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Penetration, development and emigration of juveniles of the nematode *Meloidogyne arenaria* in Myrobalan plum (*Prunus cerasifera*) clones bearing the *Ma* resistance genes

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Abstract

Penetration, development and emigration of *M. arenaria* in the roots of three Myrobalan plum (*Prunus cerasifera*) clones genetically characterized for their resistance to root-knot nematodes (RKN) were studied during the 10 (penetration) and 15 (emigration) days following the date of inoculation (D) of 2500 juveniles (J2s) per plant into the soil. Miniaturized tests were conducted on the two resistant clones P.2175 (*Ma1* gene) and P.1079 (*Ma2* gene) and the susceptible clone P.2032 (recessive for both genes), obtained from micropropagated plantlets and grown in mini-containers under controlled conditions at 25 °C in a growth chamber. For penetration and development studies, nematodes in the roots were recovered by the acid fuchsin-lactophenol staining technique. Equivalent numbers of J2s were recovered in all the clones at D + 1 and D + 2. Subsequently, the numbers increased rapidly in P.2032 and were significantly different from those in P.1079 and P.2175 that remained at a low level. No swollen larvae were observed in the resistant clones. In P.2032, the first swollen larvae were observed at D + 4, the first females were observed at D + 12, whereas the first females with attached egg sacs and the first new-generation J2s were obtained between D + 21 and D + 28. Our data suggest that the resistance phenomenon does not act on the very early nematode penetration but acts later by preventing feeding-site induction and development into the third-stage. For emigration studies, plants in which J2s had been allowed to penetrate for two days (from D to D + 2) were washed free of soil, repotted and then, after various periods of growth, soil-free roots were placed under a mistifier to evaluate the numbers of emigrating individuals. Emigration of J2s from the roots occured mainly from D + 2 to D + 4 in all the genotypes and was very limited from D + 4 to D + 10. There was no significant differences in the number of emigrated juveniles between the resistant and susceptible clones, indicating that emigration cannot explain the difference in the numbers of nematodes recovered in the roots.

Introduction

Root-knot nematodes (RKN) *Meloidogyne* spp. reduce fruit and nut production in several economically important *Prunus* species. Currently RKN are managed primarily by costly preplant fumigations. Because of their environmental impact, most fumigants are being progressively removed from the market and breeding for RKN resistant *Prunus* rootstocks appears to be a sound alternative. One of the sources of RKN resistance is Myrobalan plum *P. cerasifera*. Among Myrobalan plum, host suitability to the predominant RKN species and particularly to *M. arenaria*, the most frequent RKN species in France, ranges from highly

resistant to very susceptible (Scotto La Massese et al., 1990). The clones P.2175 and P.1079 express a high resistance level to all *Meloidogyne* species and populations tested (Esmenjaud et al., 1994, 1997) and, thus, can be used as wide-spectrum resistance sources. Their resistance is controlled mainly by the *Ma* genes (Esmenjaud et al., 1996b; Lecouls et al., 1997) that are completely dominant and confer a non-host behaviour that completely prevents the nematode multiplication.

The mechanisms of RKN resistance in *Prunus* spp. has been poorly investigated; however, as in other crops (Williamson and Hussey, 1996), a hypersensitive reaction is hypothetized. In the peach rootstock Nemaguard, resistance can be either complete and early as for *M. incognita* for which no development of infective second-stage juveniles (J2s) into swollen larvae occurs or delayed as for *M. javanica* for which juveniles and giant cells begin to develop but rarely evolve into adult females and normal feeding sites (Malo, 1967; Marull et al., 1994). In Myrobalan plums bearing the *Ma* resistance genes, J2s do not develop, even at high temperatures and under a high inoculum pressure (Esmenjaud et al., 1996a). The high-level and broad-spectrum resistance conferred by the *Ma* genes justifies examination of the biological mechanisms. The present study, based on genetically characterized resistant and susceptible clones (Esmenjaud et al. 1996b), determined penetration, development in the roots and emigration from the roots of RKN juveniles. Myrobalan plum is an interesting perennial model species because it can be clonally propagated and a method using plantlets grown from *in vitro* culture has been developed for accurate studies on this aspect (Esmenjaud et al., 1993).

