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Factors Affecting Egg Hatch of *Heterodera mediterranea* and Differential Responses of Olive Cultivars to Infestation[1]

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Abstract: The influence of temperature and olive root exudates on *Heterodera mediterranea* egg hatch and the effects of *H. mediterranea* on the growth of two olive cultivars (Arbequina and Picual) were investigated. Egg hatch occurred over a temperature range of 10 to 30 °C and was optimal at 20 to 25 °C. There were no differences in egg hatch between sterile deionized distilled water or root exudate dilutions (undiluted, diluted 1:1, and 1:2) of Arbequina and Picual at 20 °C. *Heterodera mediterranea* reproduced on both olive cultivars in growth chambers at 25 °C. Soil and root final nematode populations, as well as total number of cysts per plant and reproduction rate, were significantly higher in Arbequina than in Picual. Shoot dry and root fresh weights as well as increases of shoot height, trunk diameter, and numbers of nodes were significantly suppressed by infection with 10,000 eggs + second-stage juveniles/pot in Arbequina but not in Picual.

Key words: Arbequina, hatching, *Olea europaea,* olive cyst nematode, pathogenicity, Picual, reproduction, root exudates, Spain, temperature.

Olive trees (*Olea europaea* L. subsp. *europaea*), native to western Asia, are grown extensively in the Mediterranean Basin, the subtropical regions of Australia, southern Africa, and North and South America. Some 750 million trees are grown on approximately 8.5 million ha, of which about 97% are in Mediterranean countries (Anonymous, 1991). In Andalusia, southern Spain, olive trees cover more than 1.3×10^6 ha (Barranco, 1999). Several plant-parasitic nematode species have been found associated with olive trees, including *Criconemella xenoplax* (Raski) Luc and Raski, *Helicotylenchus* spp., *Heterodera mediterranea* Vovlas, Inserra and Stone, *Meloidogyne* spp., *Pratylenchus* spp., and *Rotylenchulus* spp. (Castillo et al., 1999; Diab and El-Eraki, 1968; Lamberti and Vovlas, 1993). Some of these species, such as root-knot and lesion nematodes, cause decline of young olive trees (Lamberti, 1969; Lamberti and Baines, 1969). The olive cyst nematode, *H. mediterranea*, occurs in commercial olive orchards in the Seville province of Andalusia, southern Spain (Castillo et al., 1999). Population levels of this nematode range from 465 to 1,336 eggs and second-stage juveniles (J2) per 250 cm^3 of soil (Castillo et al., 1999). The potential of these nematode infestations to damage olive orchards is not known. There is also a lack of information on the ability of olive cultivars to tolerate *H. mediterranea* infections. Selections from wild olive trees are commonly used as rootstocks in the olive industry of the Mediterranean region, but some improved olive cultivars, such as Arbequina and Picual, are grown on their own rootstocks. In Spain, the improved cultivars Arbequina and Picual are widely adopted by the olive industry because of their high yield and oil content.

The efficiency with which host root exudates stimulate egg hatching of cyst nematodes (e.g., *Globodera pallida* Stone, *G. rostochiensis* (Wollenweber) Behrens) sometimes provides indications of host susceptibility or resistance to these nematodes (Arntzen et al., 1994; Farrer and Phillips, 1983). Poor stimulation of egg hatch was suggested as a component of host tolerance to potato cyst nematode by potato cultivars (Evans, 1983). However, with other cyst nematodes, no differences in hatching stimulation have been observed between resistant and susceptible cultivars, e.g., *Globodera tabacum solanacearum* (Miller and Gray) Behrens (Wang et al., 1997).

The objectives of this study were to: (i) evaluate the influences of temperature and olive root exudates of cvs. Arbequina and Picual on hatching of *H. mediterranea* eggs and (ii) investigate the pathogenicity of *H. mediterranea* to these two olive cultivars under controlled conditions.

Materials and Methods

Inoculum preparation: The nematode population used in the study was obtained from feeder roots of olive (cv. Manzanilla) collected in fields at Utrera (Seville), southern Spain, in May 1998 (Castillo et al., 1999). Starting from a single mature female cyst, inoculum was increased several times on olive planting stocks, cv. Arbequina, which were maintained in a growth chamber at 24 °C. Cysts were extracted from soil by thoroughly mixing infested soil with water in a plastic bucket and settling for 15 seconds. The supernatant was poured through a 750-µm-pore sieve nested over a 250-µm-pore sieve. Cysts were collected from the finer sieve for egg hatch and pathogenicity experiments.

