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Biogas digestate as potential source for nematicides

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ABSTRACT

Returning biogas digestate to the fields as biofertilizer is an established agricultural practice which has been promoting plant resistance to soil borne pests. Root-knot nematodes (*Meloidogyne spp.*) are widely distributed Plant Parasitic Nematodes (PPNs), constituting a major threat to the world's food supply. Novel PPNs control methods are needed in the context of sustainable agriculture to replace or to complement the use of hazardous synthetic nematicides. The identification of nematicidal compounds in biogas digester effluents could provide a win–win solution for digestate' costly disposal and for renewable crop-protection products formulation. However, the understanding of PPNs control mechanisms by digestate is still scarce. In this study, we evaluated the nematicidal activity of digestate samples from two on-farm biogas plants both *in vitro* and *in planta* on *Meloidogyne incognita* second stage juveniles (J2). Over 60 % J2 mortality was observed *in vitro* after only 24 h exposure to diluted digestate (10 % v/v), and reached > 90 % after 7 days. In greenhouse trials, digestate application at 10 % and 5 % dilution was effective in preventing root galling caused by *M. incognita* in highly susceptible cultivars (tomato and cucumber), sustaining plant biomass. Liquid–liquid fractionation(s) on the crude digestate mixture allowed the isolation of extracts of different polarity for untargeted compound analysis by Gas Chromatography–Mass Spectrometry (GC–MS). Our findings confirm the value of biogas digestate as PPNs suppressive amendment and introduce a novel approach to PPNs control, moving towards more circular and sustainable agri-food systems.

1. Introduction

Sustainably ensuring global food security is a key challenge for agricultural markets and policy makers worldwide (FAO, 2016; Pérez-Escamilla, 2017; FAO, 2021). Plant pathogens and pests, including plant parasitic nematodes (PPNs), represent a significant constraint on the productivity of cropping systems, causing pre-harvest yield losses up to over one-third of crops in affected fields (Oerke, 2006; IPPC Secretariat, 2021). Soil-borne PPN outbreaks – often overlooked – have a significant impact on marketable crops, undermining the availability and quality of staple foods and causing annual economic losses of about USD 80 to USD 157 billion worldwide (Abad et al., 2008; Nicol et al., 2011; Hassan et al., 2013). This is quite daunting as over 80% of the food for human consumption, as well as most of the livestock feeds, are plant sourced (FAO, 2021). Moreover, in the near future, the increased food demand from the growing world population and

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Climate Change will further increase the pressure that PPNs exert on global agriculture (IPPC Secretariat, 2021). Rising temperatures, changes in precipitations patterns, soil degradation and agricultural intensification are likely to favor the spread of PPNs populations on to previously unaffected areas and to lower plant defenses and to break resistance in resistant plants, worsening PPN outbreaks in areas of current distribution (Ruess et al., 1999; Ghini et al., 2008; Wang et al., 2009; IPPC Secretariat, 2021). Thus, more efficient and up-to-date management strategies are needed to face current and future challenges in PPN control (Isman, 2019).

Despite of the environmental drawbacks, field application of synthetic pesticides is a common phytosanitary measure in most countries (Chen et al., 2020). Traditional PPN control strategies have been relying on the extensive use of highly toxic, broad-spectrum agrochemicals such as pre-plant fumigants and organophosphates or carbamates (neural toxins), thus undermining the establishment of more sustainable agro-systems (Sasanelli et al., 2021). However, the increasing regulatory pressure on use of phytosanitary products, along with societies' growing environmental awareness, have sparked research into alternative, more environmentally friendly agricultural practices and pest control strategies to foster the transition to more resilient and sustainable food production systems (Wezel et al., 2018). Organic soil amendments such as animal manures and composts are considered a promising tool for integrated PPN management as they can contribute in building a natural suppressiveness of the soil against nematode infestation (Linford et al., 1938; Oka, 2010; Thoden et al., 2011; Renčo, 2013; Silva et al., 2018; Rosskopf et al., 2020).

Biogas digestate constitutes a cheap and readily available organic amendment obtained by the anaerobic digestion of biodegradable organic matter (i.e., organic fraction of municipal solid waste, animal manure, agro-industrial waste, and energy crops). In the anaerobic reactor, substrates are fermented by complex microbial consortia to obtain energy-rich biogas, collected as a renewable energy source, and a nutrient-rich digestate or effluent as a residual by-product (Weiland, 2010; Hijazi et al., 2016). Digestate is a mixture of digested inert organic materials and bacterial biomass that needs to be disposed of as waste or, if managed properly, can be recycled as valuable and inexpensive soil conditioner or biofertilizer (Dahlin et al., 2015; Barzee et al., 2019; Pastorelli et al., 2021). About 19,000 anaerobic digestion plants are currently operating in Europe, producing over 180 Mt of digestate each year (Arnau et al., 2021). In virtue of carbon neutral policies, the biogas energy is a fast-growing market, expected to quadruple its capacity by 2050, raising concerns about the non-hazardous disposal of larger volumes of digested residues (Fuchs and Drosg, 2013; Arnau et al., 2021; Korbag et al., 2020). Thus, alternative uses are urgently needed. The identification of novel, more sustainable pest control strategies and the recovery of resources from agricultural wastes are two challenging research frontiers in agricultural studies, fundamental for meeting the sustainability goals of modern agro-systems (Selvaraj et al., 2022). In this regard, the identification of naturally occurring molecules or metabolites with biocontrol properties in agricultural wastes or digestates could provide a novel, low-cost, and environmentally safe approach to PPN management while tackling the issues of digestate disposal and natural resources scarcity.

Meloidogyne incognita is among the most widespread, economically important and widely studied root-knot nematode (RKN), directly affecting over 1700 vascular plants (Jones et al., 2013). RKN are ranked among the top five major plant pathogens and the first most important genera of plant parasitic nematode worldwide (Mukhtar, 2018). As most RKN, *M. incognita* is a sedentary endoparasite with a relatively simple life cycle consisting of: egg, four juvenile stages, adult male and female. The second stage juveniles (J2) of *M. incognita* enter the root tip and migrate intercellularly to the vascular bundle, where they establish permanent feeding sites by the induction of multinucleated 'giant' cells, visible as knotted formations in the roots (root galls or root knots). Severe infection by RKNs results in impaired water and nutrient uptake by the plant, eventually hindering crop productivity and resulting in stunted growth, yellowing of foliage, root necrosis, and in extreme cases, death of the host plant (Hussey et al., 1994; Jones et al., 2013). *M. incognita* host species include numerous staple crops such as maize and rice, as well as tomato, cucumber, soybean, and other important fiber crops such as cotton (Hedin et al., 1995; Wesemael et al., 2011; Kayani et al., 2017). It is ubiquitous in temperate, tropical, and subtropical climates and in commercial greenhouses worldwide. In Europe, RKNs outbreaks are increasingly relevant and are regulated according to international standards (Wesemael et al., 2011; EPPO, 2021).

Suppressive effects on *M. incognita* and other RKNs upon pot and land application of biogas digestate (BD), have been contrastingly documented but generally acknowledged, suggesting some nematicidal properties of the mixture (Jothi et al., 2003; Xiao et al., 2007; Min et al., 2007, 2011; Mahran et al., 2008; Wang et al., 2019). However, due to the high compositional variability of digestates, the chemical properties of this complex mixture have been scarcely researched, and the underlying mechanisms that promote the observed resistance effect to PPN infection are not yet well understood. It has been speculated that disease resistance may be promoted either indirectly by the soil mending effects of the digestate, or directly by an as yet unidentified group of molecules or metabolites that may exhibit natural antimicrobial, nematicidal and antifungal activities. To our knowledge, no study has investigated the nematicidal properties of specific degradation products in biogas digestate. However, screening for these biomolecules might contribute to a better understanding of disease suppressive effects upon digestate land application, and to develop novel 'green' formulations for integrated PPN management, eventually leading towards a full circular economy in biogas plants.

