Evaluation of the Phytopathological Reaction of Wild and Cultivated Olives as a Means of Finding Promising New Sources of Genetic Diversity for Resistance to Root-Knot Nematodes

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Abstract

Olive (*Olea europaea* L.) is one of the most important fruit crops in the Mediterranean Basin, because it occupies significant acreage in these countries and often has important cultural heritage and landscape value. This crop can be infected by several *Meloidogyne* species (*M. javanica*, *M. arenaria*, and *M. incognita*, among others), and only a few cultivars with some level of resistance to these nematodes have been found. Innovations in intensive olive growing using high planting densities, irrigation, and substantial amounts of fertilizers could increase the nematode population to further damaging levels. To further understand the interactions involved between olive and pathogenic nematodes and in the hope of finding solutions to the agricultural risks, this research aimed to determine the reaction of important olive cultivars in Spain and wild olives to *M. javanica* infection,

including genotypes of the same and other *O. europaea* subspecies. All olive cultivars tested were good hosts for *M. javanica*, but high levels of nematode reproduction found in three cultivars (Gordal Sevillana, Hojiblanca, and Manzanilla de Sevilla) were substantially different. In the wild accessions, *O. europaea* subsp. *cerasiformis* (genotype W147) and *O. europaea* subsp. *europaea* var. *sylvestris* (genotype W224) were resistant to *M. javanica* at different levels, with strong resistance in W147 (reproduction factor [Rf] = 0.0003) and moderate resistance in W224 (Rf = 0.79). The defense reaction of W147 to *M. javanica* showed a strong increase of phenolic compounds but no hypersensitive reaction.

Keyword: histopathology

Olive (*Olea europaea* L.) is one of the most important fruit crops in the Mediterranean Basin, because it occupies significant acreage in these countries and often has important cultural heritage and landscape value. Furthermore, this crop is expanding worldwide because of the high demand for olive oil related to its health benefits (Rallo et al. 2016). In addition to oil production, some cultivars are specifically cultivated for consumption as table olives and others are used for both purposes. The cultivated olive tree belongs to the *O. europaea* complex, which consists of six different subspecies associated with specific, often isolated, geographical areas (Besnard et al. 2018; Green 2002): (i) *O. europaea* subsp. *europaea* with two botanical varieties (*O. europaea* var. *sylvestris*, namely oleasters or wild olive; and *O. europaea* var. *europaea*, cultivated olive) distributed in the Mediterranean Basin, (ii) *O. europaea* subsp. *laperrinei* in the Saharan mountains, (iii) *O. europaea* subsp. *cuspidata* distributed from southern Africa to southern Egypt and from Arabia to China, (iv) *O. europaea* subsp. *guanchica* native from the Canary Islands, (v) *O. europaea* subsp. *maroccana* in southern Morocco, and (vi)

O. europaea subsp. *cerasiformis* in Madeira Island. This broad range of wild relatives, including both genotypes of other subspecies and those pertaining to the *O. europaea* var. *sylvestris* of its own subspecies, represent promising new sources of genetic diversity for resistance to plant pathogens, including root-knot nematodes (*Meloidogyne* spp.).

The olive tree rhizosphere was found to be a good habitat for many species of plant-parasitic nematodes (Ali et al. 2014; Castillo et al. 2010). However, only some were shown to be pathogenic or to directly feed on olive roots, among which one of the most widespread and damaging is the genus *Meloidogyne* (Ali et al. 2014; Archidona-Yuste et al. 2018; Castillo et al. 2010). A number of *Meloidogyne* species were found to infect olive (Abrantes and Santos 1991; Archidona-Yuste et al. 2018; Castillo et al. 2003; Minz 1961; Tarjan 1953; Yang and Zhong 1980), including *M. javanica* (Treub, 1885) Chitwood, 1949; *M. incognita* (Kofoid & White, 1919) Chitwood, 1949; *M. hapla* Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; *M. lusitanica* Abrantes & Santos, 1991; *M. baetica* Castillo, Vovlas, Subbotin & Troccoli, 2003; and *M. oleae* Archidona-Yuste, Cantalapiedra-Navarrete, Liebanas, Rapoport, Castillo & Palomares-Rius, 2018.

The aforementioned *Meloidogyne* spp. were reportedly associated with olive in 19 countries, representing a real threat for olive culture worldwide (Ali et al. 2014). Plant-parasitic nematodes can also be found in sporadic distributions in wild olives, olive nurseries, and established orchards and have been demonstrated to cause heavy root galling and a severe reduction in plant growth in pathogenicity tests (Castillo et al. 2010; Lamberti and Baines 1969; Nico et al. 2002, 2003; Sasanelli et al. 1997, 2002). Estimates of olive losses in the United States attributable to *Meloidogyne* spp. and *Tylenchulus semipenetrans* (Cobb, 1913) range from 5 to 10% (Koenning et al. 1999; Singh et al. 2013). In some cases, plant-parasitic nematodes are associated with damaging syndromes such as “drying syndrome” in newly established olive orchards in Argentina (Pérez et al. 2001) or with vascular diseases (Lamberti et al. 2001; Saeedizadeh et al. 2003). Damage in nursery plants is more severe as a result of nematode parasitism and more importantly, plant-parasitic nematode populations are disseminated from nurseries to uninfested areas (Nico

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et al. 2002). Furthermore, the olive crop is changing from traditional low-input orchards without irrigation to new, highly mechanized orchards, with irrigation, high fertilizer inputs, and high planting densities, often belonging to new cultivars adapted to these crop conditions (Rallo et al. 2016). These new high-density irrigated orchards tend to have soil conditions that are more conducive for the establishment of the diseases caused by nematodes, mainly *Meloidogyne* spp. (Ali et al. 2016).

