THE COLD-HARDINESS OF *CRYPTOLESTES FERRUGINEUS* AND THE USE OF ICE NUCLEATION-ACTIVE BACTERIA AS A COLD-SYNERGIST

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Abstract

Cold temperatures have been used for many years to control stored-product insect populations. However there are few field studies describing how these pests react to low temperatures. Cold-hardiness in insects is affected by many factors. One of the most important is the degree of cold acclimatization. The cold-hardiness of *Cryptolestes ferrugineus*, the most common and cold-hardy insect attacking Canadian grain, was examined to determine how to use low temperatures to control this insect. In an unventilated, 100 tonne granary located near Winnipeg, up to 50% of adults survived the winter. Cold-hardiness, as measured by survival at -10°C, varied from lethal time $_{50}$ = 5 days in November to lethal time $_{50}$ = 40 days in February. Dryer grain reduced overwintering survival (12% moisture content = 10%, 14-16% moisture content = 50%,) and cold-hardiness. In the laboratory, working with cold-acclimated individuals, adults > fourth instar > second instar > eggs, in their ability to survive -10°C.

Cryptolestes ferrugineus adults exposed to grain treated with the ice nucleating bacteria *Pseudomonas syringae* had increased mortality compared with controls when exposed to -10°C. Treated insects froze at -9°C, whereas control insects froze at -17°C. The potential of using *P. syringae* as a grain protectant is discussed.

Introduction

Canada has zero tolerance for live grain-feeding insects in export wheat. Each year millions of dollars are spent controlling infestations of stored-product insects. The most common grain feeding insects found in Canadian grain are the Rusty Grain Beetle, *Cryptolestes ferrugineus* (Stephens) and the Red Flour Beetle, *Tribolium castaneum* (Herbst). Most of the cosmopolition stored-product pests are also established in Canadian stored grain (Madrid *et al.* 1990, Sinha and Watters 1985).

The use of low temperatures as a means of controlling stored-product insects has been studied extensively (Armitage 1987, Evans 1983, Smith 1970). It has several advantages over insecticides currently used to control stored-product insects. Unlike the contact insecticides, there are no residues left on the grain. There is little risk to the applicators, whereas there is some concern over the safety of the fumigant phosphine (Garry

[1]Mention of a proprietary product does not constitute endorsement by Agriculture Canada.

et al. 1989). Also, low temperature treatments are effective against pesticide resistant populations and reduce the growth of moulds.

There are also major drawbacks that prevent low temperature from being used extensively. Ventilation equipment is required for ambient air cooling and in some areas of Canada the cost to supply electrical power to granaries is prohibitive. Also low ambient air temperature is only available during the winter months, and in some areas these air temperatures are not cold enough to control the pest populations. Cold treatments can also take considerable time to effectively kill most of the insects, whereas chemical control methods take a few days. Finally, in certain situations, such as flour mills, water must be drained from pipes to prevent frost damage during cold treatment.

Recent work with a lady beetle has demonstrated that the supercooling point (SCP), the temperature at which freezing begins, is greatly increased when adults are exposed to ice nucleating bacteria (Strong-Gunderson *et al.* 1990). These results suggest that ice nucleating bacteria could be used as a cold-synergist to reduce the cold-hardiness of stored-product insects enough to use low temperature control in situations where low temperatures have previously not been effective.

The purpose of this study was three-fold. Firstly, to determine in the laboratory which developmental stage of *C. ferrugineus* is the most cold-hardy. Then to examine the overwintering survival and seasonal changes in cold-hardiness in field cold-acclimated individuals. Finally, to explore the potential of using ice nucleating bacteria (Lindow 1983) as a means of making stored-product insect pests more susceptible to cold treatment.

Materials and Methods

Laboratory Cold Acclimation

To compare the cold-hardiness of different developmental stages of *C. ferrugineus*, insects were reared on unbleached, white flour with 10% brewers' yeast (w:w). Adults for tests were obtained by letting *C. ferrugineus* adults oviposit on flour for 24 hours, rearing their progeny for 6 weeks at 30 ∓ 1°C, 60 ∓ 5% RH, sifting off all the adults and holding them for 2 weeks. Adults were placed 50 insects/vial, with each vial containing 8 g of hard red spring wheat cv. Columbus plus 5% wheat germ. Larvae were reared in a similar fashion. One group was reared for 12 days (3% I instar, 92% II instar, 5% III instar, n = 165; instars determined by head capsule width). Another group was reared for 19 days (12% III instars, 88% IV instars, n = 72). Larvae were placed 50 insects/vial, with each vial containing 7 g of flour + 10% brewers' yeast. Eggs were obtained by letting adults oviposit in flour for 24 hours and sifting the eggs off the flour using a 210 micron sieve. Eggs were then placed in plastic serological trays (Becton Dickson Labware), one egg per well.