The present research aims to set a baseline for biogas digestate usage in integrated nematode management or for the development of environmentally friendly nematicides. The study includes testing of digestates under both laboratory and greenhouse conditions, an *in vitro* assay-guided chemical fractionation of the digestate mixture, and chromatographic identification of potential nematicidal compounds by GC–MS.

2. Material and methods

2.1. Biogas digestate collection

In April 2021, two biogas plants in the vicinity of Wädenswil (ZH, Switzerland) were sampled for liquid biogas digestate. Approximately 60 L of liquid digestates (solids <30 mm) were collected from each plant after phase separation, stored in PE-bottles, and preserved at −18 °C in dark conditions until use. Differences in plant operating parameters (e.g., substrate pretreatment, digester feedstock, reactor temperature, and hydraulic retention times) allowed the distinction of: (1) one agricultural digestate (AD), sourcing from mixed animal manure fermented for 140 days (mean retention time) through a 3 stages digestion; (2) one municipal digestate (MD), sourcing from municipal and industrial bio-wastes fermented for 10–14 days through a 2 stages digestion. The sampled biogas plants are regularly inspected and digestates are certified for organic food production. Details concerning the sampled digestates and digestion parameters are presented in Table S1.

2.2. Preparation of nematode inoculum

The root-knot nematode (RKN) *Meloidogyne incognita* (isolate Reichenau 2, Hallmann and Kiewnick, 2018) was cultured on *Solanum lycopersicum*, cv. Oskar (Syngenta) in a controlled environment (24 °C and 60% relative humidity).

RKN propagation was maintained by inoculation of approximately 20,000 infective second-stage juveniles (J2) on three-week-old tomato plants. Infected plants were reared under greenhouse conditions until the completion of two nematode life cycles. Freshly hatched J2 were harvested every 2–3 days for up to one week from heavily galled root systems by using a funnel spray method with a mistifier (Hallmann and Viaene, 2013). Nematode density (J2 mL^{-1}) was counted under a light microscope (Zeiss, at 5X magnification) and nematode suspensions were kept refrigerated at 4 °C until further use.

2.3. In vitro nematicidal assay

The effect of crude digestate on *M. incognita* J2 motility, was tested *in vitro*. At first, the digestates were applied without pretreatment (total crude digestate). Then, in order to remove solid particles, and allow a better visualization of the nematodes under the light microscope, crude digestates were centrifuged (6000 rpm, 10 min). Digestates were tested at 5 dilutions (0.5%, 1.0%, 2.5%, 5.0% and 10.0% BD) on an inoculum of 100 J2 mL^{-1}. An aqueous control (0% BD) was included. Tap water was used in all dilutions and controls in order to mimic the nature of interstitial soil waters and to avoid the build-up of osmotic pressure. A visual evaluation of J2 motility and fitness after incubation with the diluted BD solutions was done over a 14-days period at 5 time points (1, 2-, 3-, 7-, and 14-days post inoculation — dpi). Second-stage juveniles were counted in quadruplicates on a nematode counting slide under a light microscope and classified as: *active*, when showing a normal motile behavior (similar to the water control); *inhibited*, when showing twisted or coiled shapes, and *dead*, when motionless and straightened (Fig S1). In order to determine the infection capacity of the nematode inoculum exposed to BD, the *in vitro* assay was accompanied by plant infestation bioassays. At each time point (1, 2, 3, 7, and 14 dpi), aliquots (∼250 J2) of each treatment (0.5%, 1.0%, 2.5%, 5.0% and 10.0% BD) were applied in quintuplicates (n = 5) to pre-germinated cucumber seedlings (*Cucumis sativus*, cv. Landgurken, Bigler Samen), sown in 10 mL soil pots. Infected cucumber plants were grown for 21 days in a climate chamber (T = 24 °C, RH = 60%). Then, a classification of the infestation level of the root systems was performed after a 10-grade scale root gall rating, as described by Zeck (1971; 0: no galls, up to 10: completely galled roots).

2.4. Biogas digestate effect on Meloidogyne incognita under green house conditions

A greenhouse bioassay was performed to evaluate nematode infection in tomato roots upon soil application of BD. Perforated polypropylene pots (flowerpots 5°, 13 cm, Desch Plantpak, Netherlands) were filled with approximately 600 mL (±10 mL) volume of sand:soil mix (3:1, 1–6 mm quartz sand: sieved soil from Cadenazzo, TI, Switzerland). A filter paper disk (595 μm, Hahnemühle, Germany) was placed at the bottom of each pot to prevent soil loss. Approximately 3000 *M. incognita* J2 were inoculated homogeneously in each pot and kept moist while untreated, to allow the nematode distribution and adaptation in the soil. After two days, BDs were applied at five different concentration rates in soil (10.0%, 5.0%, 2.5%, 1.0%, and 0.5% BD, v/v; n = 12). The topsoil was mixed to mimic field application conditions. Some digestate-free pots were kept as diseased (inoculated with *M. incognita*) and healthy (not inoculated) control (n = 12). Three days after BD application, five-week-old tomato plants (cv. Oskar, Syngenta) were planted in the pots in a randomized set up and grown in standard greenhouse conditions (T = 24 °C, RH = 60%). In order to assess the potential fertilizing potential of the digestates, few plants (n = 4) were exposed only to BD (10.0%, 5.0%, 2.5%, 1.0%, and 0.5% BD, v/v) without nematode inoculum. All pots were consistently fertilized with 1 mL L^{-1} Wuxal® (Maag Profi, Syngenta) to support plant growth. Plants were harvested after 5 weeks (n = 3), and 10 weeks from transplanting (n = 9). Shoots were tested for fresh and dry weight, while root galling was indexed according to Zeck (1971). The gall rating was repeated with higher nematode pressure. For this purpose, a second set of plants (n = 5) was inoculated with approximately 8000 *M. incognita* J2, and reared for 7 weeks after BD application at the same conditions previously detailed.

2.5. Solvent fractionation of the biogas digestate

Liquid digestates were fractionated by liquid: liquid partition using solvents with different relative polarities, namely: ethyl acetate (EA, 0.228), chloroform (CHL, 0.259), and acetone (AC, 0.335) — pure solvents (Merck) against water (0.998) on a separation funnel (Reichardt, 2003). Crude biogas digestate (200 mL) was mixed with an equal volume of each extracting solvent separately (1:1; v/v), in a borosilicate glass separation funnel (500 mL). The mixture was stirred and cooled at −18 °C for 45 min, and later at 6 °C for 1 h, to allow the separation of the polar and non-polar fractions, which were finally collected in separate flasks. The procedure was done the same way for both digestates. The obtained aqueous fractions were concentrated by rotary evaporation (Buchi Brinkmann R-110 Rotavapor Rotary Evaporator) at 40 °C to approximately 50 ml, frozen and freeze-dried. Non-polar samples were concentrated at the same conditions until dryness, resuspended in methanol, transferred to glass vials, and air-dried. All resulting extracts were weighted to estimate extraction yields.