Host plant resistance could be the easiest, safest, and most cost-effective long-term approach to controlling the damage caused by plant-parasitic nematodes (Castillo et al. 2010), provided, for example, by breeding for resistant rootstocks. Previous studies identified a few olive cultivars associated with some degree of resistance to the most common species of *Meloidogyne*, such as *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla* (Al-Sayed and Abdel-Hameed 1991; Lamberti and Baines 1969; Sasanelli et al. 1997). In general, commercial cultivars are grown self-rooted, but the use of resistant rootstocks, alternative cultivars such as Allegra in California (McKenry 1994), or wild types could also be of great interest. Apart from the indications above, most preliminary reports indicate that most olive cultivars are good, rather than resistant, hosts for *Meloidogyne* spp., such as cultivars Arbequina and Picual as hosts for *M. javanica*, *M. incognita*, and *M. arenaria* (Nico et al. 2003) and Cima di Bitonto as a host for *M. javanica* (Sasanelli 2009). Knowledge of resistance or susceptibility of wild olives is lacking. Thus, the search for resistance in traditional cultivars and/or newly bred cultivars as well as in wild olive genotypes requires further attention. Progress in understanding the reaction of olive cultivars, wild olive genotypes, and other related subspecies to *Meloidogyne* spp. represents a critical step to finding new solutions for sustainable olive agriculture.

The specific objectives of this research were (i) to determine the host suitability of widely used, mainly Spanish, commercial olive cultivars, wild olive genotypes, and related subspecies of the *O. europaea* complex to *M. javanica* and (ii) to assess the histopathological plant–nematode interaction in susceptible and resistant hosts in order to better understand the mechanisms involved. *M. javanica*, currently

the most prevalent *Meloidogyne* species in both cultivated and wild olives in Spain (Archidona-Yuste et al. 2018), was used for standardized evaluation.

Materials and Methods

Nematode inocula. An isolate of *M. javanica* from a commercial orchard (cultivar Manzanilla de Sevilla) in La Campana, Sevilla Province, Spain, was identified to the species level based on features of the female perineal pattern, isozyme malate and esterase patterns, and molecular data (sequence characterized amplified region-based PCR assays, *coxII*-16S rRNA, and specific PCR) (Donkers-Venne et al. 2000; Esbenshade and Triantaphyllou 1985; Hartman and Sasser 1985). Inoculum of the *M. javanica* isolate was increased on tomato (*Solanum lycopersicon* L. ‘Tres Cantos’) grown in clay pots filled with an autoclaved (120°C, 2 h) sandy soil mixture, starting from a single egg mass in a growth chamber adjusted to 25 ± 1°C, 60 to 90% relative humidity, and a 16-h photoperiod of fluorescent light at 360 ± 25 µE m⁻² s⁻¹ for 2 months. The inoculum consisted of eggs and second-stage juveniles (J2) extracted from 2-month-old tomato plants using 1% sodium hypochlorite (Hussey and Barker 1973) followed by centrifugal flotation (Coolen 1979).

Plant material. The wild olive genotypes and the related subspecies were obtained from the ex situ wild repository established at the Centro Alameda del Obispo at the Instituto Andaluz de Investigación y Formación Agraria Pesquera Alimentaria y de la Producción Ecológica (IFAPA) (Belaj et al. 2016; León et al. 2018) in Córdoba, Spain, whereas the olive cultivars (Table 1) came from the IFAPA World Olive Germplasm Collection, which is also maintained at the same research center (Belaj et al. 2016). Although both the wild and cultivated genotypes are represented by two to three trees per genotype in their respective collections, the plant material utilized in this study and further vegetatively propagated by semihardwood cuttings was obtained from one and always the same tree. The following procedure was used to rapidly produce homogeneous plants suitable for the experiments: Cuttings from branches of the preceding year’s growth (0.5 cm in diameter, with a length of approximately 12 to 14 cm) were selected from each tree under study for vegetative propagation. The stem cuttings were surface disinfested with fungicide (a 1% CuSO₄ solution) for 5 min and washed four times in sterile distilled water to prevent fungal contamination. Afterward, the lower end of the cuttings was dipped for 5 s in 3,000 ppm of indole butyric acid powder (Rootone F; Compo, Barcelona, Spain) to promote rhizogenesis. Then the treated and dried stem cuttings were planted in propagation trays filled with perlite and kept under suitable conditions for rooting (25 ± 1°C, 60 to 90% relative humidity) for 2 months in the greenhouse chamber. The rooted cuttings were transferred to 1-liter plastic pots filled with peat and maintained for approximately 6 months in a shade house for hardening (Del Río and Caballero 2005).

