Factors Affecting *Pseudomonas savastanoi* pv. *savastanoi* Plant Inoculations and Their Use for Evaluation of Olive Cultivar Susceptibility

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ABSTRACT

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Pseudomonas savastanoi pv. *savastanoi* causes olive knot disease, which is present in most countries where olive trees are grown. Although the use of cultivars with low susceptibility may be one of the most appropriate methods of disease control, little information is available from inoculation assays, and cultivar susceptibility assessments have been limited to few cultivars. We have evaluated the effects of pathogen virulence, plant age, the dose/response relationship, and the induction of secondary tumors in olive inoculation assays. Most *P. savastanoi* pv.

savastanoi strains evaluated were highly virulent to olive plants, but interactions between cultivars and strains were found. The severity of the disease in a given cultivar was strongly dependent of the pathogen dose applied at the wound sites. Secondary tumors developed in noninoculated wounds following inoculation at another position on the stem, suggesting the migration of the pathogen within olive plants. Proportion and weight of primary knots and the presence of secondary knots were evaluated in 29 olive cultivars inoculated with two pathogen strains at two inoculum doses, allowing us to rate most of the cultivars as having either high, medium, or low susceptibility to olive knot disease. None of the cultivars were immune to the disease.

Additional keywords: *Olea europaea*, olive germplasm bank, resistance.

Olive knot disease, caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi*, seriously affects olive trees (*Olea europaea* L.) mainly in Mediterranean countries, where climatic conditions often favor spread of the disease. Disease symptoms are characterized by tumorous outgrowths on different parts of infected plants, mainly shoots and branches. The development of these galls is dependent on bacterial production of the phytohormone indoleacetic acid and cytokinins (5,13,19,21), and more recently it has been reported that olive knot formation is *hrp/hrc*-dependent (17).

Nowadays there is an increasing interest in olive-growing in many countries probably due to the benefits of olive oil for human health (1,14). Spain is the leading world producer of olive oil with about 2.5 million ha of olive trees, representing about 25% of the world's cultivated area and producing almost 50% of the world's olive oil production. Olive knot disease is considered an important problem for olive crops because of its effect on vegetative growth (24), olive yield (16), and even possibly on olive oil quality (15).

The use of resistant cultivars is considered one of the most appropriate methods of control, but unfortunately, little information is available from comparative inoculation experiments and is limited to a few cultivars from Italy, Morocco, and Portugal (3,9,11,23). Information also comes from field observations such as those reported in Spain (2,22). Because evaluation of germplasm for olive knot susceptibility has been very limited, little is known

about the occurrence of resistance (18), and germplasm assessment is the first step toward determining the feasibility of breeding for olive knot resistance. Therefore, the aim of this study was to analyze factors affecting *P. savastanoi* pv. *savastanoi* inoculations, such as pathogen virulence, dose/response behavior, plant age, and secondary tumor development, in order to assess cultivar susceptibility. The methodology developed was applied to the evaluation of 29 olive cultivars, mainly from the World Olive Germplasm Bank, Centro de Investigación y Formación Agraria (CIFA) "Alameda del Obispo," Córdoba, Spain, for resistance to olive knot disease.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. Twenty Spanish strains of *P. savastanoi* pv. *savastanoi* isolated from olive knots were obtained from the Instituto Valenciano de Investigaciones Agrarias (IVIA) collection, and the type strain CFBP 1670^T was obtained from the Collection Française de Bactéries Phytopathogènes (CFBP), INRA, Angers, France, as supplied by L. Gardan. Strains were routinely grown in King's medium B (10) at 26°C. Bacterial suspensions for plant inoculations were adjusted turbidometrically to densities of ca. 10⁸ colony forming units (CFU) per ml (optical density at 600 nm = 0.5) in phosphate buffered saline (PBS) and 10-fold dilutions in PBS were prepared when appropriate.

Plant material. One- and three-year-old olive plants from the World Olive Germplasm Bank of Spain were used (6). Plants were propagated by rooting soft-wood cuttings under mist (4), and the rooted cuttings were planted in plastic pots as single trunk trees by removing other shoots when their average height was

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around 25 to 40 cm (7). After inoculation, plants were kept in the open-air inside a screenhouse in individual pots filled with a substrate composed of peat and sand (1:0.6, vol/vol) and watered by trickle irrigation.

