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A high-throughput method for early screening of coffee (*Coffea* spp.) genotypes for resistance to root-knot nematodes (*Meloidogyne* spp.)

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Abstract Root-knot nematodes (*Meloidogyne* spp.) threaten the livelihood of millions of farmers producing coffee worldwide. The use of resistant plants either as cultivars or rootstocks appears to be the single most effective method of control. A screening method was developed to evaluate large populations of plants for resistance to root-knot nematodes. Two coffee cultivars, one susceptible and the other resistant to *Meloidogyne paranaensis,* were grown under controlled conditions in two substrates: a commercial sieved potting compost and an inert substrate containing sand with a water-absorbent synthetic polymer. Plant growth and development and nematode multiplication were compared for two inoculation dates (2 and 8 weeks after planting) and two evaluation dates (eight and 13 weeks after inoculation). Root growth, but not nematode multiplication, was influenced by the choice of substrate. Evaluation of the differences in root weight and nematode numbers between the different cultivars, substrates and dates of inoculation suggested that an optimal condition could be defined. The best discrimination between susceptible and resistant plants was found in the experiment where inoculation occurred at 2 weeks after planting and evaluation occurred at 8 weeks after inoculation. Because the total duration of this experiment was only 3 months, high-throughput evaluation was possible, opening up new possibilities for screening large germplasm collections and studying the genetic control of root-knot nematode resistance in coffee.

Keywords Coffee · *Meloidogyne* · Resistance assessment · Root-knot nematode · Screening method

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Introduction

Root-knot nematodes have become a major threat in the world's main coffee-producing countries (Campos and Villain 2005). Seventeen species of *Meloidogyne* are now acknowledged as parasitic to coffee (Carneiro and Cofcewicz 2008). Resistance to *M. exigua* Göldi (Anthony et al. 2005) and *M. incognita* Carneiro, Carneiro, Abrantes, Santos & Almeida (Albuquerque et al. 2010) has been associated with a hypersensitive response, indicating a specific response to the pathogen

infection. The main *Coffea arabica* L. cultivars grown worldwide (i.e., Caturra, Mondo Novo, Catuai) are susceptible to many pests and diseases, including nematodes. Transfer of resistance genes from resistant accessions into susceptible cultivars without affecting other traits has become the main objective of breeding programs (Van der Vossen 2001; Bertrand et al. 2003). The resistant accessions of species other than *C. arabica* can be efficiently used as rootstocks (Bertrand et al. 2000b), constituting a cheap alternative to introgressive breeding (Lashermes et al. 2000; Bertrand et al. 2001). Sources of resistance to *M. arabicida* López, *M. arenaria* (Neal) Chitwood and *M. paranaensis* have been identified in both cultivated species (Bertrand et al. 2000b, 2002; Anzueto et al. 2001; Hernández et al. 2004; Boisseau et al. 2009). Against *M. exigua* and *M. incognita* (Kofoid and White) Chitwood, resistant accessions have been detected only in *C. canephora* and progenies of interspecific hybrids (*C. arabica* x *C. canephora*) (Curi et al. 1970; Gonçalves and Ferraz 1987; Carneiro 1995; Bertrand et al. 1997; Gonçalves and Pereira 1998; Silvarolla et al. 1998). The search continues for resistance to other *Meloidogyne* species that are parasitic to coffee.

Most experiments that assess resistance have been conducted using coffee seedlings grown in greenhouses and have been evaluated on the basis of the six-class gall index defined by Taylor and Sasser (1978). Gall observations have been supplemented by nematode extraction using a blender and sieves (Barker 1985). However, no standard and reliable method has been developed so far, which has considerably limited the comparison of data between experiments. The volume of substrate (usually a mixture of compost and sand) used for plant cultivation has varied from 180 ml (Barbosa et al. 2007) to 1,300 ml (Morera and López 1988); the number of eggs and juveniles inoculated has ranged from 200 (Alpizar et al. 2007) to 15,000 (Morera and López 1988); and the number of days between inoculation and evaluation has ranged from 48 days (Morera and López 1988) to 180 days (Barbosa et al. 2007). The objective of the present study was to develop an early and rapid method for evaluating coffee plant responses to inoculation with root-knot nematodes. The hypothesis was that young coffee seedlings could express resistance to root-knot nematodes as early as the cotyledon stage.

