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Culturing and Screening the Plant Parasitic Nematode Ditylenchus dipsaci

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TITLE:

Culturing and Screening the Plant Parasitic Nematode *Ditylenchus dipsaci*

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SUMMARY:

The present protocol describes a reliable and straightforward method for culturing, collecting, and screening *Ditylenchus dipsaci*.

ABSTRACT:

Plant-parasitic nematodes (PPNs) destroy over 12% of global food crops every year, which translates to losses of roughly 157 billion dollars (USD) annually. With a growing global population and limited arable land, controlling PPN infestation is critical for food production. Compounding the challenge of maximizing crop yields are the mounting restrictions on effective pesticides because of a lack of nematode selectivity. Hence, developing new and safe chemical nematicides is vital to food security. In this protocol, the culture and collection of the PPN species *Ditylenchus dipsaci* are demonstrated. *D. dipsaci* is both economically damaging and relatively resistant to most modern nematicides. The current work also explains how to use these nematodes in screens for novel small molecule nematicides and reports on data collection and analysis methodologies. The demonstrated pipeline affords a throughput of thousands of compounds per week and can be easily adapted for use with other PPN species such as *Pratylenchus penetrans*. The techniques described herein can be used to discover new nematicides, which may, in turn, be further developed into highly selective commercial products that safely combat PPNs to help feed an increasingly hungry world.

INTRODUCTION:

Plant-parasitic nematodes (PPNs) are estimated to be responsible for the loss of 12.3% of global food production and cause an estimated 157 billion dollars in damage annually[1,2,3]. Unfortunately, the ability to control PPNs is waning because effective chemical nematicides have been banned or are facing escalating restrictions because of human safety and environmental

concerns. This is primarily due to the poor nematode selectivity of previous generations of pesticides[4]. Over the last 25 years, six new chemical nematicides have been piloted or introduced into the market[5]. One of these has already been banned in Europe, and another has been discontinued while being investigated for its impact on human heatlh[6,7]. Hence, there is a pressing need for new nematicides that are highly selective for PPNs.

The stem and bulb nematode, *Ditylenchus dipsaci (D. dipsaci)* is an economically impactful PPN[5]. *D. dipsaci* infects nearly 500 plant species across 30 biological races and targets some of the most agriculturally important crops such rye, oats, garlic, onion, and leek[8,9]. For example, *D. dipsaci* has recently scourged garlic fields in Ontario and Quebec, resulting in losses of up to 90%[10,11]. Its geographical distribution is nearly ubiquitous and includes the Americas (including California and Florida), Europe, much of Asia (including China), and Oceania[9]. *D. dipsaci* is a migratory endoparasite that enters the stomata on leaves or wounds and lenticels where they release enzymes to break down the cell wall[12]. Compounding the impact of *D. dipsaci* on crops, the damage caused by the PPN makes the plant susceptible to secondary infection[11]. Unfortunately, *D. dipsaci* shows high tolerance levels to current nematicides compared to other nematode strains[13,14].

This protocol describes the culturing of *D. dipsaci,* and its use in large-scale screens for small molecule candidate nematicides. Briefly, *D. dipsaci* populations are maintained and expanded on pea plants cultured in sterile Gamborg B-5 (GA) media[15]. Before growing seed sprouts on GA medium, the seeds must be sterilized through a series of washes and plated on nutrient agar (NA) to check for contamination. Seed sterilization is essential to detect bacterial and fungal contaminants that may be present. The non-contaminated seeds are then transferred to GA plates, where seed sprouts will grow in preparation for infection. The GA plates containing seed sprouts are infected with nematodes from a previous culture plate by transferring a piece of agar containing root tissue to the fresh plates. After 6-8 weeks, the nematodes are extracted from the GA media and are filtered through a coffee filter-lined funnel into a collection beaker. The nematodes can be used in various bioassays once a suitable number has been collected. The technique described in this protocol generates approximately 15,000 *D. dipsaci* per culture plate. Alternative protocols to cultivate *D. dipsaci* have been published[16,17].

An *in vitro* small molecule screening assay using 96-well plates is also described here[18]. As a proxy of worm health, the mobility of 20 nematodes per well is examined after 5 days of small molecule exposure. To better visualize worm mobility, NaOH is added to increase the movement of live worms[19]. This protocol allows medium-throughput screening and provides valuable data to assess the nematicidal potential of small molecules. If a different nematode collection technique is used[16,17], the small molecule screening methodology described herein can nevertheless be implemented.

PROTOCOL:

The *D. dipsaci* strain G-137 used for the present work was collected from Fish Lake 4 Variety garlic in Prince Edward County and was provided by Agriculture and Agri-food Canada. If starting a fresh culture, consult Poirier et al. for inoculation methodology[15].

1. Culturing of *D. dipsaci*

1.1. Prepare the media and plates following previously reported work[15].

1.1.1. Prepare 500 mL of Nutrient agar (NA) media with 23 g/L of NA (see **Table of Materials**) and ultrapure water. Using sterile technique, pour 25 mL of autoclaved NA media into 20 disposable Petri dishes (100 mm diameter x 15 mm deep). Allow agar to solidify at room temperature (22 °C) with the lid on for ~2 h and set aside for later use.

1.1.2. Prepare 500 mL of Gamborg B-5 (GA) media containing 3.2 g/L of GA basal medium with minimal organics, 20 g/L of sucrose, 15 g/L of agar, and distilled water (see **Table of Materials**). Using sterile technique, pour 50 mL of autoclaved GA media in 10 disposable Petri dishes (100 mm diameter x 25 mm deep). Allow agar to solidify at room temperature (22 °C) with the lid on for ~5 h and store upright at room temperature in a sterile bag until the sprout transfer.

