Entomologia Experimentalis et Applicata **89:** 103–109, 1998.

Reduction of insect cold-hardiness using ice-nucleating active fungi and surfactants

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Accepted: August 5, 1998

Key words: Insecta, supercooling capacity, cold tolerance, *Hippodamia convergens*, *Fusarium avenaceum*, *Fusarium acuminatum*, pleomorphism, biological control

Abstract

The supercooling point (SCP) of an insect model, the lady beetle *Hippodamia convergens* Guérin-Menéville (Coleoptera, Coccinellidae) was markedly elevated by treatment with aqueous suspensions of the filamentous, ice nucleation active (INA) fungi *Fusarium avenaceum* and slightly elevated by *Fusarium acuminatum*. Addition of the surfactant Tween 80 to the fungal suspensions further reduced the supercooling capacity of adult beetles. When used alone the surfactant Triton X-100 produced a greater SCP elevation than Tween 20 or Tween 80. The emulsifier gum arabic was ineffective in elevating beetle SCPs when applied alone and when added to INA fungal preparations it decreased their efficacy. Aqueous suspensions of both viable sporulating and viable pleomorphic (a permanent, degenerative, nonsporulating cultural state) forms of both fungal species were more effective in elevating the SCP than killed preparations except for the pleomorphic *F. acuminatum* suspension in which the killed form was slightly more active. Application of INA fungi applied in combination with surfactants may be useful in the development of methods for the biological control of overwintering freeze-susceptible insect pests by decreasing their capacity to avoid lethal freezing by supercooling.

Introduction

In the absence of an efficient heterogeneous ice nucleator, small volumes of pure water may supercool (i.e. remain in a liquid state) to subzero temperatures as low as −39 °C (Bigg, 1953). Many insects including most pest species in temperate regions are intolerant of freezing and must avoid exposure to subzero temperatures or survive by supercooling (Lee et al., 1993). To maximize their capacity to supercool these freezing intolerant species must avoid inoculative freezing by external ice and the action of ice nucleating agents which catalyze ice formation at relatively warm temperatures. As an insect is cooled the limit of supercooling is reached when ice forms internally, termed the supercooling point (SCP) or the temperature of crystallization (Lee, 1991).

Ice nucleation active (INA) nuclei are generally defined as those nucleators which catalyze the freezing of water at temperatures above −10 °C (Hirano et al., 1978). Bacteria were the first microbes reported to possess the ice nucleating trait (see review by Upper & Vali, 1995), while INA fungi were more recently discovered (Kieft, 1988; Pouleur et al., 1992; Tsumuki et al., 1992; Richard et al., 1996). Ice nucleating activity is known in five lichen mycobionts (Kieft & Ahmadjian, 1989), and amongst several species of one free-living fungal genus, *Fusarium*, including *F. avenaceum* (Pouleur et al., 1992; Hasegawa et al., 1994), *F. acuminatum* (Pouleur et al., 1992), *F. oxysporum* and *F. tricinctum* (Richard et al., 1996), and a *F. moniliforme* isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) (Tsumuki et al., 1992). Certain strains of these

species are highly efficient ice nucleators, initiating the freezing of water at temperatures above −5 °C.

Recently it was proposed that INA microorganisms may be used to reduce the supercooling capacity of insect pests that do not survive internal freezing and thereby reduce their overwintering survival (see reviews by Fields, 1992; Lee et al., 1993; 1995). A growing literature documents that topical application via misting or dusting of INA bacteria, and to a lesser extent INA fungi, reduces the supercooling capacity in a variety of insects including stored grain insects (Fields, 1990; Lee et al., 1992; Fields, 1993) and the Colorado potato beetle (Lee et al., 1994; Steigerwald et al., 1995). It is also known that INA bacteria and fungi are normal flora in the gut of some insects and may function, at least in one case, to promote freeze tolerance (Lee et al., 1991; Tsumuki et al., 1992).

