



# Biological enhancement of the cover crop *Phacelia tanacetifolia* (Boraginaceae) with the nematophagous fungus *Pochonia chlamydosporia* to control the root-knot nematode *Meloidogyne hapla* in a succeeding tomato plant

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**Abstract** Root-knot nematodes cause global economic losses in a wide range of crops. We investigated the potential of seed coatings of the cover crop *Phacelia tanacetifolia* (Boraginaceae) when inoculated with the nematophagous fungus *Pochonia chlamydosporia* (Hypocreales: Clavicipitaceae) to protect subsequently grown tomato plants from root galling

caused by the root-knot nematode *Meloidogyne hapla* (Tylenchida: Meloidogynidae). Therefore, seeds of *P. tanacetifolia* were coated with *P. chlamydosporia* blastospores and planted in *M. hapla*-infested pots. After 50 days of growth in infested soil, *M. hapla* eggs were extracted from *P. tanacetifolia* roots and quantified. Tomato plants grown in the remaining soil served as bioindicator of *M. hapla* infestation as expressed by the gall index. Results showed that seed coating of *P. tanacetifolia* with *P. chlamydosporia* ( $290 \pm 51$  CFU per seed) reduced the number of *M. hapla* eggs up to 95.6% in comparison to untreated controls. *Pochonia chlamydosporia* as blastospore suspension ( $5 \cdot 10^8$  blastospores per 600 ml soil) reduced the number of *M. hapla* eggs by up to 75.5%. Additionally, tomato plants grown for 50 days in substrates previously planted with *P. tanacetifolia* seeds coated with *P. chlamydosporia* showed a significantly lower gall index than plants grown in untreated pots. In conclusion, biological enhancement of *P. tanacetifolia* by seed coating with *P. chlamydosporia* successfully reduced *M. hapla* and thus provides an additional tool in the management of this nematode. The method still has potential for further improvement such as increasing blastospore viability within the seed coating by optimized formulation technology.

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## Introduction

Plant-parasitic nematodes can reduce crop yields by feeding on plant tissues, thus causing substantial economic losses of agronomically important crops (Nicol et al. 2011; Jones et al. 2013). In particular, root-knot nematodes *Meloidogyne* spp. are among the most harmful plant-parasitic nematodes, being polyphagous and distributed worldwide (Carter 1985; Jones et al. 2013). In temperate regions, *Meloidogyne hapla* (Tylenchida: Meloidogynidae) is one of the most damaging root-knot nematodes, mainly infecting vegetable crops. A survey carried out in Germany detected *Meloidogyne* in 51% of soil samples of vegetable fields, with *M. hapla* being the most prominent species (Hallmann et al. 2007). Maximum densities of *M. hapla* reached up to 3,300 juveniles in 100 ml soil. Damage by *M. hapla* is most severe in sandy soils and on susceptible crops such as carrots and onions (Hallmann et al. 2007; Wesemael et al. 2011). Control of *M. hapla* can be achieved, among other means, by crop rotation with non-host plants (e.g., cereals) or resistant cultivars (e.g., fodder radish). However, these methods are often insufficient at high infestation densities (Moens et al. 2009; Wesemael et al. 2011; Vestergård 2019). The introduction of a sanitation year as suggested by Hallmann (2021) successfully controls *M. hapla* and is cost-effective. Here, crops were exclusively grown for the purpose of reducing *M. hapla* below the economic threshold level, i.e., the farmer does not generate any income during this period. Chemical control is, in most cases, not a viable option due to increasing requirements regarding human and ecosystem safety and costs. At the same time, cover crops and biological control are of increasing interest in nematode management, raising the question whether both strategies can be used concomitantly to further improve nematode control.

Cover crops are an indispensable tool in sustainable cropping practices. One such cover crop is *Phacelia tanacetifolia* (Hydrophyllaceae), which is widely grown in temperate climates (Scavo et al. 2022), e.g., in the Czech Republic and Germany. The beneficial effect of *P. tanacetifolia* as a cover crop is mainly due to its suppression of volunteer plants and weeds (Brant et al. 2009; Brust et al. 2014; Handlřřova et al. 2017), but also its low susceptibility to plant-parasitic nematodes. The following early spring, plant residues are incorporated into the soil and the main

crop is sown. Being the only cultivated species in the subfamily Hydrophyllaceae (Boraginaceae), *P. tanacetifolia* has its own unique spectrum of pests that in most cases is different from that of other crops grown within a rotation. For *M. hapla*, *P. tanacetifolia* is a maintenance host, meaning that the nematode population will neither increase nor decrease during the cropping season (Viaene and Abawi 1998). This makes *P. tanacetifolia* an ideal candidate for biological enhancement, especially in cases where the succeeding main crop is a good host for *M. hapla* such as tomato or potato.

The potential of antagonistic microorganisms to control plant-parasitic nematodes is well documented (Hallmann et al. 2009; Stirling 2014; Peiris et al. 2020). However, only few commercial products are available for control of *Meloidogyne* spp. (Hallmann et al. 2009; Wesemael et al. 2011; Flores Francisco et al. 2021; Darling et al. 2023). One such biocontrol agent is the nematophagous fungus *Pochonia chlamydosporia* (Hypocreales: Clavicipitaceae). *P. chlamydosporia* is known to parasitize eggs and adults of various nematode taxa like *Heterodera* spp., *Meloidogyne* spp., *Globodera* spp. and *Nacobbus* spp. (Kerry et al. 1993; Tobin et al. 2008; Manzanilla-Lopez et al. 2011). Therefore, *P. chlamydosporia* is a promising candidate for plant-parasitic nematode biocontrol, and commercial products are available such as Rizotec® (Rizoflora Biotecnologia S.A., Brazil) and KlamiC® (Centro Nacional de Sanidad Agropecuaria, Cuba). In both products, *P. chlamydosporia* chlamydospores are formulated as granular nematicides. Chlamydospores are thick-walled aerial cell structures that lead to high persistence during periods of unfavourable conditions associated with formulation and application. Production of chlamydospores is usually performed on solid media. Application is done via drip irrigation, spraying or soil drenching, which requires high amounts of product and water resulting in overall high costs. One option for reducing application rates and thus costs is fungal delivery through seed coating. This would also make it possible to place the biocontrol agent directly at the root-soil interface, where protection of the young seedling is needed.