NOTE: Excess media plates are made in case contamination occurs.

1.2. Perform seed sterilization following a method modified from a previous work[15].

1.2.1. Autoclave one 2 L beaker with a stir bar, 2 forceps, a glass petri dish, and 1 L of distilled water. Prepare 200 mL of 95% EtOH solution and 200 mL of a 15% commercial bleach solution.

1.2.2. Use pea seeds (see **Table of Materials**). Pour 150 seeds into a sterile 2 L beaker with a stir bar near a Bunsen burner flame on a lab bench.

1.2.3. Add 200 mL of 95% EtOH to the seeds within the beaker, stir vigorously on the stir plate for 5 min, and then pour off EtOH in a waste container.

1.2.4. Pour bleach solution into the beaker to completely immerse the seeds. Stir vigorously on a stir plate for 20 min then pour off bleach in a waste container.

1.2.5. Pour distilled water into the beaker to immerse seeds and stir vigorously on a stir plate for 20 min. Repeat water washes three times, pouring off distilled water after each wash. After the final water wash, pour sterilized seeds into the glass Petri dish.

1.2.6. To check for contamination, transfer 6 seeds to each 10 cm NA plate (prepared in step 1.1.1) in the laminar flood hood using sterilized forceps. Arrange the seeds around the plate's circumference (plumper seeds work best). Wrap the plates individually in laboratory wrapping film and incubate in the dark for 3 days at 26 °C.

NOTE: Plating more seeds than needed will allow for selective use of non-contaminated seeds.

1.3. Perform sprout transfer following the step below.

1.3.1. In a laminar flow hood, use sterilized forceps to plate 2 non-contaminated seeds on each GA plate (prepared in step 1.1.2). Wrap the plates individually in laboratory wrapping film and incubate at room temperature for 7-10 days to allow seeds to sprout.

1.4. Perform *in vitro* rearing following a method modified from a previous work[15].

1.4.1. Prepare 50 mL of 20 g/L sucrose solution. Filter sterilize sucrose solution and set aside.

1.4.2. In a laminar flow hood, cut a piece of agar containing root tissue (~2 cm^3) from an existing culture plate. Pipette 500 µL of sucrose solution on new GA plate with pea seedlings and place agar cube on top of sucrose. Wrap the plates individually in laboratory wrapping film and maintain the culture in a box lined with aluminum foil at room temperature (~22 °C).

1.4.3. Subculture nematodes on fresh GA plates every 8-9 weeks to maintain the culture. Nematodes are ready to be extracted after ~8 weeks.

2. Extraction and collection of *D. dipsaci*

2.1. Perform the extraction of *D. dipsaci* following the steps below.

2.1.1. Autoclave a 50 mL beaker, an 80 mm funnel, 150 mL distilled water, and coffee filters. Place the funnel into the beaker and line the funnel with sterile coffee filter.

2.1.2. In a laminar flow hood, cut the agar and the root tissue into 1 cm^3 using a sterile scalpel. Transfer the agar cubes into the coffee filter-lined funnel and slowly pour distilled water on the agar to moisten the coffee filter.

2.1.3. Remove the coffee filter-lined funnel from the beaker and fill the beaker with distilled water until the water level is just touching the bottom of the filter once the coffee filter-lined funnel is replaced.

2.1.4. Cover the coffee filter-lined funnel and beaker with aluminum foil. Leave overnight (16 h) on the benchtop to allow worms to move through the coffee filter into the collection beaker.

NOTE: A nematode-culture plate is ready for extraction when worms have crawled into the agar when examined with a dissection microscope. Typically, a plate is ready for extraction 6-8 weeks after its initial inoculation.

2.2. Perform the collection of *D. dipsaci*.

NOTE: The next day, the *D. dipsaci* worms will have settled to the bottom of the beaker.

2.2.1. Remove the coffee filter-lined funnel and aspirate the top 40 mL of water from the

collection beaker; ensure not to disrupt the settled worms. Using a 10 mL plastic serological pipette, collect the remaining liquid into a 15 mL conical centrifuge tube.

NOTE: This collection can be used directly in assays. Place at 4 °C if they will not be used within 24 hours. The worms can be left for 3 days at 4 °C with no visible impact on mobility. Removing the funnel may disturb the worms in the collection beaker. Let them settle to the bottom before collecting.

3. *In vitro* small molecule screen

3.1. Prepare the assay plates following the steps below.

3.1.1. Pour autoclaved distilled water into a sterile trough and, using a multichannel pipette, dispense 40 µL of distilled water from the trough into each well of a flat-bottom 96-well plate.

3.2. Prepare pinning tool and add the chemicals

3.2.1. Set up pinner trays (see **Table of Materials**) near a Bunsen burner flame on a lab bench. Add the following to successive pinner trays: 25 mL of pin cleaning solution, 35 mL of 50% DMSO (in water), 45 mL of distilled water, 55 mL of 70% EtOH, and 65 mL of 95% EtOH. Place one piece of blotting paper in front of each tray

3.2.2. Clean the pinning tool by emersing the pins in the cleaning solution and moving the pins up and down three times (3x) in the solution. In this protocol, '3x' and '10x' are defined as moving the pinner up and down in a solution three or ten times, respectively. Blot the pins on the blotting paper. Repeat this procedure once more.

3.2.2.1. Next, rinse the pins 3x in distilled water, followed by blotting the pins. Repeat the procedure once more.

3.2.2.2. Lastly, rince the pins 3x in 95% EtOH, followed by blotting the pins. Repeat once more. Flame the pinner and allow the ethanol to evaporate.

