Comparative analysis of the chemotaxis behaviour in the root-knot nematode *Meloidogyne incognita* for tomato host and companion plants

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TABLE OF CONTENTS

ABSTRACT

Until recently, the use of pesticides was central in agriculture in order to meet the needs of the ever increasing world's population. Unfortunately, it has altered the humans and the environmental health. In this context, new generation molecules such as biopesticides seem promising to replace synthetic pesticides. This study investigated the possibility of using companion plants to fight against a soil-born phytoparasite: the root-knot nematode *M. incognita*. It is an obligate endoparasitic and polyphagous microscopic soil worm, which causes 12.3% yield loss worldwide in market garden crops. In the Mediterranean area, they are responsible for 30% crop losses in the tomato agrosystem. Methyl bromide-based nematicides were efficient at controlling this pest but have been banned in most countries due to their neurotoxicity. As an alternative, a list of plants has been established that, according to the literature, allow the control of *M. incognita* in tomato cropping systems. Among them: *Crotalaria juncea, Tagetes erecta* and *T. patula*. Tested in 2022, these plants were found to secrete root compounds called exudates that were not toxic to the stage 2 juvenile of *M incognita*, the only motile stage of the nematode, but blocked its development in the eggs. Based on the number of egg masses produced on plant, *C. juncea* was characterized as a poor host for *M. incognita*, while both *Tagetes* species were non-host, with no nematode reproduction at all. According to the null or poor level of infection observed, one can wonder if those plants could be affecting the pre-parasitic step of infection. In other words: would exudates from those plants repel these nematodes away from the roots or at the opposite attract them in order to trap them? Chemotaxis behaviour of *M. incognita* was assessed *in vitro* in kinetics using time-lapses imaging. Initiated in 2022, this protocol was further improved during the internship and tested with reference chemicals. Then, root exudates from the 3 selected companion plants were assessed for their chemotaxis effect on *M. incognita* (not all exudates were analyzed before the submission of the report but analyses will be carried out next month).

KEYWORDS: root-knot nematode, *M. incognita*, companion plants, exudate, chemotaxis behaviour

PREFACE

The use of pesticides took off in the 1930's, in connection with the development of synthetic organic chemistry. Until recently, their use was at the heart of intensive agriculture that was built to meet the needs of the ever-growing world's population. Unfortunately, the global use of phytopharmaceutical products has impacted both the human and the environmental health. Currently, one of the main challenges in agriculture is ecological intensification aiming at meeting the increasing demand for agricultural products while respecting human and environmental health. In this context, new generation molecules such as biopesticides seems promising to replace synthetic pesticides. Being of natural origin, these new molecules are supposed to be easily biodegradable and more respectful of the environment. My internship study is part of a larger investigation aiming at **using companion plants** to manage **soil-born phytoparasitic nematodes**; a project of the Priority Research Program (PPR) "Cultivating and protecting differently" in which the plant-nematode interactions team (IPN) is involved and that aims at regulating multiple pests with service plants and new crop designs. This unit is located at ISA (Institut Sophia Agrobiotech) in Sophia-Antipolis.

Plant parasitic nematodes are in most cases **microscopic-soil-born worm** infecting roots. They are impacting many crops and nowadays, nematicides such as methyl bromides that were used to control these parasites are banned or restricted[1]. As infection occurs underground, it is **difficult to detect the presence of nematodes in the field**. In addition, infected plants show a variety of non-specific symptoms that can be confused with dwarfing, wilting or even necrosis caused by other pathogens or abiotic stresses. There are already potential alternative methods to control these pests such as the use of **natural resistances introgressed in cultivars**[2,3,4,5], physical control methods such as **solarization** or **flooding**[6] of the land, and **prophylaxis** but they are either **not very effective** or **not environmentally friendly** (solarization kills every micro-organisms in the top soil, including destroying microbiota and flooding involve using lots of water), and many resistances are already overcome by these pests. In fact, varietal resistances are effective but genes are rare, notably in vegetables (only one gene exploited for tomatoes: *Mi-1*, a few genes for peppers, no total resistance for other vegetable crops) and generally heat sensitive like the tomato *Mi-1* gene that is inactive above 28°C, which could be problematic in Mediterranean regions considering the raise of the temperatures due to climate changes. Implementing crop rotations and black fallow could be effective to lower nematode populations but these management strategies are impaired by the broad host range of the *Meloidogyne* species and fallows are an economic burden for most farmers. Other investigations are in progress to develop biological control methods such as bio-fumigation with forage sorghum or rotation with trap crops (nematotoxic or bad hosts).

This internship was funded by the **GIS PICLég** (Groupement d'Intérêt Scientifiques pour la Protection Intégrée des Cultures Légumières) and carried out as part of the PPR project "**CAP Zero Phyto**" in which 5 control levers that can strengthen plants immunity against bioaggressors are tested alone and in combination: genetic resistance, service plants, biocontrol solutions promoting plants defence (bio-stimulants), UV-C flashes and nitrogen supply management. Apple and peach (for Rosaceae) and tomato and pepper (for Solanaceae) are used as model crops with their respective pests. The IPN team is particularly interested in service plants that can be used in market gardening agrosystem (tomatoes, peppers). Last year, during a previous internship at IPN, Mrs. Marielena Lahoreau Gilio tested the direct toxic effects of exudates and extracts of service plants (*Tagetes erecta, T. patula* and *Crotalaria juncea*), on the eggs and larvae of the root-knot nematode *M. incognita*. A toxic effect was observed on the eggs with a significant inhibition of hatching but no direct effect on the 2nd stage larvae[7]. In the current study their possible role in the nematode chemotaxis: **repellent or attractive effects** were therefore studied.

I. INTRODUCTION

Nematodes are **unsegmented roundworms**. They can measure from 80µm to more than 10m long and are filiform. They are multi-cellular organisms and also the most abundant and diversified group of invertebrates (over 27 000 described species). They were able to **colonize all habitats**, whether marine or terrestrial, and **move better in sandy substrates**. Some are very good assets for the organic matter cycle and are used as bioindicators of soil health[8]. There are also bacterivores, fungivores and some evolved parasitism, causing diseases on plants and animals. On the other hand, entomopathogens are used as biological control agents. For example, nematodes of the genus *Steinerma* can help to control certain plant-parasitic insect's larvae. Some nematodes are themselves **plant-parasites**, such as nematodes of the **genus *Meloidogyne*** recognized as the most damaging to plants and especially to crops worldwide[9].

1. Phytoparasitic nematodes: a focus on *Meloidogyne incognita*

1. 1. Generalities about phytoparasitic nematodes

There are about 4,500 described species of plant-parasitic nematodes[10]. The majority are invisible to the naked eye and measure maximum 5mm long for the largest. They are generally classified according to their **buccal stylet**. This one is essential because it allows to: **perforate the wall of the plant cells, suck the content of the cells** and **inject substances**. They also can be classified by their **tail shape**. Some, like *Pratylenchus*, are ectoparasites, or migratory ectoparasites like the virus vectors *Xiphinema* spp., and they carry their lifecycle outside the plant (no penetration). Nematodes could also be migratory endoparasites, like some *Ditylenchus* spp. and will carry out the cycle in several different plants, or **sedentary endoparasites**, like *Meloidogyne* species, and complete their **cycle in a single plant**. Where ectoparasites will be attracted to specific hosts, **sedentary endoparasitic** nematodes will be more generally **polyphagous**, making it difficult to find a way to eliminate them. Moreover, endoparasitic nematodes can **live for several years**[11].

1. 2. The root-knot nematodes

Nematodes of the genus *Meloidogyne* are called **root-knot nematodes** because they cause deformations named galls on plant roots. They are among the **smallest nematodes**: from 0.2 to 2mm maximum, which makes them invisible to the naked eye (Figure 1). Nevertheless, they are the first in the list of the 10 most important plant-parasitic nematodes in the world for their economic importance[9].

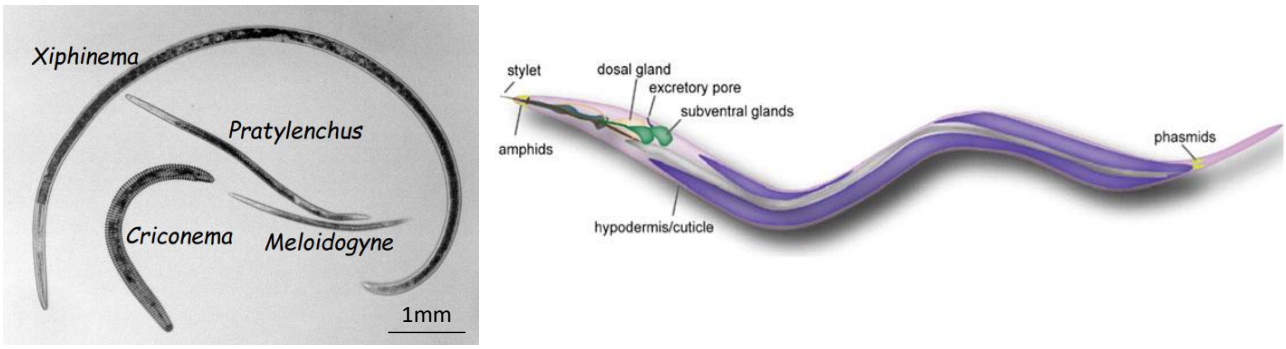


Figure 1: Comparison of the size of some phytoparasitic nematodes (from S. Fournet, INRA) and schematic overview of a plant-parasitic nematode (Shinya et al., 2013)

Root-knot nematodes have a life cycle that can vary **from 3 weeks to 3 months** depending on the temperature. The higher the temperature, the faster the cycle will be completed. Because they are **obligate endoparasites**, they must establish a **feeding site inside the root by inducing transformation of plant cells** (formation of multinucleated giant cells by cell multiplication without wall formation). One female can generate 300 to 1000 eggs and complete **several cycles per year**[12] which can rapidly spread the pathogen (4 to 7 generations in one year[13]). Our study will thus be based on root-knot nematodes, more particularly on the specie *Meloidogyne incognita*, the most widespread specie in the world.

1. 3. The life cycle of *Meloidogyne incognita*: specie used in our study

The life cycle (Figure 2) is divided into two phases: one **in the soil** (called exophytic), from oviposition to the penetration of the J2 stage juveniles into the root[14], and an **endophytic phase** of elaboration of the feeding site at the level of the **central cylinder of the root** (where the sap is transported) allowing the development of the nematode.

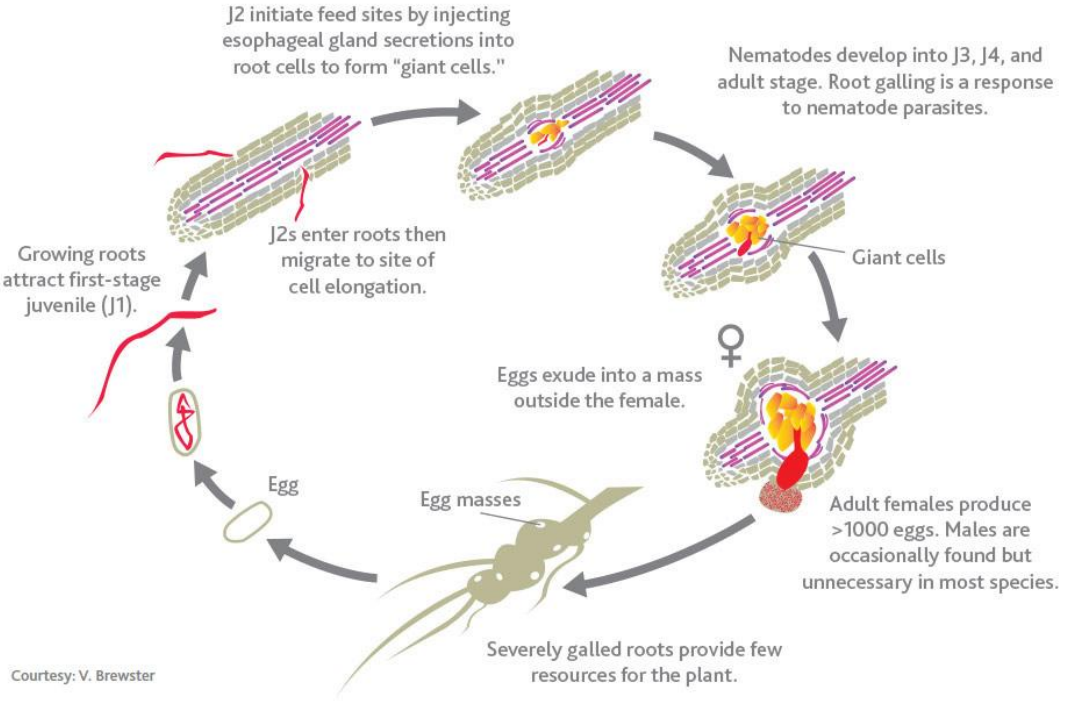


Figure 2: The life cycle of *Meloidogyne incognita* from G. Abawi and V. Brewster

In the egg, the juvenile of stage J1 makes its first moult and hatches at stage J2. The 2nd stage juveniles (J2 larvae) is elongated and thread-like and has a thin but resistant cuticle that covers it, protects it and allows it to move more easily in the presence of a film of water. It is **the only free form that spreads in the soil**. Its ability to move is **facilitated by water flows** or **by the displacement of the ground**. Under favourable conditions of humidity and temperature, most eggs hatch immediately and evolve into larvae. The optimal temperatures for the infestation and the development of the nematodes are between 20 and 25 °C[15].

Chemotaxis is one of the most common behaviour of nematodes. They possess some chemosensory organs to perceive their hosts through chemical cues released from the plant roots[16]. The first ones are a pair of **amphids**, found in the anterior (head) region at the base of the lips. They are the principal **olfactosensory organs** of nematodes[17]. Each amphid is made up of 12 sensory neurons with ciliated

dendrites that carry chemical receptors. The second type are the **phasmids**, a pair of unicellular sensilla in the lateral tail region. They are smaller than amphid sensilla and are necessary in modulation of chemorepulsion behaviour. Previous reports have shown that specific exudates from the roots of host plants attract them, whereas substances from non-host plants may repel them[18,19,20]. It was reported for example that methyl salicylate strongly attracted *M. incognita* to tomato roots[21]. Therefore, very **few plant-derived chemicals have been shown to function as root-knot nematode attractants**[22,23]. Among the attractive molecules, certain diamines such as **cadaverine** and **putrescine** are **known to be highly attractive** at a concentration of 100mM[24]. The identification of chemo-attractants can contribute to the development of root-knot nematodes control strategies[25] in agriculture.

2. Root-knot nematodes: a major problem

2. 1. Damages on all crops

Meloidogyne species are very impactful at the global economic level. Indeed, they are present in the whole world (Figure 3) and are polyphagous[26]. They are found throughout the intertropical zone and in warm temperate regions. Some species are even found quite far north in Europe.

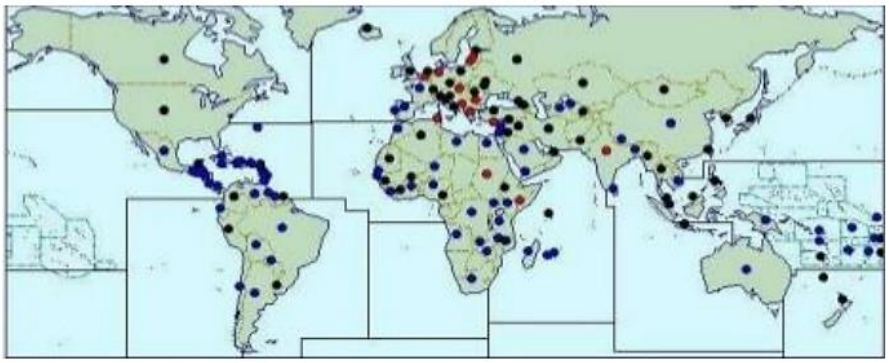


Figure 3: Worldwide distribution of *Meloidogyne incognita* (CABI 2017). In black: present; in blue: largely present; in red: localized.

Because they are polyphagous, they attack many crops such as fruits: banana trees, grapevines; vegetables: tomatoes, peppers, plums, carrots; cereals: rice (1st cereal produced and consumed in the world), maize, sorghum; coffee, cotton, sugarcane, etc[27]. They can cause **40 to 100% of yield loss depending on the crop**[28]. They cause annual **yield loss of 12.3% ($157 billion dollars) worldwide**[29], the table (Figure 4) below shows different examples.

Species	Host plant	Area	Yield loss
M. incognita	Colocasia	India	21%
M. incognita	Rice	India	60%
M. incognita	**Tomatoes**	Mediterranean	**30%**
M. incognita	Citrus	Mediterranean	20 to 30%
Total *Meloidogyne* species	Sweat potatoes	Mediterranean	Up to 25%
Total *Meloidogyne* species	Eggplant	Tropics	17-20%
Total *Meloidogyne* species	Melon	Tropics	18 to 33%

Figure 4: Some examples of yield losses due to *Meloidogyne* species[30,31]

2. 2. Symptoms and detection of the pest

On the roots, the main symptom is the formation of galls, typical of *Meloidogyne* infection (Figure 5), **reducing the ability of the plant to absorb water and nutrients** and causing mechanical injuries that facilitate infection by other pathogens. Early **galling** can be observed about **ten days after penetration** into the roots of tomato. Since Meloidogyne attack the underground organs of the plants, **there are not always visible signs of infestation** in the culture.

Figure 5: Root galls in infested tomato by *M. incognita* after 2 months of egg inoculation

Several cycles can occur in one year, **up to eight generations per year on tomato**, but generally three to four per year for *M. incognita* under plastic tunnels in southern France. When damages are visible: **clear spots in the field** (Figure 6), it is too late to save the crop. This is why it is important to find some solutions to control this pest.

Figure 6: Infested rapeseed plots by *M. incognita* from Céline ROBERT, 2019

3. Companion plants: a promising solution

3. 1. Generalities about companion plants

Service plants are a promising solution to decrease excessive use of chemical products and can be used as cover plants, companion plants or non-crop plants and can be planted in mono- or multi-species cultures, in field edges or within the field (in rotation or in association). They can **provide ecosystemic services that are not directly marketable**, for example: soil fertility or pest regulation while being vigilant to possible negative impacts (disservices) and taking into account the context (climate, pests, type of cropping system…)[32]. Some service plants can regulate nematodes by planting them with or before a crop: they are **companion plants**. Their effect can be to **repel the pest**, to **attract it away from the crop**, to **inhibit its impact or to kill it**.

To control soil-born pathogen like *M. incognita*, a potential service plant needs to act **from the roots** (Figure 7). Even before being subjected to biotic stress, the plant secretes low molecular weight compounds called **phytoanticipins** that act as a chemical barrier against a wide range of aggressors (bacteria, insects, nematodes, fungi). These compound excreted by the roots are named **exudates**.

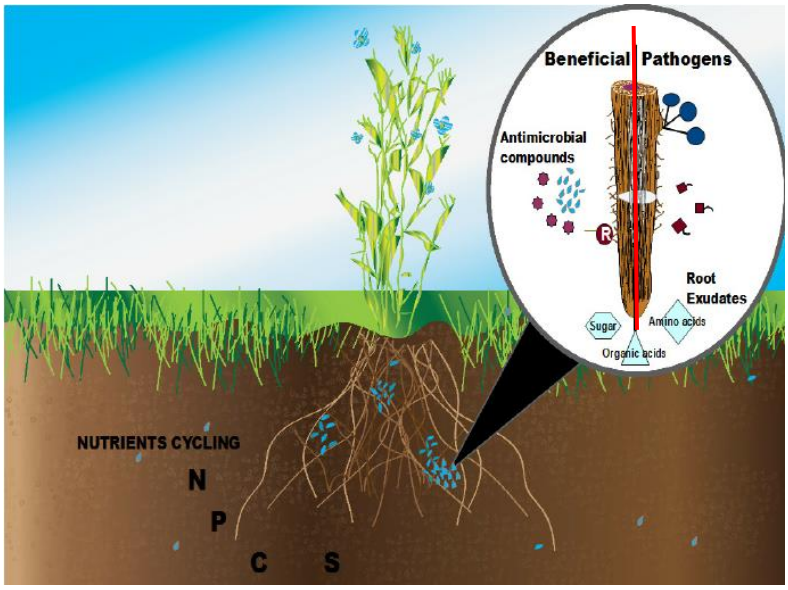


Figure 7: The root tip: an area full of interactions (Vivian A. Rincon-Florez et *al*)

When a plant is attacked, there is an **increase of the production of phytoanticipins** and of other low molecular weight antimicrobial compounds called **phytoalexins**. For example, *Glycine max* (Fabaceae) produces Glyceolin, a nematostatic phytoalexin that inhibits respiration of the *M. incognita* larvae[33,34]. Exudates can also be **phenolic compounds and terpenoids** that have high antibacterial and antifungal capacities.

3. 2. The selected companion plants to control *M. incognita*

A **list of candidate plants** was established, by Cliven Njekete, potentially allowing to control nematodes together with aerial pathogens in the frame of his phD project "Bitarget biocontrol plants against aboveground and belowground pests in the tomato agrosystem"[35], according to the literature. Finally, **5 plants were found to be potential multi-targets** for aerial pathogens and nematodes (Table 1 in annex), among them: ***Tagetes patula*** and ***Tagetes erecta*** appeared to be the most efficient. A third plant seems to be a good candidate to control *M. incognita*, it is ***Crotalaria juncea***. Previous experiments done by Njekete Cliven classified the 3 selected plants as bad or non-hosts for *Meloidogyne spp.* (Figure 8). Tomato is **susceptible** to nematodes (host), meaning that nematodes are

able to reproduce well in the roots and form a **large number of egg-masses**. A **bad hosts** plant means that **reproduction can take place but far less egg-masses will be produced compared to susceptible host plants**. This is the case for *C. juncea*, in which the **number of egg-masses is lower than in tomato**. Bad-host plants can be used as **trap plants** if they attract juveniles and limit their reproduction such as *Tagetes minuta*, or kills nematodes in the root with toxic compounds such as *Cajanus cajan* (Fabaceae)[33,34]. Then the plants can be **non-hosts**, which means that the **reproduction is not possible** in these plants. This is the case for both *Tagetes* species for which there is **no egg-mass** (Figure 8).

