University of South Bohemia in České Budějovice, Faculty of Science

Selection of bacterium for mass production of *Phasmarhabditis* spp. and its effect on the mortality of slugs

Bachelor Thesis

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Annotation:

Bacteria was collected from dead slugs and the nematode species *Phasmarhabditis apuliae*. From the isolated bacteria nine times one bacterium was selected to produce monoxenic nematode/bacteria cultures which then were tested on growth in liquid and solid growth medium and two monoxenic culture were tested on the effectiveness to kill *Deroceras* species.

Declaration of Own Work

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Place, date.
Student's signature

Abstract

The aim of this Bachelor thesis is to discover bacteria strains that grow well in liquid medium as monoxenic nematode culture and then test the monoxenic cultures on their effectiveness to kill slugs of the species *Deroceras reticulatum*. For the experiments the nematode species *Phasmarhabditis apuliae* was used in combination with different bacteria strains for the cultivation of monoxenic cultures. The method applied for mass production of the monoxenic cultures was to place 250 monoxenic nematodes into a glass flask with a 25 ml liquid or solid kidney medium and 10 grams of polyurethan foam. For the mortality tests several *D. reticulatum* were put into a box containing wet soil, food and 13 000 Dauer Juveniles of a monoxenic culture and each day the dead slugs were cumulated and counted. The growth rate of the monoxenic cultures was lower than that of cultures recorded in many other studies, especially in the liquid medium. Only three monoxenic cultures produced satisfactory results in solid medium and one in liquid medium. For the solid media the bacteria used for the monoxenic cultures with satisfactory results were *Myroides marinus* (17), *Myroides odoratus* (B1) and *Serratia marcescens* (B2) and for the liquid medium it was *Stenotrophomonas rhizophila* (10). Both of the monoxenic cultures tested on the mortality of slugs performed similarly to other studies, however, in the culture with the bacteria strain *Serratia sp.* (2) 90% of the slugs died in 18 days while it took the *Stenotrophomonas rhizophila* (10) 15 days to kill the same percentage of slugs. Our results indicate that while the method used to grow the monoxenic cultures is not viable for mass production, the tested cultures for mortality may have a future as biocontrol agents if better production methods were to be developed.

Content

1. Introduction

1.1. Slugs as agricultural pests

In European countries as well as Australasia and North and Central America, slugs are a common pest which hinder the growth of agricultural products, such as potatoes, wheat and oilseed rape; they also present significant problems for domestic gardeners and those interested in horticulture. Slugs that cause economic damage are mainly the grey garden slug *Deroceras reticulatum* (family: Agriolimacidae), as well as the species of genera *Arion* (family: Arionidae), *Tandonia*, *Milax* (family: Milacidae) and of *Boettgerilla* (family: Boettgerillidae). Mollusc not only harms plants, but they often also carry parasites which are hazardous to humans and animals. An example of this is the *Lymnaea* spp. which carries the liver fluke *Fasciola hepatica (Wilson, et al., 1993)*. Changes in agricultural practices, for example the use of cover crops and no-till systems, lead to growth of the slug population. Therefore, farmers and researchers are looking for alternative methods of slug management. These methods include biological control as well as the use of chemicals (Nermut and Půža, 2017). A commonly used chemical pesticide is a type of pellet that contains either metaldehyde, methiocarbe or iron phosphate. However, European Union regulations restrict these chemicals from being used in organic agriculture and molluscides containing metaldehyde are only allowed if they are utilised in traps containing repellents for higher animals (Speiser and Kistler, 2016). Recently, methiocarb was banned in the whole EU and metaldehyde is banned or will be banned in most EU countries soon, even in conventional agriculture.

1.2. Natural enemies of slugs and snails

In addition to nematodes, which are a subject of this thesis, there are many other natural enemies of the slug. One of the most common in Europe is the beetle *Pterostichus melanarius* (Carabidae). Thus, *P. melanarius* was tested by assessing its feeding on slugs with anti-slug antiserum and ELISA. Coupled with accumulation of *P. melanarius* in places with a high slug population, more than 80 percent of the tested *P. melanarius* contained remains of slugs. Data from over five years indicate a loose relationship between the presence of *P. melanarius* and the absence of slugs (Mckemey, et al., 2001). Additionally, other beetles such as *Carabus nemoralis*, *Nebria brevicollis, Pterostichus niger* from the Carabidae family and *Staphylinus erythropterus* from the Staphylinidae family are predators of newly hatched slugs and slug eggs (Hatteland, et al., 2010). Another predator of slugs is the fly species *Tetanocera elata* from the Sciomyzidae family, whose first and second instar

larvae feeds on slug species *D. reticulatum* and *D. leave* while the third instars feed on a variety of other slug species (Ahmed, et al., 2019).

1.3.Biocontrol agents

Agostino Bassi, Louise Pasteur and Elie Metchnikoff were pioneers in the use of microorganisms for pest control (Steinhaus, 1956, Steinhaus,1975). Entomopathogenic organisms used for microbial control of insect pests are bacteria, viruses, fungi, protozoa, and nematodes. Compared to conventional chemical pesticides, entomopathogens are environmentally safer, which not only protect non-target organisms, but also reduce the amount of pesticide residues in food and lead to an increase in biodiversity. The use of entomopathogenic organisms also has distinct advantages over other arthropod biocontrol agents because they are easily produced and can be applied with conventional equipment. Disadvantages of the use of these biocontrol agents are their persistence, the time required to kill the host, too broad or narrow specificity and the cost in comparison to chemical insecticides. Entomopathogenic organisms can mostly be used like any other biocontrol agents (Harper, 1987). The different application strategies are as follows: augmentation, in which they are used to augment the natural pathogens; conservation, in which the use conserves or activates the natural pathogens; inculcative release, in which they are used as classic biocontrol agents which establish themselves in the pest population for long-term regulation; and inundating release, in which they are used for short-term control.

Among nematodes there are seven nematode families that show potential as biocontrol agents against insects: Mermithidae, Tetradonematidae, Allantonematidae, Phaenopsitylenchidae, Sphaerularidae, Steinernematidae and Heterorhabditidae (Kaya and Stock, 1997). The steinernematids and heterorhabditids are the most promising potential biocontrol agents because they possess many attributes of pathogens and parasitoids (Kaya and Gaugler, 1993, Gaugler, et al., 1997a) and use insects as their definitive hosts. Entomopathogenic nematodes (EPN) have symbiotic bacteria of the genera *Xenorhabdus* (Steinernematidae) and *Photorhabdus* (Heterorhabditidae). This bacterium is highly virulent and a lethal pathogen of insects. The nematode/bacteria complex is known to kill the host within 48 hours (Lacey, et al.,2001). If EPN are used against pests it is crucial to match the correct nematode species to the pest (Bedding, 1990, Kaya and Gaugler 1993), because some nematode species are more effective against a certain pest and less effective against another. For example, the effectiveness of *Steinernema kushidai* against scarab grubs is high but is low against lepidopteran larvae (Mamiya, 1989). Additionally, the hunting methods of the

nematodes influence their efficiency: some are ambushers (e.g. *Steinernema carpocapsae*) and therefore tend to remain near the soil surface or on mobile hosts, others are active cruisers (e.g. *Heterorhabditis bacteriophora*) which look for their host in the soil (Lewis, et al., 1993, Campbell and Gaugler 1993, Campbell and Gaugler 1997). To make nematodes more effective than a biocontrol agent, genetic improvements may be used to increase search capacity, virulence, and resistance to environmental changes. Gaugler et al. (1997b) inserted a heat-shock protein with molecular techniques into *H. bacteriophora*, which resulted in transgenic nematodes which were eighteen times more effective at surviving stress related to high temperatures than wild types of nematodes. Due to the high production and formulation cost of these EPN, the use in niche markets and regions where chemicals cannot be used is limited (Lacey, et al., 2001).

1.4. Nematodes as biocontrol agent against slugs

There are many molluscs parasitic nematodes (MPN) in nature, and almost all come from eight different families: *Agfidae*, *Alaninematidae*, *Alloionematidae*, *Angiostomatidae*, *Cosmocercidae*, *Diplogasteridae*, *Mermithidae*, and *Rhabditidae (Stock and Goodrich-Blair, 2012)*.