3.2.3. Add chemicals from the 96-well chemical stock plates to assay plates by pinning 3x into the chemical plate, then transferring the pins 10X into the assay plate. Blot onto paper in front of the cleaning solution.

3.2.4. Clean pinning tool between plates by washing in the following order, blotting in between on blotting paper: 3x in 50% DMSO (once), 3x in distilled water (once), 3x in 75% EtOH (once), 3x in 95% EtOH (twice). Flame the pinner and allow ethanol to evaporate.

3.2.5. Repeat step 3.2.2 when all pinning is completed.

NOTE: The screening was performed at a final concentration of 60 µM.

3.3. Addition of worms

3.3.1. Count the number of nematodes from the collection by first resuspending and then pipetting 5 µL using low retention tips onto a slide for observation. Count the number of nematodes in 5 µL using a dissection microscope.

3.3.1.1. Adjust the concentration to 2 worms/µL using sterile distilled water. Using a multichannel pipette and a trough, add 10 µL (~20 worms) to each well of the 96-well plates.

NOTE: Approximately 15,000 *D. dipsaci* nematodes will be collected per culture plate using the described culture and collection method. Twenty worms are used per well because the small number facilitates the clear visualization and accounting of mobile and immobile ones.

3.3.2. Seal the plates in laboratory wrapping film and wrap with a damp paper towel. Place in box and affix on a sticky pad in 20 °C shaking incubator set at 200 rpm. Ensure that plates are stabilized in the box by adding an extra damp paper towel to ensure minimal movement of plates.

4. Data collection and analysis

4.1. Observe plates on day 5 under a dissecting microscope. Count the number of mobile and total number of *D. dipsaci* in DMSO solvent controls and drug-treated wells.

4.1.1. If the worms are relatively immobile, add 2 µL of 1 M NaOH to a final concentration of 40 mM to the well to stimulate movement[19, 20].

NOTE: After adding NaOH, worms will move instantly and need to be viewed within 5 min. The number of mobile worms and the total number of worms will be used to calculate the proportion of mobile worms. The length of the assay may change depending on the aim of the screen.

4.2. Calculate the proportion of mobile worms. In the *D. dipsaci* screens, wells that reproducibly yielded 0% mobile worms are categorized as strong hits.

NOTE: Prolonged exposure of plates on the stage of dissection microscopes is avoided because the light source can heat the plates and induce variable effects on the bioassay.

REPRESENTATIVE RESULTS:

Independent replicates reveal reproducible hits

To illustrate the expected variation between the replicate screens, the means and variation in sample mobility are plotted from three representative plates from a recent screen (**Figure 1**). Three replicates of the screen were performed on three different days. All three plates have negative (solvent-only) controls (darker bars), and samples within the set contained established nematicides. The remaining compounds are from a custom library currently being characterized

in the Roy lab. Compared to similar screens done with *C. elegans* with the same molecules (SC, JK, PJR, unpublished results), the hit rate with *D. dipsaci* is significantly lower, and many drug plates exhibit no activity (**Figure 1A**). Some plates have fully reproducible hits (**Figure 1B,C**), while others vary in activity (**Figure 1C**). Relative to other species screened (not shown), *D. dipsaci* shows less variability in its response to compounds. Regardless, replicates are considered necessary in the identification of reproducible hits.

Nematicides vary in their ability to immobilize *Ditylenchus dipsaci*

Of the seven characterized nematicides tested against *D. dipsaci,* only Fluopyram exhibits robust activity in the assay described herein (**Figure 1C**). This is consistent with previous work showing that *D. dipsaci* is tolerant of nematicides[13,14]. Fluopyram inhibits complex II of the electron transport chain in a nematode-selective manner[20] and is a commercial nematicide used to control a variety of PPNs, including *Rotylenchulus reniformis* and *Meloidogyne incognita*[4,21,22]. Fluopyram and one of the nematicides that lacked activity at the concentration tested (Oxamyl)[23] were investigated in more detail through a dose-response analysis with *D. dipsaci*. Fluopyram induces an apparent dose-dependent effect on *D. dipsaci* mobility with an EC50 of 9.3 μM (with a 95% confidence interval between 8.2 to 10.5 μM) (**Figure 2A,B**). This result is expected based on published *in vitro* results from Storelli et al., 2020[13]. Oxamyl has no significant effect on mobility up to a concentration of 120 μM (p = 0.3632, unpaired t-test) (**Figure 2C,D**).

Sodium hydroxide improves assay sensitivity

In contrast to the near-continuous swimming activity of *C. elegans* in liquid culture[18], *D. dipsaci* animals are dramatically less mobile. This is not uncommon among parasitic nematodes in culture[16]. To help distinguish 'resting' worms from sick worms, 40 mM of NaOH is used at the assay endpoint to stimulate movement in those individuals who are capable (**Figure 3**)[19]. This technique allows for the clear identification of small molecules that immobilize worms, given that all worms in negative control wells move and yield an exceptionally low background of false positives in the screen.

FIGURE LEGENDS:

Figure 1: Examples of screen output. Three biological replicates (open circles) with *D. dipsaci* were performed against the small molecules (60 μM) in each of the three plates shown. All three plates have 0.6% of DMSO solvent-only controls (darker bars). Except where otherwise noted with solvent controls or known nematicides, the wells of the three plates contain relatively uncharacterized drug-like compounds purchased from vendors (see Burns et al., 2015)[18]. (**A**) A 96 well plate from the small molecule library lacks any molecule with observable bioactivity against *D. dipsaci*. (**B**) A 96 well plate from the small molecule library has a single molecule that reproducibly disrupts *D. dipsaci* mobility. (**C**) A 96 well drug plate containing the characterized nematicides fluopyram (fluo), iprodione (ipro), abamectin (abam), fluensulfone (flue), tioxazafen (tiox), oxamyl (oxam), and wact-11. The error bars represent the standard error of the mean.