2.6. In vitro effect of non-polar and polar biogas digestate fractions on Meloidogyne incognita second stage juvenile

The solvent extracted fractions were tested *in vitro* for nematicidal activity on *M. incognita* J2. Nematode motility was evaluated in a 24-well plate containing nematode suspension and different concentrations of tested extracts. All dried extracts (EA, AC, and CHL non-polar and polar extracts) were re-dissolved in a suitable solvent (methanol or water, respectively) to be tested at three different concentrations (1.0, 0.1, and 0.01 mg mL^{-1}). The resuspended extracts (300 μL per well) were added to 24-well plates at the desired concentration and air-dried, then a 400 μL nematode suspension (approximately ∼150 J2 per well) was added to each well. Non-polar and polar treatments were kept in separate plates from the water and methanol controls. All treatments were carried out in triplicates over a period of 7 days. Plates were covered with plastic lids and sealed with Parafilm® to minimize evaporation, and incubated at room temperature (∼21 °C), in the dark. Immotility rates of *M. incognita* J2 were determined for each well as described previously (Section 2.3) at 1, 2, and 3 dpi.

2.7. Untargeted chemical characterization of selected biogas digestate solvent extracted fractions

The assessment of the chemical diversity of the digestate fractions was attempted through an untargeted approach by using a gas chromatographer coupled with mass spectrometer (GC–MS). Sample derivatization was done in 100 μL aliquots of 1 mg mL^{-1} of each of the non-polar extracts, transferred to a 2 mL reaction vial and evaporated to dryness under nitrogen flow. An equal volume of pyridine and BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) + TMCS (trimethylchlorosilane) (Sigma Aldrich) was added was used to re-dissolve and silylate the samples. The mixture was incubated at 60 °C for ∼7 h. Derivatized samples were dried and diluted (1:5) in chloroform and analyzed using a Varian 450-GC coupled to an Varian 240-MS detector (Darmstadt, Germany) using a VARIANT FactorFour Capillary column VF-5 ms (30 m, 0.25 mm, 0.25 μm). Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. Inlet temperature was set at 320 °C, and 10 μL of the sample were injected.

All analyses were carried out in technical triplicates. The NIST mass spectra library was used for compound identification, and relative abundances were calculated as percentages in relation to the total and individual peak areas determined for each of the non-polar extracts.

2.8. Statistical methods

The *in vitro* nematicidal activity of crude digestates and their derived extracts was expressed as J2 *mortality*, intended as the mean percentage of dead nematodes to the sum of the total count in the replicate test samples or as *immotile* J2 (intended as the mean percentages of immotile J2 — both inhibited, and dead). Data from *in vitro* and *in planta* viability assays of *M. incognita* J2, as well as the data on BD growth promotion effect were analyzed using one-way analysis of variance (ANOVA) with post-hoc Tukey–Kramer HSD test, or Student's t-tests (*t*-test) for statistical significance with R and RStudio softwares (R Development Core Team, 2008). The statistical significance among variables was tested using a significance level of 0.05.

3. Results

3.1. Biogas digestate decreases Meloidogyne incognita in vitro survivability and fitness

Both digestates (AD — Agricultural Digestate, MD — Municipal Digestate) showed nematicidal effects *in vitro* on *M. incognita* J2, as supported by *in planta* infestation bioassays. A significant increase of J2 mortality was observed *in vitro* over a 14-days' assay at all AD concentrations tested and at MD concentrations $\geq$ 2.5% (v/v) (Fig. 1). At 1 dpi, mortality rates of *M. incognita* were significantly affected ($p \leq 0.05$) only by the highest BD applications (5.0% and 10.0% BD v/v), reaching average mortality rates of 19.7% ± 5.82% and 60.6% ± 8.89% for AD, and 32.2% ± 5.30% and 73.1% ± 12.88% for MD, respectively, while less than 1.0% J2 mortality occurred in the respective water controls. A similar effect was observed

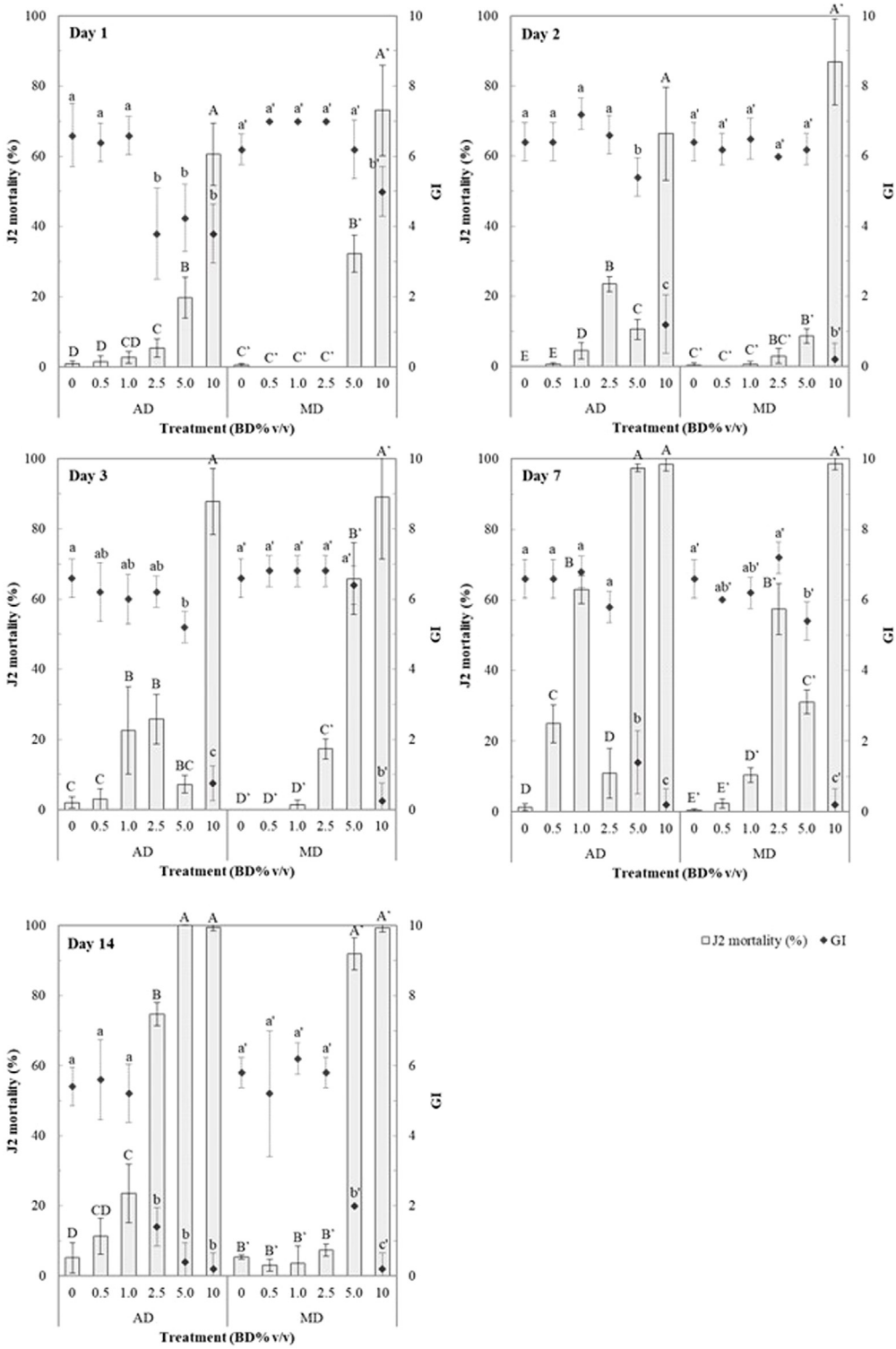

Fig. 1. *Meloidogyne incognita* second-stage juvenile (J2) mortality response (mean percentages ± standard deviations) following *in vitro* treatments at different biogas digestate rates (%, v/v), compared to the water controls (0%) at 1, 2, 3, 7, and 14 dpi ($n = 4$). Gall indexes (GI; $n = 5$) according to Zeck (1971; 0: no galls, 10: completely galled up roots), for the respective infestation bioassays, are displayed in the secondary axis (♦). AD — Agricultural Digestate, MD — Municipal Digestate. Statistical significance was calculated using one-way ANOVA with post-hoc Tukey–Kramer HSD test, $p < 0.05$. Means followed by the same letter are not significantly different.

