DIPLOMA THESIS

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NEMATICIDE AND NEMATOSTATIC POTENTIAL OF MULCH EXTRACTS ON *MELOIDOGYNE INCOGNITA* JUVENILES

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Undersigned Renáta Petrikovszki, agree that my dissertation can be used for research purposes.

Gödöllő, April 18, 2022.

signature

Contents

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Abstract

While the nematicide abilities of mulching against root-knot nematodes (*Meloidogyne* spp.) is calculated with in organic crop protection, underlying mechanisms are not yet fully explored. Experiments were set up to determine whether mulch-derived substances cause mortality directly, or deter *Meloidogyne* juveniles from crop rhizosphere. Mortality and area choice tests were conducted with mulch-derived extracts; supported by the measurements on tannic acid content and the pH values of extracts as supplementary examinations. In our study, leaf litter and straw extracts were generally found lethal to the juveniles, which is in line with their area preference. However, compost extract had no negative effect on *M. incognita* juveniles. Tannic acid content showed positive correlation with mortality only in such cases. Tannic acid and pH slightly correlated with repellent effect of used extracts. Our results have inspired further experiments to explore nematicidal components of leaf litters, together with and the development of a new approach in crop protection based on the nematostatic effect of these materials.

Keywords: leaf litter mulch, pH, tannic acid. area choice, mortality

1. Introduction

Many of the cultivated plants or their extract are used as soil amendments or for plant protection purposes worldwide. On the other hand, leaf litter, which becomes available every autumn in large amounts, has not got sufficient attention from this aspect yet, even though many leaves have a nematicidal effect. At the same time, underlying mechanisms, especially repellent or attractant modes of actions, are not completely understood yet (Spiegel et al. 2005, Sobkowiak et al. 2018). Therefore, experiments were set up under laboratory conditions to investigate whether substances leaching from mulch materials directly cause mortality, or these extracts deter *Meloidogyne incognita* juveniles from the rhizosphere.

2. Literature

One hundred billion-dollar losses are attributed annually to plant-parasitic nematode damage worldwide, which in great part is accredited to root-knot nematodes (*Meloidogyne* spp.) (Ralmi et al. 2016). *Meloidogyne* species have a wide range of host plants (Jones et al. 2013). Their damages occur mainly on the roots of vegetable and protected crops in Hungary (Dabaj et al. 1994, Bíró-Stingli & Tóth 2011, Pinheiro et al. 2015). The symptoms could be various disorders in water and nutrient uptake and translocation, yield loss, dieback, yellowing, wilting of the canopy and the whole plant. They have been shown to colonize the root system of plants and caused galls or knots on the plant roots by the formation of giant cells due to the feeding of the second-stage juveniles of *Meloidogyne* (Sankaranarayanan & Hari 2013, Khan et al. 2014).

Protection of crops against plant-parasitic nematodes is difficult because their eradication from the field is not sustainable or hardly even feasible (Khan et al. 2014). Several methods exist against *Meloidogyne*, among them chemical nematicides are often preferred by growers (Nyczepir & Thomas 2009, Djian-Caporalino 2012, Talavera et al. 2012). Soil sterilization is the most frequent technology despite its harmful effect on groundwater, fitotoxicity of cultivated plants and the risk of human health (Desaeger & Watson 2019). Therefore, the use of soil sterilization was decreased and alternative methods, like biological control, were advised by the European Directive (2009/128/EK) (Williamson & Roberts 2009). Due to the decreasing

number of available chemical pesticides, the use of environmentally friendly and biological methods has become even more encouraged.

Various plant-derived extracts are referred to as biopesticides (Godlewska et al. 2021, Nxumalo et al. 2021). Certain plants have undergone physical or chemical development in order to protect themselves against pests and pathogens. These chemical compounds (e.g. phenols, polyphenols, terpenoids, alkaloids) can be extracted from the plant by various methods, from simple aqueous soaking to the use of organic solvents to distillation (Dubey 2011, Suteu et al. 2020). These extracts may have antifungal, antimicrobial activity against plant pathogens (Šernaitė 2017) and a number of mechanisms have been developed against plant pests. These modes of actions may include feeding inhibition, physical repellent, paralysis of nervous system, or inhibition of cellular respiration (Pavela 2016, Suteu et al. 2020).