Virulence of *P. savastanoi* pv. *savastanoi* strains. One-year-old plants of cvs. Arbequina, Blanqueta, Borriolenca, Manzanilla, Picual, and Villalonga were used to select the *P. savastanoi* pv. *savastanoi* strains for further experiments. Olive plants were wounded at three sites on the main stem and at two sites on lateral branches. Wounds of 0.5 cm long from the stem surface into the cambial area were made with a sterile scalpel. Each wound site was inoculated with 10^6 CFU of *P. savastanoi* pv. *savastanoi* in 10- μ l aliquots of PBS. Two plants of each cultivar were used for each of the 21 assayed strains (60 wound sites per strain). Another two plants were used as noninoculated control plants. The number of inoculated wound sites developing knots was recorded 3 months after inoculations.

Dose/response relationship. Olive plants of cvs. Nevadillo blanco de Jaén-cj, Lechín de Sevilla, and Mollar de Cieza were wounded at 10 sites on the main stem as described previously. Increasing 10-fold doses of *P. savastanoi* pv. *savastanoi* strain IVIA 1628 from ca. 10^2 to 10^6 CFU were applied to wounds in 5- μ l aliquots of PBS. Five to ten plants were inoculated per dose (50 to 100 wound sites per dose) in two independent experiments. Two plants were used as noninoculated control plants. The disease response for each dose was measured as the proportion of inoculated wound sites developing knots 3 months after inoculations.

Effect of plant age. One- and three-year-old olive plants of cvs. Callosina, Dulzal de Carmona-cj, Nevadillo blanco de Jaén-cj, Picual, and Picudo were compared for olive knot reaction. Plants were wounded at four sites on the main stem and inoculated with *P. savastanoi* pv. *savastanoi* strains IVIA 1628 and IVIA 1657-8 at low (10^2 CFU/wound) and high (10^6 CFU/wound) doses. Eleven plants of each cultivar were used per strain at each dose (44 inoculation sites per dose and strain). The disease reaction was measured as described previously.

Secondary knots. One-year-old olive plants of cvs. Lechín de Granada, Gordal sevillana, and Picual and 3-year-old plants of cvs. Callosina, Cornicabra, Dulzal de Carmona, and Nevadillo blanco de Jaén-cj were wounded at one site on the main stem, 10 cm above the crown. The initial wounds (wound site number 1, Fig. 1) were inoculated with *P. savastanoi* pv. *savastanoi* strains IVIA 1628 or IVIA 1657-8 at a high inoculum dose (10^6 CFU/

wound). On the same inoculation day, another wound (site number 2) was aseptically made on the main stem, 10 cm from the initial wound site (20 cm above the crown), but was not inoculated (Fig. 1). Fifteen days after inoculation of the initial wound, two new wounds were made 40 and 60 cm above the crown (site numbers 4 and 6), but were not inoculated. Thirty days after inoculation of the initial wound, two new wounds were made 30 and 50 cm above the crown (site numbers 3 and 5), but were not inoculated (Fig. 1). Eight to thirteen replicate plants of each cultivar were used per strain. Two plants per strain and cultivar were used as noninoculated control plants. The number of noninoculated wound sites developing knots was recorded 3 months after each wounding.

Cultivar susceptibility. Twenty-six olive cultivars were used for the evaluation of the development and weight of primary knots and the presence of secondary knots after inoculations with *P. savastanoi* pv. *savastanoi*. Two-year-old plants were wounded at five sites on the main stem and inoculated with *P. savastanoi* pv. *savastanoi* strains IVIA 1628 and IVIA 1657-8 with low (10^2 CFU/wound) and high (10^6 CFU/wound) inoculum doses. Ten plants per cultivar, strain, and dose were used (50 inoculation sites per dose and strain with each cultivar). All inoculations were performed in late spring. Two to four plants of each cultivar were inoculated with buffer as control plants. The disease response was measured as described above 3 months after inoculations. The next spring, knots were removed and weighted. Five new wounds between the previous ones were aseptically made in order to evaluate the appearance of secondary knots 3 months after wounding. Two independent experiments were done, one for the evaluation of 16 cultivars in 1999 and a second one in 2001 with 25 cultivars, including 12 replicate cultivars already analyzed in the previous experiment, in order to evaluate the reproducibility of the analyses.

Each cultivar included in the susceptibility analysis was rated with respect to the development and weight of primary knots and the presence of secondary knots. In each experiment, a given cultivar was considered as having high susceptibility for the production of primary and secondary knots when in a given treatment the percentage of wounds developing knots showed no significant differences with the highest obtained value. On the other hand, a given cultivar was considered as having low susceptibility when the percentage of knots showed no significant differences with the smallest obtained value. A given cultivar was considered as having medium susceptibility when the percentage of knots fell in the middle of the previous two groups. Cultivars were also rated according to the average weight of knots per diseased plant, in three categories: high, medium, and low susceptibility, considering medium as those cultivars with no significant differences with the average value.

