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Resistance verification in *Prunus* selections to a mixture of thirteen *Meloidogyne* isolates and resistance mechanisms of a peach-almond hybrid to *M. javanica*

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Summary – Thirteen root-knot nematode isolates from Spain that included *Meloidogyne incognita* (four), *M. arenaria* (six), and *M. javanica* (three) were used to verify the resistance level of twelve *Prunus* rootstocks. The majority of the rootstocks were peach-almond hybrids in the process of selection. In a first trial, the hybrids G × N Nos 3, 7, 14, 15, and Cachirulo, the almond D-3-5, and the peach Nemared exhibited different levels of resistance to a mixture of twelve isolates of *Meloidogyne*. Cv. Garrigues was susceptible. In a second trial, the hybrids G × N Nos 2, 9, and Nemaguard were highly resistant to ten isolates of *Meloidogyne*, although the almonds D-3-5 and Moncayo, the peach GF-305 and the hybrid GF-677 were susceptible. A nematode penetration and development bioassay indicates that an hypersensitive reaction is involved in the resistance mechanism of G × N No 9 infected with *M. javanica*. The nematode induces a necrotic reaction in the surrounding cortical parenchyma isolating the dead tissue by a compact barrier formed by several cell layers in which callose was detected in its cell walls. In F2 progenies of a seedling population of G × N, 92 % of the plants showed resistance.

Résumé – ***Vérification de la résistance de sélections de* Prunus *à un mélange de treize isolats de* Meloidogyne *et mécanismes de résistance d'un hybride pêcher-amandier à* M. javanica** – Treize isolats de *Meloidogyne* originaires d'Espagne comprenant *M. incognita* (six), *M. arenaria* (six) et *M. javanica* (trois) ont été utilisés pour vérifier le niveau de résistance de douze porte-greffe de *Prunus*. La majorité de ces porte-greffe était constituée d'hybrides pêcher-amandier en cours de sélection. Lors d'un premier essai, les hybrides G × N Nos 3, 7, 14, 16 et Cachirulo, l'amandier D-3-5 et le pêcher Nemared ont montré différents niveaux de résistance à un mélande de douze isolats de *Meloidogyne*. Le cv. Garrigues s'est révélé sensible. Un second essai a montré que les hybrides G × N Nos 2, 9 et le cv. Nemaguard sont hautement résistants à dix isolats de *Meloidogyne*, encore que les amandiers D-3-5 et Moncayo, le pêcher GF-305 et l'hybride GF-677 se soient révélés sensibles. La pénétration des nématodes, ainsi que des bio-essais concernant le développement, ont indiqué qu'une réaction d'hypersensibilité est impliquée dans le mécanisme de résistance de G × N No 9 infesté par *M. javanica*. Le nématode induit une réaction nécrotique dans le parenchyme cortical l'entourant, isolant ainsi le tissu mort par une barrière compacte formées de plusieurs couches de cellules dont les parois contiennent de la callose. Dans la descendance (F2) d'une population de plants de G × N, 92 % de ces derniers se sont montrés résistants.

Key-words : Hypersensitivity, *Meloidogyne arenaria, M. incognita, M. javanica,* pathogenic variability, *Prunus,* resistance, root-stocks.

Recent studies in Spain indicate that several almond (*Prunus amygdalus* Batsch.) and peach-almond hybrid (*P. persica* Stock. × *P. amygdalus* Batsch.) rootstocks in selection phase, have a high level of resistance to single isolate inoculations of *Meloidogyne incognita, M. arenaria* and *M. javanica* (Marull *et al.,* 1991; Marull & Pinochet, 1991). The most interesting selections derive from crosses between the Spanish almond Garfí and the root-knot resistant peach Nemared (Ramming & Tanner, 1983). These genotypes (G × N selections) have good vigor, red leaf, are resistant to iron chlorosis, and most of them adapt well to poor soils and dryland conditions, such as those that prevail in Mediterranean environments.

Testing a *Prunus* rootstock to one or a few species of *Meloidogyne* is insufficient to ensure a desired resistance. The pathogenic variability of the nematode seems to be the most accepted explanation (Triantaphyllou, 1987; Roberts, 1990). In some cases resistance to the nematode was shown to be species specific (Scotto La Massèse, 1989; Esmenjaud *et al.,* 1993), while in other cases a variable plant response has been observed within populations of the same root-knot species (Sharpe *et al.,* 1969; Pinochet *et al.,* 1989; Esmenjaud *et al.,* 1993).

