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Establishment of *Arthrobotrys flagrans* as biocontrol agent against the root pathogenic nematode *Xiphinema index*

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Abstract

Plant-parasitic nematodes cause devastating agricultural damage worldwide. Only a few synthetic nematicides can be used and their application is limited in fields. Therefore, there is a need for sustainable and environment-friendly alternatives. Nematode-trapping fungi (NTF) are natural predators of nematodes. They capture and digest them with their hyphae and are starting to being used as bio-control agents. In this study, we applied the NTF *Arthrobotrys flagrans* (*Duddingtonia flagrans*) against the wine pathogenic nematode *Xiphinema index*. *A. flagrans* reduced the number of *X. index* juveniles in pot cultures of *Ficus carica*, an alternative host plant for *X. index*, significantly. Sodium-alginate pellets with *A. flagrans* spores were produced for vineyard soil inoculation under laboratory conditions. The NTF *A. conoides*, *A. musiformis* and *A. superba* were enriched from several soil samples, showing their natural presence. Trap formation is an energy-consuming process and depends upon various biotic and abiotic stimuli. Here, we show that bacteria of the genus *Delftia*, *Bacillus*, *Pseudomonas*, *Enterobacter* and *Serratia* induced trap formation in NTF like *A. conoides* and *A. oligospora* but not in *A. flagrans* in the absence of nematodes. The application of NTF along with such bacteria could be a combinatorial way of efficient biocontrol in nematode-infested soil.

INTRODUCTION

Over 4100 species of plant-parasitic nematodes have been described, and it is estimated that 15% of the global crop yield (about $170 billion) is lost due to those parasites (Coyne et al., 2018; Jones et al., 2013; Singh et al., 2015). Besides causing physical damage, nematodes may also transmit bacterial, fungal, or viral pathogens (Garcia et al., 2019; Hajji-Hedfi et al., 2019).

The ectoparasitic nematode *Xiphinema index* is a parasite of grapevine (*Vitis vinifera*) and threatens viticulture worldwide (Belval et al., 2019; Jones et al., 2013; Villate et al., 2008). Injury to root cells results in the formation of root galls. However, most damage is caused by serving as a vector for the grapevine fanleaf virus (GFLV) (Andret-Link et al., 2004; Van Ghelder et al., 2015). This disease causes shortened bud spacing, reduced fruit quality and yield loss up to 80% (Andret-Link et al., 2004; Rubio et al., 2020; van Zyl et al., 2012). Although GFLV-resistant and economically feasible rootstocks are currently developed, the main strategy is to control outbreaks through nematode vector control (Djennane et al., 2021; Schurig, Ipach, Hahn, & Winterhagen, 2021; Schurig, Ipach, Helmstätter, et al., 2021). An economically devastating fallow period of up to 7 years is the most common treatment used to date and the only direct method available for controlling *X. index* in the field, as most synthetic nematicides are banned from applying in the environment. The nematodes can survive in vineyard soil for at least 4 years without the presence of a host plant, and virus transmission remains possible even after a 5-year fallow period (Demangeat et al., 2005; Meng et al., 2017; Nguyen et al., 2019; Villate et al., 2008).

Nematode-trapping fungi (NTF) could be used as an ecological and cost-effective alternative for the control of nematodes. In this regard, the fungus *Arthrobotrys flagrans* (formerly *Duddingtonia flagrans*) shows great potential as a biological nematicide (Araújo et al., 2008; Braga & de Araújo, 2014; da Silva et al., 2013; Mostafanezhad et al., 2014; Vilela et al., 2012). The fungus forms three-dimensional sticky trap networks to trap nematodes. Under laboratory conditions, the fungus forms resistant chlamydospores in addition to normal conidiospores, which gives the fungus an advantage in the competition with other fungi in the application area (Arora et al., 2016). The fungus has been used mainly to control nematode infestations in animals by adding fungal spores to food pellets (Longo Ribeiro Vilela et al., 2016; Vilela et al., 2012). The robust chlamydospores pass through the intestinal tract of the animals and are excreted. They cannot germinate at body temperature and thus do not harm the animal. Therefore, the spores germinate only in the animal's faeces where also nematodes hatch from the eggs and start their development (Saumell et al., 2016). The presence of NTF in the faeces contaminated with nematode eggs reduces the number of nematodes and thereby the re-infection risk of the animals grazing around their pastures. The Research Institute of Organic Agriculture (FiBL) has patented the use of *A. flagrans* for biological pest control in livestock (WO2017125468 A1). The product Bioverm® contains *A. flagrans* spores and is commercially available in Brazil. The product reduced the nematode egg counts per gram of faeces by around 75% in a recent 6-months study on controlling gastrointestinal nematodes in sheep (Rodrigues et al., 2022). Initial trials with potted crop plants and in greenhouses showed the potential of NTF against plant parasitic nematodes. For example, *A. oligospora* has been successfully used to reduce *M. incognita* infestations on tomato plants (Soliman et al., 2021). Interestingly, plant roots are even colonized by *A. oligospora*, and the interaction resembles those of endophytic fungi (Bordallo & Lopez-Llorca, 2002). Experiments with *A. flagrans* and tomato plants also showed a positive effect of the fungus on plant growth. Co-cultivation resulted in increased uptake of nutrients by the plants (Monteiro et al., 2018). The addition of 8200 *A. flagrans* chlamydospores per gram of soil reduced the number of *Meloidogyne javanica* juveniles by 73% (Monteiro et al., 2020) These observations provide a basis for the control of plant-parasitic nematodes by NTF.