Insects were either non-cold-acclimated (always held at 30°C) or cold-acclimated, before being tested for cold-hardiness by placing insects at -10°C for various durations, warming them up (5°C for 24 hours, then 30°C for 4 hours) and assessing their mortality. Twenty-five different cold acclimation temperature regimes were tested, but only the one (20°C for 7 days, 15°C for 21 days, 10°C for 21 days, 5°C for 21 days) that resembled fall granary temperatures and also gave the most cold-hardy individuals will be reported here. At all temperatures, once insects were placed in vials, they were held in desiccators over a saturated NaCl solution which maintains the relative humidity at 75%. Non-cold-acclimated

insects were left at -10°C for 1, 2, 7, 14, and 21 days. Cold-acclimated insects were left at -10°C for 0, 7, 14, 21, and 28 days before being warmed up and their mortality assessed. Eggs were considered dead if they did not hatch after 6 days at 30°C (control egg hatch was 97%, n = 150). Larvae and adults were judged dead if they were incapable of coordinated movement (never greater than 6%) or did not move at all.

Field Cold Acclimation

Adult *C. ferrugineus* laid eggs on hard red spring wheat moisterized to 16% MC (moisture content) with 5% wheat germ at 30°C, 60% RH and were sifted out 17 days later. Adults emerging from this wheat were sifted off 21 days later and held for 7 days before being placed into vials (100/vial), which contained 15 g of cracked hard red spring wheat moisturized to 16, 14 or 12% MC. The vials were placed into plastic tubes (6 vials/tube) and the remaining space in the tube filled with wheat (approximately 350 g) of the same moisture content as the vials. In late October 1989, the tubes were pushed 1 m down below the surface of a 100 tonne hard red spring wheat mass, which was held in a metal granary (5.8 m diameter, 4.6 m high). At this time the grain temperature around the tubes was 19.5°C. For each moisture content one tube was pulled from the granary at the beginning of each month. To estimate overwintering survival, one vial (100 insects) was placed directly into the warm-up regime (-5, 0, 5, 10°C each for 1 day and 30°C for 4 hours). To estimate cold-hardiness, the other 5 vials were placed at -10°C and held there for 7, 14, 21, 28, or 35 days, before being warmed up. The mortality assessment used was the same as for the laboratory cold acclimation experiments.

Thermocouples were placed in the grain mass at 1 m, 2 m, and 3 m below the top surface in the centre, on the tubes that contained the insects and outside the granary on the north side under the roof edge out of direct sunlight. Temperatures were taken every 5 minutes and daily maximums and minimums stored in a datalogger (CR10, Campbell Scientific).

At monthly intervals the grain 1 m below the top surface was sampled for moisture content. The moisture content remained stable throughout the study at 13.3 ∓ 0.1% MC (× ∓ SEM, n = 6). When tubes were pulled from the granary the moisture content of the grain surrounding the vials in the tube was measured. Grain that was initially moisturized to 16% MC had an average moisture content of 14.6 ∓ 0.1% MC, 14% MC had an average moisture content of 13.6 ∓ 0.2% MC and 12% MC had an average moisture content of 11.9 ∓ 0.1% MC (× ∓ SEM, n = 6).

Ice Nucleation - Active Bacteria

The source of ice nucleators was the same product that is used commercially in snowmaking and was provided by the Snomax Technologies Division of Genencor International Inc. (Rochester, N.Y., U.S.A.). To obtain this potent source of ice nucleators, *Pseudomonas syringae* (strain 31a), a common foliar bacterium isolated from corn leaves, is grown under conditions that maximize its ice nucleating activity. It is then concentrated, freeze-dried and killed with electron beam irradiation. The live end product has been shown to be non-toxic (acute oral LD_{50} for rats greater than 5 g/kg) and non-pathogenic to mammals or plants (Goodnow *et al.* 1990a,b). Pellets of *P. syringae* (ice nucleating activity

= 11.0) were placed into vials containing 8 g of hard red spring wheat cv. Columbus (MC = 16% with 10% cracked wheat) at 10, 100, 1000 ppm. Non-cold-acclimated *C. ferrugineus* adults (100/vial) were held on the treated wheat and a control without *P. syringae* for 1 or 7 days at 30°C, 75% RH. One set of insects was placed at -10°C for 1 day, 5°C for 1 day, and 30°C for 4 hours before having their survival assessed. Another set of insects was taken directly from 30°C and their supercooling point measured using a 42 channel data logger (CR7, Campbell Scientific).

The INA (ice nucleating activity) of the wheat was measured using a technique modified from Vali (1971). A weighed amount of wheat was placed in 100 ml of phosphate buffer (0.01 M potassium phosphate, pH between 6.95 and 7.20) and stirred for 20 minutes at 0°C. One hundred 10 μl droplets are placed on a cold plate covered with aluminum foil coated with a thin, even layer of petroleum jelly. The solution is diluted until about 50% of droplets freeze after 5 minutes at -5°C. Ice nucleating activity is the log of the number of ice nucleating sites per gm, and calculated by the following formula:

$$INA = \log\left(\frac{\ln(N/F)}{D/V \times W}\right)$$

Where N is the total number of drops spotted, F is the number of drops not frozen, D is drop volume, V is the total volume of buffer used to dilute sample, and W is the weight of the sample.