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A resistance verification process that would consider the use of many populations of the most common root-knot species from different origins and hosts (incorporating pathogenic diversity) would allow a more rigorous test that perhaps, would ensure an acceptable broad resistance (Scotto La Massèse *et al.*, 1984). This aspect is especially important with material in the process of selection, as well as with commercial rootstocks, supposedly resistant and introduced from other countries into Spain. The purpose of this research was to verify the resistance of several experimental genotypes of peach-almond hybrids and new *Prunus* introductions in the country of thirteen isolates of *Meloidogyne*. A second objective was to study the resistance mechanisms in G × N selections, and obtain information of the inheritance of the nematode resistant character in F2 segregating lines.

Table 1. *General information on host and geographic origin of thirteen* Meloidogyne *isolates used in resistance verification tests on* Prunus *rootstocks in Spain.*

Isolates/species*	Host	Geographic origin
A1 *M. arenaria*	Melon	Tarragona, Tarragona
A2 *M. javanica*	Fig	Cabrils, Barcelona
A3 *M. incognita*	Kiwi	Tordera, Barcelona
A4 *M. arenaria*	Tomato	Cabrera, Barcelona
A5 *M. arenaria*	Tomato	Canet, Barcelona
A6 *M. incognita*	Peach-almond	Montañana, Zaragoza
A7 *M. arenaria*	Peach	Sastre, Tarragona
A8 *M. javanica*	Almond	Reus, Tarragona
A9 *M. incognita*	Citrus	Amposta, Tarragona
A10 *M. arenaria*	Peach	Caspe, Zaragoza
A11 *M. javanica*	Carnation	Seville
A12 *M. arenaria*	Peach	Abarán, Murcia
A13 *M. incognita*	Potato	Longares, Zaragoza

* Populations were identified by morphologic, electrophoretic (PAGE) and cytogenetics methods. PAGE and cytogenetics identifications were conducted at North Carolina State University (Cenis *et al.*, 1992).

Materials and methods

Resistance verification

Seven resistant peach-almond hybrids (G × N Nos 2, 3, 7, 9, 14, 15, and Cachirulo), two almonds (Moncayo and D-3-5) were evaluted in two separate experiments. Five additional rootstocks with variable response to root-knot nematodes were included as reference rootstocks. These were the peach rootstocks Nemaguard and Nemared (resistant), GF-305 (susceptible to moderately resistant), the peach almond hybrid GF-677 and the almond Garrigues (susceptible). Plant material was provided by the Programa de Fruticultura del Servicio de Investigación Agraria de la Diputación General de Aragón in Zaragoza, Spain.

The peach-almond hybrids were propagated from wood cuttings (Hansen & Hatman, 1967). Almond and peach were propagated from seeds (Marull & Pinochet, 1991). Rooted material was planted in 100 cm^3 trays containing a 2:1 (v:v) sand-peat mixture previously pasteurized at 80 °C. After several weeks plant material was transplanted to 1.6 l containers filled with a pasteurized soil (80 % sand, 16 % loam, 4 % clay) with a pH 7.6, less than 1 % organic matter, and a cation exchange capacity of less than 10 meq/100 g soil. Plants were kept in the greenhouse for 7 weeks before inoculation.

Thirteen populations including *M. incognita, M. arenaria* and *M. javanica* were isolated from different Spanish localities and hosts (Table 1) and reared monoxenically from single egg mass cultures on transformed tomato (*Lycopersicon esculentum* Mill.) roots by *Agrobacterium rhizogenes* (Verdejo *et al.*, 1987). Isolates were identified by perineal patterns (20 females per isolate). These were confirmed by isoenzyme electrophoresis in acrylamide gel and cytogenetic techniques (Cenis *et al.*, 1992) with exception of isolates A9 and A13. Four populations (A1, A2, A8, and A11) showed differences in the identification to species level. In these cases of discrepancy, the electrophoretic and cytogenetic techniques were considered as more reliable identification criteria. Inoculum from each isolate was prepared by extracting nematodes in transformed tomato roots in a 0.12-0.15 NaOCl solution (Hussey & Barker, 1973).