Therefore, this study aimed to enhance the effectiveness of the cover crop *P. tanacetifolia* by combining it with a biological control agent to target *M. hapla*. This was done by coating seeds of *P. tanacetifolia* with *P. chlamydosporia*. In contrast to

commercial products based on fungal chlamydo-spores, we used blastospores of *P. chlamydo-sporea* as active ingredient. Blastospores are yeast-like vegetative cells produced by budding of the hyphae within their host or are formed during in vitro growth at nutrient-rich and oxygen-rich culture conditions in liquid media as shown for *Beauveria bassiana* (Mascarin et al. 2015). Blastospores have several advantages over aerial conidia, such as a higher infectivity, as shown for the endoparasitic fungus *Esteya vermicola* controlling the pinewood nematode *Bursaphelenchus xylophilus* (Wang et al. 2013). Additionally, the fermentation process for producing blastospores appears cheaper and more efficient than that of chlamydo-spores, as shown for various filamentous and dimorphic fungi (Jaronski and Mascarin 2017; Dietsch et al. 2021; Silva et al. 2022). Conversely, *P. chlamydo-sporea* blastospores have a thinner cell wall than chlamydo-spores and are therefore be more sensitive to the drying processes (Dietsch et al. 2021). We did not compare the survival rate of chlamydo-spores and blastospores after drying, but blastospores were found to successfully survive the drying process and are thus considered a suitable delivery system for this fungus. Here, we hypothesize, that (1) application of *P. chlamydo-sporea* as a seed coating on a cover crop will reduce the number of *M. hapla* eggs on a succeeding tomato plant compared to treatments without *P. chlamydo-sporea*, and (2) the resulting lower nematode inoculum level will limit gall formation on roots of subsequently grown tomato plants.

## Material and methods

### *Pochonia chlamydo-sporea* blastospore production

*Pochonia chlamydo-sporea* strain Pc001, deposited in the fungal culture collection at the Julius Kühn Institute, Germany, was originally isolated from surface-sterilized cysts of *Heterodera schachtii* collected from sugar beet fields located in North Rhine-Westphalia, Germany (Nuaima et al. 2021). Chlamydo-spores were cultivated on potato dextrose (PDA, 29 g l<sup>-1</sup>, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Petri plates with 14-day-old fungal cultures were washed several times with 0.1% Tween 80 to collect the chlamydo-spores. The chlamydo-spores were

counted using a Thoma cell counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Chlamydo-spores were used as inoculum to produce blastospores. To cultivate blastospores, sterile 250 ml flasks with baffled base were filled with 100 ml sterile potato dextrose broth (PDB, 26.5 g l<sup>-1</sup>, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), inoculated with 10<sup>6</sup> *P. chlamydo-sporea* chlamydo-spores and incubated at 26 °C on a rotary shaker at 150 rpm (IKA KS 4000 ic, Staufen, Germany) (Wang et al. 2015, modified). After seven days, the PDB liquid culture was filtered through a 5–10-µm Whatman filter (VWR, Darmstadt, Germany) under sterile conditions to separate blastospores from mycelium. The blastospores were washed twice with 0.9% sterile NaCl solution by centrifugation (3600 g, 20 °C, 10 min) and resuspended in a 0.9% sterile NaCl solution. The number of blastospores was counted under a light microscope (AxioStar plus; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) in a Thoma cell counting chamber at 200 × magnification.

### Seed coating

Seed coating was performed in two ways: a custom-made formulation and a commercial formulation. The custom-made seed coating of *P. tanacetifolia* was performed in a self-constructed drum dryer. Seeds were surface-sterilized in 70% ethanol, 3% (v/v) NaClO and 70% ethanol for 3 min each, and washed thrice in sterile ddH<sub>2</sub>O. 25 g surface-sterilized *P. tanacetifolia* seeds (approx. 12,500 seeds) were mixed with 30 g potato starch superior (Emslandstärken, Emlchheim, Germany) and 12 g bentonite (Tonsil 510ff, Clariant AG, Muttenz, Switzerland) in the drum under constant rotation of 33 rpm. The biopolymer suspension for seed coating was prepared by mixing the blastospores with 0.9% NaCl and 0.5% gellan gum (AppliChem GmbH, Darmstadt, Germany). This resulted in 10<sup>9</sup> (FH Bi, Experiment I) or 5·10<sup>7</sup> (FH Bi+, Experiment II) blastospores in 20 ml of 0.5% gellan gum that were finally added to the *P. tanacetifolia* seeds. After thorough mixing of all components, the coated seeds were dried by warm air. Maximum temperature on the seed surface was 30 °C, measured with an infrared sensor. The drying process was finished when the water activity (a<sub>w</sub>) of the treated seeds was below 0.55 (LabMaster-aw, Novasina, Lachen, Switzerland), which was reached

after 30–45 min. The target density of vital blastospores was at least 200 CFU per seed in Experiment I and 50 CFU per seed in Experiment II. The commercial seed coating Mantelsaat® (MS– and MS+) used in Experiment II was produced and provided by Feldsaaten Freudenberger GmbH & Co. KG (Krefeld, Germany). To produce MS–, fertiliser (carbonic acid lime), rock flour (mixture of feldspar, mica, and kaolinite), clay mineral (calcium bentonite), clay, perlite, humic acids, cellulose, and adhesives were coated onto 50 kg seeds (approx. 25,000,000 seeds) of *P. tanacetifolia*. *Pochonia chlamydosporia* was incorporated into MS+ as a biopolymer suspension prepared by mixing the blastospores with 0.9% NaCl and 0.5% gellan gum. The seed coating process was also performed in a rotating drum. The targeted spore density of MS+ was 50 CFU per seed, the same as for FH Bi+ in Experiment II. To investigate the coating efficiency and survival of the *P. chlamydosporia* blastospores within the process, three replicates of ten seeds each of freshly coated and dried seeds were washed in 0.9% NaCl and plated on PDA (39 g l<sup>-1</sup> PDA) containing 0.1 g l<sup>-1</sup> streptomycin, 0.05 g l<sup>-1</sup> tetracycline, 0.1 g l<sup>-1</sup> dodine, and 0.05 g l<sup>-1</sup> cycloheximide (Strasser et al. 1996). Plates were incubated for five days at 23 °C in the dark and CFU were counted.

#### *Meloidogyne hapla* inoculum

The *M. hapla* inoculum was obtained from galled tomato roots (*Solanum lycopersicum* cv. Money-maker) grown under greenhouse conditions. The nematode is part of the living nematode collection hosted at the Julius Kühn Institute, Germany. The *M. hapla* juveniles are routinely checked for purity using the protocol of Adam et al. (2007) and using the species-specific IGS primers JMV1 and JMVhapla. For the extraction of nematode eggs, the tomato shoot was cut off and discarded and the substrate with roots soaked in tap water for a few minutes. Next, the roots were carefully separated from the substrate and rinsed under tap water to remove adhering soil particles. The roots were then cut into 1–2 cm pieces and transferred to a 500 ml polyethylene bottle. The bottles were filled with 250 ml of 1% NaClO solution (Dan Klorix household bleach diluted 1:1.8 with tap water) and shaken at 450 rpm for 3 min. Finally, the suspension was passed through a 250 µm sieve to remove the roots and placed over a 20 µm sieve to

collect the nematode eggs. The egg suspension on the 20 µm sieve was rinsed with tap water for a few seconds to remove residual NaClO. Eggs on the 20 µm sieve were transferred to a beaker and adjusted to 600 eggs ml<sup>-1</sup>. The ratio of embryonal and juvenile egg stages was 1:1.