These extracts may also be derived from parts of herbs used in human medicine, cultivated crops or ornamental plants (Pavela 2016, Suteu et al. 2020, Godlewska et al. 2021). In addition, compounds that may affect not only plant pests but also non-target organisms may be released from organic mulching materials (Akhtar & Alam 1992, Anderson 2005).

3. Methods

3.1. Preparation of agar medium

Agar of 10 g was diluted with 500 ml of distilled water and heat sterilized. Since agar substrate is hot in liquid state, water from condensation appears on the inner side of the lid of the Petri dish. By prior experiences, this condensation water can drop down to the surface of agar, which may cause nematode-locomotory difficulties. To obtain a dry surface agar, the lid of Petri dishes was put down to the bottom part so as to leave a 5 mm-wide gap to allow for evaporation. Petri dishes were left for 15 hours in a sterile laminar box. UV light was switched on for 30 minutes before use.

3.2. Preparation of mulch-derived extracts

The following mulch materials were used: common walnut (*Juglans regia*), Norway maple (*Acer platanoides*), sycamore (*Platanus* × *hybrida*), common oak (*Quercus robur*) leaf litters, green yard waste compost (‘Zöld Híd Komposzt’ 04.2/3245-2/2017 NÉBIH, 2019), and common wheat (*Triticum aestivum*) straw.

Collected mulch materials were dried at 25 ° C and 20% relative humidity for 2 days. 2.5 g per each mulch material was grinded in a coffee grinder (Bosch MKM 6000) for 15 seconds.

Then 50 ml of Milli-Q was added for the powder, mixed together and covered with aluminum foil and then let them soak at room temperature. After 24 hours, the stock solutions (5% w / v) were filtered through cotton wool, and additional concentrations (1; 0.5 and 0.1%) were diluted by adding Milli-Q water (Figure 1).

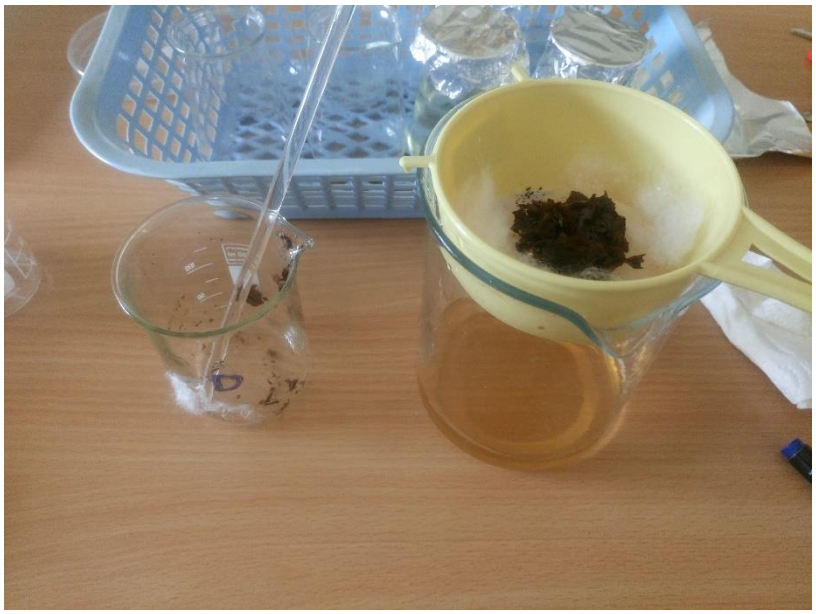

Figure 1. Filtering of walnut leaf litter extract through cotton wool

3.3. *Meloidogyne incognita* culture

Egg masses of *Meloidogyne incognita* were collected from tomato roots (*Solanum lycopersicum* "Dány"). Egg masses were suspended in tap water and kept at 24 ± 1°C for hatching. After 7 days, hatched second-stage juveniles were checked under a dissecting microscope with transmitting illumination at a 30× magnification (Figure 2). Only viable *M. incognita* juveniles were used in the experiments.