Figure 2: Example of positive and negative results using mobility assay. (**A**) Examples of the terminal phenotypes after the exposure of *D. dipsaci* to increasing concentrations of fluopyram

after 5 days before addition of NaOH. (**B**) A dose-response analysis of the movement of *D. dipsaci* after 5 days of exposure to the indicated concentrations of fluopyram. (**C,D**) The same as A,B, except for oxamyl. Each graph shows trials done on two separate days with three replicates each day. The y-axis of each graph indicates the fraction mobile of the worms in each well calculated as the number of animals moving relative to the total number of animals in the well. The error bars on both graphs represent the standard error of the mean. The scale bar represents 1 mm.

Figure 3: Mobility of *D. dipsaci* after adding 40 mM of NaOH. The effect of NaOH addition to *D. dipsaci* samples in the presence of solvent-only control (**A**), tioxazafen (**B**), or fluopyram (**C**) after 5 days of co-incubation. The scale bar represents 1 mm.

DISCUSSION:

Critical steps

Despite the protocol's simplicity, there are critical steps in the protocol that deserve additional attention to maximize the likelihood of success. First, overbleaching the seeds can disrupt their growth. Therefore, limiting the seeds' time in the bleaching solution to 20 minutes or less is essential. Second, as previously noted by Storelli et al., the apparent health of the nematodes decreases over time when stored at 4 °C[16]. Using the nematodes soon after their collection provides additional confidence that optimal screening conditions can be achieved. Should longer-term storage be necessary, ensure that the tube's lid is not tight to allow oxygen exchange. Third, ensuring that the peas grow on the NA plates for the suggested time enables the experimenter to judge which seeds are contaminated. Fourth, overgrowing the peas on the GA plates before adding the nematodes will weaken infection and reduce the nematode yield. Finally, many factors that are difficult to control can impact screening results. Therefore, it is essential to perform multiple independent replicate screens on different days and ideally with PPNs collected from different culture plates to ensure the reproducibility of results.

Limitations of method

A limitation to the protocol is that it fails to synchronize the developmental stage of the collected worms, which range from juveniles to adults. Hence, strong hits revealed by any screen are likely effective at multiple stages. However, the protocol increases the risk of overlooking effective stage-specific hits. A second consideration is that *in vitro* screening should be considered the first step in a nematicide-discovery pipeline; soil-based assays are an excellent addition to a pipeline to test the translatability of the hits.

Significance and application of the protocol

The protocols described herein are simple and easily replicated. Furthermore, this protocol has been successfully applied to other PPNs in the lab, including *Pratylenchus penetrans*, by making only slight modifications. Developing new and safe PPN control measures is essential to ensure global food security. This is especially true for species like *D. dipsaci* that are generally tolerant to a wide variety of currently acceptable chemical nematicides[13,14]. Hence, the protocols outlined here have the potential to make an important contribution to human health on a global scale.

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DISCLOSURES:

The authors declare no conflicts of interest.

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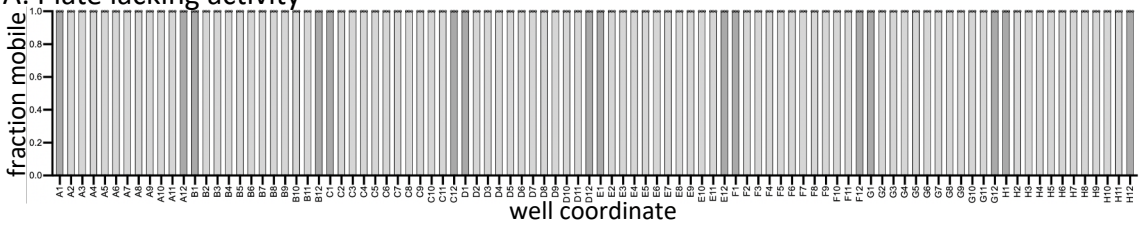
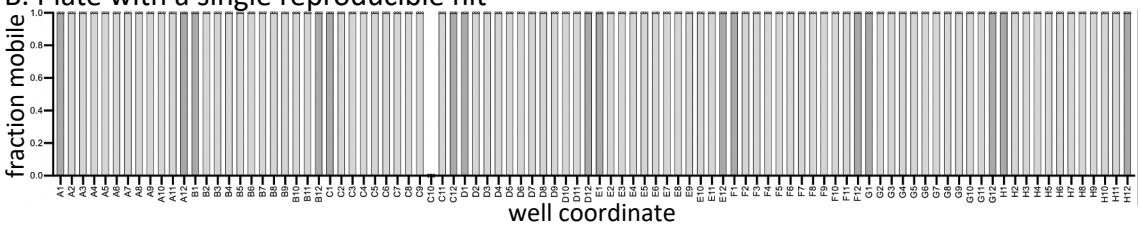
Figure 1

