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Improved Control of *Otiorhynchus sulcatus* at 9°C by Cold-stored *Heterorhabditis megidis* UK211

PAUL F. L. FITTERS,[1] RICHARD DUNNE[2] AND CHRISTINE T. GRIFFIN[1]

[1] *Institute of Bioengineering and Agroecology, Department of Biology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland;*
[2] *Teagasc, Kinsealy Research Centre, Malahide Rd, Dubin 17, Ireland*

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The effect of storage temperature (9 and 20°C) on North West European Heterorhabditis megidis *isolate UK211 for control of* Otiorhynchus sulcatus *larvae at 9°C is assessed.* O. sulcatus *mortality increased from −5.3% (corrected mortality) using freshly produced nematodes, to 27.1% using nematodes that had been cold-stored for 12 weeks. The number of nematodes invading the insect larvae increased almost 27-fold. Nematode storage at 9°C for 11 to 12 weeks weeks resulted in significantly higher* O. sulcatus *mortality (41%) than storage at 20°C for 2 to 3 weeks (12%). Thus, cold storage does enhance nematode infectivity for* O. sulcatus *larvae.*

Keywords: *entomopathogenic nematodes,* Heterorhabditis megidis*, biocontrol,* Otiorhynchus sulcatus*, bioassay,* Galleria mellonella*, lipids*

INTRODUCTION

The black vine weevil, *Otiorhynchus sulcatus*, is a serious pest of soft fruits (e.g. strawberries, cranberries), ornamental plants and hardy ornamental nursery stocks. In the past, *O. sulcatus* in containerized plants was controlled by the incorporation of the persistent organochlorine aldrin into the potting compost. This gave effective and lasting control. Aldrin has been banned in the EU and the USA since the nineties for environmental and toxicological reasons (Cross *et al*., 1995). Since the banning of aldrin, a slow release granular formulation of chlorpyrifos is the most effective chemical, but control has not been as good as with aldrin (Cross *et al*., 1995). Chemical control has also been a problem in soft fruit. The inadequacies of chemical pesticides, the banning of persistent chlorinated hydrocarbons and the increasing dispersal of the insect by infested potted plants has led to an urgent need to develop new and safer pest control methods (Zimmermann, 1996).

Entomopathogenic nematodes (EPN) are very promising for the control *O. sulcatus* (e.g.

Correspondence to: P. F. L. Fitters. Tel.: +353 1 682 5222 x3136; Fax: +353 1 708 3845; E-mail: Paulus.F.Fitters@may.ie

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van Tol, 1993a,b; Blackshaw, 1994; Kakouli-Duarte *et al.*, 1997). There are two families of EPN commercially in use, Steinernematidae and Heterorhabditidae, of which the latter is more effective against *O. sulcatus* larvae (van Tol, 1993b). Infective juveniles (IJs) of *Heterorhabditis* spp. can be reared in fermenters and applied inundatively to the soil, where they actively search for host insects. However, late in autumn and early in spring, when *O. sulcatus* larvae cause the most damage, the soil temperatures are often below 12°C, which limits the activity and thus the efficacy of EPN, particularly *Heterorhabditis* spp. Much research is therefore focussed on finding cold active EPN strains (e.g. Griffin & Downes, 1994; Steiner, 1996; Richardson *et al.*, 1999), which would be effective against *O. sulcatus* at low soil temperatures.

Alternatively, improved infectivity at low temperature could be achieved by long-term low temperature storage. Several authors have found that cold storage of EPN has a profound effect on IJ infectivity (Fan & Hominick, 1991; Griffin, 1996). Several observations were made in relation to storage time and temperature of North West European (NWE) *Heterorhabditis megidis* isolates. Firstly, infectivity (percentage nematodes invading the insects) increased with increased storage time when IJs were stored and tested at both high and low temperatures (Griffin, 1993, 1996; Griffin *et al.*, 1994). Highest infectivity levels of *H. megidis* isolates UK211 and HF85 for *Galleria mellonella* were found after 3 weeks storage at 20°C and after 11 weeks storage at 9°C (Griffin, 1996). Secondly, maximum infectivity was highest when IJs were tested at the storage temperature. For example, maximum infectivity of IJs stored and tested at 9°C was higher than that of IJs stored at 20°C and tested at 9°C. A dramatic 120-fold increase in infectivity from time of harvest was observed for *H. megidis* stored and tested at 9°C (Griffin, 1996).