Materials and methods

Inoculum

A clonal population of *M. paranaensis* was established from a single egg mass of nematodes originally collected from coffee plants in Apucarana (Paraná State, Brazil) and then maintained in culture by Embrapa Recursos Genéticos e Biotecnologia (Boisseau et al. 2009). The population was maintained in a confined greenhouse in Montpellier on cv. Caturra, a coffee cultivar susceptible to *M. paranaensis*. For inoculation, nematodes (eggs and juveniles) were extracted in a mist chamber (Seinhorst 1950).

Plant material

Two cultivars that have been characterised as susceptible or resistant to *M. paranaensis* were used in this study. *C. arabica* cv. Caturra (Carvalho et al. 1991) is susceptible to *M. paranaensis* (Boisseau et al. 2009). Seeds of cv. Caturra were produced in the Doka farm (Costa Rica). The rootstock cv. Nemaya of *C. canephora* is resistant to *M. paranaensis* (Bertrand et al. 2000b). Seeds of cv. Nemaya were produced in seed gardens in the Coffee Research Centre of Guatemala (ANACAFE). For germination, batches of 12 seeds were placed on 18 g of vermiculite fully saturated with 45 ml of sterile water in closed plastic boxes (Magenta®) (Dussert et al. 2003). Germination of cv. Caturra seeds required 2 weeks, while that of cv. Nemaya required 3 weeks.

Coffee seedlings were grown following the procedure described for screening resistance of rice to *Heterodera sacchari* Luc & Merni (Réversat and Destombes 1998). After germination, seedlings were planted in 110 ml culture tubes made of PVC cylindrical tubing (26 mm inner diameter and 210 mm in length) suspended from plastic boxes (Fig. 1). Each box (0.6×0.4 m) held 30 culture tubes. The bottoms of the tubes were closed with a 0.25 mm mesh stainless steel sieve welded into the PVC. Each tube was lined with a transparent polyethylene sheet and filled with about 100 ml of substrate. The plastic sheet extended 5 mm over the top of the PVC tube, which made it easy to pull the cylinder of substrate out of the tube without damaging the roots and the nematodes at the end of the experiment. Two substrates were compared: a sieved (size of the sieve: 2 mm)

Fig. 1 Automatic management of coffee plants inoculated with *M. paranaensis* in the confined greenhouse of IRD, including control of light, temperature, humidity and water supply

potting compost (Neuhaus® type S) and a mixture of sand (particle size: 0.1–0.4 mm) with a water-absorbent synthetic polymer (Réversat et al. 1999). The seedlings were kept in a confined greenhouse in Montpellier at a temperature ranging from 22 to 28°C and at 70–90% relative humidity. Soil was automatically watered daily (1 min during the first month, then 2 min) with excess water drained off. Two mg of slow-release fertiliser (Basacote Plus 6MK, Compo®) were given to each plant 3 days after inoculation.

Inoculation and evaluation

Two inoculation dates were compared: 2 and 8 weeks after planting in culture tubes. Single seedlings were inoculated three times, 2 days apart, with a total of about 1,500 eggs and second-stage juveniles (J2) into 1-cm deep holes around the collar region. Evaluation occurred after two periods of nematode multiplication: at eight and 13 weeks. Plants were carefully removed from the pots. The number of leaf pairs was counted and the aerial part was weighted. The root systems were gently washed with tap water and weighed. Roots were cut into 3–5 mm fragments and then placed on 1 mm–mesh sieves over open funnels that were individually supported by a 1-litre polyethylene bottle. Nematodes were separated from plant tissue by incubation in a mist chamber (Seinhorst 1950). Nematodes were collected three times, 5 days apart, and their number was estimated in 1 ml aliquots under a microscope. Eggs and juveniles were counted separately.