A. Plate lacking activity



B. Plate with a single reproducible hit



C. Plate with multiple reproducible hits and intermediate responses

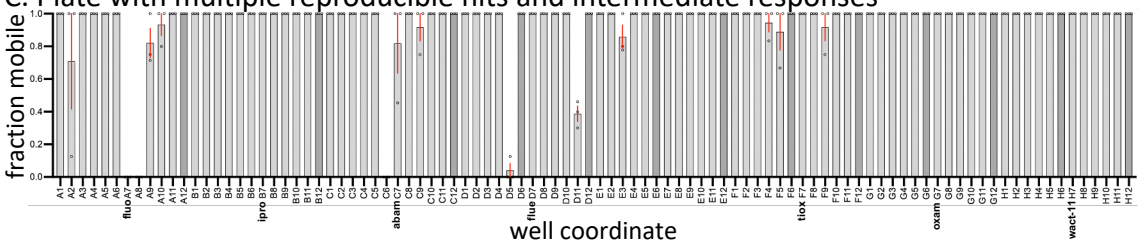


Figure 2

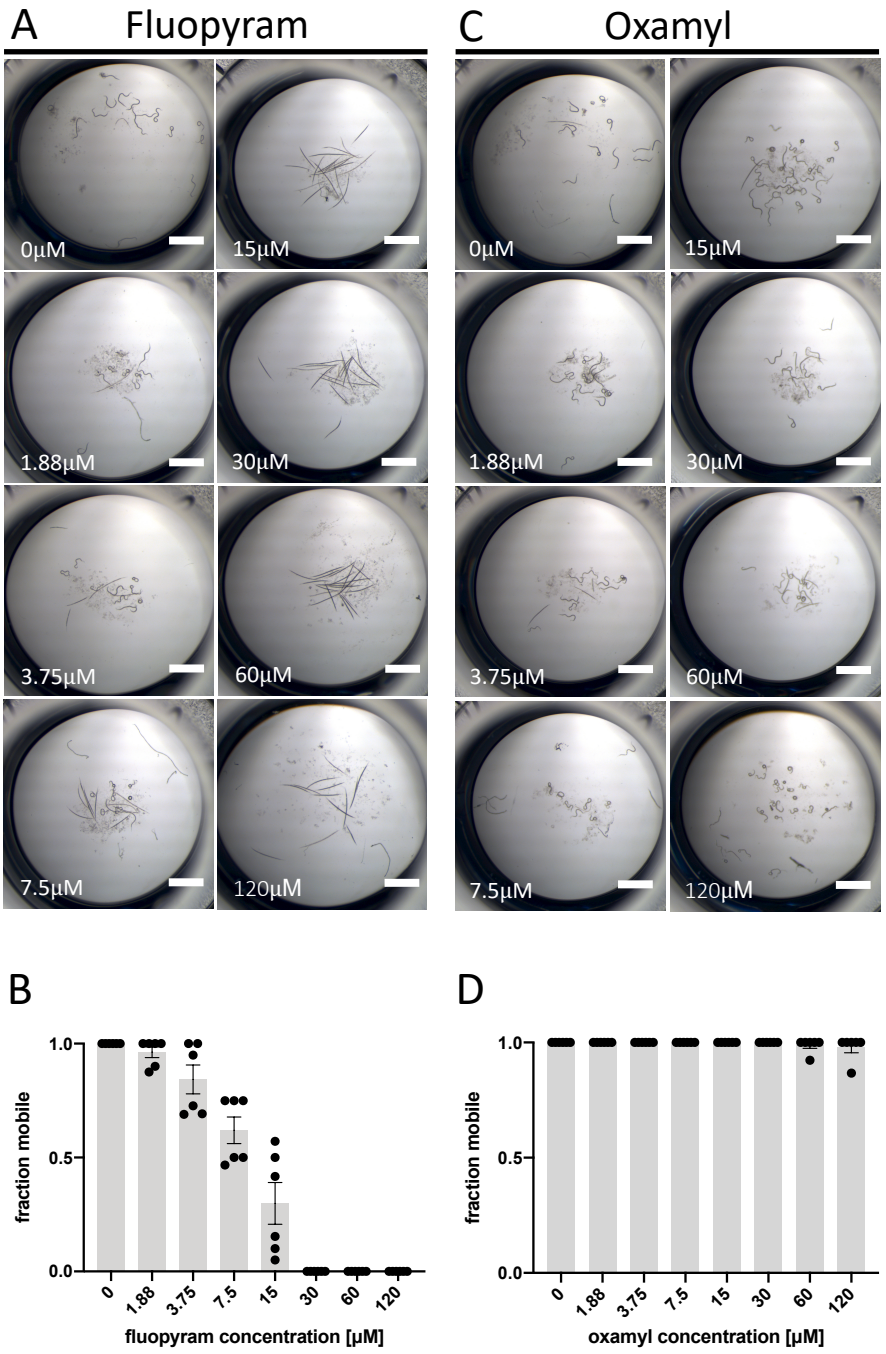
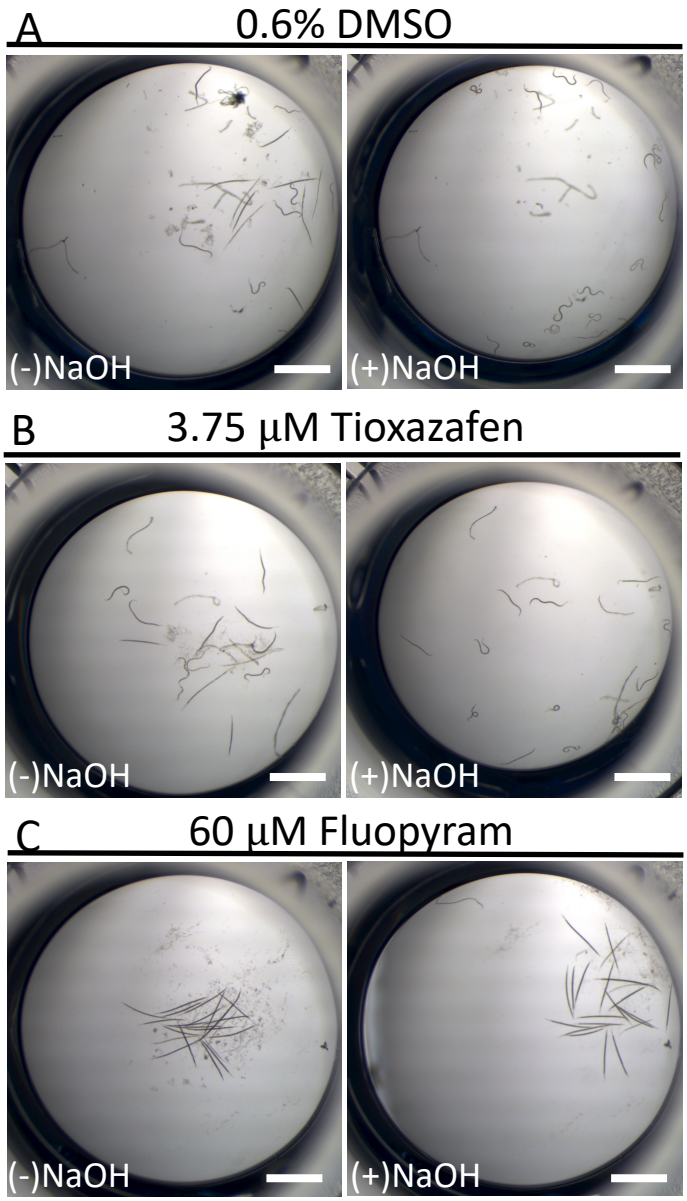