Cold storage could be a practical way to improve the infectivity of *Heterorhabditis* spp. against *O. sulcatus* at low soil temperatures. Griffin's (1996) experiments demonstrated that cold storage improved the ability of *H. megidis* IJs to invade the highly susceptible *G. mellonella* larvae in sand at 9°C, but insect mortality at that temperature was not tested. The aim of the experiments reported here was to test whether cold stored *H. megidis* would also be more effective at controlling *O. sulcatus* larvae under more natural conditions (Exp. 1) and to test whether improved infectivity obtained by long-term cold storage could also be acquired by short-term storage at 20°C (Exp. 2).

MATERIALS AND METHODS

Nematode and Insect Cultures

H. megidis isolate UK211 were cultured *in vivo* on larvae of the greater wax moth *G. mellonella* (The Mealworm Co., Sheffield, UK) at 20°C. After harvest, the IJs were washed three times by sedimentation in tap water.

O. sulcatus larvae were reared at room temperature on potted *Primula* (cv Quantum) (pot diameter 16 cm), which were supplemented with carrot disks (diameter 3–5 cm, height 2 cm, 3 disks/pot) placed on top of the soil (Masaki & Sugimoto, 1991) when plants had died. Excess *O. sulcatus* larvae were transferred to pots with fresh *Primula* plants and stored in a growth room at 9°C (14 h light/10 h darkness).

Experiment 1: Effect of Long-term Storage of IJs at 9°C on *O. sulcatus* Control at 9°C

Nematodes. IJs were stored in tap water (1000 IJs ml^{-1}; 100 ml/dish) in Para film-sealed Petri dishes (diameter 20 cm) at 9°C in the dark for up to 16 weeks. Dishes were shaken once a week. Every two weeks, IJs were tested for control of *O. sulcatus* in potted *Primula* and assessments were made of the lipid content of the IJs (Table 1). The experiment was run three times, each with a different IJ culture batch, to compensate for possible changes in insect or other experimental variation over the 16-week period of the trial. The testing of the batches was staggered, starting at two-weekly intervals. Thus, after four weeks all three

TABLE 1. Overview of nematode storage conditions and use

Exp.	Nematodes: Batch numbers	Nematodes: Storage temperature (°C)	Times when tests were performed (weeks): *O. sulcatus* control at 9°C	Lipid analysis	*G. mellonella* control at 9°C
1	1–3	9	0, 2, 4, 6, 8, 10, 12, 14 and 16	0, 2, 4, 6, 8, 10, 12, 14 and 16	
2	1–3	9	11 and 12		0, 1, 3, 5, 7, 9, 11 and 12
	1–3	20			0, 1, 2, 3, 4 and 5
	4–6	20	2 and 3		0, 1, 2, 3, 4 and 5

batches were tested together, testing weeks 4, 2 and 0 of storage for batches 1, 2 and 3, respectively. Similarly, batches were tested simultaneously in subsequent weeks.

Control of O. sulcatus *larvae in* Primula *at 9°C.* Pots (diameter 16 cm) with well established *Primula* (c.v. Quantum) plants were infected with five last instar *O. sulcatus* larvae each and placed in a growth room (14 h light/10 h darkness) at 9°C (soil temperature, ±0.4°C), 3 days before IJ application. For the first 6 weeks, all the larvae used were freshly cultured, but from week 8 onwards, stored larvae were used in some or all of the pots. The pots were arranged in a randomized block design, surrounded by one row of buffer pots. Where nematodes from two or three batches were tested together, the pots were organized in a single randomized block design. There were 10 pots for each nematode treatment and 10 pots served as controls. Eight ml of IJ suspension (1000 IJs ml^{-1}) at 9°C was added to the soil surface of each pot. Eight ml of water was applied to the control pots. Soil temperature was monitored at 30 min intervals in two pots, using a Squirrel digital data logger (1200 series, Grant Instruments, Cambridge, UK).