Materials and methods

Plant material

Three Myrobalan plum clones were used: P.2175 and P.1079, both highly resistant to *Meloidogyne* spp., and the suceptible P.2032 (Esmenjaud et al., 1994; 1996a). The resistance to RKN, and in particular to the isolate *M. arenaria* Monteux, is controlled by single dominant genes: the clone P.2175 is heterozygous for the *Ma1* gene (genotype *Ma1 ma1*, *ma2 ma2*), the clone P.1079 is homozygous for the *Ma2* gene (genotype *ma1 ma1*, *Ma2 Ma2*) and the clone P.2032 (cv. Myrabi) is homozygous recessive for both genes. The most probable hypothesis is that the genes *Ma1* and *Ma2* are the same or allelic (Lecouls et al., 1997).

Nematode material

The isolate *M. arenaria* Monteux, identified by its electrophoresis phenotype (Janati et al., 1982) and reared fm a single egg mass culture on tomato cv. St Pierre, was used. The first study on the genetics of resistance in Myrobalan plum (Esmenjaud et al., 1996b) has been based on this nematode. Second stage juveniles (J2s) were obtained in mistifier from tomato roots previously inoculated with this isolate.

Experimental procedure

The three Myrobalan plum clones were propagated and rooted on Murashige and Skoog (1962) medium at 22 °C with a 16-h photoperiod as described by Esmenjaud et al. (1993). The plantlets were transplanted for acclimatization into 1 : 1 (v/v) fine sand and perlite sterilized substrate in tanks ($50 \times 30 \times 15$ cm) in a growth chamber (70–90% R.H.; 100 W/m^2; 16L : 8D) for 2 weeks at 20–22 °C and 2 weeks at 23–27 °C. Homogeneous plantlets of the three clones ranging from 6 to 7 cm high with an equivalent top and root development were selected and transplanted into 0.25 l individual containers filled with sterilized fine sand and loamy soil (3 : 1, v/v) potting medium and grown for one more month before inoculation for tests. A sand particle size of 0.1–0.5 mm was used based on the results of Wallace (1969). Containers were drip irrigated daily with a commercial nutrient solution for ligneous plantlets (Algoflash, Algochimie, Tours, France) at pH 5.8 and maintained in the growth chamber at 25 ± 1 °C with a 16 h photoperiod during the tests.

Penetration and development experiment

For each clone, plantlets were grouped in six sets of 13 (one set for each date of harvest). The whole plantlets were inoculated individually on the same date (D) with 2500 freshly hatched juveniles (24–48 h old), deposited into four 2-cm-deep holes 1 cm from the base of the stem. On the bases of a previous methodological study on the same Myrobalan plum clones (Esmenjaud et al., 1993), this level of inoculum was chosen to be sufficient to allow a better differentiation of the nematode penetration and emigration between clones whilst avoiding intraspecific competition. Penetration was studied during the 10-day period following inoculation. Five replicates per clone were harvested for root staining on each of the dates D + 1, D + 2, D + 4, D + 6, D + 8 and

D + 10. Additional sets of the susceptible clone P.2032 (5 replicates per date) were also harvested and stained at D + 12, D + 16, D + 21, D + 28, D + 35, D + 42 and D + 49.

Plantlet roots were recovered by individual immersion of containers into a small bucket and were washed with caution under tap water. Nematodes were stained using the acid fuchsin-lactophenol method described by Hooper (1986). This method was preferred to the simpler method described by Daykin and Hussey (1985) as this resulted in less marked and durable nematode staining in *Prunus*. Stained white rootlets were then dissected from the roots, spread over a transparent glass to separate them, covered with another watch glass and crushed between both glasses for viewing the nematodes in the plant tissues. Nematodes (J2s, swollen J2s, J3–J4s, females and males) were counted for each individual plant by examining the whole root system under a stereomicroscope.

