

# Host-Finding and Invasion by Entomopathogenic and Plant-Parasitic Nematodes: Evaluating the Ability of Laboratory Bioassays to Predict Field Results

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**Abstract:** Directly viewing soil-dwelling entomopathogenic and plant-parasitic nematodes in situ is difficult, if not impossible. As a result, researchers have developed a diverse array of bioassays which assess nematode behavioral traits within arenas designed to simulate various aspects of the natural habitat. However, reliably rendering what we can see in the laboratory into accurate predictions of how nematodes achieve their objectives in the field is challenging. In the current review, we systemically assessed the goals and attributes of several of the assays most commonly used to investigate nematode host finding and host invasion behavior. By illuminating the relative strengths and limitations of each assay, we hope to improve our ability to develop meaningful predictions for the field.

**Key words:** behavior, chemotaxis, ecology, electrophysiology, olfactometer, soil column

Laboratory bioassays are used to understand nature via a simplified version of the real world. In the act of simplification, some important aspects of realism are compromised. Useful bioassays are simple enough to enable accurate measurement of the biological phenomenon of interest, while still reflecting to some degree the natural situation where the phenomenon actually occurs. The balance of simplicity to realism in bioassays is a difficult one to define; it is the seeking of this balance that is the topic of our review. Here we focus on bioassays that are used to understand the relationships between soil-dwelling stages of entomopathogenic and plant-parasitic nematodes and their respective hosts. It is our hope that our systematic treatment of the diversity of these assays, and their various goals, will illuminate the attributes and liabilities of various types of laboratory- and greenhouse-based assays.

Soil-dwelling nematodes are a group of organisms for which laboratory bioassays have been designed out of necessity. Directly viewing these nematodes in situ is nearly impossible because they are small, usually less than 1mm in length, often transparent, and located in an opaque soil matrix. As an alternative to watching tiny, clear organisms in the dark, numerous simulations of their habitats have been designed, all with the goal of enabling us to understand their behavioral attributes. However, translating what we can see in the laboratory to accurate predictions of how nematodes achieve their objectives in the field is challenging and often not successful. In other words, often our hypotheses about nematode behavior formed in the laboratory are rejected when tested in the field. Thus, a central question to designing bioassays and interpreting their results re-

mains “How much reality can be sacrificed in a bioassay without altering behavior to the point where it is not reflective of nature?”

The answer to the question posed above depends on the aims of the research. For example, extremely simple assays can be used to measure accurately behavioral responses to various chemicals. The basic assay system that has been used most frequently is a petri dish containing a flat base of agar, on which the nematodes can move towards or away from a test compound placed at a set point on the agar and left to set up a concentration gradient. The modifications of this agar-plate attraction bioassay are far too numerous to detail here. Agar plates have been instrumental in research to analyze components of nematode movement (Croll, 1970) and have been central to the extensive and detailed analysis of behavioral responses of *Caenorhabditis elegans*. The problems with the assay are more apparent when responses of parasitic nematodes to putative host attractants/repellants are being examined. As will be discussed in the sections below, disadvantages include: the test compound has to diffuse through the agar to set up a gradient and the time taken for this is unknown and may vary among different compounds, and the concentration of the test compound to which the nematode responds is unknown but will be less than the concentration spotted on to the agar. In addition, the nematodes are moving on their sides in a two-dimensional system, which is quite unlike their natural environment. However, as will be discussed, the main problem is the sometimes unjustified extrapolation from these in vitro tests to the situation in the soil environment.

Carbon dioxide attracts many species of entomopathogenic and plant-parasitic nematodes (reviewed by Lewis et al., 2006; Robinson and Perry, 2006). The bioassays used in the experiments that determined this for entomopathogenic nematodes were first conducted on plain agar over which hosts were suspended. Considerable refinement of this assay has greatly increased the details of what we know about entomopathogenic nematode host finding but the assay developed by

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Gaugler et al. (1980) remains a standard benchmark bioassay today in the study of entomopathogenic nematode behavior. So in the context of determining the attraction of entomopathogenic nematodes to carbon dioxide, this simple assay was extremely useful and by-and-large accurate. On the other hand, simple assays can be misleading. Another example from the entomopathogenic nematode literature is illustrative. Petri dish assays of entomopathogenic nematode host range are extremely common (reviewed by Lewis et al., 2006). Briefly, putative insect hosts are housed in petri dishes lined with moist filter paper, and tens to hundreds of entomopathogenic infective juveniles (IJ) are pipetted into the dish. After a few days, the hosts are examined to determine whether they were infected, and, if so, they are included as 'hosts' for that species of entomopathogenic nematode. Here, simplicity resulted in some inaccurate hypotheses of what species of insect entomopathogenic nematodes could control. For example, *Steinernema carpocapsae* Weiser can kill scarab larvae in petri dishes, given a high enough dose. However, when applied to field infestations of scarabs, *S. carpocapsae* fails to reduce their populations. What was not tested in petri dishes was the ability of the nematode to find the host. Since IJ of *S. carpocapsae* forage on the soil surface and scarab grubs are subterranean, this pairing had little chance of success.