#### Greenhouse experimental set up

Two greenhouse experiments with either three (Experiment I) or six (Experiment II) treatments were set up to study the biocontrol potential of *P. chlamydosporia* towards *M. hapla*. Each of the two experiments was performed once and consisted of two stages: first, the “cover crop” stage with *P. chlamydosporia*-treated or non-treated *P. tanacetifolia* (cv. Balo, provided by Feldsaaten Freudenberger GmbH & Co. KG, Krefeld, Germany) grown in *M. hapla* infested substrate and, second, the “host plant” stage with tomato plants as bioindicator for nematode infestation.

Experiment I consisted of three treatments: (1) uncoated seeds without *P. chlamydosporia* (control), (2) uncoated seeds with *P. chlamydosporia* as blastospores suspension (BS), (3) coated seeds *P. chlamydosporia* blastospores (FH Bi) (Table 1). Seeds were sown in 600 ml pots containing a sterile sand/vermiculite substrate (4:1, v/v) amended with 0.125% slow-release osmocote fertilizer. One half of the treatments was inoculated with 6000 eggs of *M. hapla* per pot after sowing via the same 2 cm deep holes that were made for sowing. The other half received no nematode inoculation. Directly afterwards, *P. chlamydosporia* was added as blastospore suspension with 5·10<sup>8</sup> spores per pot in 0.9% NaCl via the same holes (BS). Each treatment consisted of ten replicates. Pots were randomly set up in the greenhouse at 22 °C:19 °C day:night temperature and watered as required. The total number of germinated seeds per pot was counted after 14 days.

The first stage of Experiment I was terminated and evaluated 50 days after inoculation, when about 50% of the newly formed *M. hapla* eggs had reached juvenile stages. The shoots of *P. tanacetifolia* were harvested and fresh and dry weights were determined. Next, the roots were separated from the substrate by gentle shaking and root fresh weight was recorded. The *M. hapla* eggs were then extracted from roots with 1% NaClO as described above. The number of

**Table 1** List of treatments and initial concentration of *P. chlamydosporia* blastospores in Experiments I and II

	Acronym	Treatment	Number of sown seeds per pot	Initial concentration of <i>P. chlamydosporia</i> blastospores
Experiment I	control	Without <i>P. chlamydosporia</i>	10	–
	BS	<i>P. chlamydosporia</i> as blastospore suspension in 0.9% NaCl	10	5·10 <sup>8</sup> per pot
	FH Bi	Seed coating FH Bi with <i>P. chlamydosporia</i>	10	290 ± 51 per seed
Experiment II	control	Without <i>P. chlamydosporia</i>	10	–
	BS	<i>P. chlamydosporia</i> as blastospore suspension in 0.9% NaCl	10	5·10 <sup>8</sup> per pot
	FH Bi–	Seed coating FH Bi without <i>P. chlamydosporia</i>	10	–
	FH Bi+	Seed coating FH Bi with <i>P. chlamydosporia</i>	10	73 ± 6 per seed
	MS–	Seed coating Mantelsaat® without <i>P. chlamydosporia</i>	10	–
	MS+	Seed coating Mantelsaat® with <i>P. chlamydosporia</i>	10	51 ± 14 per seed

Each seed set was either inoculated with *M. hapla* (600 specimen 100 ml soil<sup>-1</sup>) or left uninoculated (n = 10). Acronyms: BS: blastospore suspension, FH Bi: seed coating FH Bi with *P. chlamydosporia* (Experiment I), FH Bi– : seed coating FH Bi– without *P. chlamydosporia* (Experiment II), FH Bi+ : seed coating FH Bi with *P. chlamydosporia* (Experiment II), MS– : Mantelsaat® without *P. chlamydosporia*, MS+ : Mantelsaat® with *P. chlamydosporia*

healthy as well as parasitized eggs, identifiable by fungal outgrowths, was counted in 1 ml aliquots with an inverted microscope at 100× magnification. The number of *M. hapla* eggs per root system for single pots was analysed, but for better legibility the unit eggs per pot was chosen. Finally, the reproduction factor (RF) of *M. hapla* was calculated as the quotient of the final number ( $P_f$ ) of extracted *M. hapla* eggs 50 days after inoculation divided by the inoculated number of *M. hapla* eggs ( $P_i = 6000$ ).

For the second stage of Experiment I, the substrate of each pot was mixed by hand and a 200 ml aliquot filled into folded plastic boxes (4 × 4 × 12 cm, Seufert, Rodgau, Germany) in which a 10-day-old tomato seedling (cv. Moneymaker) was transplanted. The folded boxes were set up in the greenhouse at equal conditions as described above. After another 50 days, the above-ground plant parts were discarded and roots separated from the substrate. The boxes were soaked for 5 min in water and the soil was then gently washed from roots. Tomato root gall was assessed using the 0 (no galls visible) to 10 (all roots severely galled, plant usually dead) index according to Bridge and Page (1980).

Experiment II was performed as described above, but with modifications of the seed coating (Table 1). The experiment consisted of six treatments: (1) uncoated *P. tanacetifolia* seeds (control), (2) uncoated seeds with *P. chlamydosporia* blastospore suspension (BS), (3) custom-made coated seeds without *P.*

*chlamydosporia* (FH Bi–), (4) custom-made coated seeds with *P. chlamydosporia* blastospores (FH Bi+), (5) commercially coated seeds without *P. chlamydosporia* (MS–), and (6) commercially coated seeds with *P. chlamydosporia* blastospores (MS+). In the first stage of the experiment, seeds were sown and inoculated with *M. hapla* as described above. Treatments FH Bi– and MS– without *P. chlamydosporia* were employed to investigate the effect of seed coating itself. Each treatment consisted of ten replicates. The termination and evaluation of the first and second stages of Experiment II were performed as described above for Experiment I.