In the first experiment the G × N selections Nos 3, 7, 14, 15, Cachirulo, and D-3-5 were evaluated. Nemared and Garrigues were used as resistant and susceptible controls, respectively. Inoculum consisted in a mixture of 12 isolates (A1 to A12) that included 500 nematodes from each isolate (6000 nematodes per plant). Tomato plants cv. Roma were inoculated to confirm the infectivity of each isolate at inoculation time.

In a second experiment the G × N selections Nos 2 and 9, Moncayo and D-3-5 were evaluated. Nemaguard and GF-677 were used as resistant and susceptible controls, respectively. Inoculum consisted in a mixture of ten isolates (A1, A2, A4, A6-A11, A13) and was prepared as in the previous experiment (5000 nematodes per plant), although in this case, okra *(Hibiscus esculentum)* was used to check the infectivity of each isolate instead of tomato.

Plants were harvested 120 days after inoculation. Gall indices, final nematode population per plant in soil and in roots, and the numbers of nematodes per gram of root were determined. Gall index was determined by using the 1-6 scale recommended by Barker (1985) for evaluating resistance to *Meloidogyne* spp. The resistance rating of each rootstock was estimated according to the scale suggested by Taylor and Sasser (1978) based on nematode reproduction and root galling : I = immune; HR = highly resistant; R = resistant; MR = moderately

resistant; and S = susceptible. Nematodes in soil were obtained from 250 cm³ sub-samples (Marull & Pinochet, 1991). Nematodes then were extracted by differential sieving and sugar flotation (Jenkins, 1964). Nematodes in the roots were recovered by macerating whole roots in a blender in a solution of NaOCl (0.25-0.30 %) for three periods of 15 s, separated by two intervals of 10 s. Nematodes were then concentrated using sieves with pore sizes of 0.150, 0.074 and 0.025 mm. Root tissue collected on the 0.150 mm sieve was discarded.

Plants were watered daily and fertilized with Hoagland's nutrient solution (Hoagland & Arnon, 1950). Experiments were conducted in a greenhouse with temperatures ranging between 20-32 °C. Inoculated pots were placed in a sand bed to avoid temperature and humidity fluctuations. Each material was replicated seven times in a completely randomized design. Data were analyzed by one-way ANOVA; data for gall index, final nematode population, and nematodes per gram of root were log_{10} $(x + 1)$ transformed. Means were compared by Duncan's Multiple Range Test ($P < 0.05$).

Penetration, development and reproduction bioassay

The G × N No 9 selection was chosen for this experiment. GF-677 was used as a susceptible reference rootstock. Both materials were propagated from herbaceous cuttings. After rooting, plantlets were transferred to 150 cm³ pots containing pasteurized quartz sand. Inoculum from tomato transformed roots of the A2 isolate *(M. javanica)* was prepared as previously described. Inoculum was adjusted to deliver aliquotes of 1000 eggs per plant through five holes at 3 cm from the stem. Inoculated plantlets were maintained at 20-28 °C in a greenhouse and were irrigated daily with water and fertilized with Hoagland's nutrient solution twice a week. Plants were harvested in sets of five at 0, 7, 14, 21, 28, 35, 42, and 49 days after inoculation. At each harvest date, the numbers of nematodes in each stage in the roots and in the soil were assessed as described for verification trials. For histology, selected pieces of roots with galls were fixed in FAA, dehydrated in a tertiarybutyl alcohol series, embedded in a 56 °C paraffin wax and sectioned in a microtome at 15-18 µ. Sections were stained with safranin and fast green (Daykin & Hussey, 1985). Galled root tissue stained with decolourised aniline blue were mounted and observed in a fluorescent microscope to detect the presence of callose (Martin, 1959).

Resistance inheritance

In a fourth experiment, 34 individual plants from a G × N F2 seedling population were assessed for galling and nematode reproduction in the same way as described in previous tests. Progenies had been obtained by open pollination in an isolated compact block of F1 plants. Nemared and GF-677 were used as resistant and susceptible controls, respectively. Inoculum consisted in 1000 *M. javanica* eggs per plant (A2 population). Plants were evaluated 90 days after inoculation.