Statistical analysis. For the selection of the most appropriate inoculum dose, the standard error of the probability that an inoculated wound develops a knot was obtained using the normal approximation after the formula

$$\sigma_{\hat{p}} = \sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$$

For cultivar susceptibility, two different approaches were used according to the type of distribution of the variable. For the percentage of wounds developing knots and secondary knots, a two-way generalized linear model (12) assuming binomial distribution was used. All possible contrasts were calculated to compare means. When an interaction was significant, the analyses were done individually for each strain. In all cases, due to the high number of mean comparisons, the Bonferroni correction was used to maintain the type I error to nominal 5% levels although the power of the test was very low. In order to know if there was a relationship between the number of inoculated wound sites developing knots and the number of knots that appeared in the non-

Fig. 1. Diagram showing the position and time of wounding for the secondary knots assay.

inoculated wound sites, a Spearman correlation test was calculated for each combination of assay, dose, and strain. For the average weight of knots, a two-way analysis of variance (strain by cultivar) within each experiment and dose was performed. Mean comparisons were obtained by the DGC test (8) that allows a disjoint classification of the means.

RESULTS

Virulence of *P. savastanoi* pv. *savastanoi* strains. Differences in virulence among 21 *P. savastanoi* pv. *savastanoi* strains were investigated in order to select representative strains for further cultivar susceptibility analyses. Two strains, CFBP 1670^T and a Spanish strain, were less virulent than the others, causing knots only in 25 and 6.6% of inoculated wound sites, respectively. Two other strains were intermediate in virulence (62 and 55% of inoculations developing knots, respectively). The other 17 strains were highly virulent, producing knots in more than 80% of the inoculated wounds. These strains caused symptoms in all inoculated cultivars, except one strain that did not cause knot formation in one cultivar. None of control plants developed knots at any wound site. Two of the highly virulent strains (IVIA 1628 and IVIA 1657-8) were considered representative of the majority of Spanish strains and they were selected for further use.

Dose/response relationship. Six levels of inocula were applied to olive plants to determine the optimum doses for symptom development and evaluation of cultivar susceptibility. For a given cultivar, the disease response (measured as the probability that an inoculated wound develop a knot) was strongly dependent on the inoculum dose applied (Fig. 2). At the highest inoculum dose (10^6 CFU/wound) almost all inoculation sites developed knots in the three cultivars assayed. The disease response decreased with decreasing amounts of bacteria applied to each wound site. As is shown in Figure 2, the greatest difference between cultivars was found after inoculating 10^2 CFU/wound. None of control plants produced knots in any wound site. Thus, we selected the inoculum dose of 10^2 CFU/wound for the susceptibility analysis of cultivars. A high inoculum dose (10^6 CFU/wound) was also included in the analysis as a positive control, in order to identify low susceptible cultivars.

Effect of plant age. Preliminary inoculation experiments performed on 1-year-old plants indicated that the assayed cultivars were all highly susceptible (data not shown), and then the disease response for 1- and 3-year-old plants was compared. After inocu-

Fig. 2. Dose/response effect for three olive cultivars after inoculation with different concentrations of *Pseudomonas savastanoi* pv. *savastanoi* strain IVIA 1628. Data represent the probability that a wound site develops a knot based on 50 to 100 inoculated wound sites from two independent experiments. Bars represent the standard error.

lation with the high dose of bacteria (10^6 CFU/wound), the percentage of inoculated sites developing knots was greater than 80 and 60% using 1- and 3-year-old plants, respectively, in all cultivars inoculated with both *P. savastanoi* pv. *savastanoi* strains. However, when inoculating with the low dose of bacteria (10^2 CFU/wound), a different reaction between 1- and 3-year-old plants was observed for some cultivars (Fig. 3). One-year-old plants developed knots in more than 80% of inoculated wounds, precluding the differentiation of cultivar susceptibility. However, when 3-year-old plants were inoculated with the low inoculum dose, the proportion of wounds developing knots ranged from 25 to 98% depending on the cultivar, allowing us to establish different susceptibility groups. Consequently, 1-year-old plants were not used in further cultivar susceptibility experiments.