Twenty-seven days after nematode application, the pots were searched for *O. sulcatus* larvae. The number of live, dead and infected larvae was recorded. From these data, mortality values, corrected for natural mortality in the control pots (Abbott, 1925), were calculated. All retrieved larvae were rinsed immediately through three containers of tap water, to remove IJs adhering to the cuticle, dried on tissue paper and incubated at 20°C. After 5 days the number of infected (red colouring) larvae was recorded. To assess the number of nematodes that invaded the insect larvae, the infected cadavers were dissected in quarter-strength Ringer solution (Oxoid, Hampshire, UK) and the adult nematodes were counted.

Lipid estimation. On each of the dates on which nematodes were applied to the potted *Primula*, lipid reserves of the IJs were estimated. This was done by measuring optical density (OD) using an image analyser, according to the method of Fitters *et al.* (1997). Nematode OD and OD per unit area were assessed for twenty IJs.

Experiment 2: Effect of Storage of IJs at 9 and 20°C on their Infectivity for *O. sulcatus* and *G. mellonella* at 9°C

Nematodes and insects. IJs were stored in plastic food containers with snap on lids (diameter 15 cm, Roundstone Catering, Melksham, Wiltshire, UK) at 9 and/or 20°C. Six batches of nematodes were reared (Table 1). Infectivity for *O. sulcatus* at 9°C was assessed after storage of IJs for 11 and 12 weeks at 9°C (nematode batches 1–3) and after 2 and 3 weeks at 20°C (nematode batches 4–6) (Table 1). The assessment weeks were chosen as the ones most likely to be the ones in which peak infectivity would be expressed, based on the work of Griffin (1996) and Dempsey (pers. comm. NUI Maynooth) and results of Exp. 1.

Control of O. sulcatus *at 9°C.* Infectivity for *O. sulcatus* larvae was assessed in potting compost with carrots as a food source. Three carrot slices (15 cm long) were placed vertically in plastic pots (diameter 16 cm) filled with fresh potting compost, with less than 2 cm of the carrot remaining above the compost. Ten last instar *O. sulcatus* larvae were added per pot. The pots were placed at 9°C and left for 5 days for the larvae to enter the soil and acclimatize. Nematodes (1000 IJs ml^{-1}; 8 ml/pot) were applied, spread evenly around the carrots. Controls received 8 ml of tap water. There were five pots per treatment. The treatments were: IJs stored at 9°C for 11 weeks (batches 1–3); IJs stored at 20°C for 2 weeks (batches 4–6); and a control (no nematodes). A second trial was conducted the following week using the same batches of nematodes that had now been stored for 12 weeks at 9°C and 3 weeks at 20°C.

The pots of each trial were placed in a completely random design at 9°C, and were covered with tin foil to minimize evaporation. After 28 days, the pots were searched for living and dead *O. sulcatus* larvae and mortality (corrected for natural mortality (Abbott, 1925)) was assessed.

Bioassay with G. mellonella *at 9°C.* Infectivity of IJs for *G. mellonella* at 9°C was assessed at weekly intervals (week 0 to 5) for the IJs stored at 20°C and at week 0, 1, 3, 5, 7, 9, 11 and 12 for those stored at 9°C. One *G. mellonella* larva was placed in the middle of a 5 cm Petri dish and covered with moist (8% w/w) sand (particle diameter < 425 μm). The dishes were thermo-equilibrated at 9°C overnight. The next day, 100 IJs per dish in 100 μl of water were added, the dishes were covered, sealed with Para film and placed at 9°C in the dark. There were 10 insects (dishes) per treatment. After 72 h the *G. mellonella* larvae were retrieved from the sand and washed in tap water to remove externally adhering IJs. The larvae were dried in tissue paper and incubated at 20°C. Infectivity was assessed 5 days later by dissecting the infected larvae in quarter strength Ringer solution and counting the number of adult nematodes.

Statistics

Statistical tests were performed using Sigma Stat 1.0 (1993) or Minitab (release 13.1). Regression analysis was performed on insect mortality (corrected) and on proportion established IJs (log-transformed) (Exp. 1). Analysis was repeated while weeks were subtracted until the lowest *P*-value was obtained. Other data (Exp. 1 and 2) were either analysed by ANOVA (followed by an all pair-wise multiple comparison procedure, either Student-Newman-Keuls (SNK) or Bonferroni, both at $P < 0.05$), or by using a *t*-test ($P = 0.05$).