Results

Resistance verification trials

All the tested materials, with the exception of Garrigues, exhibited different levels of resistance to the twelve isolates of *M. javanica, M. incognita* and *M. arenaria* in the first experiment (Table 2). The G × N selections Nos 7, 3, 15, D-3-5, and Nemared showed no differences ($P \leq 0.05$) in galling (between 1.0 and 1.7). G × N No 14 differed significantly from the other resistant rootstocks reaching a higher galling index (2.4) and from the susceptible Garrigues (6.0). Nematode reproduction was low in resistant materials. The final nematode population of G × N Nos 7, 3, D-3-5, and Nemared were significantly lower than in Cachirulo and

Table 2. *Gall indices and reproduction of* Meloidogyne *on* Prunus *rootstocks at 120 days after inoculation with a mixture of 12 isolates (6000 nem/plant) that included* M. incognita *(2),* M. javanica *(4), and* M. arenaria *(6).*

Rootstocks*	Gall index (1-6)	Final population (soil and roots)	Nematodes g/root	Resistance rating**
G × N No 7	1.0 *a*	15 *ab*	2 *ab*	HR
G × N No 3	1.1 *a*	75 *ab*	0 *a*	HR
G × N No 15	1.4 *ab*	225 *ab*	0 *ab*	R
D-3-5	1.4 *ab*	0 *a*	0 *a*	R
Nemared	1.7 *ab*	105 *ab*	8 *ab*	R
Cachirulo	2.0 *b*	775 *c*	35 *c*	MR
G × N No 14	2.4 *c*	370 *c*	15 *bc*	MR
Garrigues	6.0 *d*	245 390 *d*	10 130 *d*	S

* Data are means of seven replications. Actual data are presented but data were transformed to log_{10} $(x + 1)$ for analysis. Means in columns not followed by the same letter do not differ according to Duncan's multiple range test ($P \leq 0.05$).

** HR = highly resistant ; R = resistant; MR = moderately resistant; S = susceptible.

Table 3. *Gall indices and reproduction of* Meloidogyne *on* Prunus *rootstocks at 120 days after inoculation with a mixture of ten isolates (5000 nem/plant) that included* M. incognita *(3),* M. javanica *(3), and* M. arenaria *(4).*

Rootstocks*	Gall index (1-6)	Final population (soil and roots)	Nematodes g/root	Resistance rating**
G × N No 2	1.1 *a*	110 *ab*	8 *a*	HR
G × N No 9	1.4 *a*	0 *a*	0 *a*	HR
Nemaguard	2.0 *a*	0 *a*	0 *a*	HR
GF-305	4.0 *b*	480 *b*	80 *b*	S
Moncayo	4.1 *b*	4770 *c*	1715 *d*	S
GF-677	6.0 *cd*	1930 *c*	230 *c*	S
D-3-5	5.8 *d*	17 700 *d*	7450 *d*	S

* Data are means of seven replications. Actual data are presented but data were transformed to $log_{10}(x + 1)$ for analysis. Means in columns followed by the same letter do not differ according to Duncan's multiple range test ($P \leq 0.05$).
** HR = highly resistant ; S = susceptible.

G × N No 14. Garrigues reached the highest final population. Nematodes were absent from the roots of three rootstocks, low in G × N no 14 and Cachirulo, and high in Garrigues.

In the second trial, G × N Nos 2 and 9, and Nemaguard had a highly resistant response against ten isolates of *M. javanica, M. incognita,* and *M. arenaria* (Table 3), while GF-305, Moncayo, D-3-5, and GF-677 were susceptible. G × N Nos 2, 9, and Nemaguard exhibited a significantly lower galling index than the rest of the tested materials. GF-305 and Moncayo showed similar galling levels, whereas D-3-5 and GF-677 presented the highest. The final nematode population was significantly lower in G × N Nos 2 and 9, and Nemaguard as compared to the rest, although G × N No 2 did not differ from GF-305. GF-677, Moncayo, and D-3-5 had higher population increases. In relation to nematode densities, G × N Nos 2, 9, and Nemaguard had lower numbers of nematodes per gram of root. The remaining rootstocks showed different levels of population buildup in the roots.

All tomato and okra plants used for checking infectivity on each individual isolate were heavily galled 60 days after inoculation.