#### Statistical analysis

Data were statistically analysed with R version 7.2 (R Core Team 2022). Figures were created using the ggplot2 package (Wickham, 2009). First-stage data (“cover crop”) were analysed with (generalized) linear models. Linear models were fitted to predict above-ground and root dry weight with *P. chlamydosporia* inoculum, *M. hapla* inoculum and seed treatment. Standardized parameters were obtained by fitting the model on a standardized version of the dataset. 95% confidence intervals and p-values were computed using a Wald t-distribution approximation. A generalized linear model using Poisson distribution with a log link function was fitted to test differences between treatments with *P. chlamydosporia*

and control in the total number of *M. hapla* eggs. A generalized linear model using binomial distribution with a logit link function was fitted to model the percentage of parasitized *M. hapla* eggs with *P. chlamydosporia* inoculum and seed treatment. Standardized parameters were obtained by fitting the generalized linear models on a standardized version of the dataset. 95% confidence intervals and p-values were computed using a Wald test. Estimated marginal means (EMM, R package emmeans, by Lenth 2022) were calculated based on these models. EMM and contrasts were calculated with emmeans and the `trt.vs.ctrl()` function. Germination of *P. tanacetifolia* and gall indices of tomato roots (second stage data “host plant”) were compared using Kruskal–Wallis test with pairwise Wilcoxon rank sum tests with Benjamini & Hochberg (1995) correction as post-hoc tests.

## Results

The *P. chlamydosporia* blastospores remained viable after coating and drying in both Experiments I and II (Table 1). The survival rates, as compared to the initial blastospore concentration during the seed coating process, were  $0.72 \pm 0.13\%$  for FH Bi and  $1.64 \pm 0.51\%$  for FH Bi+, respectively. For MS+, the CFU per seed indicated a survival rate of  $17.40 \pm 4.90\%$  in relation to the initial number of blastospores. The number of CFU per seed in the seed coatings FH Bi+ and MS were not significantly different from each other. Therefore, the effects of these two seed coatings in Experiment II could be directly compared.

### Experiment I

Seed germination was significantly lower for FH Bi in comparison to the control without *P. chlamydosporia* and BS treatment ( $\chi^2 = 33.74$ ,  $df = 2$ ,  $p < 0.001$ ). *M. hapla* inoculum had no effect on seed germination ( $\chi^2 = 0.11$ ,  $df = 1$ ,  $p = 0.74$ ) (Table 2). In contrast, above-ground dry weight of FH Bi-treated plants was significantly higher than in control plants with a difference of 1.0 g ( $\beta = 1.00$ , 95% CI [0.65, 1.34],  $t_{56} = 5.82$ ,  $p < 0.001$ ) regardless of *M. hapla* inoculation ( $\beta = -0.07$ , 95% CI [-0.35, 0.21],  $t_{56} = -0.49$ ,  $p = 0.63$ ). The model explained a statistically significant and substantial proportion

of variance ( $R^2 = 0.47$ ,  $F_{3,56} = 16.42$ ,  $p < 0.001$ ). The model's intercept, corresponding to no *M. hapla* inoculation and no treatment with *P. chlamydosporia* was at 1.07 g (95% CI [0.79, 1.35],  $t_{56} = 7.62$ ,  $p < 0.001$ ). Root dry weight was not significantly affected by *M. hapla* inoculation ( $\beta = -0.09$ , 95% CI [-0.20, 0.03],  $t_{56} = -1.52$ ,  $p = 0.14$ ), but root dry weight was significantly higher in FH Bi compared to the control with a difference of 0.25 g ( $\beta = 0.25$ , 95% CI [0.11, 0.39],  $t_{56} = 3.51$ ,  $p < 0.001$ ). The same trend was observed in the presence of *M. hapla*, but differences of 0.09 g were not statistically significant ( $\beta = 0.09$ , 95% CI [-0.05, 0.23],  $t_{56} = 1.29$ ,  $p = 0.20$ ). The model explained a statistically significant and moderate proportion of variance ( $R^2 = 0.21$ ,  $F_{3,56} = 4.96$ ,  $p = 0.004$ ). The model's intercept, corresponding to no *M. hapla* inoculation and no treatment with *P. chlamydosporia* was at 0.33 g (95% CI [0.21, 0.44],  $t_{56} = 5.73$ ,  $p < 0.001$ ). The average RF of *M. hapla* in *P. tanacetifolia* without *P. chlamydosporia* was  $1.83 \pm 0.53$ . The RF of *M. hapla* on tomato plants without *P. chlamydosporia* was  $33.60 \pm 8.04$ . Direct inoculation of *P. chlamydosporia* blastospores (BS) and application of blastospores as seed coating (FH Bi) significantly reduced the total number of *M. hapla* eggs on *P. tanacetifolia* by 75.50% ( $2683 \pm 1194$  eggs per pot;  $\beta = -1.41$ , 95% CI [-1.42, -1.39],  $p < 0.001$ ; EMM:  $t_{27} = -2.81$ ,  $p = 0.02$ ) and 95.60% ( $485 \pm 122$  eggs per pot;  $\beta = -3.12$ , 95% CI [-3.15, -3.09],  $p < 0.001$ ; EMM:  $t_{27} = -3.56$ ,  $p = 0.003$ ), respectively, in comparison to the control without *P. chlamydosporia* (Fig. 1a). The model's intercept, corresponding to no *P. chlamydosporia* inoculation was at  $10,962 \pm 3201$  eggs per pot ( $\beta = 9.30$ , 95% CI [9.30, 9.31],  $p < 0.001$ ). The parasitization rate of *M. hapla* eggs after coating the seeds with *P. chlamydosporia* was more than fourfold higher ( $114 \pm 25$  parasitized eggs per pot,  $37.20\% \pm 10.58\%$ , RF:  $0.11 \pm 0.02$ ;  $\beta = 0.67$ , 95% CI [0.63, 0.71],  $p < 0.001$ ; EMM:  $F_2 = 932.51$ ,  $p < 0.0001$ ) than in the control ( $1017 \pm 349$  parasitized eggs per pot,  $8.67\% \pm 0.85\%$ ; Fig. 1b). For BS, the parasitization rate was more than two-fold higher ( $447 \pm 192$  parasitized eggs per pot,  $20.53\% \pm 6.29\%$ ; RF:  $0.45 \pm 0.20$ ,  $\beta = 0.67$ , 95% CI [0.63, 0.71],  $p < 0.001$ ) than in the control (Fig. 1b). The model's explanatory power was substantial (Nagelkerke's  $R^2 = 0.39$ ). The model's intercept,

**Table 2** Effect of seed coating with *Pochonia chlamydosporia* on *Phacelia tanacetifolia* germination and plant performance 50 days after sowing in Experiment I and II (mean  $\pm$  standard deviation (SD), n = 10)