Figure 3



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2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Authors Response: Done.

3. Please remove citations from the Abstract and include them in the Introduction section.
Authors Response: Done.

4. Please ensure that all the abbreviations are defined at the first instance.
Authors Response: Done.

5a. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead.
Authors Response: Done.

5b. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Parafilm, Falcon
Authors Response: Done; we have included a Table of Materials.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Authors Response: We have done our best to ensure ‘the how’ is clear.

7. Please add more details to your protocol steps:
Original Line 77/96: Please include the citation in the Reference list and include the citation number here.
Authors Response: We have included the citation in the reference list.

Original Line 80: Please mention the quantity of pea seeds to be taken.
Authors Response: Done.

8. Please include one line space between the protocol steps and highlight that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Authors Response: Done.

9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manuscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of

the step includes at least one action that is written in imperative tense. However, the NOTEs cannot be filmed, so please do not highlight.
Authors Response: Done.

10. Figure 1: Please include explanation for the individual Figure panels, (A), (B) and (C).
Authors Response: Done.

Reviewer #1:
Manuscript Summary:
The manuscript describes the techniques required for screening in vitro nematicidal activity using Ditylenchus dipsaci. The nematode culture was also included. The nematode culture method is used previously and in vitro assay is optimized for the nematode. Supporting nematode immobility observation using new literature is good.

Major Concerns:
for reliability and applicability of the protocols are;
1a. culture method is rely on sterile culture medium in a petri dish which is very prone to aerial contamination during culture studies.
Authors Response: We have added a line at the end of the ***Protocol*** Section 2 that notes that plating more seeds than needed will allow for selective use of non-contaminated seeds.

1b. Plant roots are easily could be decay on the moist medium.
Authors Response: We thank the reviewer for noting that roots could easily decay on moist medium, but this has not yet been our experience.

1c. Additionally, medium preparation is labor and expensive relative to carrot culture which is another technique used commonly for migratory endoparasitic plant nematodes.
Authors Response: We have included a line in the revised introduction that draws attention to alternative methods and cite the carrot culture technique.

2. 20 nematodes per well could be lower even the nematode species has lower variation in response to chemical application. I suggest using at least 100 nematodes per well. Because there could be false results depending on the nematode fitness.
Authors Response: We address this point in the revised section ***In Vitro Small Molecule Screen*** step 3.1, where we write, '20 worms are used because the small number facilitates the clear visualization and accounting of those that are mobile and immobile.'

Minor Concerns:
1. line 69-70: for use in seed sterilisation- please check this phrase for the meaning of the sentence.
Authors Response: We have corrected the sentence by writing, '…set aside for later use.'

2. indicate the amount of both medium poured in petri dishes.
Authors Response: We have made the requested change.

3a. line 99: incubation in room temperature for nematode culture- better to say 20 C in dark.
Authors Response: 20°C would be fine, except that we don't do it that way. We have modified the sentience to read, '…maintain culture in a box lined with aluminum foil at room temperature (approximately 22 °C).

3b. 8-9 weeks for culture development is appropriate considering the nematode life cycle.
Authors Response: We interpret the reviewer's comment to mean that we should harvest the animals for processing/screening at 8-9 weeks. Anecdotal reports from other members of the community suggest 4-6 weeks, but our in-house protocol is 6-8 weeks, which is what we describe in the manuscript.

4. line 136 and later: 3x did not make sense. Could you add some clear explanation for this.

Authors Response: We are not certain whether the reviewer is asking about what '3X' means (it is shorthand for 'three times', which we have now defined upon its first introduction in the ***In Vitro Small Molecule Screen*** Section 2.2), or why we would repeat the action three times. If the latter, we have also clarified by modifying the sentence in the ***In Vitro Small Molecule Screen*** Section 2.2 as follows: 'Clean the pinner by pinning three times (3X) (moving the pinner up and down 3X) in cleaning solution and blot.'