As a morphological differentiation, trap formation is associated with increased energy requirements and therefore represents a major investment by the fungus that must be well adapted to environmental conditions. Therefore, trap formation of most NTF is induced only by specific signals (Vidal-Diez de Ulzurrun & Hsueh, 2018; Yu et al., 2021). Conserved nematode signalling molecules called ascarosides were identified to be sensed by NTF and show trap-inducing properties (Hsueh et al., 2013; Yu et al., 2021). Additionally, a nutrient-poor environment and nitrogen deficiency are prerequisites for the switch from saprotrophic growth to a predatory lifestyle (Vidal-Diez de Ulzurrun & Hsueh, 2018). While the direct presence of nematodes strongly induces trap formation, certain amino acids and dipeptides such as phenylalanine-valine are reported as inducers in *A. oligospora* (Nordbring-Hertz, 2004; Su et al., 2017). Some bacteria are also capable of inducing trap formation in some NTF (Wang et al., 2014). It was proposed that the bacteria recruit NTF as a defence mechanism to protect themselves from bacterivorous nematodes. Furthermore, diketopiperazines secreted by bacteria of the genera *Stenotrophomonas* and *Rhizobium* have been identified as triggers of trap formation in *A. oligospora* (Li et al., 2016). In the case of the genus *Chryseobacterium* and *A. oligospora* direct bacterial contact was required to induce trap formation in the fungus although the inducing compound was not identified (Li et al., 2011). Here, we investigate the potential of NTF as biocontrol agent against the plant parasitic nematode *X. index*. We show the presence of different *Arthrobotrys* species and growth of *A. flagrans* in vineyard soil. *A. flagrans* significantly reduces the number of *X. index* juveniles in pot cultures of *Ficus carica*. Additionally, we demonstrate that bacteria of several genera induce trap formation in *A. conoides* and *A. oligospora*. Bacteria appeared to be in close contact with hyphae and actively moved along them.

EXPERIMENTAL PROCEDURES

Cultivation of microorganisms

Cultivation of *A. flagrans* (CBS 349.94), *A. conoides*, *A. oligospora*, *A. musiformis*, *D. stenobrocha* (Liu et al., 2014) and *D. haptotyla* (CBS 200.50) was performed on potato dextrose agar (PDA) at 28°C.

Cultivation of *B. subtilis*, *M. luteus*, *E. cloacae* and *P. mirabilis* was performed on LB at 37°C. Cultivation *of Delftia*, *P. stutzeri* and *S. marcescens* was performed on solid or liquid LB at 28°C. *Caenorhabditis elegans* was cultivated on nematode-growth medium and *E. coli* OP50 lawn at 20°C.

Isolation of NTF from soil samples

Soil samples were collected from 20 to 50 cm depth of the root zone at five different sites of the State vineyard Karlsruhe. To isolate fungal spores, approximately 10 g

of soil were mixed with 10 ml of water and allowed to mix at 180 rpm for 30 min. Then, 50 µl of the suspension was spread on water agar containing 100 µg/ml ampicillin and 100 µg/mL chloramphenicol. Trap formation of NTF was induced by adding circa 2000 *C. elegans* larvae and incubated at room temperature. After 5 to 7 days, individual spores of the isolates were located under a stereomicroscope and transferred to fresh agar plates using a thin needle.

Cultivation and extraction of *X. index*

A *X. index* culture was obtained from the culture of the Dienstleistungszentrum Ländlicher Raum in Neustadt and were kindly provided by Dr. Ulrike Ipach and Dr. Juliane Schurig.

The cultivation of *X. index* was carried out on *F. carica* according to Ipach (2014). Fig plants were grown from wood cuttings in water and transferred to perlite after successful rooting. Cultivation was done at room temperature and 16 h light day (Hydroca LED Blue Line 416). Plants were fertilized once a week with Long-Ashton nutrient solution.

For nematode culture, individual fig plants were transferred to 500 g sand (2 mm grain size) after 2 to 3 months and infected with around 100–300 adult nematodes. Infected plants were watered daily to 40% of maximum water capacity (WC_{max}). Nematodes were isolated from the sand at the earliest after 6 months of incubation. For this purpose, the sand was placed in a beaker and washed with water several times. Subsequently, the supernatant was decanted onto an analytical sieve (80 µm mesh size) with a total of 5 L of water. Sieve residue was collected in a new beaker and nematodes were extracted using a modified Baerman funnel. A funnel was extended with a 20 cm rubber tube and connected at the end with a tube clamp closed. A layer of miracloth (VWR, EM475855-1R) was placed in the funnel and filled with nematode suspension and incubated overnight. Individual nematodes were examined with a stereomicroscope and transferred with a fine metal wire for further experiments.

In vitro infection assay

A. flagrans spores were harvested with a sterile loop from a 7-day old culture plate and transferred in 1 ml of sterile water. The number of spores was quantified using a counting chamber and the 10× objective of a light microscope (Nikon Eclipse e200). Twenty microliters of spore suspension containing 1000 spores were added on thin water agar pads (1% agar, 1.5 × 1.5 cm size) and co-inoculated with 10 *X. index* adults for up to 72 h. Microscopic observations were performed using a Zeiss AxioImager Z.1 microscope.

Infection experiments with *X. index*

Infection experiments with *X. index* were performed in 80 g of sterilized quartz sand (grain size 2 mm) in 50 ml reaction tubes according to the substrate test (Schaaf, 1999). Before planting, *F. carica* seeds were cold stratified overnight at 4°C and sterilized in 70% ethanol for 1 min. Then, they were washed three times with sterile water and incubated in chlorix solution (5% sodium hypochlorite) for 10 min. Afterward, they were washed three times with sterile water and planted in the reaction tubes, covered with a thin layer of sand. The seeds germinated after 14 to 21 days at room temperature and a 16-h light cycle. The seedlings were grown for 8 weeks. Plants were watered daily to 60% WC_{max} and fertilized once a week with Long-Ashton nutrient solution. For the infection experiments, each plant was infected with 25 hand-picked *X. index* females. To do this, a small hole was dug in the sand with the back of a Pasteur pipette and the nematodes were pipetted near the root zone. 4×10^4 *A. flagrans* spores/g sand were further added to the tested plants as a spore suspension 24 h after the addition of the nematodes. After 40 days, the number of larvae was quantified. For this, the sand of each sample was washed with a total of 2 L of water as described above. All nematodes in a sample were quantified in a Petri dish using a stereomicroscope. Five replicates were prepared per experimental condition. The experiment was performed twice. The weight and length of plant roots and shoots were measured after each experiment.