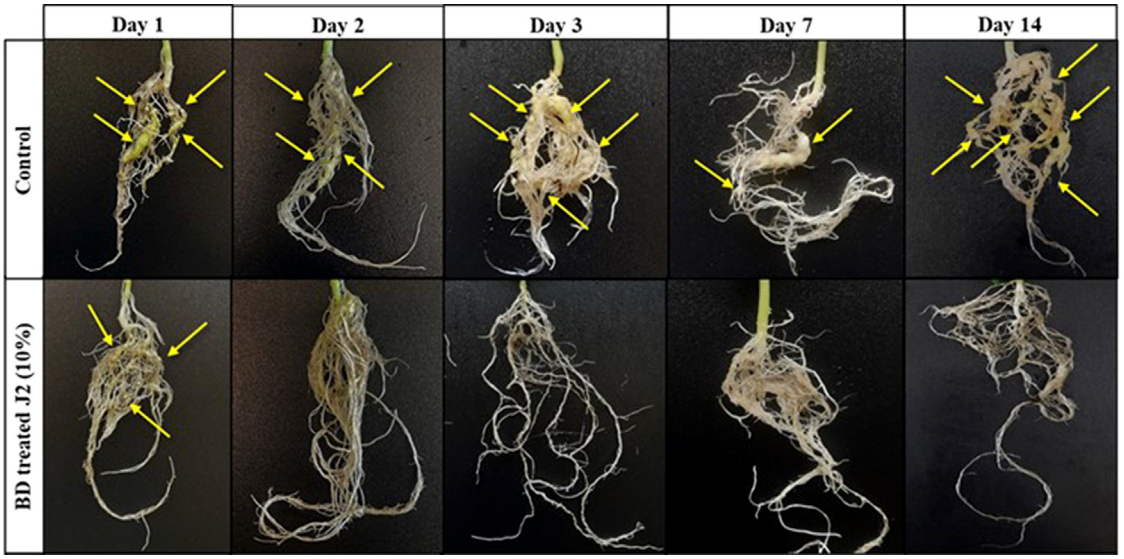

Fig. 2. Observed gall formation on cucumber roots cv. Landgurken (Bigler Samen) 21 days post inoculation (dpi) with *Meloidogyne incognita* previously exposed to 10.0% of biogas digestate (BD) (v/v) for 1, 2, 3, 7, and 14 days. Pre-germinated seedlings were inoculated with ~250 *M. incognita* second-stage juveniles (J2).

at lower application rates over the incubation time. At 2 and 3 dpi, AD did not show significant nematicidal effect at 5.0% but lower concentrations (2.5% at 2 dpi; 1.0% and 2.5% at 3 dpi) showed significant increase in mortality with respect to the control. In fact, at these concentrations, at 3 dpi, J2 mortality reached 22.5% ± 12.42% and 25.8% ± 7.07% respectively. At 7 and 14 dpi, all AD concentrations showed significant increase in mortality rates with respect to the water control, reaching a near total of dead J2 in the two highest treatment rates (5.0% and 10.0% BD). This was not consistent for the MD treatments, where at day 7, 2.5%, 5.0%, and 10.0% treatments showed significant differences from the control, while at day 14 only 5.0%, and 10.0% treatments were significantly different from the water control. Both digestate application rate and time of exposure (dpi) were found to be significant parameters by linear models calculated on average percentages of dead J2. Significant differences in J2 mortality rates were also observed when comparing both BD, but only at 2.5% (1, 2, 7 and 14 dpi) and 5.0% (3, 7 and 14 dpi) (Table S1).

In planta infestation assays, conducted at each time point by inoculation of cucumber seedlings with the BD-exposed nematode suspensions (Section 2.3), confirmed the ability of 5.0% and 10.0% AD and MD treatments of mitigating the damage from *M. incognita* infection. A strongest reduction of root galling was observed at 10.0% BD treatment 2 dpi for both digestates [mean GI from 6.4 ± 0.55 (control) to 1.2 ± 0.84 (AD) and 0.2 ± 0.45 (MD)] matching the high mortality recorded *in vitro*. At this dilution, from 2 dpi onwards, replicate plants with gall indexes of zero were also found for both digestates. A comparable nematode suppression was obtained with 5.0% BD treatment, after 7 dpi for AD (GI 1.4 ± 0.89), and after 14 dpi for MD (GI 2.0 ± 0.0). A suppression on root gall formation was observed also for the 2.5% treatment at 14 dpi (GI 1.4 ± 0.55), but only for AD. In general, a less prominent but significant effect could already be seen at 1 dpi for 2.5%, 5.0%, and 10.0% AD treatments (Figs. 1 and 2; Table S2).

3.2. Biogas digestate supports plant growth and decreases M. incognita infection efficiency under greenhouse conditions

Effects on plant growth parameters and root gall index were tested in a greenhouse bioassay over 5 and 10 weeks (Fig. 3). Digestate application did not show phytotoxic effects on tomato plants. On the contrary, taller shoots were observed in the pots treated with higher BD concentrations. In fact, at both time points, fresh and dry weight showed an increasing trend with increasing BD application rates. Plants treated with 10.0% BD showed fresh weights more than double that of the control, with AD providing the highest biomass gain (Fig. 3a). Dry weight followed a similar trend (Fig. 3b), ad significant differences were even observed between AD and MD at 10.0% after 5 weeks (*p*-value 0.03) treatment and at 5.0% after 10 weeks treatment (*p*-value 0.00). Furthermore, application of diluted biogas digestate showed a positive effect on preventing root galling, at 5.0% and 10.0% (GI 1.0 ± 0.00 and 1.7 ± 1.15 for AD and 1.7 ± 0.58 and 1.3 ± 0.58 for MD, respectively) after 5 weeks treatment. After 10 weeks treatment, 5.0% showed a higher impact in root gall formation for AD (GI 1.6 ± 0.73), while 10.0% BD application promoted a reduction in root gall for MD (2.2 ± 0.58). As a tendency, increasing BD application rate promoted progressively lower root galling for both digestates (Fig. 3c). When comparing both BD, significant differences were observed only after 10 weeks treatment at 2.5% (*p*-value 0.03) and 5.0% (p-value 0.02) BD application.