Reviewer #2:
Manuscript Summary:
This manuscript is welcomed in this field of research. Although publications already address these issues, this manuscript has the advantage of presenting detailed protocols. I am actively working with this nematode and look forward to using this protocol when it is published.

Major Concerns:
I recommend that the authors briefly introduce the major steps of their protocol. Indeed, it is not always logical for any reader of the goal of certain steps. For example, it would be to introduce the step of seed preparation. The reader may not know that the protocol is for a pea agar culture.
Authors Response: It was unclear to us as to whether the reviewer wanted an expanded introduction section, or an introduction at each step of the protocol. Given the latter may be unorthodox (but a good idea nonetheless), we went with the former and have revised the introduction accordingly.

Minor Concerns:
Line Comments
Abstract The abstract should not contain references
Authors Response: We have corrected the abstract.

54 add references of established protocols
Authors Response: We have revised the text and no longer refer to previous protocols in that sentence, but refer to them elsewhere.

70 How long ?
Authors Response: We have corrected the passage and now indicate the time (2 hours).

70 Do we have to let the lid open?
Authors Response: We have corrected the passage and now indicate that we keep the lid closed.

74 How long ?
Authors Response: We have corrected the passage and now indicate the time (5 hours).

74 Do we have to let the lid open?
Authors Response: We have corrected the passage and now indicate that we keep the lid closed.

80 Justify the use of this cultivar
Authors Response: The literature and our experience indicates that different pea cultivars work. For example, Poirier et al. paper uses yellow pea seeds and we use the Thomas Laxton cultivar, which worked well. Regardless, we have changed the passage to no longer refer to a specific cultivar because we do not think the cultivar is critical at this point.

80 How much seed to use. Does the beaker have to be completely filled?
Authors Response: We have clarified both of these issues in section ***D. dipsaci Culture*** Section 2.2 by writing, 'Pour 150 seeds into sterile 2L beaker with stir bar.' Section ***D. dipsaci Culture*** steps 2.1 and 2.3 make it clear how much of the 2 litre beaker is filled.

77 Is the sterilization of seeds done under sterile conditions in a sterile bench - laminar flow hood?
Authors Response: The revised protocol makes it clear in the ***D. dipsaci Culture*** Section steps 2.2. to steps 2.6 that seed sterilization is done on the bench near a flame and that we only use the laminar flow hood when transferring seeds onto NA plates.

89 Do we have to sterilize the forceps after each seed transfer
Authors Response: We find it unnecessary to sterilize after each transfer.

97 Does the piece of agar contain sprouted seeds?
Authors Response: Yes, the piece of agar should have some root tissue. We have clarified this issue in the ***D. dipsaci Culture*** Section 4.2.

106 funnel size ?
Authors Response: In section ***Extraction and Collection of D. dipsaci*** Section 1.1, we have indicated the funnel size to be 80 mm in diameter.

111 You have to be clearer. You indicated that we have to remove the funnel which contained the coffee filter. Now you mention that the filter is not anymore in the funnel?
Authors Response: We have clarified the passage by writing, 'Remove coffee filter-lined funnel from beaker and fill the beaker with distilled water until the water level is just touching the bottom of the filter once the coffee filter-lined funnel is replaced.'

112 How is the filter held at the beaker? I expect that the funnel hold the filter?
Authors Response: Yes, the funnel holds the filter. See above response.

113 Are you suggesting to wait a specific time (Overnight = 16 h ? Or 24 h?)
Authors Response: We address this question in the revised section ***Extraction and Collection of D. dipsaci*** step 1.4: Cover the coffee filter-lined funnel and beaker with aluminum foil. Leave overnight (16 hours) on benchtop to allow worms to move through the coffee filter into the collection beaker.

118 Would it not be more appropriate to work with 50-ml falcon tube (instead of a 50 ml beaker presented in line 106) in order to centrifuge them?
Authors Response: If we were to use a 50 mL falcon tube, the coffee filter would not be able to touch the water within the falcon tube unless a very small funnel would be used, but then, this would not allow for much agar to be placed in the funnel for collection. This is why we do it like we do it.

65-124 How many nematode individuals can be gain with this method (this information is only available in line 153)? Have you consider other rearing methods (Kühnhold et al. 2006; Storelli et al. 2021)?
Authors Response: The rearing methods we describe are those that we use. In the revised introduction, we cite alternative culture protocols.

126 Before starting with the protocol, it would be useful to have a brief introduction about the aim of this preparation.
Authors Response: We have revised the introduction as requested.

160 Why waiting 5 days? Litterature mentionned between 2 h and 72 h (Faske and Hurd 2015, Storelli et al. 2020)
Authors Response: We have found that our negative controls to be healthy at day 5 while our positive control (fluopyram) kills all the nematodes at that time point. We have therefore routinely used this timepoint as the assay endpoint. That said, different time points may be appropriate depending on the goal of the screen. In our work, 72 hours is sufficient to observe killing by fluopyram, but we identify additional reproducible hits by waiting another 48 hours. We assume that operators will modify aspects of the protocol as needed.

Results To understand results, a chapter material and methods is required. In this chapter please describe active ingredient and concentrations used.
Authors Response: The JOVE format does not allow a materials and methods section, only a Table of Materials, which we have provided.