Plants of uniform root system and shoot size were selected and transplanted into 75 × 77 × 180-mm plastic pots (one plant per pot) filled with an autoclaved (120°C, 1 h, twice) soil mixture (sand/clay loam, 2:1, vol/vol). Plants were watered on alternate days with 100 ml of sterilized tap water and fertilized with 100 ml of a 0.1% solution of a 20-5-32 (N-P-K) plus micronutrient fertilizer (Poly-Feed, Haifa, Israel) and pruned to maintain a single shoot every week. The plants were inoculated with *M. javanica* inoculum after a 7-day recovery period.

Growth chamber experiments. The experiments were conducted in a growth chamber under the conditions described above, which are considered optimal for the development and reproduction of *M. javanica* (Trudgill and Perry 1994). Plants were inoculated individually by adding 10,000 eggs plus J2 of *M. javanica* in 10 ml of sterile distilled water. The nematode suspension, corresponding to a theoretical inoculum density of 10 nematodes/cm³ soil, was added to four holes in the soil around the base of the plant. The nematode inoculum density of the water suspension was determined by counting nematode specimens in ten 1-ml aliquots.

Plants were watered with 100 ml of water on alternate days and fertilized weekly with 100 ml of the previously mentioned nutrient

Table 1. Olive (*Olea europaea*) wild genotypes, related subspecies, and cultivars included in this study with their respective passport code in the ex situ wild repository and IFAPA World Olive Germplasm Collection and geographic origin^z

Plant material type	Passport code	Geographic origin
Wild olive genotype/related subspecies		
<i>Olea europaea</i> subsp. <i>cerasiformis</i>	W147	Portugal, Madeira
<i>O. europaea</i> subsp. <i>cuspidata</i>	W158	Ethiopia
<i>O. europaea</i> subsp. <i>europaea</i> var. <i>sylvestris</i>	W224	Morocco
	W223	Morocco
	W166	Spain, Extremadura
	W19	Spain, Jaén
<i>O. europaea</i> subsp. <i>guanchica</i>	W33	Spain, Canary Islands
	W46	Spain, Canary Islands
	W1048	Spain, Canary Islands
<i>O. europaea</i> subsp. <i>maroccana</i>	W215	Morocco
	W228	Morocco
Cultivar (<i>O. europaea</i> subsp. <i>europaea</i> var. <i>europaea</i>)		
Arbequina	231	Spain
Ayvalik	97	Turkey
Gordal Sevillana	234	Spain
Hojiblanca	v 2	Spain
Koroneiki	218	Greece
Lechín de Sevilla	5	Spain
Manzanilla de Sevilla	21	Spain
Picual	9	Spain
Sikitita	1920	Spain

^z IFAPA = Instituto Andaluz de Investigación y Formación Agraria Pesquera Alimentaria y de la Producción Ecológica.

solution. Each genotype was replicated eight times and the experiment was arranged in a completely randomized design. The experiment was conducted twice, with a duration of 120 days after nematode inoculation.

Assessment of plant growth variables and data analyses. Plant growth, root galling, and nematode reproduction were rated at the end of both trials. Plant growth of the genotypes was assessed by comparing root fresh weight with that of uninoculated control plants. Before assessment of root weight, the root system of a plant was gently washed free of adhering soil and debris and root galling was rated on a 0 to 6 scale as follows: 0 indicates no galls, 1 indicates 1 to 10, 2 indicates 11 to 20, 3 indicates 21 to 40, 4 indicates 41 to 70, 5 indicates 71 to 90, and 6 indicates ≥ 91 galls (Nico et al. 2003).

Final soil and root nematode population densities (P_f) were determined. Soil was washed thoroughly with tap water through a 710- μ m mesh sieve and the filtered water was collected in a beaker and thoroughly mixed with 4% kaolin (vol/vol). This mixture was centrifuged at $1,100 \times g$ for 4 min, the supernatants were discarded, pellets were resuspended in 250 ml of $MgSO_4$ ($\delta = 1.16$), and the new suspensions were centrifuged at $1,100 \times g$ for 3 min. The new supernatants were sieved through a 5- μ m mesh, and nematodes collected on the sieve were washed with tap water, transferred to Petri dishes, and counted under a stereomicroscope (Coolen 1979). To assess the nematode population in *Meloidogyne*-infected roots, the complete root system of a plant was washed free of soil and cut into 1- to 2-cm segments, and *M. javanica* (eggs, sedentary stages, and J2s) were extracted by maceration followed by centrifugation. Root tissues were homogenized in 250 ml of a 1% solution of NaOCl using a Waring blender at $1,800 \times g$ for 1 min, and homogenates were centrifuged and extracted as described above (Coolen 1979; Hussey and Barker 1973). Population densities were used to calculate the reproduction index (reproduction factor [Rf] = final population density in soil and roots [P_f] divided by initial population density [P_i]). All data on root symptom severity, nematode reproduction, and root fresh weight were transformed into $\log_{10}$ values ($X + 1$) before analyses (Gómez and Gómez 1984). Similarity between the experiment repetitions was tested by preliminary analyses of variance (ANOVAs) using experimental runs as factors, which determined that the experiment $\times$ genotype interaction was not significant ($P \geq 0.05$) and thus permitted combining the data of both experimental runs for further analyses (Gómez and Gómez 1984). ANOVAs were carried out using Statistix 10.0 software (NH Analytical Software, Roseville, MN). Significant differences among means of root weight, gall rating, and nematode reproduction were estimated using the least significant difference multiple range test ($P = 0.05$). To avoid the use of zero in the ANOVA, data from uninoculated control treatments were not included in analyses of gall ratings and nematode reproduction.