	Treatment		Total number of germinated seeds <sup>#</sup>	Above-ground dry weight per plant (g) <sup>x</sup>	Root dry weight per plant (g) <sup>x</sup>
			Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Experiment I	– PC	– MH	5.50 $\pm$ 1.08 <sup>a</sup>	1.16 $\pm$ 0.23	0.31 $\pm$ 0.15
		+MH	6.40 $\pm$ 1.17 <sup>a</sup>	0.91 $\pm$ 0.21	0.26 $\pm$ 0.08
	BS	– MH	6.60 $\pm$ 1.43 <sup>a</sup>	0.97 $\pm$ 0.24	0.41 $\pm$ 0.12
		+MH	6.40 $\pm$ 0.97 <sup>a</sup>	0.94 $\pm$ 0.22	0.34 $\pm$ 0.13
	FH Bi	– MH	3.40 $\pm$ 1.26 <sup>b</sup>	2.00 $\pm$ 0.85 <sup>*</sup>	0.60 $\pm$ 0.40 <sup>*</sup>
		+MH	2.80 $\pm$ 1.48 <sup>b</sup>	2.07 $\pm$ 0.94 <sup>*</sup>	0.46 $\pm$ 0.29
Experiment II	– PC	– MH	8.50 $\pm$ 1.51 <sup>A</sup>	0.84 $\pm$ 0.35	0.10 $\pm$ 0.04
		+MH	8.20 $\pm$ 1.69 <sup>A</sup>	0.92 $\pm$ 0.24	0.11 $\pm$ 0.02
	BS	– MH	8.80 $\pm$ 1.14 <sup>A</sup>	0.80 $\pm$ 0.20	0.12 $\pm$ 0.03
		+MH	7.40 $\pm$ 0.97 <sup>A</sup>	1.00 $\pm$ 0.20	0.17 $\pm$ 0.06
	FH Bi–	– MH	8.60 $\pm$ 1.51 <sup>A</sup>	0.86 $\pm$ 0.24	0.12 $\pm$ 0.05
		+MH	8.90 $\pm$ 1.52 <sup>A</sup>	0.82 $\pm$ 0.24	0.08 $\pm$ 0.03
	FH Bi+	– MH	6.44 $\pm$ 1.59 <sup>A</sup>	1.09 $\pm$ 0.29	0.13 $\pm$ 0.07
		+MH	6.80 $\pm$ 1.23 <sup>A</sup>	1.17 $\pm$ 0.26	0.17 $\pm$ 0.05
	MS–	– MH	7.30 $\pm$ 1.70 <sup>A</sup>	1.01 $\pm$ 0.37	0.13 $\pm$ 0.04
		+MH	6.40 $\pm$ 1.71 <sup>A</sup>	1.08 $\pm$ 0.21	0.14 $\pm$ 0.06
	MS+	– MH	8.40 $\pm$ 1.65 <sup>A</sup>	0.83 $\pm$ 0.18	0.13 $\pm$ 0.05
		+MH	8.10 $\pm$ 0.99 <sup>A</sup>	0.83 $\pm$ 0.19	0.15 $\pm$ 0.09

– MH: without *M. hapla*, +MH: with *M. hapla*, – PC: without *P. chlamydosporia*, BS: blastospore suspension, FH Bi: seed coating FH Bi (Experiment I), FH Bi–: seed coating - without *P. chlamydosporia* (Experiment II), FH Bi+: seed coating FH Bi with *P. chlamydosporia* (Experiment II), MS–: Mantelsaat® without *P. chlamydosporia*, MS+: Mantelsaat® with *P. chlamydosporia*

<sup>#</sup>Total number of germinated seeds per pot: values with no letter in common are significantly different. Small letters were added for statistics of Experiment I and capital letters were added for statistics of Experiment II. (Kruskal–Wallis-test with pairwise Wilcoxon rank sum tests with Benjamini & Hochberg (1995), correction as post hoc tests,  $p < 0.05$ )

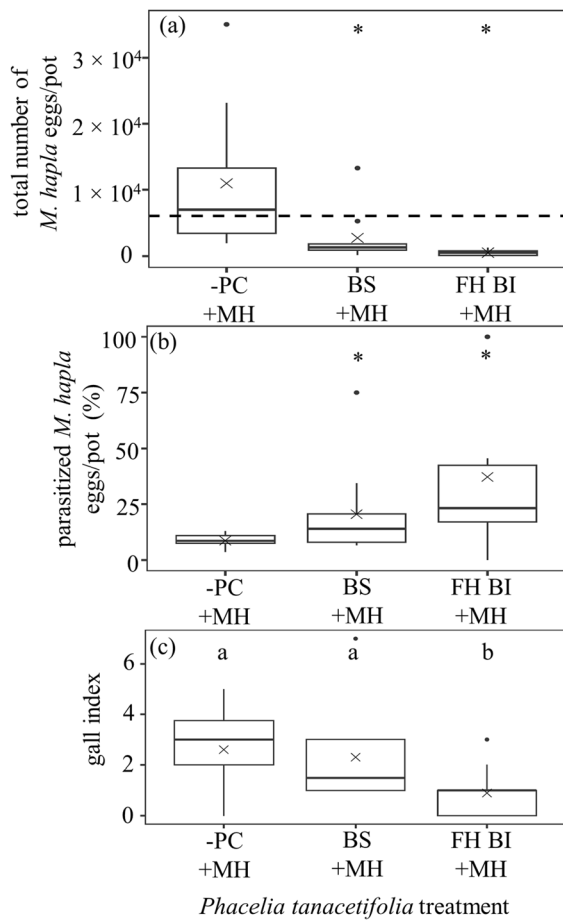
<sup>x</sup>Above-ground dry weight and root dry weight of *P. tanacetifolia* per plant: linear models (LM) and estimated marginal means (EMM) ( $p < 0.05$ ), ‘\*’ indicate significant differences in comparison to the control (– PC – MH) of the respective experiment at the  $p < 0.05$  level according to LM and EMM

corresponding to no *P. chlamydosporia* inoculation was at – 2.28% (95% CI [– 2.30, – 2.26],  $p < 0.001$ ).

In the subsequently grown tomato plants, the root gall index of individual plants ranged from 0 (no galls visible) to 7 (galls appear on main roots—majority of main roots galled). Average gall indices were 3 in the control, 1.5 in treatment BS and 1 in treatment FH Bi (Fig. 1c). The lower gall index compared to the control was significant for FH Bi ( $\chi^2 = 6.87$ ,  $df = 2$ ,  $p = 0.025$ ; Fig. 1c), but not for BS ( $\chi^2 = 6.87$ ,  $df = 2$ ,  $p = 0.44$ ). Percentage of parasitized *M. hapla* eggs and gall index were negatively correlated (Spearman’s  $\rho = -0.58$ ,  $p < 0.05$ , data not shown).

