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Effect of an Ice-Nucleating Activity Agent on Subzero Survival of Nematode Juveniles

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Abstract: Juveniles of five species of nematodes, *Caenorhabditis elegans, Panagrellus redivivus, Pratylenchus agilis, Pristionchus pacificus,* and *Distolabrellus veechi,* were added to solutions with (treatment) and without (control) a commercial ice-nucleating activity (INA) agent. Ten-microliter droplets of the solutions containing the juveniles were placed on glass microscope slides and transferred to a temperature-controlled freeze plate where the temperature was reduced to −6 to −8 °C. At this temperature, the droplets containing the INA agent froze while those without the agent remained liquid. After 2 minutes, the temperature of the plate was raised to 24 °C, and the slides were examined with a light microscope to determine the viability of the juveniles. The results showed that usually most juveniles (43% to 88%, depending on species) in solutions that did not contain the INA agent (controls) were active, indicating that the juveniles were capable of supercooling and were thereby protected from the subzero temperatures. Alternatively, less than 10% of the juveniles that had frozen for 2 minutes in solutions containing the INA agent remained viable, indicating that inoculative freezing of the solution was lethal to the supercooled juveniles. Our results suggest that, in geographical areas where winter temperatures may not be sufficiently low or sustained to freeze soil, the addition of an INA agent may help induce ice nucleation and thereby reduce the populations of nematode species that are unable to survive when the soil solution is frozen.

Key words: *Caenorhabditis elegans,* cryobiology, *Distolabrellus veechi,* freeze susceptibility, freeze tolerance, ice-nucleating activity agent, INA, nematode, *Panagrellus redivivus, Pratylenchus agilis, Pristionchus pacificus,* Snomax, supercooling.

The freezing of water involves a phase change (i.e., liquid is converted to a solid). This process, which normally occurs at subzero temperatures, usually is initiated by nucleation particles consisting of organic or inorganic materials that are also known as ice-nucleating activity (INA) agents (Vali, 1995). Lindow et al. (1975, 1976) reported that a strain of the bacterium, *Pseudomonas syringae,* was an active INA agent. Further studies with INA bacteria, namely *Pseudomonas syringae* and *Erwinia herbicola,* indicated that the active particle was localized on the outer membrane of certain gram-negative bacteria (Lindow et al., 1989; Wolber et al., 1986). This information has been used to produce a commercial INA agent, Snomax, which is used worldwide by the ski industry. Adding this product to a supercooled water source raises the freezing temperature so that artificial snow can be made more effectively at higher temperatures.

The INA agents may impact agriculture. The presence of INA bacteria on leaves can render plants susceptible to frost injury when temperatures fall below freezing, whereas plants with leaf bacteria that do not have INA activity are less susceptible to frost injury. Many insects survive subzero temperatures by a process known as supercooling (reducing the temperature at which ice crystals form). However, spraying them with solutions of INA bacteria limits their capacity to survive (Strong-Gunderson et al., 1989, 1992). In fact, using INA bacteria and fungi to reduce survival of supercooled insects has been documented in more than 15 species among four different orders (Fields et al., 1993; Lee et al., 1993). This procedure currently is being used to control insects on pears and in grain silos.

Recent studies in our laboratory (Wergin et al., 1999) have indicated that lowering soil temperature by using a cryogen, such as

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dry ice, reduced by more than 95% the numbers of *Meloidogyne incognita* eggs that could be recovered from susceptible plants growing in infested soil, suggesting that dry ice might have potential as a cryonematicide. However, the cost of this procedure could be prohibitive. Consequently, the current study evaluates whether an INA agent would affect the viability of supercooled juveniles and could thereby be used to increase the efficiency and economics of potential cryonematicides.

Materials and Methods

Nematode cultures: Five species of nematodes having active juveniles were chosen so that viable and non-viable individuals could be readily distinguished. *Caenorhabditis elegans* and *Panagrellus redivivus* were grown axenically in sterile medium (Chitwood et al., 1995), *Distolabrellus veechi* and *Pristionchus pacificus* were cultured monoxenically on NGM agar plates (Wood, 1988), and *Pratylenchus agilis* was obtained from corn (*Zea mays,* cv. IO Chief) explant cultures (Huettel and Rebois, 1985). Juveniles from each species were concentrated in 10 ml of either the medium (*C. elegans* and *P. redivivus*) or distilled water (*P. agilis, P. pacificus,* and *D. veechi*), which was subsequently diluted for the evaluation procedure described below.