Histopathological study. Galled roots from the different subspecies of olive and cultivated plants infected by *M. javanica* and controls (similar root zones from uninoculated plants) were selected at the end (120 days) of the experiments (two to three roots per plant from three to four plants in every genotype and treatment for each experiment repetition). The galled roots were then gently washed free of adhering soil and debris, fixed in FAE solution (formalin, acetic acid, 95% ethanol, and distilled water [10:5:50:35 vol/vol/vol/vol]) for at least 48 h, dehydrated in a tertiary butyl alcohol series (70–85–90 to 100%), and embedded in paraffin (58°C melting point; Merck, Darmstadt, Germany) for histopathological observations. Embedded tissues were sectioned longitudinally and transversely at 12 μ m with a rotary microtome and stained with a combination of tannic acid–ferric chloride, safranin, and fast green, by which nuclei, chromosomes, and lignified or suberized cell walls stain red, cytoplasm and cellulosic cell walls stain green, and the tannic acid–iron chloride aids in cell wall definition and is considered to be a general test for phenols (Jensen 1962; Reeve 1951; Ruzin 1999). The stained sections were examined microscopically (Leica DMRBFHC optical microscope; Leica Microsystems, Heerbrugg, Switzerland) and photographed (Leica DFC450C digital camera).

Two genotypes (cultivar Ayvalik and the wild genotype W19) developed typical galls but were damaged during histological processing, so no microphotographs could be obtained. The wild olive genotype from Morocco (W224) was not included in the final histological analysis because of the few and small galls produced. Similarly, genotype W147 from Madeira Island and belonging to *O. europaea* subsp. *cerasiformis* was also excluded from this analysis because roots were not galled in the two experimental repetitions.

In an additional experiment, 12 plants from genotype W147 were inoculated with a high inoculum level of 15,000 J2s in order to further explore early interactions with the host plant, such as a possible hypersensitive reaction or repellence of the nematode by the root. These plants were sampled at 4, 11, 25, and 70 days after inoculation (DAI) (three plants per sampling time). Half of the roots for each plant were processed according to the histopathological procedure described above, and the other half were processed for fuchsine acid staining following Byrd et al. (1983). Unfortunately, wild olive plantlets of genotype W224 were not available for the additional study of resistance.

Results

Suitability of wild and cultivated olive genotypes as hosts of *M. javanica*. Symptoms on aboveground plant parts did not appear on either nematode-inoculated or uninoculated plants. In some cases, differences in root fresh weight were detected between inoculated and uninoculated plants. For the wild olives, W166 was the only genotype with a significant difference in root fresh weight between inoculated and uninoculated plants, with greater root fresh weight in uninoculated plants (Table 2). Root fresh weights were high for the inoculated cultivars Hojiblanca and Manzanilla de Sevilla (Table 3), whereas cultivars Gordal Sevillana and Picual had lower root fresh weight when inoculated with *M. javanica*. There was no difference in fresh root weights between the inoculated and uninoculated plants in the other five cultivars (Table 3). Variation in fresh root weights was observed, however, among the genotypes of wild olive and commercial cultivars, likely related to the original plant size and growth level at the beginning of the experiment (Tables 2 and 3).

Although relative levels of root galling and Rf values differed among olive genotypes, they followed similar patterns. The wild genotype W158 belonging to *O. europaea* subsp. *cuspidata* had the highest levels of root galling and the highest Rf value, followed by the two genotypes (W1048 and W46) belonging to *O. europaea* subsp. *guanchica* as well as the subsp. *maroccana* genotype W215 (Table 2). Most of the other wild genotypes and related subspecies had statistically lower levels of root galling and Rf values than the corresponding noninoculated plants. Two genotypes, W147 and W224, belonging to *O. europaea* subsp. *cerasiformis* and subsp. *europaea* var. *sylvestris*, respectively, had exceptionally low levels of root galling and Rf values (Rf < 1) when inoculated with *M. javanica*. The commercial cultivars all had high to moderate root galling (from 0.95 to 3.00) and high Rf values (from 2.17 to 8.64) (Table 3). However, substantially higher Rf values were observed in Gordal Sevillana, Hojiblanca, and Manzanilla de Sevilla compared with the other cultivars (Table 3).

Root morphological and histopathological reaction of olive genotypes to *Meloidogyne* spp. The root-system morphology had typical galls produced by *M. javanica* (Fig. 1), characterized by galling at the tips of growing roots. Histopathological sections of the root galls of wild olives (Fig. 2) and olive cultivars (Fig. 3) infected by *M. javanica* had typical feeding sites with five to six giant cells close to each nematode female, with bigger galls having more than one female per gall (Figs. 2 and 3). The giant-cell cytoplasm was dense, granulated, and homogenous and contained numerous hypertrophied nuclei. Disruption of xylem vessels was detected close to the massively enlarging giant cells.