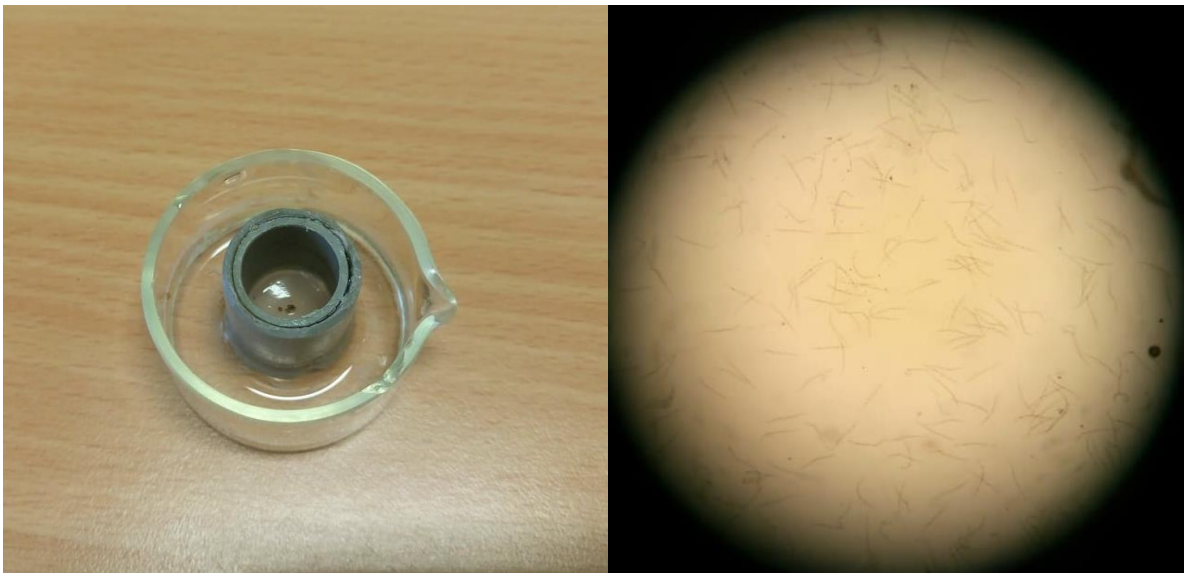

Figure 2. Hatching of *Meloidogyne incognita* eggs through a sieve (left), hatched juveniles at 30× magnification (right)

3.4. Mortality test with mulch-derived extracts

Mortality tests were performed in flat-bottom 96-well microplates (Kartell S.p.A., Italy) with the same methodology as in Petrikovszki et al. (2019) [65]. Five *M. incognita* juveniles were placed into each well. MQ-water (Milli-Q) served as control with 8 replications, and 4 replications/concentrations were used for each extract. Nematode viability was checked after 24 hours under a transmission stereomicroscope.

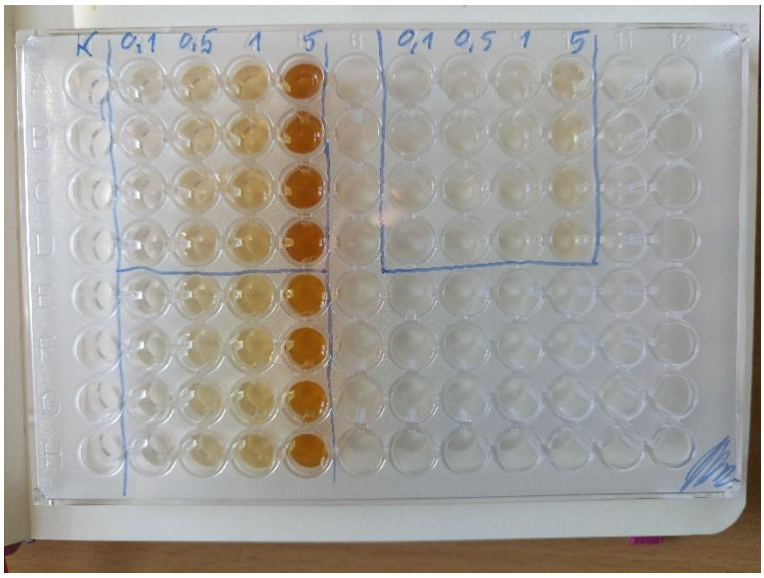

Figure 3. Mortality test set in a 96-well microplate

3.5. Area choice test with mulch-derived extracts

The setup was based on the combined and modified version the method described of Hewlett et al. (1997) and Zhai et al. (2018): Petri dishes of 6 cm diameter and 10 % water agar were used.

The 10% aqueous agar was poured into Petri dishes of 6 cm diameter, then two holes, each 5 mm diameter, were cut into the agar plate. One hole was always the control, into which 50 µl of Milli-Q water was pipetted, while the hole on the opposite side always contained 5% of the mulch-derived extract to be treated, also in a volume of 50 µl.