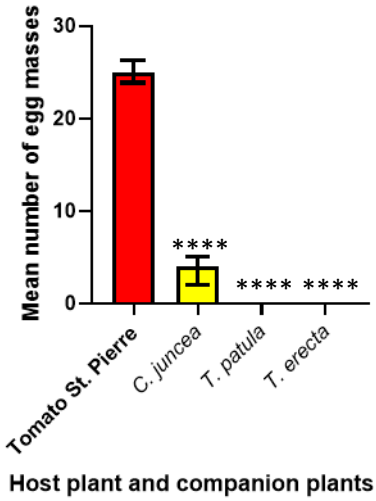


Figure 8: Mean number of egg-masses of *M. incognita* on roots of companion plants 6 weeks after inoculation with 2,000 eggs (means (n=6) ± standard deviation), from a study of Njekete Cliven (2021), significant differences found according to an ANOVA and post-hoc Tukey test

In addition, C. Njekete showed that the 2nd stage juveniles were able to **penetrate the roots of *Crotalaria juncea***, significantly **much more than in the tomato host**, but **very few nematodes were found in the roots of *Tagetes* species** (Figure 9). This inability to penetrate in roots could be explained by pre-infection defence mechanisms: the root epidermis can act as a physical barrier or by the root exudates that could also be attractive, repellent or even toxic for eggs and/ or juveniles.

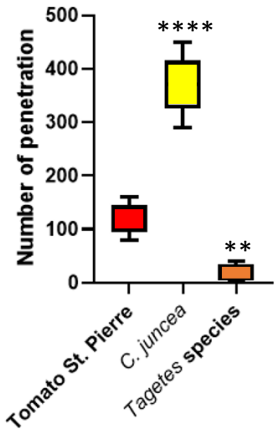


Figure 9: Number of *M. incognita* J2 larvae that penetrate the roots of companion plants 8 days after inoculation of 600 J2 larvae per plant (n=10), from a study of Nekjete Cliven (2022), significant differences found according to an ANOVA and post-hoc Tukey test

According to the study from M. Lahoreau[7], it is known that the **exudates of these three plants are not toxic for the J2 larvae** (only 3% of mortality observed during the tests) but they were toxic on the eggs (up to 60% of egg hatching inhibition). Thus, the chemotaxis behaviour of nematodes larvae against these 3 companion plants was studied using concentrated root exudates to better understand if these plants can affect the pre-invasion step of the infection.

II. Objectives

This study was divided in two phases:

The first one was to improve and validate the set-up designed by Sophie Mantelin in 2022 to study the chemotaxis behaviour of *M. incognita.* The set-up had to be evaluated and adapted using **reference chemicals whose attractive powers on *M. incognita* are known**[24] (Figure 10).

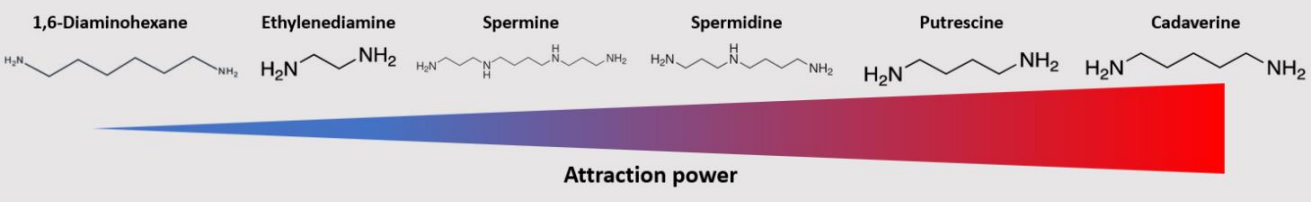


Figure 10: Attraction power of diamine molecules adapted from Oota et *al* 2019[24]

Repellent molecules were also sought, thanks to the literature[36,37], during this training course in order not to test only neutral or attractive molecules.

The second phase (once the protocol was improved) was to **test the chemotaxis of *M. incognita* towards different exudates from companion plants** and the tomato host control, that were either collected in 2022 or freshly prepared, in order to answer two questions:

- Are there molecules produced by the *Tagetes* in the root exudates that **repel** the J2 nematodes that can explain the weak level of penetration of the nematodes in that non-host plant?

- Does *C. juncea* **attract** these nematodes? And if this is the case, **is it more attractive than the tomato** since it shows a higher level of penetration by *M. incognita* despite the poor reproduction observed at the end of the cycle?

Since root exudates from companion plants have already been tested for toxicity to *M. incognita* J2 in 2022, only **the reference molecules were tested for their toxicity** (diamines and repellent molecules) and their LD50 were defined in order to better understand the behaviour of nematodes observed in the time-lapses kinetic chemotaxis experiments.

III. Materials and Methods

1. Biological material

1. 1. The nematodes

The nematodes: ***M. incognita*** (population Morelos) were propagated in greenhouses **on susceptible tomato** plants (*Solanum lycopersicum* cultivar St-Pierre) **in soil**. For the larvicidal test with diamine molecules and for chemotaxis experiments, J2 nematode larvae were collected from 3-month-old infected tomato plants, **2 months after nematode inoculation** which allow to have an end of cycle egg

masses on roots. The roots were washed from soil, cut into pieces and placed in a perforated collecting bucket in the **mist chamber** maintained at 25°C for egg hatching. Nematodes were collected from the mist and **filtered on a 4-layersKleenex® tissue** (Figure 11) **over-night** at room temperature in the laboratory (20-24°C) to purify the suspension from root debris and soil particles, and to only **keep the active larvae** (the dead larvae don't pass through the chaotic mesh of the Kleenex®). The container was covered with aluminium foil to keep the nematodes in the dark.

Figure 11: Filtration of the suspension of nematodes on a 4-layers of Kleenex® tissues

The suspension was then **sampled to evaluate the nematode density** by counting 10µl drops of J2s under the binocular magnifier (Figure 12), and the volume adjusted to obtain about **15,000 J2s per mL of tap water sterilised by filtration on 0.2µm filter** (concentration of nematodes used to perform all the experiments)**.**

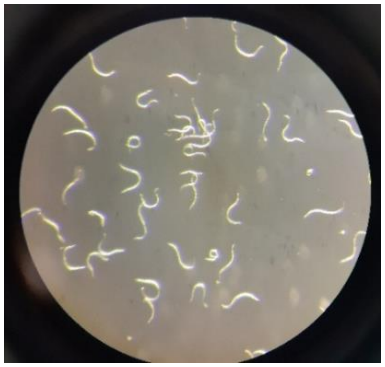


Figure 12: Counting of 10µl drops of the filtered nematode suspension

1. 2. The diamine molecules

The five diamine molecules tested came from Sigma Aldrich and were provided in the form of ampoule or powder that were used to prepare **stock solutions**. Here is the table (Figure 13) summarizing all the characteristics of the molecules.

Molecule	Reference	Purity	Quantity	Linear formula	Density	CAS number	Molecular weigh
Cadaverine	SIGMA D22606-5G	95%	5g in solution	$NH_2(CH_2)NH_2$	0.873g/mL at 25°C	462-94-2	102.18 g/mol
Putrescine	SIGMA 51799-100mg	>98.5%	100mg powder+ ampoule	$NH_2(CH_2)_4NH_2$	0.877 g/mL at 25°C	110-60-1	88.15g/mol

Spermidine	SIGMA 49761-100mg	>97.5%	0.1g in ampoule	$NH_2(CH_2)_3NH(CH_2)_4NH2$	0.925g/mL at 25°C	124-20-9	145.25 g/mol
Ethylenediamine	SIGMA 410086-1mL-F	99.8%	1 mL	$NH_2CH_2CH_2NH_2$	0.899 g/mL at 25°C	107-15-3	60.10 g/mol
1,6-Diaminohexane	SIGMA 8.04323	>99%	5g powder	$H_2N(CH_2)_6NH_2$	0.873 g/mL at 25°C	124-09-4	116.20 g/mol

Figure 13: Characteristics of the diamine molecules tested

Stock solutions (Figure 14) were **prepared in double distilled water** (all diamines used are soluble in water according to the Merk Index) when the diamines were received in powder form.

Molecule	Volume in stock	Concentration in stock
Cadaverine	5.73mL	8.54 mol/L
Putrescine	1ml + 200µl in ampoule	C= 1.13 mol/L
Spermidine	108 µl	C = 6.37 mol/L
Ethylenediamine	1 mL	C = 14.96 mol/L
1,6-Diaminohexane	5mL	C= 8.6 mol/L

Figure 14: Characteristics of the stock solutions realised in March 2023

Each solution was **freshly diluted to 100mM** (Table 2 in annex) the day of use, vortexed to homogenize well and placed in the refrigerator until use.

1. 3. The root exudates

During a previous internship in 2022, pre-flowering plant root exudates were prepared from the three companion plants and tomato using a **hydroponic method** as described in Figure 15.

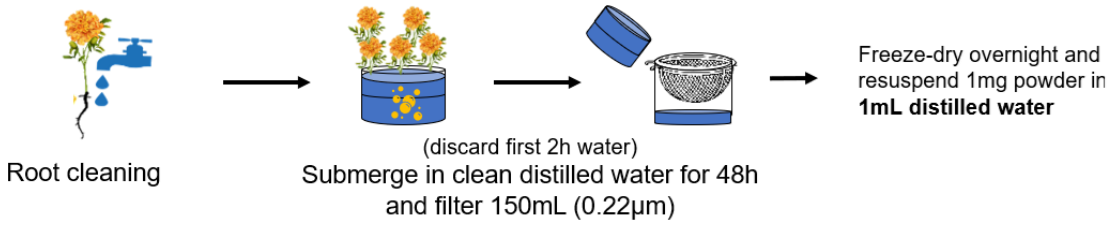


Figure 15: Synthetic scheme of the root exudates collection (made by M. Lahoreau)

In addition **fresh pre-flowering root exudates** were prepared as only a small quantity was available if any (Figure 3 in annex), **using a derivative of the hydroponic method**. Roots attached to the aerial parts were **washed with tap water** and **suspended in sterile glass jars** (covered with aluminium foil to avoid light interference). Roots were **immersed in 600mL of mineral Volvic® water** for **48h** before collection of root exudates. The solutions were then filtered using a vacuum pump on glass fiber filters). **100 mL per plant species were filtered** again homogeneously with non-pyrogenic Acrodisc syringe filters using **first 0.45µm** and **finally 0.22µm diameter under sterile hood**. The solutions were kept **in 50mL hawks stored at 4°C until freeze-dried overnight**. The collected powder was **weighed**

and then **1 mg/mL** was resuspended in pure water for all tests. This quantity of powder was chosen because this is the one used last year to test the toxicity of the root exudates on eggs and J2, that showed no effects on J2 while inhibiting eggs hatching .

2. The macroscope set-up

2. 1. Imaging protocol

To complete the chemotaxis experiments **I-Luer µ-slides from Ibidi®** (Figure 16) were used (10 slides per experiments). The channel represents a fixed volume of **200 µL** and the Luers on each side represent a volume of **80 µL**. The Luers were used to fill-up the channel with nematodes, always by the same side (right hand-side), and to put the solution to test.

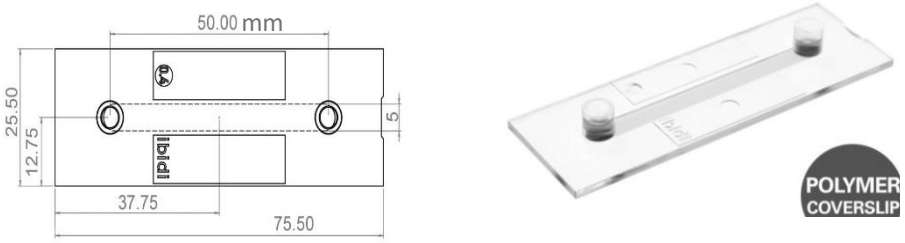


Figure 16: Dimensions (mm) and picture of a standardized slide

For the assays, **1mL of suspension of J2 larvae** of *M. incognita* (concentration: 15 000 J2/mL) was diluted in **3mL of Pluronic® F-127 gel at 32%** (1:3 ratio) to obtain a final gel at 24% with the nematodes. This gel is **non-toxic for the nematodes** and has a **neutral pH**. It **allows nematodes to freely move** in three dimensions and to **establish a chemical gradient** in the system by diffusion from the wells. Because the gel is liquid at cold temperature and solidify above 15°C, the slides were prepared **in the cold room** at 4°C with **cold materials** (pipette tips stored in the cold room and slides previously washed and placed in a distilled water bath in the refrigerator until use). Each channel was then filled using a 1-mL pipette, allowing the gel containing the nematodes to flow gently from one side to the other (representing ca. 750 J2 in the 200µL of the channel-containing volume), and cautiously to **avoid introducing air bubbles** in the system. The **excess of gel was removed** from each well and they **were capped** to prevent the gel from drying. Next, the slides were placed on a glass plate, in the laboratory at around 20-25°C, to let the gel set and the nematodes spread in the system for at least 3 hours.

The next step was to fit up 10 slides at a time, on the platform of the **macroscope**, to be imaged in time-lapses using the Zen Blue software (from Zeiss).

For each chemotaxis experiment, 10 slides maximum could be imaged, previously annotated from 1 to 10 to associate each slide with the corresponding image file after the imaging. First of all, to place the 10 slides, a "**preview scan**" was realised to see the field of view of the macroscope. After, the imaging parameters were set: imaging in **transmitted light** using **brightfield** (nematodes were dark) contrast method, with a **16x magnification zoom** and **objective X1**. Channels were delimited by creating 10 scenes in "Tiles" mode. A slide corresponds to a scene and the scenes are named like the slides from 1 to 10. The macroscope has been set to generate a **time-lapse of 16 hours** by **taking image series every 3 minutes**.

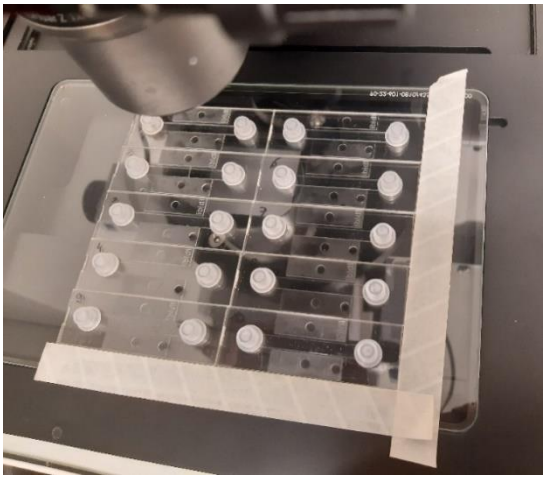



Figure 17: Picture of the ZEISS macroscope AxioZoom.v16 and the capped slides organization

Images to be taken correspond to the red squares (6 images with 10% overlap to cover the channel between the two Luer wells). The yellow rectangles are the boundaries of each channel, named "scenes" and yellow dots are the focus points placed on each channel (Figure 18).

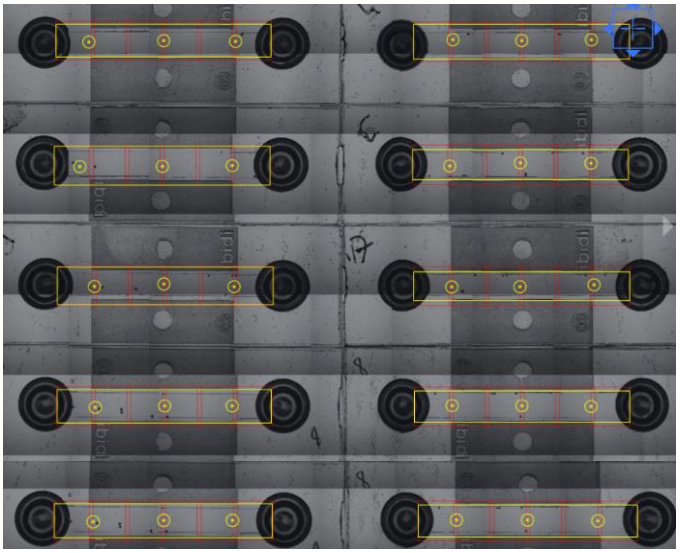


Figure 18: Delimitation of the 10 slides with the focus points

Once the parameters were set and the channels visible, **50µL** of water or chemicals was added in each Luer: **pure water on the left-hand side of each slides** (from 1 to 10), **control molecule** (cadaverine 100mM) **on the right-hand side of the slides 1 to 4** and **9** and **another molecule** was added **to compare with the control on the right-hand side of the slides 5 to 8** and **10**. Slides **9 and 10** were filled with Pluronic 24%, **without nematodes**; to measure the background noise that maybe caused by the chemical gradient forming in the gel when the products are not colourless; if this is the case, the signal will be subtracted from the other slides. To finish, a **15 µL drop of mineral oil** was added on top of the solutions to avoid evaporation during the course of the 16 hours experiment as the slides were imaged uncapped. Finally, "start experiment" has been launched after turning off the light in the room.

At the end of the run, the **slides were cleaned to be reused**.

2. 2. Post-imaging analysis

After the 16h of recording, the created file, containing the imaging of the 10 channels, was **split into individual scenes** to reduce the size of the file for analysis (time-series of single chemotaxis slide). The

6 images composing each channel were then stitched together to reconstitute 1 image per slide and per time point. To collect the light signal (Figure 19), a **region of interest** (ROI; 10x5 mm) was outlined next to the Luers on each side of the channel, to observe and quantify the nematode passing through or accumulating in the frames. Water being neutral, if the chemical gradient is attractive, the nematodes will move to the side of the tested molecule and if it is repulsive, they must rather leave the ROI. The presence of J2s in the ROI was quantified as **a signal inversely proportional to the light intensity** measured (in brightfield imaging nematodes appear in black on a white background so the nematodes are detected as objects masking the light). So **a decrease in light intensity measured was related to an increase in nematode density**. These data were then exported in Excel files.

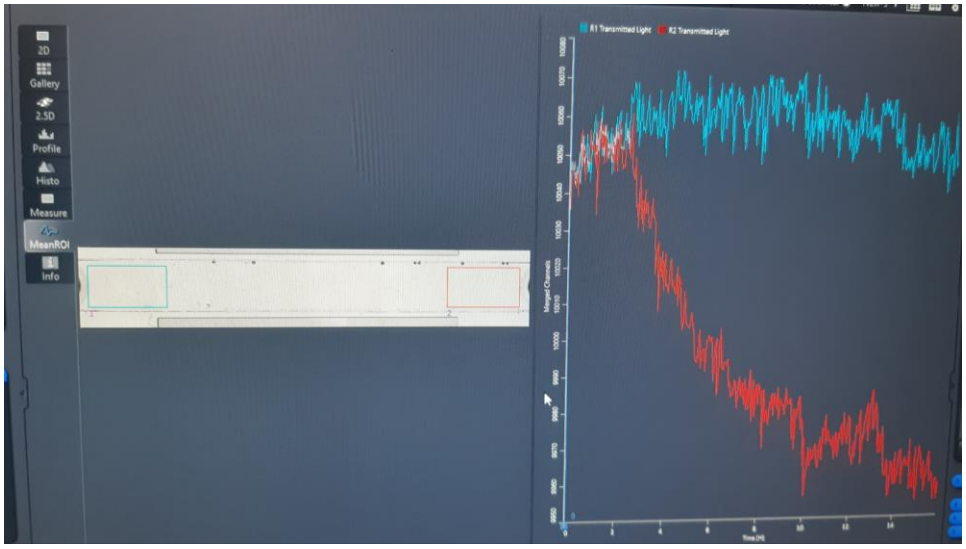


Figure 19: Example of signal measured in the areas of interest; in blue: side of the water; in red: side of the cadaverine

3. The *in vitro* toxicity tests

Toxicity tests were carried out with the 5 diamines first at the concentration used in chemotaxis: 100mM, then at lower concentrations (10mM, 15mM, 20mM, 25mM and 30mM) to find their lethal dose. Bithiophene (a compound produced by *Tagetes* and known to be toxic at a dose around 6mM) will also be used for the tests because it will be evaluated for its repulsive effect in chemotaxis tests.

To assess toxicity tests on juveniles, a suspension of **5µL with 100 J2 of *M. incognita***, were placed per well of 12 multi-well plastic plates (Figure 20), immediately followed by the **addition of 0.5mL of each tested compounds or control** (tap water). The first 6 wells contained the molecule to be tested and the last 6 contained the control. If a compound was volatile, another box was used to make only the control.

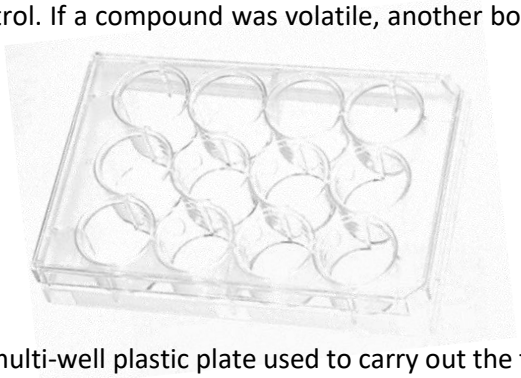


Figure 20: Picture of the 12 multi-well plastic plate used to carry out the tests (Cell Culture Plate from Costar®)

Each treatment was replicated 6 times and each control also 6 times. Juvenile paralysis was assessed **after 24h** by stimulating immobile larvae with a needle. Paralyzed nematodes were recovered with a small dedicated pole or with a pipette if they were a lot and placed in a new box containing 0.5 mL of tap water per well. They were left overnight in aluminium-covered boxes in an incubator at 25°C to keep a constant temperature and the next day, mortality was evaluated by **counting juveniles that did not show motility after second stimulation**.

4. Statistical analysis

4. 1. Analysis of the chemotaxis results

The signal relative to the light intensity measured in the ROI areas was first inverted, transformed as (1/signal) and corrected to have the two curves obtained from the same slide starting at the same value at T0. A **Chemotaxis Index** was then calculated CI = (B-A)/(A+B) for each time point, where B was the signal related to the amount of nematodes detected in the ROI on the right-hand side of the channel (side of the chemical or plant root exudate) and A the signal detected on the left-hand side of the channel (water control side). The chemotaxis index values were plotted in function of the time and a model response curve was fitted using **GNLS** (Fit Nonlinear Model Using Generalized Least Squares) **model** which allow to correlate errors and a one-way **ANOVA** was used to check if the chemical factor has an effect on the chemotaxis of the nematode. Then, a **post-hoc Tukey test** was realised to check the differences between the controls and the tested molecules or exudates.