Genotypes W147 and W224 produced no or minimal nodulation in response to *M. javanica* infection and had small rates of reproduction (Table 2). Genotype W147 (*O. europaea* subsp. *cerasiformis*) at 4 DAI had very few juveniles in the few root tips. During this period,

Table 2. Host suitability of wild olive (*Olea europaea*) genotypes (including subspecies other than *europaea*, and var. *sylvestris* of subsp. *europaea* with their respective passport code in the ex situ wild repository and IFAPA World Olive Germplasm Collection) to *Meloidogyne javanica* and effects of root growth^w

Host genotype	Passport code	Inoculation treatment	Variables ^x		
			Root fresh weight (g)	Root symptoms ^y	Rf ^z
<i>Olea europaea</i> subsp. <i>cerasiformis</i>	W147	Uninoculated control	23.43 ± 5.08 CD	–	–
		<i>M. javanica</i>	18.31 ± 7.02 F	0.5 ± 0.0000 G	0.0003 ± 0.0005 F
<i>O. europaea</i> subsp. <i>cuspidata</i>	W158	Uninoculated control	69.80 ± 1.98 A	–	–
		<i>M. javanica</i>	92.68 ± 15.17 A	4.13 ± 0.44 A	20.64 ± 9.12 A
<i>O. europaea</i> subsp. <i>europaea</i> var. <i>sylvestris</i>	W224	Uninoculated control	26.10 ± 3.88 BCD	–	–
		<i>M. javanica</i>	32.77 ± 7.10 CD	0.60 ± 0.21 G	0.79 ± 0.43 E
	W223	Uninoculated control	30.48 ± 2.18 B	–	–
		<i>M. javanica</i>	40.01 ± 11.30 BC	1.08 ± 0.34 F	3.98 ± 2.27 D
	W166	Uninoculated control	81.50 ± 21.38 a A	–	–
		<i>M. javanica</i>	49.40 ± 19.01 b B	2.34 ± 0.60 CD	7.45 ± 2.24 BC
	W19	Uninoculated control	25.93 ± 6.46 BCD	–	–
		<i>M. javanica</i>	25.09 ± 8.04 E	1.73 ± 0.47 E	3.33 ± 1.14 CD
<i>O. europaea</i> subsp. <i>guanchica</i>	W33	Uninoculated control	19.55 ± 3.89 D	–	–
		<i>M. javanica</i>	24.58 ± 12.63 E	1.28 ± 0.55 F	3.78 ± 2.29 D
	W46	Uninoculated control	11.90 ± 0.36 E	–	–
		<i>M. javanica</i>	12.36 ± 3.86 G	3.15 ± 1.11 B	3.43 ± 0.86 CD
<i>O. europaea</i> subsp. <i>maroccana</i>	W1048	Uninoculated control	11.67 ± 1.24 E	–	–
		<i>M. javanica</i>	12.36 ± 3.86 G	3.15 ± 1.11 B	3.43 ± 0.86 BCD
	W215	Uninoculated control	26.78 ± 3.99 BCD	–	–
		<i>M. javanica</i>	26.86 ± 9.69 DE	2.68 ± 0.59 BC	12.60 ± 8.57 AB
	W228	Uninoculated control	28.90 ± 5.66 BC	–	–
		<i>M. javanica</i>	24.21 ± 14.28 EF	2.28 ± 1.35 DE	10.80 ± 10.17 ABC

^w Data are the mean of 16 replicated plants per treatment combination from two replicated experiments. Inoculated plants received 10,000 eggs plus J2 (*Pi*) of *M. javanica* while uninoculated plants did not receive nematodes. For each *O. europaea* genotype, lowercase letters refer to differences between uninoculated and inoculated treatments within each genotype and are only shown when differences are significant ($P \geq 0.05$) according to the least significant difference (LSD) test. Uppercase letters refer to comparisons of all means of the same treatment (either *M. javanica* inoculated or uninoculated control) among the different genotypes. Means followed by the same uppercase letter do not differ significantly ($P \geq 0.05$) according to the LSD test. IFAPA = Instituto Andaluz de Investigación y Formación Agraria Pesquera Alimentaria y de la Producción Ecológica.

^x Mean percentage and standard deviation of each variable during the experiment. Dashes indicate no symptoms.

^y Root symptoms were assessed on a 0 to 6 rating scale according to the number of root galls (0 = no galls, 1 = 1 to 10, 2 = 11 to 20, 3 = 21 to 40, 4 = 41 to 70, 5 = 71 to 90, and 6 = ≥ 91).

^z Nematode reproduction factor (Rf) = Final nematode numbers per plant (*Pf*)/Initial nematode inoculum per plant (*Pi*).