Treatment pairs within a Petri dish were the following:

- MQ-water – MQ-water (control)
- Maple leaf litter extract – MQ-water
- Oak leaf litter extract – MQ-water
- Sycamore leaf litter extract – MQ-water
- Walnut leaf litter extract – MQ-water

- Straw extract – MQ-water
- Compost extract – MQ-water

Then 20–30 individuals of *M. incognita* juveniles were added with 20 µl water to the centre of the Petri dish. Petri dishes were randomized on a tray and incubated for 8 hours in a thermostat (20 °C ± 1 °C) in dark. Treatment pairs were replicated ten times. After 8 hours, the number of juveniles on both sides was counted under a transmission stereomicroscope (Olympus SZH 10) on ×30 magnification.

The sectors of the agar plate were drawn previously on a foil. The foil was placed under the Petri dish in order to determine the position of the juveniles. Juvenile individuals in 'sector 0' were not included on either side (Figure 4).

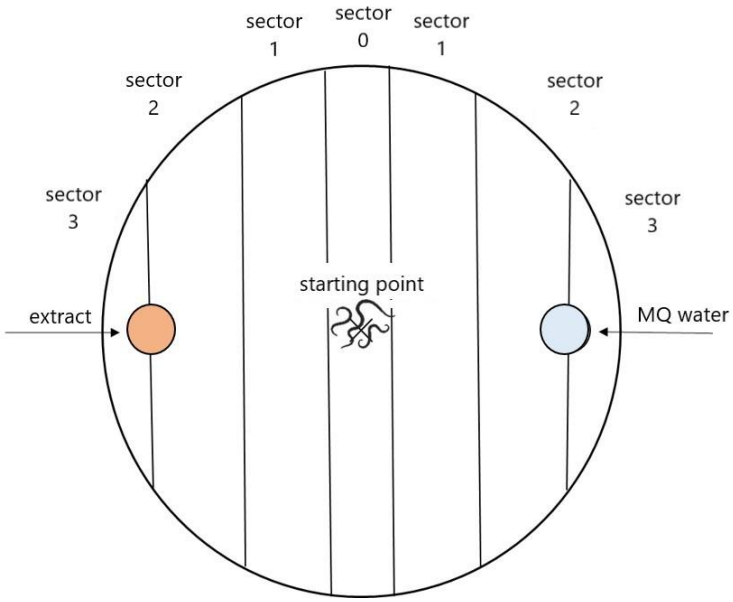

Figure 4. Representation of the sectors identified during the reading of the area choice test

3.6. Determination of tannic acid content of mulch derived-extracts

Determination of the tannin content of the mulch-derived extracts in our experiments followed the Hungarian Standard protocol for tannin determination by spectrometer 'MSZ ISO 9648' and was carried out by Corvinus Fitolabor Kft. of Szent István University (Budapest, Hungary). Tannic acid content of mulch-derived extracts was in mg/ml.

3.7. Determination of pH values of mulch-derived extracts

The pH measurements were repeated five times by Voltcraft pH-212. Before measuring, pH meter calibration was performed by the two-point standardization method (pH 7 and pH 4) according to the instructions.

3.8. Statistical analysis

Data of percentage values were square root arcsine-transformed in MS Excel 2016 before being analysed by PAST3 (Hammer et al. 2001). One-way ANOVA and pairwise Tukey tests were used to analyze the average mortality values caused by various concentrations of each mulch-derived extract in the case of mortality tests. Treatment pairs within Petri dishes were compared to each other by Independent Samples T-test in the case of every preference tests. $P \leq 0.05$ significant level was determined for every statistical analysis.

For linear regression, analyses were made to reveal the relationship between the pH/tannic acid (independent factor) and the mortality of *M. incognita* juveniles as dependent factor. In addition, in the case of fungi area choice test, pH values served as independent factor and the percentage of avoidance of *M. incognita* larvae as dependent factor.

4. Results

4.1. Mortality test

Mortality was less than 20% in the 0% control treatment. A steep rise started in 0.5% of maple and oak leaf litter extracts, and then reached a peak at concentrations of 1% which caused 100% mortality. Walnut leaf litter extract showed lower values at 0.1 to concentrations of 1% compared to the previous extracts. At 0.5% concentration, mortality hit a lower value, then increased and remained constant at the value of 100% mortality. In the case of sycamore leaf litter extract, there was a slight fall at 0.5% concentration, then raised 29.6% at 1% concentration and reached the top of mortality value at the highest concentration. Lower (0.1; 0.5 and 1%) concentrations of straw extract showed lower mortality. However, the 5% concentration, mortality value soared and peaked at 100%. As a contrast, compost extracts showed very low mortality values at every each of examined concentrations.