The effectiveness of INA fungi in raising insects' SCPs appears limited compared to the effectiveness of INA bacteria to reduce insect's supercooling capacity possibly due to a reduced level of fungal ice nucleating activity compared to bacterial potency in dry-application studies (Fields et al., 1995), and due to their larger size or other characteristics that may limit their access to the insect's body water across its hydrophobic cuticle or via natural body orifices (Lee et al., 1993). The potential use of INA fungi as a biological control agent would be enhanced if it were possible to increase the efficacy of INA fungi in elevating the SCP and extend the duration of its effectiveness by use of different strains or morphologic forms of INA fungi or improved methods of delivery that promote colonization or improve adherence of microorganisms (Lee et al., 1993).

In the present study we continued our use of the lady beetle *Hippodamia convergens* (Strong-Gunderson et al., 1992) as an insect model to answer the following questions: Can surfactants/emulsifiers enhance the effectiveness of topical exposure to INA fungi in decreasing the supercooling capability? Is the cultural degenerative loss of sporulating capacity, termed fungal pleomorphism, linked to reduced fungal ice nucleating activity? Is the duration of reduced supercooling capacity by topically applied INA fungi extended, or the killing capacity enhanced, by addition of surfactants to the inoculum?

Materials and methods

Insects, INA fungi, surfactants and emulsifyng agents. Field-collected, overwintering adult *H. convergens* were received from Fountain's Sierra Bug Company, Rough and Ready, CA, USA. This species is freezing intolerant and supercools to approximately −15 °C (Lee, 1980). To simulate their natural overwintering condition beetles were held unfed at 4 °C. The surfactants Tween 20, Tween 80, Triton X-100 and the emulsifier gum arabic, were purchased from Sigma Chemical Company, St. Louis, MO, USA. INA strains of the filamentous fungal species *Fusarium acuminatum* Ellis and Everhart strain 303 and *F. avenaceum* (Corda ex Fr.) Sacc. strain 411 were kindly provided by Stephan Pouleur and Claude Richard, Agriculture Canada, Sainte-Foy, Canada. A non-INA strain of *Fusarium oxysporum*, obtained from Miami University's culture collection, served as the negative control. Unless otherwise noted these fungi were grown on parafilmed potato flake agar (PFA) plates (Rinaldi, 1982) at 25 °C in the dark throughout the experiment. Pleomorphic mycelia of each strain were selected from nonpigmented, nonsporulating degenerative areas of the thallus, confirmed microscopically and maintained on PFA.

INA detection and SCP determinations. Aqueous fungal suspensions of *F. acuminatum*, *F. avenaceum* and *F. oxysporum* were made from cultures grown at 25 °C for 7 days on PFA (Rinaldi, 1982) by scraping 30 mg of mycelium harvested from ca. 1 cm^2 area of dense, pigmented if sporulating, or white if pleomorphic, aerial mycelium into 3 ml sterile distilled water. Killed fungi were obtained by soaking undisturbed 1 cm^2 sections of mycelium from 7 day-old colonies in full strength chloroform for 10 min and rinsing in sterile water. Firm pressure was applied manually to pieces of mycelium by squeezing and rubbing them against the inner side of a test tube with a sterile cotton swab for one minute, resulting in a uniform pink to lavender suspension from sporulating fungi. One minute settling time was allowed prior to misting. Microscopic examination of the supernatant confirmed the presence of hyphae, and, in sporulating cultures, macroconidia and microconidia. A solid remnant of dense mycelium remained unsuspended. Ice nucleating activity of the fungal suspensions was determined according to the freeze-drop method of Vali (1971) as modified by Lindow (1982), in which 160 ten-μl droplets of fungal suspension were placed

in aluminum boats floating on a refrigerated ethanol bath with an initial temperature of 0 °C and lowered at ca. 0.3 °C/min. The ice nucleation temperature was determined visually, as the temperature at which the liquid drop became cloudy, opaque and solid.

Supercooling point values were determined by positioning adult beetles in contact with a 30-gauge copper-constantan thermocouple within a 1.5 ml polypropylene tube (Strong-Gunderson et al., 1990). These tubes were placed in glass test tubes suspended in a 0 °C refrigerated bath and allowed to equilibrate for 5 min before cooling at ca. 0.3 °C/min. The lowest temperature reached prior to the release of the latent heat of fusion was recorded as the SCP.