Other tests were also tested in the course of this study, in order to find an alternative means of analysis, notably the binomial GLM model.

4. 2. Analysis of the toxicity tests

The difference of toxicity between the water control and the tested chemical were tested using a **generalized linear model** (GLM) created on R. This model allows to compare each concentrations tested with the control (tap-water), returning p-values. A post-hoc test: **estimated marginal means with Tukey adjustment** was then performed to correct any analysis errors and to compare each concentrations with each other's, using R package "emmeans**"**.

In order to find the lethal dose 50 (LD50) for each molecule, a **survival curve was drawn on Graph-Pad** and not a log-normal law as usually performed for LD50. In general LD50 is obtained by testing the pharmacogical effect of chemical drugs using 10x serial dilutions. With nematodes it is impossible to obtain a LD50 in unit mass of substance per body mass (mg/kg) such as those performed on rats. In addition, the microscopic worms have proven to be sensitive to slight variations in concentration of toxic chemicals. We therefore tested fairly close doses, every 5mM.

IV. Results and Discussion

1. Chemotaxis results

1. 1. With the reference molecules (diamines and repulsive molecule)

Different concentrations (10mM vs 100mM) of cadaverine were tested to see whether even at low concentrations cadaverine would be attractive or not. The first experiment (Figure 21.A) was carried out with 8 slides (according to the initial protocol created in 2022).

During this experiment, we realized that diamines could **cause a dark gradient** during imaging, when diffusing into the gel, thereby distorting the results. This partly explains the marked increased signal observed in the first 5 hours, particularly with highly concentrated cadaverine (100mM). We therefore decided to make two additional slides in the protocol (slides 9 and 10) without nematodes (see section III. 2. 1) in order to image the chemical gradients and then subtract it from the signal of the chemotaxis slides. After removing the gradient signal (Figure 21.B and Figure 21.C), we noticed that the chemotaxis index values do not increase exponentially as observed on Figure 21.A. This solution seems to work to compensate for gradient imaging.

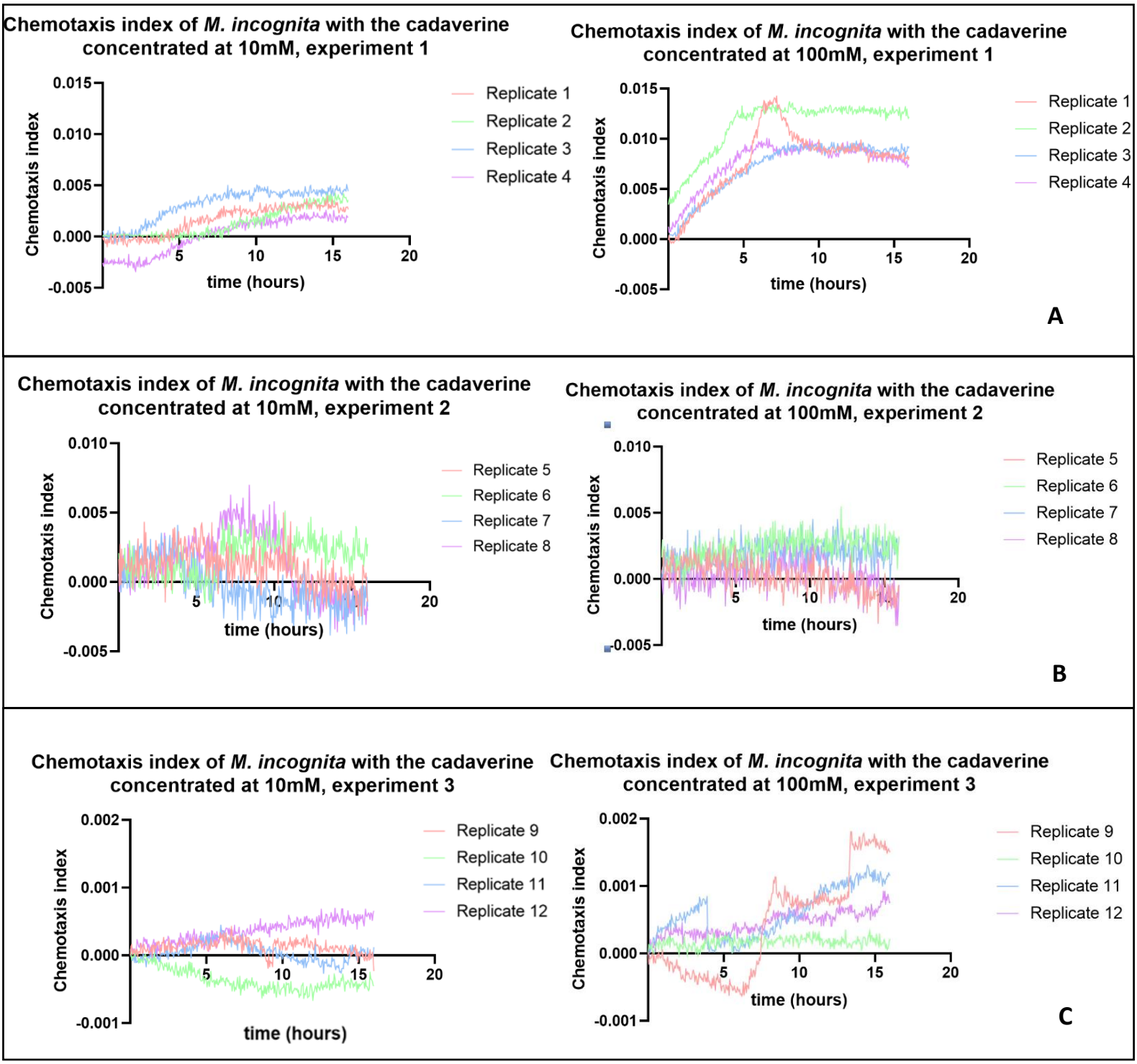


Figure 21: Kinetics of the chemotaxis index for the three experiments performed with cadaverine 10mM and 100mM for 16 hours, A: The signal used to calculate the chemotaxis index was not corrected as in the initial protocol established in 2022; B and C: The signals were corrected by subtracting the chemical gradient background signal before calculating the chemotaxis index.

Ideally, the 4 replicates obtained for each chemical tested in the 3 repeated experiments would be combined for analysis (n=12). Before doing, we needed to know whether the replicates belonged to the same group, or whether they were significantly different and could therefore not be combined. After performing an **ordinary one-way ANOVA with post-hoc Tukey test** (Figure 22 and Figure 23), we noticed that the 12 replicates cannot be merged, only a few of them are similar.

Replicates	Group
1, 2	A
3	B
4	C
5, 6, 8, 9, 11, 12	D
7	E
10	F

Figure 22: Summary of the multiple pairwise comparisons realised following an ANOVA of the different replicates obtained after testing with cadaverine 10mM

Replicates	Group
1', 3', 4'	A'
2'	B'
5'	C'
6', 9', 10', 11', 12'	D'
7'	E'
8'	F'

Figure 23: Summary of the multiple pairwise comparisons realised following an ANOVA of the different replicates obtained after testing with cadaverine 100mM

When comparing the chemotaxis indexes obtained with 10mM and 100mM cadaverine, we also notice a **strong heterogeneity of results**. Some attractions are significantly different between cadaverine 10mM and 100mM, such as replicate 1 (10mM) which is significantly different from each replicate made at 100mM (p-values <0.001). But many other replicates are not significantly different despite the difference in concentration, such as replicate 5 versus replicates 5', 8', 9', 10', 11' and 12' (p-values >0.01).

An experiment using another contrast method on the macroscope: the **darkfield mode** (Figure 24) was done, were nematodes appeared as white (lightning signal) on a dark background.

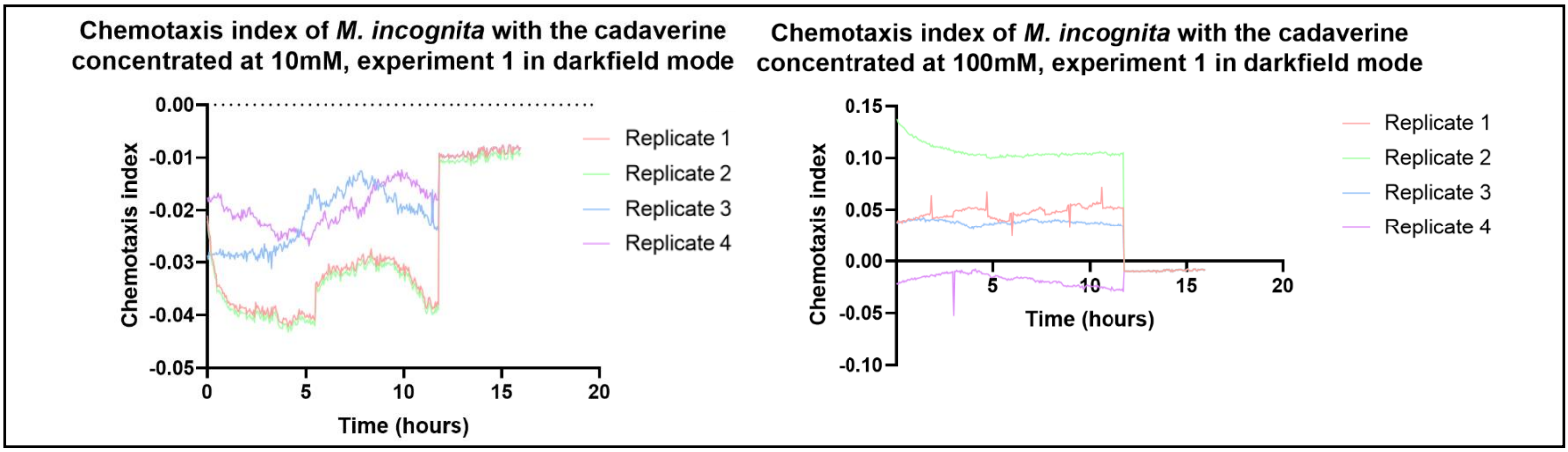


Figure 24: Kinetics of the chemotaxis index for the experiment performed with cadaverine 10mM and 100mM for 16 hours (imaging in darkfield mode).

In darkfield mode **the gradient formed by the cadaverine** (the most colourful diamine used) was not detected, however **all the dust particles** (present in the gel or on the slide) **are detected** (small peaks on the graphs). This requires even **more data processing than in brightfield mode** to remove the non-homogenous background noise. The results obtained for the chemotaxis indexes were even **more heterogeneous**. Thus, we decided to **continue imaging in brightfield mode** with background correction using the control slides without nematodes, as shown in the figures 21.B and 21.C.

Since we observed a **strong variability in the results**: the replicates themselves were significantly different in all experiments with the 5 diamines (see statistical tests tables 4, 5, 6 and 7 in annex), we will simply comment the observed chemotaxis index curve in order to describe the behaviour of nematodes. A series of slides testing cadaverine at 100mM were used in each experiment to be compared to the other diamines tested. Based on the curves of the chemotaxis indices, putrescine (Figure 25) seems to be as attractant (positive chemotaxis index with a maximum of 0.005) than cadaverine (positive index as well, with a maximum at 0.004, Figure 25). The same trend is observed for the kinetic of the indexes. Spermidine also showed a positive chemotaxis index and (Figure 26) seems attractive but maybe less than cadaverine (maximum values around 0.008 and 0.018 for spermidine and cadaverine, respectively). Regarding ethylenediamine and diamino-hexane (Figures 27 and 28), their indexes are close to zero. These molecules seem as neutral as water. The observed results for *M. incognita* behaviour towards the different diamines tested seem to corroborate the effect described for those chemicals on the nematode chemotaxis in Oota *et al*. study[24], although statistical comparisons cannot be performed on our dataset since replicates could not be combined. A way to adapt the data for statistical analysis was discussed in the section IV. 3.

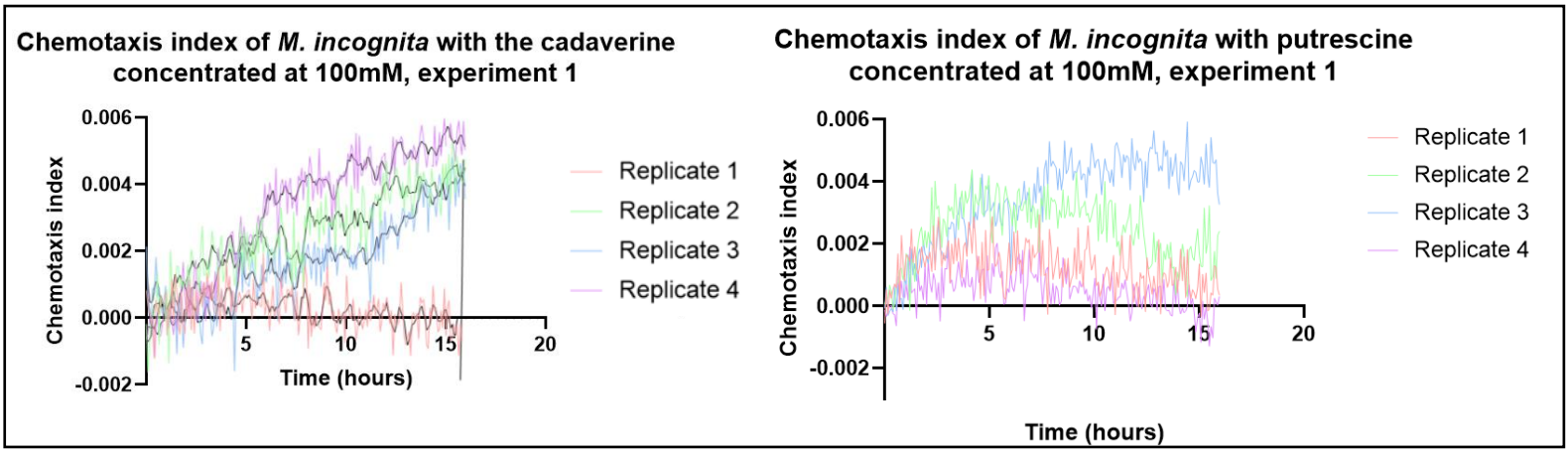


Figure 25: Kinetics of the chemotaxis index for the experiment performed with cadaverine 100mM (n=4) and putrescine 100mM (n=4) for 16 hours (the signal of the shaded gradient has been removed).

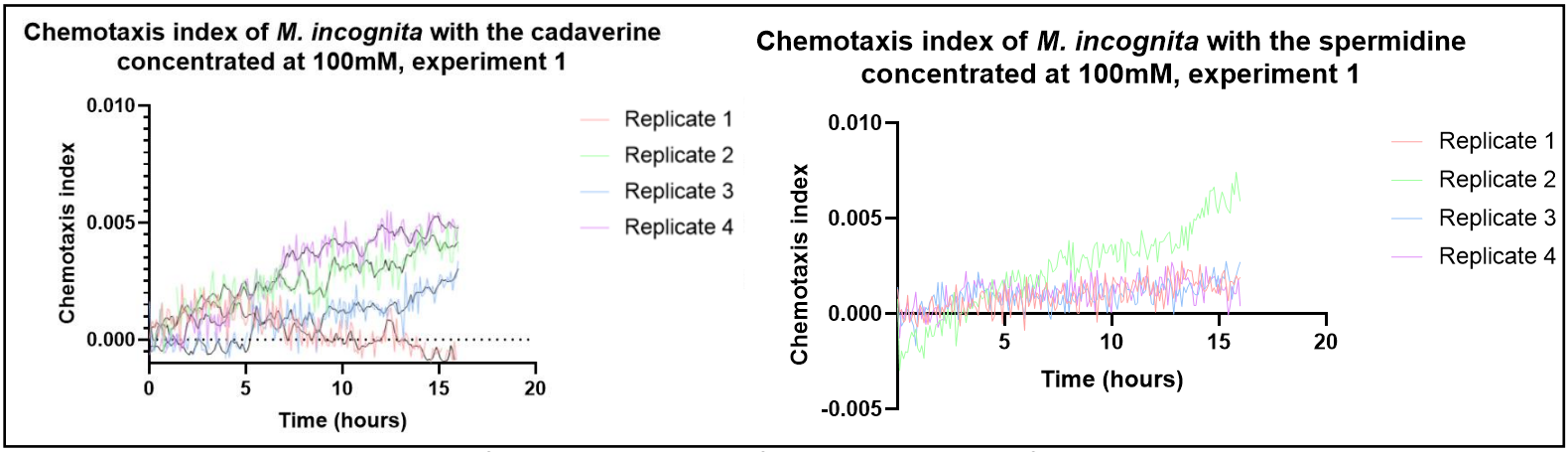


Figure 26: Kinetics of the chemotaxis index for the experiment performed with cadaverine 100mM (n=4) and spermidine 100Mm (n=4) for 16 hours (the signal of the shaded gradient has been removed)

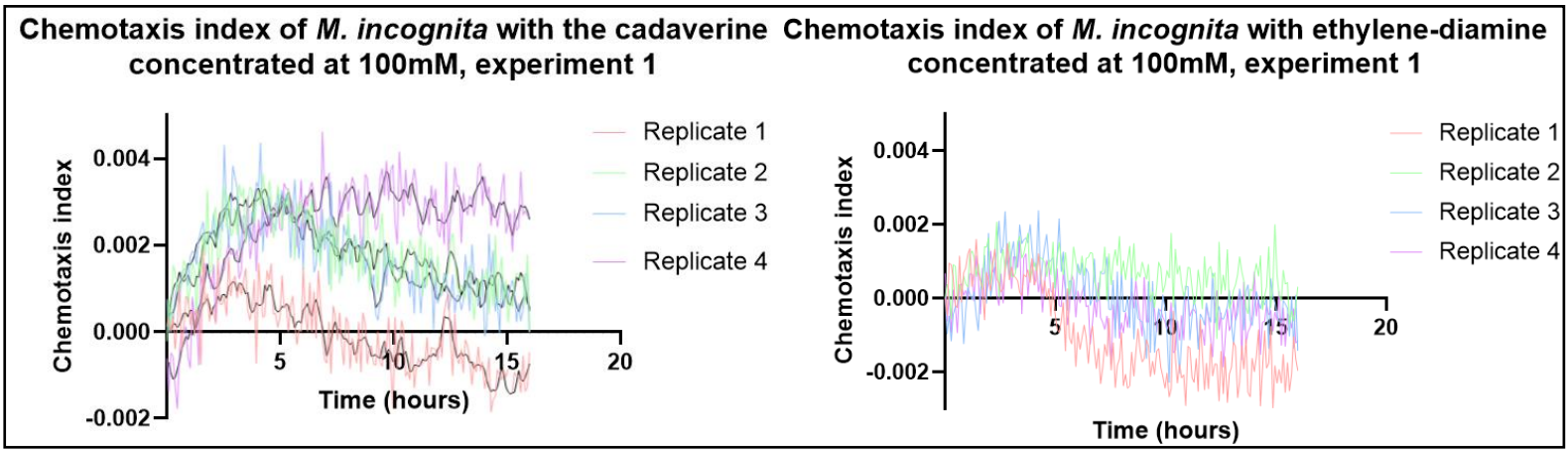


Figure 27: Kinetics of the chemotaxis index for the experiment performed with cadaverine 100mM (n=4) and ethylene diamine 100mM (n=4) for 16 hours (the signal of the shaded gradient has been removed).

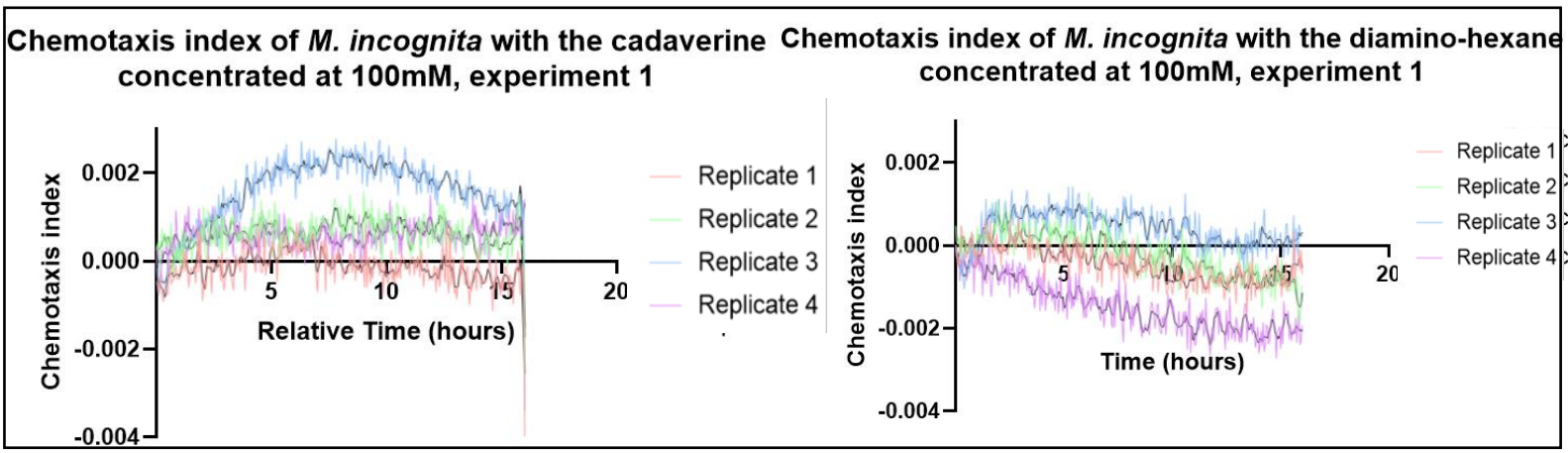


Figure 28: Kinetics of the chemotaxis index for the experiment performed with cadaverine 100mM (n=4) and diamino-hexane 100mM (n=4) for 16 hours (the signal of the shaded gradient has been removed).

This heterogeneity between replicates maybe explained by **the number of nematodes that varies in each slide in addition to an ever-changing number of worms that die during the experiment.** Indeed, after watching the time-lapses videos, we realized that far less than the ca. 700 nematodes originally injected in the channel were still moving after the gel set: **only ca. 100 were still motile**, the rest looked rigid. We thought that gel matrix prepared with distilled water may not be suitable for them. Therefore, various gels were tested using **distilled water (as control)**, a **Volvic mineral water** and **M9 nematode culture medium** (protocol P. 39). In addition, the **older distilled water-based gel** (the one used until now) was also tested. Three slides per gel (except for the older gel were only one slide was imaged), containing ca. 700 nematodes each, were imaged at time 0 for 1 hour. The slides were then imaged for 3 hours after 16 hours incubation at room temperature, to be able to compare the state of the nematodes in each gel after the duration of the usual chemotaxis experiment.