RESULTS

Experiment 1

During the assessment of unstored (week 0) nematodes of batch 1, the temperature went up to 12°C. Therefore the data of nematode batch 1 for that week were excluded from the results.

Corrected *O. sulcatus* mortality (at time of harvest) increased from −5.3% using freshly produced nematodes, to 27.1% using nematodes that had been cold-stored for 12 weeks (Figure 1). Slightly more *O. sulcatus* larvae were retrieved from the control pots (4.8 larvae/pot) than from the nematode treated pots (4.6 larvae/pot).

The relationship between storage time and insect mortality was most significant ($P = 0.0002$) when regression analysis was performed on the data up to week 12. Most of the insects recorded as dead were already infected and coloured red at time of harvest. Less than 1% of the *O. sulcatus* larvae that were alive at time of harvest died and contained IJs after 5 days at 20°C.

The average number of nematodes recovered per infected cadaver, with a maximum of 6.1 nematodes/cadaver, did not increase significantly with prolonged storage (Figure 2). How-

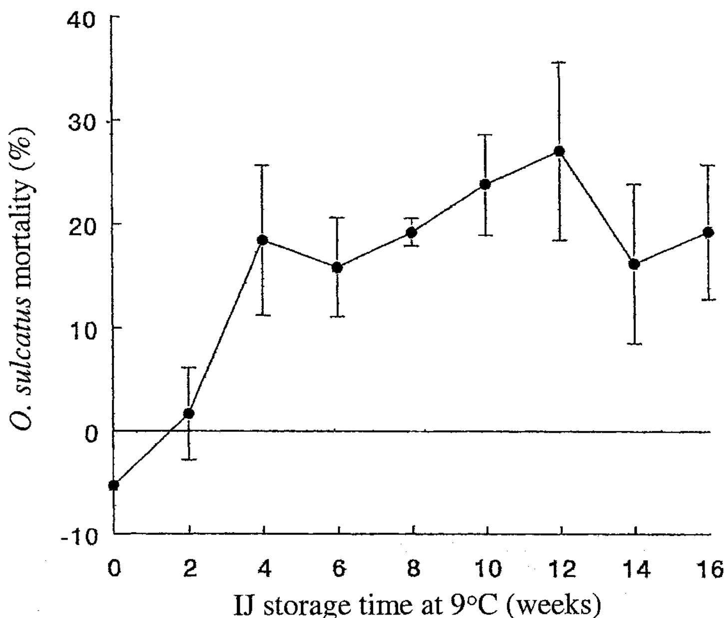

FIGURE 1. *Otiorhynchus sulcatus* mortality (%) (corrected for natural mortality) following exposure (28 days at 9°C) to *Heterorhabditis megidis* isolate UK211 IJs that had been stored at 9°C for up to 16 weeks prior to application. Each point represents the mean (±SE) of three nematode batches.

ever, the total number of nematodes in all the insects of 10 pots of each assessment week showed an almost 27-fold increase from 2.5 in week 0 to 67 in week 12 (Figure 2). This relationship between storage time and number of IJs invading the insects was most significant ($P = 0.0007$) when regression analysis on the data was performed up to week 12.

Stored *O. sulcatus* larvae had significantly lower (*t*-test, $P = 0.012$) mortality levels compared to freshly reared *O. sulcatus* larvae when exposed to *Heterorhabditis* IJs. At weeks 12 and 14 of nematode batch 1, weeks 10 and 12 of batch 2 and weeks 8 and 10 of batch 3, two to three out of 10 pots contained stored larvae and the comparison is based on these weeks. All later assessment weeks only had stored larvae.

Estimated lipid reserves of IJs, measured as OD, decreased gradually with increased storage time (Figure 3). OD values of week 0 were significantly (ANOVA, SNK, $P < 0.05$) higher than those found after 12 to 16 weeks. OD was also expressed per unit area (OD/area), which effectively gives the mean grey level of the object (IJ). Similarly to OD, OD/area of IJs that were stored for 0 and 2 weeks was significantly higher (ANOVA, Bonferroni, $P < 0.05$) than that of IJs that were stored 12 or more weeks (Figure 3).

We have previously shown that for *H. megidis* UK211 the maximum possible OD/area depletion, representing 'available density', is 0.2 units μm^{-2} (Fitters, 1999). The decrease in OD/area after 16 weeks storage at 9°C, expressed as a percentage of the available density, was 21%.

