Pathogenicity of wild and commercial *Phasmarhabditis hermaphrodita* exposed to the pestiferous slug *Deroceras invadens*

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Abstract

Many terrestrial gastropods are pestiferous and pose a significant threat to agriculture, horticulture and floriculture. They are usually controlled by metaldehyde based pellets but an alternative control method is the slug parasitic nematode *Phasmarhabditis hermaphrodita* which has been formulated into a biological control agent (Nemaslug®) for use by farmers and gardeners to kill certain pestiferous slug species in 4-21 days. The current strain of *P. hermaphrodita* (called DMG0001) has been used in commercial production since 1994, but there is little information about the pathogenicity of wild strains of *P. hermaphrodita* towards slugs. Here, we exposed the pestiferous slug *Deroceras invadens* to nine wild isolated strains of *P. hermaphrodita* (DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011) and the commercial strain (DMG0001) to three doses (0, 500 and 1000 nematodes per ml). Survival and feeding were recorded over 14 days. All wild *P. hermaphrodita* strains (other than DMG0010) and *P. hermaphrodita* (DMG0001), applied at 500 nematodes per ml caused significantly mortality to *D. invadens* compared to an uninfected control. Similarly, all *P. hermaphrodita* strains (apart from DMG0003) caused significant mortality to *D. invadens* when compared to an uninfected control at 1000 nematodes per ml. Overall, all wild *P. hermaphrodita* strains (other than DMG0011) caused significantly more mortality than *P. hermaphrodita* DMG0001 at one or both dose rates. In summary, we have found some wild *P. hermaphrodita* strains were more virulent than *P. hermaphrodita* (DMG0001). Ultimately, these strains could potentially be developed as alternative, efficient biological control agents for use against slugs.

Keywords: Biological control; Molluscicide; Pests; Slugs

1. Introduction

On average, 32% of crop production is lost on a global scale due to pests (Dhawan *et al.,* 2010). Terrestrial gastropods (slugs and snails) inhabit all continents and are important agricultural pests in Europe, America and Australia (South, 1992; Barker, 2002). Uncontrolled slug damage could result in £43 million loss of oilseed rape and winter wheat product in the UK (Nicholls, 2014). In some cases, whole fields have to be re-sown with economic repercussions (Willis *et al.,* 2006). Slugs can also act as vectors for pathogens and parasites such as Metastrongyloidae nematodes (Grewal *et al*., 2003). The main method to control slugs is by using metaldehyde based slug pellets (Castle *et al.*, 2017). In the UK, it was estimated that 1640t of metaldehyde was used between 2008 and 2014 (Fera, 2016). Metaldehyde pellets can harm non-target organisms including canines and other vertebrates (Cope, 2006), and due to leaching into watercourses it is now considered an emerging pollutant of concern (Stuart *et al.*, 2012). There are other chemical slug control options available to growers and farmers, including iron phosphate based products (Koch *et al*., 2000; Iglesias *et al*., 2001; Kozlowiski *et al*., 2014) Iron phosphate based products containing chelating agents however, affect earthworm activity, growth and may be toxic (Langan and Shaw, 2006; Edwards *et al*., 2009).

An alternative to chemical control is the slug parasitic nematode *Phasmarhabditis hermaphrodita*, which is capable of killing several pestiferous slug species (Wilson *et al*., 1993). In 1994, this nematode was developed into a biological control agent and is sold by BASF Agricultural Specialities under the product name 'Nemaslug®'. Nemaslug® is now sold to gardeners and farmers across Northern Europe and has a market value of £1 million per annum (Pieterse *et al*., 2017). Nematodes are formulated into a water dispersible formulation and upon soil application dauer larvae (the infective stage of the lifecycle) locate slugs via faecal and mucus cues (Rae *et al.,* 2006), enter host slugs via the dorsal

integumental pouch and migrate to the shell cavity (Wilson *et al*., 1993). They then develop into self-fertilising hermaphrodites and proliferate, killing the host in 4-21 days (Wilson *et al*., 1993; Tan and Grewal, 2001a). Nematodes proliferate on the cadaver until the food source is depleted, then new dauers enter the soil to locate a new host. Commercial *P. hermaphrodita* is produced in monoxenic conditions with the bacterium *Moraxella osloensis*. It is thought that *P. hermaphrodita* vectors *M. osloensis* into a slug host, causing death via septicaemia (Tan and Grewal, 2001b; 2002). One common symptom of *P. hermaphrodita* infection is host-feeding inhibition, which may be a reason why fast efficient control against slug damage is seen (Glen *et al*., 2000). *P. hermaphrodita* has been used to control slug damage to many crops including winter wheat (Wilson *et al*., 1994), cabbage (Grubišič *et al*., 2003, 2018; Kozolowski *et al*., 2012) and sugar beet (Ester & Wilson, 2005).