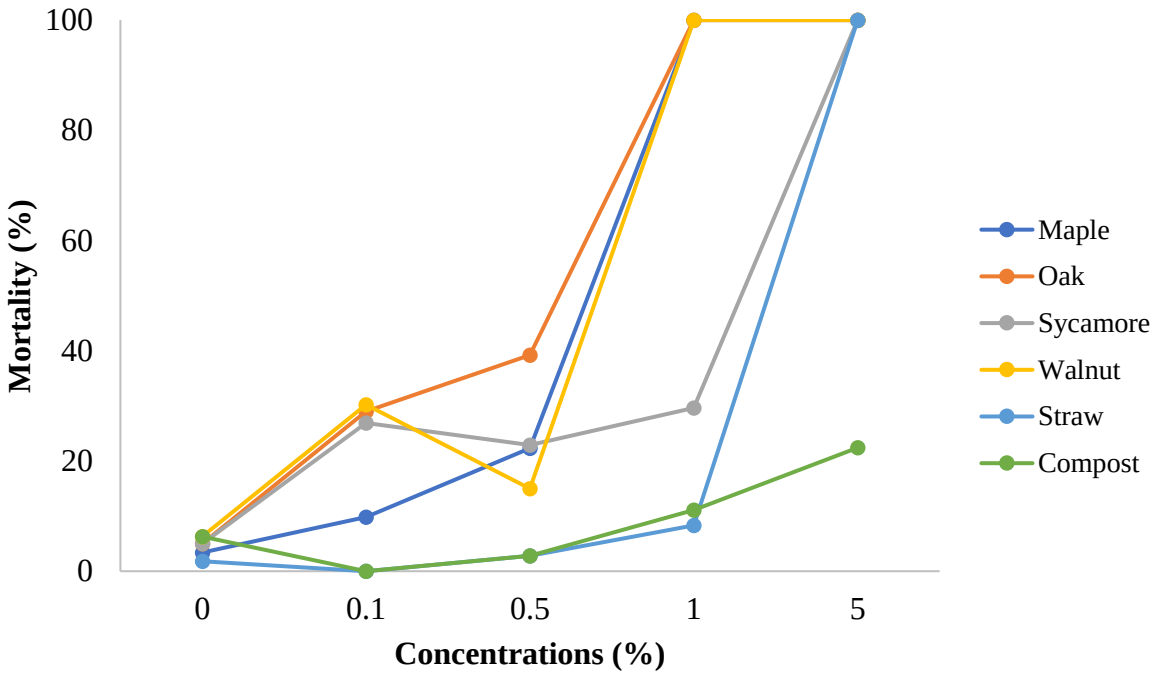

Figure 5. Mortality rates (mean ± CI 95%) of *Meloidogyne incognita* juveniles of different concentrations (0.1, 0.5, 1 and 5%) of various leaf litter, straw and compost extracts (CI 95%: 95% of confidence interval)

4.2. Area choice test

In the area choice study of *M. incognita* juveniles, both of the sides of the Milli-Q water control were selected almost equal percentage (46% –54%). No difference was made between the compost extract and the Milli-Q water treatment pairs (50% –50%). However, *M. incognita* juveniles avoided the sides treated with leaf litter extracts in the following percentage relative to the MQ water side: oak leaf litter 59%, walnut leaf litter 63%, maple leaf litter 68%, sycamore leaf litter 75%. Straw extract, similarly to walnut leaf litter, repelled the 63% of the juveniles.