Thirteen sets of 4 seedlings (corresponding to the following sampling and staining dates: D + 1, D + 2, D + 4, D + 6, D + 8, D + 10, D + 12, D + 16, D + 21, D + 28, D + 35, D + 42 and D + 49) of the RKN susceptible tomato cv. St Pierre, grown in 50-ml plastic tubes, were inoculated at the 3-leaf stage on the same date (D) as *Prunus* plantlets with 250 J2 of the Monteux isolate of *M. arenaria*. They were used as controls to verify the good infectivity of the juveniles and as reference to follow the nematode development in tomato in comparison with Myrobalan plum.

Emigration experiment

For each clone, 5 sets (corresponding to 5 dates) of 4 plantlets (4 replicates), inoculated with the same level of inoculum (2500 J2s), were processed 2 months after the penetration experiment as follows. Two days after inoculation (D + 2), the whole plantlets were individually immersed into a small bucket and carefully washed free of soil particles with caution under tap water. Four of the 5 sets were immediately repotted into 0.25 l containers filled with nematode-free soil for later processing. The fifth set was immediately processed by placing the whole plant (top + roots) individually on a large-mesh sieve inside a plastic container having a lateral 5-mm diam. hole located 10 mm above the bottom. Each sieve+container was placed in a dark mist chamber enabling collection of the emigrating J2s in the bottom of the container. Juveniles were recovered daily and counted. The second set was washed free of soil at D + 4 and processed by the same method. The three other sets were similarly processed at D + 6, D + 8 and D + 10. For each plantlet, daily countings were stopped when no nematodes had been recovered in the container for three consecutive days.

Results

Penetration and development

Equivalent numbers of J2s were recovered in all the clones at D + 1 and D + 2. Afterwards the numbers increased rapidly in P.2032 whereas they stayed the same in P.1079 and P.2175 (Figure 1). Differences between the suseptible and the two resistant clones were significant from D + 4. No swollen J2s were observed in the resistant clones. In the susceptible clone, after 10 days, total penetrated J2s represented about 60% of the initial inoculum. In this latter clone, the first swollen J2s were observed at D + 4 and in additional lots of P.2032, the first females were observed at D + 12, the first males at D + 16 and the first females with attached egg sacs and the first J2s from the new generation were obtained between D + 21 and D + 28 (data not shown). These values correspond to the same speed of development as the nematodes inoculated on the susceptible tomato St Pierre.

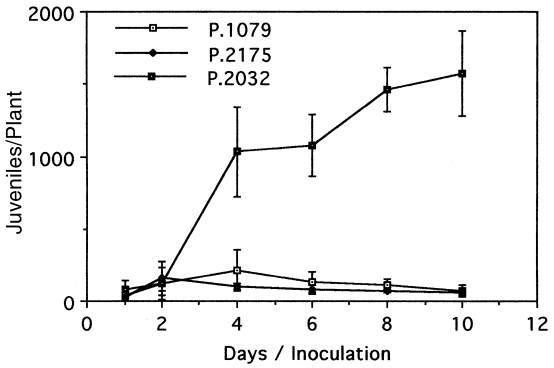

Figure 1. Numbers of juveniles per plant in the roots of resistant (P.1079 and P.2175) and susceptible (P.2032) clones of *P. cerasifera* during the 10 days following the inoculation of 2500 individuals into the soil. Nematodes were stained by the acid fuchsin-lactophenol technique before countings. Vertical bars represent the standard deviation values.

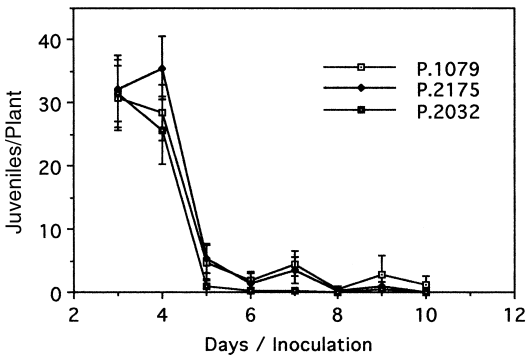

Figure 2. Daily numbers of juveniles recovered after emigration from the roots of resistant (P.1079 and P.2175) and susceptible (P.2032) clones of *P. cerasifera.* Juveniles inoculated at D into the soil were submitted to the emigration process after 2 days (D + 2).