Secondary knots. After wound inoculation with *P. savastanoi* pv. *savastanoi* at one position on olive stems, secondary tumors developed in new wounds subsequently performed away from the primary inoculation site, and the frequency of the appearance of the secondary knots varied among cultivars and strains (Table 1). Noninoculated control plants did not produce any knot in the preformed wounds. *P. savastanoi* pv. *savastanoi* migrated and produced secondary knots more easily on cvs. Picual and Gordal sevillana than on other cultivars. On Picual, 17 noninoculated wound sites developed secondary knots with the following distribution: 10 knots developed on wounds made on the first day (10 cm from primary knots), three knots developed on wounds made on day 15 (30 cm from primary knots), and four knots developed on wounds made on day 30 (20 cm from primary knots). Secondary tumors did not appear further than 40 cm from the primary inoculation site. On Gordal sevillana, 16 non-inoculated wound sites developed secondary knots with the following distribution: eight knots developed on wounds made on the first day, five knots developed on wounds made on day 15, and three knots developed on wounds made on day 30. Two knots developed in noninoculated wounds 50 cm from the primary inoculation site. The rest of the cultivars developed between two and four secondary knots. On Cornicabra, secondary knots were not produced with either strain.

Cultivar susceptibility. The development and weight of primary knots and the presence of secondary knots were evaluated after inoculations with *P. savastanoi* pv. *savastanoi* in 29 olive cultivars in two experiments. In the first experiment, 16 olive cultivars were evaluated (Table 2). As expected, when using a high inoculum dose, all cultivars, except one, developed knots at more than 90% of inoculation sites, and no significant differences were observed between the two *P. savastanoi* pv. *savastanoi* strains used as inoculum (data not shown). However, when inoculating with a low dosage inoculum, we found large differences in disease response among cultivars using both pathogen strains. The percent-

Fig. 3. Percentages of inoculated wound sites developing knots in 1- and 3-year-old plants of five olive cultivars inoculated with the pathogen strain IVIA 1628 at 10^2 bacteria per wound. Cultivars were as follows: 1, Callosina; 2, Picudo; 3, Nevadillo blanco de Jaén-cj; 4, Dulzal de Carmona-cj; and 5, Picual.

age of inoculated sites developing knots ranged from 0 to 100% depending on the cultivar (Table 2). None of control plants produced knots in any wound site. Mean values of the average weight of knots per diseased plant on each cultivar inoculated with both *P. savastanoi* pv. *savastanoi* strains at the low inoculum dose are also shown in Table 2. Six cultivars produced an average weight of knots per diseased plant significantly larger than the average of all cultivars ($\bar{x} = 0.33$). Significant differences in the presence of secondary knots were found among cultivars after inoculating the primary wound sites with a low inoculum dose of either *P. savastanoi* pv. *savastanoi* strain (Table 2). The percentages of non-inoculated sites developing knots ranged from 0 to 86% depending on the cultivar. A positive correlation was found between the number of primary and secondary knots inoculated with either *P. savastanoi* pv. *savastanoi* strain.

In the second experiment, 25 olive cultivars were evaluated, including 12 cultivars already analyzed in the first experiment (Table 3). When inoculating with low dose inoculum, we found

again significant differences in disease response with both challenge pathogens. The percentages of inoculated sites developing knots ranged from 0 to 100%, allowing us to make a rating of cultivars as shown below. None of the control plants produced knots in any wound site. Only cvs. Frantoio, Koroneiki, and Oblonga produced an average weight of knots per diseased plant significantly greater than the average of all cultivars ($\bar{x} = 0.58$). With respect to the presence of secondary knots in noninoculated wound sites, differences among the cultivars were found only when inoculating the initial wound sites with high dose inoculum using strain IVIA 1628. Noninoculated sites developed knots at rates of 0 to 65.5%, depending on the cultivar (data not shown).

Each cultivar evaluated in both performed experiments was rated for the development and weight of primary knots and the presence of secondary knots in each experiment using the low inoculum dose (Table 4). Considering the development of primary knots as the main factor to assess cultivar susceptibility, cv.

TABLE 1. Number of secondary knots appearing at wound sites 3 months after each wounding

Cultivar	<i>Pseudomonas savastanoi</i> pv. <i>savastanoi</i> strain	No. of plants inoculated ^y	No. of primary (1) and secondary knots at different wound locations (2 to 6) ^z						Total no. of secondary knots
			1	2	3	4	5	6	
Picual	IVIA 1628	12	12	4	2	1	0	0	7
	IVIA 1657-8	11	11	6	2	2	0	0	10
Gordal sevillana	IVIA 1628	8	8	2	0	0	0	0	2
	IVIA 1657-8	13	13	6	3	3	0	2	14
Nevadillo blanco de Jaén-cj	IVIA 1628	13	13	0	0	0	0	0	0
	IVIA 1657-8	13	12	2	1	1	0	0	4
Lechín de Granada	IVIA 1628	13	13	1	0	0	0	0	1
	IVIA 1657-8	12	12	1	0	1	0	0	2
Dulzal de Carmona	IVIA 1628	12	11	1	1	0	0	1	3
	IVIA 1657-8	13	13	0	0	0	0	0	0
Callosina	IVIA 1628	13	13	1	0	0	0	0	1
	IVIA 1657-8	13	12	1	0	0	0	0	1
Cornicabra	IVIA 1628	13	9	0	0	0	0	0	0
	IVIA 1657-8	13	13	0	0	0	0	0	0

^y Two plants per strain and cultivar were used as noninoculated control plants. None of those control plants produced knots in any wound site.