Dry exposure versus misting; and enhancement with surfactants. To determine the effect of dry exposure to INA fungi, lady beetles were inoculated cuticularly with *F. acuminatum* by rubbing 10 mg of harvested, dense, aerial, pigmented mycelium upon their dorsa and ventra. Supercooling points were measured immediately and compared to a control group of non-inoculated beetles.

Aqueous fungal suspensions (10 mg mycelium/3 ml sterile, distilled water) were misted onto the beetles placed in a sterile 100 × 15 mm petri dish. A mister, held approximately 10 cm above the lady beetles, was used to deliver droplets ca. 3 mm diameter that totaled 0.5 ml of misted fungal suspension. The insects were held at 25 °C after inoculation. Additional test groups of beetles were misted with 0.01% aqueous suspensions of surfactants Tween 80, Tween 20, Triton X-100, and the emulsifier gum arabic, and with inocula of 10 mg fungi per 3 ml 0.01% aqueous suspension of each of these surfactants/emulsifiers. Control SCP values were determined on dry, noninoculated insects and insects misted with sterile distilled water alone. SCP values for test and control groups of insects held at 25 °C were determined immediately, at 1 and 3 days post-misting.

Examination of the beetles' cuticle for the presence of INA microorganisms. Cuticles from ten untreated, control lady beetles were cultured on PFA aerobically at 25 °C for 7 days to determine whether *F. acuminatum*, *F. avenaceum* or other INA microorganisms were present naturally. Aqueous fungal and bacterial suspensions, $A_{550\,nm}$ 0.80 using a Gilford 250 spectrophotometer, of each bacterial and fungal colony cultured from the cuticle were tested via the freeze-drop assay (Vali, 1971; Lindow, 1982) at −10 °C to detect the ice nucleation phenotype.

Table 1. Ice nucleating activity of viable sporulating mycelia, viable pleomorphic mycelia, and killed sporulating mycelia of INA fungal suspensions of *Fusarium acuminatum* and *Fusarium avenaceum*, and non-INA *Fusarium oxysporum* using the freeze-drop assay. T_{max}, T_{50} and T_{90} indicate respectively the temperatures at which the first droplet froze, 50% or 90% of droplets had frozen

Fungal suspension	Temperature (°C)		
	T_{max}	T_{50}	T_{90}
Ice nucleating active			
Fusarium acuminatum			
sporulating	−2.8	−5.9	−6.9
pleomorphic	−6.0	−14.9	−16.5
sporulating & killed	−3.1	−6.3	−8.7
pleomorphic & killed	−7.4	−15.9	−18.0
Fusarium avenaceum			
sporulating	−2.7	−4.7	−6.5
pleomorphic	−2.2	−3.9	−5.3
sporulating & killed	−3.1	−5.2	−7.6
pleomorphic & killed	−2.9	−3.7	−4.6
Non-ice nucleating active			
Fusarium oxysporum	−9.5	−17.9	−20.2

Results

Relative potencies of fungal suspensions revealed that the ice nucleating activity of viable sporulating whole mycelium were similar for both *F. acuminatum* and *F. avenaceum*, as indicated by T_{50}s near −5 °C with T_{max} greater than −3 °C and T_{90}s greater than −6.9 °C (Table 1). In contrast, the non-INA fungus, *F. oxysporum* had a T_{50} of −17.9 °C. Interestingly, although pleomorphic mycelia of *F. avenaceum* exhibited significant ice nucleation activity at warm temperatures, pleomorphic mycelia of *F. acuminatum* had dramatically reduced ice nucleating activity compared to the sporulating form, suggesting that sporulation enhances the ice nucleation potency of this species. In general, the ice nucleating activity of killed preparations was similar to that of the sporulating or pleomorphic preparations (Table 1).