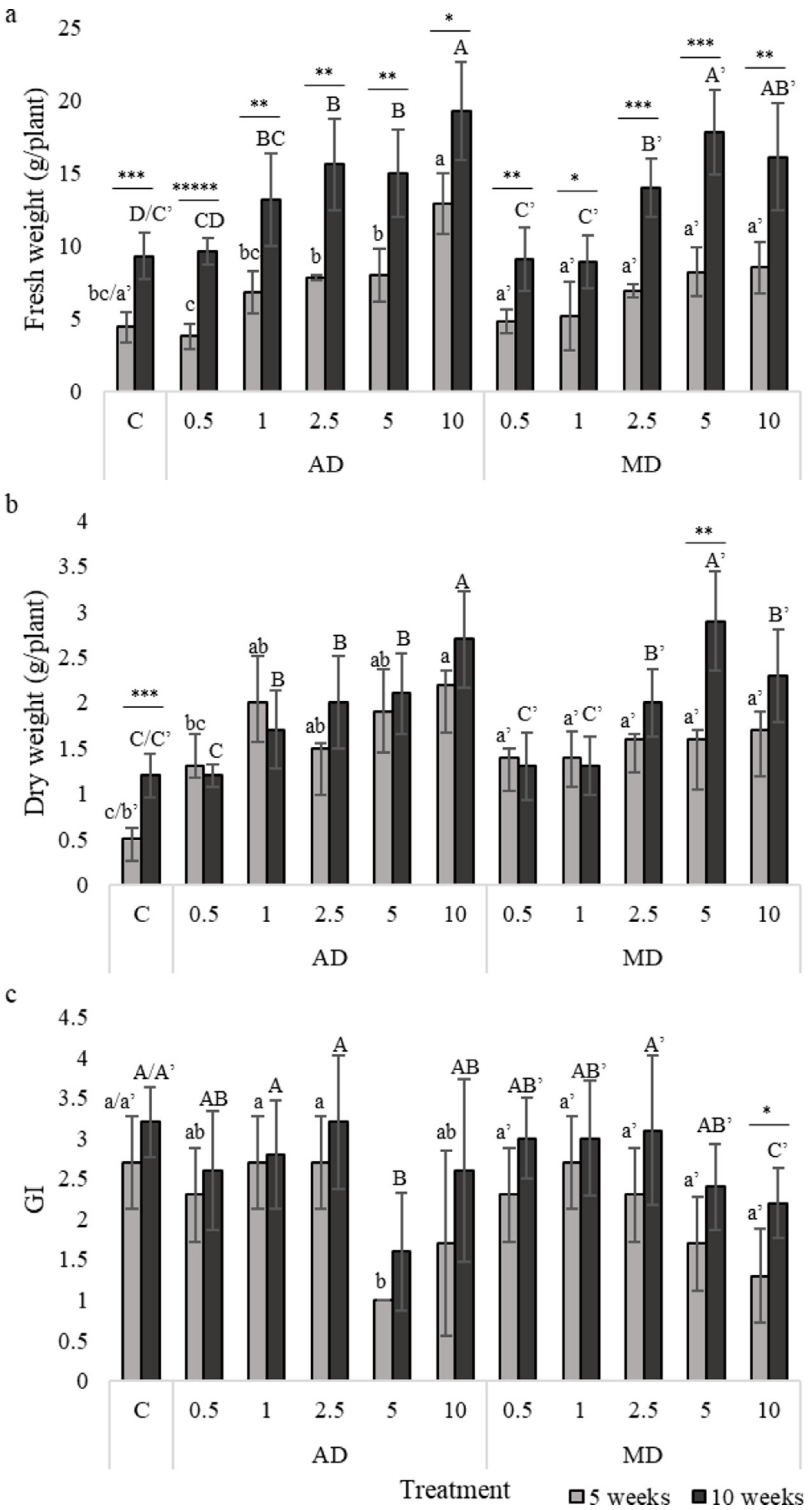

Fig. 3. Effect of biogas digestate (AD — agricultural digestate, MD — Municipal digestate) application on *Meloidogyne incognita* infected *Solanum lycopersicum* (cv. Oskar). Plant growth parameters, fresh (A) and dry (B) weight and root gall index recorded according to Zeck's (1971) scale of root-knot infection (C) after 5 ($n = 3$) and 10 ($n = 9$) weeks are displayed. Statistical significance was calculated using one-way ANOVA with post-hoc Tukey–Kramer HSD test, $p < 0.05$. Means followed by the same letter are not significantly different. * Is used to denote statistically significant differences between 5- and 10-weeks treatments: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ***** $p < 0.0000$.