^z Figure 1 provides wounding time and site locations.

TABLE 2. Development and weight of primary knots and appearance of secondary knots after *Pseudomonas savastanoi* pv. *savastanoi* inoculations in 16 olive cultivars evaluated in experiment 1

Cultivar	Percentage of primary knots for <i>P. savastanoi</i> pv. <i>savastanoi</i> strains ^w		Weight of primary knots ^x	Percentage of secondary knots for <i>P. savastanoi</i> pv. <i>savastanoi</i> strains ^y	
	IVIA 1628	IVIA 1657-8		IVIA 1628	IVIA 1657-8
Arróniz	100 a	81.8 ab	0.43 a	34.5 abc	68.2 abc
Changlot real	100 a	90.9 a	0.41 a	32.7 abc	47.3 abc
Nevadillo blanco de Jaén-cj	100 a	95.4 a	0.26 b	50.0 abc	60.0 ab
Picudo	100 a	100 a	0.31 b	n.d. ^z	n.d.
Arbequina	97.7 a	100 a	0.43 a	86.0 a	56.3 abc
Morisca	95.4 a	100 a	0.67 a	32.7 abc	24.0 bc
Lechín de Sevilla	47.7 b	68.2 ab	0.55 a	29.9 abc	43.6 abc
Oblonga	38.6 bc	2.5 d	0.19 b	30.0 abc	4.0 bc
Gordal sevillana	34.4 bc	n.d.	0.46 a	78.0 ab	n.d.
Frantoio	6.8 d	50.0 bc	0.19 b	14.5 c	60.0 ab
Manzanilla cacereña	6.8 d	6.8 d	0.17 b	0.0 c	10.9 bc
Chemlali	0.0 d	0.0 d	n.d.	1.8 c	0.0 c
Dulzal de Carmona	0.0 d	0.0 d	n.d.	0.0 c	5.5 bc
Lechín de Granada	0.0 d	0.0 d	n.d.	0.0 c	0.0 c
Picual	0.0 d	4.5 d	0.05 b	0.0 c	82.0 a
Villalonga	0.0 d	27.3 cd	0.27 b	0.0 c	38.2 abc

^w Percentages of inoculated wound sites developing knots with low dose inoculum (10^2 bacteria per wound) with each challenge *P. savastanoi* pv. *savastanoi* strain. Within each column, the different letters indicate significant differences based on a two-way generalized linear model ($P < 0.0013$).

^x Mean values of the average weight of primary knots per diseased plant (grams) of each cultivar inoculated with both IVIA 1628 and IVIA 1657-8 *P. savastanoi* pv. *savastanoi* strains with low inoculum dose (10^2 bacteria per wound). Cultivars with different letters vary significantly based on the DGC test ($P < 0.05$).

^y Percentages of noninoculated wound sites developing knots when primary wound sites were inoculated at low dose inoculum (10^2 bacteria per wound) with each challenge *P. savastanoi* pv. *savastanoi* strain. Within each column, the different letters indicate significant differences based on a two-way generalized linear model ($P < 0.0007$).

^z Not determined.

Nevadillo blanco de Jaén-cj was rated as highly susceptible for production of primary knots in the first experiment when both *P. savastanoi* pv. *savastanoi* strains were used, and also in the second experiment only when strain IVIA 1657-8 was used.