INA agent toxicity: The INA agent (Snomax) used in these studies was obtained from Snomax, Snow Inducer (York Snow, Victor, NY) and consisted of *Pseudomonas syringae* strain 31a. The commercial product is subjected to low-dose beta irradiation to reduce cell viability without causing significant loss of INA. To evaluate the potential toxicity of Snomax, a solution was prepared by dissolving one 3-mm-diam. pellet (less than 0.01 g) of Snomax in 200 ml of distilled water. Next, stock solutions were prepared by combining aliquots of the growth medium containing juveniles of *C. elegans* with equal aliquots of either distilled water (controls) or the INA solution (treatment) in glass vials. After 30 minutes, 10-µl droplets of the control or treated stock solutions, each containing 100 to 300 juveniles, were put on microscope slides and observed with a compound microscope at ×250. The first 100 juveniles randomly encountered on each slide were evaluated. Those juveniles that were curved or sinuous, displayed movements characteristic of the species, and exhibited homogeneous body fluids throughout their lengths were considered living. Juveniles that were relatively straight, did not move, and had aggregated body fluids were deemed dead. This procedure was repeated six times to allow viability evaluations of 600 control (no INA) and 600 treated (INA) juveniles.

Freezing experiment: Aliquots of juvenile suspensions were combined with equal parts of distilled water (controls) or Snomax solution in plastic petri dishes. Next, 10-µl droplets of these control or treated solutions, each containing 100 to 300 juveniles, were put on glass slides, which were then placed on the aluminum platform of a temperature-controlled freeze plate apparatus (Thermoelectrics Unlimited, Wilmington, DE). This apparatus consisted of a temperature control module, a polished aluminum work platform, and a transparent Plexiglas cover (Fig. 1). The work platform could be actively heated or cooled over a temperature range of +27 °C to −17 °C by using a single selectable temperature set-point control. The platform, which measured 90 × 130 mm, accommodated four glass slides (two controls and two with Snomax for each species). A Plexiglas cover enclosed and protected the samples from ambient air. Video microscopy and recording were used to view the samples directly through the Plexiglas cover.

The temperature set-point of the freeze plate, which was initially at 24 °C, was lowered to a subzero reading of -6 to -8 °C, which was reached within 2 minutes; during this time, the droplets containing the INA agent froze. The subzero temperature was maintained for 2 minutes after freezing and then reset to 24 °C. By the time the freeze plate reached 24 °C (about 2 minutes), the frozen droplets had thawed; cover slips were added and the slides were removed for microscopic observations. The condition of the

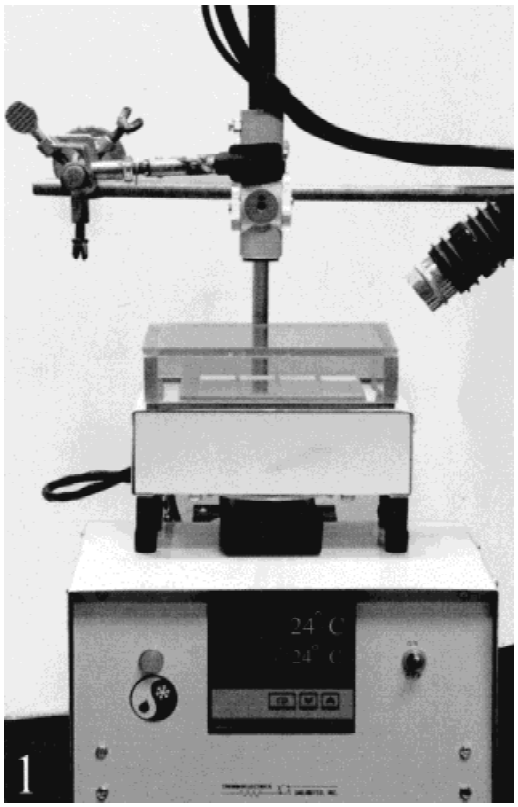


FIG. 1. The freeze plate apparatus used in these experiments, consisting of a temperature control module, a polished aluminum work platform, and a transparent Plexiglas cover to protect the platform from external environmental factors.

juveniles was evaluated as in the toxicity experiment. This procedure was repeated six times until 600 juveniles of each species had been evaluated in water and in Snomax.