The time-lapses generated at T0 and T16 hours were then imported into Fiji in order to **count the total number of motile nematodes** at the two time points. The results obtained with each gel were then compared to find the gel matrix that better sustains *M. incognita* viability.

Images taken at T0 (Figure 29) with the old distilled water gel (Figure 29.D) show that a large proportion of the nematodes were already rigid/paralyzed (nematodes appear as sticks on the image) compared with images obtained with the other three gels (nematodes appear curved, Figure 29.A,B,C). Looking at the time-lapses, we could see that the nematodes were very active in the distilled and fresh mineral water gels, whereas they were slower in the M9 gel.

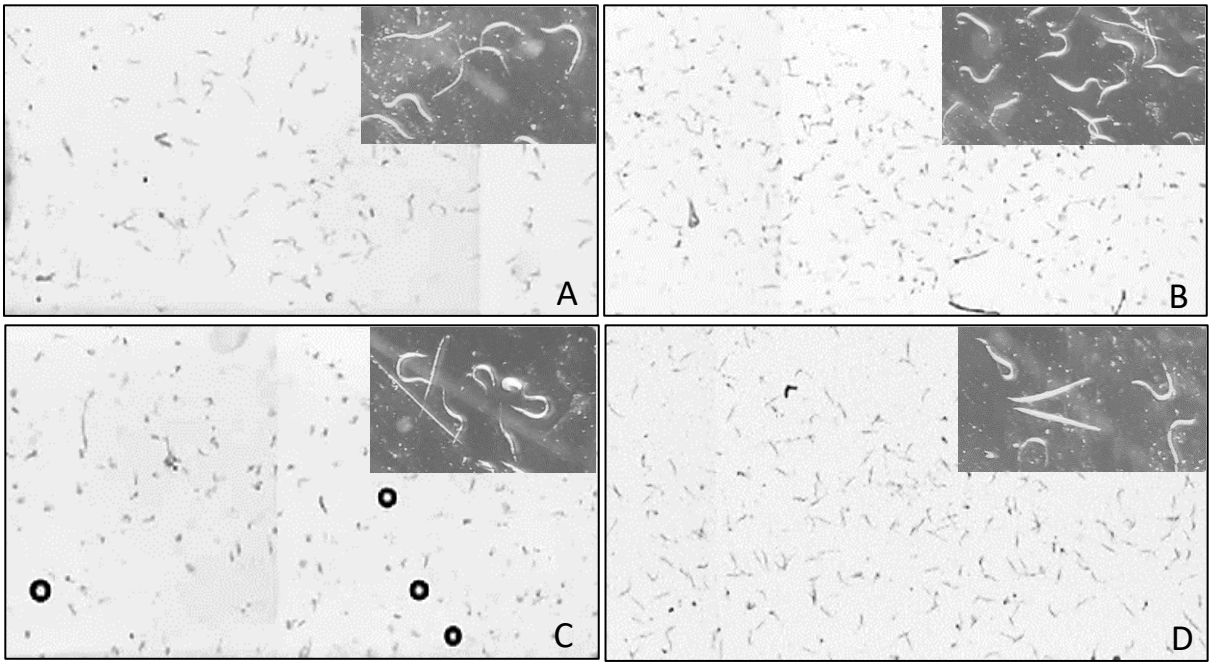


Figure 29: Images taken with the macroscope AxioZoom with 16x magnification zoom of the state of nematodes in A: fresh distilled water gel; B: mineral water gel; C: M9-based gel; D: 5 month old distilled water gel and additional pictures taken with a binocular loop x8 (top right hand corner)

After 16 hours (Figure 30), the nematodes in the fresh distilled and mineral water gels (Figure 30.A,B) were still very mobile, whereas in the M9 medium (Figure 30.C), many were immobile and just as many were paralyzed or dead in the old distilled water gel (Figure 30.D).

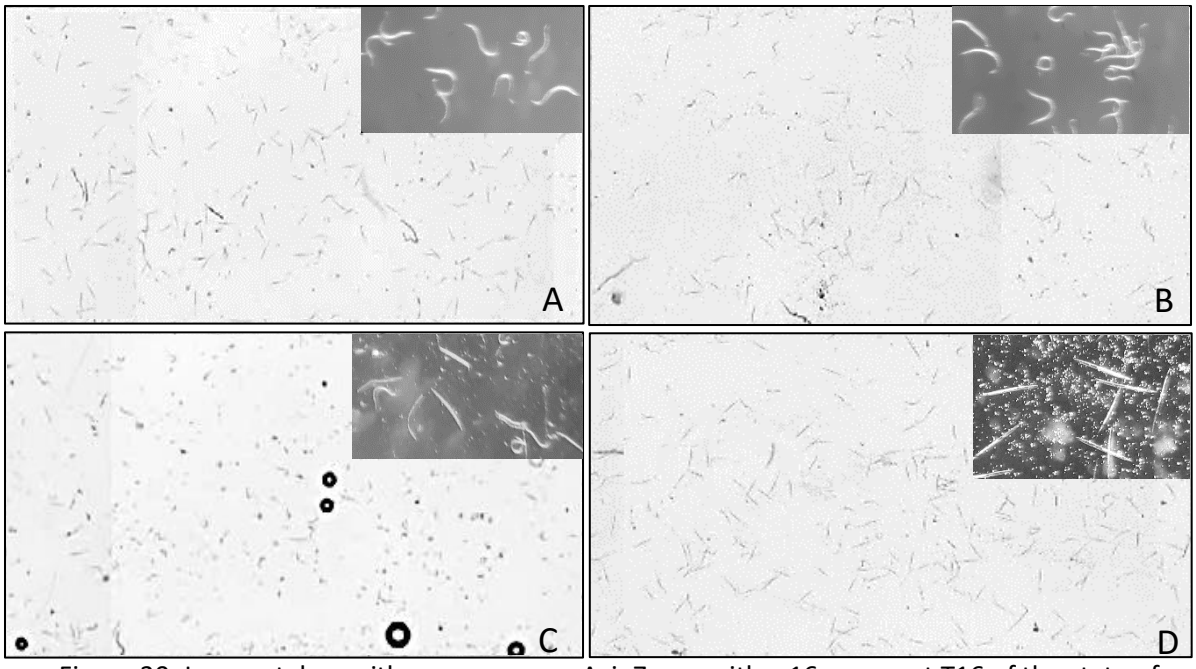


Figure 30: Images taken with a macroscope AxioZoom with a 16x zoom at T16 of the state of nematodes in A: fresh distilled water gel; B: mineral water gel; C: M9 based gel; D: 5 month old distilled water gel and additional pictures taken with a binocular loop (top right corner)

At the end of the 16h incubation period, the nematodes seemed **better in the mineral water-based gel and in the fresh distilled water gel** compared to the older gel used until now (Figure 31). However, the number of motile nematodes was the most significantly different in the mineral water-based gel and we decided to **continue experiments with the mineral-water gel**.

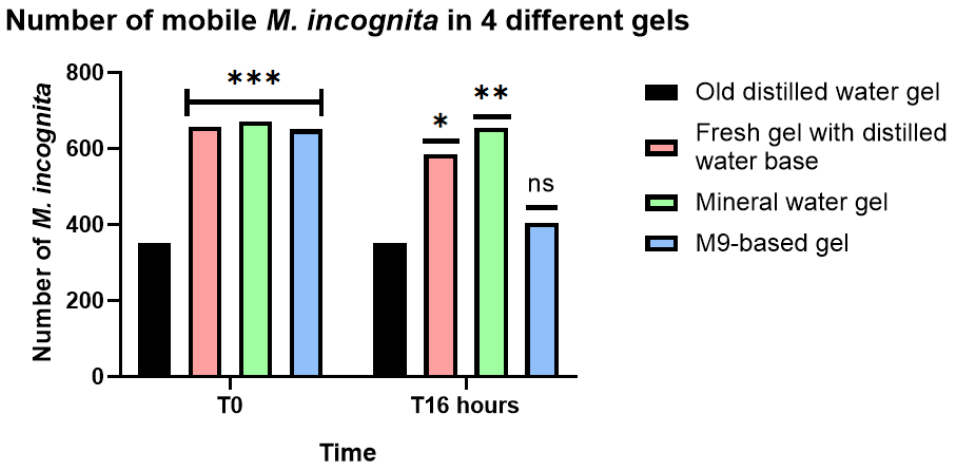


Figure 31: Differences in *M. incognita* motility between the 5 old month distilled water gel and the new gels (n=3, statistical differences obtained by ANOVA and post-hoc Tukey test), non-significant difference was denoted using "NS", significant codes: p-value <0.001 ***, p-value <0.05 ** and p-value <0.1 *.

Before testing the root exudates, we did an experiment including nematodes in the mineral water-based gel and testing distilled water (n=4) and putrescine (n=4) as attractant rather than cadaverine (the results obtained previously with putrescine seemed to be more consistent than those obtained with cadaverine). The kinetics of the chemotaxis indexes for the 4 replicates in each condition showed a similar trend (Figure 32): for water indexes were all stable and for putrescine they were all increasing the first 4h of kinetics and then they decrease. However, when we looked at the ANOVA result (Table 8 in annex), it again indicated that not all the replicates were similar. Despite this, we decided to start the test with exudates in the mineral-water based gel until we find a way to analyse it.

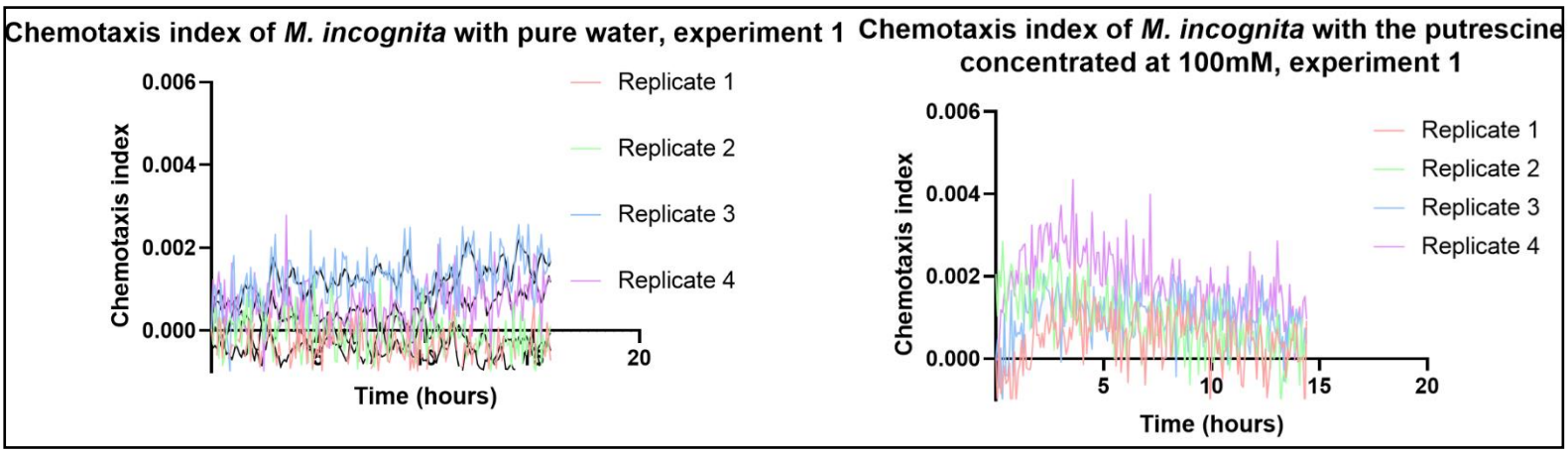


Figure 32: Kinetics of the chemotaxis index for the experiment performed with distilled water and putrescine 100mM for 16 hours (performed in the mineral water based gel)

A repellent molecule to *M. incognita* was found in the literature[37], which is the **lauric acid, repellent at 4mM** according to the article. This molecule is awaiting order and will be tested as a reference

repellent molecule during the remainder of this internship, along with bithiophene, which will also be tested.

1. 2. With the root exudates

For the time being, only a **preliminary test to visualize the attraction of *M. incognita* to tomato root exudates compared to the effect of putrescine as a control** (Figure 33) was carried out. Tomato root exudates appeared to be attractive (with a positive chemotaxis index) and even more attractive than putrescine (maximum chemotaxis index of 0.002 and 0,004 for putrescine and tomato, respectively). Moreover, the attraction observed for the tomato root exudates was constantly increasing, unlike that of putrescine, with a chemotaxis index that was decreasing after 8 hours.

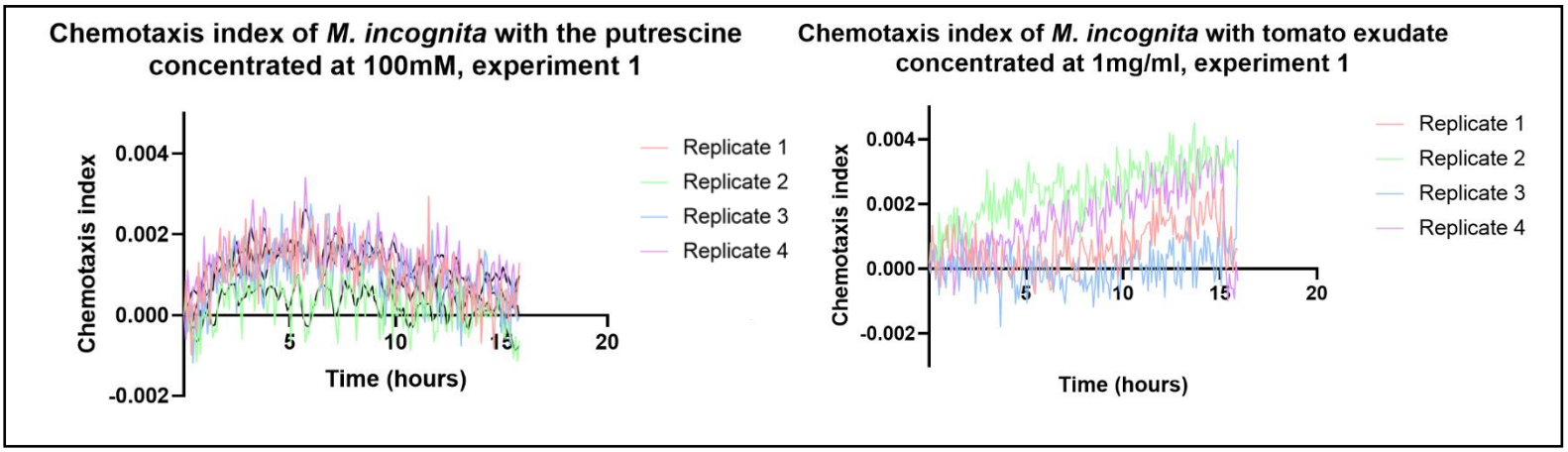


Figure 33: Kinetics of the chemotaxis index for the experiment performed with putrescine 100mM and tomato root exudates for 16 hours (in the mineral water-based gel).

2. Results of the toxicity tests

2. 1. With the diamine molecules

First, the toxicity of the diamines was tested at the concentration used in chemotaxis: 100mM. As shown in the Figure 34, this concentration is **toxic for nematodes**: 0 survivors with each diamine.

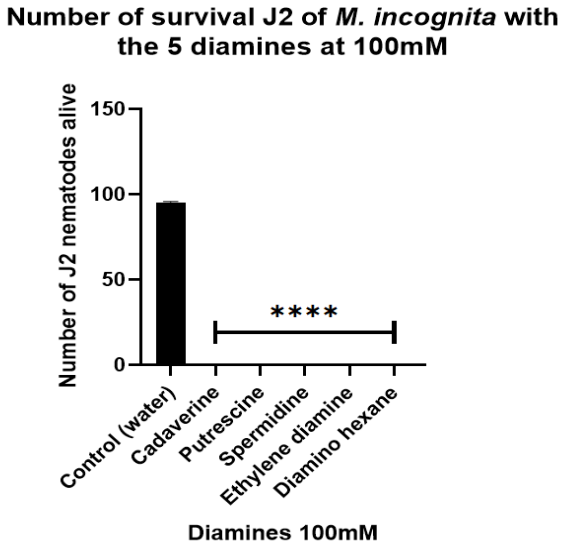
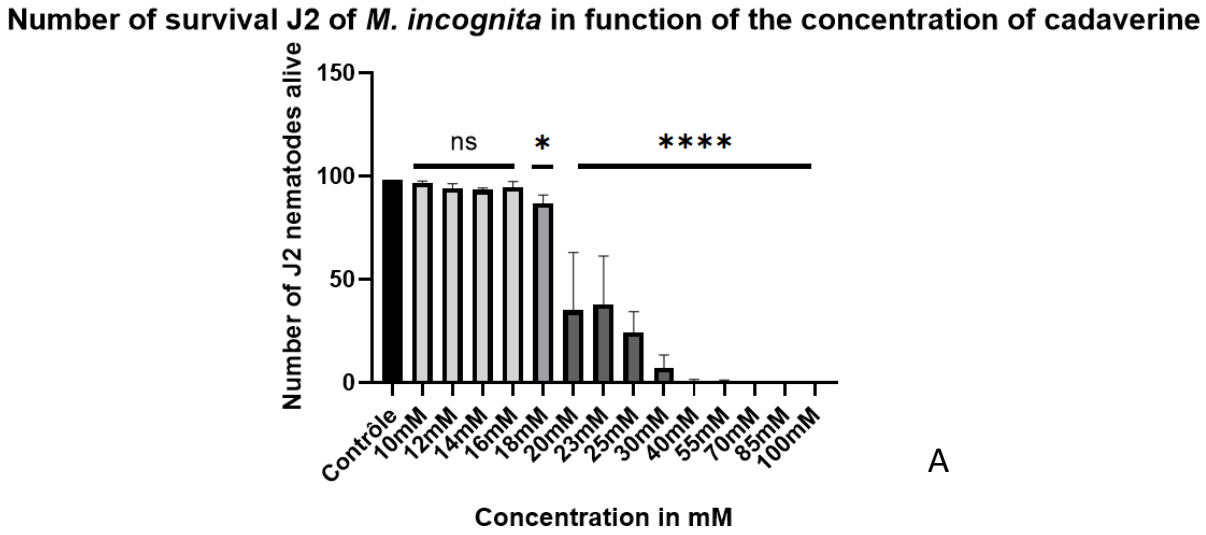


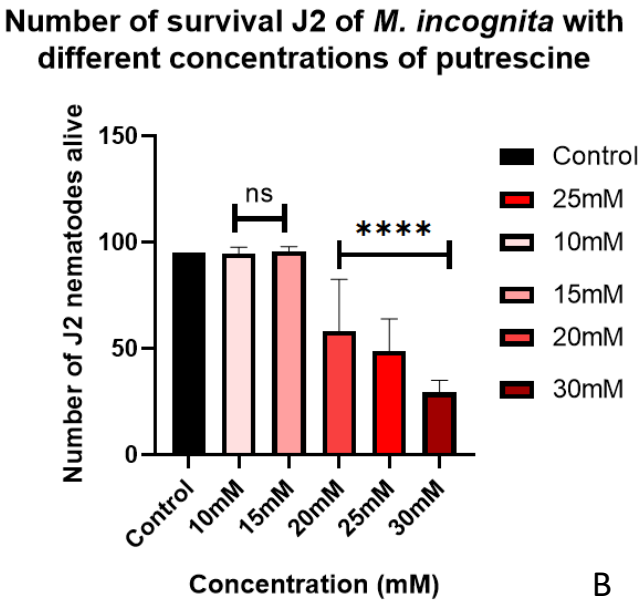
Figure 34 *In-vitro* toxicity test of 5 diamine molecules at 100mM in *M. incognita*, after 24h incubation(n=6 for each diamine tested), significant codes: p-value <0.0001 ****

This concentration for the chemotaxis assays was originally used firstly because it is the one used by the reference article[24] and secondly because when the diamines were added in the chemotaxis slide system they were slowly diffusing in the gel and were therefore less concentrated so nematodes were not directly immersed in pure diamine.

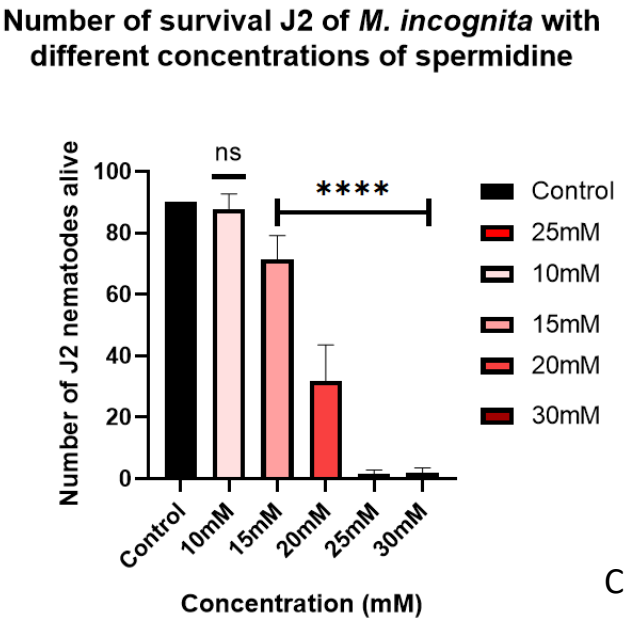
Other concentrations were then investigated (Figure 35), in order to find a **non-toxic dose**. Cadaverine was tested with more concentrations to see if the nematodes were very sensitive to small changes in concentration or not. This allowed us to decide to **test concentrations every 5mM** because there was no difference every 2mM.