The range of behavioral assays discussed in this review reflects the expertise of the authors; we concentrate on assays designed to help understand the behaviors associated with host finding and invasion site selection by plant-parasitic and entomopathogenic nematodes. We have structured the review into four categories; the first addresses the context of interactions between the nematode and the host and the remaining three concern responses to long-range cues, responses to short-distance cues and responses to contact, or 'local' cues. Further, we classify assays according to the 'goal' of the parasite (i.e., host habitat finding) and according to the physical make-up of the assay arena.

#### *Physical and biological context of nematode-host interactions*

Translating the results of laboratory assays into reasonable expectations for the field is challenging under any condition, but perhaps impossible without an awareness of both the physical and biological context within which nematode-host interactions occur. Plant-parasitic nematodes must locate a host plant and feed before energy reserves are exhausted. Several gradients exist around physiologically active roots. Although agar plate experiments may indicate response to a gradient of a test compound, it cannot be assumed that gradients exist similarly in the soil. For example, much of the early work on the responses to salts was undertaken using unbalanced salt solutions on agar plates, a situation far removed from soil conditions. In addition, gra-

dients of test compounds in a test arena may not have the temporal or spatial attributes in the soil that are needed to provide a consistent attractant for nematodes (Perry, 2005). Thus, the root influence on the surrounding rhizosphere is central to the nematode-plant interaction, but more research needs to be done in association with plant physiologists and soil biochemists to enhance our understanding of the likely situation *in vivo*. Interactions with entomopathogenic nematodes (EPN) and their hosts pose similar problems for *in vitro* assessment, especially since the foraging strategies of EPN span a spectrum from sessile ambushers to ranging cruisers. Behavioral assays conducted in the laboratory show that foraging strategy is an important aspect of the biological context of the nematode-host interaction, having a profound effect on this association. For example, on agar, *S. carpocapsae* shows minimal response when naïve juveniles are exposed to CO<sub>2</sub> from host insects while other species, including *Heterorhabditis bacteriophora* Poinar and the congeneric *S. glaseri* Steiner, are consistently attracted by CO<sub>2</sub>. These results might lead one to expect that the latter two species would be more promising control agents in the field. However, due to its foraging strategy, *S. carpocapsae* only responds to host volatile cues either after contact with host cuticle (Lewis et al., 1995) or during bouts of standing on their tails (Campbell and Kaya, 2002). The various ecological and behavioral filters that influence host-parasite relationships are excluded from these tests, and therefore extrapolation from them should be done with caution.

Plant roots, and thus the insects and nematodes that feed on them, are not uniformly distributed throughout the soil, but rather occur at specific depths or in association with certain soil features such as pockets of moisture or nutrient-rich areas. Plant morphology also affects distribution of roots and their herbivores. For example, grass roots tend to grow shallowly, while other plants like yellow star thistle send their roots deep into the soil profile. Insects are influenced by soil features, by the roots of plants and by other environmental aspects. For example, fungus gnats (Diptera: Sciaridae) favor moist, poorly drained soils, whereas ghost moth caterpillars eschew the soil entirely in favor of galleries within bush lupine stems. In addition to depth and moisture preferences, host occurrence will be influenced by soil texture, fertility and temperature regimes. Laboratory assays often ignore the context of where in the environment the host species of the nematode occurs.

Lack of consideration for natural host location and behaviors has resulted in petri dish assays that suggest unrealistic root attractants for plant-parasitic nematodes and, especially, do not differentiate between long-distance, short-distance and local attractants in the test arena. For experiments on EPN, the petri dish assays have indicated overly broad host ranges for many spe-

cies. The hosts that are most likely to be attacked by EPN in the field are those that occur in the right place in the environment. For example, ambushers like *S. carpocapsae* locate and kill a wide variety of insect species in laboratory assays. However, in the field they are most effective against hosts that frequent the soil surface, which is the nematodes' preferred foraging habitat (Campbell and Lewis, 2002; Lewis et al., 2006). Laboratory assays can also go awry due to the opposing problem of underestimating the host range of a nematode. Nematode foraging strategy should be kept in mind when choosing the assay medium, for example. Agar is a suitable substrate for the evaluation of host finding or attachment of cruisers, but ambushers like *S. carpocapsae* and *S. scapterisci* Nguyen and Smart are able to engage successfully in their full range of infection behaviors only when provided a more complex substrate such as sand, filter paper or leaf litter.