Statistical analysis: The percentage of living nematodes was analyzed as a two-factorial general linear model using PROC MIXED (SAS/STAT, V6.12, SAS Institute Inc., Cary, NC).

RESULTS

INA agent toxicity: After 30 minutes, 97% and 98% of the *C. elegans* larvae were considered alive in the distilled water and Snomax, respectively. Consequently, no indication of Snomax toxicity was evident after 30 minutes.

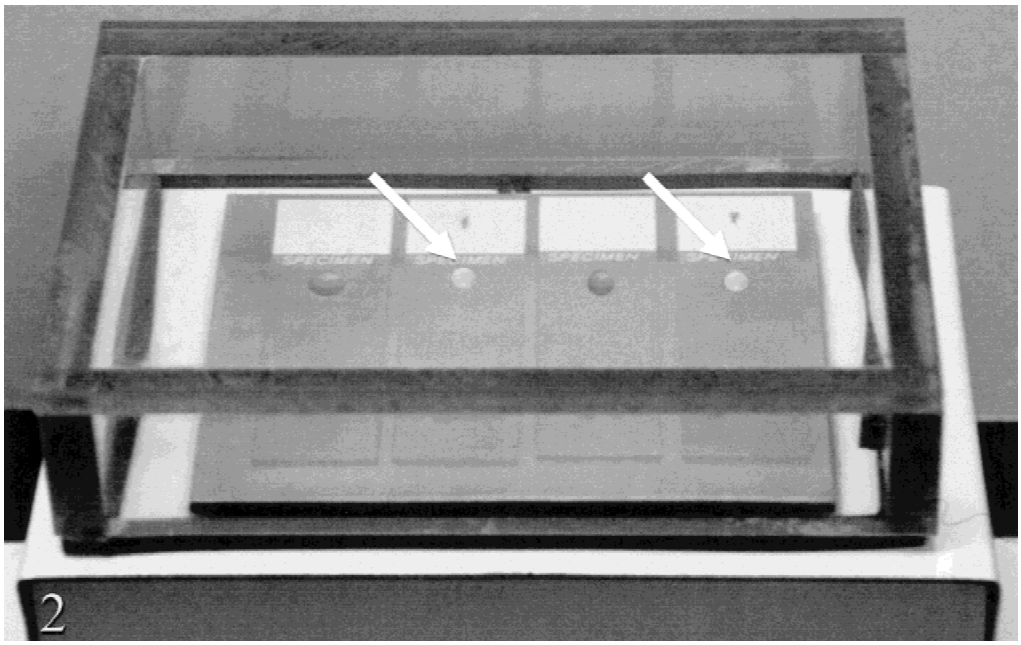
Freezing experiments: Evaluation of the juveniles from one set of slides cycled on the freeze plate was also accomplished in about 30 minutes. After the temperature set-point was lowered to subzero temperatures, droplets of juvenile solutions containing Snomax froze and became opaque while droplets without Snomax remained liquid (Fig. 2). After thawing, about 90% of the juveniles in droplets that had frozen were relatively straight and motionless, and appeared to have coagulated cellular contents (Fig. 3). These juveniles were assumed dead. About 10% of the juveniles were curled or coiled but active and appeared normal internally; these juveniles were counted as alive. In water droplets without Snomax, 43–91% of the juveniles survived the subzero temperatures (Table 1).

Statistical analysis: Pair-wise contrasts indicated that *C. elegans, P. agilis, and P. redivivus* were somewhat more tolerant to supercooling than the other two species and that their survival rate was not significantly different from one another. The least-tolerant species to subzero temperatures was *P. pacificus,* and *D. veechi* was intermediate.