Dry beetles or ones treated with misted water supercooled extensively with SCPs below −14 °C (Table 2). This result is consistent with the fact that no

INA microbes were detected from cultures of the beetle's cuticular surface. Similarly, treatment with the non-ice nucleating active fungus *F. oxysporum* or a 1% solution of Tween 80 had little effect on the beetle's supercooling capacity. In contrast, misted aqueous suspensions of *F. acuminatum* and *F. avenaceum* significantly elevated the SCPs to −11.0 and −6.2 °C respectively (Table 2), whereas cuticular inoculation by rubbing dry mycelial of *F. acuminatum* onto the dorsa and ventra of beetles did not elevate the SCP (−15.0 °C ± 0.2, $n = 22$). Within 24 h the elevation of SCPs caused by the application of *F. acuminatum* was lost. The effect of treatment with *F. avenaceum* also decreased with time as SCPs decreased to −10.8 °C within 24 h and to −11.6 °C after 3 days.

In an attempt to further enhance the effectiveness of INA fungi in decreasing the beetles' supercooling capacity several surfactants and an emulsifier were tested alone or added to the fungal suspensions. When tested alone the surfactant Triton X-100 solution elevated the SCP (−9.2 °C) to a greater extent than the other three treatments (Figure 1). An aqueous solution of Tween 80 misted onto the surface of beetles caused a slight elevation of the supercooling point to −13.6 °C (Table 2). Similarly, when Tween 80 was added to the *F. avenaceum* suspension the SCPs increased slightly to −5.4 °C compared to −6.2 °C for the *F. avenaceum* suspension alone (Table 2). Likewise the addition of Triton-X and Tween 20 enhanced the action of the *F. avenaceum* suspension. When Tween 80 was added to the *F. acuminatum* suspension the SCP was markedly elevated to −5.9 °C compared to the fungal suspension alone (−11 °C). The addition of gum arabic to the two fungal preparations did not enhance their efficacy; in fact it reduced the SCP elevation caused by *F. avenaceum* alone from −5.9 °C to −12.1 °C and had a similar but less dramatic effect on *F. acuminatum* (Figure 1, Table 2).

We also examined the effect of using viable *versus* killed and sporulating *versus* pleomorphic forms of the fungi on their capacity to elevate the SCP. Regardless of specific formulation *F. avenaceum* was more efficient in reducing supercooling capacity than *F. acuminatum* (Table 3). In the absence of surfactant, comparisons of the viable preparations of sporulating or pleomorphic fungi were significantly more effective in elevating the SCP than killed fungal preparations except for the pleomorphic *F. acuminatum* suspension in which the killed form was slightly more active. Sporulating *versus* pleomorphic preparations generally produced less than a 2 °C difference in the mean

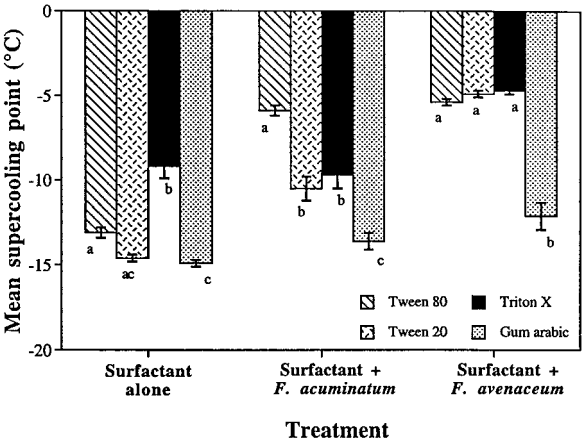

Figure 1. Effect of misting with commercial surfactants and an emulsifier, gum arabic, with and without INA fungi on the supercooling point of *Hippodamia convergens*. Values reported as means ± SEM. Each value is based on 22–24 individuals. Within each treatment mean supercooling points were compared using ANOVA and the Bonferroni Multiple Comparisons Test. Values followed by different letters are significantly different, $P<0.05$.

SCP values except for the Tween 80 with *F. acuminatum* treatment in which the sporulating preparation produced a 7.8 °C elevation (Table 3).