## Experiment II

This experiment compared two different seed coatings for *P. chlamydosporia* blastospores with direct blastospore application. Seed germination was similar in all treatments, including the control ( $\chi^2 = 3.73$ ,  $df = 3$ ,  $p = 0.29$ ), regardless if *P. chlamydosporia* ( $\chi^2 = 0.95$ ,  $df = 1$ ,  $p = 0.33$ ) or *M. hapla* ( $\chi^2 = 2.03$ ,  $df = 1$ ,  $p = 0.15$ , Table 2) was inoculated or not. In contrast to Experiment I, for above-ground dry weight the linear model explained a statistically not significant and weak proportion of variance ( $R^2 = 0.04$ ,  $F_{5,113} = 0.83$ ,  $p = 0.53$ ). The model’s intercept, corresponding to no *P. chlamydosporia* inoculation, no *M. hapla* inoculation, and no seed treatment was at 0.85 g (95% CI

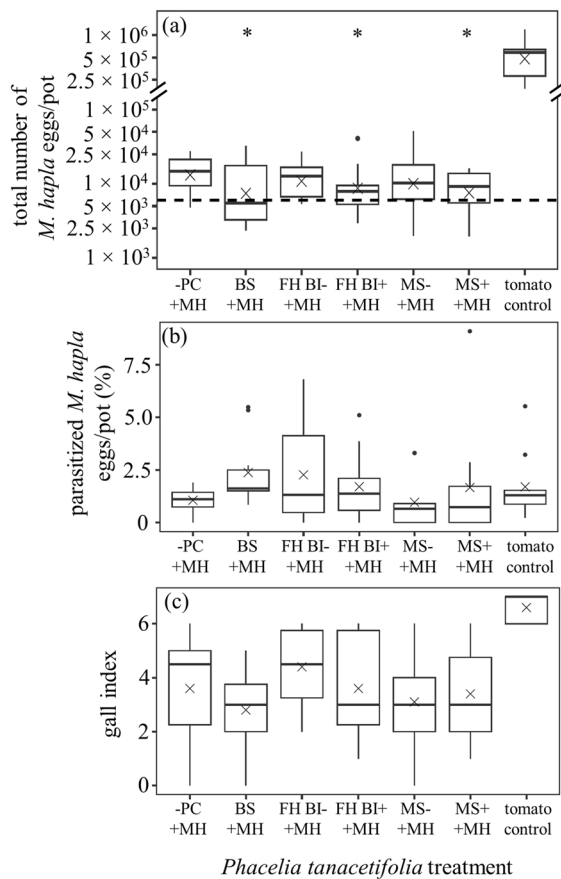


**Fig. 1** Effect of cover crop *Phacelia tanacetifolia* seed coating with *Pochonia chlamydosporia* on *Meloidogyne hapla* population density, parasitism, and gall index of subsequently planted tomato plants (Experiment I). Boxplots show the extremes, the upper (75%) and lower (25%) quartiles, the median (line), mean (X), and outliers (dots). Abbreviations: +MH: with *M. hapla*, -PC: without *P. chlamydosporia*, BS: blastospore suspension, FH Bi: seed coating FH Bi. **a** Total number of *M. hapla* eggs per plant 50 days after inoculation. Dotted line indicates number of inoculated eggs at experimental start ( $n=10$ ). **b** Percentage of parasitized *M. hapla* eggs 50 days after inoculation ( $n=10$ ). **a** and **b**: “\*” indicates significant differences of treatments in comparison to the control (-PC+MH) at the  $p<0.05$  level according to generalized linear models and estimated marginal means. **c** Gall index of tomato plants 50 days after sowing in previously inoculated substrate ( $n=10$ ). Values with no letter in common are significantly different (Kruskal–Wallis test with pairwise Wilcoxon rank sum tests with Benjamini & Hochberg (1995) correction, as post-hoc tests,  $p<0.05$ )

[0.72, 0.98],  $t_{113}=12.94$ ,  $p<0.001$ ). For root dry weight the linear model explained a statistically significant and weak proportion of variance ( $R^2=0.10$ ,  $F_{5,113}=2.48$ ,  $p=0.036$ ). The model’s intercept, corresponding to no *P. chlamydosporia* inoculation, no *M. hapla* inoculation, and no treatment was at 0.10 g (95% CI [0.07, 0.12],  $t_{113}=7.31$ ,  $p<0.001$ ). Adding *P. chlamydosporia* increased the root dry weight statistically significant by 0.03 g ( $\beta=0.03$ , 95% CI [0.01, 0.05],  $t_{113}=2.08$ ,  $p=0.04$ , EMM:  $F_{1,113}=4.318$ ,  $p=0.04$ ). The number of *M. hapla* eggs was reduced in all treatments with *P. chlamydosporia* compared to the *P. tanacetifolia* control without *P. chlamydosporia* ( $15,074 \pm 2,270$  eggs/pot, RF:  $2.51 \pm 0.28$ ;  $\beta=-0.35 \pm 0.003$ ,  $z_{55}=-114.51$ ,  $p<0.001$ ). The model’s intercept, corresponding to no *P. chlamydosporia* inoculation and no seed treatment was at 15,074 eggs per pot ( $\beta=9.62$ , 95% CI [9.62, 9.63],  $p<0.001$ ): for treatment BS by 26% ( $11,206 \pm 3,151$  eggs per pot, RF:  $1.87 \pm 0.53$ ;  $\beta=0.05 \pm 0.005$ ,  $z_{55}=10.59$ ,  $p<0.001$ ), for seed coating FH Bi +by 29% ( $10,658 \pm 3464$  eggs per pot, RF:  $1.78 \pm 0.58$ ;  $\beta=-0.16 \pm 0.004$ ,  $z_{55}=-43.6$ ,  $p<0.001$ ), and in MS +by 49% ( $7669 \pm 1809$  eggs per pot, RF:  $1.28 \pm 0.30$ ;  $\beta=-0.14 \pm 0.004$ ,  $z_{55}=-39.58$ ,  $p<0.001$ ) (Fig. 2a). The percentage of parasitized *M. hapla* eggs in Experiment II (max. 9%) was generally lower than in Experiment I (up to 100% in some replicates). The model’s intercept, corresponding to no *P. chlamydosporia* inoculation and no seed treatment was at -4.54% (95% CI [-5.60, -3.75],  $p<0.001$ ). Inoculation of *P. chlamydosporia* increased the proportion of parasitized eggs, but not significantly ( $\beta=0.04$ , 95% CI [-0.69, 0.78],  $p=0.91$ ). The same trend was calculated for treatments BS ( $\beta=0.78$ , 95% CI [-0.49, 2.16],  $p=0.24$ ), FH Bi ( $\beta=0.62$ , 95% CI [-0.41, 1.81],  $p=0.25$ ), and MS ( $\beta=0.20$ , 95% CI [-0.89, 1.42],  $p=0.73$ ).