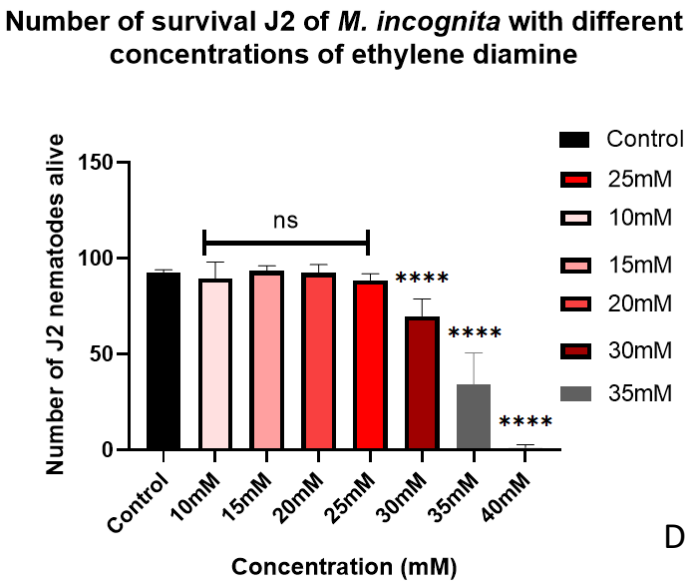
A



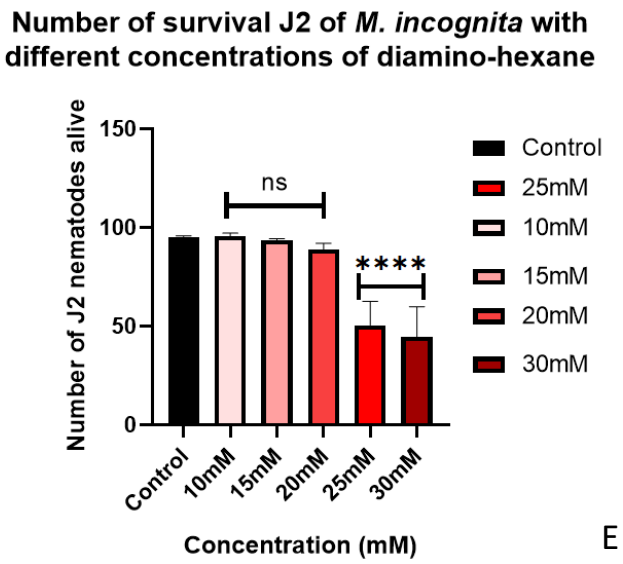
B



C



D



E

Figure 35: Number of *M. incognita* J2 survivals relative to the diamine concentration after 24h incubation. A: Cadaverine; B: Putrescine; C: Spermidine; D: Ethylene diamine; E: Diamino-hexane. Bars represent the mean±SE for n=6. Difference between means of diamine treatments compared to water control were tested using post-hoc Tukey test (non-significant difference was denoted using "NS", significant codes: p-value <0.0001 ****, p-value <0.001 ***, p-value <0.05 ** and p-value <0.1 *).

After plotting the survival curves for each molecule (Figure 9 in annex), we could see that the LD50was around 19mM for cadaverine, 21mM for putrescine, 18mM for spermidine and 25mM for diamino-hexane. Tests at higher concentrations had to be carried out for ethylene diamine, as the concentrations tested did not induce lethality. The LD50 is 33mM for ethylene-diamine.

2. 2. With the repulsive molecule

For bithiophene, the LD50 was slightly below 0.3mM. Lauric acid will also be tested for toxicity, once the product has been received. According to the article[32], lauric acid attracts nematodes at low concentrations (0.5-2.0mM) and consequently causes death (because it is toxic), while high concentrations (4.0mM) repel *M. incognita*.

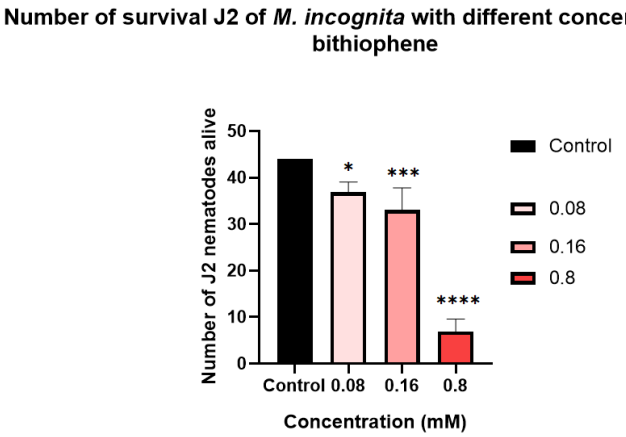


Figure 36: Number of *M. incognita* J2 survivals relative to the bithiophene concentration after 24h incubation. Difference between means of diamine treatments compared to water control were tested using post-hoc Tukey test (non-significant difference was denoted using "NS", significant codes: p-value <0.0001 ****, p-value <0.001 *** and p-value <0.1 *).

3. Discussion

Although the protocol first developed makes it possible to **clearly image *M. incognita*** and to **see through time-lapses the direction they go**, the chemotaxis results obtained with diamines were very heterogeneous. This heterogeneity could be for part attributed to the varying amount of active nematodes in slides, an effect that was dependent on the gel matrix used to include the nematode. Once we had prepared a better gel for the nematodes, we also realized that the **oxygenation of the gel** could also be important and therefore its freshness too. Indeed, we realized that despite the use of the new mineral water-based gel, if we did not aerate it beforehand using a magnetic stirrer left overnight, the nematodes were once again paralyzed in large number.

Furthermore, when we watched the time-lapses, we saw that the nematodes were **highly motile for the first 6h**, then accumulated and **became rigid in the vicinity of the cadaverine and putrescine wells** for the rest of the experiment. The fact that diamines are **toxic at 100mM** (section IV. 2) could explain that at some point in the experiment they become motionless or start to swim in the opposite direction as it was observed for groups of nematodes in response to cadaverine and putrescine. In addition, observation of the behaviour of nematodes in response to tomato root exudates showed that they **continued to move towards the well for the 16h kinetics without being paralyzed**. This can be explained by the fact that in the tests carried out last year by. Lahoreau, it was observed that the root exudates from either tomato or companion plants **were non-toxic to the J2 larvae**. Regarding spermidine, it was observed that attraction occurred after longer time than for cadaverine and putrescine. This could be due to a difference in the speed of diffusion of the chemical and thus a delay in the gradient formation compared to the other diamines, or a lesser attraction effect of this chemical on the nematode attraction.

As the chemotaxis data were particularly variable, we thought of another way we could transform the data to analyse the results, which would no longer allow us to quantify attraction but rather give an indication to whether a molecule is attractive or not. As an example (Figure 37), we propose that if on the one hand, the index of a molecule tested is at least 5% higher than the index observed for water, then it takes the value of 1 and the molecule is attractive. On the other hand, if the index is more than

5% lower than for water, then it takes the value -1 and the molecule could be considered as repulsive. In other case, the index is not different from the water and it therefore takes the value 0.

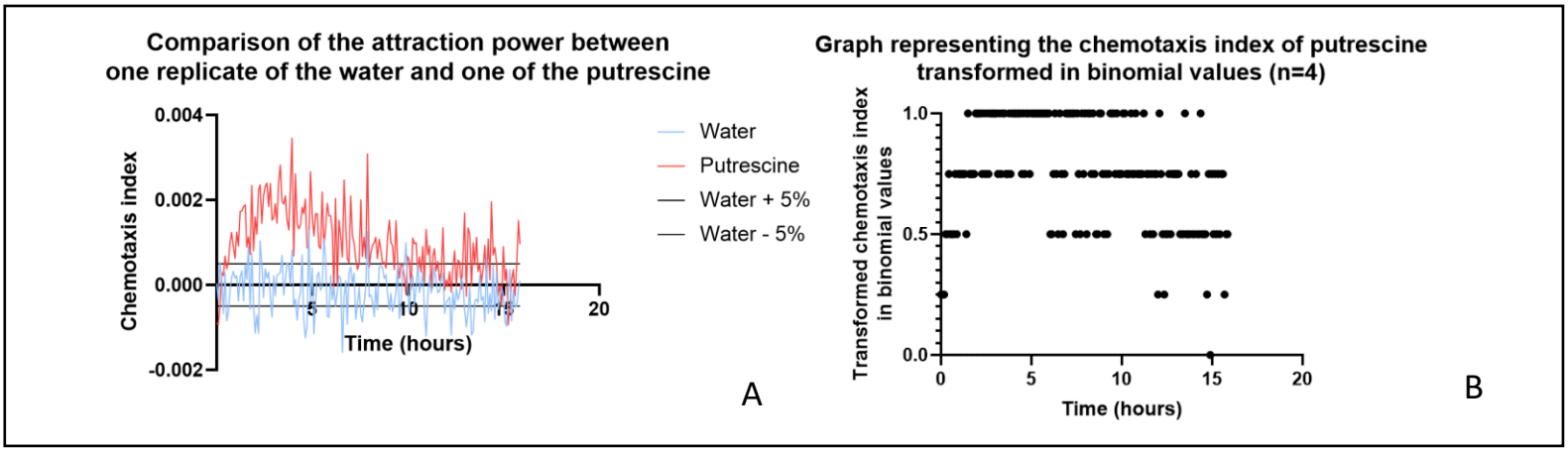


Figure 37: Comparison of the attraction power between water and putrescine at 100mM; A: illustration of the chemotaxis index values with the black lines corresponding to upper and lower 5% for the water index; B: transformed chemotaxis index in binomial values (plotted as the mean of 4 replicates against time).

In this way, we will be able in the future, to run a binomial GLM model using the 1 and 0 values and see which time-points had similar attraction over the 5 diamines (diamines must be re-tested according to the modified protocol before). According to this figure, putrescine can be considered attractive from 1-8h.This is an arbitrary way of interpreting data to at least know whether a molecule can be considered attractive, neutral or repulsive.

The last solution found to analyze the data was to only use replicates that could be grouped based on statistics (e.g. 5 of 12 found similar for each diamine). The average chemotaxis index could then be plotted (Figure 38.A), and an one-way ANOVA with pairwise comparison according to Tukey post-hoc test (Table 10 in annex) could be applied. Ethylene diamine and diamino hexane are **similar** and are **similar to water** (as described in the article by Oota et *al.*[24]). Spermidine, cadaverine and putrescine are all **significantly different from water**. However, we expected cadaverine and putrescine to be very different from water and spermidine a little less (according to Oota et *al*), but over the 16 hours they are all 3 equally different from water (p-value<0.0001).

If we look at the behaviour of nematodes over the first 6 hours only (Figure 38.B) and test the effect of the diamines by ANOVA only at that timepoint (Table 11 in annex), cadaverine and putrescine are similar (as attractive). Furthermore, spermidine is both different from water and from the cadaverine and putrescine, so spermidine showed an intermediate attraction power on *M. incognita*.

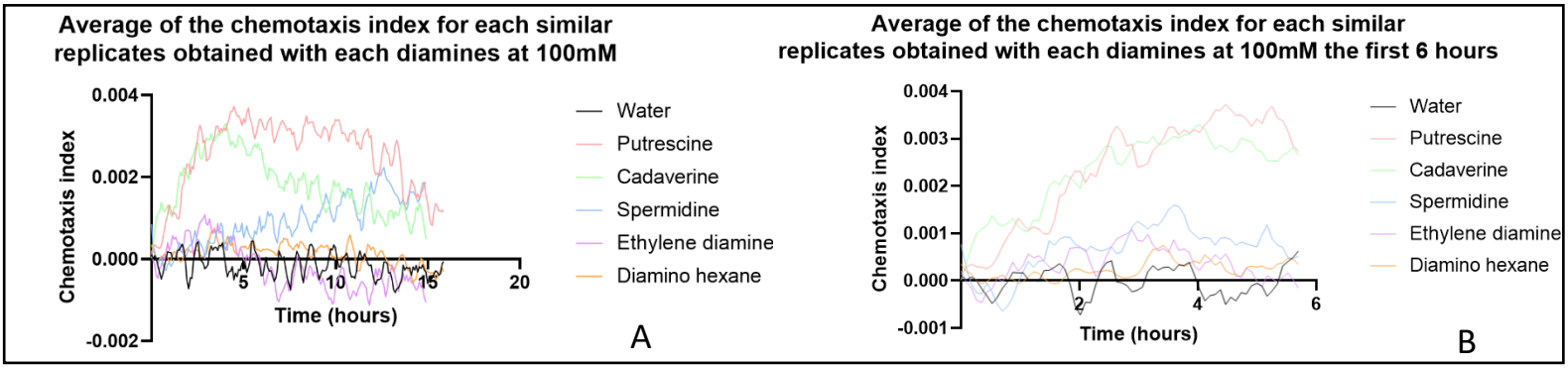


Figure 38: Average of the chemotaxis index for each replicates obtained with each diamines at 100mM, A: during the 16 hours of experiment; B: during the first 6 hours of experiment

This analysis of the data seems to be the most suitable found for the moment although we do not exploit the 12 replicates. Further experiments are required to obtain more than 5 similar replicates for analysis.

If we now compare the preliminary test carried out with putrescine and tomato using this method of analysis, we find that the chemotaxis index of tomato is significantly different (p-value<0.0001) from that of putrescine if we compare them during the 16 hours (Table 12 in annex). However, if we compare them from the first 6 hours (Table 13 in annex), we notice that they are not as much different (p-value = 0.0126).

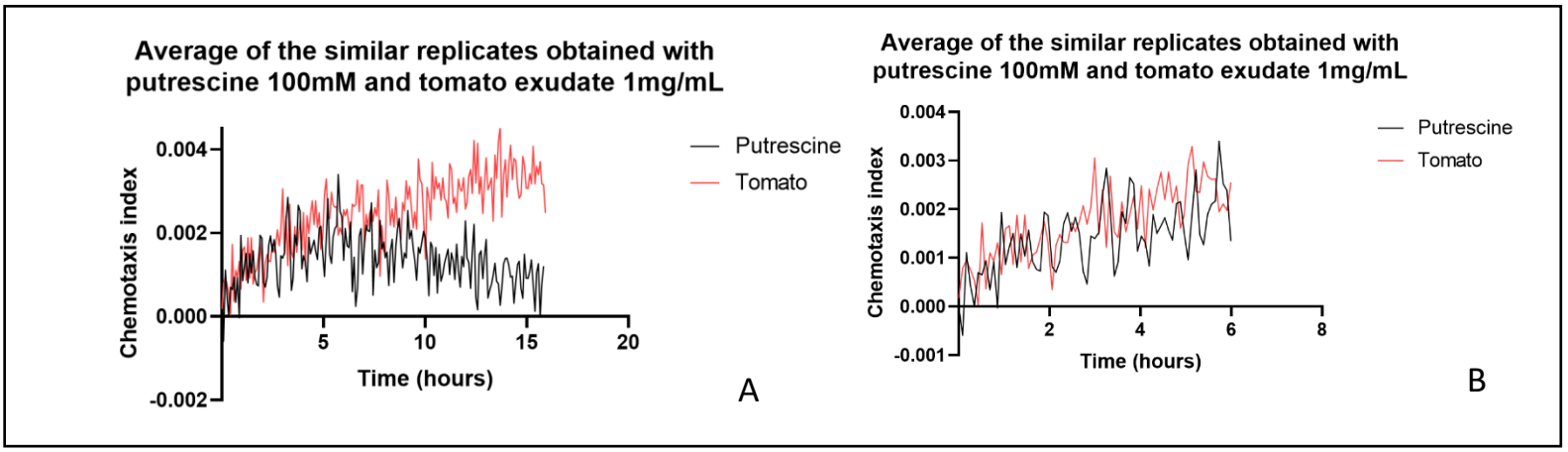


Figure 39: Average of the chemotaxis index for a mean of n=5 replicates obtained with putrescine and tomato exudate at 100mM, A: during the 16 hours of experiment; B: during the first 6 hours of experiment

Despite replicates that are not always similar, the macroscope remains an **effective tool for imaging nematode behaviour** over a period of time. The protocols developed until now to study *M. incognita* consist in imaging them at time T0 and after a certain time, in petri dishes divided into several areas. Like in the experiment carried out by Pinyi Wang et *al* (2019)[38], where around 100 to 150 J2 were placed in the middle of a petri dish divided into three parts and compounds were deposited on the other parts. After 4 h in the dark at 28°C, the nematodes were counted under the microscope and the chemotactic index (CI) was calculated. In these cases, nematode behaviour is not assessed kinetically, so it cannot be observed over the entire duration of contact with the chemical as is the case with the macroscope, where the entire kinetics can be observed.

Other protocols exist and involve placing nematodes in modified Y-chamber olfactometers filled with Pluronic gel. By proposing two routes to a source of attractant, one long and the other short, or to one attractive source and the other neutral, the routes taken by the nematodes can be imaged, as explained in the study from Andy M. Reynolds (2010)[39]. However, once again, only images at T0 and Tn are taken, and not over the entire experiment.

V. Conclusion and perspectives

This study made it possible to **improve the imaging protocol** and the analysis of the chemotaxis behaviour response of nematodes with the macroscope. The gel used so far has also been improved to compensate for the heterogeneity of the results. For the time-being, **no conclusions can be drawn from the tests with the root exudates**, however these will be tested during the rest of this internship

as well as the diamines that will be imaged again but this time with a more adapted protocol and allowing to hopefully get more consistent results with more motile nematodes. **Cf annex pages 41 to 48.**

However, we can conclude that the time-lapses taken with the macroscope are good ways for observing the kinetics of the nematode response: we can see them quite clearly for a long period of time compared to other protocols.

Perspective for improving the analysis of results obtained using the protocol will be discussed in partnership with researchers from the phenotyping platform, in order to **find a way to better analyze the time-lapses and maybe use an analysis by nematode using tracking**. It would also be interesting to carry out tests to determine the speed at which gradients are established for each molecule tested by coloring molecules, for example using Dansylcadaverine, an autofluorescent dye that can be added to the cadaverine solution.

An improved protocol was produced during the first part of this course and is available on page 44.

EXPERTISE GAINED

This internship allowed me to acquire a lot of economic and physiological knowledge on root-knot nematodes, especially on *Meloidogyne incognita*. I learned how to use the macroscope for studies of the chemotaxis behaviour of nematodes and also for many other functions that Olivier taught me such as programming it to observe fluorescent samples or to take photographs and not time-lapses. I also learned to use some functionality of the R statistics software and I learned how to choose some statistical models based on my data. This internship also allowed me to strengthen my ability to work in a team by having to share biological material (nematodes) and sometimes compromise when there was not enough for the whole team. This was also the case when booking the macroscope, much used by other teams. I was lucky enough to be surrounded by caring people and to be able to carry out most of my experiences without any setbacks.

Finally, this internship enabled me to improve my writing in English, thanks to the many corrections made by my supervisors.

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ANNEX

PdS	NEMATICIDE				INSECTICIDE								
	M. incognita	*M. arenaria*	*M. javanica*	autres nematodes	Aleurode des serres (*Trialeurodes vaporariorum*)	Aleurode du tabac (*Bemisia tabaci*)	Autres aleurodes	Pucerons	Noctuelle de la tomate (*Helicovepa armigera*)	Araignée rouge des serres (*Tetranychus urticae*)	Thrips du tabac et de l'oignon (*Thrips tabaci*)	Punaises (*Lygus hesperus ou Apolygus lucorum*)	Chenille foreuse de l'aubergine (*Lecuinodes orbonalis*)
Tagetes erecta CrackerJack, Inner Mongolia, Golden Age	+ (toxique)	+ (toxique)		+ (*Pratylenchus penetrans, Nacobbus aberrans*)			+	+ (pucerons ailés & *Myzus persicae*)	+ (attractif & piège)	+ (attractif)			+ (répulsif COV)
T. patula Nana, LIDEA, Single Gold, Creole, Sparky, Golden Guardian, French Dwarf Double, Mix	+ (toxique)	+ (toxique)	+ (toxique)	+ (*P. penetrans, Rotylenchulus renifomis, Heterodera glycines*)	+ (répulsif COV)	+ (toxique)		+ (*Myzus persicae*)	+		=?	+ (toxique)	+ (répulsif COV)
Foeniculum vulgare Rondo, Fino	+ (toxique)	+ (toxique)	+ (toxique)				+ (attractif, favorise parasitoïde *Peristenus spretus*)	+ (répulsif)		+ (répulsif)		+	
Fagopyrum esculentum	+ (toxique)	+ (toxique)				+ (réduit)	+ (attractif, favorise parasitoïde *Peristenus spretus*)	+				+	
Datura stramonium			+ (toxique)				+						

Figure 1: Service plants used to control certain soil and airborne pathogens in tomato crops found in the literature

Exudate	Quantity of powder
T. erecta	0.010g
T. patula	0.00g
C. juncea	0.00g

Figure 2: Quantity of pre-flowering exudates available from the exudate collection dated to 2022

Cadaverine	$Ci \times Vi = Cf \times Vf \rightarrow Vi = (Cf \times Vf)/Ci$ $= (0.1M \times 0.5.10^{-3}L)/8.54M = 6.10^{-6}L =$ **6ul in 0.5mL of filtered water**
Putrescine	**44ul of stock in 0.5mL of filtered water**
Spermidine	**7.8ul of stock in 0.5mL of filtered water**
Ethylenediamine	**3.3ul of stock in 0.5mL of filtered water**
1,6-Diaminohexane	**5.8ul of stock in 0.5mL of filtered water**

