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Biological Interactions Between Root-knot Nematode (*Meloidogyne incognita*) and Pepper (*Capsicum annuum*)

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Root-knot nematodes (RKNs; *Meloidogyne* spp.) are soilborne plant parasites that severely decrease productivity of susceptible pepper (*Capsicum annuum*) varieties. Genetic resistance to RKNs serves as a nonchemical alternative to nematode management strategies. Although previous studies of pepper resistance genes to RKNs demonstrate a hypersensitive reaction-based mechanism, an advanced inbred line of pepper ('UFRJ107(6)A3', named 'Ruby') completely inhibits penetration to *M. incognita*. To gain insight into the mechanism of resistance in 'Ruby', responses of M. incognita infective juveniles to root tissues and root extracts from pepper test lines were evaluated in vitro using Pluronic F-127 gel. First, infective second-stage juveniles (J2) were able to penetrate root tips of *C. annuum* cv. Jimmy Nardello Italian (JNI, a susceptible cultivar), and 'Ruby' when 3-week-old seedlings were used. The ability of J2 to penetrate root tips of 'Ruby' suggests that resistance in 'Ruby' could be associated with plant growth developmental stage. Second, hydrophilic compounds from root extracts of both 'JNI' and 'Ruby' showed a negative effect on chemoreception by J2s. The results of the current study can be used to better understand the multiple components of RKN resistance unique to 'Ruby'.

Pepper (*Capsicum annuum*) is a vegetable crop with high economic impact and nutritional value. Root-knot nematodes (RKN; *Meloidogyne* spp.) are the most widely distributed plant-parasitic nematode that severely decreases productivity of susceptible crops including peppers. Damage caused by nematodes in agricultural crops results in an estimated annual loss of 80–157 billion USD globally (Coyne et al., 2018). The primary method to manage RKNs in pepper production has has been broad-spectrum soil fumigants. Genetic resistance to RKNs serves as a nonchemical alternative to nematode management strategies involving fumigants.

Currently in pepper, there are 10 reported genes controlling resistance to RKN with varying species specificity (Djian-Caporalino et al., 1999; Hendy, et al., 1985; Wang and Bosland, 2006). Two resistance genes, *Me3* and *Me7*, inherent in the lines HDA149 and 'Criollo de Morellos' (CM334), respectively, are effective against *M. incognita, M. arenaria, M. javanica,* and *M. hapla*. The resistance mechanisms of *Me3* and *Me7* have been reported to act through a hypersensitive response (HR) with some differences. In HDA149, RKN root penetration induces an immediate HR in the root epidermis while a delayed HR localized to root vasculature is observed in CM334 after penetration. Furthermore, resistance in CM334 is uniquely triggered after a feeding site has been established (i.e., giant cells have formed) (Bleve-Zacheo et al., 1998; Pegard et al., 2005). In contrast to these HR-based mechanisms of resistance, 6-week-old seedlings of an advanced inbred pepper cultivar, UFRJ107(6)A3 ('Ruby'), showed complete inhibition of *M. incognita* penetration based on the results of root staining 7 d after inoculation (Maquilan et al., 2020). Thus, the mechanism and genes involved in resistance unique to 'Ruby' are unknown.

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As one of many avenues of enquiry, we aimed to test whether plant root extracts made in specific solvents likely containing chemical compounds from roots in high enough concentrations could influence nematode behavior. Others have successfully used such methods. For example, infective J2 of *M. incognita* and *Heterodera glycines* were highly repelled and attracted, respectively, to extracts of three different plant species, including pepper, in an in vitro assay (C. Wang et al., 2018). An understanding of the role of various plant root extracts on nematode behavior has implications for differentiating resistance mechanisms and may allow future identification of metabolites that function as components of RKN resistance.

To gain an understanding of the mechanisms involved during the early interactions between *M. incognita* and the pepper genotypes tested, an in vitro, Pluronic F-127 gel-based assay was conducted. Pluronic F-127 gel, a copolymer that forms a gel at room temperature, allows for free movement of J2s, is transparent, and allows for diffusion of chemical gradients. The objectives of the current study were to: 1) compare attraction or repulsion of *M. incognita* to the roots of 'Ruby' and a susceptible cultivar [Jimmy Nardello Italian (JNI)] and 2) evaluate the response of *M. incognita* to root extracts of 'Ruby' and 'JNI' in a choice experiment.