Discussion

Fields et al. (1995) reported that *F. avenaceum* could be used to reduce the supercooling capacity of the rusty grain beetle (*Cryptolestes ferrugineus*). By mixing a freeze-dried mycelial preparation with grain containing beetles they raised the SCP of *C. ferrugineus* from −17 to −9 °C. We obtained similar results by misting aqueous suspensions of the INA fungi *F. acuminatum* and *F. avenaceum* on adults of *H. convergens*. However, in some cases in the present study beetle SCPs were increased to −6 °C or higher. Nonetheless these SCP values are lower than achieved with *H. convergens* and other species using INA bacterial suspensions (Strong-Gunderson et al., 1990, 1992; Fields, 1992; Lee et al., 1992, 1994). This difference is not surprising since the bacterial preparations had a significantly higher ice nucleating potency than the fungal preparations used in the present study. The extent of the SCP elevations observed in the beetles (Table 2) did not reach the ice nucleating activity of the fungal suspensions alone (Table 1). For example, the suspensions of *F. acuminatum* had a T_{50} of −4.9 °C while the SCP of beetles was −11.0 °C, and *F. avenaceum*'s T_{50} of −4.7 °C corresponded to

Table 2. Influence and duration of treament with viable, sporulating fungi on the supercooling point (SCP) of adult *Hippodamia convergens*. Values are reported as mean ± SEM (sample size)

Treatment	Supercooling point (°C)		
	Immediate	24 h post misting	72 h post misting
Untreated, dry	−14.9 ± 0.3 (21)	ND*	ND
Water only	−14.5 ± 0.3 (22) a	−14.8 ± 0.3 (23) a	ND
1% Tween 80	−13.6 ± 0.5 (11) a	−15.2 ± 0.2 (21) b	−14.6 ± 0.4(11) ab
F. acuminatum in water	−11.0 ± 0.7 (24) a	−15.3 ± 0.2 (24) b	−15.0 ± 0.3 (12) b
F. acuminatum in 1% Tween 80	−5.9 ± 0.3 (24) a	−13.9 ± 0.5 (23) b	−15.7 ± 0.2 (11) c
F. avenaceum in water	−6.2 ± 0.5 (23) a	−10.8 ± 0.6 (24) b	−11.6 ± 0.8 (24) b
F. avenaceum in 1% Tween 80	−5.4 ± 0.2 (24) a	−9.9 ± 0.8 (23) b	−13.4 ± 1.2 (11) c
F. oxysporum in water	−14.7 ± 0.3 (20)	ND	ND
F. oxysporum in 1% Tween 80	−15.0 ± 0.3 (24)	ND	ND

*ND–not determined. Within rows mean values were compared using ANOVA and the Bonferroni Multiple Comparisons Test. Values followed by the same letter are not significantly different, P>0.05. The row containing 'Water only' was analyzed with an unpaired, two-tailed Student *t*-test.

Table 3. Effect of misting with viable *versus* killed INA fungi in the presence or absence of 1% Tween 80 on the supercooling point of adult *Hippodamia convergens*

Fungal Strain	Supercooling point (°C)						
	Viable fungus		Killed fungus			ANOVA table	
	Without Tween 80	With Tween 80	Without Tween 80	With Tween 80	Surfactant	Viability Status	Interaction
F. acuminatum	−11.0 ± 0.7	−5.9 ± 0.3	−13.5 ± 0.6	−8.4 ± 0.6	F=83.8	F=20.8	F=0.001
Sporulating					P<0.0001	P<0.0001	P=0.97
F. acuminatum	−12.8 ± 0.7	−13.7 ± 0.5	−11.3 ± 0.8	−10.8 ± 0.7	F=0.1	F=10.1	F=1.17
Pleomorphic					P=0.75	P<0.005	P=0.28
F. avenaceum	−6.2 ± 0.5	−5.4 ± 0.2	−9.3 ± 0.8	−4.9 ± 0.1	F=27.5	F=6.7	F=13.9
Sporulating					P<0.0001	P<0.01	P<0.0005
F. avenaceum	−6.2 ± 0.5	−4.3 ± 0.2	−7.1 ± 0.7	−6.8 ± 0.7	F=3.8	F=9.0	F=1.9
Pleomorphic					P=0.056	P<0.005	P=0.17

Values are reported as means ± SEM. Each value is based on 22–24 individuals. Within rows mean SCPs were compared using a two-way ANOVA.

a beetle SCP of −6.2 °C. Comparison studies using bacterial preparations that may have a T_{max} of greater than −2 °C found that insects misted with 10^8 INA *Pseudomonas syringae*/ml raised *H. convergens*' SCP to values as high as −3 °C, with 70% of misted insects freezing by −5 °C, and 100% by −9 °C (Strong-Gunderson et al., 1992).