The current strain of *P. hermaphrodita* (called DMG0001) has been used in the production of Nemaslug® for 25 years and there is little information on the pathogenicity of wild strains of *P. hermaphrodita*. Therefore, we investigated the pathogenicity and host feeding inhibition caused by nine recently isolated *P. hermaphrodita* strains (Supplementary Table 1) towards the common pest slug *Deroceras invadens* and compared them to *P. hermaphrodita* (DMG0001) to help understand if there is natural variation in the pathogenicity of these nematodes. We chose *D. invadens* as the host in our studies as it has been reported invading new areas over the last century and matures faster than other common pest slugs, such as *D. reticulatum* (Hutchinson *et al.*, 2014). It is now an important pestiferous slug of UK agricultural crops (Williams *et al*., 2010). Ultimately, by identifying more pathogenic strains of *P. hermaphrodita* further studies could investigate how these nematodes kill slugs by first focussing on potential bacterial symbionts.

2. Materials and methods

2.1 Source and maintenance of invertebrates

P. hermaphrodita commercial strain DMG0001 (Nemaslug®) was supplied by BASF Agricultural Specialities and stored at 10ºC before use. Wild *P. hermaphrodita* strains (DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011) were isolated from slugs collected from locations around Liverpool, UK (Supplementary Table 1). They have been in culture at Liverpool John Moores University (LJMU) since 2014 on modified White traps (White, 1927). Each strain began as an isogenic line from a single hermaphrodite mother and was identified to species via amplification and sequencing of the ITS1, 18SrRNA and the D2-D3 domain of large subunit (LSU) rDNA genes (Andrus and Rae, 2018). For experimentation the nematodes were grown up on decaying slug (*Limax flavus*) on modified White traps until they had reached the dauer stage and were then stored in cell culture flasks at 10°C (see Andrus and Rae, 2018 for more details). *L. flavus* were frozen at -80°C before use to kill any existing nematodes. For each experiment, fresh dauers were grown.

D. invadens (mean weight 0.70 g ± 0.55, n = 900) were collected from parks around Liverpool and stored at 10°C in the dark. Slugs were fed lettuce *ad libitum* and kept for 1 week before use to screen for any previous nematode infection.

2.2 Survival and feeding inhibition of D. invadens *exposed to* P. hermaphrodita

An adapted method by Wilson *et al*. (1993) was used to test the pathogenicity and feeding inhibition of *D. invadens* exposed to *P. hermaphrodita*. Two *D. invadens* were placed in a 20 ml plastic tube with a cotton wool bung pushed to the bottom and 2 ml of water was added to the tube. Slugs were exposed to 2 ml of 500 or 1000 nematodes per ml (or 2 ml of

water as a control). A cotton wool bung was used to stop the slugs escaping and the lid was loosely screwed on to allow airflow. Slugs were then incubated at 10°C in the dark for 5 days. After 5 days of infection, each slug was placed on a 5 cm Petri dish containing a 3 cm diameter disk of lettuce (area 700mm^2). Petri dishes were then incubated at 10°C for 9 days. Mortality was recorded every 2-3 days and the volume of lettuce disk eaten was recorded 8 and 14 days after initial infection by tracing the remaining lettuce disk on 1 mm^2 graph paper (Rae *et al.,* 2009). Ten *D. invadens* were used per experiment and it was repeated three times for each *P. hermaphrodita* strain (DMG0001, DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011). A no nematode control (dose rate 0) and *P. hermaphrodita* (DMG0001) (dose rates 500, 1000) were run with each group of wild *P. hermaphrodita* tested.

2.3 Statistical analysis

Survival of *D. invadens* exposed to *P. hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011) at 0, 500 and 1000 nematodes per ml was analysed using a Log Rank test in OASIS (Yang *et al.*, 2011). A One-Way ANOVA with Tukey's post hoc test was used to compare the amount of lettuce eaten by *D. invadens*.