Hatching tests—influence of temperature on egg hatch: Hatching was monitored in chambers composed of 20-mm-diam. microsieves (75-µm aperture) enclosed in petri dishes containing sufficient sterile deionized distilled water (SDDW) to cover the cysts. The SDDW was

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renewed at 2-day intervals in each hatching chamber throughout the period of the experiment. Ten mature and uniformly sized cysts with a mean viable egg content of 175, produced on plants in the growth chamber, were placed in each of 10 replicate hatching chambers for each treatment. Hatching chambers were maintained at 10, 15, 20, 25, and 30 °C (±1 °C) in the dark for three weeks. Numbers of J2 that emerged were recorded at 2-day intervals for 3 weeks. Second-stage juveniles were removed from the hatching chambers after counting. After 22 days, cysts from each hatching chamber were crushed to estimate the numbers of unhatched eggs, and the numbers of hatched J2 were expressed as a cumulative percentage of viable J2. Hatching chambers for each treatment were completely randomized within each incubator.

Hatching tests—influence of olive root exudates on egg hatch: Root exudates were collected from 6- to 8-month-old olive planting stocks of cvs. Arbequina and Picual. Roots were gently washed free of soil, and 25 g fresh weight of roots from three plants was soaked for 4 hours in a 1-liter beaker filled with SDDW at 24 °C in the dark. Undiluted root exudate solutions were collected by filtering through filter paper and subsequently sterilized by passage through a sterile 0.22-mm Millipore (Chicago, IL) filter. Root exudates were diluted 1:1 or 1:2 (v/v) with SDDW. All root exudate solutions were kept at 4 ± 1 °C until use. Root exudate treatments were organized in a complete factorial design of cultivars and dilutions with 10 replicates. Three exudate concentrations (undiluted, 1:1, and 1:2) from each olive cultivar (Arbequina and Picual) were used in the experiment. An SDDW treatment was used as the control for the two cultivars. Hatching was monitored in hatching chambers as previously described, and the hatching chambers were incubated at 20 °C (±1 °C) in the dark. Root exudate solutions and SDDW were renewed and emerged J2 counted at 2-day intervals in each chamber during the experiment.

Pathogenicity test: The damaging effects of *H. mediterranea* on olive cvs. Arbequina and Picual were evaluated under controlled conditions. Single 6-month-old olive plants of uniform size were transplanted into clay pots containing 1 liter of an autoclaved potting mixture (sand: clay loam, 2:1, v/v). Plants were infected by adding 10,000 nematodes (eggs + J2) (Pi) in 10 ml of sterile distilled water around the root ball of each plant at transplanting. Nematode concentrations were determined in 1-ml aliquots of water suspension containing eggs + J2 released from crushed cysts. The number of nematodes used to infect the olive plants was similar to that occurring naturally in olive orchards infested by the nematode. Control plants were treated similarly but without adding nematodes. All plants were grown in a growth chamber adjusted to 25 ± 1 °C, 60 to 90% relative humidity, and a 14-hour photoperiod of fluorescent light at 360.5 ± 24.7 $\mu Em^{-2} s^{-1}$. Plants were watered on alternate days with 100 ml of water and fertilized weekly with 100 ml of a nutrient solution (Hoagland and Arnon, 1950). Treatments were replicated 14 times in a randomized complete block design.

The experiment was terminated 9 months after inoculation. Each plant was cut at soil level and the roots washed free of soil on a sieve to collect the cysts detached from the roots. Shoot dry and root fresh weights; increases in shoot height, trunk diameter, and node numbers (expressed as percentages); and final soil and root populations of cysts were determined. Cysts were extracted from soil by Fenwick can and counted (Fenwick, 1940; Seinhorst, 1974). Cysts were detached from roots with a high-pressure water spray and counted using the same procedure as for the soil. Cysts from soil and roots were crushed manually to determine final population (Pf) values, which were expressed as number of eggs +J2 per 100 cm^3 soil or g fresh roots. From the Pf values, the nematode reproduction factors (Rf = Pf/Pi) were calculated. No attempt was made to determine the number of nematode life stages inside the roots.