TABLE 3. Development and weight of primary knots after *Pseudomonas savastanoi* pv. *savastanoi* inoculations in 25 olive cultivars evaluated in experiment 2

Cultivar	Percentage of primary knots for <i>P. savastanoi</i> pv. <i>savastanoi</i> strain ^y		Weight of primary knots ^z
	IVIA 1628	IVIA 1657-8	
Vallesa	100.0 a	51.1 cde	0.34 c
Royal de Cazorla	90.9 a	32.7 cdef	0.72 c
Ascolana tenera	70.9 ab	7.3 f	0.27 c
Mollar de Cieza	54.5 bc	21.8 def	0.87 c
Nevadillo blanco de Jaén-cj	54.0 bcd	100.0 a	0.67 c
Gordal sevillana	51.4 bcd	35.0 cdef	1.18 c
Pajarero	50.9 bcde	100.0 a	0.31 c
Changlot real	40.0 bcdef	0.0 f	0.18 c
Gordal de Hellín	32.7 cdef	21.8 def	0.38 c
Lechín de Granada	29.1 cdef	14.5 f	0.08 c
Picual	29.1 cdef	78.2 ab	0.74 c
Nevadillo negro	25.4 cdef	14.5 f	0.07 c
Chemlali	25.4 cdef	9.1 f	0.45 c
Lechín de Sevilla	21.8 cdef	58.0 bc	0.26 c
Dulzal de Carmona	20.0 cdef	16.4 ef	0.13 c
Manzanilla cacereña	20.0 cdef	16.4 ef	0.19 c
Cerezuela	18.5 def	5.4 f	0.32 c
Frantoio	16.4 ef	30.9 cdef	2.91 a
Manzanilla de Sevilla	16.4 ef	18.2 ef	0.09 c
Azapa	7.3 f	18.2 ef	0.10 c
Morisca	7.3 f	5.4 f	0.12 c
FS-17	0.0 f	0.0 f	0.00 c
Gordal de Archidona	0.0 f	4.4 f	0.19 c
Koroneiki	0.0 f	49.1 cde	1.86 b
Oblonga	0.0 f	61.8 bc	1.47 b

^y The inoculum dose was 10^2 bacteria per wound. Within each column, different letters indicate significant differences based on a two-way generalized linear model ($P < 0.0002$).

^z Mean values of the average weight of primary knots per diseased plant (grams) of each cultivar inoculated with both IVIA 1628 and IVIA 1657-8 *P. savastanoi* pv. *savastanoi* strains at 10^2 bacteria per wound. Weights followed by a different letter vary significantly based on the DGC test ($P < 0.05$).

Gordal sevillana, Oblonga, and Frantoio were rated for primary knots as medium and low in susceptibility depending on the experiment and *P. savastanoi* pv. *savastanoi* strain used. Chemlali, Dulzal de Carmona-cj, Lechín de Granada, and Manzanilla cacereña were rated as low susceptibility with respect to primary knot formation in both experiments with both *P. savastanoi* pv. *savastanoi* strains. Changlot real, Morisca, Lechín de Sevilla, and Picual varied greatly between experiments or strains used. Considering the weight of primary knots, 6 out of the 12 cultivars produced knots with a weight similar to that of the average weight in both experiments and were rated as medium in susceptibility. The other six cultivars produced knots with a greater weight than average, but only in one of the two experiments performed. With respect to the appearance of secondary knots, most of the cultivars were rated as medium or low in susceptibility to both *P. savastanoi* pv. *savastanoi* strains. Nevadillo blanco de Jaén-cj, Gordal sevillana, Frantoio, and Picual were rated as high but only when inoculated with one *P. savastanoi* pv. *savastanoi* strain.

Seventeen cultivars were analyzed just in one of the two performed experiments (Table 5). Considering the development of primary knots as the main factor to assess cultivar susceptibility after inoculations with *P. savastanoi* pv. *savastanoi*, in experiment 1, Arbequina, Arróniz, and Picudo were rated as having high susceptibility and Villalonga as having low susceptibility. For cultivars that were analyzed in experiment 2, Pajarero and Vallesa were classified as high or medium in susceptibility for primary knots depending on the strain used for inoculum. Mollar de Cieza and Koroneiki were classified as having medium or low susceptibility depending on the strain used. Azapa, Gordal de Hellín, FS-17, Nevadillo negro-cj, Cerezuela, Manzanilla sevillana, and Gordal de Archidona were classified as having low susceptibility for production of primary knots when inoculated with either *P. savastanoi* pv. *savastanoi* strain. Ascolana tenera and Royal de Cazorla were rated in different categories depending on the strain used for inoculum. Considering the weight of primary knots, all cultivars, except Arbequina, Arróniz, and Koroneiki, produced knots with a weight similar to that of the average. With respect to secondary knots, only cv. Arbequina was rated as highly susceptible in experiment 1 when inoculating strain IVIA 1628.