Nematodes have 3 associations with molluscs. They can be either paratenic, intermediate or definitive hosts. Molluscs, which are paratenic hosts, serve the nematode as a vector to another host with no development taking place (Anderson, 2000) and are the most common for vertebrate parasitic nematodes. In intermediate hosts the nematode completes a part of its life cycle in the molluscs until it develops into the infective stage, during which it infects their final host after the intermediate host has been eaten by the final host. Nematodes which use the molluscs as definitive host complete their life cycle in the host.

There are 3 different associations between definitive hosts and nematodes. The first is for nematodes which have parasitic juveniles, and the adults are free living and may kill the host after leaving it (e.g. *Mermis albicans*). The second association is when nematodes complete their life cycle in the host but do not kill it (e.g. *Agfa flexilis*). Lastly, the third association is with nematodes which lead to a high mortality rate in the infected hosts (e.g. *Phasmrhabditis* hermaphrodita) (Nermut and Půža, 2017).

1.4.1. *Phasmarhabditis hermaphrodita* used as biocontrol agent

The parasitic nematode *P. hermaphrodita,* infective juveniles (IJs) or also called dauer juveniles (DJs) act as a vector for the bacteria (known as *Moraxella osloensis* in commercial

products) that is transported to the shell cavity of *D. reticulatum* or other hosts by entering the slug beneath the area of the mantle and releasing the bacteria. The bacteria start to multiply, kill the mollusc and serve as a source of nutrition for the nematodes that develop into hermaphrodites. There is then a high probability that the infected slug will die within 7-21 days. *P. hermaphrodita* does not strictly associate with one bacterium as EPN do, but rather feeds on several different common bacteria species in its habitat. This bacteria species also influences the life of the *P. hermaphrodita* in many additional ways (Nermut and Půža, 2017). *P. hermaphrodita* has 3 different life cycles depending on the slug species it encounters: saprobic, necromenic and parasitic.

Saprobic life cycle

Experiments conducted by Tan and Grewal (2001a) show it is possible to grow nematodes on homogenized slugs and slug faeces, which suggests that nematodes can live in saprobical environments. Unfortunately, no data is available that shows this occurring in nature, but the ability of *P. hermaphrodita* to live in saprobical environments is supported by several studies. Tan and Grewal 2001a further suggest that *P. hermaphrodita* may persist even when no live hosts are present and, therefore, may serve as a potential method of long-term inoculative slug control. Long term persistence in the soil environment was also proven by Nermut (2012).

Necromenic life cycle

In the necromenic life cycle the DJs enter the slug and eventually stop developing in its mantle cavity. When the slug dies, the DJs recover and, using the slug cadaver as a food source, start to further develop and reproduce until the food source is no longer available. Then, new DJs are developed. Nematodes enter this life cycle if they infect a large slug species (e.g. *Limax maximus* or *Arion vulgaris*) or if the associated bacteria is not able to cause septicaemia and kill the host.

Parasitic life cycle

In the parasitic life cycle, the nematodes DJs enter the slug through the dorsal integumental pouch, then travel through a short canal into the slug's shell cavity below the mantle. If many nematodes invade, they spread to several of the slugs' body parts. Inside the slug, the nematodes develop into adults and reproduce, which typically leads to swelling of the rear half of the slugs' mantle. *P. hermaphrodita* typically develop 250-300 offspring, known as the second generation, which then spreads throughout the body. When the slug dies, a third

generation is produced, which then uses the cadaver of the slug as nourishment and, in turn, forms new DJs (Wilson and Grewal, 2005).
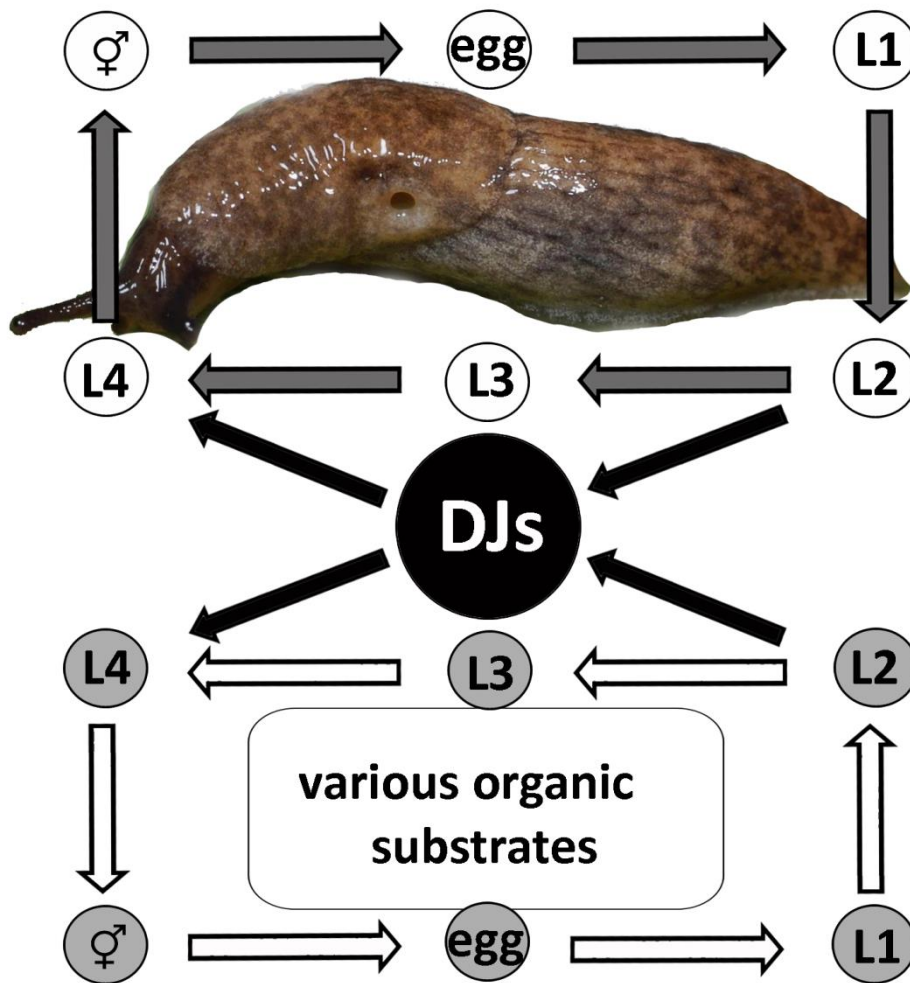

Figure 1: The saprobic and parasitic life-cycle of Phasmarhabditis hermaphrodita (Nermuť, Půža, 2017)

1.4.2. Formulation and production of biocontrol agent

There are two types of methods to cultivate nematodes either *in vivo* or *in vitro*. The *in vivo* method of production yields inconsistent results compared to the *in vitro* method. Both the nematodes species *P. hermaphrodita* and *Alloionema appendiculatum* yield high levels of production. While *P. hermaphrodita* is produced commercially by a method involving liquid media and high-volume fermenters, *A. appendiculatum* is produced placing a homogenised pig kidney on a 2% agar plate and can survive many years in its saprophytic form at 15 °C. The kidney is the only item that must be changed every two weeks (Nermut, et al., 2014b, Nermut and Půža, 2017). Nematodes can be grown in xenic cultures, but these cultures may contain harmful bacteria, which can inhibit nematode growth or can be pathogenic to

nematodes. To produce more predictable and constant nematode yields, nematodes can be grown in monoxenic cultures with only a single bacterium present. The nematodes that are grown in such monoxenic culture can differ in presence or absence of toxic metabolites, growth, and reproduction rate, as well as their ability to attract bacteria, ingest, and digest; all these factors are strongly dependent on which bacteria are used. In order to use nematodes as commercial molluscicide, Friedman (1990) reviewed mathematical models for several production techniques and concluded that a liquid medium is the best medium for large-scale, commercial production. Therefore a monoxenic bacteria-nematode culture that can be cultivated in a liquid medium is needed (Wilson, et al., 1995b). EPN are commercially viable because methods for large-scale fermentation have previously been developed and, with a slightly different composition of the culture medium, it is also possible to produce *Phasmarhabditis spp.* by utilizing very similar methods (Wilson, et al., 2001). Fermenters of the e-nema Gmbh produce nematodes in up to 60,000 l (e-nema, 2021) and in suitable media, nematode yield is estimated to be 100,000 DJs/ml (Nermut and Puza, 2017). The commercial production of nematodes through fermentation leads to large quantities of media with viable DJs, dead or non-dauer juvenile nematodes, spent media, particulate debris, and the associated bacteria of the nematode. The presence of non-DJs in the formulation allows for the growth of contaminants, which leads to unpleasant odours and a shorter shelf-life. A method to increase shelf-life is to add antimicrobial agents, however, this increases the production and, thus, retail cost and makes it impossible to market the product as a natural product. To separate waste components from the DJs downstream, operations such as centrifugation, gravity settling, and floatation are used. It is important for the downstream operation that a high concentration of the DJs accumulate in the suspension before formulation (Wilson, et al., 2003). A good method to recover the DJs of the use of floatation that adjusts the continuous phase density, but for *P. hermaphrodita,* the flocculation of insoluble spent media, which can be reduced by adding to the air supply, can present problems (Rae, et al., 2007). Another method requires the use of vibrating membrane filters (VMF), which provides a flexible operation for separation, cleaning and concentration of the nematodes from the fermentation broth (Wilson, et al., 2003).