Similarly, the host ranges of plant-parasitic nematode (PPN) species may be misinterpreted in laboratory tests. Many species from genera such as *Pratylenchus*, *Meloidogyne* and *Heterodera*, for example, are thought to have wide host ranges, but attraction to a plant species may not necessarily mean that a plant is a good host. Robinson (2002) considered that most PPN respond to general cues and that this may lead to non-specific attraction; root penetration may occur irrespective of whether the plant species is an appropriate host. Thus, it is important to differentiate between attraction assays and invasion assays. Assays to determine the host status of a plant species frequently involve inoculating large numbers of the invasive stages of the PPN next to roots of the test plant(s), covering the roots with soil or sand and later determining the invasion and development. In such assays, there is no component of root attraction involved, and, thus, discussion of such tests can only be couched in terms of root penetration and development.

On the other hand, there are some examples where nematodes respond to specific host attractants that accurately predict their host ranges. *Ditylenchus phyllobius* (Thorne) Filipjev, a foliar parasite of certain *Solanum* spp., is attracted to an unknown compound, apparently unique to the host, which accumulates in leaves and is leached by rain to accumulate at the base of stems and establish a gradient in the surrounding soil; the infective fourth-stage juvenile moves up this attractant gradient to locate host stems (Robinson et al., 1979). This is an excellent example of a host-specific short-distance attractant. Similarly, the number of *M. naasi* Franklin J2 attracted to a resistant species of grass was fewer than were attracted to susceptible plants, and Balhadère and Evans (1994) considered that this may be associated with a less acidic environment produced by roots of the resistant cultivar. Thus, with this species of nematode, pH may be one factor acting as a local attractant. Luc et al. (1969) were among the first to demonstrate root

diffusate-mediated attraction when they used radioactive phosphorus to track nematodes in soil and demonstrated that diffusates were attractants and stimulated nematode activity. There is evidence that potato root diffusate (PRD) may aid J2 of *G. rostochiensis* (Wollenweber) Behrens to locate host roots. Electrophysiological analysis demonstrated sensory responses of *G. rostochiensis* J2 to PRD, but not to root diffusate from the non-host sugar beet, thus indicating host-specific responses (Rolfe et al., 2000). In-depth analysis of host-parasite interactions may provide evidence of more species of plant-parasitic nematodes showing responses to host-specific attractants; however, as indicated by Robinson (2002), it may be the case that species with wide host ranges respond to general plant cues, which lead to movement to host and non-hosts as well. While entomopathogenic *Steinernematidae* spp. and *Heterorhabditiidae* spp. responding to general cues may also move toward non-hosts, the infection decision is conditional on specific host selection; infecting a non-host may reduce fitness to zero since EPN IJ cannot reverse the infection decision. Not surprisingly, EPN are fairly discriminating in their choice to infect insects, demonstrated in host recognition studies with *S. carpocapsae* (Lewis et al., 1996b). Host evaluation (both identity and condition) is a continuous and underlying part of the infection process for EPN.

In the previous sections and in those that follow, it is not our intention to disparage any particular assay or technique that has been used to study nematode host-finding behavior. Each has its strengths and weaknesses, and there is an unavoidable tension between assay complexity and data clarity. This tension pervades science, but is particularly challenging in soil ecology where increasing complexity often means limiting our ability to make direct observations (e.g., agar vs. soil).

#### *Long-range host finding*

Long-distance cues (relevant to the scale of several cm) serve to guide the nematode to areas likely to contain hosts. Putative cues include CO<sub>2</sub> and other compounds associated with hosts, either plant roots or insects, or in the case of EPN, the plant upon which the host is feeding. Several types of assays with numerous variants have been used to study long-distance attraction of parasitic nematodes to hosts and related cues. Of these, agar petri dish assays have been used the most extensively in the study of PPN and EPN behavior. Usually, solid water agar (at 0.5–2% w/v) is used so that nematodes may move along the surface, but the medium can be made more dilute so that nematodes can move within the agar matrix. To assess long-range host finding, volatile cues are added to the assay arena using a variety of techniques, including: cue-impregnated filter paper (Stamps and Linit, 2001) or ethylene sheets (Zhao et al., 2007); wells of cue solution (Shapiro et al., 2000); and suspended hosts (Lewis and Gaugler, 1994).