Emigration

On the first set of plantlets for which emigration was studied from D + 2, numbers of emigrated juveniles were high at D + 3 and D + 4 and decreased drastically at D + 5, whatever the clone (Figure 2). Emigration curves of the resistant and susceptible clones were similar and no significant differences were evidenced. In the other sets concerning emigration measured from D + 4, D + 6 and D + 8, emigrated numbers were very low and confirmed data obtained from D + 2 sets. The emigration curves (Figure 3) were also similar for all clones. Although differences were observed, numbers recovered from the three clones were not significantly different. After D + 12 onwards, emigrated numbers were negligible or null.

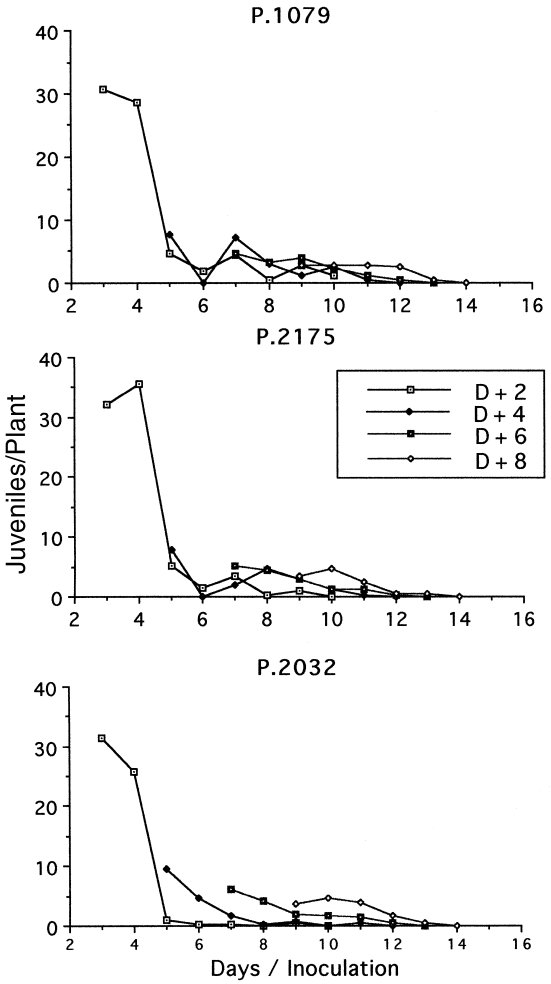

Figure 3. Daily numbers of juveniles recovered after emigration from the roots of resistant (P.1079 and P.2175) and susceptible (P.2032) clones of *P. cerasifera.* The soil containing the Myrobalan plum plantlets were inoculated at D, then all plantlets were repotted in nematode-free soil at D + 2 days (D + 2) and different sets of each clone were submitted to the emigration process at D + 2 days (D + 2), D + 4 days (D + 4), D + 6 days (D + 6) and D + 8 days (D + 8).

Discussion

As expected, the staining technique yielded high nematode numbers (80% after 10 days in P.2032). Our data show that the nematode development and the corresponding life cycle duration are similar in the susceptible *Prunus* clone and in a susceptible tomato reference. This can be attributed to the abundance of young rootlets provided by the micropropagated plantlets.

The absence of major differences between numbers recovered in the roots of the two resistant clones and the susceptible clone during the 48 h following inoculation indicates that no marked early resistance phenomenon as a barrier to penetration seems to occur (Huang, 1985; Cook and Evans, 1987). Differences in nematode numbers in the roots after D + 2 suggest that juveniles continue to penetrate into the roots, select a feeding site, settle and develop into swollen J2s and J3s in the susceptible clone, whereas they either no longer penetrate or penetrate but fail to settle and either die or leave the roots in the resistant clones. In a previous work on peach rootstocks, Meyer (1977) also did not observe any significant difference in the penetration numbers by *M. javanica* between the susceptible