Statistical analysis: Cumulative percentages of hatch at each temperature were subjected to linear regression analysis over period of incubation. Data were transformed into ln $[1/(K - Y)]$ for linear regression analysis, where Y = cumulative hatched eggs and K = the maximum Y reached for each temperature. Regressions were performed using 10 replicates for each treatment combination. Data were analyzed using Statistix (NH Analytical Software, Roseville, MN). Coefficients of determination (R^2), coefficients of determination adjusted for degree of freedom (R^2_a), and patterns of residuals plotted against expected values were used to indicate appropriateness of the model to describe the data (Campbell and Madden, 1990). Following regression analyses, slopes of linear regressions for egg hatch at different temperatures were compared by t-tests at $P = 0.05$ (Gomez and Gomez, 1984). Additionally, at the end of the experiment, the influence of temperature on egg hatch was fitted to a quadratic model, $Y = \beta_0 + \beta_1 T + \beta_2 T^2$, in which Y = final cumulative percentage hatch and T = temperature (°C).

In the root exudate experiment, for each treatment, the area under cumulative percentage hatch (AUCPH) was estimated by trapezoidal integration (Campbell and Madden, 1990). The AUCPH and final cumulative percentage hatch were analyzed by ANOVA. Treatment means of AUCPH and final cumulative egg hatch at each temperature or olive root exudate were compared using Fisher's protected least significant difference test (LSD) at $P = 0.05$ (Gomez and Gomez, 1984).

Similarly, in the pathogenicity test, data were subjected to analysis of variance (ANOVA) and treatment means were compared using Fisher's protected least significant difference test (LSD) at $P = 0.05$. All data on nematode population density (X) and relative plant

growth were transformed into $\log_{10}(X+1)$ and to arcsine-square root for analysis, respectively (Gomez and Gomez, 1984).

Results

Hatching tests—influence of temperature on egg hatch: Percentage hatch of *H. mediterranea* eggs in SDDW was influenced ($P < 0.05$) by temperature throughout the experiment (Fig. 1). The percentage of egg hatch over time was described by the general equation $\ln [1/(K - Y)] = \beta_0 + \beta_1 T$, in which Y = cumulative hatched eggs, K = the maximum Y reached for each temperature, and T = period of incubation. Regression lines were: (10 °C) $\ln [1/(K - Y)] = -2.732 + 0.157T$, $R^2 = 0.88$; (15 °C) $\ln [1/(K - Y)] = -4.215 + 0.152T$, $R^2 = 0.91$; (20 °C) $\ln [1/(K - Y)] = -4.852 + 0.212T$, $R^2 = 0.96$; (25 °C) $\ln [1/(K - Y)] = -4.661 + 0.200T$, $R^2 = 0.95$; (30 °C) $\ln [1/(K - Y)] = -3.748 + 0.145T$, $R^2 = 0.90$. All coefficients of determination were significant at $P < 0.001$ (Fig. 1). The intrinsic egg hatch rate (β_1) at 20 °C was higher ($P < 0.05$) than that at 10, 15, and 30 °C but did not differ from that at 25 °C (Fig. 1). Similarly, the final cumulative percentage hatch after 22 days of incubation was higher ($P < 0.05$) at 20 and 25 °C (76.0% and 74.7%, respectively) than that at 10, 15, and 30 °C (9.4%, 42.4%, and 25.4%, respectively). There were differences in the final cumulative percentage hatch ($P < 0.05$) among 10, 15, and 30 °C but not between 20 and 25 °C (Fig. 2). The hatch of *H. mediterranea* eggs increased with temperature up to about 20 °C and then decreased (Fig. 2). The relationship between final cumulative egg hatching of *H. mediterranea* and temperature was fitted to the quadratic equation $Y = -179.67 + 24.09T - 0.57T^2$; $R^2 = 0.93$, in which Y = final cumulative percentage hatch and T = temperature (°C) (Fig. 2).