Topical application of misted *F. avenaceum* increased the insects' SCPs to a greater extent than topical application of *F. acuminatum* (Table 2). However, when Tween 80 was added to the *F. acuminatum* suspension its effectiveness in elevating the SCP (−5.9 °C) was similar to that of *F. avenaceum* alone. A comparison of using sporulating *versus* pleomorphic forms for both fungal species revealed little difference in their effectiveness in reducing the capacity of the beetles to supercool. A difference was evident, however, when Tween 80 was added to the *F. acuminatum* suspension in which the sporulating form had greater activity than the pleomorphic one. Viable fungal preparations were generally more active than killed ones (Table 3).

Surfactants, which lower the surface tension of water and are soluble in both water and organic solutions, were used in an attempt to improve contact between the INA fungi and the insect. Zidack et al. (1992) successfully used a surfactant to promote bacterial infection of weeds for biological control. They suggested that the surfactant facilitated the penetration of leaf stomata by the bacterial pathogens. Surfactants may enhance the action of the fungi by decreasing the surface tensions of the INA inoculum and thereby fa-

cilitating contact with the largely hydrophobic cuticle of the insect and allowing penetration of the fungi into previously inaccessible anatomic sites on the insect such as cuticular pores, spiracles or other body openings (Steigerwald et al., 1995). The presence of the surfactant Tween 80, although reported to reduce ice-nucleation activity *in vitro* in a bacterium isolated from gemmisphere of tea trees (Makino, 1983), was particularly effective in augmenting the effectiveness of the INA fungal preparations, especially for *F. acuminatum*, in increasing the SCPs of *H. convergens*.

The efficacy of fungal ice nuclei observed in this study is lower than that of the most active bacterial ice nuclei reported in previous studies (Lee et al., 1993; Fields et al., 1995). For a number of treatments in the present study the SCP elevations were in the range of -5 ° to -6 °C. It is likely that SCPs in this range would not be sufficient to reduce overwintering survival for some species that overwinter in protected hibernacula because the environmental temperatures may not decrease this low. However, it is possible that the fungal ice nucleating activity might be enhanced by modifying their culture conditions or selecting for increased ice nucleating potency as has been done with *P. syringae* (LaDuca et al., 1995).

On the other hand these INA fungi have characteristics that favor their potential use for biological control (Kieft & Ruscetti, 1990; Pouleur et al., 1992). Unlike bacterial ice nuclei, fungal ice nuclei are stable at temperatures as high as 60 °C and over a wide pH range of 1.5 to 12.0, and, additionally, their activity does not require a lipid component (Kieft & Ruscetti, 1990). This stability could help solve one of the major problems that must be overcome for INA microorganisms to be used for biological control; these agents must retain ice nucleating activity at relatively warm temperatures since it may be necessary to apply the microbes weeks or months before environmental temperatures decrease (Lee et al., 1993).

With respect to the ultimate objective of using INA microbes for biological control of overwintering pests this study represents an initial step in evaluating the use of INA fungi and surfactants. This laboratory study demonstrated that the extent of SCP elevation is influenced by fungal species and its growth form as well as the addition of surfactants to these preparations. However, further study is needed on efficient ways to deliver the microbes to pests in the field that also minimize effects on non-target, beneficial species. Investigations of interactions between the insect and its overwintering microhabitat are also required. For example, the supercooling capacity of overwintering adults of the Colorado potato beetle is reduced in moist soils (Costanzo et al., 1997). Perhaps this effect of inoculative freezing in moist soils could be further enhanced by the use of surfactants alone as well as INA agents.

Acknowledgements

This research was supported by USDA CSRS grant 96–35302–3419 and by NSF IBN-9728573.

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