The addition of Snomax during supercooling was lethal to all nematode species (Table 1) and killed 93% or more of the juveniles. *Pristionchus pacificus* was the most susceptible, with less than a 0.2% survival rate. The overall treatment means showed that less than 4% of the juveniles survived Snomax-induced freezing. The average of the combined survival rate for each nematode species was similar to the ranking of the survival rate for the supercooling treatment.

DISCUSSION

We have shown that an INA agent can be used to induce freezing of supercooled solutions of juveniles and thereby reduce their viability by more than 90%. These results are not inconsistent with previous studies where freezing has been used to preserve the viability of a wide range of free-living and plant- and animal-parasitic nematodes (Bridge and Ham, 1985; Curran et al., 1992; Folkertsma et al., 1997; Ham et al., 1981; James, 1981; Popiel and Vasquez, 1991; Riga and Webster, 1991; Smith et al., 1990; Sulston and



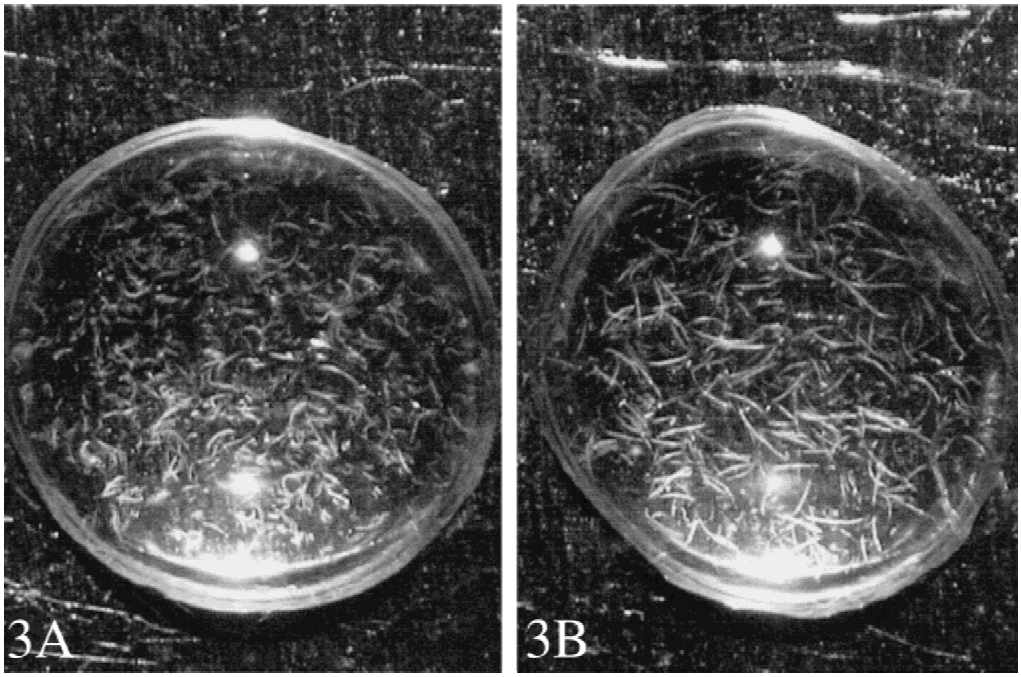


FIG. 2. Work platform (90 mm × 130 mm) for accommodating up to four microscope slides holding droplets of solutions containing juvenile nematodes. At subzero temperatures, droplets of juvenile solution containing an INA agent froze and became opaque (arrows) while droplets without the agent remained liquid.

FIG. 3. Appearance of *Caenorhabditis elegans* juveniles in droplets after being subjected to subzero temperatures on the freeze plate apparatus, then thawed. Most of the juveniles in the unfrozen droplet (Fig. 3A) appeared normal, while more than 90% of those from the frozen droplet (Fig. 3B) were relatively straight and motionless, and appeared to have coagulated cellular contents.

TABLE 1. Percentage survival for juveniles of five nematode species subjected to subzero temperatures in distilled water (control) and distilled water containing Snomax, an ice-nucleating activity (INA) agent.