1.4.3. Storage of nematodes

Temperature is a deciding factor in the survival rate of nematodes stored in water. The optimal water temperature depends on the climate of the nematodes' natural habitat; the storage temperature should be below this temperature. One method to obtain storage stability

for entomopathogenic nematodes is the use of anhydrobiosis. Grewal (2000) found that the longevity of *Steinernema carporcaspae* and *Steinernema riobrave*-IJs increased when stored in water dispersed with granules at 25°C in comparison to nematodes stored in pure water. However, desiccation at 5°C decreased the nematodes' longevity. Because steinernematids and heterorhabitids showed that they tolerate osmotic stress, Glazer and Salame (2000) evaluated the use of osmolytes to desiccate nematodes at 45°C, which proved to increase their survival rate, compared to non-desiccated nematodes. Additionally, air-dried and osmotic desiccated ones behave similarly (Glazer and Salame 2000). In their experiments on the nematode *P. hermaphrodita*, Wilson et al (1993) showed that all nematodes died after 2 hours in culture flasks at 100 DJs/ml and 35°C, while only half of them died in 6-7 hours if the temperature was between 26-29°C. In another experiment only 6 percent of the nematodes survived 2 months at 10°C in 100ml water with 5000 DJs stored in 250 ml culture flasks (Grewal and Grewal, 2003).

1.4.4. Application of nematodes

The nematode *P. hermaphrodita* navigates through heterogeneous soil to its host and, therefore, its efficiency as a biological control agent can be increased with a better understanding of its dispersal abilities, as well as the interactions between host and parasite. It can, however, present a challenge to fully gain this understanding, especially regarding soil habitats, because they are complicated to characterise and simple changes in these characteristics can result in a significant impact on the biological functions of soil organisms. Physical changes in the soil influence nematodes' chances of survival, as well as their patterns of movement within the soil. Therefore, the physical characteristics of the soil as well as the host density have a significant influence on whether *P. hermaphrodita* is a successful biological control agent. For example, in sandy loam soil the nematode *P. hermaphrodita* moved 12 cm in 3 days. Because there is only a small amount of literature on this topic there are no models for the spatial spread of nematodes in soil (Hapca, et al., 2007). Nematodes require a high application rate, which further increases the cost of this method. To effectively use nematodes in suitable soil conditions, it is necessary to apply at least 3×10^9 IJs/ha to protect the crops from slug damage (Glen and Wilson, 1997). If improved application methods are used instead, the application rate can be reduced (Grewal et al., 2001). One of these methods, described by (Hass, et al., (1999 a,b) is to position nematodes around the plants which are being fed upon by slugs. However, this method requires a substantial amount of time to be effective and, therefore, does not protect the

plants in the initial stage of application. A new approach, which involves a reduced application of the amount of nematodes, targets slug shelters as opposed to the aforementioned application at the entire feeding sites. The slug, which is a nocturnal forager, has developed the habitual behaviour of making a shelter and returning to it. As an example, the slug *Ariolimax columbianus* digs a shelter by excavating a depression into the soil and from this shelter it forages the area of about 4.5m^2 around it (Ingram and Adolph, 1943). Because slugs have a trail-following behaviour, they can relocate these shelters via mucous trails, which is also a common behaviour for gastropods (Wells and Buckley, 1972). The created shelters provide the slug with good conditions for slug activity: mating as well as protection against environmental conditions such as sunlight and heat. In Grewal et al. (2001) experiments, the method of targeting slug shelters proved to be effective, however, Wilson et al. (1999) suggested in another study that slugs avoid nematode treated areas (Grewal, et al., 2001), which was proven in an additional study by Wynne et al. (2016) using a concentration of 120 nematodes/cm^2 (Wynne, et al., 2016). In comparison to that study, such behaviour was not found if the nematode concentration remained below 38 IJs/cm^2 (Grewal, et al., 2001). A further method is the repeated application of a smaller dose of nematodes. An application of 50,000 IJs/m^2 three times a month can reduce the overall dose by 50% compared to a single time application. The efficacy of this method is almost the same as when using metaldehyde pellets (Ester et al., 2003). Furthermore, *P. hermaphrodita* application can also be combined with the use of metaldehyde, because it has no negative effects on nematode survivability (Nermut and Půža, 2017).

2. Goal of this thesis

The goal of this thesis is to identify and isolate bacteria species that are associated with the nematode species *Phasmarhabditis apuliae*, thereby establishing several monoxenic cultures with the isolated bacteria species and *P. apuliae*. Further, it is intended to test the effectiveness of the obtained monoxenic cultures on the feeding activity and mortality rate of slugs (*Deroceras reticaltum* or *Arion vulgaris*), as well as to test the growth of monoxenic cultures in solid and liquid medium.

3. Materials and methods

3.1. Collection of slugs

Deroceras reticulatum and *Arion* slug species were collected near the University of South Bohemia in České Budějovice by using wooden boards that were laid out to serve as slug

shelters. The slugs were collected in the morning or evening, placed into plastic containers measuring 17cm x 12 cm, and covered with a lid that had holes for air flow. In each container there was a (roughly) 2 cm layer of slightly wet garden soil and food, such as carrots, cucumber and dog food. The slugs in the container were stored at room temperature and controlled for contamination, such as the growth of fungus and the state of the food each day. Eggs laid by the slugs in the container were collected and transferred into a separate container to obtain juveniles.

3.2. Isolation and identification of bacteria

Bacteria for the monoxenic cultures were isolated through the collection of samples from dead *Arion* or *Deroceras* species, as well as from the nematode species *Phasmarhabditis apuliae* (strain BAR), which were cultivated on a homogenized pig kidney or on a freeze-killed slug species. In order to isolate the bacteria, plates consisting of standard nutrient agar I or kidney agar were prepared in a 1 litre flask and then autoclaved at 121°C for 15 minutes (see Appendix). After the autoclaved process was complete, the agar was poured into 90 mm Petri dishes in a sterile flow box, while the kidney agar was first filtered with a muslin to separate the solution from the meat pieces. Then, after adding the agar, the flask was autoclaved a second time at 121°C for 15 minutes and poured into 90 mm Petri dishes in a sterile flow box. The bacteria were first collected by using a bacteriological loop to stroke the dead slug or the pig kidney and was then placed on a Petri dish with kidney or standard nutrient agar. After the bacteria were transferred to agar plates, the Petri dishes were closed and sealed with parafilm. The sealed Petri dishes were stored at room temperature for 24 hours and then checked for development of bacteria colonies. A single bacteria colony was then taken from these Petri dishes using a sterile bacteriological loop (inoculating loop) and transferred into a sterile YS medium. The YS medium used for bacteria culturing was prepared in a 1 litre flask and dispersed into several Erlenmeyer 50 ml flasks (see Appendix). To serve as a stopper for the Erlenmeyer flask, a suitably sized cork with an aluminium foil cover was used. To sterilize the YS medium, the Erlenmeyer flask was put into an autoclave and autoclaved at 121°C for 15 minutes. The procedure for putting the bacteria in the YS medium or extracting solution was the following: 1. Under sterile conditions (Flow box that was UV-sterilized) the aluminium cork was removed. 2. With a sterile tip of an automatic pipette, 100 µl of the medium was taken out or with a sterile bacteriological loop, the bacteria colony was put into the YS medium and stirred. 3. The end of the cork that was meant to be inserted into the flask was sterilised using a Bunsen burner. The YS medium

with the bacteria was put onto a shaker (180 rpm) for 24 hours. Bacterial cultures were soaked in a 15% glycerol solution in a freezer at -20°C for later use. All the bacterial strains were obtained and processed by this method.