Table 3. Host suitability of several olive (*Olea europaea*) commercial cultivars to *Meloidogyne javanica* and effects of root growth^w

Host genotype	Cultivar	Inoculation treatment	Variables ^x		
			Root fresh weight (g)	Root symptoms ^y	Rf ^z
<i>O. europaea</i> subsp. <i>europaea</i> var. <i>europaea</i>	Arbequina	Uninoculated control	3.08 ± 0.71 CD	–	–
		<i>M. javanica</i>	3.91 ± 1.36 E	1.75 ± 0.72 B	2.40 ± 2.89 B
	Ayvalik	Uninoculated control	38.23 ± 3.68 A	–	–
		<i>M. javanica</i>	38.33 ± 12.29 A	0.95 ± 0.16 C	2.61 ± 1.56 B
	Gordal Sevillana	Uninoculated control	5.08 ± 1.28 a BC	–	–
		<i>M. javanica</i>	3.05 ± 1.30 b DE	2.83 ± 0.38 A	7.11 ± 8.99 A
	Hojiblanca	Uninoculated control	3.05 ± 0.66 a CD	–	–
		<i>M. javanica</i>	7.51 ± 2.57 b B	3.00 ± 0.84 A	7.26 ± 5.22 A
	Koroneiki	Uninoculated control	3.20 ± 1.37 CD	–	–
		<i>M. javanica</i>	2.85 ± 1.00 E	2.08 ± 0.63 B	2.17 ± 1.93 B
	Lechín de Sevilla	Uninoculated control	2.15 ± 1.08 D	–	–
		<i>M. javanica</i>	3.00 ± 1.88 E	1.88 ± 1.11 B	3.27 ± 4.49 B
	Manzanilla de Sevilla	Uninoculated control	2.35 ± 0.73 a D	–	–
		<i>M. javanica</i>	5.63 ± 1.38 b C	2.78 ± 0.47 A	8.64 ± 6.03 A
	Picual	Uninoculated control	7.40 ± 6.10 a B	–	–
		<i>M. javanica</i>	3.91 ± 1.36 b D	1.75 ± 0.72 B	2.40 ± 2.90 B
	Sikitita	Uninoculated control	6.93 ± 2.52 B	–	–
		<i>M. javanica</i>	8.33 ± 5.84 B	1.75 ± 0.80 B	3.33 ± 3.74 B

^w Data are the mean of 16 replicated plants per treatment combination from two replicated experiments. Inoculated plants received 10,000 eggs plus J2 (*Pi*) of *M. javanica*, while uninoculated plants did not receive nematodes. For each *O. europaea* genotype, lowercase letters refer to differences between uninoculated and inoculated treatments of that genotype and are only shown when differences are significant ($P < 0.05$) according to the least significant difference (LSD) test. Uppercase letters refers to comparisons of all means of the same treatment (either *M. javanica* inoculated or uninoculated control) among the different genotypes. Means followed by the same uppercase letter do not differ significantly ($P \geq 0.05$) according to the LSD test.

^x Mean percentage and standard deviation of each variable during the experiment. Dashes indicate no symptoms.

^y Root symptoms were assessed on a 0 to 6 rating scale according to the number of root galls (0 = no galls, 1 = 1 to 10, 2 = 11 to 20, 3 = 21 to 40, 4 = 41 to 70, 5 = 71 to 90, and 6 = ≥ 91).

^z Nematode reproduction factor (Rf) = Final nematode numbers per plant (*Pf*)/Initial nematode inoculum per plant (*Pi*).

nematodes were moving within the root tip, searching for specific suitable cells in order to induce feeding sites. These juveniles were surrounded by high levels of phenolic compounds and some necrotic cells (Fig. 4L, M, and N). At 11 DAI, a few nematodes were sedentary but the others were still looking for appropriate cells for inducing the feeding site. The root tissue where nematodes were present was darker than other areas of the root, owing to the presence of phenolic compounds. Some root tips were very slightly galled (Fig. 4B and C). At 25 DAI, the nematodes in feeding sites with giant cells visible were crowded in specific galls and exhibited different stages of development inside the gall ranging from J2 sedentary, J3 (Fig. 4D, E, and F), or J4 (Fig. 4G). Males were also detected at this time. Nematodes viewed in histopathological sections were surrounded by feeding sites, but these were saturated with very dark areas as a result of the presence of phenolic compounds. In addition, the cytoplasm of the giant cells was generally denser and darker than the surrounding uninfected tissues. However, the nematodes were feeding on those cells and clearly undergoing development (Fig. 4D, E, P, Q, S, and T). At 70 DAI, only a few small females had developed, which were solitary and occupied tip positions (from two to five females per plant), and only a small number of eggs were produced inside them and in the egg mass (Fig. 4H and K). Overall, fewer nematodes were detected in the roots than observed at 25 DAI using fuchsine acid. Because of the low number of females present within the root tissue, it was not possible to locate females in the histological sections.