Pelleting and germination of *A. flagrans* spores in sodium alginate

A. flagrans spores from a 7-day-old PDA agar plate were isolated using sterile water and a Drigalski spatula. The spore suspension was filtered through a single layer of miracloth and collected in a 50 ml reaction tube. After centrifugation at 5000 rpm for 10 min, the supernatant was carefully removed, and the spores resuspended in 3 ml of water. A counting chamber was used to quantify the number of spores. The fungal spores were then placed in a 1% sodium alginate (w/v) solution and pipetted dropwise into a 0.1 M $CaCl_2$ solution. The resulting pellets were collected and used for further experiments.

Germination in soil and restriction fragment length polymorphism

The germination capacity of *A. flagrans* sodium alginate pellets was checked in vineyard soil. For this purpose,

the soil was placed in a Petri dish and inoculated with *A. flagrans* pellets. After 14 days of incubation at room temperature, DNA was isolated from soil using a NucleoSpin Soil Mini kit (Macherey-Nagel) according to the manufacturer's instructions. The isolated DNA was used as a template to amplify the internal transcribed spacer (ITS) region using the primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCG CTTATTGATATGC). The generated PCR product was purified using the Zymoclean Gel DNA extraction kit (Zymo Research), ligated into the cloning vector pJET1.2 using T4 DNA ligase, and transformed into *E. coli* TOP10 cells. The insert of individual clones was checked using the pick & patch method using the primer pair pjet_kill_fwd (CGACTCACTATAGGGAGA GCGGC) and pjet_kill_rev (AAGAACATCGATTTTCC ATGGCAG). A total of 24 colonies were checked. Five microliters of each PCR product was digested with the restriction enzyme *Alu*I (New England Biolabs) in the manufacturer's recommended buffer in a total volume of 10 µl for 1 h and then checked in a 2% agarose gel. The *A. flagrans* ITS region was used as a control. The inserts of matched clones were further sequenced for further verification.

Root clarification and WGA-FITC staining

Mycelial growth on roots was visualized after 40 days of co-incubation of *A. flagrans* and *F. carica* seedlings in sand. Roots were rinsed with water, cut into small pieces, and incubated with KOH (10%) at 80°C for 1 h. Subsequently, samples were washed three times with 1× PBST-buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4, +0.02% Tween20) and incubated with HCl (1%) for 5 min at room temperature. Roots were again washed three times with water and three times with 1× PBST-buffer. To visualize the mycelium, the samples were incubated with Wheat Germ Agglutinin, Alexa Fluor 488 (WGA-FITC) dye overnight at 4°C and in darkness. The next day, roots were washed three times with 1× PBST buffer (+0.02% Tween20) and imaged on a fluorescent microscope (Zeiss AxioImager Z.1).

Statistical analysis

Statistical analyses were performed using GraphPad Prism. In the pot trials, each biological replicate of the bar graphs is colour-coded (blue and orange). The averages are shown as triangles calculated from the corresponding data points depicted as dots. The *p*-value of the number of *X. index* larvae (Figure 3C) was calculated using the total 10 replicates of both experimental rounds and a *t*-test with Welch's correction.