Experiment 2

O. sulcatus *bioassay.* There was no significant difference in *O. sulcatus* mortality between treatments where IJs had been stored for 11 or 12 weeks at 9°C (44 and 38%, respectively), nor between IJs stored 2 or 3 weeks at 20°C (14 and 11%, respectively). When the data for each storage temperature were pooled, IJs stored for 11 to 12 weeks at 9°C gave 41%

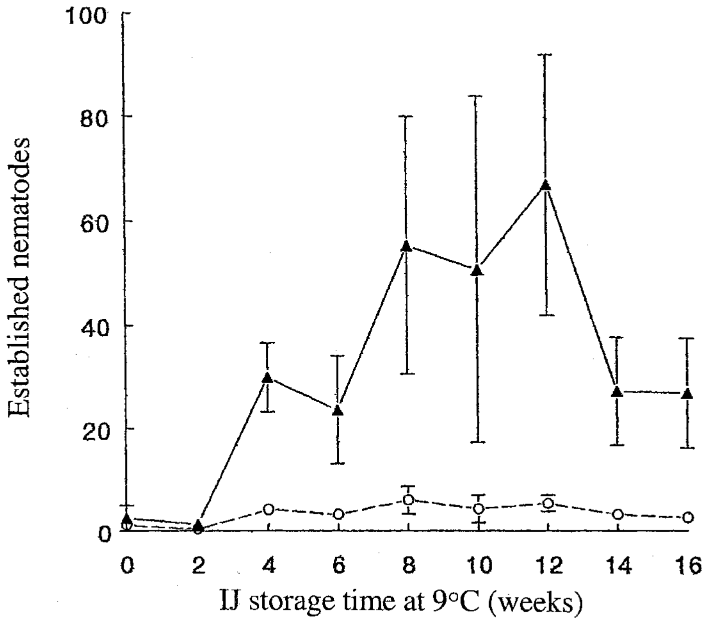

FIGURE 2. Number of nematodes established per *O. sulcatus* larva (- -○- -) and total number of nematodes established in all *O. sulcatus* larvae of 10 pots (—▲—) following exposure to *Heterorhabditis megidis* isolate UK211 IJs that had been stored at 9°C for up to 16 weeks. Each point represents the mean (± SE) of three nematode batches.

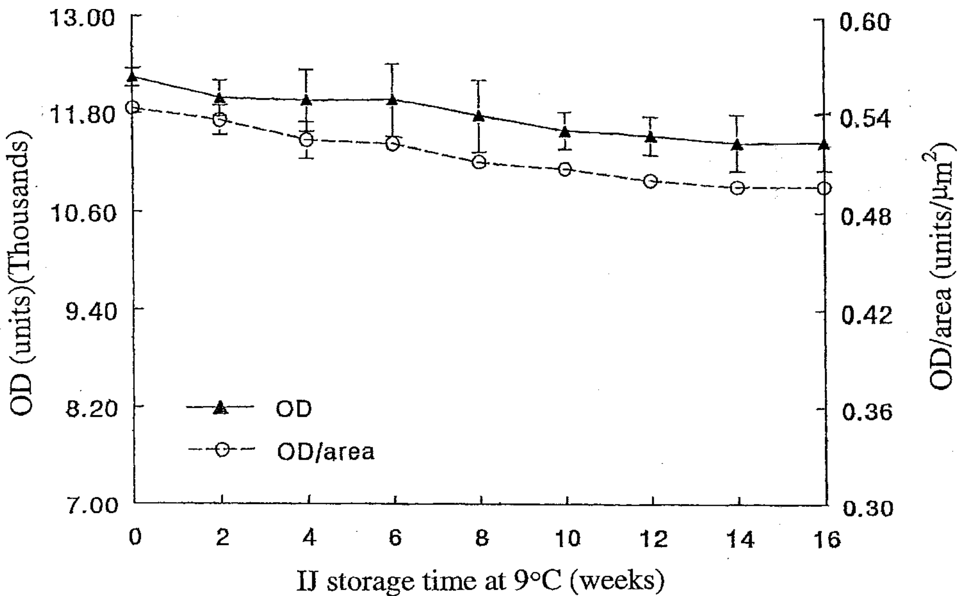

FIGURE 3. Optical density (OD) (—▲—) and OD per unit area (- -○- -) of *Heterorhabditis megidis* UK211 infective juveniles that had been stored at 9°C for up to 16 weeks. Each point represents the mean (± SE) of three nematode batches.