TABLE 4. Rating of 12 olive cultivars assayed in two independent experiments with respect to the development and weight of primary knots and appearance of secondary knots after *Pseudomonas savastanoi* pv. *savastanoi* inoculations^u

Cultivar	Reference number ^v	Susceptibility rating based on primary knots ^w				Susceptibility rating based on secondary knots ^y			
		Experiment 1		Experiment 2		Weight of primary knots ^x		Experiment 1	
		IVIA 1628	IVIA 1657-8	IVIA 1628	IVIA 1657-8	Experiment 1	Experiment 2	IVIA 1628	IVIA 1657-8
Nevadillo blanco de Jaén-cj	R-44	H	H	M	H	M	M	M	H
Changlot real	R-15	H	H	L	L	H	M	M	M
Morisca	R-17	H	H	L	L	H	M	M	L
Gordal sevillana	R-72	M	n.d. ^z	M	L	H	M	H	n.d
Lechín de Sevilla	R-5	M	H	L	M	H	M	M	M
Oblonga	R-210	M	L	L	M	M	H	M	L
Frantoio	R-80	L	M	L	L	M	H	L	H
Picual	R-9	L	L	L	H	M	M	L	H
Chemlali	R-744	L	L	L	L	n.d.	M	L	L
Dulzal de Carmona	R-31	L	L	L	L	n.d.	M	L	L
Lechín de Granada	R-54	L	L	L	L	n.d.	M	L	L
Manzanilla cacereña	R-430	L	L	L	L	M	M	L	L

^u Using the data shown in Table 2 (experiment 1) and Table 3 (experiment 2), cultivars were rated for each treatment in each experiment as H = high susceptibility, M = medium susceptibility, and L = low susceptibility as described in the text.

^v The registration number from the World Olive Germplasm Bank of CIFA, Córdoba, Spain, is indicated for each cultivar.

^w Percentages of inoculated wound sites developing knots with low dose inoculum (10^2 bacteria per wound) with each challenge *P. savastanoi* pv. *savastanoi* strain.

^x Mean values of the average weight of primary knots per diseased plant (grams) of each cultivar inoculated with both IVIA 1628 and IVIA 1657-8 *P. savastanoi* pv. *savastanoi* strains at 10^2 bacteria per wound.

^y Percentages of noninoculated wound sites developing knots when initial wound sites were inoculated with low dose inoculum (10^2 bacteria per wound) with each challenge *P. savastanoi* pv. *savastanoi* strain.

^z Not determined.

DISCUSSION

Information on the susceptibility of olive to olive knot disease is limited to few cultivars. Varvaro and Surico (23) compared the behavior of six Italian cultivars after *P. savastanoi* pv. *savastanoi* inoculation and did not find any differences because more than 95% of the inoculated wound sites developed knots in all of them. Considering information obtained in the present study, their results were probably due to the high inoculum dose applied ($>10^6$ bacteria per wound) and to the use of young plants (1-year-old). Benjama (3) inoculated six olive cultivars from Italy, Morocco, and Spain with eight *P. savastanoi* pv. *savastanoi* strains at different doses. Although, he did not report any statistical analysis of the data, he concluded that cv. Gordal sevilana was more tolerant to olive knot disease than the other cultivars. Marcelo et al. (11) evaluated six Portuguese cultivars by inoculating with only one *P. savastanoi* pv. *savastanoi* strain and found slight differences among cultivars. The percentage of inoculated sites with knots ranged from 36 to 66% depending on the cultivar, but the results were not statistically analyzed. Finally, Hassani et al. (9) evaluated five Italian cultivars inoculated with five *P. savastanoi* pv. *savastanoi* strains at an inoculum dose of ca. 5×10^7 bacteria per wound. Although they did not report the percentage of inoculated wound sites that developed knots, it is likely that with the high inoculum dose used, more than 90% of wounds developed knots in all cultivars. Consequently, they evaluated cultivars only by determining the knot size with a visual scale. As far as we know, no more information on this subject is available.

Our data from *P. savastanoi* pv. *savastanoi* virulence assays suggested that most of the analyzed strains of *P. savastanoi* pv. *savastanoi* were highly virulent on olive plants. Two strains were used as challenge pathogens in cultivar susceptibility to indicate the possibility of interactions between a given strain of the pathogen and a given cultivar and to minimize the error in cultivar evaluation due to interactions. Pathogen-cultivar interactions were

inferred from previous *P. savastanoi* pv. *savastanoi* inoculation experiments (3). Our results from dose/response assays with three cultivars indicate that the disease response was strongly dependent on the inoculum dose applied to the wounds, and confirm previous data indicating that inoculation with more than 10^4 to 10^5 bacteria per wound obscures differences between cultivars, because more than 75% of inoculated wound sites developed knots regardless of the cultivar (3,23). The fact that small differences in the inoculum dose resulted in big differences in the disease response for the same cultivar might explain possible discrepancies among cultivar evaluation from different sources. We found that an inoculum dose of 10^2 CFU/wound was the most appropriate for assaying cultivar susceptibility. We also determined that young olive plants (1-year-old) were too susceptible to olive knot disease for discrimination of susceptibility among cultivars. Probably, low susceptible phenotypes are only expressed in olive plants after a certain period of time or vegetative development.