Penetration, development and reproduction bioassay

Hatching of *M. javanica* eggs in the soil was gradual, occuring between 7 and 14 days after inoculation in G × N No 9 resistant rootstock (Fig. 1). Penetration in roots by J2 (32 nematodes) was first recorded at 14 days. Occasional and visible galls (1 to 2 mm-diameter) were detected after 21 days, as well as the presence of J2, J3 and J4 within the roots. After 28 days and onwards no further nematodes were extracted from the roots. Few larvae remained in the soil up to 35 days. Thereafter, low numbers of unhatched eggs (less than 50) were found at 42 and 49 days. During the bioassay,

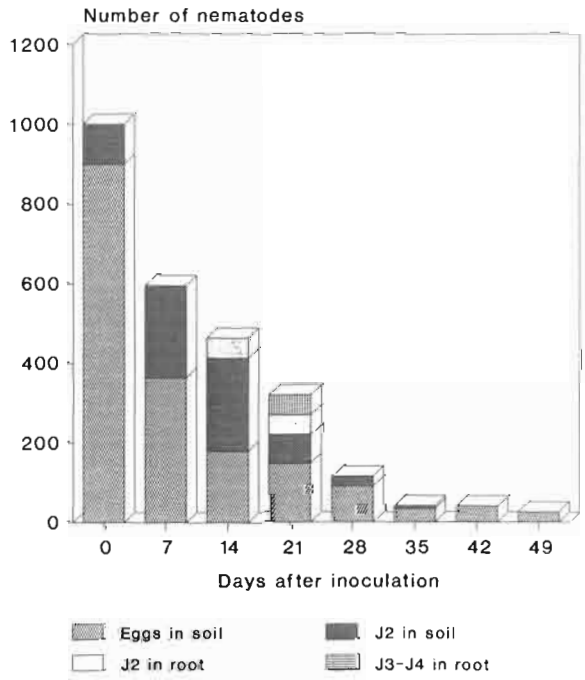

Fig. 1. *Penetration and development of* Meloidogyne javanica *in the resistant peach-almond hybrid rootstock G × N No 9 during a 7 week period.*

adult stages, egg masses or individual eggs were neither found nor extracted from G × N No 9 roots.

Histological sections revealed that a few cortex cells were collaped as a result of J2 migration 15 days after inoculation, although no histological changes were observed for this period. After 21 and 28 days, J3 and J4 were found in incipient galls (1-2 mm) inducing giant cell formation in G × N No. 9 (Fig. 2A). Hyerplasia and cell disorientation was evident. Giant cells and nuclei within giant cells of G × N No. 9 were smaller and less