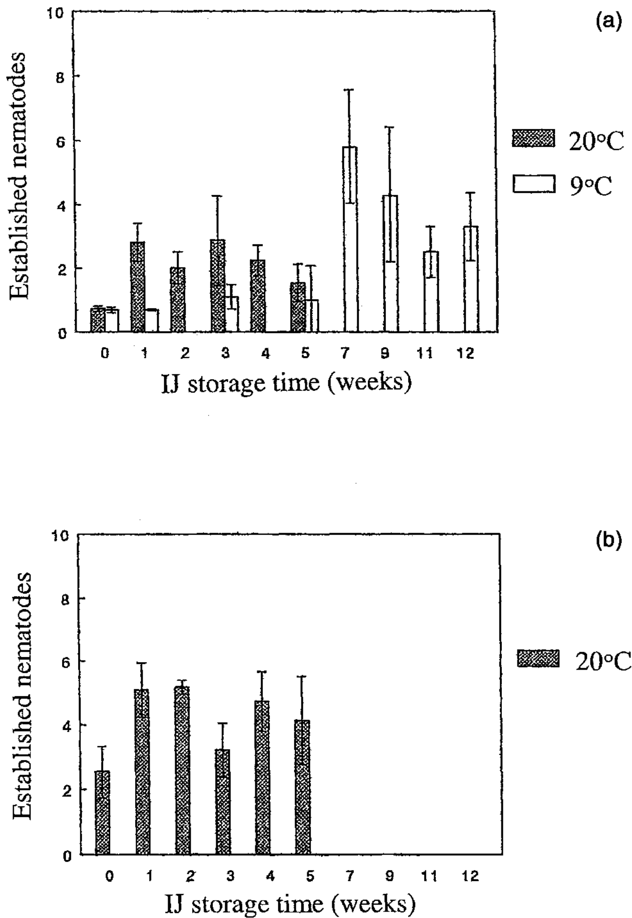

FIGURE 4. Number of established nematodes per *Galleria mellonella* larva in a sand dish bioassay at 9°C for 3 days, of *Heterorhabditis megidis* UK211 that had been stored in tap water for (a) 5 weeks at 20°C or 12 weeks at 9°C (batches 1–3) and (b) 5 weeks at 20°C (batches 4–6). Bars represent SE.

O. sulcatus mortality, which was significantly higher (t-test, $P < 0.001$) than that of IJs that were stored 2 to 3 weeks at 20°C (12% mortality). Percentage infected larvae (red colouring) at the day of harvest was similar to the mortality values. When the harvested larvae were kept for 3 days at 20°C, average infection levels increased by 10% for the 9°C stored IJs and by 6% for the 20°C stored IJs.

G. mellonella *bioassay*. IJs stored at 9°C underwent a significant (ANOVA, SNK, $P < 0.05$) increase in infectivity for *G. mellonella* in the sand dish bioassay, with a maximum of 6.4 nematodes/insect after 7 weeks storage (Figure 4(a)). For the IJs stored at 20°C there was no significant difference between storage weeks. IJs batches 4–6 (20°C storage) (Figure 4(b)) were significantly more infective than the 20°C stored IJs of batches 1–3 (Figure 4(a)) (ANOVA, SNK, $P < 0.05$).

DISCUSSION

This study clearly demonstrated that the control of *O. sulcatus* larvae at low temperatures by *H. megidis* IJs was improved due to cold storage. Exp. 1 shows that 12 weeks cold-

storage significantly improved infectivity of laboratory-cultured *H. megidis* isolate UK211, resulting in a dramatic increase in *O. sulcatus* mortality at 9°C in potting soil. The reported increase in mortality is likely to be an under-estimate for two reasons. Firstly, fewer larvae were recovered in the nematode treated pots compared to the control pots; most likely, the missing larvae were dead ones that could not be found in the potting compost due to their red-brown colouring and/or decomposition. Secondly, the stored *O. sulcatus* larvae, that were used progressively from week 8 onwards, were less susceptible to nematodes. If 'fresh' larvae were used in the later weeks of the experiment, an even higher mortality would most likely have been found.