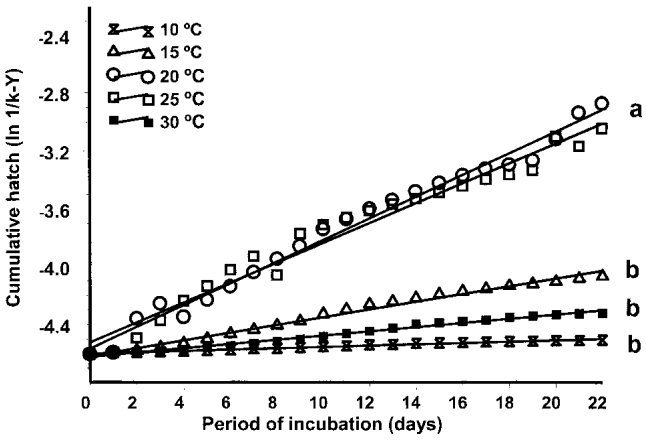

FIG. 1. Linear regression analysis of cumulative percentage hatch (Y) of *Heterodera mediterranea* eggs over 22 days of incubation at different temperatures. Symbols represent a mean of 10 replicates. Regressions were performed on replicate values. Data were transformed to $\ln [1/(K - Y)]$ for regression analysis, where Y = cumulative hatched eggs and K = the maximum Y reached for each temperature. Slopes of linear regressions with a letter in common do not differ ($P = 0.05$) according to a t-test.

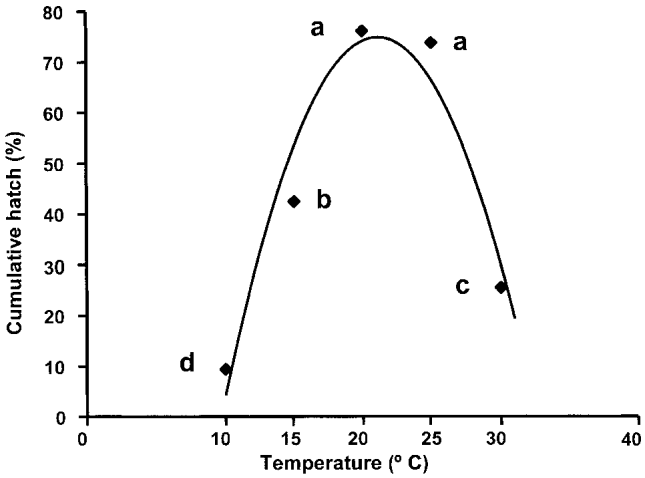

FIG. 2. Influence of temperature on egg hatch (Y) of *Heterodera mediterranea* over 22 days of incubation at different temperatures. Symbols represent a mean of 10 replicates. Symbols with a letter in common do not differ ($P = 0.05$) according to Fisher's protected LSD test. Regressions were performed on replicate values. Final cumulative egg hatch of *H. mediterranea* fit the quadratic equation $Y = 179.67 + 24.09\ T - 0.57\ T^2$; $R^2 = 0.93$, where Y = final cumulative percentage of hatched eggs and T = temperature (°C).

Hatching tests—influence of olive root exudates on egg hatch: Hatching of *H. mediterranea* eggs occurred in SDDW and olive root exudates at 20 °C, irrespective of the source of exudate (cv. Arbequina or Picual) or exudate dilution (Fig. 3). Percentage hatch, determined by the AUCPH, did not differ ($P > 0.05$), irrespective of the source of exudate (cv. Arbequina or Picual) or exudate dilution (Fig. 3). Similarly, the final cumulative percentage hatch after 22 days of incubation was not different ($P > 0.05$) at 20 °C, irrespective of exudate or dilution.

Pathogenicity test: Both olive cultivars (Arbequina and Picual) were infected by *H. mediterranea*. Nevertheless, no disease symptoms on aboveground plant parts were observed in either *H. mediterranea*-infected or uninfected control plants. Infected feeder roots showed white females and cysts attached to the surface, and necrotic areas near *H. mediterranea* infection sites were observed. Plant growth, assessed by root fresh and shoot dry weights, and percentage increases in shoot height and trunk diameter were suppressed ($P < 0.05$) by infection of *H. mediterranea* in Arbequina but not in Picual (Table 1). In neither olive cultivar were there significant differences ($P > 0.05$) in numbers of nodes detected between infected and uninfected plants (Table 1). Although both olive cultivars tested allowed reproduction of *H. mediterranea*, final nematode populations in soil and roots, as well as total number of cysts per plant and reproduction rate, were higher ($P < 0.05$) for Arbequina than for Picual (Table 1).