3.3. Identification of collected bacteria

To identify the bacteria, a small amount of a YS medium with the bacteria was transferred in an Eppendorf tube of 1.5 ml, which was then centrifuged at 1000g for 60 seconds, following which the supernatant was removed. This process was repeated at least 3 times until a approximately 0,1cm high pellet with the bacteria was visible at the bottom of the tube. Then, the DNA of each bacterial strain was extracted with the DEP-25 DNA Extraction kit (Top-Bio) according to the producer´s manual. The obtained DNA extract was used as a template for PCR (Polymerase Chain Reaction). For the identification, a 16S marker was used. This marker was amplified with the primers 10F (5´-AGTTTGATCATGGCTC AGATTG-3´) and 1507R (5´- ACCTTGTTACGACTTCACCCCAG-3´), designed by Sandström (2001). The PCR profile was as follows: primary denaturation at 94°C for 60 seconds, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds, elongation at 72°C for 2 minutes and final elongation at 72°C for 3 minutes. The product of the PCR was checked on a 1% agarose gel in 1x concentrated TAE buffer in an electrophoresis (100 V, 40 min.). After that, the DNA was stained in 1xTAE with ethidium bromide and visualized with UV light in transilluminator. PCR products of the right size (compared with DNA marker 200-1500 bp Top-Bio) were purified by using 0.5 µl of Exonuclease I and 1 µl of thermosensitive Alkaline Phosphatase in a PCR cycler with the profile 37°C for 30 minutes followed by 80°C for 15 minutes. After that, the PCR products were sent to sequencing (GATC company). Obtained sequences were edited and compared with those presented in the GenBank (Nucleotide BLAST: Search nucleotide databases using a nucleotide query (nih.gov)).

3.4. Monoxenic nematode culture

To develop the monoxenic cultures, a colony of nematodes was cultivated by using a White trap (White, 1927), which necessitates a small piece of homogenized pig kidney on wet muslin. The pig kidney served as a food source for the nematodes. Larvae were then sprayed around the kidney. The nematode species used was *P. apuliae*, strain BAR, originally isolated on the University of Bari in Apulia campus in Italy in 2012. Using this method, several nematode colonies were made and stored in a refrigerator. After 72 hours the colony was searched for the presence of adult nematodes. Nematodes from large colonies were then

transferred to a new White trap with a fresh pig kidney to form a new colony. The gravid adult females were collected using a small needle and placed into the Eppendorf tube, along with a small amount of tap water. To obtain a monoxenic culture from the collected adult gravid females, the following procedure was used: First the gravid adult females were placed in a sterile glass tube filled with Ringer´s solution and then ruptured by adding small razor blades and vortexing the glass tube or homogenising them with a glass rod or homogeniser. Then the eggs were collected into a sterile 1.5 ml Eppendorf tube by filtering the solution with an Uhelon 130T filter with loops of 40 µm. The tubes with eggs dispersed in Ringer´s solution were centrifuged for 2 minutes at 2000 rpm. The supernatant was then removed and sterilization solution (10 ml H2O, 1.5 ml 12% NaClO, 0.5 ml 4M NaOH) was added (from this moment on all work was conducted in sterile conditions in flow box). The solution was gently shaken for 4 minutes. Then the solution was centrifugated a second time for 2 minutes at 4000 rpm. Next, the supernatant was removed and sterile YS solution was added; the solution was then centrifuged at 4000 rpm again. This step was repeated at least twice. Then a sterile pipet was used to suck up 300 µl of concentrated eggs, which were then distributed into multi well plates (24 wells). After 48 hours, each well was checked for contamination and hatched eggs. The uncontaminated hatched nematodes (without turbidity) were then placed on and kept to one side of a sterile petri dish, already containing pig kidney agar, while one isolated bacteria culture was placed on the other half of the petri dish. After sealing the petri dish with parafilm and leaving a small opening for air, it was placed in a climatic box for cultivation at 16°C. Eleven monoxenic nematode cultures were obtained using this procedure. (Table 1).

3.5. Growth of monoxenic culture in liquid and solid media

After a monoxenic culture had grown enough that nematodes were visible on the surface of the pig kidney agar to the naked eye, all the nematodes from the monoxenic cultures with the same bacteria were transferred into a tube by using a 1ml automatic pipet. This was done in a flow box by spraying sterile water with the automatic 1ml pipet on the Petri dish and pouring the nematodes with the water into a 100 ml tube. If all the nematodes from the Petri dishes with the same monoxenic culture were transferred to the 100ml tube and 100ml was not reached, the amount of the missing volume was added in sterile water by using a measuring cylinder. The full 100ml tubes were mixed by shaking the tube. Then a 250 µl sample was extracted from the nematode/water suspension and put onto a microscope glass slide to count the nematodes. This was repeated for each prepared tube 10 times and then an average

nematode count was calculated. To measure the growth rate of the nematodes in the time span of 2 months, 250 nematodes were then inoculated into a growth flask (100 ml) with a solid or liquid medium that was slightly open for air ventilation and wrapped by aluminium foil. These flasks were stored in a climatic box at 16°C. For the experiment due to the limited number of flasks available was the first batch prepared with4 growth flasks and the second batch with 6 growth flasks per bacteria strain and medium. With the second batch having a lower number of different bacteria strains. The growth flasks were prepared by putting 10 grams of polyurethan foam into the flask then a solution of solid or liquid kidney growth medium was prepared. From the kidney medium, 25 ml was added into the flask with the polyurethan foam and thoroughly shaken so that it was evenly distributed. The flasks were marked if liquid or solid kidney medium was used and autoclaved at 121°C for 15 minutes. The 250 nematodes from the monoxenic culture were then put into a flow box and into the autoclaved and cooled growth flask. After 2 months, the juvenile nematodes in the flask were counted. Before counting, nematodes were extracted from the polyurethane foam by using sieves that were made of Uhelon 90T with loops of 67 µm. These sieves were placed on a water surface and the nematode juveniles migrated through the sieves into the water onto a Petri dish below. When all juvenile nematodes had settled at the bottom of the Petri dish, they were collected in a 100 ml flask and the volume in the flask was noted. Following this, the flask was thoroughly mixed and 5 droplets were made with an automatic pipet on the same volume setting; the nematodes in each droplet were then counted. The total number of nematodes in the growth flask was calculated by the average number of nematodes in the droplets.

3.6. Mortality of the slugs

To see the effectiveness of the nematode/bacteria combination on the mortality rate of slugs, plastic boxes measuring 17cm x 12cm were filled with nematode-free (heated in microwave for 5 min.) fresh wetted soil. The following procure was applied for each box. Seven slugs of the *Deroceras* or *Arion* species were put into the plastic box, then the nematodes from the monoxenic culture were distributed by using an automated pipet with a total nematode concentration of 13,000 DJ/box (which would be about 64 DJ/cm^2). Finally, the granulated dog food was put into the box and it was closed with a lid with holes for air ventilation. The slugs were checked each day for the number of deaths and possible contaminants, such as fungus, as well as for the condition of the food supply. The same procedure was conducted for the control group, except that no nematodes were added to the box. For each monoxenenic nematode/bacteria combination and control group, 3 boxes were used.

4. Results

4.1. Bacteria

Seventeen bacterial strains from the nematode *P. apuliae* and dead *Deroceras* and *Arion* slugs were isolated and identified. All bacterial strains are listed in Table 1 with the respective identification number that was given to them. All the isolated bacterial genera and species are common in soil environments or are associated with molluscs and other invertebrates. All the bacteria are gram negative, aerobic or facultative anaerobic rod-shaped bacteria that are not dangerous for humans or farm animals (Reimer et al.,2019).