RESULTS AND DISCUSSION

Soil samples of a vineyard are rich in NTF

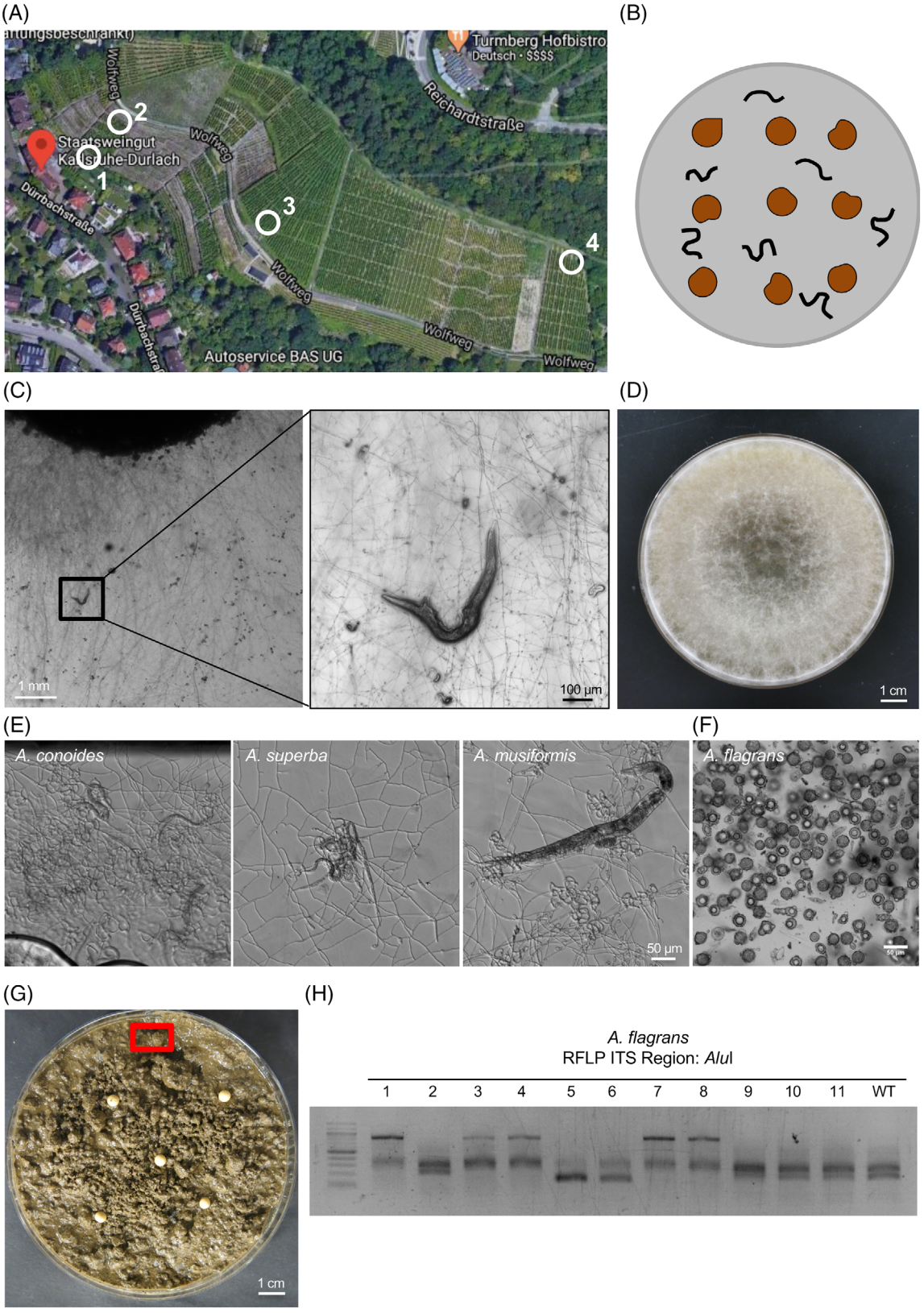
To investigate NTF abundance in vineyard soil and confirm that biological control with *A. flagrans* would not introduce an unnatural organism into the soil, soil samples were collected from 20 to 50 cm depth of the root zone at five different sites of the State Vineyard Karlsruhe (Figure 1A). NTF were enriched in all samples and pure cultures of the isolates were established (Figure 1B–D, see Section 2 for details). Sequencing of the ITS region assigned all isolates to the genus *Arthrobotrys*, which form sticky three-dimensional trap networks. A total of seven isolates were obtained. *A. conoides* was identified in all soil samples. The species *A. musiformis* and *A. superba* were additionally isolated in samples 1 and 3. All isolates formed trap networks after 24 h co-incubation with *C. elegans* on water agar (Figure 1E). In agreement with our results, it was reported recently that 68% of 178 soil samples from Taiwan contained NTF (Yang et al., 2020).

To verify that *A. flagrans* germinates and establishes a mycelium in vineyard soil, *A. flagrans* spores were incorporated into sodium alginate pellets and incubated in a Petri dish with soil of sample 1 at room temperature for 14 days (Figure 1F,G). Approximately 3 cm away from the pellet, a small soil sample was removed, DNA isolated, and *A. flagrans* was detected via ITS1 PCR, restriction fragment length polymorphism and sequencing (Figure 1H). *A. flagrans* was not detected before in this soil sample (see above). These results indicate that NTF are present in vineyard soil but could also be added into the soil for efficient pest control. The advantage of pellets compared with spore suspensions is that they can be stored dry and activated by rehydration. The alginate additionally protects the spores from other environmental factors like UV radiation which increases the overall shelf-life of spores.

A. flagrans decreases the number of *X. index* adults in vitro

To test the ability of *A. flagrans* to capture and digest *X. index*, the interaction was tested in vitro on water agar. Ten *X. index* adults were co-incubated with approximately 1000 *A. flagrans* spores for 72 h. After 12 h of incubation, first traps were visible. After 24 h, first trapped worms were observed, and at the latest after 72 h, they were completely digested (Figure 2A). This result further strengthens the ability of *A. flagrans* as a biocontrol agent against plant-parasitic nematodes as previously described (Youssar et al., 2019). Additionally, *A. flagrans* had a positive impact on growth and nutrient uptake in a greenhouse study with tomato plants, making the species a valuable addition in the

field (Monteiro et al., 2018). If *A. flagrans* would positively interact with plants like the NTF *A. oligospora*, it would be an ideal biocontrol agent for plant parasitic nematodes because it would not only reduce the pathogens but also promote plant growth (Monteiro et al., 2018). The resistant chlamydospores which



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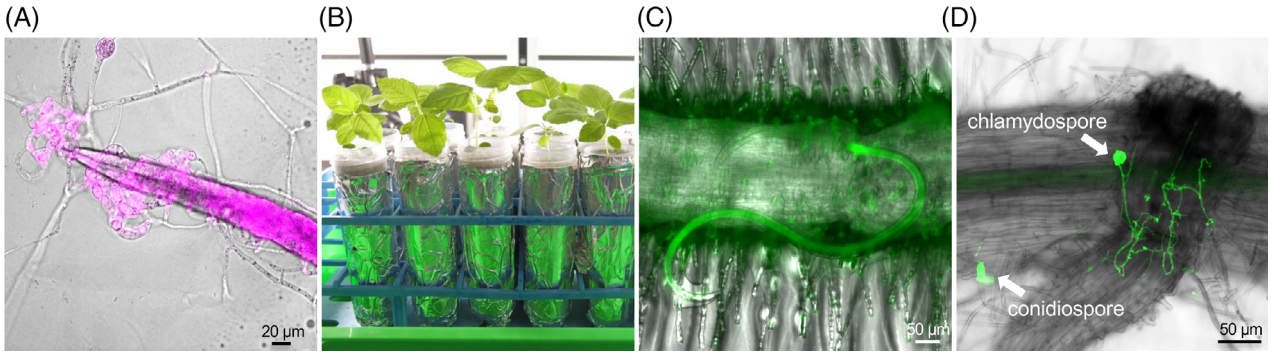