Discussion

The primary objectives of this study were to determine the influences of temperature and olive root exu-

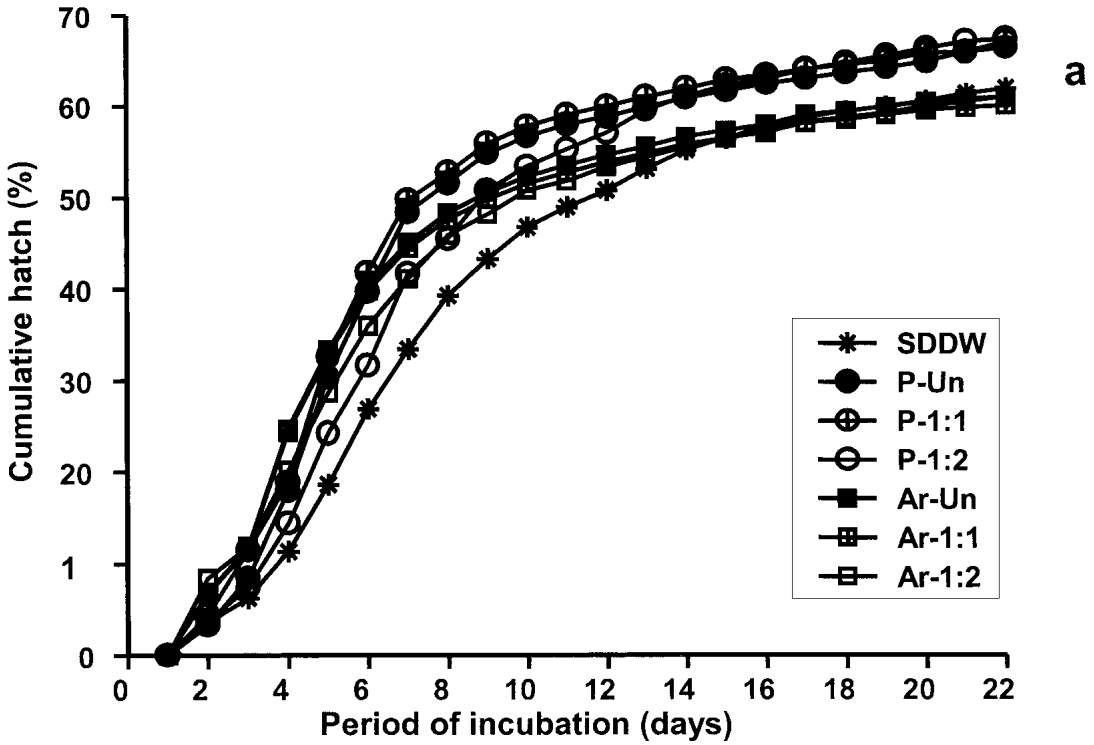

FIG. 3. Cumulative hatch of *Heterodera mediterranea* over 22 days of incubation at 20 °C in olive root exudates. The AUCPH and final cumulative means followed by the same letter do not differ ($P = 0.05$) according to Fisher's protected LSD test.

dates on *H. mediterranea* egg hatch, and the effects of *H. mediterranea* on the growth of two olive planting stocks. Our results indicate that *H. mediterranea* egg hatch is greatly influenced by temperature, with greatest cumulative percentage hatch between 20 and 25 °C (Fig. 1). This study covers the temperature range that occurs in olive orchards where the olive cyst nematode was found in southern Spain (Castillo et al., 1999). Temperatures that favor hatch of *H. mediterranea* (between 20 and 25 °C) occur in late spring and early summer in southern Spain. However, higher temperatures during summer, which frequently exceed 30 °C in the top soil layers, would be expected to reduce hatching (Fig. 1). Temperatures between 10 and 15 °C (which occur in soils of southern Spain during late autumn and winter) still permitted egg hatching, although final cumulative hatch was between only 12% and 56%—much lower than that occurring at 20 or 25 °C. These results indicate that *H. mediterranea* is well adapted to a Mediterranean environment (Castillo et al., 1999; Vovlas and Inserra, 1983). The hatching found at the highest temperature (30 °C) agrees with data reported for other cyst-forming nematode species, i.e., *Heterodera humuli* Filipjev (Von Mende and McNamara, 1995), *Heterodera cruciferae* Franklin (Koshy and Evans, 1986), and *Globodera rostochiensis* (Salazar and Ritter, 1993). Egg hatch of these nematodes is adversely affected by high temperatures, probably because of J2 quiescence or mor-