Lovell and the resistant Nemaguard from 12 h to 4 days after soil inoculation. However, the penetration difference appeared earlier in the present study than reported by Meyer (1977). A phenomenon of hypersensitive reaction as observed on the RKN resistant Nemaguard peach by Malo (1967) may explain the lack of development of J2s in the resistant genotypes. However, this phenomenon has to be confirmed by demonstrating the cell necroses at the histological level on *P. cerasifera*. We have performed such histological sections at random in the root tips of resistant Myrobalan plum clones but have not yet found evidence of any cell necrosis (unpublished data). Resistance to *M. hapla* in alfalfa (Griffin and Elgin, 1977) and *M. incognita* in maize (Windham and Williams, 1994) similarly does not appear to be related to differences in J2 penetration and our results also agree with those of Herman et al. (1991) for *M. incognita* in soybean. These authors even observed by 2 days after inoculation a higher penetration rate in the roots of the highly resistant material in comparison with the susceptible soybean reference. In peach, Kochba and Samish (1971) hypothesized that hormonal levels may be involved in differential nematode development between resistant and susceptible material. These authors have observed that the exogenous auxin (NAA) and cytokinin (kinetin) supplied to seedlings allowed to *M. javanica* to develop on the resistant Nemaguard rootstock.

Determination of juvenile emigration by our process appears satisfactory. This is confirmed by the good agreement, for the same clone, of the curves of emigrated numbers obtained for the different sets of plants submitted to the emigration process at different dates (Figure 3). Even when the curves did not match very well as in P.2032, differences were not significant. Such differences might be attributed to the fact that, in the soil, a proportion of the juveniles that emigrate probably re-penetrate the roots soon after, whereas these juveniles cannot re-penetrate when the roots are exposed to the mistifier.

Numbers of juveniles allowed to emigrate from 2 days after inoculation were similar for the different clones. As nematode numbers were the same at D + 2 in all clones in the penetration experiment, this period of time has been chosen to allow nematode penetration and it is assumed that initial numbers of juveniles in the roots for the emigration experiment were also equivalent in all clones. On this basic hypothesis, our results indicate that the post-infectional reduction in numbers of J2s in roots of resistant clones is not correlated with emigration from roots. Although a positive correlation between resistance and emigration has often been observed (Reynolds et al., 1970; Griffin and Elgin, 1977; Herman et al., 1991), it has not been systematic and McClure et al. (1974) had already found non-significant differences in *M. incognita* numbers between resistant and susceptible cotton cultivars.

From our data, it appears that emigration occurs mainly between D + 2 and D + 4 whatever the clone. The authors previously mentioned, studying diverse non-ligneous plant species, observe a more progressive pattern of emigration and not an emigration phenomenon limited in the time as we did. In particular, Herman et al. (1991) obtained equivalent numbers of *M. incognita* juveniles leaving the roots of resistant and susceptible genotypes, when emigration was evaluated for 5 days beginning 2 and 4 days after inoculation. Differences in the root maturation process of ligneous and herbaceous plant species might explain this particular pattern of emigration.

Further studies will examine the specific effect of the *Ma* genes themselves and will have to be conducted on more genetically close plant material such as the resistant and susceptible brother clones used for the study of the genetics for resistance in this *Prunus* species (Lecouls et al., 1997).

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References

Cook R and Evans K (1987) Resistance and tolerance. In: Brown RH and Kerry BR (eds). Principles and practice of nematode control in crops (pp 179–231). Academic press, Marrickville, Australia

Daykin ME and RS Hussey (1985) Staining and histopathological techniques in nematology. In: Barker KR, Carter CC and Sasser JN (eds). An advanced treatise on *Meloidogyne*. Vol II: Methodology (pp 39–46). North Carolina State University, Raleigh

Esmenjaud D, Minot JC, Voisin R, Salesses E, Poupet R and Onesto JP (1993) Assessment of a method using plantlets

grown from *in vitro* for studying resistance of *Prunus cerasifera* Ehr. (Myrobalan plum) to *Meloidogyne* spp. Nematropica 23: 41–48

Esmenjaud D, Minot JC, Voisin R, Pinochet J and Salesses G (1994) Inter- and intraspecific resistance variability in Myrobalan plum, peach, and peach–almond rootstocks using 22 root-knot nematode populations. Journal of the American Society for Horticultural Science 119: 94–100