Discussion

Olive production in the Mediterranean Basin is threatened by soil pathogens such as *Verticillium dahliae* and *Meloidogyne* spp. and their interactions with soil microorganisms. In the case of nematodes of the genus *Meloidogyne*, extensive sampling in Morocco indicated that this group of nematodes was present in 23 and 52% of olive orchards and nurseries, respectively (Hamza et al. 2017). Studies in Andalusia, Spain, revealed the frequent presence of *Meloidogyne* spp. in both cultivated and wild olives, with *M. javanica* being the most prevalent species in Spain (Archidona-Yuste et al. 2018). Furthermore, the recent transition to intensive olive growing, with

high-density planting, irrigation, and substantial amounts of fertilizers, could increase the nematode population densities to further damaging levels. Based on confirmed levels of pathogenesis (Sasanelli 2009) and frequent association with olive (Archidona-Yuste et al. 2018; Hamza et al. 2017), *M. javanica* was used as a standard species for the study. This species proved to be suitable for this role and produced levels of infection readily measurable by root galling and nematode reproduction after controlled inoculations. Overall, both the cultivated and wild olive genotypes were suitable hosts, although some appeared to be particularly susceptible among the cultivars studied, and within the wild genotypes there may be candidates for resistance or for better understanding resistance mechanisms.

Consistent with the findings of this study, Mediterranean olive cultivars are generally considered good hosts for *Meloidogyne* spp. (Castillo et al. 2010; Nico et al. 2003). However, interestingly, statistically significant higher Rf values were found in three olive cultivars (Gordal Sevillana, Hojiblanca, and Manzanilla de Sevilla) compared with other cultivars used in Spain and others tested in this study. These cultivars, in particular Hojiblanca and Manzanilla de Sevilla, are planted in extensive areas of Spain (MAGRAMA 2017). Information on the susceptibility of cultivars to nematodes is critical when establishing orchards in areas infested with *Meloidogyne* spp., as the plants could increase populations up to damaging levels in only a few years after planting. Furthermore, the nematode damage risk is particularly high when the plant is still young and more susceptible. Other olive cultivars considered in this study, such as those associated with high-density plantings in hedgerows (Arbequina, Sikitita, and Koroneiki), had similarly lower Rf values but were still good hosts for *M. javanica*. The high-intensity field conditions in which these cultivars are often grown commercially could be particularly risky for nematode infection and long-term experiments are necessary to assess nematode pressure under these conditions.

Perennial plants such as the olive present a particular challenge for combating infection. Resistance genes are exposed to much longer periods of continual pressure than plants in annual production systems (Saucet et al. 2016), increasing the risk that nematodes will “break” the plant resistance (Lespinasse et al. 2003). Nematicides

Fig. 1. Root systems of olive (*Olea europaea* subsp. *europaea* var. *europaea*) cultivars **A**, Picual and **B**, Gordal Sevillana infected by *Meloidogyne javanica* showing typical galls. **C**, **D**, **E**, and **F**, Details of severely nodulated roots in olive cultivar Gordal Sevillana.

are restricted in their use in woody plants and are not economically feasible in a low-income crop such as olive, and thus alternative control measures are required. Wild olives offer the possibility of resistance sources for *Meloidogyne* spp., specifically as rootstocks. The use of resistant rootstocks for other diseases (Verticillium wilt) has

proven useful, even in the presence of *Meloidogyne* spp. (Palomares-Rius et al. 2016).

In contrast to the majority of the interactions studied in the experiments, the genotype W147, which belonged to subsp. *cerasiformis*, showed a resistant reaction to *M. javanica*. This genotype had a

Fig. 2. Light micrographs of root cross-sections of wild olives (subspecies other than *Olea europaea* subsp. *europaea* and var. *sylvestris* of subsp. *europaea*) including the feeding site induced by *Meloidogyne javanica*. **A, B, C, and D**, *Olea europaea* subsp. *cuspidata* W158. **E, F, G, and H**, *O. europaea* subsp. *europaea* var. *sylvestris* W223. **I, J, K, and L**, *O. europaea* subsp. *europaea* var. *sylvestris* W166. **M, N, O, and P**, *O. europaea* subsp. *guanchica* W33. **Q, R, S, and T**, *O. europaea* subsp. *guanchica* W46. **U, V, W, and X**, *O. europaea* subsp. *guanchica* W1048. **Y, Z, A', and B'**, *O. europaea* subsp. *maroccana* W215. **C', D', E', and F'**, *O. europaea* subsp. *maroccana* W228. Asterisks indicate individual giant cells. gc = giant cell in a feeding site and N = nematode. Scale bars = 200 μ m in A, B, E, F, I, J, M, N, Q, U, V, Y, Z, C', and D'; and 100 μ m in C, D, G, H, K, L, O, P, R, S, T, W, X, A', B', E', and F'.

minimal number of nematodes in the roots and surrounding soil, and the slight gall-like swellings observed were likely attributable to the presence of very few females developing at a slow rate, as we found in the fuchsine acid staining experiment (Fig. 4H to K). Other cultivars with resistance specific to *Meloidogyne* spp. (e.g., Coratina and Leccino) (Sasanelli 2009) had low Rf values (<1), which differed

from the resistance behavior observed in the genotype of the subsp. *cerasiformis*, in which the Rf value was extremely low (Rf = 0.0003). In that genotype, only when cultivars were inoculated with high numbers of juveniles did very few of them penetrate the roots at 4 and 11 DAI and they seemed aggregated to only certain roots. This parameter, however, could be difficult to assess in experimental conditions