Materials and Methods

Inoculum preparation. *Meloidogyne incognita* race 3 originated from cotton grown in an agricultural field in South Georgia. An isolate was derived by making a single egg mass isolate and then grown on tomato in a greenhouse in the Entomology and Nematology Department, University of Florida at 27 ± 5 °C at 75% relative humidity (RH). Host plant maintenance and egg

extractions were conducted as described in (Maquilan et al., 2020) for the preparation of freshly hatched J2 suspensions. Freshly hatched J2s were collected on a sieve with 500 μm openings, then transferred to a 50-mL Falcon tube with 20 mL of water. The J2-Pluronic F-127 suspension was prepared through an adapted protocol as described by (Williamson and Čepulytė, 2017). Pluronic F-127 23% (wt/vol) (PF-127; Sigma-Aldrich, USA) was first prepared in 10 mM Tris- morpholinoethanesulfonic acid (MES) buffer. The solution was stirred with a magnetic stir bar at 4 °C overnight until dissolved, then kept in a refrigerator at 4 °C. Freshly hatched J2 suspended in 1–2 mL H¬$_2$O were added to the PF-127, then stirred for 10 min. at 4 °C with a magnetic stir bar. Solutions were kept on ice before adding to the wells.

Pepper genotypes. Seeds of *M. incognita* 'Jimmy Nardello Italian' ('JNI') were purchased from Baker Creek Heirloom Seeds (Mansfield, MO). 'JNI' is an heirloom cultivar that is highly susceptible to *M. incognita* race 3. Advanced inbred line UFRJ107(6)A3 ('Ruby') is a specialty pepper that shows high resistance to *M. incognita*, *M. arenaria,* and *M. javanica* based on RKN reproduction and penetration measurements (Maquilan et al., 2020). An F_1 derived from a cross between 'JNI' and 'Ruby' was also evaluated for root attraction assays.

Preparation of root extract. Root extracts were prepared through an adapted protocol as described by C. Wang et al., (2018). Entire root systems of 4-week-old seedlings were rinsed and cut into pieces. Roots were ground and frozen in a mortar and pestle with liquid nitrogen into a fine powder. Approximately 0.042 g of root material was transferred to 1.5-mL microcentrifuge tubes, in which 250 μL of extraction solvents were added. The two extraction solvents used in this study were distilled water (H_2O) and methanol (MeOH) 80% (v/v). Extracts were centrifuged (20 min., 13,000 ×*g*) and supernatant volumes were transferred to a separate 1.5-mL microcentrifuge tube.

Root attraction and penetration assay. Root systems from seedlings of each pepper genotype were removed from vermiculite after 3 weeks and rinsed with sterile water. Terminal 5-mm of root tips were excised individually and placed in a cell of a 12-well tissue culture plate (Nunc™ Non-Treated Multidishes, Thermo Scientific, Waltham, MA) at room temperature. One mL of PF-127 23% (wt/v) and 10 mM Tris-MES (pH 8) containing 100 J2 was delivered to each well. The media was allowed to solidify over 10 min before recording any observations. Plates were covered in aluminum foil and kept at room temperature. The total number of J2s in contact with the root were then counted under a stereomicroscope equipped with an overhead illuminator at 2 h, 4 h, 6 h, 19 h, and 24 h. At the end of the root attraction assay, the root tips were immediately removed from the well, stained with fuchsin-acetic acid (Byrd et al., 1983), and the number of J2 were counted. Six replicate assays were used for each data point.

Root extracts choice test. For the root extracts choice test, each of the test and control solutions were suspended in PF-127 23%. The test controls used in the root extracts choice test were salicylic acid (50 ng/mL) (SA; attractant), acetic acid 1% (v/v in H¬$_2$O) (HOAc; repellent), H¬¬$_2$O , and MeOH 80% (v/v in H_2O). For each well of a 12 well tissue culture plate, approximately 330 freshly hatched J2 suspended in 1 mL of PF-127 23% was added. After the gel solidified at room temperature, 3.5 μL of test solution and 3.5 μL water or other control as indicated were injected into the gel at marked points 5.5 mm apart. Plates were covered in aluminum foil and kept at room temperature. The number of J2 within a 5 mm circle centered on the injection site of the test solution or control was counted under a stereomicroscope after 6 h and 24 h. Three replicate assays were used for each data point.

Statistical analysis. A completely randomized design was used during all experiments. Quantitative data were used to calculate means and the standard error. The statistical significance of the difference in data collected from all experiments was tested using Student's *t* test ($P \leq 0.05$) with the RStudio program (RStudio, Boston, MA).

Results and Discussion

Root tips of 'Ruby', 'JNI', and the F_1 hybrid were studied to evaluate any differences in attraction by J2 between the genotypes (Fig. 1A). The number of J2 in contact with root tips of 'Ruby' was significantly lower compared to root tips of 'JNI' at 6 h, while the opposite trend was seen at 24 h (Fig. 1B). In addition, the number of J2 in root tip in contact with the F_1 hybrid resembled that of 'Ruby'. The decrease in number of J2 in contact with root tips of 'JNI' suggested that J2 were unable to penetrate roots of 'Ruby' but able to penetrate roots of 'JNI'. To confirm this, the root tips were then removed from the wells and subjected to fuchsin-acetic acid staining to determine the number of J2 inside the root. The J2 were able to penetrate the root tips of both 'JNI' and 'Ruby' (Fig. 1C). Furthermore, the difference in mean number of J2 inside the roots of both genotypes was insignificant.