TABLE 1. Effect of *Heterodera mediterranea* on the growth of two olive cultivars exposed to initial nematode densities (Pi) of 10 eggs + J2/cm^3 soil (10,000/pot) during a 9-month experimental period, and increase of nematode populations.[a]

Nematode inoculum (eggs + J2)	Root fresh weight (g)	Shoot dry weight (g)	Growth increase during the experiment (%)[b]			Number of cysts/plants	Eggs + J2 (Pf)		Rf[c]
			Shoot height	Trunk diameter	Node number		Soil (100 cm^3)	Roots (g)	
ARBEQUINA									
0	11.8 a	6.5 a	3,739.2 a	193.2 a	393.5 a	0	0	0	0
10,000	8.8 b	5.1 b	2,737.1 b	161.0 b	388.0 a	1,963.1 A	67.6 A	15,544.0 A	12.03 A
PICUAL									
0	9.3 a	5.6 a	387.8 a	113.3 a	649.5 a	0	0	0	0
10,000	9.4 a	5.3 a	382.2 a	110.4 a	612.9 a	64.5 B	19.3 B	1,609.0 B	1.62 B

[a] Data are the mean of 14 replicates, each replicate consisting of one plant per treatment combination. For each olive cultivar, means followed by the same letter do not differ ($P = 0.05$) according to Fisher's protected LSD test. Actual data are presented for each treatment, but numbers of nematodes and percentages were transformed to $\log_{10}(X + 1)$ and to arcsine-square root, respectively, for analysis. Upper-case letters refer to mean comparisons between olive cultivars.

[b] Average percentage growth of each parameter during the experiment; *significantly different at $P < 0.05$.

[c] Rf (nematode reproduction factor) = Pf (final nematode population per plant)/Pi (initial nematode inoculum per plant).

tality (La Mondia and Brodie, 1986). Our results suggest that, under the field conditions in southern Spain, egg hatch and root invasion by *H. mediterranea* J2 would occur mainly in late spring and early summer.

Our experimental data revealed that *H. mediterranea* egg hatch was not dependent on host root exudates as reported for other cyst-forming nematodes, including the potato and tobacco cyst nematodes, which depend on root exudates from their annual plant hosts to stimulate hatch (Wang et al., 1997). Similarly, numerous studies on the soybean cyst nematode, *Heterodera glycines* Ichinohe, have demonstrated that exudates from host plants stimulate hatching (Caballero et al., 1986; Schmitt and Riggs, 1991), whereas nonhosts do not stimulate hatching (Schmitt and Riggs, 1991; Tsutsumi and Sakurai, 1966). We hypothesize that the large eclosion of *H. mediterranea* J2 in water and the lack of egg-hatching stimulus by olive root exudates are a consequence of adaptation of the nematode to parasitize perennial hosts (olive and pistachio), which provide roots for invasion by J2 throughout the year. In contrast, cyst-forming nematodes infecting annual plants are deprived of roots for long periods during the year and have a greater need for an egg-hatching stimulus by root exudates at any new cycle of their annual host.

Results of the test of *H. mediterranea* on olive growth under controlled conditions demonstrated that *H. mediterranea* was able to suppress growth of Arbequina but not Picual. Similarly, results on the reproduction of *H. mediterranea* indicated that Arbequina was more susceptible to infection than Picual. This effect needs to be verified under field conditions and checked for other olive genotypes.

Geographical distribution of this nematode in Spain is limited to a small area of sandy soils in the province of Seville (Castillo et al., 1999), and there are no records in commercial olive nurseries in Andalusia (unpubl. data). Precautionary regulatory measures are desirable to prevent *H. mediterranea* spread into olive nurseries and also from infested to non-infested olive orchards.

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