Figure 3: Table of the dilutions to be realised to obtain 100mM diamines

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CAD 1 vs. CAD 2	0.004211	0.003354 to 0.005067	Yes	****	<0.0001
CAD 1 vs. CAD 3	0.005773	0.004917 to 0.006630	Yes	****	<0.0001
CAD 1 vs. CAD 4	0.002334	0.001478 to 0.003191	Yes	****	<0.0001
CAD 1 vs. PUTR 1	0.005971	0.005115 to 0.006828	Yes	****	<0.0001
CAD 1 vs. PUTR 2	0.004707	0.003850 to 0.005563	Yes	****	<0.0001
CAD 1 vs. PUTR 3	0.003723	0.002867 to 0.004580	Yes	****	<0.0001
CAD 1 vs. PUTR 4	0.007187	0.006331 to 0.008044	Yes	****	<0.0001
CAD 2 vs. CAD 3	0.001562	0.0007057 to 0.002419	Yes	****	<0.0001
CAD 2 vs. CAD 4	-0.001877	-0.002733 to -0.001020	Yes	****	<0.0001
CAD 2 vs. PUTR 1	0.001760	0.0009036 to 0.002617	Yes	****	<0.0001
CAD 2 vs. PUTR 2	0.0004960	-0.0003606 to 0.001353	No	ns	0.6498
CAD 2 vs. PUTR 3	-0.0004876	-0.001344 to 0.0003690	No	ns	0.6698
CAD 2 vs. PUTR 4	0.002976	0.002120 to 0.003833	Yes	****	<0.0001
CAD 3 vs. CAD 4	-0.003439	-0.004295 to -0.002582	Yes	****	<0.0001
CAD 3 vs. PUTR 1	0.0001980	-0.0006586 to 0.001055	No	ns	0.9970
CAD 3 vs. PUTR 2	-0.001066	-0.001923 to -0.0002097	Yes	**	0.0041
CAD 3 vs. PUTR 3	-0.002050	-0.002906 to -0.001193	Yes	****	<0.0001
CAD 3 vs. PUTR 4	0.001414	0.0005575 to 0.002271	Yes	****	<0.0001
CAD 4 vs. PUTR 1	0.003637	0.002780 to 0.004493	Yes	****	<0.0001
CAD 4 vs. PUTR 2	0.002372	0.001516 to 0.003229	Yes	****	<0.0001
CAD 4 vs. PUTR 3	0.001389	0.0005323 to 0.002246	Yes	****	<0.0001
CAD 4 vs. PUTR 4	0.004853	0.003996 to 0.005709	Yes	****	<0.0001
PUTR 1 vs. PUTR 2	-0.001264	-0.002121 to -0.0004077	Yes	***	0.0002
PUTR 1 vs. PUTR 3	-0.002248	-0.003104 to -0.001391	Yes	****	<0.0001
PUTR 1 vs. PUTR 4	0.001216	0.0003596 to 0.002073	Yes	***	0.0005
PUTR 2 vs. PUTR 3	-0.0009836	-0.001840 to -0.0001270	Yes	*	0.0118
PUTR 2 vs. PUTR 4	0.002480	0.001624 to 0.003337	Yes	****	<0.0001
PUTR 3 vs. PUTR 4	0.003464	0.002607 to 0.004321	Yes	****	<0.0001

Table 4: Results from one-way ANOVA pairwise comparison with Tukey post-hoc test of the fourth replicates made with cadaverine 100mM (named CADn) and putrescine 100mM (named PUTRn)

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CAD 100mM 4 vs. CAD 2	-0.002073	-0.002494 to -0.001652	Yes	****	<0.0001
CAD 100mM 4 vs. CAD 3	-0.0004423	-0.0008633 to -2.136e-005	Yes	*	0.0314
CAD 100mM 4 vs. CAD 4	-0.002488	-0.002909 to -0.002068	Yes	****	<0.0001
CAD 100mM 4 vs. SPER 1	-0.0005150	-0.0009360 to -9.407e-005	Yes	**	0.0052
CAD 100mM 4 vs. SPER 2	-0.001908	-0.002329 to -0.001487	Yes	****	<0.0001
CAD 100mM 4 vs. SPER 3	-0.0005731	-0.0009940 to -0.0001521	Yes	***	0.0010
CAD 100mM 4 vs. SPER 4	-0.0006777	-0.001099 to -0.0002568	Yes	****	<0.0001
CAD 2 vs. CAD 3	0.001631	0.001210 to 0.002052	Yes	****	<0.0001
CAD 2 vs. CAD 4	-0.0004154	-0.0008363 to 5.602e-006	No	ns	0.0562
CAD 2 vs. SPER 1	0.001558	0.001137 to 0.001979	Yes	****	<0.0001
CAD 2 vs. SPER 2	0.0001653	-0.0002557 to 0.0005862	No	ns	0.9344
CAD 2 vs. SPER 3	0.001500	0.001079 to 0.001921	Yes	****	<0.0001
CAD 2 vs. SPER 4	0.001395	0.0009744 to 0.001816	Yes	****	<0.0001
CAD 3 vs. CAD 4	-0.002046	-0.002467 to -0.001625	Yes	****	<0.0001
CAD 3 vs. SPER 1	-7.270e-005	-0.0004937 to 0.0003483	No	ns	0.9995
CAD 3 vs. SPER 2	-0.001466	-0.001886 to -0.001045	Yes	****	<0.0001
CAD 3 vs. SPER 3	-0.0001307	-0.0005517 to 0.0002902	No	ns	0.9818
CAD 3 vs. SPER 4	-0.0002354	-0.0006564 to 0.0001856	No	ns	0.6890
CAD 4 vs. SPER 1	0.001973	0.001552 to 0.002394	Yes	****	<0.0001
CAD 4 vs. SPER 2	0.0005806	0.0001597 to 0.001002	Yes	***	0.0008
CAD 4 vs. SPER 3	0.001915	0.001494 to 0.002336	Yes	****	<0.0001
CAD 4 vs. SPER 4	0.001811	0.001390 to 0.002232	Yes	****	<0.0001
SPER 1 vs. SPER 2	-0.001393	-0.001814 to -0.0009718	Yes	****	<0.0001
SPER 1 vs. SPER 3	-5.803e-005	-0.0004790 to 0.0003629	No	ns	0.9999
SPER 1 vs. SPER 4	-0.0001627	-0.0005837 to 0.0002583	No	ns	0.9395
SPER 2 vs. SPER 3	0.001335	0.0009138 to 0.001756	Yes	****	<0.0001
SPER 2 vs. SPER 4	0.001230	0.0008091 to 0.001651	Yes	****	<0.0001
SPER 3 vs. SPER 4	-0.0001047	-0.0005256 to 0.0003163	No	ns	0.9952

Table 5: Results from one-way ANOVA pairwise comparison with Tukey post-hoc test of the fourth replicates made with cadaverine 100mM (named CADn) and spermidine 100mM (named SPERn) in red: similar replicates

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CAD 1 vs. CAD 2	-0.001237	-0.001464 to -0.001010	Yes	****	<0.0001
CAD 1 vs. CAD 3	-0.001165	-0.001392 to -0.0009380	Yes	****	<0.0001
CAD 1 vs. CAD 4	-0.002745	-0.002972 to -0.002518	Yes	****	<0.0001
CAD 1 vs. ETHYL 1	0.001146	0.0009187 to 0.001373	Yes	****	<0.0001
CAD 1 vs. ETHYL 2	-0.001108	-0.001335 to -0.0008806	Yes	****	<0.0001
CAD 1 vs. ETHYL 3	0.0005264	0.0002994 to 0.0007534	Yes	****	<0.0001
CAD 1 vs. ETHYL 4	0.0009985	0.0007714 to 0.001225	Yes	****	<0.0001
CAD 2 vs. CAD 3	7.243e-005	-0.0001544 to 0.0002993	No	ns	0.9788
CAD 2 vs. CAD 4	-0.001508	-0.001735 to -0.001281	Yes	****	<0.0001
CAD 2 vs. ETHYL 1	0.002383	0.002156 to 0.002610	Yes	****	<0.0001
CAD 2 vs. ETHYL 2	0.0001297	-9.731e-005 to 0.0003568	No	ns	0.6654
CAD 2 vs. ETHYL 3	0.001764	0.001537 to 0.001991	Yes	****	<0.0001
CAD 2 vs. ETHYL 4	0.002236	0.002009 to 0.002463	Yes	****	<0.0001
CAD 3 vs. CAD 4	-0.001580	-0.001807 to -0.001353	Yes	****	<0.0001
CAD 3 vs. ETHYL 1	0.002311	0.002084 to 0.002538	Yes	****	<0.0001
CAD 3 vs. ETHYL 2	5.730e-005	-0.0001697 to 0.0002843	No	ns	0.9948
CAD 3 vs. ETHYL 3	0.001691	0.001464 to 0.001918	Yes	****	<0.0001
CAD 3 vs. ETHYL 4	0.002163	0.001936 to 0.002390	Yes	****	<0.0001
CAD 4 vs. ETHYL 1	0.003891	0.003664 to 0.004118	Yes	****	<0.0001
CAD 4 vs. ETHYL 2	0.001638	0.001410 to 0.001865	Yes	****	<0.0001
CAD 4 vs. ETHYL 3	0.003272	0.003044 to 0.003499	Yes	****	<0.0001
CAD 4 vs. ETHYL 4	0.003744	0.003517 to 0.003971	Yes	****	<0.0001
ETHYL 1 vs. ETHYL 2	-0.002253	-0.002481 to -0.002026	Yes	****	<0.0001
ETHYL 1 vs. ETHYL 3	-0.0006193	-0.0008465 to -0.0003921	Yes	****	<0.0001
ETHYL 1 vs. ETHYL 4	-0.0001473	-0.0003745 to 7.994e-005	No	ns	0.5052
ETHYL 2 vs. ETHYL 3	0.001634	0.001407 to 0.001861	Yes	****	<0.0001
ETHYL 2 vs. ETHYL 4	0.002106	0.001879 to 0.002333	Yes	****	<0.0001
ETHYL 3 vs. ETHYL 4	0.0004721	0.0002449 to 0.0006993	Yes	****	<0.0001

Table 6: Results from one-way ANOVA pairwise comparison with Tukey post-hoc test of the fourth replicates made with cadaverine 100mM (named CADn) and ethylenediamine 100mM (named ETHYLn), in red: similar replicates

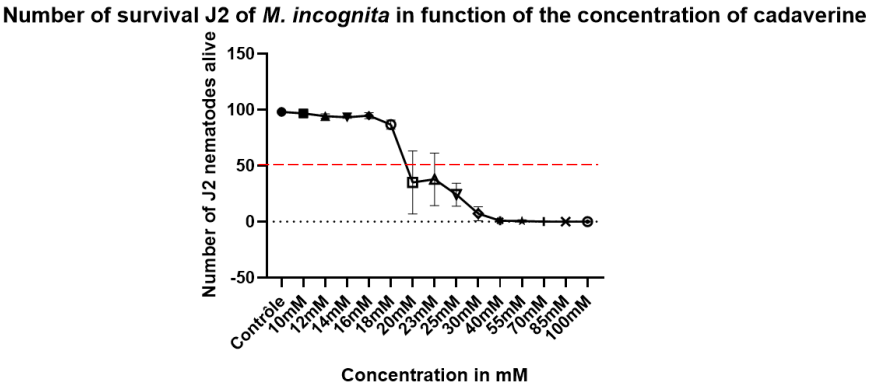
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CAD 1 vs. CAD 2	-0.0007821	-0.0009062 to -0.0006580	Yes	****	<0.0001
CAD 1 vs. CAD 3	-0.001728	-0.001852 to -0.001604	Yes	****	<0.0001
CAD 1 vs. CAD 4	-0.0007526	-0.0008767 to -0.0006285	Yes	****	<0.0001
CAD 1 vs. DIAMINO 1	0.0003054	0.0001813 to 0.0004295	Yes	****	<0.0001
CAD 1 vs. DIAMINO 2	5.824e-005	-6.588e-005 to 0.0001824	No	ns	0.8466
CAD 1 vs. DIAMINO 3	-0.0005588	-0.0006829 to -0.0004347	Yes	****	<0.0001
CAD 1 vs. DIAMINO 4	0.001311	0.001187 to 0.001435	Yes	****	<0.0001
CAD 2 vs. CAD 3	-0.0009462	-0.001070 to -0.0008221	Yes	****	<0.0001
CAD 2 vs. CAD 4	2.950e-005	-9.463e-005 to 0.0001536	No	ns	0.9964
CAD 2 vs. DIAMINO 1	0.001087	0.0009634 to 0.001212	Yes	****	<0.0001
CAD 2 vs. DIAMINO 2	0.0008403	0.0007162 to 0.0009645	Yes	****	<0.0001
CAD 2 vs. DIAMINO 3	0.0002233	9.915e-005 to 0.0003474	Yes	****	<0.0001
CAD 2 vs. DIAMINO 4	0.002093	0.001969 to 0.002217	Yes	****	<0.0001
CAD 3 vs. CAD 4	0.0009757	0.0008516 to 0.001100	Yes	****	<0.0001
CAD 3 vs. DIAMINO 1	0.002034	0.001910 to 0.002158	Yes	****	<0.0001
CAD 3 vs. DIAMINO 2	0.001787	0.001662 to 0.001911	Yes	****	<0.0001
CAD 3 vs. DIAMINO 3	0.001170	0.001045 to 0.001294	Yes	****	<0.0001
CAD 3 vs. DIAMINO 4	0.003039	0.002915 to 0.003164	Yes	****	<0.0001
CAD 4 vs. DIAMINO 1	0.001058	0.0009339 to 0.001182	Yes	****	<0.0001
CAD 4 vs. DIAMINO 2	0.0008108	0.0006867 to 0.0009350	Yes	****	<0.0001
CAD 4 vs. DIAMINO 3	0.0001938	6.965e-005 to 0.0003179	Yes	****	<0.0001
CAD 4 vs. DIAMINO 4	0.002064	0.001940 to 0.002188	Yes	****	<0.0001
DIAMINO 1 vs. DIAMINO 2	-0.0002472	-0.0003713 to -0.0001230	Yes	****	<0.0001
DIAMINO 1 vs. DIAMINO 3	-0.0008642	-0.0009883 to -0.0007401	Yes	****	<0.0001
DIAMINO 1 vs. DIAMINO 4	0.001006	0.0008816 to 0.001130	Yes	****	<0.0001
DIAMINO 2 vs. DIAMINO 3	-0.0006171	-0.0007412 to -0.0004929	Yes	****	<0.0001
DIAMINO 2 vs. DIAMINO 4	0.001253	0.001129 to 0.001377	Yes	****	<0.0001
DIAMINO 3 vs. DIAMINO 4	0.001870	0.001746 to 0.001994	Yes	****	<0.0001

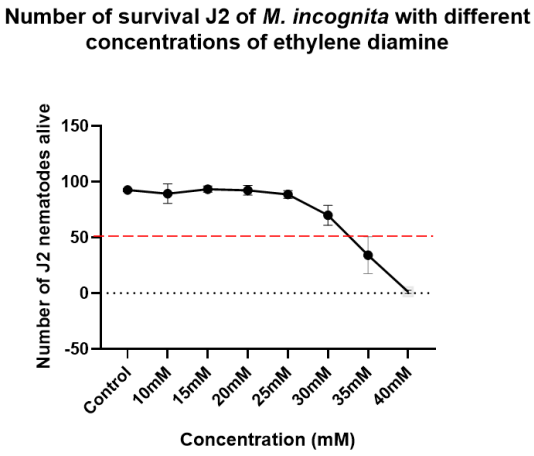
Table 7: Results from one-way ANOVA pairwise comparison with Tukey post-hoc test of the fourth replicates made with cadaverine 100mM (named CADn) and diaminohexane 100mM (named DIAMINOn), in red: similar replicate

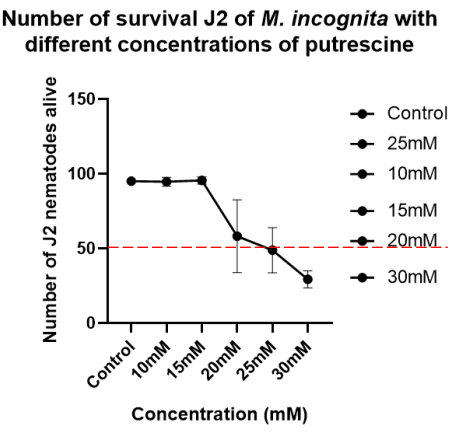
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
water 1 vs. water 2	-0.0003607	-0.0005658 to -0.0001556	Yes	****	<0.0001
water 1 vs. water 3	-0.001751	-0.001956 to -0.001546	Yes	****	<0.0001
water 1 vs. water 4	-0.001116	-0.001321 to -0.0009104	Yes	****	<0.0001
water 1 vs. PUTR 1	-0.0009683	-0.001173 to -0.0007632	Yes	****	<0.0001
water 1 vs. PUTR 2	-0.001423	-0.001628 to -0.001218	Yes	****	<0.0001
water 1 vs. PUTR 3	-0.001528	-0.001733 to -0.001323	Yes	****	<0.0001
water 1 vs. PUTR 4	-0.002352	-0.002557 to -0.002147	Yes	****	<0.0001
water 2 vs. water 3	-0.001391	-0.001596 to -0.001186	Yes	****	<0.0001
water 2 vs. water 4	-0.0007548	-0.0009600 to -0.0005497	Yes	****	<0.0001
water 2 vs. PUTR 1	-0.0006076	-0.0008127 to -0.0004025	Yes	****	<0.0001
water 2 vs. PUTR 2	-0.001062	-0.001267 to -0.0008571	Yes	****	<0.0001
water 2 vs. PUTR 3	-0.001167	-0.001372 to -0.0009621	Yes	****	<0.0001
water 2 vs. PUTR 4	-0.001991	-0.002197 to -0.001786	Yes	****	<0.0001
water 3 vs. water 4	0.0006358	0.0004307 to 0.0008409	Yes	****	<0.0001
water 3 vs. PUTR 1	0.0007830	0.0005779 to 0.0009882	Yes	****	<0.0001
water 3 vs. PUTR 2	0.0003284	0.0001233 to 0.0005335	Yes	****	<0.0001
water 3 vs. PUTR 3	0.0002234	1.829e-005 to 0.0004286	Yes	*	0.0217
water 3 vs. PUTR 4	-0.0006008	-0.0008059 to -0.0003956	Yes	****	<0.0001
water 4 vs. PUTR 1	0.0001472	-5.790e-005 to 0.0003524	No	ns	0.3653
water 4 vs. PUTR 2	-0.0003074	-0.0005125 to -0.0001023	Yes	***	0.0002
water 4 vs. PUTR 3	-0.0004124	-0.0006175 to -0.0002072	Yes	****	<0.0001
water 4 vs. PUTR 4	-0.001237	-0.001442 to -0.001031	Yes	****	<0.0001
PUTR 1 vs. PUTR 2	-0.0004546	-0.0006598 to -0.0002495	Yes	****	<0.0001
PUTR 1 vs. PUTR 3	-0.0005596	-0.0007647 to -0.0003545	Yes	****	<0.0001
PUTR 1 vs. PUTR 4	-0.001384	-0.001589 to -0.001179	Yes	****	<0.0001
PUTR 2 vs. PUTR 3	-0.0001050	-0.0003101 to 0.0001002	No	ns	0.7781
PUTR 2 vs. PUTR 4	-0.0009292	-0.001134 to -0.0007240	Yes	****	<0.0001
PUTR 3 vs. PUTR 4	-0.0008242	-0.001029 to -0.0006191	No	ns	0.3653

Table 8: Results from one-way ANOVA pairwise comparison with Tukey post-hoc test of the fourth replicates made with pure water versus putrecine 100mM (named PUTRn), in red: similar replicates

Survival curves obtained with the *in-vitro* toxicity tests:







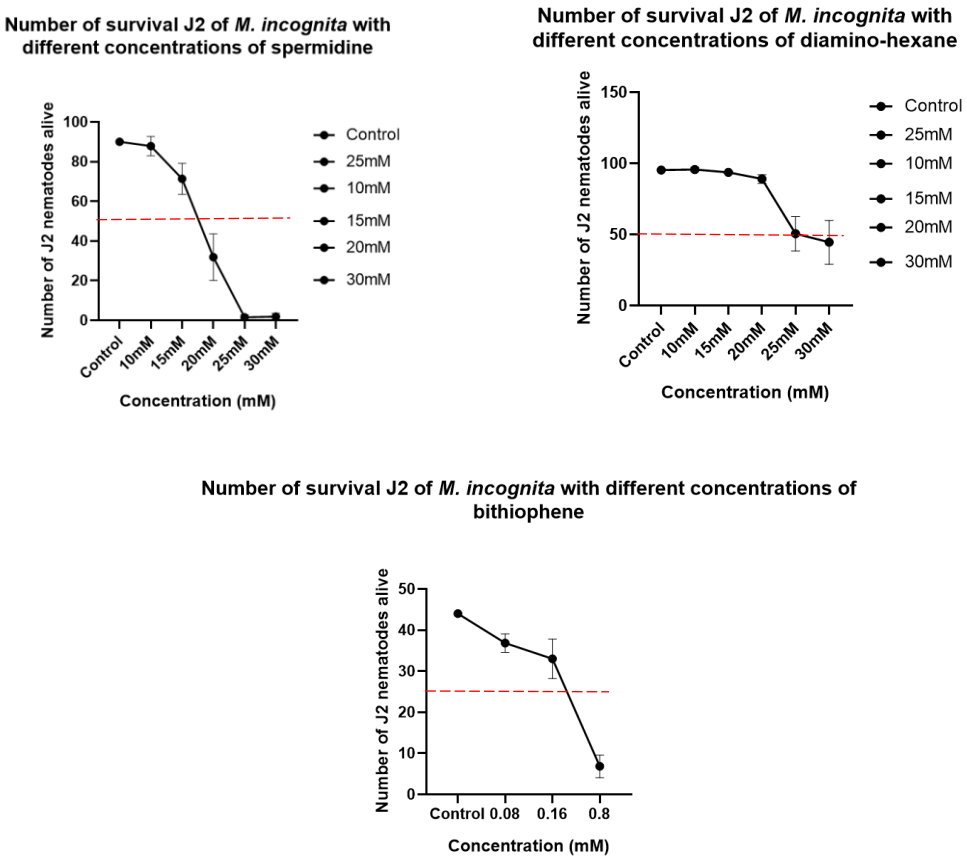


Figure 9: survival curves obtained with the *in-vitro* toxicity tests with all the molecules tested after 24 hours

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Water vs. Putrescine	-0.002606	-0.002822 to -0.002389	Yes	****	<0.0001
Water vs. Cadaverine	-0.001971	-0.002196 to -0.001746	Yes	****	<0.0001
Water vs. Spermidine	-0.001071	-0.001296 to -0.0008463	Yes	****	<0.0001
Water vs. Ethylene diamine	5.649e-005	-0.0001685 to 0.0002815	No	ns	0.9800
Water vs. Diamino hexane	6.732e-005	-0.0001287 to 0.0002633	No	ns	0.9242
Putrescine vs. Cadaverine	0.0006345	0.0004145 to 0.0008545	Yes	****	<0.0001
Putrescine vs. Spermidine	0.001534	0.001314 to 0.001754	Yes	****	<0.0001
Putrescine vs. Ethylene diamine	0.002662	0.002442 to 0.002882	Yes	****	<0.0001
Putrescine vs. Diamino hexane	0.002673	0.002483 to 0.002863	Yes	****	<0.0001
Cadaverine vs. Spermidine	0.0008999	0.0006715 to 0.001128	Yes	****	<0.0001
Cadaverine vs. Ethylene diamine	0.002028	0.001799 to 0.002256	Yes	****	<0.0001
Cadaverine vs. Diamino hexane	0.002039	0.001839 to 0.002239	Yes	****	<0.0001
Spermidine vs. Ethylene diamine	0.001128	0.0008993 to 0.001356	Yes	****	<0.0001
Spermidine vs. Diamino hexane	0.001139	0.0009386 to 0.001339	Yes	****	<0.0001
Ethylene diamine vs. Diamino hexane	1.083e-005	-0.0001892 to 0.0002108	No	ns	>0.9999

Table 10: Comparison of the average chemotaxis index obtained with each diamines at 100mM during the 16 hours of experiment using one-way ANOVA pairwise comparison with Tukey post-hoc test, non-significant difference: “NS”, significant codes: p-value <0.0001 ****

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Water vs. Putrescine	-0.002347	-0.002735 to -0.001960	Yes	****	<0.0001
Water vs. Cadaverine	-0.002302	-0.002689 to -0.001914	Yes	****	<0.0001
Water vs. Spermidine	-0.0006845	-0.001072 to -0.0002969	Yes	****	<0.0001
Water vs. Ethylene diamine	-0.0003713	-0.0007589 to 1.638e-005	No	ns	0.0693
Water vs. Diamino hexane	-0.0002470	-0.0006346 to 0.0001407	No	ns	0.4511
Putrescine vs. Cadaverine	4.546e-005	-0.0003422 to 0.0004331	No	ns	0.9994
Putrescine vs. Spermidine	0.001663	0.001275 to 0.002050	Yes	****	<0.0001
Putrescine vs. Ethylene diamine	0.001976	0.001588 to 0.002364	Yes	****	<0.0001
Putrescine vs. Diamino hexane	0.002100	0.001713 to 0.002488	Yes	****	<0.0001
Cadaverine vs. Spermidine	0.001617	0.001230 to 0.002005	Yes	****	<0.0001
Cadaverine vs. Ethylene diamine	0.001930	0.001543 to 0.002318	Yes	****	<0.0001
Cadaverine vs. Diamino hexane	0.002055	0.001667 to 0.002442	Yes	****	<0.0001
Spermidine vs. Ethylene diamine	0.0003132	-7.440e-005 to 0.0007009	No	ns	0.1908
Spermidine vs. Diamino hexane	0.0004375	4.989e-005 to 0.0008252	Yes	*	0.0167
Ethylene diamine vs. Diamino hexane	0.0001243	-0.0002634 to 0.0005119	No	ns	0.9417

Table 11: Comparison of the average chemotaxis index obtained with each diamines at 100mM during the first 6 hours of experiment using one-way ANOVA comparison with Tukey post-hoc test, non-significant difference: "NS", significant codes: p-value <0.0001 ****, p-value <0.1 *.