The results from this experiment contrasts with previous findings that J2 of *M. incognita* were unable to penetrate roots of 'Ruby' (Maquilan et al., 2020). In comparing variables between the previous and current experiment, two that differed are the medium used and seedling age. It is possible that the PF-127 23% media exogenously induced sensitivity to penetration in 'Ruby'. The media used for 'Ruby' seedlings may play a role in the components of resistance unique to 'Ruby'. In regard to seedling age, the previous and current study evaluated roots of Ruby seedlings at 8 weeks and 3 weeks, respectively. The results of both experiments imply that different developmental plant-growth stages are associated with penetration inhibition in 'Ruby'. For example, resistance that is developed at later stages of plant-growth is referred to as adult plant resistance. In contrast to highly specific HR-based resistance, adult plant-growth resistance confers broad-spectrum resistance and is quantitative in nature (Johnson, 1984). Further studies are needed to evaluate differences between penetration ability in 'Ruby' at different plant-growth developmental stages.

Evaluating root extract activity on J2 behavior serves to test chemical components of resistance. Thus, root extracts of 'Ruby' and 'JNI' were compared to evaluate any effects on J2 behavior at 6 h and 24 h. Salicylic acid (SA) served as an attractant control (Wuyts et al., 2006). Although acetic acid 1% (v/v) acted as a repellent control in a preliminary experiment, the same effect was not observed in the root extracts choice test (data not shown) possibly due to diffusion of the solution into the gel to below optimal concentrations after placement. Both 'Ruby' and 'JNI' root extracts obtained through ddH2O extraction demonstrated significantly reduced attractance compared to H_2O (Table 1). Furthermore, the reduced attractance was observed at 24 h, suggesting that the compounds in both roots are stable in solution. In contrast, both 'Ruby' and 'JNI' root extracts obtained through MeOH 80% (v/v in ddH_2O) extraction did not show any reduced attraction except for 'Ruby' at 6 h (Table 1). In summary, roots