Esmenjaud D, Minot JC and Voisin R (1996a) Effects of durable inoculum pressure and high temperature on root galling, nematode numbers and survival of Myrobalan plum clones (*Prunus cerasifera* Ehr.) highly resistant to *Meloidogyne* spp. Fundamental and Applied Nematology 19: 85–90

Esmenjaud D, Minot JC, Voisin R, Bonnet A and Salesses G (1996b) Inheritance of resistance to the root-knot nematode *Meloidogyne arenaria* in Myrobalan plum. Theoretical and Applied Genetics 92: 873–879

Esmenjaud D, Minot JC, Voisin R, Pinochet J, Simard MH and Salesses G (1997) Differential response to root-knot nematodes in *Prunus* species and correlative genetic implications. Journal of Nematology 29: 370–380

Griffin GD and Elgin JH (1977) Penetration and development of *Meloidogyne hapla* in resistant and susceptible alfalfa under different temperatures. Journal of Nematology 9: 51–56

Herman M, Hussey RS and Boerma HR (1991) Penetration and development of *Meloidogyne incognita* on roots of resistant soybean genotypes. Journal of Nematology 23: 155–161

Hooper DJ (1986) Preserving and staining nematodes in plant tissues. In: Southey JF (ed). Laboratory methods for work with plant and soil nematodes (pp 81–85). MAFF, London

Huang JS (1985) Mechanisms of resistance to root-knot nematodes. In: Barker KR, Carter CC and Sasser JN (eds). An advanced treatise on *Meloidogyne*. Vol I: Biology and control (pp 165–175). North Carolina State University, Raleigh

Janati A., Bergé JB, Triantaphyllou AC and Dalmasso A (1982) Nouvelles données sur l'utilisation des isoestérases pour l'identification des *Meloidogyne*. Revue de Nématologie 5: 147–154

Kochba J and Samish RM (1971) Effect of kinetin and 1-naphthylacetic acid on root-knot nematodes in resistant and susceptible peach rootstocks. Journal of the American Society for Horticultural Science 96: 458–461

Lecouls AC, Salesses G, Minot JC, Voisin R, Bonnet A and Esmenjaud D (1997) Spectrum of the *Ma* genes for resistance to *Meloidogyne* spp. in Myrobalan plum. Theoretical and Applied Genetics 98: 1325–1334

Malo SE (1967) Nature of resistance of 'Okinawa' and 'Nemaguard' peach to the root-knot nematode *Meloidogyne javanica*. Proceedings of the American Society for Horticultural Science 90: 39–46

Marull J, Pinochet J, Felipe A and Cenis JL (1994) Resistance verification in *Prunus* selections to a mixture of 13 *Meloidogyne* isolates and resistance mechanisms of a peach–almond hybrid to *M. javanica*. Fundamental and Applied Nematology 17: 85–92

McClure MA, Ellis KC and Nigh EL (1974) Post-infectional development and histopathology of *Meloidogyne incognita acrita* in resistant cotton. Journal of Nematology 6: 21–26

Meyer AJ (1977) Penetration of peach cultivars 'Kakamas' and 'Nemaguard' by the larvae of the root-knot nematode *Meloidogyne javanica*. Phytophylactica 9: 73–74

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiologia Plantarum 15: 473–497

Reynolds HW, Carter WW and O'Bannon JH (1970) Symptomless resistance in alfalfa to *Meloidogyne incognita acrita*. Journal of Nematology 2: 131–134

Scotto La Massese C, Esmenjaud D, Minot JC, Voisin R (1990) Host suitability in the genus *Prunus* to *Meloidogyne arenaria*, particularly clones and intraspecific hybrids of *P. cerasifera*. Acta Horticulturae 283: 275–284

Wallace HR (1969) The influence of nematode numbers and of soil particle size, nutrients and temperature on the reproduction of *Meloidogyne javanica*. Nematologica 15: 55–64

Williamson VM and Hussey RS (1996) Nematode pathogenesis and resistance in plants. Plant Cell 18: 1735–1745

Windham GL and Williams GP (1994) Penetration and development of *Meloidogyne incognita* in roots of resistant and susceptible corn genotypes. Journal of Nematology 26: 80–85