Table 1 Bacteria species and their assigned identification number

Identification number	Bacteria species	Used bacteria	Family	Phylum
1	*Serratia sp.*		Yersiniaceae	Proteobacteria
2	*Serratia sp.*	x	Yersiniaceae	Proteobacteria
3	*Enterobacter cloacae*	x	Enterobacteriaceae	Proteobacteria
4	*Serratia sp.*		Yersiniaceae	Proteobacteria
5	*Acinetobacter johnsonii*		Moraxellaceae	Proteobacteria
6	*Pseudomonas fragi*	x	Pseudomonadaceae	Proteobacteria
7	*Pseudomonas putida*	x	Pseudomonadaceae	Proteobacteria
8	*Pseudomonas sp. 1*	x	Pseudomonadaceae	Proteobacteria
9	*Pseudomonas sp. 1*		Pseudomonadaceae	Proteobacteria
10	*Stenotrophomonas rhizophila*	x	Xanthomonadaceae	Proteobacteria
11	*Pseudomonas fragi*		Pseudomonadaceae	Proteobacteria
12	*Myroides profundi*		Flavobacteriaceae	Bacteroidetes
14	*Acinetobacter johnsonii*		Moraxellaceae	Proteobacteria
16	*Myroides marinus*		Flavobacteriaceae	Bacteroidetes
17	*Myroides marinus*	x	Flavobacteriaceae	Bacteroidetes
B1	*Myroides odoratus*	x	Flavobacteriaceae	Bacteroidetes
B2	*Serratia marcescens*	x	Yersiniaceae	Proteobacteria

4.2. Solid and liquid growth media

From the 17 identified bacteria species (Table 1) nine were used in the liquid and solid kidney medium experiment. The bacteria used for the monoxenic cultures were *Serratia sp.* (2), *Enterobacter cloacae* (3), *Pseudomonas fragi* (6), *Pseudomonas putida* (7), *Pseudomonas sp.* 1 (8), *Stenotrophomonas rhizophila* (10), *Myroides marinus* (17), *Myroides odoratus* (B1) and *Serratia marcescens* (B2). The experiments showed that there were, on average, more DJs per ml in the solid growth flask than in the liquid growth flask, and according to an ANOVA analysis (d.f: 1; 55 F = 20.34 and p < 0.001) the difference is significant. Figure 1 shows that there are apparent differences among the bacterial strains, but due to the high variability (e.g standard error of mean from strain 10 in liquid media) of the data, an ANOVA analysis (d.f: 8, 55 F= 1.98 and p = 0.06) was run, which determined no significant difference among the bacterial strains. Figure 1 also depicts the mean yield of DJs for liquid and solid media, as well as the respective bacteria strain. It shows that in solid media the bacteria strain *Myroides marinus* (17) with 4 382 DJs/ml produced the highest yield/ml followed by *Myroides sp, Myroides odoratus* (B1) 2 752 DJs/ml and *Serratia marcescens* (B2) 2 786 DJs/ml. In conclusion, all three of these strains did not grow well in the liquid media, with 168 DJs/ml, 120 DJs/ml, and 30 DJs/ml respectively, while *Stenotrophomonas rhizophila* (10) had poor yields in solid media 78 DJs/ml but the highest yields in liquid media 2 931 DJs/ml.

4.3. Mortality rate of slugs

The mortality rate of the *Deroceras reticulatum* was significantly influenced by not only the bacterial strain used (df: 2, 70 F = 13.64 p<0,001) but also time (df: 13, 70 F = 17.43 p<0,001). There was also a significant difference regarding the interaction of the bacteria strain with the nematode and the mortality rate (df: 26, 70 F = 1.66 p < 0,05). Figure 2 shows the cumulated mortality rate of the control group and the two bacteria strain groups in percentages. There is an obvious difference concerning the mortality rate between the control group and the bacteria strains, namely that the control group exhibits a stepwise cumulative mortality rate, while the mortality rate of the bacteria strains grows exponentially. Looking closely at the bacteria strains, it can be seen that the cumulative mortality rate for the monoxenic culture with the *Stenotrophomonas rhizophila* (10) strain increases at a higher pace after the eleventh day, while it takes 15 days with the *S. rhizophila* (10) strain and 18 days with the *Serratia sp.* (2) strain to kill about 90 % of the slugs. This indicates that *S. rhizophila* (10) is slightly better at killing *Deroceras* reticulatum than

Serratia sp. (2). Figure 2 also shows that the effect of the nematodes on the mortality rate of slugs is visible only 4 days after the application of the nematodes, after which it increases rapidly.
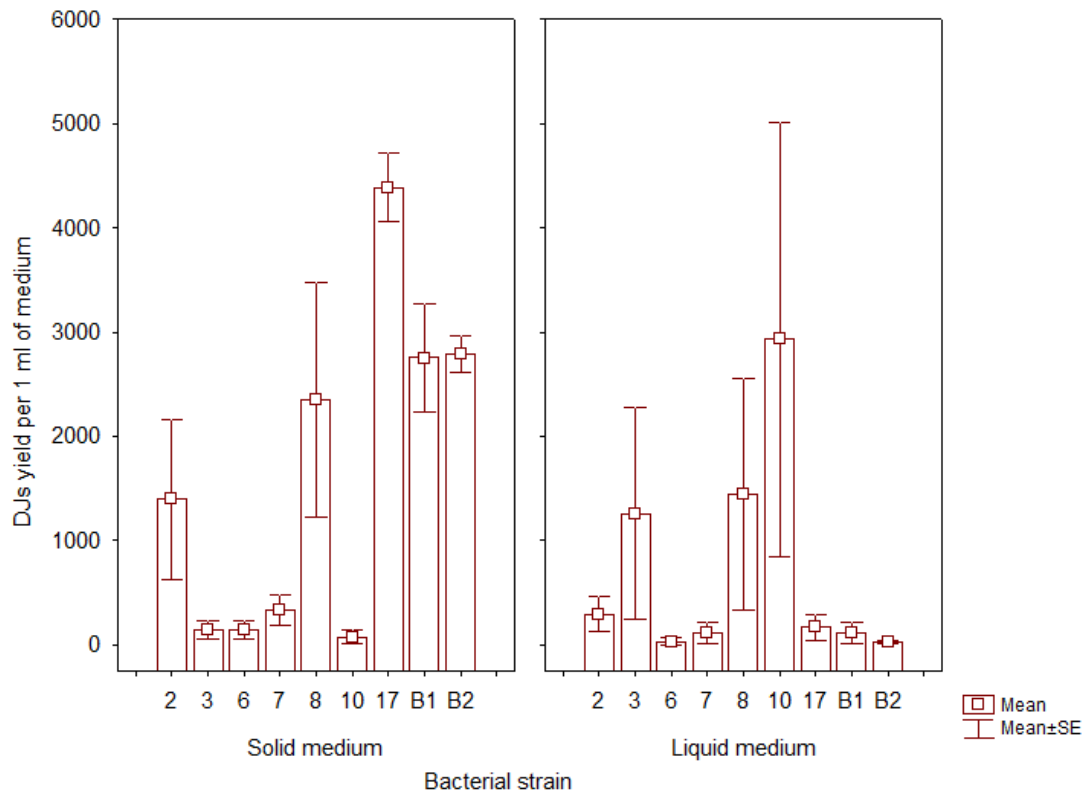

Figure 1 Nematodes yield/ml in each monoxenic nematode/bacteria culture for liquid and solid kidney medium. Bacterial strains: Serratia sp. (2) Enterobacter cloacae (3), Pseudomonas fragi (6), Pseudomonas putida (7), Pseudomonas sp. 1 (8), Stenotrophomonas rhizophila (10), Myroides marinus (17), Myroides odoratus (B1) and Serratia marcescens (B2).
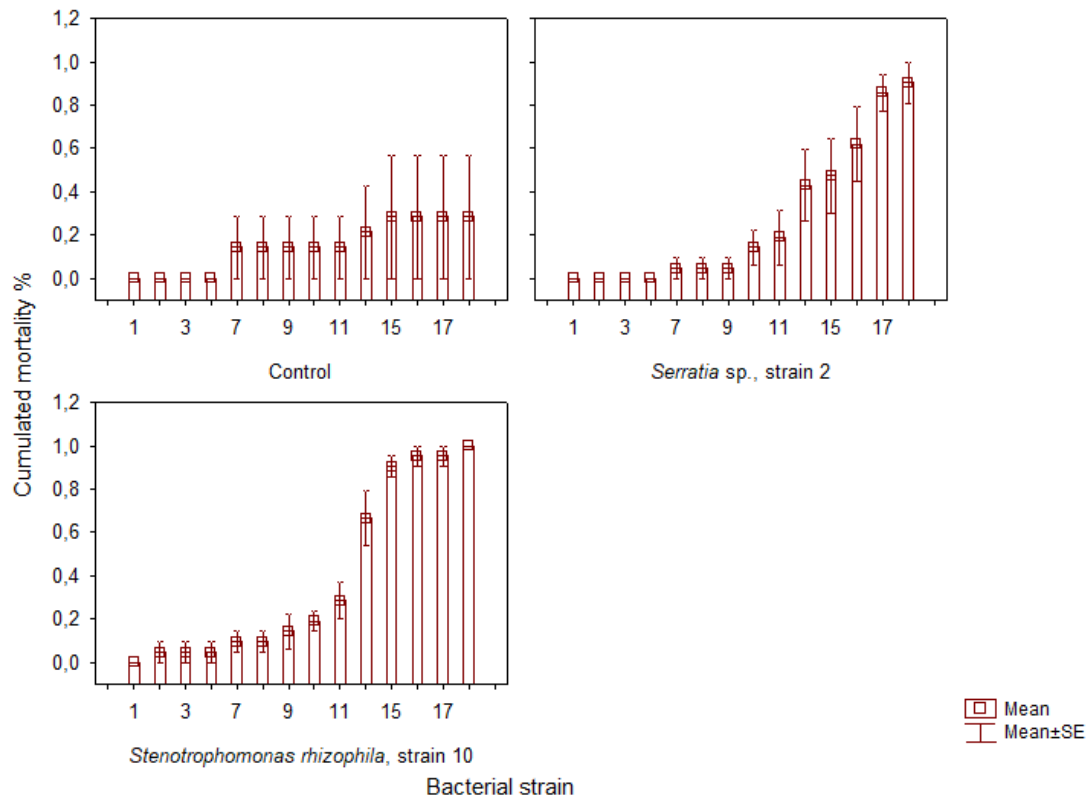
Figure 2 Cumulated mortality of slugs *Deroceras reticulatum* for different monoxenic nematode/bacteria culture. Bacterial strains: *Serratia sp. (2), Stenotrophomonas rhizophila* **(10)**.