Experimental design

Four experiments were defined on the basis of the inoculation dates (2 or 8 weeks after planting) and the evaluation dates (eight or 13 weeks after inoculation). The experiments consisted of 14 seedlings of each cultivar grown on either inert substrate or sieved potting compost. Within each experiment, the plants of both cultivars were randomly distributed in the plastic boxes (Fig. 1).

Statistical analysis

The number of leaf pairs and the weights of the aerial part and root system were transformed using the log(x) function, while the number of nematodes extracted from a plant was transformed using the $\sqrt{(x)}$ function. Relationships between variables were examined using Pearson's correlation coefficient. The effects of cultivar, substrate and dates of inoculation and evaluation, as well as the effects from multiple interactions were estimated using multi-factor ANOVA. Means were compared using the Newman and Keuls test at $P=0.05$. The distribution of extracted nematode numbers was given per cultivar and experiment using a categorised box-whisker plot, wherein the median value is presented as a point inside a box, 25–75% values are presented as the box, and minimum and maximum values are presented as a pair of whiskers. Statistical analyses were carried out using Statistica software (©StatSoft, Inc.).

Results

Plant growth and development

Observation of the root systems before nematode extraction revealed differences in growth for both cultivars depending on the substrate (Fig. 2). With the inert substrate composed of a mixture of sand and water-absorbent synthetic polymers, the coffee roots were concentrated in the upper half of the culture tubes. With the organic substrate, the roots occupied the entire volume of the tubes. Sand weight could have caused a subsidence of the inert substrate to the lower part of culture tubes, which may have caused the negative effect on root growth. Other differences were observed in the root systems depending upon the susceptibility or resistance of the plants (Fig. 2).

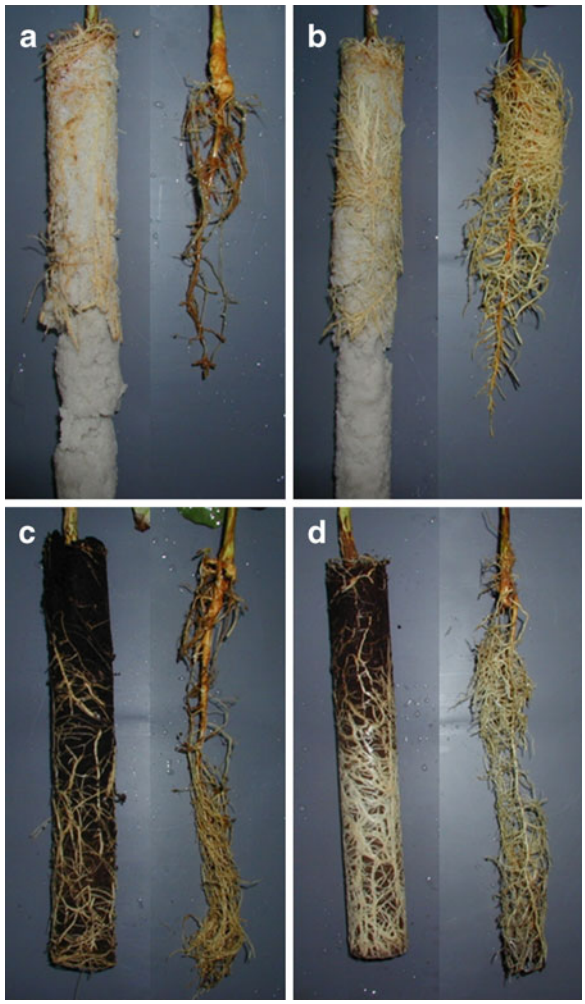

Fig. 2 Root systems of cv. Caturra and cv. Nemaya grown on inert (Fig. 2a and 2b, respectively) and organic (Fig. 2c and 2d) substrates, inoculated 8 weeks after planting and evaluated 13 weeks after inoculation. Symptoms of corky-root are visible on the root system of the susceptible cv. Caturra (Fig. 2a and 2c)