Treatment	*Caenorhabditis elegans*	*Pratylenchus agilis*	*Panagrellus redivivus*	*Pristionchus pacificus*	*Distolabrellus veechi*
Distilled water	86.5a[1]*	88.5a*	91.2a*	43.5c*	67.3b*
INA agent	2.5	3.7	5.5	0.2	6.5

[1] Nematode means with different letters differ at $P < 0.05$; * indicates value for distilled water that differs from the corresponding INA agent value at $P < 0.01$.

Hodgkin, 1988). In these previous studies, the procedures used generally required pretreatments with cryoprotectants, the duration and the temperature had to be precisely controlled, and success frequently depended on the age and vigor of the nematodes and the rate of thawing (Sayre and Hwang, 1975; Triantaphyllou and McCabe, 1989; van der Beek et al., 1996). Our experiments were meant to maximize the lethal effects of freezing. Consequently, juveniles were frozen quickly in nutrient media or in water containing Snomax; no cryoprotectants were used.

Low-temperature effects on nematode viability have been studied for more than 40 years. Ichinohe (1955) reported that eggs of *Heterodera glycines* remained viable in cysts that were stored at −40 °C for as long as 7 months. Slack and Hamblen (1961) found that juveniles would emerge from cysts that were maintained at −24 °C for 18 months. However, juveniles were killed at −40 °C when ice crystals formed in the water (Slack et al., 1972). Investigations with *Meloidogyne* spp. led Sayre (1963, 1964) to suggest that nematodes could be divided into three classes based on their response to low temperatures: (i) those that are susceptible to chilling injury and die at temperatures above freezing, (ii) those that are capable of supercooling (freeze-tolerant), and (iii) those that are not injured by freezing (freeze avoidance). Most nematodes appear to be freeze-tolerant, i.e., they are capable of surviving subzero temperatures by depressing their supercooling points (Ash and Atkinson, 1986; Mabbett and Wharton, 1986; Wharton, 1995; Wharton and Block, 1993; Wharton and Brown, 1991). However, we suggest that if ice formation occurs in their surrounding environment, ice nucleation is also likely to occur in the nematode and will be fatal. The five species of nematodes that were used in the present study appear to be in this class.

Wharton and co-workers have done extensive studies on freeze-tolerant nematodes (Wharton, 1995). Adults of *Panagrellus silusiae,* third-stage juveniles of *Trichostrongylus colubriformis,* and fourth-stage juveniles of *Ditylenchus dipsaci* could supercool to −20 °C, −30 °C, and −21 °C, respectively (Wharton et al., 1984). Perry and Wharton (1985) indicated that second-stage juveniles of *Globodera rostochiensis* could supercool to −29.5 °C. Another study with *Heterorhabditis zealandica* indicated that ensheathed juveniles of this species could supercool to −25 °C and that the presence of the sheath prevented ice crystal formation or inoculative freezing of the juveniles (Wharton and Surrey, 1984). Our study was not intended to determine the lowest supercool temperature that juveniles can survive but rather to measure the reduction in viability that occurs when inoculative freezing is induced in a nematode suspension. However, the freeze plate apparatus used in our experiments appears to be a simple technique that could be used to determine supercool temperatures in freeze-tolerant nematodes.

In conclusion, our results indicate that each of the species examined was capable of supercooling to some degree and that they survived subzero temperatures only when no external ice nucleation was induced, i.e., they remained in a supercooled liquid. However, exogenous ice nucleation of the liquid, which was triggered by introducing an INA agent, was lethal to 90% of the juveniles. Wharton (1986) has stated that nematodes

are essentially aquatic organisms and that a film of water must be present for their feeding, growth, and reproduction to occur. Therefore, in nature, exogenous ice nucleation that is induced in the water film could significantly reduce the population of the large class of nematodes that are freeze-tolerant. Years ago Bouyoucos (1920) demonstrated that soils vary widely in their capacity to supercool before freezing. More recently, Rosinski et al. (1976) found that ice nucleation is not initiated in cultivated soils from Montana until −12 °C, whereas nucleation in soils from Colorado did not occur even at −20 °C. These observations and our results lead us to speculate that the introduction of an INA agent to soils in areas that remain unfrozen but supercooled could induce ice nucleation and thereby significantly reduce the populations of nematodes that are freeze-tolerant.

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