FIGURE 2 Characterization of the interactions of *Arthrobotrys flagrans*, *Xiphinema index* and *Ficus carica* in the experimental setup. (A) *X. index* is trapped and digested by *A. flagrans* wildtype on water agar. The cell wall of the fungus is labelled by calcofluor-white and depicted in magenta. (B) *F. carica* seedlings were grown in sand for 40 days. (C) A root of a fig seedling infected with *X. index* on water agar. (D) Hyphal growth of *A. flagrans* around roots of a fig seedling. Fig roots were prepared for imaging after 40 days of co-incubation with *A. flagrans*, and the mycelium was fluorescently labelled with the dye WGA-FITC (depicted in green). Arrows indicate a conidiospore and a chlamydospore.

A. flagrans produces readily, are also advantageous for the fungal application in the field and not only to resist the passage through the intestine of animals.

Establishment of a *X. index* infection system on *Ficus carica*

To further test the potential of *A. flagrans* as a biocontrol agent, a culture of *X. index* was established on the fig tree *Ficus carica* and an infection study was conducted on seedlings adapted from (Schurig, Ipach, Hahn, & Winterhagen, 2021; Schurig, Ipach, Helmstätter, et al., 2021) (Figure 2B). Fig is not a host for GFLV, so it can be ensured that the culture remains virus-free (U. Ipach, personal communication). The attraction of *X. index* adults to the roots was confirmed on water agar (Figure 2C).

Prior to the infection study, the germination ability of *A. flagrans* was checked in quartz sand. Fig seedlings were inoculated together with about 10^5 fungal spores and growth was checked after 40 days. WGA-FITC staining was used to confirm the growth of fungal mycelium around the roots by fluorescence microscopy (Figure 2D).

Infection studies were conducted according to Schaaf's substrate test (Schaaf, 1999). The number of nematode larvae was determined after 40 days of co-incubation. Each plant was infected with a total of 25 hand-picked *X. index* females. After 1 day, a total of 4×10^4 *A. flagrans* spores/g sand were added to each plant. After 40 days of co-incubation, plants infected with *X. index* showed gall formation and lesions on the roots (Figure 3A,B). The addition of *A. flagrans* decreased the number of *X. index* larvae by 50 ± 10% compared to the control infection (Figure 3C). No significant changes in root weight and length, or shoot weight and length, were detected by the addition of *A. flagrans*, or *X. index*, respectively (Figure S1). These results highlight the potential of *A. flagrans* for the biological control of root pathogens.

Improving biocontrol capacity using bacteria is a common but less conserved trait of NTF

The formation of traps is a very energy-consuming process (Yang et al., 2011). Although some NTF species seem to form traps spontaneously under laboratory conditions, many require specific abiotic or biotic stimuli (Vidal-Diez de Ulzurrun & Hsueh, 2018). In addition, there is evidence that a quorum-sensing-like mechanism is used to ensure that traps are only formed if many nematodes are present (Yu et al., 2021). Hence, it would be desirable to further improve the trapping

FIGURE 1 Enrichment of nematode-trapping fungi (NTF) from vineyard soil and verification of germination of *Arthrobotrys flagrans* in sodium alginate pellets. (A) Marking of the four sampling sites at the State Vineyard Karlsruhe, Germany. The fifth sampling site was at a vineyard in Grötzingen, Germany. (B) Enrichment strategy for NTF. Isolated soil was incubated on water agar with circa 2000 *Caenorhabditis elegans* larvae at room temperature. (C) Captured nematode after 5 days of incubation. (D) Growth of the isolated species *A. conoides*. (E) Trap networks of *A. conoides*, *A. superba* and *A. musiformis* isolated from vineyard soil after 24 h of co-incubation with *C. elegans* on water agar. (F) Chlamydospores of *A. flagrans*. (G and H) Germination of *A. flagrans* in vineyard soil. Spores were dispersed in soil using sodium alginate pellets. After 14 days of incubation, DNA was isolated from a section (marked in red) and the presence of *A. flagrans* was confirmed by RFLP and sequencing. DNA from the ITS sequences was digested with the restriction enzyme *Alu*I. An ITS sequence amplified from *A. flagrans* wildtype was used as a control. Samples 2, 9, 10 and 11 show the same restriction pattern as the control. The ITS region of sample 11 was sequenced and *A. flagrans* was confirmed as the species.