3. Results

3.1 Survival of D. invadens *exposed to wild* P. hermaphrodita *strains and commercial* P. hermaphrodita *strain.*

At a dose rate of 500 nematodes per ml the commercial strain of *P. hermaphrodita* (DMG0001) and all wild strains (DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, and DMG0011), other than *P. hermaphrodita* DMG0010, caused significant mortality to *D. invadens* when compared to an uninfected control after 14 days ($p < 0.05$) (Fig. 2A) (Supplementary Table 2A). When compared to the commercial *P. hermaphrodita* strain (DMG0001) the wild *P. hermaphrodita* strains DMG0002, DMG0005, DMG0007, and DMG0008 were significantly more pathogenic. DMG0002 and DMG0008 caused rapid and consistent mortality, killing 12.2% and 10.6% of slugs per day respectively. ($p < 0.05$) (Fig. 2A) (Supplementary Table 2A).

At the higher dose rate of 1000 nematodes per ml *P. hermaphrodita* commercial strain (DMG0001) and all wild strains (DMG0002, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011), other than *P. hermaphrodita* DMG0003, caused significant mortality to *D. invadens* when compared to an uninfected control after 14 days ($p < 0.05$) (Fig. 2B) (Supplementary Table 2B). Wild *P. hermaphrodita* strains DMG0002, DMG0003, DMG0006, DMG0009 and DMG0010 caused significantly more *D. invadens* mortality than the commercial *P .hermaphrodita* strain (DMG0001) at 1000 nematodes per ml after 14 days. The fastest mortality rate was seen in *P. hermaphrodita* strains DMG0009 (15% per day) and DMG0010 (13.9% per day) at 1000 nematodes per ml ($p < 0.05$) (Fig. 2B) (Supplementary Table 2B).

3.2 Feeding inhibition of D. invadens *caused by* P. hermaphrodita *infection*

There was a significant difference between the amount of lettuce consumed by *D. invadens* exposed to all treatments after 8 days (F (10, 272) = 3.716, $p < 0.0001$, and after 14 days (F (10, 227) =5.922, $p < 0.0001$ at a dose rate of 500 nematodes per ml. After 8 days

only *P. hermaphrodita* DMG0008 (43.3 ± 18.3mm², p = 0.007) and DMG0009 (114.9 ± 32.4mm², p = 0.044) caused significant feeding inhibition to *D. invadens* compared to the uninfected control (317.6 ± 38.6mm²) at a dose rate of 500 nematodes per ml (Fig. 3A). After 14 days *P. hermaphrodita* DMG0003 (297.4 ± 58.5mm², p = 0.007), DMG0005 (215.4 ± 47.5mm², p = 0.001), DMG0006 (296.8 ± 60.9mm², p = 0.014), DMG0007 (255.4 ± 64.8mm², p = 0.009), DMG0008 (81.6 ± 25.1mm², p < 0.001), DMG0009 (208.9 ± 60.2mm², p < 0.001), DMG0010 (244.8 ± 49.6mm², p < 0.001) and DMG0011 (301.7 ± 42.4mm², p = 0.031) caused significant feeding inhibition to *D. invadens* when compared with the uninfected control (534.5 ± 33.6mm²) at a dose rate of 500 nematodes per ml (Fig. 3B). Only *P. hermaphrodita* DMG0008 (81.6 ± 25.1mm², p = 0.040) significantly inhibited *D. invadens* feeding more than commercial strain DMG0001 (354.9 ± 45.9mm²) after 14 days at 500 nematodes per ml (Fig. 3B).

After a dose rate of 1000 nematodes per ml there was a significant difference between the amount of lettuce consumed by *D. invadens* exposed to all treatments after 8 days (F(10, 246) =11.890, p = < 0.0001) and after 14 days (F(10, 169) =9.156, p < 0.0001. Feeding was inhibited significantly more than the uninfected control (348.9 ± 36.4mm²) after 8 days by *P. hermaphrodita* DMG0001 (180.4 ± 30.5mm², p = 0.008) and the wild strains DMG0002 (76.8 ± 22.6mm², p = 0.001), DMG0003 (57.0 ± 22.3mm², p = 0.007), DMG0005 (28.6 ± 9.6mm², p =0.032), DMG0006 (139.8 ± 34.2mm², p < 0.001), DMG0007 (111.5 ± 25.5mm², p < 0.001) and DMG0009 (30.3 ± 14.5mm², p < 0.001) (Fig. 3C). After 14 days *P. hermaphrodita* DMG0001 (214.5 ± 38.7mm², p < 0.001) and the wild strains DMG0002 (164.1 ± 46mm², p < 0.001) , DMG0003 (148.1 ± 41.3mm², p < 0.001), DMG0005 (138.4 ± 52.8mm², p < 0.001) , DMG0006 (283.4 ± 68.5mm², p < 0.001), DMG0007 (185.4 ± 43.8mm², p < 0.001), DMG0009 (85.0 ± 59.7mm², p = 0.005) and DMG0011 (285.7 ± 48.2mm², p = 0.002) caused more feeding inhibition compared to the uninfected control