Considering all of the plants (N=112), root weight was highly correlated with leaf-pair number (r=0.76, P<0.0001) and leaf weight (r=0.88, P<0.0001). This finding suggested that plants with a weak root system can be discarded by observing the vegetative parts. Leaf-pair number and leaf weight were significantly different between the two cultivars (P<0.0001 and P<0.001, respectively), but the root weights were similar (Table 1). The substrate also influenced leaf-pair number (P<0.05), leaf weight (P<0.0001) and root weight (P<0.001). Multiple differences in root weight between cultivars, substrates and dates of inoculation and evaluation suggested that it should be possible to find an optimal condition for root development. Separation of the interacting effects between the cultivars and substrates for root weight showed that the sieved potting compost was more favourable to root development than the inert substrate for both cultivars (Table 2). According to the data dispersion, cv. Nemaya appeared more sensitive to the effect of substrate than cv. Caturra.

Nematode multiplication

The number of extracted nematodes varied according to the cultivars and the dates of inoculation and evaluation (Table 1). Several interactions between main effects were significant, in particular between cultivar and dates of inoculation and evaluation (P<0.001). This highlights the influence of inoculation and evaluation dates on the coffee plant's response to nematode infection. The substrate had no effect on nematode multiplication unless the inoculation date was taken into account (P<0.05). Data from the two substrates were thus grouped for each experiment in the following analyses. Comparison of the means of nematode multiplication showed a homogeneous response of cv. Nemaya at all dates considered (Table 3). Low reproduction of root-knot nematodes has already been reported in cv. Nemaya after inoculation with *M. paranaensis* (Boisseau et al. 2009) and in other coffee cultivars resistant to *M. exigua* (Anthony et al. 2005; Barbosa et al. 2007) or *M. incognita* (Albuquerque et al. 2010). In contrast, cv. Caturra showed two groups of means that were statistically separated, with a higher number of nematodes (>33,000 nematodes/plant) being obtained from the longest experiment. Examination of the data distribution clearly indicated that the best separation of nematode numbers per cultivar was obtained at the earliest and latest dates of inoculation and evaluation (Fig. 3). The data obtained from the earliest dates was less scattered than that from the latest dates.

Discussion

Until now, the slow growth of coffee seedlings and the long duration of experiments have constituted a major bottleneck in the development of rapid and large-scale tests of resistance. The work reported here defines a systematic method for evaluating resistance

Table 1 Significant *F* values and associated probabilities from multi-factor ANOVA for leaf number, leaf weight, root weight and nematode number, with cultivar, substrate and dates of inoculation and evaluation as the sources of variation (112 plants)

Source of variation	Leaf-pair number[a]	Leaf weight[a]	Root weight[a]	Nematode number[b]
Cultivar	236.1 ($P<0.001$)	12.0 ($P<0.001$)		293.7 ($P<0.001$)
Substrate	4.0 ($P<0.05$)	61.9 ($P<0.001$)	20.9 ($P<0.001$)	
Inoculation date	1154.1 ($P<0.001$)	1035.2 ($P<0.001$)	772.2 ($P<0.001$)	23.6 ($P<0.001$)
Evaluation date	276.1 ($P<0.001$)	174.6 ($P<0.001$)	139.9 ($P<0.001$)	8.0 ($P<0.01$)
Cultivar × Substrate		9.0 ($P<0.01$)	5.4 ($P<0.05$)	4.7 ($P<0.05$)
Cultivar × Inoculation date				13.1 ($P<0.001$)
Cultivar × Evaluation date	4.6 ($P<0.05$)		5.4 ($P<0.05$)	8.3 ($P<0.01$)
Substrate × Inoculation date			5.4 ($P<0.05$)	6.3 ($P<0.05$)
Substrate × Evaluation date				
Inoculation date × Evaluation date	8.8 ($P<0.01$)		4.5 ($P<0.05$)	11.0 ($P<0.01$)
Cultivar × Substrate × Inoculation date				
Cultivar × Substrate × Evaluation date	12.0 ($P<0.001$)			
Cultivar × Inoculation date × Evaluation date	13.9 ($P<0.001$)			18.9 ($P<0.001$)
Substrate × Inoculation date × Evaluation date		5.4 ($P<0.05$)		