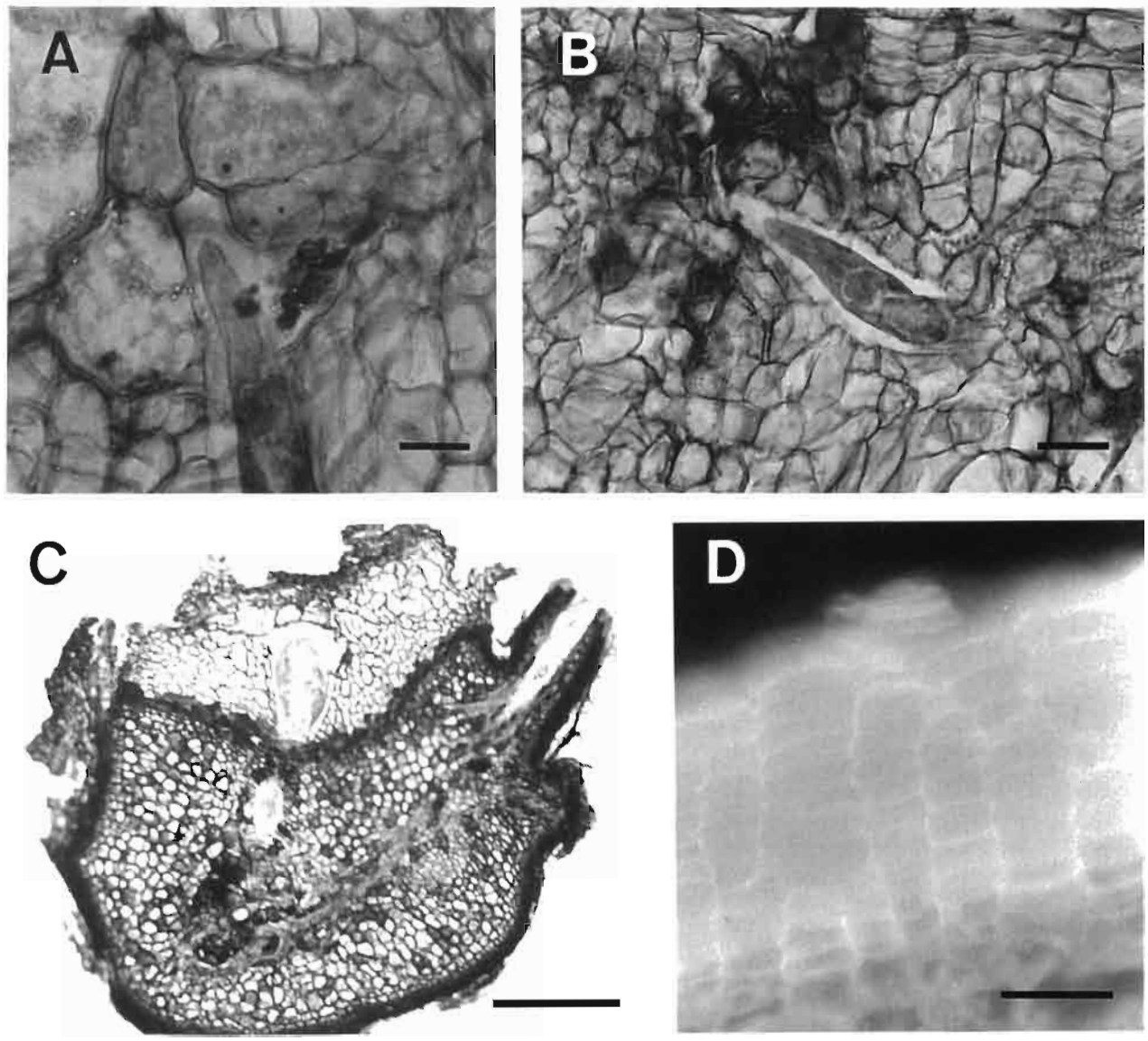

Fig. 2. *Histological changes in roots of resistant peach-almond hybrid G × N No 9 rootstock infected with* Meloidogyne javanica. *A : A third stage larvae inducing giant cell formation at 21 days after inoculation; B : Collapse of giant cells and absorbtion of stain (safranin) in sorrounding nematode feeding site at 28 days; C : Hypersensitive reaction in a sectioned gall showing necrotic tissue and a compact barrier of cells (walling off) isolating dead tisue (anterior end of the nematode) from live tissue (posterior end); D : Presence of callose detected in compact cell barrier by fluorescent microscope technique.* (Bar scale = 30 μm in A; 40 μm in B; 0.25 mm in C; 30 μm in D).

numerous (nuclei) as compared to those found in susceptible GF-677 rootstock. In some cases, giant cells collapsed and the adjacent feeding site stained heavily with safranin (dead tissue) (Fig. 2B). After 35 days several galls were sectioned, but in all of them the nematode had disintegrated, leaving an empty cavity (no traces). Only in one gall was a J4 encountered showing a necrotic reaction with collapsed giant cells and a compact barrier of 4 to 8 layers of cells isolating live tissue in contact with the posterior end of the nematode from the dead tissue in contact with the anterior end (Fig. 2C). A high concentration of callose was detected in this compact barrier (Fig. 2D). The nematode reproduced readily on GF-677 (susceptible) plants producing histological changes similar to those described for other susceptible *Prunus* hosts (Malo, 1967; Simeone, 1978).

Resistance inheritance

In this experiment, two plants of the 34 F2 seedling population of G × N were susceptible (plants 14 and

Table 4. *Inheritance of the nematode resistance character in 34 plants of an F2 population of the peach-almond hybrid G × N (Garfi × Nemared) at 90 days after inoculation with 1000 nematodes of* Meloidogyne javanica *per plant.*

Selection/Rootstok	Plant No*	Galls/plant	Nem./g of root	Rating**
Garfi × Nemared F2	1-2	0	0	R
	3	1	0	R
	4-8	0	0	R
	9-13	1	0	R
	14	80	2275	S
	15	1	0	R
	16-17	0	0	R
	18	2	0	R
	20	0	0	R
	21	1	0	R
	22-33	0	0	R
	34	100	3500	S
GF-677 (susceptible)***		85	910	S
Nemared (resistant)***		0	0	R

* G × N plants Nos 7, 19, 24, and 30 died during testing.
** Rating : R = resistant; S = susceptible.
*** Data for GF-677 and Nemared are mean of four replications.

34), showing considerable galling and a high level of parasitism (Table 4). The remaining plants had a resistant response with no evidence of nematode reproduction. Five plants showed occasional galls. No intermediate level of resistance occured. Reference rootstock Nemared (parent) was resistant.