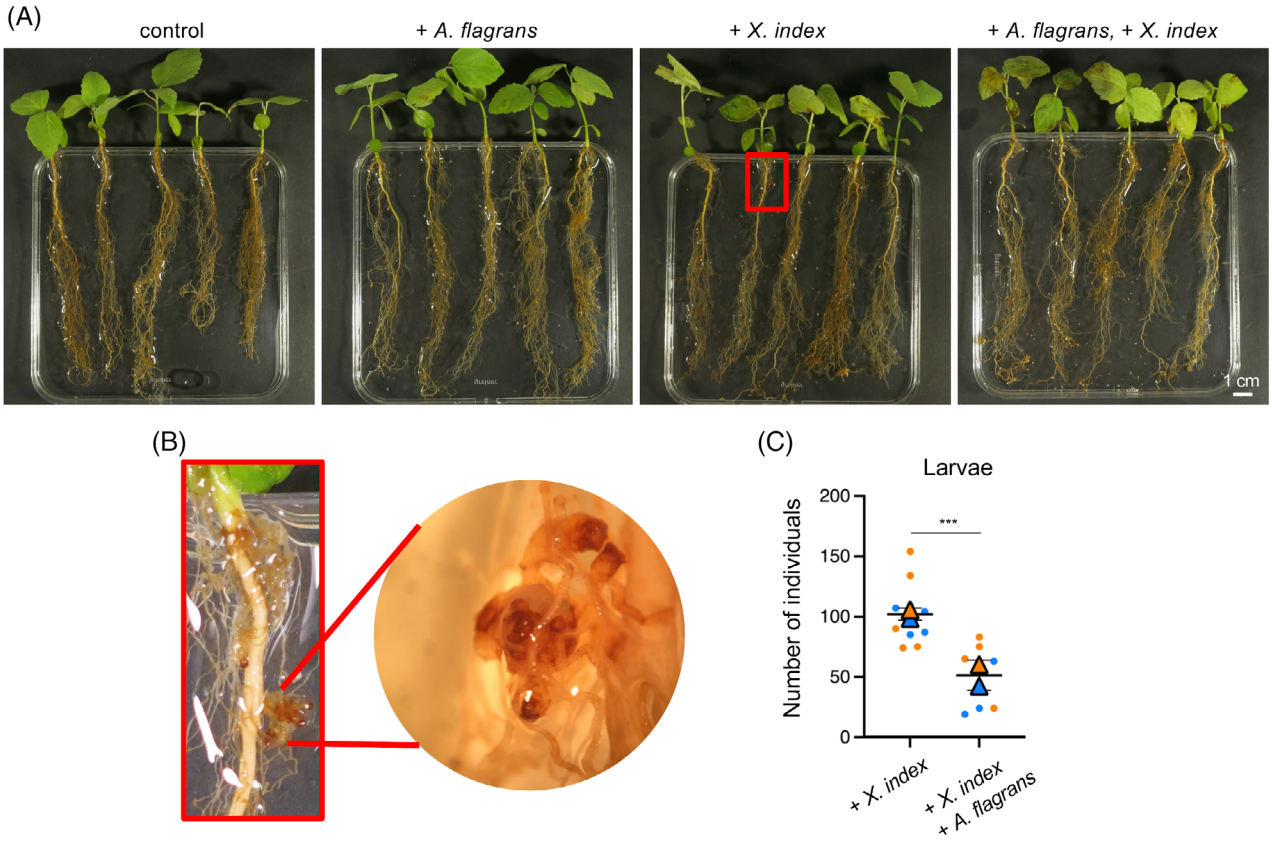

FIGURE 3 *Arthrobotrys flagrans* reduces the *Xiphinema index* larval count in pot trials. (A) Photographs of *Ficus carica* plants after the completion of the infection experiment with different conditions. The red-marked area is shown enlarged in (B). (B) Root damage caused by the infection of *X. index*. (C) Quantification of *X. index* larvae after 40 days of incubation. The data points are depicted as dots, the averages are shown as triangles. Error bars indicate the standard deviation. The experiment was replicated twice with five replicates each. $p = 0.0002$, *t*-test with Welch's correction

ability of NTF as a biocontrol agent by combining them with inducing substances.

During experiments with the *A. flagrans* Δ*sofT* (*so*) mutant we observed trap formation after 2 days of incubation on water agar without the presence of nematodes: a behaviour that was never observed in wild-type (WT) samples (Figure 4). The SO protein is only found in filamentous ascomycetes and is involved in cell-to-cell communication. It is also associated with the response to various stresses (Fleissner et al., 2009; Haj Hammadeh et al., 2022). In search for possible inducing substances and signalling pathways, bacterial contamination was identified on the Δ*sofT* mutant containing water agar samples. Certain bacteria induce trap formation and produce trap-inducing molecules like urea or diketopiperazines (Li et al., 2016; Wang et al., 2014). To characterize the trap-inducing bacteria in our experiment, their ribosomal 16 S rDNA region was sequenced. Sequence comparisons placed the bacteria to the genus *Delftia*. Interestingly, trap formation was not induced in *A. flagrans* WT or the Δ*sofT* re-complemented strain (Figure 4A). Cell-free culture supernatant or heat-inactivated bacteria did not induce trap formation in the Δ*sofT* mutant, indicating a necessity of physical contact. The bacteria showed indeed active locomotion along the fungal hyphae of the Δ*sofT* mutant and heavy swarming around traps (Figure 4B, Movies S1 and S2). Next, we screened additional bacterial strains available to the lab for trap induction in different NTF by inoculating the bacteria with *A. flagrans*, *A. oligospora*, *A. conoides*, *A. musiformis* (all form three-dimensional trap networks), *Drechslerella stenobrocha* (constringing ring traps), or *Dactylelina haptoyla* (adhesive knob traps). *A. conoides* and *A. oligospora* formed traps upon co-cultivation of *Bacillus subtilis*, *Pseudomonas stutzeri*, *Enterobacter cloacae*, *Serratia marcescens*, or *Delftia* for 48 h whereas the other tested NTF did not (Table 1, Figure 5A). *Delftia* bacteria have been shown to have a positive effect on plant growth and defensive properties against plant pathogenic fungi, rendering them interesting candidates for combinatorial use with NTF (Han et al., 2005; Janahiraman et al., 2016). NTF and trap-inducing bacteria may even be used in combination with other plant-growth promoting bacteria or fungi and may be introduced into the environment using coated seed (Requena et al., 1997; Rocha et al., 2019).