5. Discussion

The results indicate that all monoxenic nematode cultures from the experiments grow in the solid and liquid kidney media, but the yields were lower than in similar studies, such as those performed by Wilson et al. (1995a); especially different were the yields in the liquid medium. This discrepancy may be due to the different method used and to the fact that more samples would have led to a more valid evaluation. Another issue is the variability and consistency of the obtained data, particularly the liquid cultures, which was likely caused by either incompatible bacteria nematode culture or the experimental design. For this study, the nematode cultures with the bacteria strains *Pseudomonas fragi* (6) and *Pseudomonas putida* (7) had very low yields in liquid and solid media and are, therefore, not suitable for cultivation, which may be due to the nematode/bacteria complex's lack of compatibility with each other. This could be related to the fact that the bacteria did not grow in this type of media or that the bacteria themselves inhibited nematode growth. Further experiments (Table 2 and Figure 3 in Appendix) reinforced the statement that *Pseudomonas fragi* (6) and *Pseudomonas putida* (7) did not support nematode growth in liquid media. Similarly, the bacteria strains *Myroides marinus* (17), *Myroides odoratus* (B1) and *Serratia marcescens* (B2) are not suitable for liquid media cultures. The probable reason for this is, as subsequent

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experiments revealed, that bacteria in intensely shaken (160 rpm) Erlenmeyer flasks overgrow and kill the nematodes, because liquid media environments are very well suited to the growth of bacteria. In comparison, the growth in solid media is not favoured and, thus, nematodes can benefit from the bacteria when placed in this type of medium. Therefore, it is only possible to grow this type of nematode/bacteria culture well in solid media. The bacteriophagous *Phasmarhabditis spp. (P. apuliae)* lives in association with different bacteria species, which leads to differing growth rates in liquid and solid media (Wilson et al., 1995a; Rae et al., 2010). The reason for this is that these associate bacteria species have a significant influence on nematode fertility (Wilson et al., 1995a, Nermut et al., 2014a), as well as the pathogenicity of the host (Wilson et al., 1995b).

The experimental design, which involved using polyurethan foam to absorb the liquid medium, may have led to issues, because the flasks were not shaken continuously. Even though the polyurethan foam was fully soaked in the liquid medium, this led to liquid forming at the bottom of the flask, which caused the nematodes and bacteria to also concentrate in this area of the flask. This led to the worst possible condition for nematode growth and a possible reason for generating inconsistent results. In comparison, the recorded yields of the solid kidney medium are very consistent. Therefore, continuously shaking the flask would have likely solved this issue for the liquid cultures (Buecher and Popiel, 1989).

Although *P. apuliae* is a recently discovered species and not much about its ecology is known, it has the potential to influence the mortality rate of slugs, similarly to other *Phasmarabditis* species (Wilson et al., 1993; Glen et al., 2000a). This study used 2 bacteria, *Serratia sp.* and *Stenotrophomonas rhizophila,* to test of the effectivity of *P.apuliae* on the mortality rate of slugs, and both monoxenic nematode bacteria cultures performed similarly to other experiments testing slug mortality rates (Rae, R.G., et al., 2010, Mc Donnell, et al., 2018). This indicates that the monoxenic *P.apuliae* cultures with these two bacteria strains are effective parasites/pathogens of slugs, but before using these two cultures as biocontrol agents, their effectiveness in field conditions needs to be tested and an improved method for their production in a liquid culture needs to be developed. The experiments in this study indicate that possible development of a *S. rhizophila* (10) bacteria/nematode culture in a liquid medium is more promising than *Serratia sp.* (2). The reason for the differences in mortality rates achieved between the two nematode/bacteria cultures is that the nematode associated bacteria influenced the pathogenicity of the culture differently (Wilson *et al*., 1995a).

6. Conclusion

In conclusion, our research proves that *P. apuliae* has the potential to be an effective bioagent against slugs. The nematode/bacteria complex of *P.apuliae* and *Stenotrophomonas rhizophila* (10) is best suited for mass production in liquid medium and leads to a satisfactory mortality rate of the slug *D. reticulatum*, while the strains *Myroides marinus* (17), *Myroides odoratus* (B1) and *Serratia marcescens* (B2) are only suited for production in solid media. For the bacteria strains *Pseudomonas fragi* (6) and *Pseudomonas putida* (7), it can be concluded that they cannot be used as nematode/bacteria complex for cultivation in either liquid or solid kidney medium. Furthermore, the method of using polyurethan foam, even though it provides a very airy environment, leads to a variable and results in lower yields than is the case when the flasks are continuously shaken.

Appendix

Preparation of kidney medium for growth of *Phasmarhabditis* spp. in solid or liquid cultures (According to Wilson, 2012)

1. Prepare an aqueous suspension of 3.5% homogenized pig kidney, 2.5% yeast extract and 3% sunflower oil (% w/v) and autoclave at 121 °C.
2. During the heating, proteins congeal. Filter out the precipitated lumps through muslin.
3. Dispense the desired volume into culture flasks, plug with bungs of non-absorbent cotton wool and re-autoclave at 121°C.
4. To make kidney agar, add technical grade agar at 2% (w/v) to the muslin filtered medium and re-autoclave. Pour the medium aseptically into Petri dishes using standard methods.

Preparation of standard nutrient agar

37g Standard-I-Nutrient Agar (Merck)
1000 ml aqua distilled

Luria Bertani Broth (for 1 l) (YS medium)

5 g yeast extract
10 g tryptone
5 g NaCl

1. Place all solid ingredients into a 2-l Erlenmeyer flask.
2. Add 1 l ddH2O.
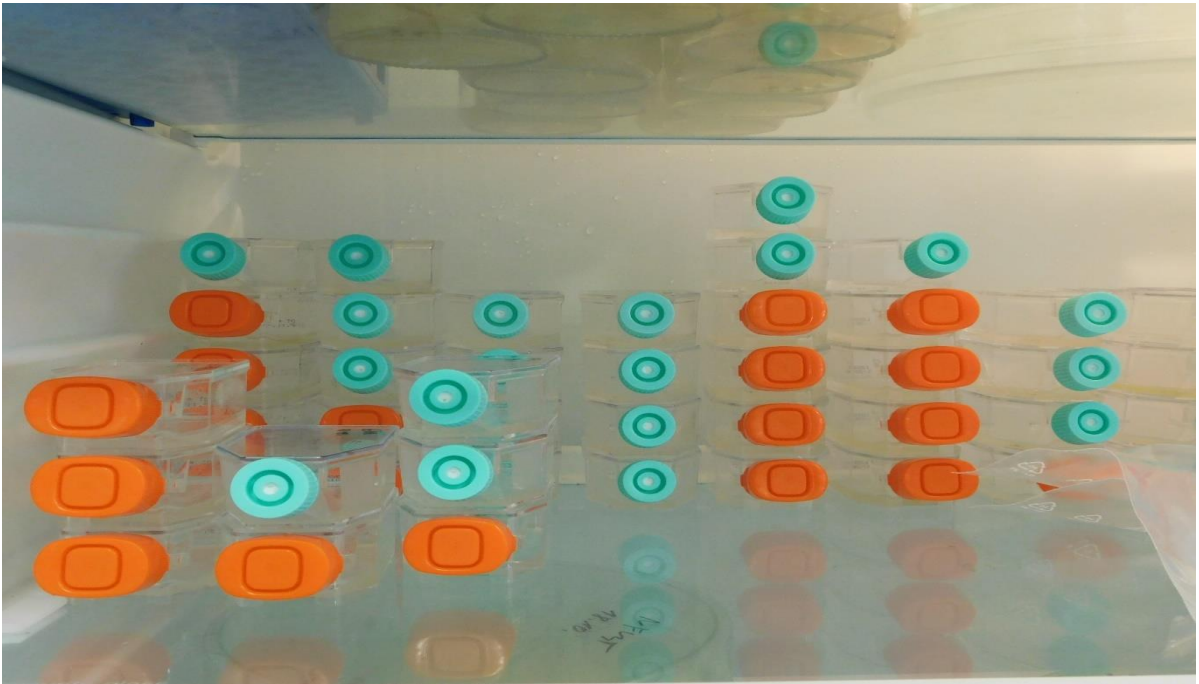3. Autoclave at 120°C for 3 min on using liquid setting.