Results It would be interesting to mention what are the doses used in the field. Indeed, your results suggest that these active ingredients have nematicidal activity. However, is it thinkable to work with such concentration in a field?
Authors Response: The concentrations in the field vary depending on the crop that is being managed. Some crops are treated with up to 90.0 ppm fluopyram solution. The fluopyram concentration that we use as a control is at 23.8 ppm. Wrt other molecules screened, at the standard 60 uM concentration tested, the ppm will vary according to MW, but will be in the same ballpark as flyuopryam (typically below 100 ppm) given that most molecules we screen have a MW <500 Da. Any hit obtained in vitro would require rounds of chemical optimization, together with potted plant trials etc, so the in vitro screen is just an early step in the pipeline. We consider these issues to be beyond the scope of the manuscript.

Figure and table legends You have to give complete nematode name -> Ditylenchus dipsaci
Authors Response: In the revised abstract and introduction, we use the term *Ditylenchus dipsaci* and then use the shortened form *D. dipsaci* thereafter.

218 Indicate in your legend the meaning of "fraction mobile"
Authors Response: We have made the requested change.

224 nematode name has to be wirtten fully in a legend
Authors Response: See response to comment above.

224 Please mention time (5 days)
Authors Response: We have made the requested change.

237 Storelli et al 2021 reported similar issue and recommended to use nematode directly after extraction.
Authors Response: We have made the requested citation.

249 Do you have nematode stage proportion present in you extraction sample ? Can we consider that if the rearing duration is shortened, the proportion of juvenile nematode increase ?
Authors Response: In our work, we have not quantified proportions of developmental stages. What the reviewer is suggesting is logical, but we would rather not comment on the issue because we have not rigorously investigated it.

260 Please indicate not only the genus but also the species -> dipsaci (this comment has to be

considered for the whole manuscript)
Authors Response: In the revised abstract and introduction, we use the term *Ditylenchus dipsaci* and then use the shortened form *D. dipsaci* thereafter.

Reviewer #3:
Manuscript Summary:
This manuscript provides useful information on in vitro culture of plant parasitic nematodes and screening protocol of small molecules.
Overall, this manuscript is well written but some basic information is missing as indicated below.

Minor Concerns:
Line 97: "cut a piece of agar (roughly a 2.5 cm circle) from an existing culture plate and place on surface of new GA dish". Only agar? Should not root tissues be contained in the piece of agar? How about stem tissues?
Authors Response: Yes, the piece of agar should have some root tissue. We have clarified this issue in section the ***D. dipsaci Culture*** Section 4.2.

Line 99: There is no description of nematode inoculation. Describe nematode inoculation before nematode subculture.
Authors Response: We did not do an initial inoculation/start the culture ourselves (transferring worms from soil sample to the plates); the worms were sent to us on cultured agar plates. We have instead cited the paper that describes nematode inoculation in the note below section the ***D. dipsaci Culture*** Section 4.2 as follows: If starting fresh culture, consult Poirier et al. for inoculation methodology[15].

Soon after the agar transfer, nematodes can be inoculated? Or, how long should we wait before nematode inoculation? How many nematodes should be inoculated?
Authors Response: Again, we have not started our own culture from a soil sample. Hence, we elect to simply refer to the Poirier et al. (2019) publication as described above for inoculation methodology.

How were nematodes surface-sterilized? Establishing nematode culture without microbe contamination is rather difficult than seed sterilization.
Authors Response: We have not initiated our own sterile culture; as explained above, it was given to us. We have, however, cited literature that explains how a culture freshly isolated from the field can be cultured in section the ***D. dipsaci Culture*** Section 4.2.

Line 108: How can you know that a nematode-culture plate is ready for extraction of nematodes? How long should we wait for after inoculation of nematodes to harvest nematodes?
Authors Response: In the notes of the revised ***Extraction*** section, we note the following: A nematode-culture plate is ready for extraction when worms have crawled into the agar when examined with a dissection microscope. Typically, a plate becomes ready for extraction 6-8 weeks after its initial inoculation.

Reviewer #4:
Manuscript Summary: Very nicely done presentation of a protocol to evaluate chemicals against Ditylenchus dipsaci. A detailed descriptions of the methods is provided which will make it easy to reproduce; something that is not common in this field of study. The utility of the assay was demonstrated with small molecules as well as established nematicides. The authors do an excellent job of highlighting the positives and negatives of the assay.

Major Concerns:
None

Minor Concerns:
1. The entire manuscript should be focused exclusively on D. dispaci, not the genus. Many of the statements that are made are specific to D. dipsaci.
Authors Response: In the revised abstract and introduction, we use the term *Ditylenchus dipsaci* and then use the shortened form *D. dipsaci* thereafter.

2. In the protocol part of the manuscript, are there original references for D. dipsaci culturing and the 96-well assay that were modified? If yes, provide. The NaOH methodology was first created by Chen and Dickson (2000) which should be referenced.
Authors Response: Original references for *D. dipsaci* culturing are included in the revised manuscript in several relevant sections. For the 96 well assay, we modified the protocol from our previous work (Burns et al., 2015), which we have now cited in the revised introduction section. Finally, the revised manuscript cites the Chen and Dickinson work where appropriate.

3. Figure 1 provides a nice presentation of the results, very easy to synthesize. However, it is difficult to understand what the bars represent as far as chemicals. Revise to make this clearer.
Authors Response: We have provided clarification in the legend of Figure 1 by writing: Except where otherwise noted with solvent controls or known nematicides, the wells of the three plates contain relatively uncharacterized drug-like compounds purchased from vendors (see Burns et al, 2015 for example).

4. Figure 3. The use of the word "control" is misleading because the reader thinks that it is an untreated control, not just the well without NaOH added. Change to (-)NaOH.
Authors Response: We have made the requested change.