Discussion

The choice of nematode isolates for inocula is a critical part of any selection program (Boerma & Hussey, 1992). Inoculations with a mixture of isolates can be a rigorous and reliable method to ensure resistance against root-knot nematodes in a rootstock selection scheme. This procedure allows confirmation that the high level of resistance observed in evaluations with single isolates is also present against many isolates of the same species, as well as to different species of *Meloidogyne*. The use of mixed inocula also allows detection of virulent and (or) resistant breaking forms of the nematode with a similar success as with individual population testing. In a breeding program it would be desirable to be able to evaluate interesting genotyes to as many individual root-knot populations as possible (extensive testing) to assure a broad resistance against the nematode but practical considerations in rootstock breeding and selection tend to limit resources that require large infrastructure and considerable human imput.

The response of the almond D-3-5 is perhaps, one of the most surprising results. This selection proved resistant in a previous screening test with an isolate of *M. javanica* (Marull *et al.*, 1991). The high level of resistance was confirmed in the first trial with a mixture of 12 root-knot nematode isolates. However, in the second verification test, D-3-5 showed extensive galling and high population buildup. This susceptible reaction was probably due to the presence of isolate A 13 (*M. incognita* race 1) not included in the first verification test. This result also suggests a case of typical vertical resistance in which D-3-5 showed a high level of resistance to most of the isolates but unfortunately, one isolate was pathogenic and capable of overcoming resistance (non-durable type resistance). Similarly, the peach GF-305 and the autocompatible almond Moncayo had shown a moderately resistant response to *M. hapla* and *M. incognita* in past evaluations (Pinochet, 1989) but were susceptible in this study. GF-305 has also shown a variable response to different root-knot species in France (Scotto La Massèse *et al.*, 1984; Esmenjaud *et al.*, 1993) and Italy (Tacconi & Santi, 1987). The low final population of the susceptible hybrid GF-677 (Table 3) was probably due to the destruction of the root system in less than 120 days. Galling index was very high and plants in general, were in poor conditions at the end of the experiment.

Meloidogyne javanica is capable of penetrating the roots of resistant G × N No 9 rootstock, establishes parasitic life by inducing giant cell formation, feeds and develops from J2 to J3 and seldom to J4. However, the nematode is incapable of reaching adult stages and reproducing, resulting in the disruption of the life cycle. In this process occasional galling takes place. The resistance mechanism involved appears to be a slow forming hypersensitive reaction which suppresses nematode development. As the nematode feeds, a progressive degradation of giant cells takes place. The physiological alterations observed with *M. javanica* on G × N

peach-almond hybrid are similar in many aspects to those described by Malo (1967) on peach CVS Nemaguard and Okinawa infected with the same nematode species. However, the physical separation of giant cells from normal cells by a continuous barrier, described as " walling off ", differed in size and structure in our study. The " walling off " observed in G × N No 9 clearly circumscribed the giant cells an surrounding cells of the parenchyma and conductive tissues (Fig. 2C).

These results confirm a high level of resistance in the G × N Nos 2, 3, 7, 9 and 15, and a moderate resistance in G × N No 14 and Cachirulo to 13 isolates of *M. javanica, M. incognita* and *M. arenaria.* The resistance level in most of these hybrids is similar to that observed in its nematode resistant parent, Nemared peach, indicating a high transmissibility of this character in F1 derived from crosses with the female parent Garfí (almond). The inheritance of the nematode resistant character evaluated in 34 individual plants (F2 population) to a *M. javanica* isolate indicates that the full expression of this character is transmitted in approximately 92 % of the plants. This also suggests that this character is determined by a few dominant genes, similar to other cases of root-knot nematode resistance inheritance bitter almond selections and in peach (Kochba & Spiegel-Roy, 1975; Cook & Evans, 1987; Yoshida, 1989). Callose is a hardened mucilaginous constituent of cell walls that avoids the loss of electrolites and does not allow the diffusion of active phenols (Wheeler, 1974). The presence of callose in peripheric cells (compact barrier) of necrotic tissue in G × N No 9 is a good indication of a hypersensitive reaction as a response to the invading nematode pathogen (Bleve Zacheo, 1982). The hypersensitive reaction observed in G × N No 9 is likely to be the same that occurs in other G × N selections. These genotypes have shown comparable resistance levels in previous testing cycles against this and other *Meloidogyne* species (Pinochet *et al.,* 1989; Marull & Pinochet, 1991).

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