(534.6 ± 33.5mm²) at 1000 nematodes per ml ($p < 0.05$) (Fig. 3D). None of the wild strains inhibited feeding significantly more than *P. hermaphrodita* DMG0001 after 8 or 14 days at a dose rate of 1000 nematodes per ml.

4. Discussion

Here we show wild isolated *P. hermaphrodita* from the UK are highly pathogenic towards *D. invadens*. Little research has investigated the pathogenic potential of wild *P. hermaphrodita* strains, even though *P. hermaphrodita* has been isolated globally (Pieterse *et al*., 2017). Our findings corroborate those of Wilson *et al*. (2012), Tandingan De Ley *et al.* (2020) and McDonnell *et al*. (2018), who found wild *P. hermaphrodita* strains that were pathogenic to the slug *D. reticulatum*, the snail *Theba pisana* and neonate life stages of the snail *Lissachatina fulica* respectively. Interestingly, commercial *P. hermaphrodita* DMG0001 did not kill 12 week old *L. fulica* (Williams and Rae, 2015), but a wild *P. hermaphrodita* strain (called ITD290) was highly virulent (McDonnell *et al.,* 2018). The reasons for this difference could be due to the larger snails used by Williams and Rae (2015) as bigger snails and slugs tend to be more resistant to nematode infection, which has been observed in infection studies using *P. hermaphrodita* exposed to *Cornu aspersum*, *Arion lusitanicus* and *Arion ater* (Glen *et al*., 1996; Speiser *et al*., 2001; Grimm, 2002). Only *P. hermaphrodita* DMG0002 caused significantly more mortality than *P. hermaphrodita* DMG0001 at both dose rates. *P. hermaphrodita* DMG0005, DMG0007 and DMG0008 only caused significantly more pathogenicity at the lower dose rate of 500 nematodes per ml. Higher pathogenicity at a lower dose rate indicates that these wild isolates are highly virulent, and a lower worm burden is required for infection, inducing mortality and proliferation. Other factors may be influencing this result as well, such as the ability of each strain to locate a host. For example,

wild strains may display better chemoattraction, a trait that has diminished in the commercial strain (Andrus *et al.*, 2018; Andrus and Rae, 2019). In a study by Andrus and Rae (2019), using the same wild strains as above, it was found that when exposed to multiple slug mucus samples the commercial strain responded poorly to all mucus, with the majority of the nematodes remaining at the application point. Differences in chemoattraction between the wild isolated *P. hermaphrodita* strains were also seen. Interestingly, DMG0010 caused no mortality at 500 nematodes per ml, yet was highly virulent at 1000 nematodes per ml. Presumably a higher worm burden is required for DMG0010 to establish a lethal infection and when such infection is established this strain kills more rapidly than others. Natural variation in pathogenicity between nematode strains is well documented in entomopathogenic nematodes, such as wild isolated strains of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* , that were found to be more virulent to their targeted hosts (*Cephalcia tannourinensis* and *Agrotis ipsilon*, respectively) than the commercial formulations (Noujeim *et al*., 2015; Bélair *et al*., 2013). Further host range testing of the more virulent wild isolated *P. hermaphrodita* strains is needed.

We found wild *P. hermaphrodita* strains (DMG0002 DMG0005, DMG0007 and DMG0008) were more pathogenic than the commercial strain (DMG0001) (depending on dose). The reasons for this pathogenic difference could be due to their associated bacteria. Infectivity and pathogenicity of *P. hermaphrodita* are strongly influenced by bacterial diet (Wilson *et al*, 1995a,b). One bacterium, *Moraxella osloensis*, was chosen for commercial production from a selection of 16 isolates as it constantly produced high yields of pathogenic nematodes (Wilson *et al.*, 1995 a,b). *P. hermaphrodita* is still grown on this bacterium. It is thought that *M. osloensis* is introduced and released into the slug's haemocoel by dauer stage nematodes and causes septicemia by production of an endotoxin (Tan and Grewal, 2001a, b; 2002). However, there is evidence to show that *M. osloensis* is not vertically transmitted to

the next generation of pathogenic nematodes (Rae *et al*., 2010; Nermut' *et al*., 2014). Therefore, the current relationship *P. hermaphrodita*, and especially these wild strains, have with bacteria warrants further investigation. One such approach could be to use 16S metagenomics of bacteria present in the nematode gut, which has worked well in profiling the associated microflora in other nematodes like *Caenorhabditis elegans* (Dirksen *et al*., 2016).