Column B	Tomato
vs.	vs.
Column A	Putrescine
Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.060, df=371
How big is the difference?	
Mean of column A	0.001070
Mean of column B	0.0006923
Difference between means (B - A) ± SEM	-0.0003779 ± 7.470e-005
95% confidence interval	-0.0005248 to -0.0002311
R squared (eta squared)	0.06455

Table 12: Comparison of the average chemotaxis index obtained with putrescine at 100mM and tomato exudate during the 16 hours of experiment using student test (only two columns to analyse)

Column B	Tomato
vs.	vs.
Column A	Putrescine
Unpaired t test	
P value	0.0126
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.529, df=140
How big is the difference?	
Mean of column A	0.001406
Mean of column B	0.001720
Difference between means (B - A) ± SEM	0.0003143 ± 0.0001243
95% confidence interval	6.858e-005 to 0.0005600
R squared (eta squared)	0.04368

Table 13: Comparison of the average chemotaxis index obtained with putrescine at 100mM and tomato exudate during the 6 first hours of experiment using student test (only two columns to analyse)

PROTOCOL TO MAKE THE 3 DIFFERENT PLURONIC GELS

To make 100mL of M9 medium: put 1.2g of Na_2HPO_4 + 0.6g of KH_2PO_4 + 1.0g of NaCl + 0.05g of $MgS0_47H_2O$ in a prewashed autoclaved beaker. Then add 100mL of mineral water and check the pH. If the pH is not equal to 7, adjust by adding a base or acid. Then filter to 0.2µm and place in 4°C tubes.

Then, prepare 100mL of mineral water + 100mL of distilled water + 100mL of M9 medium in 3 different autoclaved beakers with a magnet.

Place the beakers on magnetic stirrers in a cold room at 4°C and rotate the magnets.

Gradually add 32g of Pluronic powder in each beaker .

If some powder clumps that are difficult to mix appear, wait a few hours and then add the rest.

Stir overnight and bottle in glass at 13°C.

IMPROVED PROTOCOL TO REALISE CHEMOTAXIS EXPERIMENTS

- To complete the chemotaxis experiments **I-Luer µ-slides from Ibidi®** are used: **10 slides per experiments**
- For the assays, **1mL of suspension of J2 larvae** of *M. incognita* (concentration: 15 000 J2/mL) is included in **3mL of Pluronic® F-127 gel at 32%, already prepared with mineral water.** The slides are prepared **in the cold room** at 4°C with **cold materials** (pipette tips stored in the cold room and slides previously washed and placed in a distilled water bath in the refrigerator until use)
- Each channels are then filled using a 1-mL pipette, allowing the gel containing the nematodes to flow gently from one side to the other, and cautiously to **avoid introducing air bubbles** in the system. The **excess of gel is removed** from each well and they **are capped** to prevent the gel from drying
- Next, the slides are placed on a glass plate, in the laboratory at around 20-25°C, to let the gel set and spread for at least 3 hours
- Place the 10 slides under the macroscope and run a "**preview scan**"
- Set imaging parameters: **transmitted light** using **brightfield** contrast method, with a **16x magnification zoom** and **objective X1**
- Delimit each channels by creating 10 scenes in "Tiles"
- Set the macroscope to generate a **time-lapse of 16 hours** by **taking an image every 3 minutes**
- **Add the molecules** to test in slides 1 to 8. Slides **9 and 10** were filled only with 3mL of Pluronic 32% gel mixed with 1mL of tap water, **without nematodes** in order to see if the tested molecules darken the gel. If this is the case, the perceived signal will be removed from the other slides, with nematodes, because the dark molecule gradient can distort the result by considering the shadow gradient as nematodes
- **Add 15 µL drop of mineral oil** on top of the solutions to avoid evaporation during the course of the 16 hours experiment as the slides were imaged uncapped
- Click on "start experiment" has been launched after turning off the light in the room

No changes have been made to the post-imaging analysis section.

However, for statistical analysis, many tests have been carried out, and the most effective seems to be to **keep only similar replicates** for each molecule and compare them using a one-way ANOVA followed by a Tukey post-hoc test for multiple comparisons.

RESUME DU STAGE en français: Analyse comparative du comportement de chimiotaxie chez les nématodes à galles *Meloidogyne incognita* entre tomate hôte et plantes de service compagnes

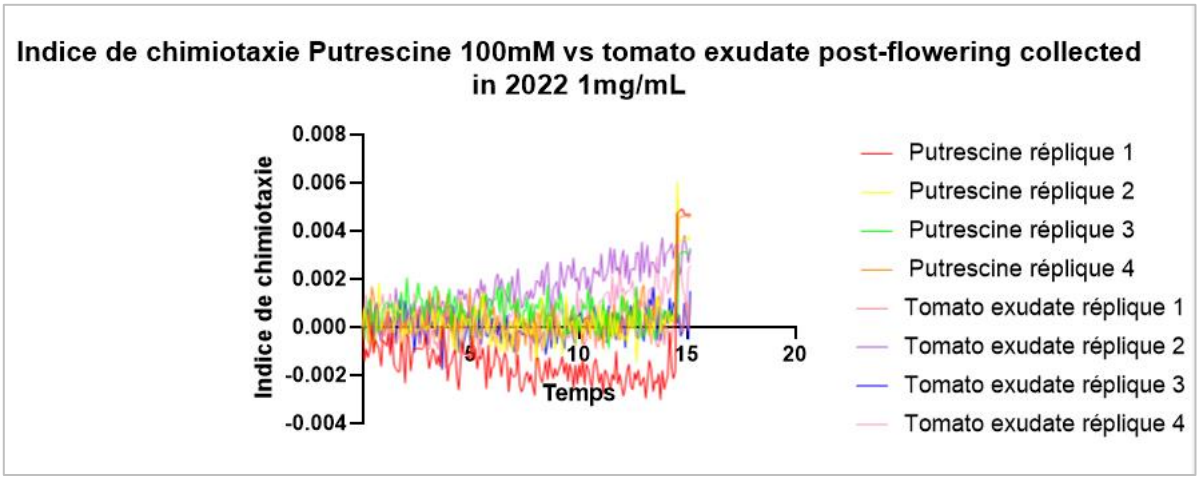
La chimio-sensation est une modalité sensorielle importante pour les nématodes phytoparasites qui leur donne la capacité de répondre aux signaux chimiques émis par les plantes pour localiser l'hôte potentiel et éviter les autres. Pour approfondir nos connaissances sur le mode d'action des plantes de service et comprendre pourquoi elles sont mauvais ou non-hôtes de nématodes à galles et comparer leur attractivité par rapport à une tomate hôte, Victoria a mis au point un dispositif expérimental de chimiotaxie (basé sur une chaine de µ-lames avec les juvéniles de nématodes inclus dans une matrice de gel) en cinétique à l'aide d'un macroscope et d'une imagerie accélérée. Les premiers tests réalisés avec les exsudats de plantes semblent montrer la répulsion des exsudats de *T. erecta,* l'attractivité des exsudats de tomate et leur perte d'attractivité lorsque la plante est en co-culture avec la tagète. Ces résultats devront être confirmés et les exsudats d'autres plantes de service testés.

Test de chimiotaxie avec les exsudats

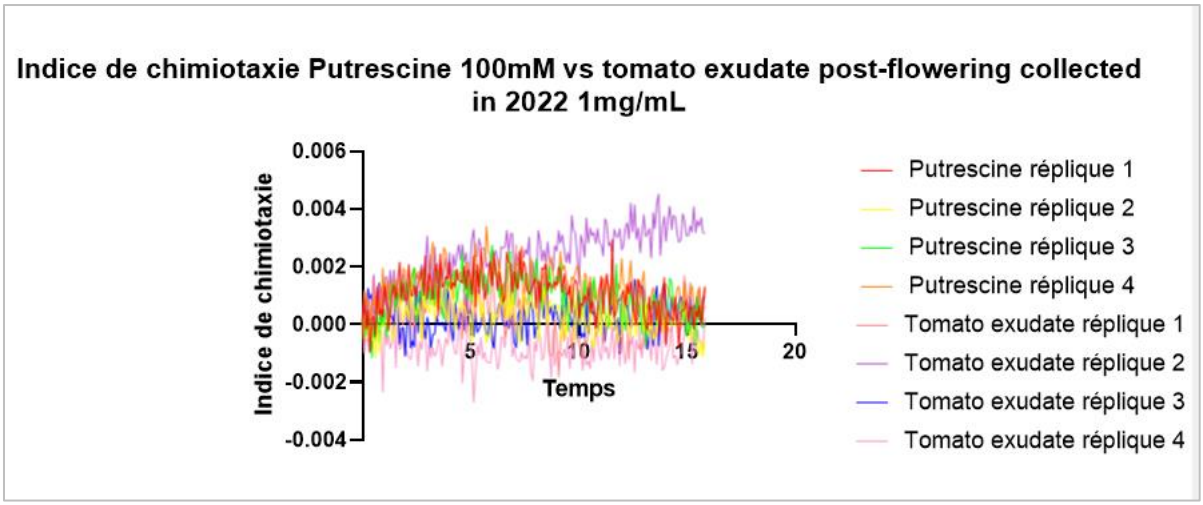
Tomato exudate

Selon les répliques, cela arrive que la putrescine soit neutre mais la plupart du temps elle est **attractive comme l'exsudat de tomate.**

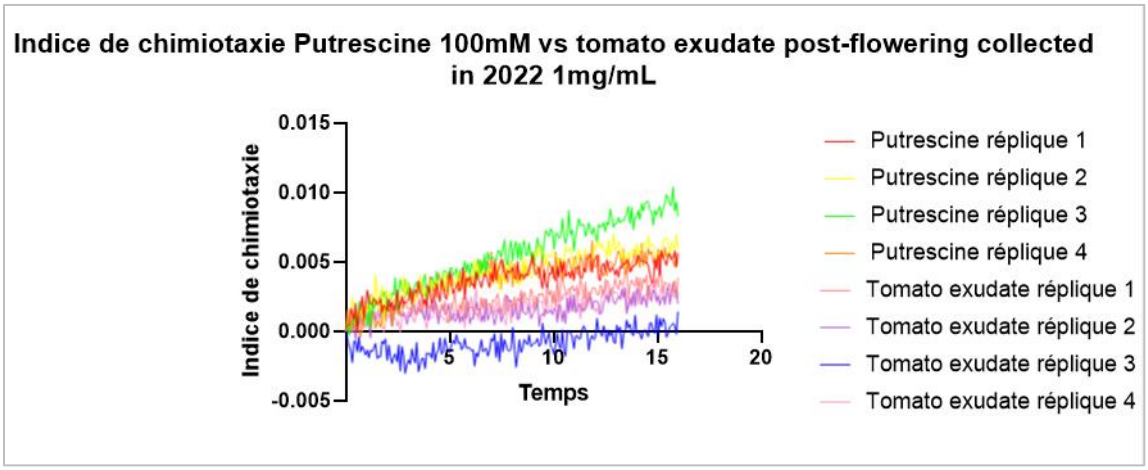
Expérience du 07/06/2023 :



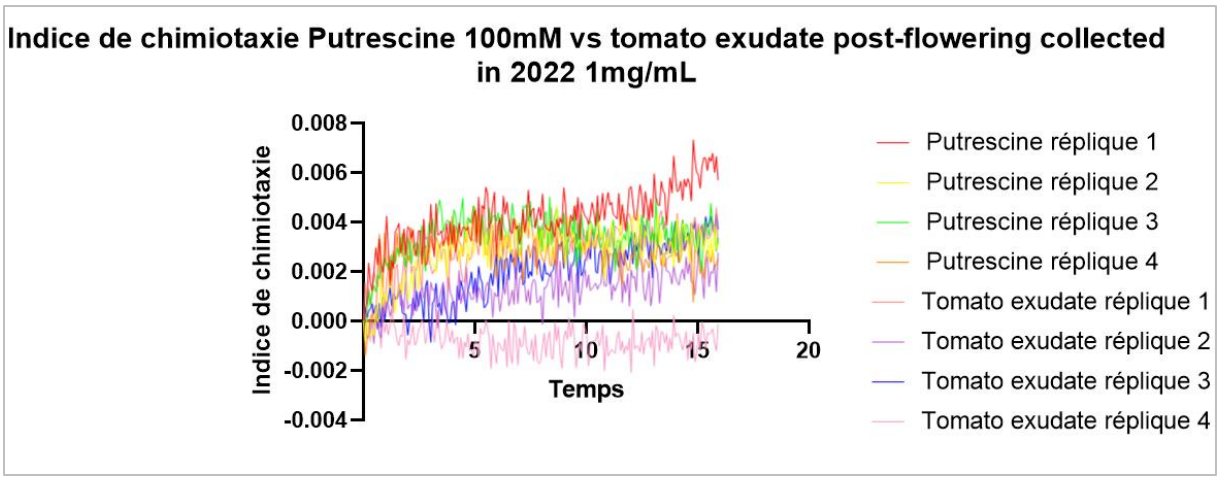
Expérience du 07/06/2023 :



Expérience du 11/07/2023 :



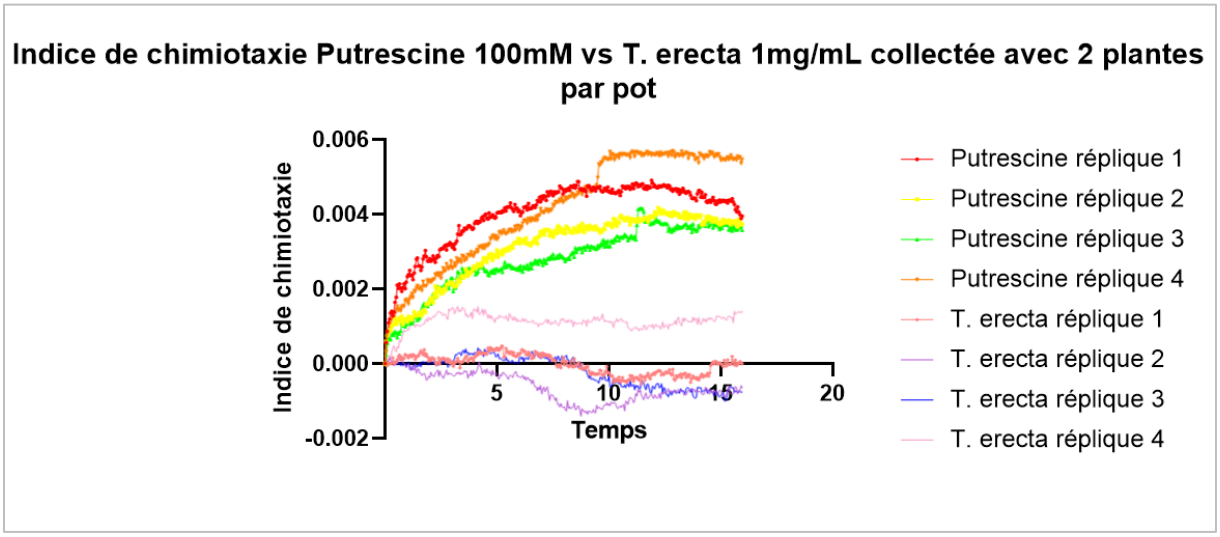
Expérience du 12/07/2023 :


Indice de chimiotaxie Putrescine 100mM vs tomato exudate post-flowering collected in 2022 1mg/mL
Indice de chimiotaxie
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
5
10
15
20
Temps
Putrescine réplique 1
Putrescine réplique 2
Putrescine réplique 3
Putrescine réplique 4
Tomato exudate réplique 1
Tomato exudate réplique 2
Tomato exudate réplique 3
Tomato exudate réplique 4

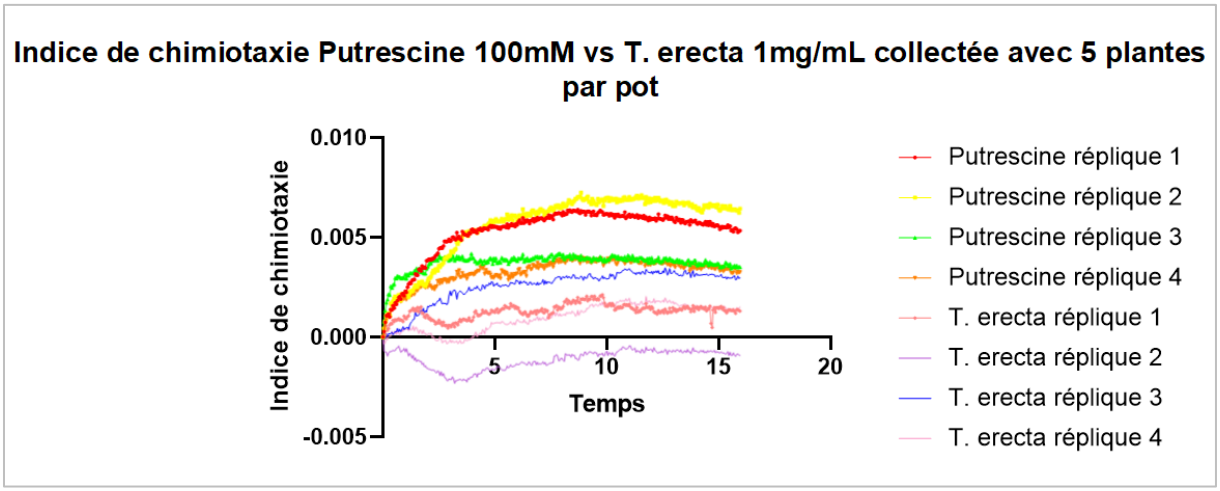
T. erecta exudate

Lors des tests de chimiotaxie réalisés avec *T. erecta* 1mg/mL collectés par Mariel Lahoreau en 2022, tous les nématodes se retrouvaient paralysés dans le gel. J'avais donc effectué un puit de test de toxicité et pourtant l'exsudat était non toxique pour les J2 (voir tableau excel sur clé USB).

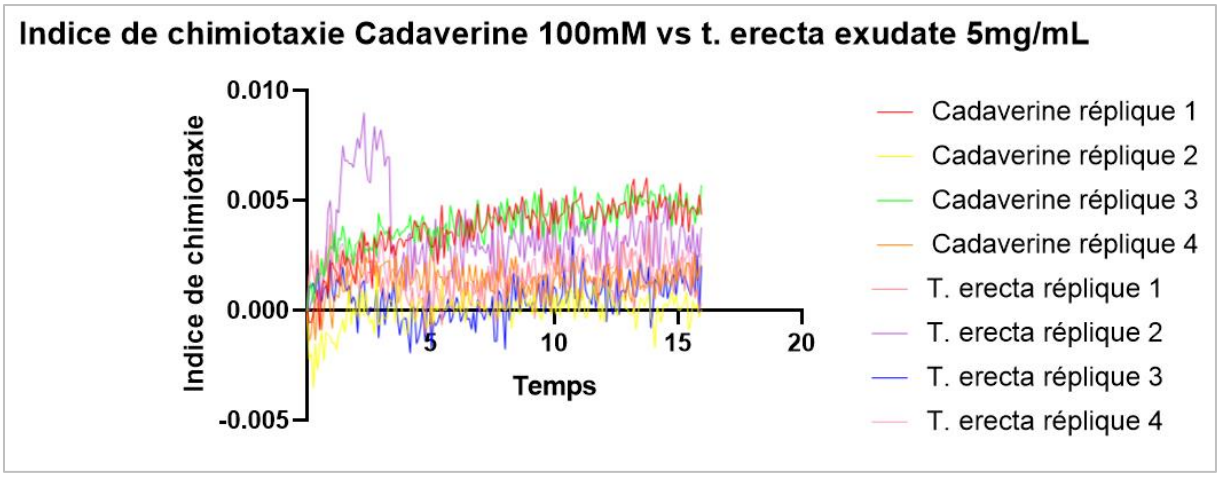
Lors des tests réalisés avec les exsudats collectés par Sharmistha Aryal en 2023 avec 2 plantes par pots, ceux-ci étaient neutres.