The gall index of tomato roots was not significantly affected in any of the treatments compared to the control, regardless of *P. chlamydosporia* inoculation ( $\chi^2=1.09$ ,  $df=1$ ,  $p=0.29$ ) or seed coatings ( $\chi^2=3.57$ ,  $df=3$ ,  $p=0.31$ ) (Fig. 2c). However, in all treatments with *P. chlamydosporia*, the median gall index was reduced 1.5-fold compared to the control. The negative correlation of parasitized *M. hapla* eggs in *P. tanacetifolia* roots with the gall index of subsequently grown tomato plants was weaker compared to





**Fig. 2** Effect of cover crop *Phacelia tanacetifolia* seed coating with *Pochonia chlamydosporia* on *Meloidogyne hapla* population density, parasitism, and gall index of subsequently planted tomato plants (Experiment II). Boxplots show the extremes, the upper (75%) and lower (25%) quartiles, the median (line), mean (X), and outliers (dots). Abbreviations: +MH: with *M. hapla*, -PC: without *P. chlamydosporia*, BS: blastospore suspension, FH BI- : seed coating FH Bi without *P. chlamydosporia*, FH BI+ : seed coating FH Bi with *P. chlamydosporia*, MS- : Mantelsaat® without *P. chlamydosporia*, MS+ : Mantelsaat® with *P. chlamydosporia*. **a** Total number of *M. hapla* eggs per plant 50 days after inoculation. Dotted line indicates number of inoculated eggs at experimental start ( $n=10$ ). ‘\*’ indicates significant differences of treatments in comparison to the control (-PC+MH) at the  $p<0.05$  level according to generalized linear models and estimated marginal means. **b** Percentage of parasitized *M. hapla* eggs 50 days after inoculation ( $n=10$ ). No significant differences for treatments in comparison to control (-PC+MH) for all treatments were found (generalized linear models and estimated marginal means  $p>0.05$ ). **c** Gall index of tomato plants 50 days after sowing in previously inoculated substrate ( $n=10$ ). Central tendency is not significantly different ( $p>0.05$ ) for all treatments (Kruskal–Wallis test with pairwise Wilcoxon rank sum tests with Benjamini & Hochberg (1995) correction, as post-hoc tests)

Experiment I (Spearman’s  $\rho=-0.09$ ,  $p<0.05$ , data not shown).

## Discussion

This study tested the hypothesis whether *P. chlamydosporia* blastospores, either applied as suspension to the soil or by coating seeds of *P. tanacetifolia*, could reduce the number of newly produced *M. hapla* eggs and increase egg parasitism. Unfortunately, our results did not provide a clear picture. While Experiment I clearly supported our hypothesis with up to 95% reduction in *M. hapla* eggs and up to 49% egg parasitism, Experiment II only resulted in up to 49% reduction in *M. hapla* eggs and a maximum of 9% egg parasitism. Thus, reduction in *M. hapla* eggs was confirmed in both experiments, whereas egg parasitism was not or only partly confirmed. Regarding nematode reduction, the results are in line with previously published studies. For example, de Leij et al. (1993) reported over 90% reduction of *M. hapla* on tomato following application of *P. chlamydosporia*.

*Pochonia chlamydosporia*’s efficiency and biocontrol potential

The status of the host plant, *P. tanacetifolia*, appears important as it is considered a maintenance host and *M. hapla* is less able to reproduce on *P. tanacetifolia* than on other hosts such as tomato or potato (Viaene and Abawi 1998). Average RF of *M. hapla* on *P. tanacetifolia* was between 1.8 and 2.5 in the absence of *P. chlamydosporia*, which was far less than for tomato (RF=33.6–93.6) used as an internal control (see Fig. 2a). Here, we were able to show that even on a maintenance host like *P. tanacetifolia*, seed enhancement with *P. chlamydosporia* blastospores can effectively reduce *M. hapla* reproduction below the initial inoculation level. In the present study, control of *M. hapla* was shown for one initial nematode density. As reproduction of nematodes is inversely related to their initial densities (Seinhorst 1967), future experiments need to test different *M. hapla* densities to confirm the biocontrol potential of *P. chlamydosporia* over a broad spectrum of infestation scenarios.

Another aspect concerning the effectiveness of *P. chlamydosporia* towards *M. hapla* might be the fungal isolate itself, or the fungus–nematode interaction.

Isolates of *P. chlamydosporia* as well as populations of *M. hapla* can vary in their specific characteristics, i.e., some populations of *M. hapla* might be more suitable for certain *P. chlamydosporia* isolates than others. The *P. chlamydosporia* P001 studied here was originally isolated from *H. schachtii* cysts and selected for its virulence against *H. schachtii* (Nuaima et al. 2021). Stirling (2014) concluded from several studies that *P. chlamydosporia* is most pathogenic to the host from which it was originally isolated. Now, we could show that *P. chlamydosporia* P001 also affects *M. hapla* eggs indicating a broad effect as biocontrol agent. Nonetheless, further studies with different *M. hapla* populations tested under different environmental conditions are needed to confirm the biocontrol potential on a broader basis.

The biocontrol potential of *P. chlamydosporia* has been investigated under various conditions against different plant-parasitic nematode species. Studies with *M. incognita* tentatively show a much lower reduction by *P. chlamydosporia* than for *M. hapla*. For example, De Leij et al. (1992) achieved a reduction of about 40% in number of eggs, juveniles, and galls caused by *M. incognita* on tomato following treatment with *P. chlamydosporia* at 50 chlamydo-spores per gram of soil. On okra, Dhawan and Singh (2010) reported a reduction of *M. incognita* by *P. chlamydosporia* ranging from 6.9 to 46.5%, depending on application method and application rate. In general, *P. chlamydosporia* is considered one of the most important players in root-knot nematode control in natural agroecosystems. Viaene & Abawi (2000) reported colonization rates of *M. hapla* eggs by *P. chlamydosporia*, measured as egg mass, between 15.5 and 42.6% in *M. hapla* infested soil. In their study, egg parasitism was associated with reduced penetration of lettuce roots by *M. hapla*. Furthermore, when lettuce was replanted into the same soil, up to 69.2% of *M. hapla* egg masses were found to be colonized by *P. chlamydosporia*. In the present study, the biocontrol potential of *P. chlamydosporia* was tested under greenhouse conditions and the effect of the tested isolate in the field still has to be evaluated. Field performance of other *P. chlamydosporia* isolates was evaluated by van Damme et al. (2005) against *M. javanica* in lettuce and tomato over two consecutive years, showing that a one-time application of *P. chlamydosporia* delayed the population build-up of *M. javanica* by five to seven months.