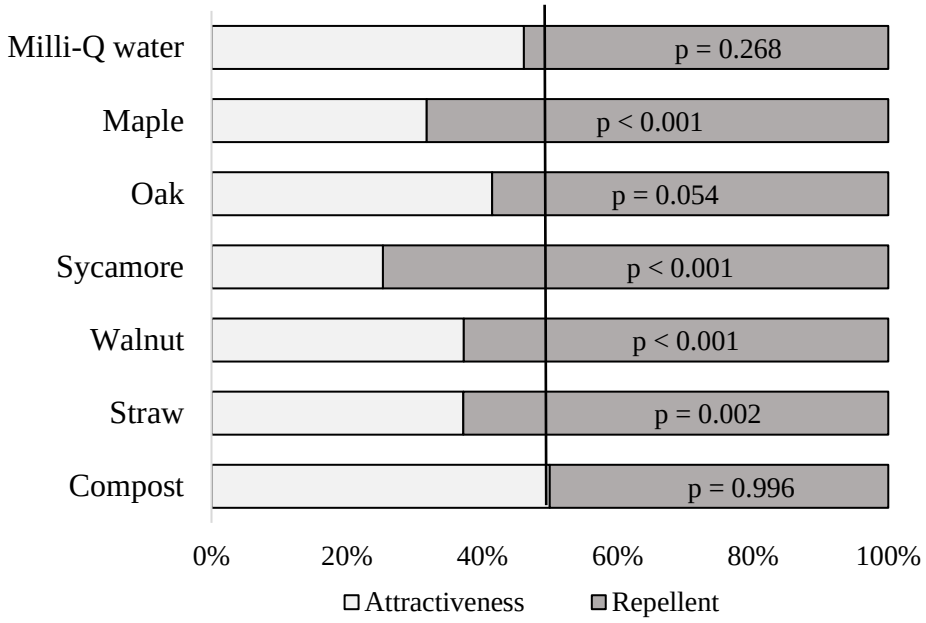

Figure 6. Area choice of *Meloidogyne incognita* juveniles with various leaf litter, straw and compost extracts (treated) and MQ-water (control). (Welch-test, $p \leq 0.05$, p-value means that the number of *M. incognita* juveniles counted on the treated side is significantly different from that on the control side)

4.3. Determination of tannic acid content and pH value of mulch derived-extracts

Among the extracts used in the experiments, the tannin content of the maple leaf litter extract was the highest, which was followed by the extract of oak leaf litter, sycamore leaf litter, walnut leaf litter, and finally straw extract. No tannin content could be measured from the compost extract (Table 1).

The pH of the concentrations of 5% of the tested leaf litter and straw extracts fell into the slightly acidic category, except of compost extract. Within this, the most acidic value was given by the 5% concentration of maple leaf litter, then oak leaf litter, sycamore leaf litter, walnut leaf

litter, and finally straw extract. The compost extract was fell in the slightly alkaline category with a pH value of 7.79 (Table 2).

Table 1. Values of tannic acid content of different concentrations (0.1, 0.5, 1 and 5%) of various leaf litter, straw and compost extracts (n.d.: non detectable)

	Tannic acid content (%)				
Concentrations (%)	0	0.1	0.5	1	5
Extracts					
Maple leaf litter	n.d.	0.00521	0.02605	0.0521	0.2605
Oak leaf litter	n.d.	0.00241	0.01205	0.0241	0.1205
Sycamore leaf litter	n.d.	0.00209	0.01045	0.0209	0.1045
Walnut leaf litter	n.d.	0.0008	0.004	0.008	0.04
Straw	n.d.	0.00002	0.0001	0.0002	0.001
Compost	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2. Values (mean ± CI 95%) of pH of different concentrations (0.1, 0.5, 1 and 5%) of various leaf litter, straw and compost extracts (CI 95%: 95% of confidence interval)

	pH value				
Concentrations (%)	0	0.1	0.5	1	5
Extracts					
Maple leaf litter	7.02 ± 0.04	5.33 ± 0.01	4.81 ± 0.02	4.78 ± 0.03	4.43 ± 0.03
Oak leaf litter	7.02 ± 0.04	5.95 ± 0.01	5.42 ± 0.02	5.23 ± 0.03	4.99 ± 0.03
Sycamore leaf litter	7.02 ± 0.04	6.42 ± 0.01	6.09 ± 0.03	5.36 ± 0.03	5.12 ± 0.02
Walnut leaf litter	7.02 ± 0.04	6.30 ± 0.01	6.63 ± 0.03	6.68 ± 0.03	6.21 ± 0.04
Straw	7.02 ± 0.04	6.37 ± 0.02	6.41 ± 0.04	6.44 ± 0.01	6.46 ± 0.03
Compost	7.02 ± 0.04	6.90 ± 0.01	7.29 ± 0.03	7.57 ± 0.03	7.79 ± 0.06

Based on the values of the regression, the pH value of the oak leaf litter extracts may have been more correlated ($r = 0.854$) with the mortality values. The lethal effects of sycamore leaf litter extracts were increased by concentration ($r = 0.919$) and tannin content ($r = 0.919$). There is a similar trend for straw extract: concentration ($r = 0.982$) and tannin content ($r = 0.982$). In the case of compost extracts, there is no strong correlation with mortality for any of the parameters studied (Table 3).