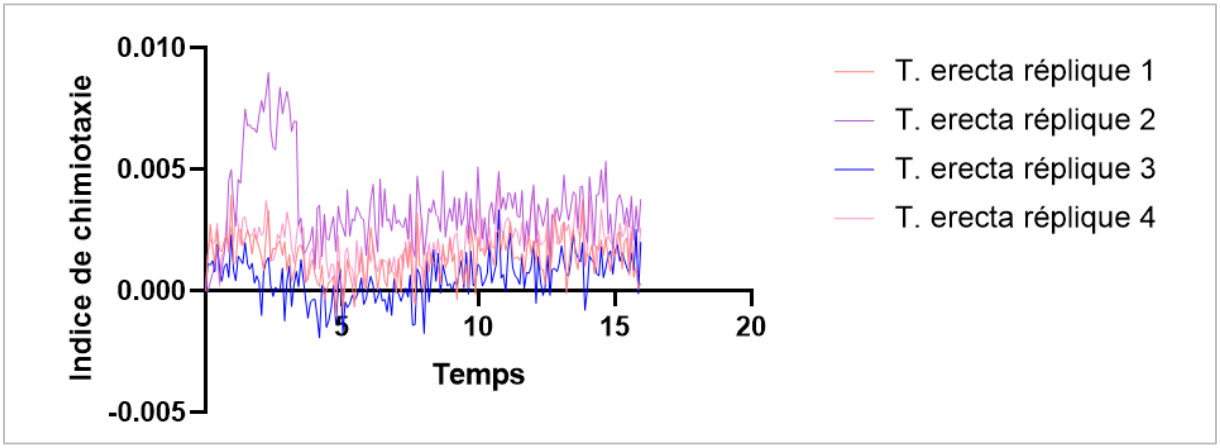
Lors des tests réalisés avec les exsudats que Sharmistha a collecté avec 5 plantes par pot à 5mg/mL, on observe une légère **répulsion** : l'indice de chimiotaxie décroît les 5 premières heures (sauf pour le réplicat 3) puis après le signal devient constant car les nématodes se sont paralysés (manque d'oxygénation ?). Lors de cette expérience il n'y avait pas assez de nématodes dans les lames (normalement il doit y en avoir 700 et là il n'y en avait que la moitié).



J'ai donc refait des répliques avec cette fois-ci plus de nématodes dans les lames :



Voici la même expérience mais seulement les graphes de *T. erecta*:



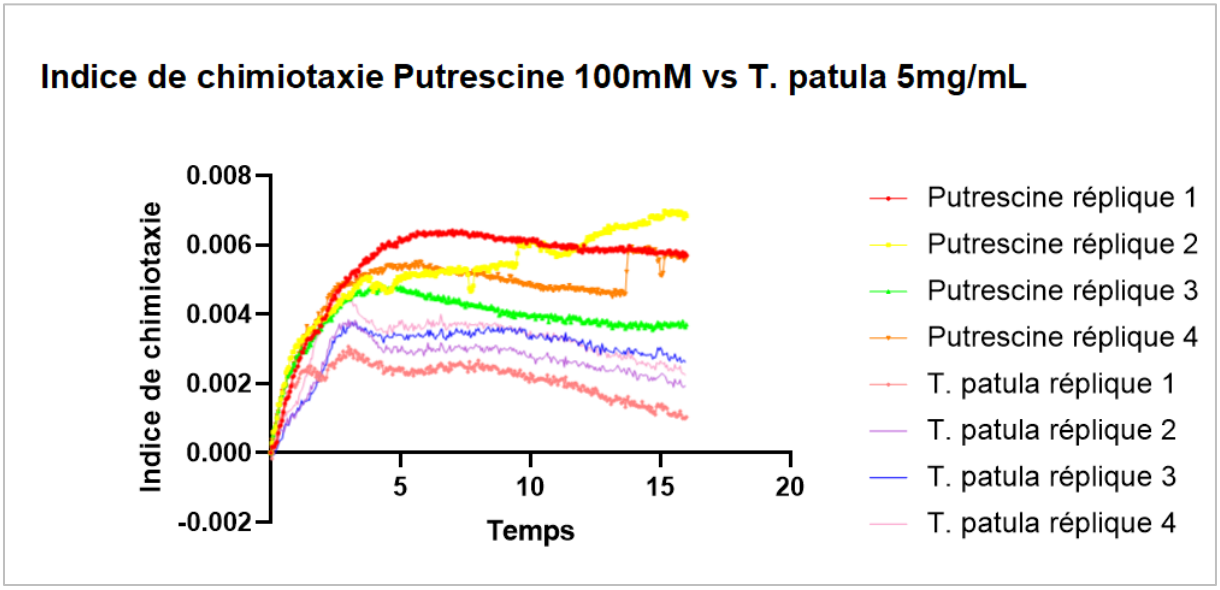
On observe que le signal décroît les 5 premières heures (hormis pour la réplique 2 où il devait y avoir une bulle qui perturbe le signal). Le signal devient neutre après car les nématodes sont paralysés après les 5 heures.

T. patula exudate

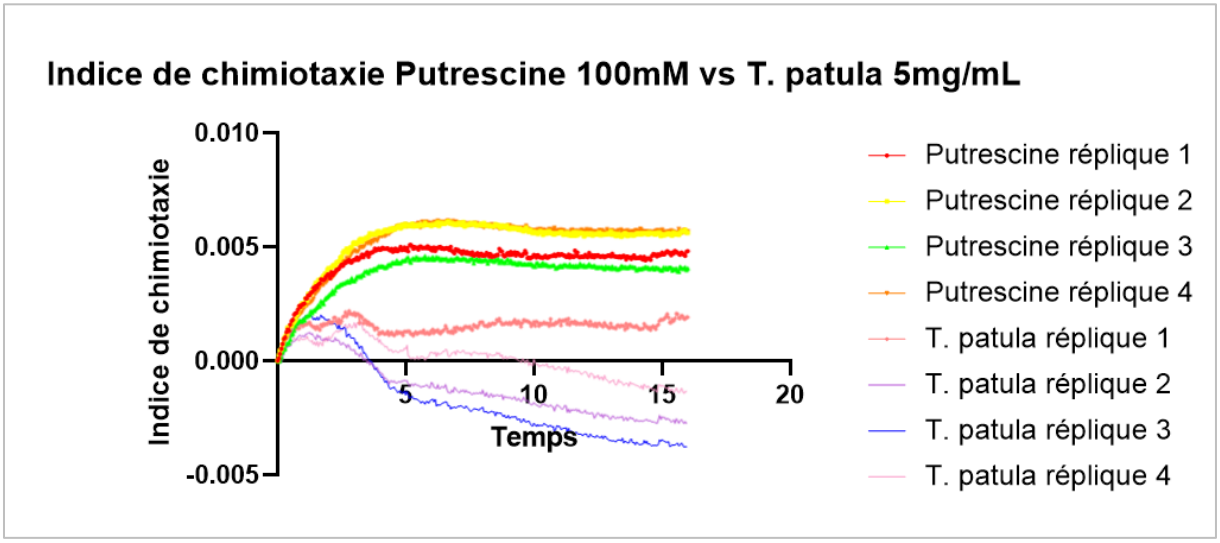
Pour T. *patula*, nous observons clairement sur les films de cinétique que celle-ci est répulsive. En réalité si nous observons que l'indice de chimiotaxie augmente les 3 première heures, c'est parce-que les nématodes qui se trouvaient près du puit de *T. patula* passent par l'aire d'intérêt pour fuir dans l'autre sens (vers le côté de l'eau).

Au bout de 3-4 heures il y a moins de nématodes dans l'aire d'intérêt de *T. patula* et donc l'indice de chimiotaxie décroît.

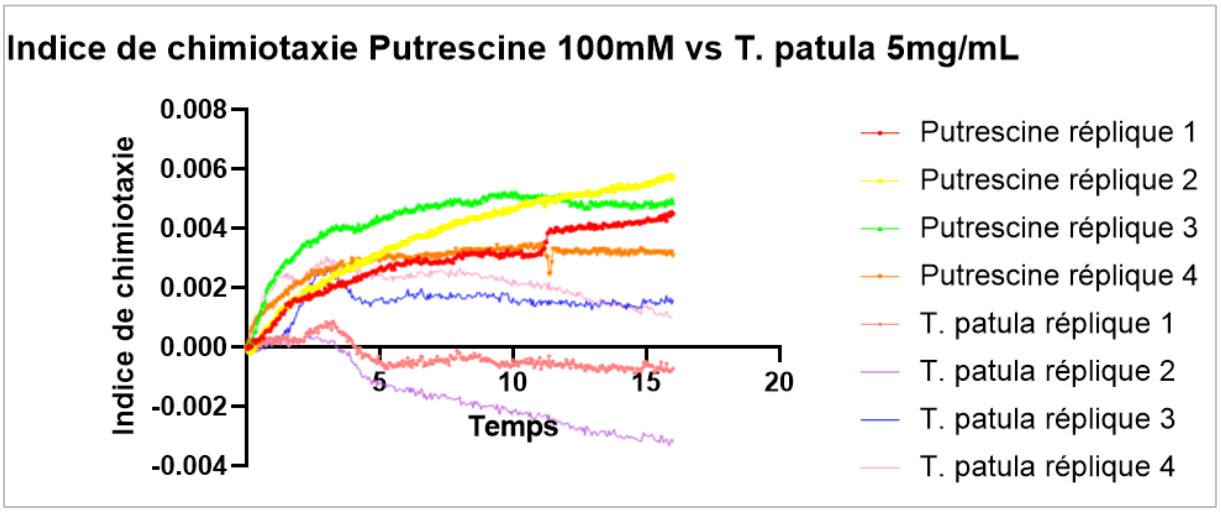
Expérience du 21/07/2023



Répliques du 22/07/2023 :



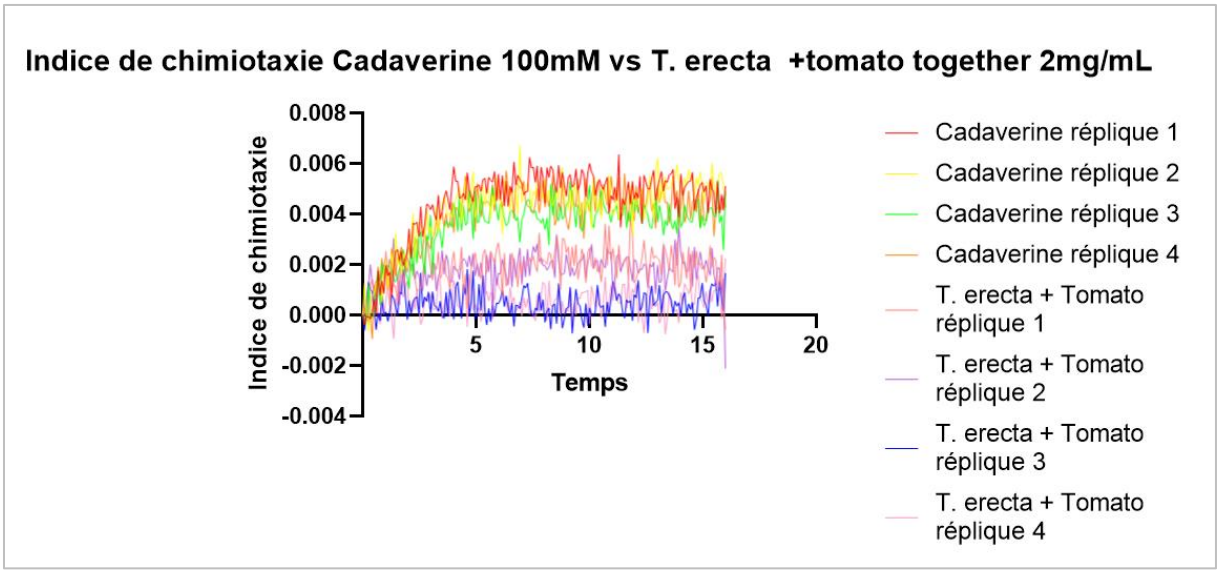
Répliques du 20/07/2023 :



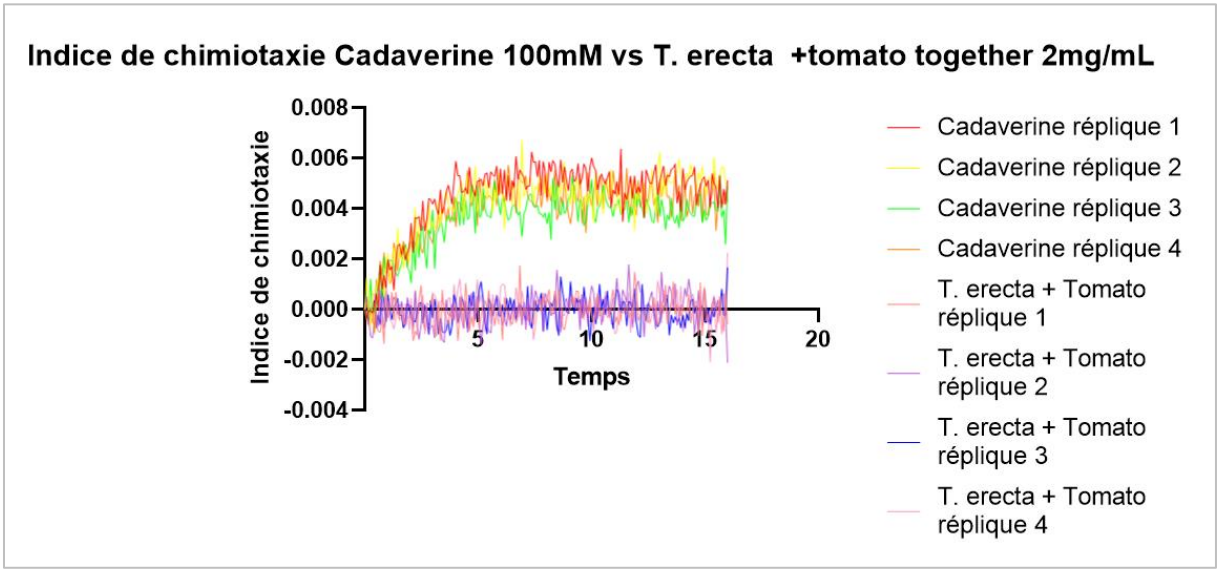
T. erecta + Tomato st.Pierre collected together

L'indice de chimiotaxie est **neutre** voir légèrement attractif selon la réplique avec l'exsudat tomate + *T. erecta* ensemble. Puisque les plantes poussaient ensemble, il pourrait y avoir eu une modification des métabolites secondaires.

Expérience réalisé le 27/07/2023.



Expérience réalisée les 28/07/2023 :

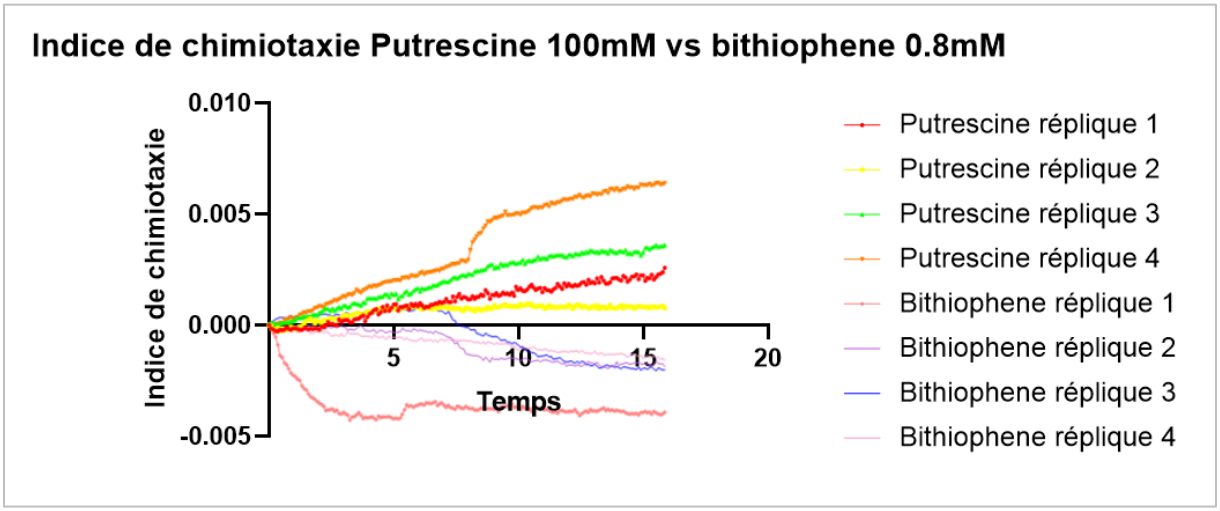


La réponse à l'exsudat est **complétement neutre** suggérant la **perte d'attractivité de la tomate lorsque la plante est en co-culture avec la tagète**.

Bithiophène 0.8mM

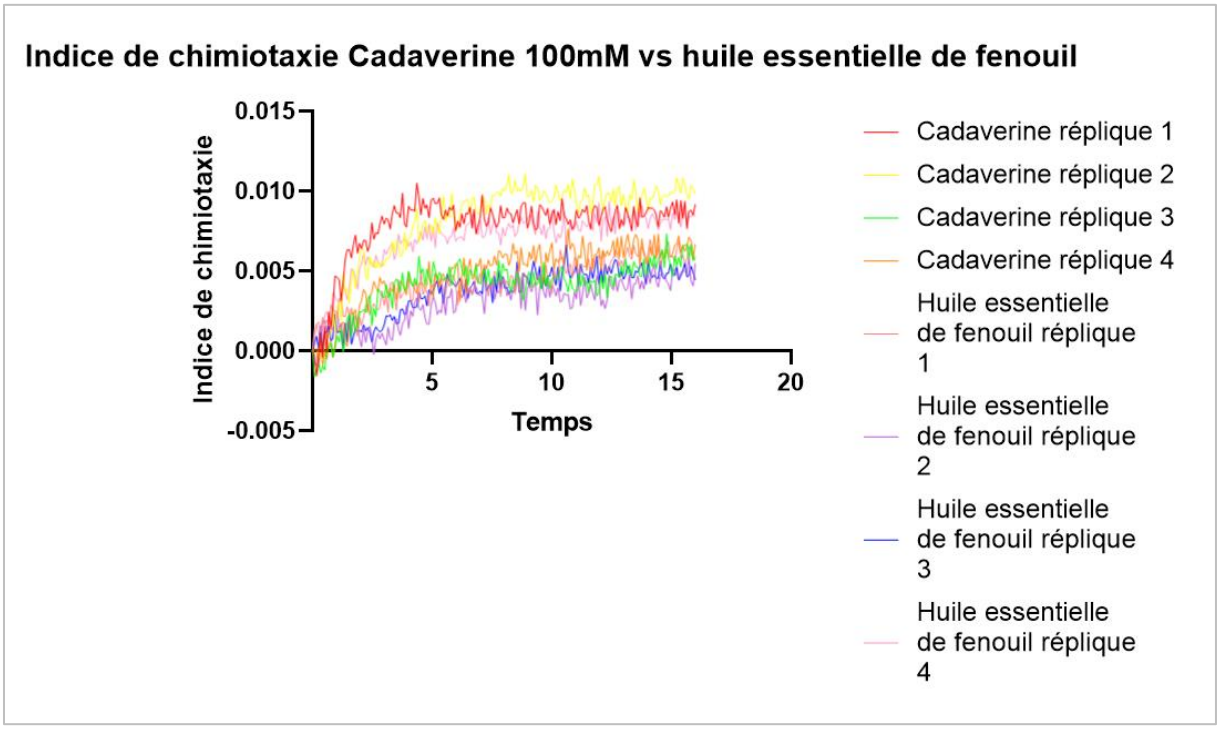
Le signal est très variable avec le bithiophène. Les expériences réalisées les autres jours avec ce composé sont très variables, avec de nombreuses bulles qui sont venues perturbaient le signal récupéré par le macroscope. En visionnant les films le bithiophène ne semble **ni répulsif ni attractif mais plutôt neutre** (voir répliques 2, 3 et 4).

Experience du 21/06/2023 :

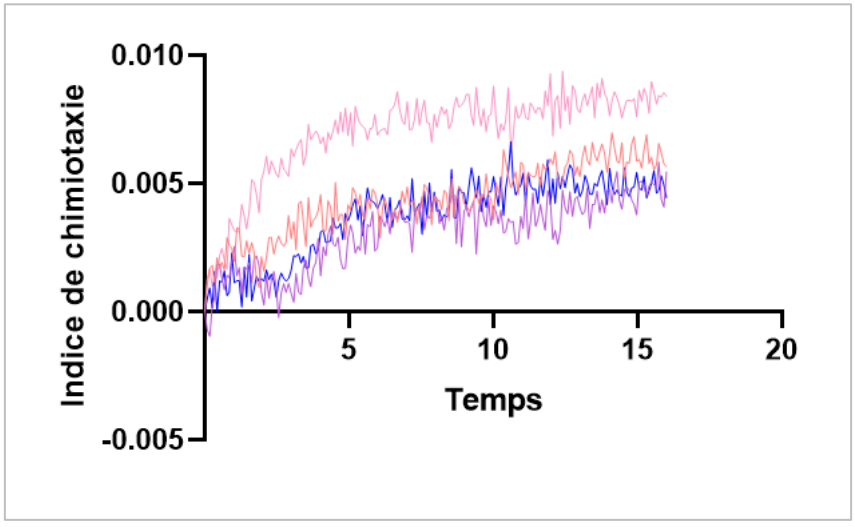


Huile essentielle de fenouil (bitter fennel)

Ici aussi le signal collecté sur le macroscope ne reflète pas ce que l'on perçoit. En effet, on observe clairement sur les films que le fenouil répulse fortement les nématodes mais ici l'indice de chimiotaxie ne décroit que les 3 première heures. Un tracking des nématodes serait donc la solution : compter à chaque images le nombre de nématodes présents dans l'aire d'intérêt pour constater ou non que le nombre de nématodes diminue ou augmente, ce qui traduirait une attraction ou une répulsion.



Voici un zoom seulement sur les indices avec l'huile essentielle de fenouil :

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