Blastospores as inoculum may be superior to chlamydo-spores

All of the references on *P. chlamydosporia* cited above applied persistent chlamydo-spores as fungal inoculum, whereas the present study applied fast-growing but non-persistent blastospores. This raises the question whether blastospore application might be a preferred method of application. In our study, blastospore application resulted in up to 49% parasitism of *M. hapla* eggs. This was in the range of what has been reported by Viaene & Abawi (2000) for parasitism of *M. hapla* eggs using chlamydo-spores. Properties relevant for choosing blastospores instead of chlamydo-spores are faster germination rates, shorter fermentation times, better production scalability, and overall less labour (Morales-Reyes et al. 2018). Additionally, biological factors like establishment and survival of blastospores in soil are relevant. Assuming that blastospores are more virulent than chlamydo-spores, as previously shown for other nematophagous fungi (Wang et al. 2013), blastospores may be able to establish in the soil as well as or better than chlamydo-spores in presence of host nematodes due to faster colony formation.

Formulation improves effectiveness of biocontrol

In Experiment I, the custom-made seed coating led to a reduced seed germination compared to uncoated seeds. This was most likely due to the inhomogeneous formulation of *P. chlamydosporia* to the *P. tanacetifolia* seeds. The material around the seeds was likely too rigid and firm and thus affected seed germination. Despite the reduced seed germination and the subsequent lower number of *P. tanacetifolia* plants in the seed coating, the biomass production was greater compared to the other treatments. This can be best explained by higher nutrient availability per plant and less intraspecific competition. In accordance with our second hypothesis, the reduced soil infestation level with *M. hapla* upon *P. chlamydosporia* treatment should benefit the subsequent plant. Our results confirmed this with the reduced root gall index of the subsequently grown tomato. These effects were stronger in the first experiment than in the second, which might be explained by the higher number of blastospores applied in the first case. This could indicate that the number of applied

blastospores is relevant for the performance of *P. chlamydosporia* controlling *M. hapla*. Bontempo et al. (2017) described a dose–response dependence for *P. chlamydosporia* against *M. incognita* on carrots under field conditions. According to this, an amount of 3 kg ha<sup>-1</sup> (3·10<sup>8</sup> chlamydospores per gram<sup>g</sup>, in total 9·10<sup>11</sup> chlamydospores per hectare) of the powder formulation Rizotec® with *P. chlamydosporia* chlamydospores (Rizoflora Biotecnologia S.A., Brazil) is required to reduce the population of *M. incognita* and increase the quality and yield of carrots. In order to reach this concentration, 2.25 10<sup>5</sup> to 3.6 10<sup>5</sup> blastospores of *P. chlamydosporia* per *P. tanacetifolia* seed are necessary (extrapolated to a sowing density of 2.5·10<sup>6</sup> to 4.0·10<sup>6</sup> seeds ha<sup>-1</sup>). In this study, under greenhouse conditions and limited competition with other microorganisms, the reduction of *M. hapla* eggs in the first experiment was already significant at a concentration of 290 blastospores per seed. Those numbers refer to 7.25 10<sup>8</sup> to 1.16 10<sup>9</sup> blastospores per hectare, which is less than the application rates mentioned above for chlamydospores. Such lower application rates might be caused by a higher virulence of blastospores over chlamydospores (Wang et al. 2013). However, the viability and virulence of blastospores under field conditions need further attention regarding the effect of heat, UV light, water potential, osmotic stress (Vieira dos Santos et al. 2012; Bernardo et al. 2020), soil environment (Luambano et al. 2015), and microbial competition (Siddiqui et al. 2009).

The comparison of Experiment I and II suggests that the number of blastospores per seed is relevant. One method of increasing the number of viable blastospores in seed coatings is to add a higher amount of blastospores during the production process. Even if the same or a larger amount of blastospores is required compared to chlamydospores, the fermentation process of *P. chlamydosporia* blastospores in liquid culture is presumably more cost-effective and efficient compared to solid fermentation of chlamydospores.

The efficiency of biological control agents depends on environmental factors like temperature, pH, soil structure, UV intensity, and food and water availability (Shields et al. 2019; Peiris et al. 2020). To minimize the influence of these factors, improvement of survival rates and root rhizosphere colonisation of applied biological control

organisms through formulations in capsules, granules, or seed coatings is necessary (Vemmer and Patel 2013; Afzal et al. 2020). Drying blastospores is still a challenge and a lot of research will be necessary to identify low-cost and reliable techniques for up-scale production (Dietsch et al. 2021). Here, we showed a new and effective formulation of *P. chlamydosporia* in which blastospores were dried directly within the (commercial) seed coating.

In conclusion, seed coating with *P. chlamydosporia* blastospores showed successful control of *M. hapla* in the cover crop *P. tanacetifolia* and a subsequent tomato cycle. The commercial Mantelsaat® application proved to be a good delivery system for *P. chlamydosporia*. In any case, blastospore numbers per seed need to achieve sufficient densities for proper nematode control. To further develop the biocontrol potential of such treatment for optimum performance under field conditions, focus should be given to the improvement of blastospore vitality after drying, shelf life of the coated seeds and fungal establishment in the root zone.

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**Author contributions** JU, DJ-S, JHS and JH designed the experiment; JHS and JH conducted the experiment; JU analysed the results; JU prepared a manuscript draft; JHS and JH edited the manuscript; DJ-S, K-JD and AP revised the manuscript for technical and scientific accuracy; DJ-S and AP acquired funding, and supervised the project. All authors approved the manuscript.

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#### Declarations

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**Research involving human and animal rights** This article does not contain any studies with human participants or animals performed by any of the authors.

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- Jana Uthoff** focuses on the development of seed treatments with beneficial fungi to control plant-parasitic nematodes.
- Desiree Jakobs-Schönwandt** performs research on the formulation of biological control agents against various plant parasites.
- Jan Henrik Schmidt** develops sustainable methods for the control of plant-parasitic nematodes with special emphasis on antagonistic microorganisms and (induced) plant resistance.
- Johannes Hallmann** works on the biology and epidemiology of plant-parasitic nematodes with a focus on vegetable crops and organic agriculture.
- Karl-Josef Dietz** focuses on molecular and physiological mechanisms of damage development and efficient stress acclimation.

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