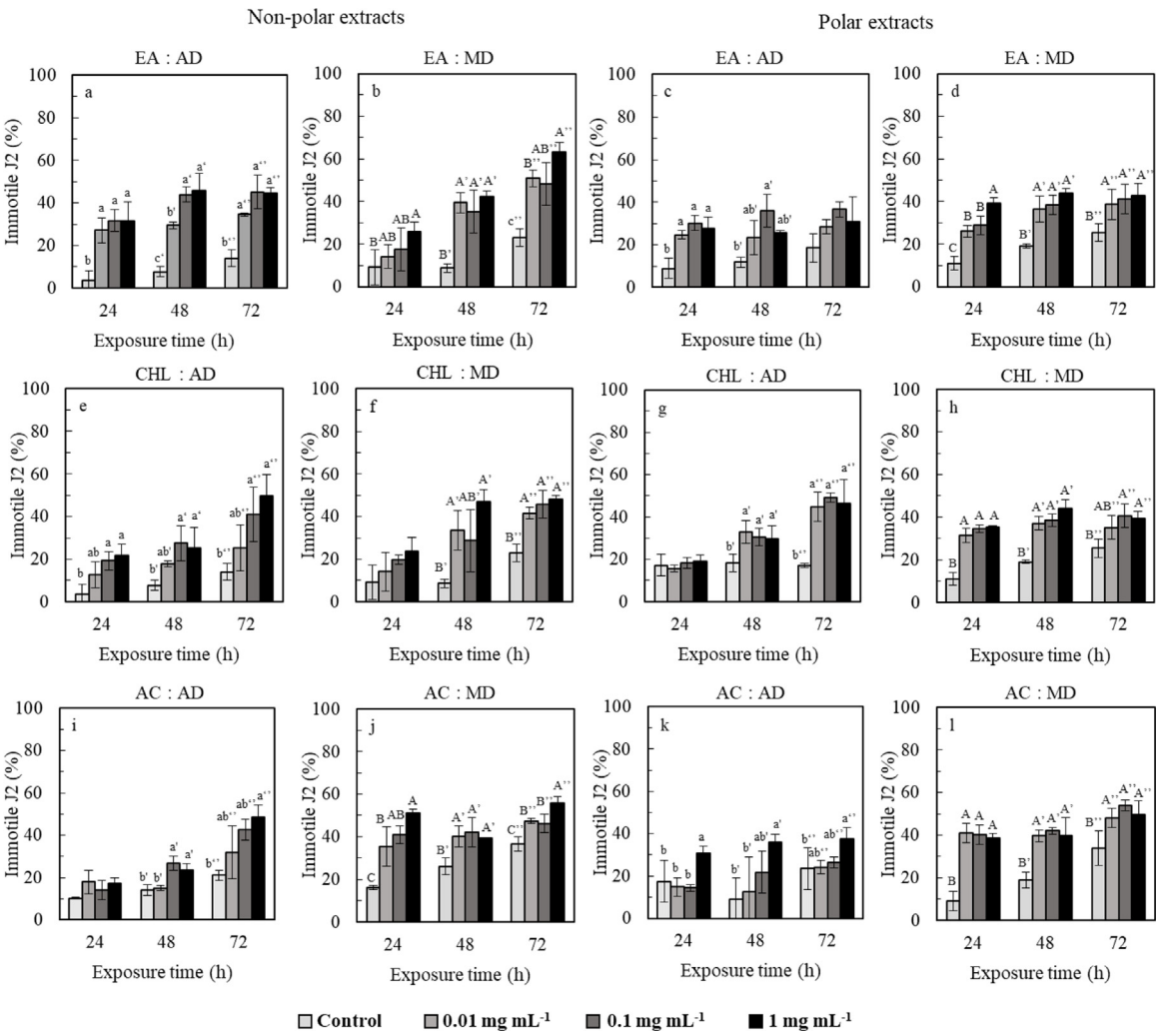

Fig. 4. Immotile (inhibited + dead) *Meloidogyne incognita* second-stage juveniles (J2; mean percentages and standard deviations) following *in vitro* treatment with non-polar and polar extracts from liquid: liquid fractionation (1:1) of AD and MD (at 0.01, 0.1 and 1.0 mg mL^{-1}, after 24, 48 or 72 h) and the respective controls are shown for all solvent separations. Statistical significance was calculated using one-way ANOVA with post-hoc Tukey–Kramer HSD test, $p < 0.05$; $n = 3$ biological replicates. Means followed by the same letter are not significantly different. Ethyl acetate (EA) — organic phase (A, A′) and water phase (B, B′); chloroform (CHL) — organic phase (C, C′) and water phase (D, D′); and acetone (AC) organic phase (E, E′) and water phase (F, F′), for AD — Agricultural Digestate and MD — Municipal digestate, respectively.

3.3. Biogas digestate solvent extracts decrease *Meloidogyne incognita* in vitro survivability

After liquid: liquid partitioning, extraction yields of the non-polar fractions were significantly lower than the respective polar yields. From the non-polar extracts, acetone (AC) partition yielded the highest amount for both BDs (approximately 22% of the total extracted mass), while chloroform (CHL) and ethyl acetate (EA) non-polar extracts accounted only for less than the 0.4% of the total extracted mass. Among the polar extracts the highest yield was achieved by the partitions with AC and EA.

Overall, nematodes incubated for 24, 48, and 72 h at all concentrations of both non-polar and polar extracts showed a significantly higher number of immotile J2 (inhibited and dead) than nematodes in the control (Fig. 4).

In vitro testing of non-polar fractions derived from AD and MD showed slightly higher percentages of immotile J2 than the respective polar fractions. Within the non-polar extracts, EA accounted for the highest inhibition rate, by reaching 45.7% ± 8.05% for AD (Fig. 4a) and 63.4% ± 3.87% immotile J2 for MD (Fig. 4b), at 1 mg mL^{-1}, after 48 h and 72 h of treatment, respectively, while CHL and AC extracts affected 49.7% ± 9.90% (AD) and 48.0% ± 1.86% (MD) (Fig. 4e and f), and 48.4% ± 12.51% (AD) and 55.7% ± 3.22% (MD) (Fig. 4i and j) of the nematodes over 72 h respectively.

Table 1
Summary of compounds detected in the two different biogas digestates by GC–MS analysis.

Compounds	m/z	Relative abundance (%)						Nematicidal properties	
		AD			MD			Plant parasitic nematode species	Reference
		AC	EA	CHL	AC	EA	CHL		
4-hydroxy-4-methylpentan-2-one	29, 43, 59, 101, 41, 58	3.4 ± 0.32^{e}	12.1 ± 0.66^{c}	3.8 ± 0.25^{de}	29.7 ± 1.42^{a}	5.4 ± 0.61^{d}	21.1 ± 0.18^{b}	n.d.	–
Nonanoic acid	117, 215, 129, 131, 132	1.4 ± 0.25^{c}	3.0 ± 0.31^{b}	–	7.3 ± 0.13^{a}	1.3 ± 0.09^{c}	1.5 ± 0.57^{c}	*Meloidogyne javanica, Heterodera glycines, Meloidogyne incognita*	Davis et al. (1997)
Nonadecanoic acid	117, 129, 132, 145, 355	20.0 ± 0.55^{a}	–	–	–	12.4 ± 0.23^{b}	8.0 ± 0.16^{c}	n.d.	–
Octanoic acid	73, 75, 117, 41, 29	5.0 ± 0.13^{c}	11.9 ± 0.10^{a}	10.0 ± 0.95^{b}	6.2 ± 0.36^{c}	1.7 ± 0.37^{d}	6.0 ± 0.10^{c}	*Heterodera tabacum, M. incognita*	Loos (1958), Da Silva et al. (2021) and Zhang et al. (2012)
Tetradecanoic acid	73, 117, 75, 129, 41	4.7 ± 0.26^{b}	5.9 ± 0.05^{b}	9.7 ± 0.98^{a}	5.4 ± 0.38^{b}	3.0 ± 0.34^{c}	6.0 ± 0.27^{b}	*M. incognita*	Zhang et al. (2012)
Decanoic acid	73, 117, 69, 75, 55, 132, 41	4.2 ± 0.09^{a}	3.6 ± 0.03^{b}	1.2 ± 0.05^{c}	4.0 ± 0.41^{ab}	1.5 ± 0.19^{c}	1.2 ± 0.04^{c}	*Bursaphelenchus xylophilus, M. incognita*	Faria et al. (2021) and Zhang et al. (2012)
Dodecanoic acid	73, 75, 117, 41, 129, 43	20.4 ± 0.51^{c}	34.1 ± 1.46^{b}	44.0 ± 0.47^{a}	12.9 ± 0.78^{e}	20.5 ± 0.69^{c}	16.7 ± 0.35^{d}	*B. xylophilus, M. incognita*	Faria et al. (2021), Dong et al. (2014) and Zhang et al. (2012)
Tetracosanoic acid	73, 75, 117, 129	10.2 ± 0.57^{c}	–	1.8 ± 0.16^{d}	22.3 ± 0.96^{a}	16.0 ± 0.81^{b}	11.5 ± 0.19^{c}	n.d.	–
Dehydroabietic acid[1]	239, 73, 240, 173, 143	–	–	5.1 ± 0.75^{a}	2.2 ± 0.14^{b}	1.2 ± 0.35^{b}	5.1 ± 0.35^{a}	*B. xylophilus*	Bolla et al. (1989)
[2-(benzenesulfinylmethyl)-3-phenylcyclopropyl]benzene	91, 117, 115	11.2 ± 0.12^{a}	6.6 ± 0.12^{b}	5.3 ± 0.82^{c}	–	6.3 ± 0.31^{bc}	7.0 ± 0.39^{b}	n.d.	–
Benzoic acid	281, 223, 73, 283, 207	2.7 ± 0.21^{b}	0.8 ± 0.06^{c}	5.1 ± 0.35^{a}	0.7 ± 0.11^{c}	0.4 ± 0.02^{c}	0.6 ± 0.06^{c}	*M. incognita*	Khan et al. (2019)
Decanedioic acid	41, 43, 55, 57, 83, 185	6.0 ± 0.51^{a}	3.8 ± 0.36^{b}	4.2 ± 0.18^{b}	2.6 ± 0.49^{c}	1.2 ± 0.07^{d}	2.5 ± 0.11^{c}	n.d.	–
Others		11.0 ± 0.10^{d}	18.2 ± 1.28^{b}	9.7 ± 0.70^{d}	6.6 ± 0.64^{e}	29.2 ± 1.64^{a}	14.3 ± 0.18^{c}	*M. javanica (Isoxazoles)*	Chopra et al. (2006)

[1] (1R,4aS,10aR)-1,4a-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-1-carboxylic acid n.d. — not determined
Statistical significance was calculated using one-way ANOVA with post-hoc Tukey-K HSD test, $p < 0.05$. Statistical differences within the same BD and extract are shown by lowercase letters, while between the same extract from a different BD are shown by uppercase letters. Means followed by the same letter within the same line are not significantly different, where 'a/A' has been given to the highest value. AC — acetone, EA — ethyl acetate, CHL — chloroform. AD — Agriculture digestate; MD — Municipal digestate

The highest inhibition rate within polar extracts was observed for CHL from AD (49.2% ± 2.18%; Fig. 4 g) and for AC from MD (54.0% ± 2.49%; Fig. 4l), both after 72 h treatment at 0.1 mg mL^{-1}. Overall, MD displayed a consistent nematode suppressing effect after short-term exposure, which was found to be less marked in the nematodes treated with AD.