Fig. 3. Light micrographs of root cross-sections including the feeding site induced by *Meloidogyne javanica* in commercial olive cultivars (*Olea europaea* subsp. *europaea* var. *europaea*). **A, B, C, and D,** Arbequina. **E, F, G, and H,** Gordal Sevillana. **I, J, K, and L,** Hojiblanca. **M, N, O, and P,** Koroneiki. **Q, R, S, and T,** Lechín de Sevilla. **U, V, W, and X,** Manzanilla de Sevilla. **Y, Z, A', and B',** Picual. **C', D', E', and F',** Sikitita. Asterisks indicate individual giant cells. gc = giant cell in a feeding site and N = nematode. Scale bars = 200 μ m in **A, B, E, I, J, M, Q, R, U, Y, Z, C', and D';** and 100 μ m in **C, D, F, G, H, K, L, N, O, P, S, T, V, W, X, A', B', E', and F'.**

as a result of root heterogeneity in woody plantlets. However, this is not the normal situation in field soil, in which the majority of the inoculum is in the form of eggs that will hatch sequentially. Genotype W147, even with the high inoculation number of juveniles, did not break resistance and only a few females were detected. Nonetheless, the fuchsin staining and histological sections showed a substantial nematode reaction to the plant once inside the root, with strong

staining of phenolic compounds in tissues close to the nematodes and, in some cases, necrosis of some cells (Fig. 4L to N).

The mechanisms providing resistance to nematodes may be too complex to easily determine, and genotype W147 does not seem to be an exception. One concern is that the nematodes only penetrated a few roots in this genotype when high numbers of juveniles were used for inoculation in the histopathological and fuchsin acid stain

Fig. 4. Light micrographs of **A to K**, fuchsin acid-stained roots and **L to T**, root cross-sections of the wild olive genotype W147. **A**, Gall at 4 days after *Meloidogyne javanica* inoculation (DAI). **B and C**, Galls at 11 DAI. **D and E**, Galls at 25 DAI. **F and G**, Nematodes at 25 DAI. **H and I**, galls at 70 DAI. **J**, Female with eggs at 70 DAI. **K**, Eggs at 70 DAI. **L, M, and N**, Gall and root sections at 4 DAI. **O, P, Q, R, S, and T**, Gall and root sections at 25 DAI. Asterisks indicate individual giant cells. gc = giant cell in a feeding site and N = nematode. Scale bars = 100 μ m in J; and 200 μ m in A through I and K through T. Accumulation of phenolic compounds is marked with arrows.

study. This might have occurred because of low attraction to the juveniles for the large proportion of roots that were not rapidly growing, a situation often found in woody plant, especially olive, root systems that are characterized by highly varied rates of growth. This hypothesis, however, is difficult to clearly test.

We suggest that competition for use of the feeding sites and the presence of phenolic compounds could lead to nematode death or lack of completion of the adult stage for the majority of nematodes in the roots, allowing only a few of them to create a proper feeding site and enable minimum reproduction. The few observed female nematodes were found alone in isolated positions of the root, supporting the hypothesis that strong competition among nematodes for scarce resources could induce the death of developing nematodes in egg and/or juvenile inoculated plants. This resistance is not associated with the typical hypersensitive response of giant cell death, such as occurs with the gene *Mi* (Williamson and Hussey 1996), because the few giant cells detected in W147 contained developed nuclei and vacuolization was not present. A similar reaction to that observed in W147 has been observed in resistant grape rootstocks (RS-3 and 10-23B), which express genetic resistance during *Meloidogyne* spp. penetration, development, and reproduction (Anwar and McKenry 2000). Resistance to *M. exigua* Göeldi, 1887 in *Coffea canephora* L. (gene *Mex-1*) is also expressed during nematode penetration and development (Alpizar et al. 2007).

Among the wild olives, it was interesting to observe the variability in host suitability among different genotypes of the same subspecies. This was seen with *O. europaea* subsp. *europaea* var. *sylvestris* to which the majority of the tested genotypes belonged. The second most resistant genotype to *M. javanica* was accession W224 (*O. europaea* subsp. *europaea* var. *sylvestris*). In this case, similar to W147, only a few and small galls were detected. For W224 *M. javanica*, Rf values were also low but not as low as W147 (Table 2), as some females were able to reproduce on the roots. A small Rf value (<1) was also reported for olive cultivars (Coratina, Leccino, Ascolana, and Moraiolo) resistant to *Meloidogyne* spp. and a selection of the wild olive DA 121 (Sasanelli 2009).

In conclusion, this research provides considerable new data about the broad susceptibility of olive cultivars used in commercial groves and new and valuable sources of resistance that require future study. Future studies on these olive genotypes should include other species of *Meloidogyne* (*M. arenaria*, *M. incognita*, and *M. lusitanica*) and other kinds of nematodes (*Xiphinema* spp. and *Rotylenchus* spp.) affecting olive. The potential of the two genotypes W147 and W224, belonging to *O. europaea* subsp. *cerasiformis* and subsp. *europaea* var. *sylvestris*, respectively, as resistant rootstocks is promising. Further effort is necessary in the characterization of the defensive reaction, grafting capabilities, and adaptability of these genotypes to the olive agricultural system.

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