The level of vine weevil control at 9°C obtained in the present experiments compares favourably with reports of similar trials at 9°C. Maximum *O. sulcatus* mortality levels obtained were 28% (Exp. 1) and 44% (Exp. 2) in pot trials lasting 4 weeks. In comparison, *O. sulcatus* mortality never exceeded 20% when exposed for 6 weeks to *Heterorhabditis bacteriophora* Hi and *H. megidis* HW79 and HL81 (Simons & van der Schaaf, 1986) or to *H. megidis* UK211, HSH and HF85 for 8 weeks (van Tol, 1994). In neither case was information given regarding IJ storage time and temperature. Westerman and van Zeeland (1994) found varying levels of *O. sulcatus* mortality, with 3 of the 26 isolates (UK211, HF85, selected for cold activity and *Steinernema kraussei*) giving almost 50% mortality. The high *O. sulcatus* control levels in the work of Westerman and van Zeeland (1994) could have been a result of the longer exposure time; 6 weeks compared to 4 weeks in this work. Long term cold storage could also have attributed to the high mortality levels, but again, no information on storage conditions was given.

Although the mean number of nematodes per infected *O. sulcatus* larva remained low, the total number of nematodes invading the insect larvae increased dramatically with storage time. This means that the additional invaders entered new insect larvae and not already infected hosts. This might suggest that *O. sulcatus* larvae invaded by UK211 become less attractive to subsequent invasion, as was reported for lepidopteran hosts invaded by *Steinernema* spp. (Glazer, 1997). However, Westerman (1997) found that the distribution of *Heterorhabditis* spp. in *O. sulcatus* larvae was aggregated, especially at 9°C, and suggested that this would result in a lower proportion of infected insects. Thus while it is not clear why the IJs invaded additional rather than already parasitized hosts, it means that the increased infectivity of the IJs was efficiently translated into increased control of the target.

IJs that had been stored for 11 and 12 weeks at 9°C caused higher *O. sulcatus* mortality than did IJs that had been stored for 2 to 3 weeks at 20°C, supporting the finding of Griffin (1996) that optimal improvement of *Heterorhabditis* infectivity was obtained by storing the IJs at the test temperature. The improvement due to cold storage possibly happens in addition to maturation which is independent of temperature (Griffin, 1996). Different nematode batches were used to test infectivity for *O. sulcatus* larvae following storage of IJs at 9°C (batches 1–3) and 20°C (batches 4–6). Therefore batch differences could have been responsible for the differences in *O. sulcatus* mortality. However, three separate culture batches of IJs were used for each test temperature. Furthermore, the IJs of batches 4–6 were generally more infective for *G. mellonella* than those of batches 1–3 stored under similar conditions. Therefore the lower *O. sulcatus* mortality attributable to the batches 4–6 stored at 20°C compared to the batches 1–3 stored at 9°C is most likely not caused by a general lower infectivity of these batches (due to, e.g. culture conditions), but to the specific conditions (time and temperature) of their storage.

The results of the *G. mellonella* bioassay would suggest that maximum infectivity of nematodes stored at 9°C occurred after 7 weeks, rather than 10 or 12 weeks as was found in the work of Griffin (1996) and in Exp. 1. As only selected storage weeks were tested for *O. sulcatus* control in Exp. 2, the maximum control potential of cold stored IJs may have been missed.

Starvation is unlikely to be related to either the improved infectivity or the subsequent drop in infectivity during storage at 9°C, as only an estimated 21% of available energy

reserves was used up over the 16 week storage period (Exp. 1). However, it is possible that a drop in lipid reserves below a certain critical percentage could act as a signal for changes in infectivity.

The strain used here, UK211, is the active ingredient of the commercial product Nemasys-H® (MicroBio, UK). Cold storage could potentially improve this and similar commercial products for the control of *O. sulcatus*. However, although commercially produced *Heterorhabditis* spp. are generally stored at low temperatures (approximately 5°C in the case of Nemasys-H®), storage times depend on supply and demand, rather than on optimal low temperature infectivity (pers. comm. Roma Gwynn, MicroBio). Testing for low temperature infectivity with commercially produced nematodes would be the next step to ascertain whether cold storage is a viable option for improving low temperature efficacy.

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