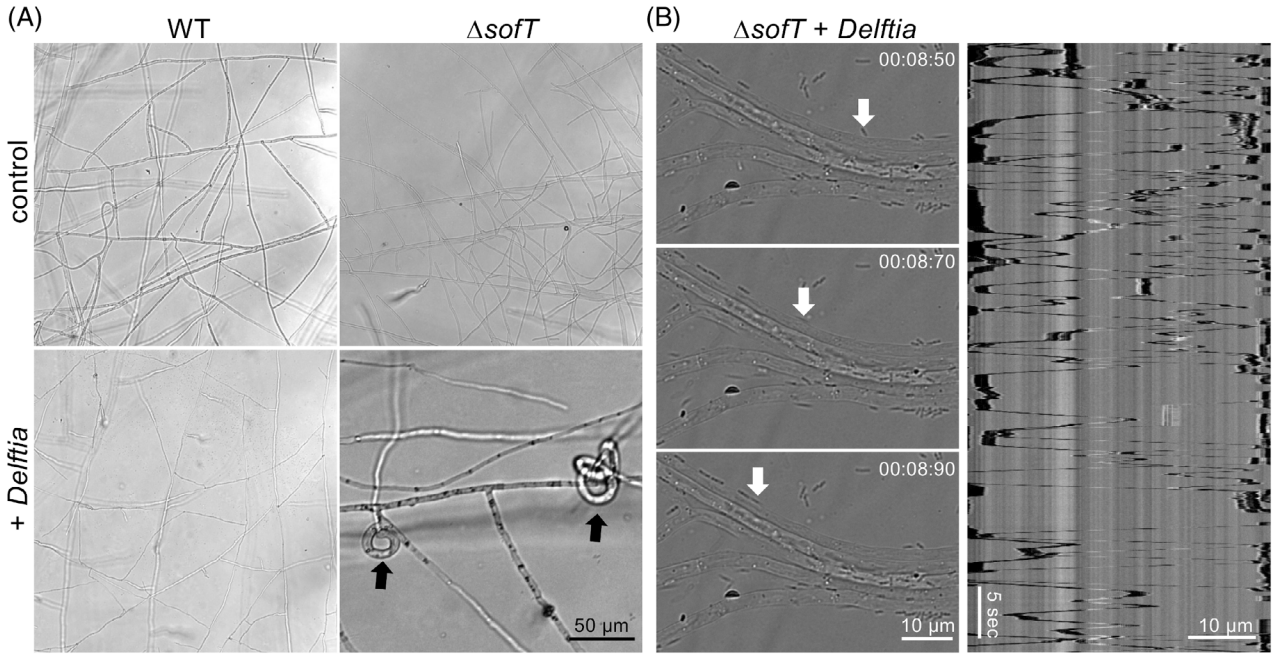

FIGURE 4 Bacteria of the genus *Delftia* induce trap formation in the *Arthrobotrys flagrans* Δ*sofT* mutant. (A) Bacteria were added after 24 h of incubation of fungal samples at 28°C and analysed after 24 h of co-incubation. In control samples, distilled water was pipetted onto the samples. The arrows mark formed traps in the ΔsofT mutant. (B) Bacteria travelling along fungal hyphae. Time-lapse images show the movement of Delftia along hyphae of the Δ*sofT* mutant. The white arrow indicates the movement of a bacterium along the hypha. A line was drawn along the hypha to create a kymograph to show bacterial movement for 1 min.

TABLE 1 Trap induction in nematode-trapping fungi by various bacteria

	A. flagrans	*A. conoides*	*A. musiformis*	*A. oligospora*	*D. stenobrocha*	*M. haptotyla*
B. subtilis	−	+	−	+	−	−
P. stutzeri	−	+	−	+	−	−
E. cloacae	−	+	−	+	−	−
S. marcescens	−	+	−	+	−	−
Delftia	−	+	−	+	−	−
P. mirabilis	−	−	−	−	−	−
M. luteus	−	−	−	−	−	−

The bacteria were also in close association with hyphae of *A. oligospora* using them as 'highway' (Figure 5B). This close interaction seemed to be essential for trap induction since culture supernatant or autoclaved bacteria did not induce trap formation in any tested fungus. In which way bacteria and hyphae profit from the close association with hyphae remains to be determined although there are examples for vitamin supply by the bacteria (Abeysinghe et al., 2020; Steffan et al., 2020). We speculated that the *A. flagrans* laboratory WT strain might have lost the ability to form bacteria-induced traps through continuous cultivation in artificial conditions for 4 years. However, three tested *A. flagrans* wild isolates (CBS143.80, CBS343.94 and CBS565.50) did not show bacteria-induced trap formation (Figure S2). These findings suggest that this specific bacterial perception is a common but not conserved trait of NTF and could be potentially used as a combinatorial biocontrol agent. Indeed, recent studies show that free-living bacteria colonize fungal chlamydospores induced by bacterial lipopeptides (Spraker et al., 2016; Venkatesh et al., 2022). Additionally, the *A. conoides* strain isolated in this work, trapped and digested *X. index* in vitro, making it a possible strategy to enrich NTF wild isolates directly from an infected field and use them in combination with trap-inducing bacteria for pest control (Figure 6). The application of local isolates, rather than laboratory strains, is likely to be advantageous because of their adaptation to the specific environmental conditions (Requena et al., 1997).

Our work expands the application of nematophagous fungi from the control of nematodes in grassing