3.4. Untargeted characterization of bioactive metabolites in selected biogas digestate-derived fractions

After derivatization and GC–MS analysis, over forty different compounds were identified in the different non-polar extracts. The distribution of chromatographic peaks was similar in all tested extracts, however, MD-derived EA and CHL extracts showed the highest diversity of compounds. The main compounds identified are summarized in Table 1. From the twelve main components, seven were previously described as having nematicidal properties against different PPN such as *M. incognita* and *Meloidogyne javanica*, *Heterodera glycines* and *Heterodera tabacum*, and *Bursaphelenchus xylophilus* (Loos, 1958; Davis et al., 1997; Chopra et al., 2006; Zhang et al., 2012; Dong et al., 2014; Da Silva et al., 2021; Faria et al., 2021), with the exception of benzoic acid, 4-hydroxy-4-methylpentan-2-one, [2-(benzenesulfinylmethyl)-3-phenylcyclopropyl]benzene, and (1R,4aS,10aR)-1,4a-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-1-carboxylic acid (dehydroaboietic acid) all the others are medium- or long-chain fatty acids (Table 1).

Dodecanoic acid appeared as the most abundant compound for both EA extracts and for AD-derived CHL extract, while for AD-derived AC extract, dodecanoic acid and nonadecanoic acid were the most abundant, and for MD-derived AC and CHL extracts, 4-hydroxy-4-methylpentan-2-one and tetracosanoic acid appeared as the most abundant.

4. Discussion

Natural product-based pesticides have gained great relevance in the pesticide market (Cantrell et al., 2012) and enriched bioactive extracts from agricultural residues and plant exudates (e.g. oil-based or plant-derived), fungal-derived formulations, as well as the incorporation of a variety of organic residues into the soil (e.g. crop residues, oil cakes, agro-industrial wastes and animal and urban wastes) have shown some success as integrated plant parasitic nematode (PPN) management techniques (Renčo, 2013; Ntalli et al., 2020; Rosskopf et al., 2020; Das et al., 2021). However, their efficiency in PPN control was not always been satisfactory (Oka, 2010).

In this context, anaerobically digested organic materials from biogas plants have shown promising but yet poorly researched suppressive effects for a wide range of agronomic important pathogens and pests (Min et al., 2011; Westphal et al., 2015; Baştabak and Koçar, 2020; Eberlein et al., 2020). Biogas digestate (BD), which is commonly recycled as a renewable fertilizer, has been found effective against at least 23 different plant diseases and 14 different pests, including PPN (Jothi et al., 2003; Feng et al., 2011; Min et al., 2011; Alburquerque et al., 2012; Wang et al., 2019).

The root knot nematode (RKN) *Meloidogyne incognita* is a widespread pest of great economic importance, causing severe yield losses in high-value crops (Jones et al., 2013). In our study, both *in vitro* assays (assessing nematode movement and fitness) and *in planta* assays (assessing root infection) confirmed the value of digestate as both plant stimulant and suppressive composite for *M. incognita* management. Parameters such as optimal BD application rate and timing were addressed. Under *in vitro* conditions diluted BD proved harmful to *M. incognita* second stage juveniles (J2) already within 24 h exposure period (Fig. 1). In greenhouse experiments, a single application of diluted digestate was sufficient to exert control over *M. incognita* infestation of susceptible cultivars (tomato cv. Oskar, and cucumber cv. Landgurken), significantly reducing or even preventing the root galling and improving plant biomass (Fig. 3). Progressively higher J2 mortality and a decrease in root knots were observed at increasing BD application rates in all experimental setups, supporting the hypothesis of a positive correlation between BD application and *M. incognita* suppression (Fig. 1). However, phytotoxic effects can be expected with application rates higher than 10.0%. The time of application may also be critical, as slower acting and/or volatile compounds may be lost or nematodes may be able to recover. For instance, in some cases, at the initial time points, *in vitro* exposed nematode suspensions were still able to induce root galls in the cucumber bioassays, even though they appeared non-viable at the time of observation. The results suggested a 1–3-day recovery period in water for the nematode population tested. Therefore, the effect may initially be nematostatic rather than nematicidal. Overall, our results suggest that the nematicidal effect is both concentration and time dependent, with a stronger dependency on the former.

These findings are quite encouraging, as they are in line with previous investigations that reported direct impact of BD on nematode mortality and benefits on plant growth in similar laboratory and greenhouses setups (Jothi et al., 2003; Min et al., 2011; Westphal et al., 2015), and also on naturally infested soils (Valocká et al., 2000; Wang et al., 2019).

Neither the source nor the retention time seemed to imply a notable difference in efficacy between the two digestates tested in our study. However, other experimental setups may result in different outcomes based on the diversity of the digestates, soil–plant system characteristics and biochemical interactions under field conditions (e.g. microbial communities present in the soil, adsorption to soil particles), making an actual prediction of the nematode-suppressing activity of digestates uncertain. For these reasons, several authors recorded inconsistent or inconclusive results (Min et al., 2011; Eberlein et al., 2020; Das et al., 2021). Moreover, the lack of systematic and in-depth research into the control mechanisms of PPN infection makes it difficult to come to an agreement on whether and how to allow the agricultural use of biogas digestate for PPN management. Thus, more specific information on the underlying mechanisms and compounds responsible for PPN control is needed to design optimized conditions (e.g. digestion conditions, mode of application) for efficient nematode control. It has often been suggested that pest control by digestate may be linked to one of these mechanisms: enhancement of antagonistic soil microbial activity, presence of toxic compounds in the organic feedstock or presence of toxic metabolites produced during microbial degradation (Renčo, 2013). However, all these theories are still mostly speculative.

As the end product of a microbiologically mediated anaerobic digestion, biogas digestate (BD) contains a wide variety of decomposed residue materials and fermentation metabolites, including plant-available macro- and micro-nutrients (N, P, K, Ca, Cu, Fe, Zn, and Mn), ammonium nitrogen, monosaccharides, and stabilized carbon. Mineralized nutrients can easily be absorbed by crops, thus supporting plant growth, while humic acids and hard-to-degrade complex organic matter (e.g., in lignin-rich organic wastes) can regulate soil structure (Alburquerque et al., 2012; Baştabak and Koçar, 2020). This explains the fertilizing effect of digestates, but also the building of soil suppressiveness. In fact, disease resistance may be *indirectly* promoted by improved soil nutritional status, increased levels of soil organic matter, and possibly by the stimulating natural antagonists that would tend to alter the host-parasite relationship in favor of the host plant (a healthier plant is a poorer host) (Baştabak and Koçar, 2020). However, we believe that this 'organic amendment' character of the digestate is only partially contributing to the control of PPN, which is the result of more synergistic and complex mechanisms involving residual bioactive metabolites (e.g., compounds with antifungal and antibacterial properties or proteases) present in the mixture. Some studies reported that soils amended with digested manure showed more effective and long-lasting effects on nematode suppression than soils amended with the same manure but undigested (Bulluck et al., 2002; Xiao et al., 2007). This suggests that an enrichment in fermentation metabolites during the digestion process may enhance the nematicidal properties of the substrate and therefore supports the theory of a rather *direct* chemical control by a yet unidentified dissolved bioactive molecule or group of molecules (soluble fermentative end products/metabolites). A plethora of bioactive and diffusible metabolites of known nematicidal and nematotoxic properties, such as alkaloids, phenols, amides, ketones, terpenoids, natural ester compounds, and N- and O-heterocyclic compounds (e.g., triazoles) have been found in natural sources, including digestates (D'Addabbo et al., 2020; Chen and Song, 2021). These substances can permeate the nematode body wall, resulting in toxicity to the organism. This, combined with higher osmotic pressure due to the high salt content of the digestate, can disrupt the osmotic balance of the nematode and thus interfere with the integrity of the nematode cuticle or hypodermis causing its death (He et al., 2020). More attention is needed to the chemical nature of compounds that confer nematode suppression and the mechanisms of this nematicidal action of