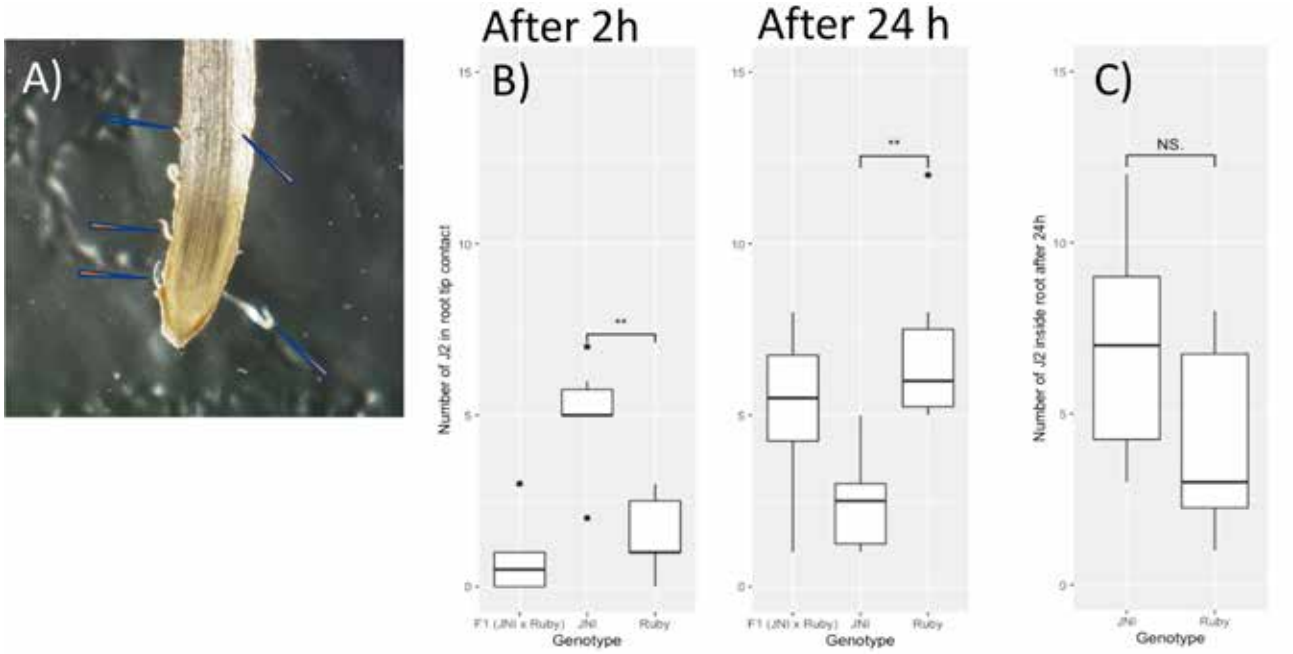

Fig. 1. **A**) Infective-stage juveniles of *Meloidogyne incognita* race 3 on a root tip of 'Jimmy Nardello Italian' ('JNI') at 2 h; triangular blocks point to individual J2s; magnification = 24.0×. **B**) Mean number of J2 in contact with root tips of 'Ruby', 'JNI', and the F_1 hybrid at 6 h and 24 h. Statistical significance was determined by mean separation by Student's *t* test at $P \leq 0.05$ (** significant at $P \leq 0.01$). **C**) Mean number of J2 inside roots of 'Ruby' and 'JNI' after staining with fuchsin-acetic acid at 24 h.

Table 1. Preferred choice of J2 between two choices presented in a pluronic gel matrix. Comparisons were made between 'Ruby' and 'Jimmy Nardello Italian' (JNI) root extracts obtained through water and MeOH 80% (v/v), as well as control solutions, at 6 h and 24 h.

Choice A	Choice B	Observation time (h)	Mean number of J2s in A + SE	Mean number of J2s in B + SE	Preferred choice	Statistical significance[z]
SA	H_2O	6	19 ± 0.882	9 ± 1.528	A	***
JNI (H2O)	H_2O	6	2 ± 0.333	14 ± 3	B	****
Ruby (H2O)	H_2O	6	5 ± 0.3333	13 ± 2.333	B	**
JNI (MeOH)	MeOH	6	5 ± 2.3333	14 ± 3.606	neither	ns
Ruby (MeOH)	MeOH	6	5 ± 2.081	9 ± 1.155	B	*
JNI (H2O)	Ruby (H_2O)	6	8 ± 1.333	3 ± 0.333	A	**
JNI (MeOH)	Ruby (MeOH)	6	6 ± 0.577	5 ± 0.882	neither	ns
SA	H_2O	24	14 ± 2.404	9 ± 1.856	A	NS
JNI (H_2O)	H_2O	24	6 ± 1.856	10 ± 1.202	B	*
Ruby (H_2O)	H_2O	24	5 ± 1.202	10 ± 0.882	B	*
JNI (MeOH)	MeOH	24	11 ± 2.186	15 ± 2.403	neither	ns
Ruby (MeOH)	MeOH	24	6 ± 1.528	7 ± 0.333	neither	ns
JNI (H_2O)	Ruby (H_2O)	24	10 ± 0.667	9 ± 1.453	neither	ns
JNI (MeOH)	Ruby (MeOH)	24	8 ± 0.882	9 ± 1.202	neither	ns

[z]Statistical significance was tested between mean comparisons between A and B using Student's *t* test.
**** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.01$, * $P \leq 0.05$, ns = nonsignificant.

of both 'JNI' and 'Ruby' contain hydrophilic compounds that negatively affect chemoreception of *M. incognita* J2.

Previous reports indicate the effects of plant metabolites on J2 behavior under different systems (Sikder and Vestergård, 2020). However, there are few studies involving pepper specifically (Bleve-Zacheo et al., 1998; Kihika et al., 2017; Pegard et al., 2005). Chlorogenic acid was identified as the primary chemical constituent of hypersensitive response sites in CM334 (Pegard et al., 2005). Nonetheless, the results in our root extracts choice test were similar to a previous study, in which extracts of a susceptible pepper cultivar did not attract *M. incognita* (C. Wang et al., 2018). Further work is required to evaluate the root extract chemical profile for identifying the primary metabolite(s) causing this repellent effect. A potential outcome that would be of great benefit is to identify genes in pepper responsible for biosynthesis of such metabolites.

Conclusions

In the current study, we evaluated the interactions between J2 of *M. incognita* race 3 and root extracts, as well as terminal root tips of resistant and susceptible pepper varieties. With the exception of *Me3* and *Me7*, RKN resistance genes in pepper have typically been characterized based on galling index, egg mass index, and reproduction factor, while the biological mechanisms of such genes generally remain poorly understood (Changkwian et al., 2019; Djian-Caporalino et al., 1999; Hajihassani et al., 2019; Hendy et al., 1985; Thies and Fery, 2000). Our aim was to test whether the RKN-resistant 'Ruby' demonstrated unique mechanisms independent of HR-based resistance as characterized in other RKN-resistance genes (e.g. *Me3* and *Me7*). Our results can be used in further studies to investigate the multiple components of RKN resistance unique to 'Ruby'.

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