aggression at sites far away from the point of attack. In these distant sites, the genes involved in defense processes are activated (e.g. PR proteins), thereby increasing the resistance of these tissues against possible further attacks (Durrant & Dong, 2004; Kessmann et al., 1994). Some products were recently evaluated for their induction of plant resistance against different pathogens such as acibenzolar-S-methyl (Bion ®), fosetyl-aluminium, calcium prohexadione or harpins. They were assayed to control some bacterial plant diseases like fire blight, citrus canker, apical necrosis of mango, etc. Most of these products do not produce phytotoxicity and their efficacy is sometimes comparable to that of antibiotics or copper-based compounds while others could not control these diseases (Brisset et al., 2000; Cazorla et al., 2006; Graham & Leite, 2004; Scortichini, 2002,). There are reports of acibenzolar-S-methyl-related activation of certain genes involved in defense responses in olive leaves of the cultivar Lechín de Sevilla (Muñoz et al., 2005). However, in one experiment acibenzolar-S-methyl did not reduce either Psv populations or the incidence of olive knot disease after two treatments per year over a four-year period (Quesada et al., 2010b). It is possible that different doses and more product applications could be required for achieve better efficacy. Curiously, vigor of cv. Picudo was significantly higher in olive trees treated with acibenzolar-S-methyl than in untreated trees, although disease incidence was similar in both treated and untreated olive trees (Quesada et al., 2010b).

### 8.2.3.2 Biological control

Biological control is another alternative to control olive knot disease, but is seldom tested against Psv. To date, biological control agents have been evaluated using isolates of *P. fluorescens* (Blightban) and Psv mutants producing bacteriocins, but without satisfactory results (Krueger et al., 1999; Varvaro & Martella, 1993). Besides, non pathogenic *Pseudomonas* sp. isolated from olive tree rhizosphere proved antagonistic against Psv (Rokni-Zadeh et al., 2008). Recently, *P. fluorescens* and *Bacillus subtilis* isolates from knots and leaves of olive trees showed antagonistic *in vitro* activity against Psv (Krid et al., 2010).

Bacteriocins are excellent candidates for using in agriculture to control plant pathogenic bacteria due to their high specificity. A bacteriocin produced by *P. syringae* pv. *ciccaronei* was shown to inhibit the proliferation and survival of epiphytic Psv form (Lavermicocca et al., 2002, 2003). Effectiveness of assays with two-year-old olive plants in a culture chamber was equivalent to that of copper hydroxide, although it would be interesting to evaluate its efficacy in nursery or field plants and determine their toxicity and persistence before advising their commercial registration.

## 9. General conclusions

Remarkable progress has been made in several aspects of the host-pathogen interaction of the causal agent of olive knot disease, recently. Additionally, studies on the epidemiology of olive knot disease, as well as on its chemical control, have also been reported to add to the scarce information available. However, further studies are needed to assess reliably the importance of the endophytic phase of Psv in the epidemiology of olive knot as well as the effect of different chemical on disease incidence. Furthermore, studies on the qualitative and quantitative effects of the disease on olive production are insufficient.

The production, maintenance and use of certified and potentially pathogen-free plant material, is one of the main preventive measures used to control plant pathogens and certification schemes based in analytical tests performed on olive plants before leaving the nurseries should be implemented. Although, so far, true resistance to this disease is uncommon among olive cultivars tested, significant differences were observed in the degree of susceptibility to the disease among them. Cultivars tolerant to olive knot and resistant/tolerant to climatic conditions, or avoiding cultural practices which favor olive knot development should be considered for planting in the new commercial fields. This is especially important for high density new plantations.

The olive knot disease integrated control should combine healthy plant material with appropriate cultural practices and the use, like preventive treatments, of chemical compounds. In such a context, copper treatments should be used regularly to achieve effective control.

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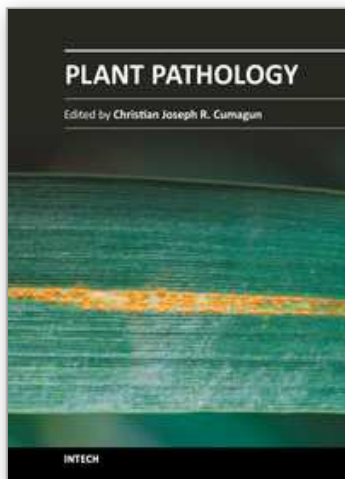
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