[a] Log(x) transformed before analysis

[b] √(x) transformed before analysis

to root-knot nematodes in coffee plants. The data allow the effects of several key factors that influence the plant-parasite interaction to be determined, such as the substrate type and the dates of inoculation and evaluation. The substrate type had a direct effect on the root weight of both cultivars, but not on nematode multiplication. Although root growth was favoured in the organic substrate, the inert substrate should be preferred for microscopic observations because of clean solutions that were obtained after extraction, and which consequently simplified counting. The fact that roots were concentrated in the upper half of the tubes increased the success of inoculations in the inert substrate and therefore the reliability of the evaluation. Comparisons between two dates of inoculation and two dates of evaluation showed that differences between the susceptible and resistant plants were more marked when inoculation and evaluation occurred early (2 weeks after planting and 8 weeks after inoculation, respectively), or conversely, late (8 weeks after planting and 13 weeks after inoculation, respectively). Early inoculation and evaluation should be preferred to the late dates because the data are more homogeneous.

Table 2 Mean (g) and minimum and maximum values (in parentheses) of root weight for the two cultivars grown on inert or organic substrates. Twenty-eight plants per cultivar were grown on each substrate

	Inert substrate	Organic substrate
cv. Nemaya	1.90 (0.31–5.00)	3.00 (0.38–11.40)
cv. Caturra	2.08 (0.59–4.80)	2.82 (0.32–8.10)

The method described in this study is rapid and does not require laborious manipulations, allowing for phenotyping of a large number of plants for resistance to root-knot nematodes. Seeds are preferable to cuttings or plantlets from in vitro culture because they are cheap to produce and easy to obtain from large field genebanks established worldwide (Dulloo et al. 2009). In addition, they represent the base propagation unit for the autogamous species *C. arabica* and can be efficiently used for identification of resistant accessions in allogamous species (Bertrand et al. 2000b). The results presented here clearly show that the resistance of coffee plants to root-knot nematodes is expressed at the earliest stages of plant development, which considerably reduces the duration of evaluation. After the seeds were sown, evaluation required only 3 months: 2 weeks for germination, 2 weeks for acclimatisation to the substrate before inoculation, and 8 weeks for nematode multiplication. This short evaluation period is important for perennial plants.

Table 3 Mean number of nematodes extracted from the two cultivars inoculated 2 or 8 weeks after planting, and evaluated eight or 13 weeks after inoculation. Means followed by the same letter are not significantly different according to the Newman-Keuls test at 5% probability. Data were transformed using the √(x) function before analysis

Sources of variation				
Cultivar	Inoculation	Evaluation	Plants (N)	Nematode number
Nemaya	2 w	8 w	14	711[a]
Nemaya	2 w	13 w	14	868[a]
Nemaya	8 w	13 w	14	1,136[a]
Nemaya	8 w	8 w	14	1,468[a]
Caturra	2 w	13 w	14	8,138[b]
Caturra	2 w	8 w	14	9,986[b]
Caturra	8 w	8 w	14	11,529[b]
Caturra	8 w	13 w	14	33,016[c]

In addition to rapidity, early evaluation reduces the space required for plants because of the small plant size. Because each plate of 30 plants occupies only 0.24 m^2, it is possible to grow 120 plants per m^2 (Fig. 1). Thus, the surface occupied by the experiments is not a limitation for the number of plants that can be evaluated simultaneously. The successive counts of nematodes extracted in the mist chamber were also not a constraint. Based on the experiments reported here, which included three counts, 200–300 plants could be evaluated by one person in a month. Thus, it is possible to evaluate several hundred plants in an acceptable period of time. The method described in this study has been successfully applied towards identifying accessions resistant to other *Meloidogyne* species, such as *M. exigua* and *M. incognita* (data not shown).