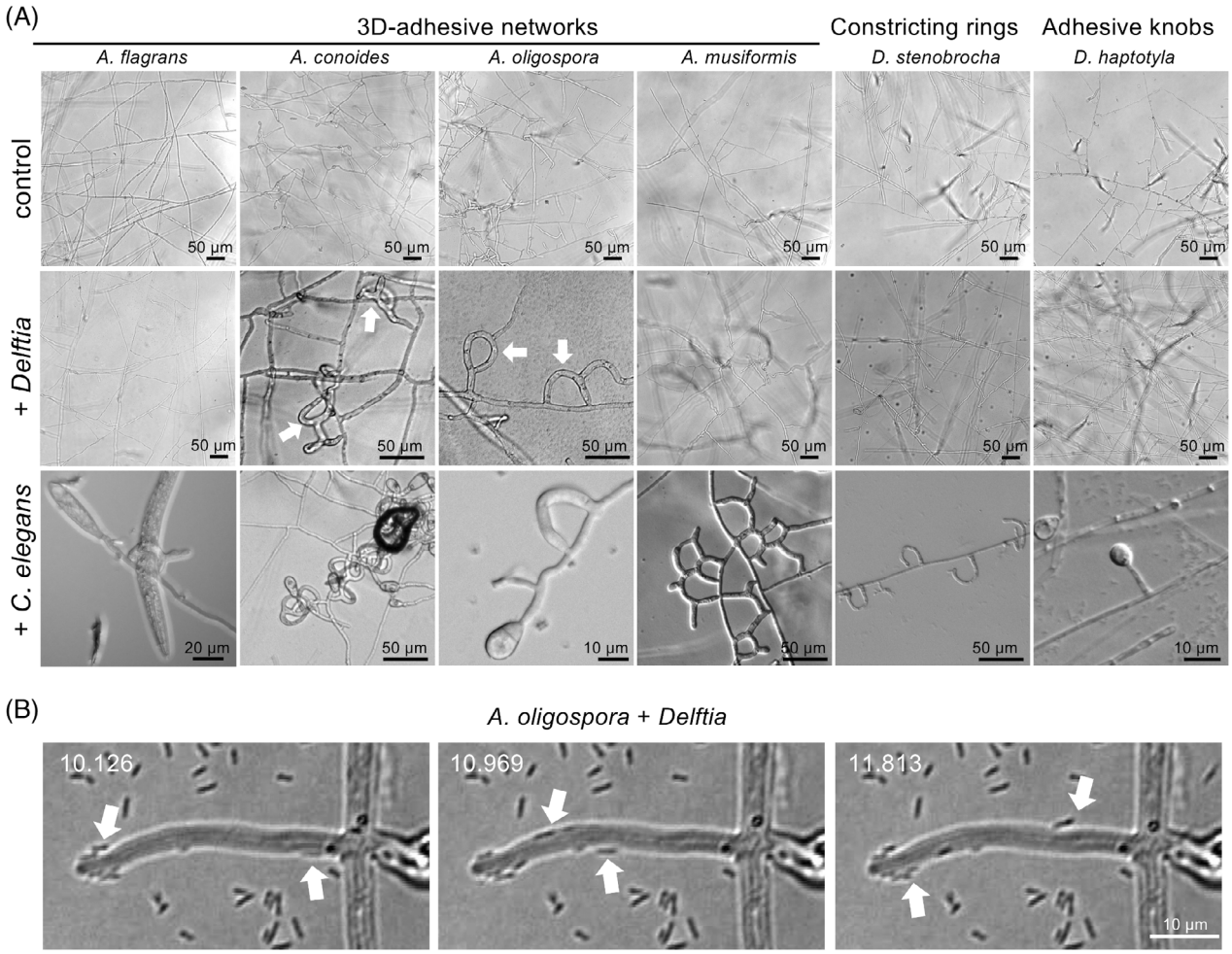

FIGURE 5 Trap induction by bacteria and nematodes in various nematode-trapping fungi. (A) Around 1000 fungal spores of each species were incubated on water agar at 28°C for 24 h and then co-incubated with the bacteria for additional 48 h. As a control, trap formation was induced by *Caenorhabditis elegans* larvae. Species forming three-dimensional trap networks, contractile rings, or sticky knobs were tested. White arrows indicate trap formation in *A. conoides* and *A. oligospora* induced by *Delftia* bacteria. (B) *Delftia* bacteria moving along a hypha of *A. oligospora*

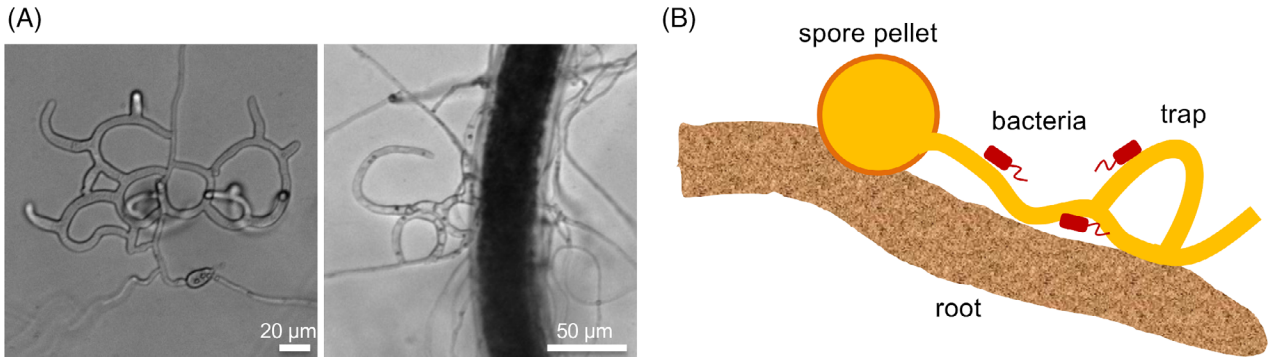

FIGURE 6 Application of natural isolates of nematode-trapping fungi (NTF) as biocontrol agent. (A) *X. index* induces traps and is digested by *A. conoides*. (B) Co-inoculating NTF and bacteria in soil is a potential strategy to increase the biocontrol efficacy of plant-parasitic nematodes.

cattle herds to the treatment of root-pathogenic nematodes in soil and presents another step in the direction of pesticide-free and sustainable agriculture.

AUTHOR CONTRIBUTIONS

Reinhard Fischer: Conceptualization (equal); funding acquisition (supporting); project administration (equal); supervision (equal); writing – review and editing

(equal). **Valentin Wernet:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); visualization (equal); writing – original draft (equal).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are included in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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