A benefit of agar over opaque media is that its transparency permits measurements of velocity, meandering and movement toward or away from cue sources for individual or groups of nematodes over time, potentially revealing nuances in the nematode responses to cues. The agar plate assay is generally limited to evaluating horizontal migration (but see Ketschek et al., 2004 for vertical migration assay).

The extreme ecological simplicity of the assay environment is an obvious constraint on how readily results can be extrapolated to field conditions. Perhaps less obvious is that the dissemination of both the cue (Perry and Aumann, 1998) and the nematodes (Robinson, 2002) are likely to be markedly different in a two-dimensional arena compared with a three-dimensional environment. Indeed, the *in vivo* cue concentration and chemical structure perceived by the nematode are often unknown (Perry, 2001) and thus cannot reliably be replicated in the simplified assay arena. Agar assays primarily assess attraction; however, attraction does not indicate penetration, nor does penetration guarantee successful establishment. Agar assays are extremely useful for developing intuition about the system and testable hypotheses, but benefit from complementation with assays with a higher degree of realism.

Developments of the basic behavioural assay include pluronic gel assays and micro-molded substrates. Both assays introduce a three-dimensional aspect into the behavioral assessment. The pluronic gel assay has proved particularly useful in investigating the short-distance attraction of plant-parasitic nematodes to host roots and their accumulation around certain sites (Wang et al., 2007). The question whether the aggregation is the result of local plant semiochemicals or an attraction pheromone released by the nematodes themselves remains to be answered. The use of micro-molded substrates has facilitated the study of the effect of pore structure on nematode behavior and migration (Eo et al., 2007). Eo et al. (2008) used micro-molded substrates to test nematode responses to gravity and found that several nematode species were not geotactic, and, thus, vertical movement in soil may be in response to factors other than gravity. These assays are more sophisticated attempts to examine nematode responses in arenas that more closely resemble the three-dimensional natural situation.

Other types of three-dimensional assays are commonly used to measure the abilities of nematodes to locate hosts that are found at a distance, either horizontally, vertically or both. In column assays, used more often with EPN than PPN to study host location (but see Prot and Vangundy, 1981; Mojtahedi et al., 1991), cylindrical tubes are generally filled either with sterilized sand or soil mixtures (a petri dish with sand may be used as a very short column). A cue is placed at one end, and nematodes are added to the opposite end. Cues may include intact hosts, host extracts (e.g., dif-

fusates), or other compounds associated with the intended host. Nematodes are allowed to migrate through the column for hours (Lewis et al., 1996a), days (Koppenhofer and Fuzy, 2003) or months (Mojtahedi et al., 1991). Useful modifications to the basic assay include using a stacked ring assembly to allow recovery of nematodes from specific column sections (Chen et al., 2003; Koppenhofer and Fuzy, 2003) and incorporation of intact growing plants (Prot and Vangundy, 1981). Relative degrees of success in host-finding have been estimated by quantifying host mortality, the number of nematodes that penetrated the host or the number within the soil of each column section. A strong point of this assay is that the behavior of the cue as it diffuses through the three-dimensional assay arena is likely to more closely reflect field conditions than two-dimensional assays. However, the inherent directionality imposed by the shape of the column influences how cue gradients are established and restricts nematode dispersal patterns. In the case where nematode penetration is used to estimate host finding ability, it is important to be aware that host acceptance and the ability to penetrate are confounding factors. In addition, we give up the possibility of tracking nematodes in real time. If nematodes infect the host, we know that they were able to move through the soil and infect the host; if the host is unmolested, either the nematodes could not reach it, the host was not susceptible, or another reason underlies the nematodes' failure to infect. Arena designs in which nematodes are able to approach but not penetrate the host are more specific for assessing host finding ability.

Olfactometers, used for choice tests between two or more cues/controls presented simultaneously, are also widely used in long-range host-finding studies (van Tol et al., 2001; Struck et al., 2004). Sand is often used as the assay medium, providing for more realistic nematode and cue migration than agar or similar substrates. One issue with this assay is that once a path is chosen, exposure to other cues may diminish or cease, removing one of the assay's strengths. For the field, results of olfactometer assays can address the initial migration decision when simultaneously presented with several cues, but less so the on-going evaluation and choices among cues confronting nematodes under natural conditions.