Our data support the concept that *P. savastanoi* pv. *savastanoi* is able to migrate and produce secondary knots in new wounds in olive plants as suggested earlier (20,24). Similar development of secondary tumors following migration of *P. savastanoi* pv. *savastanoi* from the site of inoculation in oleander stems has been reported (25). In the present study, a correlation between the number of inoculated sites developing knots and the number of secondary knots that appeared in noninoculated wound sites was found when inoculating the primary wounds with a low inoculum dose. In nature it is frequent to observe new knots mainly in branches already harboring old knots. Our data suggest that evaluation of this trait might be useful in determining cultivar susceptibility.

In summary, based on the results of our study, we propose for evaluation of olive cultivars to olive knot disease take into account the development of primary knots after application of a low inoculum dose of two *P. savastanoi* pv. *savastanoi* strains. Using this method, most cultivars were clearly placed into three susceptibility categories; however, a few of the cultivars varied greatly in

TABLE 5. Rating of 17 olive cultivars assayed for the development and weight of primary knots and appearance of secondary knots after inoculations with two *Pseudomonas savastanoi* pv. *savastanoi* strains in a unique experiment^s

Cultivar	Reference number ^t	Experiment	Susceptibility rating based on primary knots ^u		Weight of primary knots ^v	Susceptibility rating based on secondary knots ^w	
			Strain IVIA 1628	Strain IVIA 1657-8		Strain IVIA 1628	Strain IVIA 1657-8
Arbequina	R-231	1	H	H	H	H	M
Arróniz	— ^x	1	H	H	H	M	M
Picudo	R-314	1	H	H	M	n.d. ^y	n.d.
Villalonga	R-364	1	L	L	M	L	M
Pajarero	R-26	2	M	H	M		
Vallesa	R-613	2	H	M	M		
Ascolana tenera	R-62	2	H	L	M		
Royal de Cazorla	R-390	2	H	L	M		
Mollar de Cieza	R-544	2	M	L	M		
Koroneiki	— ^z	2	L	M	H		
Azapa	R-726	2	L	L	M		
Gordal de Hellín	R-567	2	L	L	M		
FS-17	— ^z	2	L	L	M		
Nevadillo negro-cj	R-45	2	L	L	M		
Cerezuela	R-331	2	L	L	M		
Manzanilla de Sevilla	R-21	2	L	L	M		
Gordal de Archidona	R-755	2	L	L	M		

^s Using the data shown in Table 2 (experiment 1) and Table 3 (experiment 2), cultivars were rated for each treatment in each experiment as H = high susceptibility, M = medium susceptibility, and L = low susceptibility as described in the text.

^t The registration number from the World Olive Germplasm Bank of CIFA, Córdoba, Spain, is indicated for each cultivar.

^u Percentages of inoculated wound sites developing knots with low dose inoculum (10^2 bacteria per wound) with each challenge *P. savastanoi* pv. *savastanoi* strain.

^v Mean weights of primary knots per diseased plant (grams) of each cultivar inoculated with both *P. savastanoi* pv. *savastanoi* strains with low inoculum dose (10^2 bacteria per wound).

^w Percentages of noninoculated wound sites developing knots when initial wound sites were inoculated with low dose inoculum (10^2 bacteria per wound) with each challenge *P. savastanoi* pv. *savastanoi* strain in experiment 1.

^x This cultivar was supplied by B. Lizar from ITGA, Navarra, Spain.

^y Not determined.

^z These cultivars were supplied by J. Pinochet from Agromillora Catalana, S.A., Barcelona, Spain.

their behavior, and could not be definitively classified in any one category. Weight of primary knots and the presence of secondary knots can be considered as secondary factors when assessing cultivar susceptibility to olive knot disease. We propose that the method can be used in further olive cultivar susceptibility assays. Based on our data, we suggest the following cultivars from the World Olive Germplasm Bank of CIFA, Spain, can be used as controls in further cultivar susceptibility analyses: Nevadillo blanco de Jaén-cj R-44 or Picudo R-314 can be considered as highly susceptible cultivars, and Dulzal de Carmona-cj R-31, Lechín de Granada R-54, or Manzanilla cacereña R-430 as low susceptible cultivars.

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