Table 3. Investigation of the regression between the concentration, the tannin content, the pH of mulch-derived extracts and the percentage mortality of the juveniles of *Meloidogyne incognita*

Extracts	Concentration/Mortality	Tannic acid/Mortality	pH/Mortality
Maple leaf litter	0.732	0.732	0.785
Oak leaf litter	0.710	0.710	0.854
Sycamore leaf litter	0.919	0.919	0.766
Walnut leaf litter	0.718	0.718	0.495
Straw	0.982	0.982	0.267
Compost	0.522	0.108	0.495

According to further regression analysis, area preferences of *M. incognita* juveniles were slightly influenced by pH (r = 0.387) and tannic acid content (r = 0.302) of mulch-derived extracts.

5. Discussion

In the case of common walnut leaf litter extracts, 100% mortality of *Meloidogyne javanica* juveniles in 20% of walnut leaf extract was observed after a 72-hour exposure (Fekrat et al. 2016), while the same result was reached even with the 1% of common walnut leaf litter extract after one day in our experiment. There can be two reasons behind the different results. One is that they used *M. javanica*, while our test species was *M. incognita* and these two species may have different sensitivity to phytochemicals. On the other hand, we prepared the extract from fallen leaves, while Fekrat et al. (2016) did not mention the exact origin of walnut leaves.

Yard waste compost did not have any nematicidal effect on Meloidogyne species in open-field experiments with several vegetable crops (McSorley & Gallaher 1995). These findings are in line with our results. At the same time, according to our previous experiments (Petrikovszki et al. 2019) and the study of Herren et al. (2018) with entomopathogenic and slug-parasitic nematodes, compost did not have any harmful effect on these beneficial nematode groups.

According to previous studies, every leaf litter and straw material contains tannic acid (Peng & Jay-Allemand 1991, Gessner & Chauvet 1994, Scutareanu & Lingeman 1994, Anderson 2005). In our experiment, straw and walnut leaf litter extracts had the lowest tannic acid contents (0.02 and 0.08%). %). In the case of straw, mortality was quite low (8.3%) at this tannic acid content, while walnut leaf litter extracts had total lethal effect (100%). It can be explained by other compounds of walnut leaves, for example juglone (Mahajan et al. 1992). It seems quite clear that besides tannic acid, many other compounds in the examined leaf litters may have nematicidal effects, resulting in 100% mortality values at higher (1 and 5%) concentration levels.

Considering the fact that while leaf materials and straw contained tannic acid, this deterrent compound was not detected in compost extracts, it has been speculated that the lack of nematicidal effect of compost can also be attributed to biodegradation: if compost contained a compound with a nematicidal effect, it may have been degraded during composting.

The results of the area choice tests are in agreement with the mortality test. Compost extract did not influence *M. incognita* juveniles. Significant repellent effects were observed in the presence of the examined leaf litter and straw extracts, however, this cannot be related to tannic acid content. In contrast, Hewlett et al. (1997) noted that tannic acid attracted *Meloidogyne*

arenaria and *M. incognita* juveniles. They used pure tannic acid for their experiments, which could have a stronger scent than our natural mulch-derived extracts contains not only tannic acid but other compounds which could influence the preference of *M. incognita* juveniles.

In the area selection experiment, except compost, extracts that caused mortality were avoided by *M. incognita* juveniles. Therefore, it can be assumed that tested extracts also have a nematostatic effect besides their nematicide effect.

Wang et al. (2009) suggests that pH between 4.5 and 5.4 has an effect of attraction on juveniles of *Meloidogyne* species. In contrast, according to Rocha et al. (2017) alkaline pH value (8) attracts, while acidic pH (5) repels *M. incognita* juveniles.

6. Conclusions

Current and similar experiments fascilitates and contributes to better understanding of nematological aspects and the mechanisms of soil surface mulching.

In the future, it would be worthwhile to study additional plant- and mulch-derived extracts not only in plant-parasitic but also in non-target nematodes and other soil-dwelling organisms in order to contribute to the development of an environmentally friendly plant protection process.

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