Pictures

Figure 3 Storage of nematodes in fridge

Figure 4 Look of the finished liquid culture flasks with polyurethane foam

Figure 5 Flask for liquid cultivation of bacteria

Preliminary results from further experiments:

Table 2 Data of used identification and the bacteria strain

Identification	Bacteria strain	Family	Phylum
2A	*Kluyvera ascorbata*	Enterobacteriaceae	Proteobacteria
1B	*Citrobacter sp.*	Enterobacteriaceae	Proteobacteria
DER t	*Acinetobacter pittii*	Moraxellaceae	Proteobacteria
DER c	*Enterobacter sp.*	Enterobacteriaceae	Proteobacteria
5	*Acinetobacter johnsonii*	Moraxellaceae	Proteobacteria
1A	*Empedobacter brevis*	Flavobacteriacea	Bacteroidetes
RET	*Enterobacter sp.*	Enterobacteriaceae	Proteobacteria

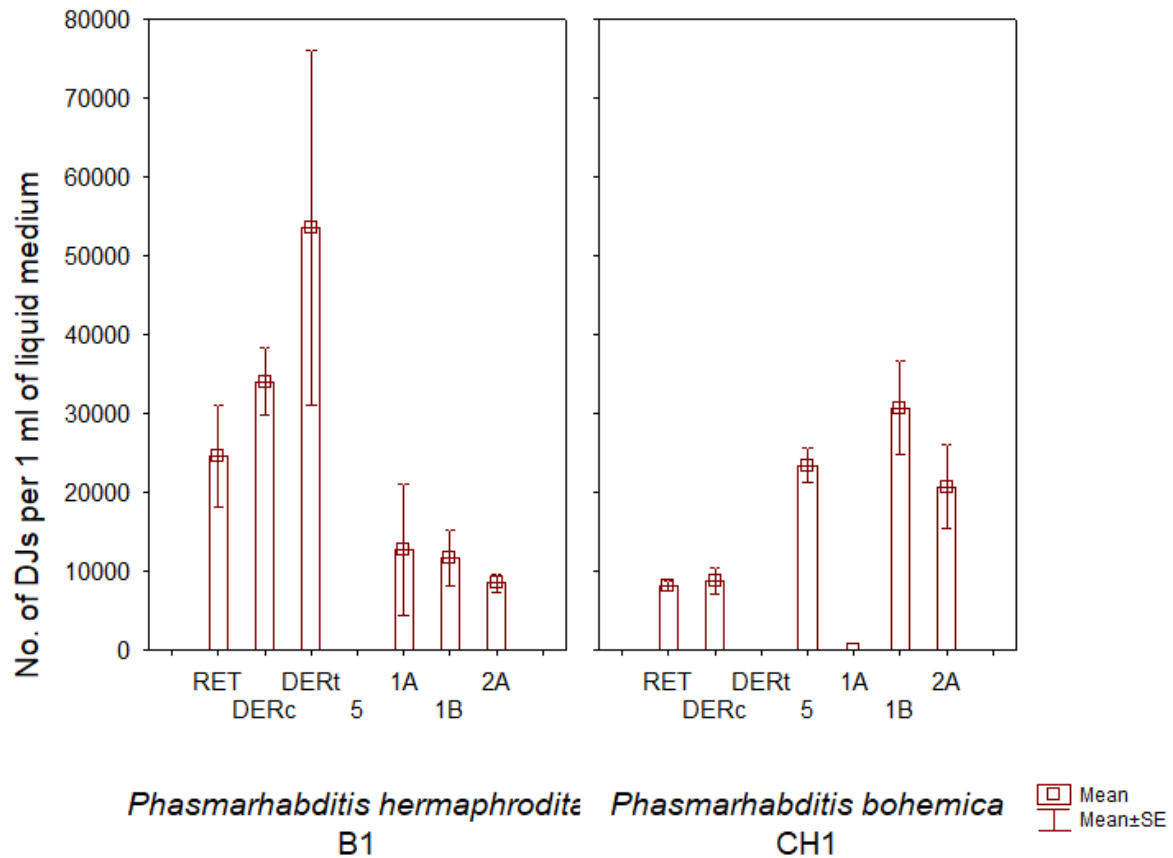*Figure 6 Nematodes yield/ml in each monoxenic nematode/bacteria culture for liquid kidney medium for P. hermaphrodita and P. bohemica nematode species. Used bacteria strains: RET (Enterobacter sp.), DER c (Enterobacter sp.), DER t (Acinetobacter pittii), 5 (Acinetobacter johnsonii), 1A (Empedobacter brevis), 1B (Citrobacter sp.), 2A (Kluyvera ascorbate)*

7. References

Ahmed K. D., Stephens C., Bistline-East A., Williams C. D., Mc Donnell R., Carnaghi M., Ó Huallacháin D., Gormally M. J., 2019, Biological control of pestiferous slugs using *tetanocera elata* (fabricius) (diptera: sciomyzidae): larval behavior and feeding on slugs exposed to *phasmarhabditis hermaphrodita* (schneider, 1859), Biological Control 135: 1-8

Anderson R. C., 2000, Nematode parasites of vertebrates: their development and transmission, CAB International, Wallingford, UK

Bedding R., 1990, Logistics and strategies for introducing entomopathogenic nematode technology into developing countries, In "Entomopathogenic Nematodes in Biological Control" (R. Gaugler and H. K. Kaya, Ed.), CRC Press, Boca Raton, Florida: 233–246

Buecher E.J., Popiel I., 1989, Liquid culture of entomogenous nematode *Steinernema feltiae* with its bacterial symbiont. Journal of Nematology 21: 500-504.

Campbell J. F., and Gaugler R. 1993, Nictation behaviour and its implications in the host search strategies of entomopathogenic FUTURE OF MICROBIAL CONTROL 241 nematodes (*Heterorhabditis* and *Steinernematidae*), Behaviour 126: 155–169.

Campbell J. F., and Gaugler R., 1997, Inter-specific variation in entomopathogenic nematode foraging strategy: Dichotomy or variation along a continuum?, Fundamental and Applied Nematology 20: 393–398.