digestate. On the other hand, enabling a more targeted identification and isolation of groups of molecules with these characteristics poses several analytical challenges. In our untargeted approach we attempted a fractionation based on the polarity of these compounds by using greener (ethyl acetate and acetone) replacement solvents to common petroleum based extractants, in comparison to chloroform. Solvent extraction is based on the relative affinity or solubility of compounds between two immiscible liquids. However, due to the narrow relative polarities of the selected solvents, and probably the similar physicochemical properties of the target compounds (e.g. ionization constant, melting point, boiling point, solubility, octanol–water partition coefficient, volatility, vapor pressure or Henry's constant) a clear difference in nematicidal effects and composition between the non-polar and polar BD-derived extracts could not be found by *in vitro* screening and the subsequent analysis of the extracts by GC–MS. In the screening, all extracts were comparably effective in suppressing J2 viability (Fig. 4), with a slightly greater effect promoted by the non-polar EA extract, while the GC–MS analysis mainly showed some differences between the two types of digestate rather than between the different extracts. To improve compound selectivity, the use of extraction solvents covering a wider range of polarity may be considered. However, it should be noted that the compounds of interest (e.g. VFAs, medium-chain carboxylic acids) are usually very similar in ionization constant, melting point, boiling point, solubility, octanol–water partition coefficient, volatility, vapor pressure or Henry's constant, which makes their separation challenging. Otherwise, some other approaches based on filtration, ultra-filtration and nanofiltration membranes could be an interesting and a more environmentally-friendly approach for separation/fractionation of biogas digestate and its derived fractions.

In order to identify the compounds, present in the non-polar extracts, the samples were derivatized (silylated). Thus, detection was limited to classes of compounds which can accept a silyl group. This resulted in a qualitative detection of mainly medium- and long-chain fatty acids or natural acid compounds (carboxylic acids and terpenoids), alkyl alcohols and enols, ketones, and esters (Table 1).

Straight chain fatty acids of intermediate length (C8–C10) and their derivatives have been reported as nematicidal to PPN and in the past have been the basis for the development of emulsions to inhibit RKN. Their biocidal effect seems to be enhanced by esterification of these compounds (Davis et al., 1997).

Emphasis has been placed in the literature on the toxicity of low molecular weight organic acids (short-chain carboxylic acids, e.g., volatile fatty acids — VFAs) and ammonia: typical fermentation metabolites produced following bacterial fermentation of amino acids under anaerobic conditions and accumulated in the digestate (Browning et al. 2006; Xiao et al., 2007; Mahran et al. 2008; Da Silva et al., 2021). Low molecular weight organic acids are known to possess antibacterial and nematicidal activity (Akhtar and Malik, 2000; McBride et al., 2000; Chantigny et al., 2004; Browning et al., 2006; Mahran et al., 2008). In addition, nematicidal activity of ammonia in organic amendments has been extensively reviewed and is known to play an important role in nematode suppression (Oka et al., 2007; Renčo, 2013). However, these compounds are easily lost in the short term and may result in the loss of long-term effect due to their volatile characteristics. More efforts in identifying more stable compounds, such as the long-chain carboxylic acids we have described (Table 1), may be a good direction for a better understanding of longer-term effects. Seven of the twelve most abundant qualitatively identified compounds were previously described as having outstanding nematicidal properties against different PPN such as *M. incognita* and *Meloidogyne javanica*, *Heterodera glycines* and *Heterodera tabacum*, and *Bursaphelenchus xylophilus* (Loos, 1958; Davis et al., 1997; Chopra et al., 2006; Zhang et al., 2012; Dong et al., 2014; Da Silva et al., 2021; Faria et al., 2021), within these, five are long-chain carboxylic acids (Table 1).

These results are not conclusive to determine the nature of PPN suppression by digestate application, as it is not possible to identify all compounds present in such a complex mixture. However, we describe a clear trend and provide a valuable insight into integrative approaches for sustainable PPN management, which can stimulate new research directions and keep digested by-products in the agricultural production chain. In fact, a bio-based production of nematicidal compounds from sludge could have significant advantages in terms of costs and sustainability compared to chemical synthesis. Thus, digested sludge could have even higher application potential when combined with the appropriate treatments and valorization process. However, more work is needed to identify innovative ways to utilize digested sludge or its conversion into less recalcitrant, less toxic digestate-based or digestate-derived plant protection products to replace or complement the use of chemical nematicides or biocontrol agents.

5. Conclusions

Biogas digestate confirmed to be a potentially valid resource for sustainable management of *M. incognita* infection in susceptible crops. Nematode survival and juveniles' infection capacity were significantly affected by the application of crude and fractionated digestate both *in vitro* and *in planta.* High mortality rates of J2 were observed *in vitro* within 14 days. In greenhouse trials, application of crude BD at 5.0% and 10.0% dilution reduced plant infection and sustained plant biomass. This suggests that digestate itself or its 'purified' compounds could partially replace traditional synthetic agrochemicals as an inexpensive biopesticide in controlling PPN infestation and hints at the possibility of an integrated control by digestate, either by direct land application or by the design of digestate-derived nematicide products. This complementary treatment would lower the environmental impact and toxicological risk linked to agrochemicals and reduce the demand for plant protection products while reducing treatment costs.

Selective separation of PPNs inhibitory compounds (e.g., volatile fatty acids, medium- and long-chain carboxylic acids, ammonia) could be a sustainable approach for the development of new nematicidal compounds. However, the mechanisms are still speculative and a more effective and broader chemical characterization of digested sludges (optimizations

of fractionation and analytic techniques) is still needed to propose new bioactive compounds as nematicides in the future. Overall, this work contributes to create awareness among growers, policy makers and researchers on the development of digestate-based renewable crop protection agents and sparks interest in better understanding the potential of digested sludge to address the environmental and economic challenges of modern agro-systems.

CRediT authorship contribution statement

Erica Oldani: Performing the experiments, Analyzing the data, Writing the draft of the manuscript. **Alessandro Cabianca:** Performing the experiments, Analyzing the data, Writing the draft of the manuscript. **Paul Dahlin:** Supported the *in vitro* and *in planta* experiments, Revised to the subsequent manuscript. **Andrea Caroline Ruthes:** Conceptualized, planned, designed the study, Revised to the subsequent manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data have been shared at https://dataverse.harvard.edu/dataverse/Spark_BD

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Appendix A. Supplementary data

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