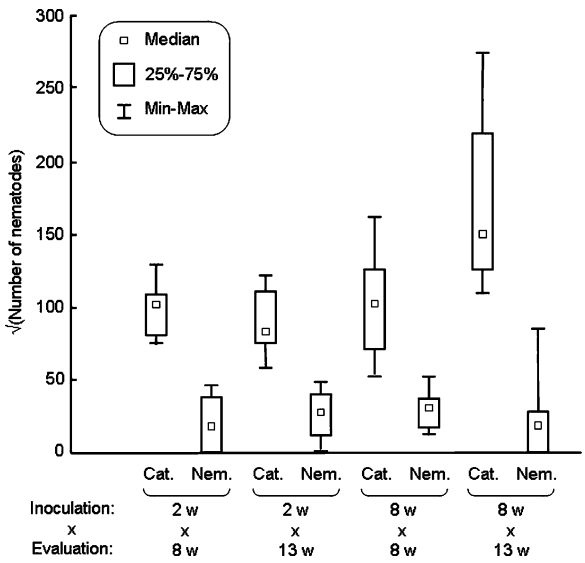

Fig. 3 Categorised box-whisker plot for the number of nematodes extracted from cv. Caturra (Cat.) and cv. Nemaya (Nem.) inoculated 2 or 8 weeks after planting, then evaluated 8 or 13 weeks after inoculation. Fourteen plants per cultivar were analysed for each experiment

Nematodes were extracted using the mist technique (Seinhorst 1950) and counted three times 1 week apart. This extraction technique is passive and produces very active infectious J2s, which are easy to count. The inoculum can thus be quantified precisely. Nematodes can also be counted using the centrifugation-flotation technique, which extracts nematodes at all stages of development (eggs, juveniles and adults) (Coolen and D'Herde 1972). However, this technique is not adaptable to high-throughput analyses because of the need for successive handling of centrifugation deposits, supernatants and filtrates recovered from sieves (Bertrand and Anthony 2008). Moreover, for inoculation purposes, exposure to sodium hypochlorite to facilitate extraction can affect the viability of extracted eggs and juveniles. Hussey and Barker (1973) estimated that only 20% of extracted eggs hatch into infectious J2s. The gall index has frequently been used to evaluate resistance to root-knot nematodes in coffee plants, but its use as a single criterion of evaluation is not recommended (Bertrand and Anthony 2008). In an F2 population segregating for resistance to *M. exigua*, two plants (out of a total of 93 plants evaluated) with a gall index of zero did not have any of the molecular markers linked to the *Mex*-1 resistance gene, and two plants with an index of four and five (on a 0–5 scale) displayed the markers of the gene (Noir et al. 2003). Moreover, observations of galls on root systems are not relevant when nematodes destroy roots in association with another pathogen (e.g., *Fusarium oxy-*

sporum), as reported for *M. arabicida* (Bertrand et al. 2000a, 2002), *M. incognita* (Negrón and Acosta 1989) and probably *M. paranaensis* (Bertrand and Anthony 2008). Populations of these nematodes decrease rapidly in susceptible plants in proportion to the destruction of the root systems, which increases the necessity of shortening the time between inoculation and evaluation.

New sources of resistance are needed to improve the level of root-knot resistance in coffee cultivars. Resistance to some poorly studied species (e.g., *M. izalcoensis* Carneiro, Almeida, Gomes and Hernández) has not yet been evaluated. The majority of the 17 *Meloidogyne* species recognised as parasitic to coffee plants have only been described in the last 50 years (Carneiro and Cofcewicz 2008), which explains why the biology and the distribution of some nematode species are still poorly documented. About 60 coffee species have been collected and are conserved in field genebanks worldwide (Dulloo et al. 2009). Few of these species have been screened for resistance (Anthony et al. 2007). New resistance genes remain to be discovered in these genetic resources. By reducing the duration of evaluation to only 3 months, faster screening of germplasm is now possible.

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