Ester A., Van Rozen K., and Molendijk L. P. G., 2003, Field experiments using the *rhabditid* nematode *Phasmarhabditis hermaphrodita* or salt as control measures against slugs in green asparagus, Crop Protection 22: 689-695

Friedman D., 1990, Commercial production and development, In "Entomopathogenic nematodes for biological control" (R. Gaugler and H. K. Kaya, Ed.), Boca Raton, Florida, CRC Press: 153-172

Gaugler R., Lewis E., and Stuart R. J., 1997a, Ecology in the service of biological control: The case of entomopathogenic nematodes, Oecologia 109: 483–489

Gaugler R., Wilson M., and Shearer P., 1997b, Field release and environmental fate of a transgenic entomopathogenic nematode, Biological Control 9: 75–80

Glazer I., Salame L., 2000, Osmotic survival of the entomopathogenicnematode *Steinernema carpocapsae*, Biological Control 18:251–257

Glen D. M., and Wilson M. J., 1997, Slug parasitic nematodes as biocontrol agents for slugs, Agro Food Industry Hi-Tech 8: 23–27

Glen D.M., Wilson M.J., Brain P., Stroud G., 2000, Feeding activity and survival of slugs, *Deroceras reticulatum*, exposed to the rhabditid nematode, *phasmarhabditis hermaphrodita*: A model of dose response, Biological Control 17: 73-81

Grewal P. S., 2000, Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (*Rhabditida*: *Steinernematidae*), International Journal of Parasitology 30: 995-1000

Grewal P. S., Grewal S. K., Taylor R. A. J., Hammond R. B., 2001, Application of molluscicidal nematodes to slug shelters: a novel approach to economic biological control of slugs, Biological Control 22: 72-80

Grewal S. K., Grewal P. S., 2003, Effect of osmotic desiccation on longevity and temperature tolerance of *phasmarhabditis hermaphrodita* (nematoda: *rhabditidae*), Journal of Parasitology 89: 434-438

Hapca S. M., Budha P., Crawford J. W., Young I. M., 2007, Movement of the nematode, *phasmarhabditis hermaphrodita*, in a structurally heterogeneous environment, Nematology 9: 731-738

Harper J. D., 1987, Applied epizootiology: Microbial control of insects, In "Epizootiology of Insect Diseases" (J. R. Fuxa and Y.Tanada, Eds.), Wiley, New York: 473–496

Hass B., Glen D. M., Brain P., Hughes L. A., 1999a, Targetingbiocontrol with the slug-parasitic nematode *phasmarhabditis hermaphroditain* slug feeding areas: A model study, Biocontrol Science and Technology 9:571–578

Hass B., Hughes L. A., Glen D.M., 1999b, Overall versus band application of the nematode *phasmarhabditis hermaphrodita* with and without incorporation into soil, for biological control of slugs in winter wheat, Biocontrol Science and Technology 9:579–586

Hatteland B., Roth S., Pianezzola E., 2010, Predation by beetles (*carabidae*, *staphylinidae*) on eggs and juveniles of the iberian slug *arion lusitanicus* in the laboratory, Bulletin of Entomological Research 100: 559-567

Ingram W. M., Adolph H. M., 1943, Habitat and observations *ofariolimax columbianus*, *Nautilus* 56

Kaya H. K., and Gaugler R., 1993, Entomopathogenic nematodes, Annual Reviews Entomology 38: 181–206

Kaya H. K., and Stock S. P., 1997, Techniques in insect nematology, In "Manual of Techniques in Insect Pathology" (L. A. Lacey, Ed.), Academic Press, London: 281–324

Lacey L. A., Frutos R., Kaya H. K., Vail P., 2001, Insect pathogens as biological control agents: do they have a future?, Biological Control 21: 230-248

Lewis E. E., Gaugler R., and Harrison R., 1993, Response of cruiser and ambusher entomopathogenic nematodes (*Steinernematidae*) to host volatile cues, Canadian Journal of Zoology 71: 765–769

Mamiya Y., 1989, Comparison of the infectivity of *Steinernema kushidai* (Nematode: *Steinernematidae*) and other *steinernematid* and *heterorhabditid* nematodes for three different host insects, Applied Entomology and Zoology 24: 302–308

Mc Donnell R., Tandingan I., Ley D., Paine T.D., 2018, "Susceptibility of *neonate Lissachatina fulica* (*Achatinidae*: Mollusca) to a US strain of the nematode *Phasmarhabditis hermaphrodita* (*Rhabditidae*: Nematoda)", Biocontrol Science and Technology 28(11): 1091-1095.

Mckemey A. R., Symondson W. O. C., Glen D. M., Brain P., 2001, Effects of slug size on predation by *Pterostichus melanarius* (*Coleoptera* : *Carabidae*), Biocontrol Science and Technology 11: 81-91

Nermut J., Půža V., 2017, Slug parasitic nematodes: biology, parasitism, production and application, CAB International, Czech Republic: 533-547

Nermut J., Půža V., Mráček Z., 2012, The response of *Phasmarhabditis hermaphrodita* (Nematoda: *Rhabditidae*) and *Steinernema feltiae* (Nematoda: *Steinernematidae*) to different host-associated cues, Biological Control 61: 201-206

Nermut, J., Půža V., Mráček Z. 2014a, The effect of different growing substrates on the development and quality of *Phasmarhabditis hermaphrodita* (Nematoda: *Rhabditidae*), Biocontrol Science and Technology 24: 1026-1038.

Nermut, J., Půža V., Mráček Z., 2014b, Some observations on morphology and ecology of mollusc-parasitic nematode Alloionema appendiculatum, 47th annual meeting of SIP, Mainz, Germany

Rae R., Verdun C., Grewal P. S., Robertson J. F., Wilson M.J., 2007, Biological control of terrestrial molluscs using *phasmarhabditis hermaphrodita*--progress and prospects, Pest Management Science 63: 1153-1164

Rae R.G., Tourna M., Wilson M.J., 2010, The slug parasitic nematode *Phasmarhabditis hermaphrodita* associates with complex and variable bacterial assemblages that do not affect its virulence, Journal of Invertebrate Pathology 104: 222-226

Reimer L. C., Vetcininova A., Sardà Carbasse J., Söhngen C., Gleim D., Ebeling C., Overmann J., 2019, BacDive in 2019: bacterial phenotypic data for High-throughput biodiversity analysis, Nucleic Acids Research 47: 631-636

Sandström J. P., Russel J. A., White J. P., Moran N.A., 2001, Independent origins and horizontal transfer of bacterial symbionts of aphids, Molecular Ecology 10: 217-228

Speiser B., Kistler C., 2016, Field tests with a molluscicide containing iron phosphate, Crop Protetion 21: 389-394

Steinhaus E. A., 1956, Microbial control: The emergence of an idea, Hilgardia 26: 107–160

Steinhaus E. A., 1975, Disease in a Minor Cord, Ohio State University Press, Columbus, OH

Stock S. P., and Goodrich-Blair H., 2012, Nematode parasites, pathogens and associates of insects and invertebrates of economic and importance, In "Manual of techniques in Invertebrate Pathalogy" (L. A. Lacey, Ed.), Academic Press, London: 373-426

Tan L., Grewal P. S., 2001a, Infection behavior of the *rhabditid* nematode *Phasmarhabditis hermaphroditato* the grey garden slug *Deroceras reticulatum*, Journal of Parasitology 87:1349–1354

Wells M. J., Buckley D. S. K. L., 1972, Snails and trails, Animal Behaviour 20: 345–355

White G. F., 1927, A method for obtaining infective nematode larvae from cultures, Science 66: 302-303

Wilson M. J., 2012, Pathogens and parasites of terrestrial molluscs, In "Manual of Techniques in Invertebrate Pathology" (L. A. Lacey, Ed.), Academic Press, London: 439

Wilson M. J., Glen D. M., George S. K., Pearce, J. D., 1995a, Selection of a bacterium for the mass-production of *Phasmarhabditis hermaphrodita* (Nematoda, *Rhabditidae*) as a Biocontrol agent for slugs, Fundamental and Applied Nematology 18: 419-425

Wilson M. J., Glen D. M., Pearce J. D., 1993, Biological control of molluscs, World Intellectual Property Organisation

Wilson M. J., Glen D. M., Pearce J. D., Rodgers P. B., 1995b, Monoxenic Culture of the Slug Parasite *Phasmarhabditis Hermaphrodita* (Nematoda, *Rhabditidae*) with Different Bacteria in Liquid and Solid-Phase, Fundamental and Applied Nematology 18: 159-166

Wilson M. J., Grewal P. S., 2005, Biology, production and formulation of slug-parasitic nematodes, Nematodes as biocontrol agents: 423-424

Wilson M. J., Hughes L. A., Jefferies D., and Glen D. M., 1999, Slugs (*Deroceras reticulatum* and *Arion ater agg.*) avoid soil treated with the *rhabditid* nematode *Phasmarhabditis hermaphrodita*, Biological Control 16: 170–176

Wilson M. J., Pearce J. D., Shamlou P. A., 2001, Scaleable downstream recovery of nematodes used as biopesticides, Biotechnology and Bioengineering 75: 733-740

Wilson M. J., Postlethwaite J., Pearce J. D., Leach G., Lye G. J., Shamlou P. A., 2003, Vibrating membrane filtration for recovery and concentration of insect killing nematodes, Biotechnology and Bioengineering 83: 235-240

Wynne R., Morris A., Rae R., 2016, Behavioural avoidance by slugs and snails of the parasitic nematode *phasmarhabditis hermaphrodita*, Biocontrol Science and Technology 26:1129-1138