As well as killing slugs, the wild strains of *P. hermaphrodita* were able to inhibit feeding of *D. invadens*. This is a common symptom of *P. hermaphrodita* infection and enhances the use of these nematodes as a biological control agent. Host feeding inhibition is also caused in resistant species (Glen *et al*., 2000), such as juvenile *A. lusitanicus* (Grimm, 2002). Variation across dose rate, day and strain was also observed. Wild *P. hermaphrodita* strain DMG0009 had the largest effect, inhibiting slug feeding more than the control at both doses and inhibiting a greater level of feeding than the commercial strain at 1000 nematodes per ml.

In conclusion, wild isolated strains of *P. hermaphrodita* are capable of killing and inducing feeding inhibition in the slug *D. invadens*, some more than the commercial strain (DMG0001). Additional research on the better performing strains will be needed, including understanding the effects associated bacteria have on yield and virulence, and optimising *in vitro* culturing techniques. This research shows that understanding natural variation between strains used for biological control purposes could result in a more effective product. Having multiple wild strains displaying genetic variation in virulence could also help with understanding which genes are associated with pathogenicity, aid in understanding the evolution of parasitism and even enhance *P. hermaphrodita* as a biological control agent (Rae, 2017).

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Figure legends

Fig. 1. *P. hermaphrodita* dauer juveniles (A) are the infective stage and develop into adult self-fertilising hermaphrodites (B). Infection in *D. invadens* (C) can cause a swollen mantle and shell ejection in *D. invadens* (D). Scale bar in A represents 100 μm, in B represents 250μm and in C represents 0.5 cm.

Fig. 2. (A) Percentage survival of *D. invadens* exposed to 0 (Black) and 500 nematodes per ml of *P. hermaphrodita* (DMG0001) (Grey), *P. hermaphrodita* (DMG0002) (Red), *P. hermaphrodita* (DMG0003) (Orange), *P. hermaphrodita* (DMG0005) (Light Green), *P. hermaphrodita* (DMG0006) (Dark Green), *P. hermaphrodita* (DMG0007) (Light Blue), *P. hermaphrodita* (DMG0008) (Dark Blue), *P. hermaphrodita* (DMG0009) (Purple), *P. hermaphrodita* (DMG0010) (Brown) and *P. hermaphrodita* (DMG0011) (Dark Grey).

Fig. 2. (B) Percentage survival of *D. invadens* exposed to 0 (Black) and 1000 nematodes per ml of *P. hermaphrodita* (DMG0001) (Grey), *P. hermaphrodita* (DMG0002) (Red), *P. hermaphrodita* (DMG0003) (Orange), *P. hermaphrodita* (DMG0005) (Light Green), *P. hermaphrodita* (DMG0006) (Dark Green), *P. hermaphrodita* (DMG0007) (Light Blue), *P. hermaphrodita* (DMG0008) (Dark Blue), *P. hermaphrodita* (DMG0009) (Purple), *P. hermaphrodita* (DMG0010) (Brown), *P. hermaphrodita* (DMG0011) (Dark Grey).

Fig. 3. Feeding inhibition of *D. invadens* exposed to 0 and 500 nematodes per ml of *P. hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010 and DMG0011) after 8 days (A) and 14 days (B) and 0

and 1000 nematodes per ml after 8 days (C) and 14 days (D). Significant differences between the amount consumed by the control and treatments at $p < 0.05$ are denoted by * and at $p < 0.001$ denoted by **. Bars represent ± 1 standard error.

Supplementary Table 1. *Phasmarhabditis hermaphrodita* strains isolated from slugs around Liverpool.

Supplementary Table 2. Mean (± s.e.) number of slugs alive 0,2,5,7,9,12 and 14 days after exposure to *P. hermaphrodita* (DMG0001, DMG0002, DMG0003, DMG0005, DMG0006, DMG0007, DMG0008, DMG0009, DMG0010, DMG0011) at 0, 500 (A) or 1000 (B) nematodes per ml.

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