#### *Short-range host finding*

A neutral response in behavioral assays designed to detect attraction or repulsion responses could be interpreted as indifference or as balanced positive and negative responses among individuals. Alternatively, the nematode may have failed to perceive the cue. A completely different type of assay to those discussed in the previous sections can examine these and other hypotheses by direct measurement of nematode neuronal responses using electrophysiology (Perry, 2001). There is

no attempt to mimic the natural environment; rather, the assay aims to examine detailed aspects of the responses of individual live nematodes to non-volatile compounds and enables investigation of concentration-specific responses and responses to sequential exposure to various different test solutions. To record electrophysiological responses, an electrode is inserted into the anterior end of a nematode bathed in an aqueous solution. Cues can be added to the solution as desired. With larger nematodes, such as the animal-parasitic nematode *Syngamus trachea*, the electrode can be inserted into individual sensory structures, such as the amphid, to allow organ-specific recordings. For smaller plant-parasitic nematodes, size limitations mean that recordings are taken from the cephalic region rather than a specific organ (Perry, 1996). The technique has been used in assays with nematode parasites of vertebrates and plants and the insect-parasitic nematode *Leidynema appendiculata* (Leidy) Chitwood, but not with entomopathogenic nematodes. The assay is not limited to the type of compound investigated; it could be a putative long-distance, short-distance or local attractant. Electrophysiological analysis provides information about the delay in response when a stimulus is provided and whether the response continues during exposure or whether the nematode becomes acclimated. As the structure and function of the nematode sensory organs may change during its development and maturation, the electrophysiology assay can examine changes in responses, and this has been done, for example, with adults and second-stage juveniles of *G. rostochiensis* (Perry, 1996). Back-up agar-plate behavioral assays are needed to determine whether the response is to an attractant or repellent, and, because the nematodes are restrained, it is not possible to link sensory responses to behavior.

The electrophysiology assay has been used to examine the feasibility of blocking sensory perception, a potentially useful novel control avenue. It is important to understand the periods that nematodes remain desensitized to specific stimuli before turnover of amphidial secretions 'unblocks' the amphids. Amphids also respond to stimulation by increasing the output of secretions. The secretion of *M. incognita* (Kofoid and White) Chitwood amphids in response to host root tip exudates were evaluated by bathing nematodes in an aqueous solution containing the root exudates and brilliant blue stain on a microscope slide (Zhao et al., 2000). The technique was also used with the pinewood nematode *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle (Zhao et al., 2007).

#### *Local/Contact cues*

The most easily scored assay of the responses of nematodes to local cues or to direct contact with hosts is penetration into the host, because penetration can be measured in any assay in which the nematode is allowed

to access the host. However, host penetration is really the last step in a complicated suite of behaviors, some of which have been described above, and can yield anomalous results. In other words, if a nematode does penetrate a host when placed in proximity, there is no way to determine whether or not it would have found the host in the field. It has also been shown (Forrest et al., 1986) that infective *G. rostochiensis* J2 will enter an unsuitable plant but subsequently exit. Thus, penetration per se is not necessarily an indicator of a good host. On the other hand, failure to penetrate a host does not necessarily mean that the host is unacceptable, but could mean that there is a stimulus required prior to penetration that was missing in a particular assay.

Again, the type of information sought is the key to interpretation. The concern of host finding is reduced where assessing host acceptability is the primary goal, and often the arena size can be restricted, by using 24- or 96-well plates, for example (Kaplan and Davis, 1991; Chen et al., 2003). In general, assays of penetration are useful in determining the acceptability of the host to a particular nematode and in determining the quality of the nematode as measured by its 'ability' to infect. These assays are of very limited value in predicting behaviors in the field.

#### *Conclusions*

Laboratory assays may be useful for generating predictions about the long-term fate of applied or natural populations of EPN. However, extrapolation of laboratory data to the field situation should be done with caution and is often unjustified. While we believe no single assay can predict the efficacy of EPN against a focal pest in the field, using laboratory assays complementarily may be a powerful approach. Assays to determine the attraction of plant-parasitic nematodes may have only limited relevance to the field situation. More realistic assessments of test arenas need to take account of whether gradients are likely to have the temporal and spatial attributes needed for an attractant to function in the natural environment. For both entomopathogenic and plant-parasitic nematodes, negative results may be as informative as positive results, perhaps more so if they help researchers reduce the pool of the hypotheses to be tested in the field.

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