To test the effect of *P. syringae* on the cold hardiness of cold acclimated insects, *P. syringae* pellets were ground into a fine powder using a mortar and pestle and mixed into wheat (16% MC) at 0, 2, 50, 100, and 1000 ppm. One hundred adult *C. ferrugineus* were placed onto 8 g of wheat and held at 30°C for 6 days, and 10°C for 28 days before being placed at -10°C for 0 to 21 days. Mortality was assessed as in the laboratory acclimation experiments.

Results

Laboratory Cold Acclimation

Non-cold-acclimated insects from all stages were all dead after 7 days at -10°C. Cold-acclimated insects survived for much longer times at -10°C than those that were not acclimated (lethal $time_{50}$: II instar = 7 d, IV instar = 14 d, adult = 57 d; lethal $time_{95}$: II instar = 14 d, IV instar = 21 d, adults = 95 d). Eggs did not survive the cold acclimation period.

Field Cold Acclimation

Despite extremely cold air temperatures of -35°C outside the granary, grain temperatures 1 m below the grain surface only reached a minimum of -6°C in March. Substantial populations of *C. ferrugineus* adults survived the winter when placed in the top 1 m of 100 tonne grain mass. After February, grain moisture content at 12% MC increased overwintering mortality (Table I). Cold-hardiness, as measured by survival time at -10°C,

Table I. The overwintering mortality and the seasonal changes in the cold-hardiness of adult *Cryptolestes ferrugineus* held at different moisture contents inside 100 tonnes of wheat over the winter of 1989-1990. Cold-hardiness is measured by survival after 7, 14, 21, 28, and 35 days at -10°C.

Date	Temperature[1] (°C)		Overwintering Mortality[2] (%)			Mortality at -10°C (Days)[3]					
			Grain Moisture Content (%)			Lethal Time$_{50}$ (95% Fiducial Limits)			Lethal Time$_{95}$ (95% Fiducial Limits)		
						Grain Moisture Content (%)			Grain Moisture Content (%)		
	Air	Grain	16	14	12	16	14	12	16	14	12
Nov	-17.0	14.6	4	0	4	5 (5,6)	6	6 (5,6)	10 (9,11)	11	11 (10,12)
Dec	-28.2	10.3	0	0	3	17 (3,27)	16 (-5,28)	11 (-11,21)	35 (25,73)	37 (25,92)	28 (19,73)
Jan	-18.0	3.8	2	3	3	29 (27,31)	35 (29,52)	31 (29,34)	50 (45,58)	62 (43,-)	51 (46,60)
Feb	-20.3	-2.2	7	7	40	40 (34,54)	30	24 (22,27)	73 (57,161)	76 (33,-)	38 (34,43)
Mar	-7.6	-5.6	17	29	47	25 (-,32)	12 (-3,18)	11 (-,26)	45 (36,-)	34 (31,39)	33
Apr	-	-	57	46	92	11 (7,14)	<7	<7	25 (23,27)	<14	<14

[1]Mean of daily minimum temperatures throughout the entire month.
[2]Adults taken from granary at the beginning of each month.
[3]Probit analysis, corrected for overwintering mortality. Those without fiducial limits were estimated from a graph.

increased until February when, as was the case with overwinter survival, the level of cold-hardiness declined until the end of the winter. Grain moisture content also affected cold-hardiness, with the insects held at the lower moisture contents generally having lower cold-hardiness.

Ice Nucleation - Active Bacteria

Pseudomonas syringae greatly reduced the cold-hardiness of non-cold-acclimated *C. ferrugineus* adults. Supercooling points rose with increasing concentrations of *P. syringae*, as did the cold-induced mortality. Survival of the adults held on *P. syringae* at 30°C was high (100 to 95%) and showed no dose dependant mortality. After 7 days at 30°C, *P. syringae* still increased mortality though not as much as after 1 day (Table II).

Table II. Supercooling points (SCP) and mortality after 24 hrs at -10°C of non-cold-acclimated adult *C. ferrugineus* held on wheat containing various concentrations of *P. syringae* (pellet form) and the ice nucleating activity (INA) of wheat held for 1 or 7 days at 30°C, 75% RH.

Period held at 30°C (days)	Concentration of *P. syringae* (ppm)	SCP[1] (°C)	SCP above -10°C (%)	Mortality[2] (%)	INA (g^{-1})
1	0	-17.0 ∓ 1.0 (34)	19	8	0.72
	10	-12.8 ∓ 0.8 (36)	38	31	4.2
	100	-14.8 ∓ 1.2 (30)	41	45	5.5
	1000	-8.1 ∓ 0.5 (36)	93	81	7.7
7	0	-16.8 ∓ 1.0 (37)	21	4	<1
	10	-14.8 ∓ 1.0 (34)	33	17	2.8
	100	-11.0 ∓ 0.6 (38)	71	33	3.3
	1000	-9.1 ∓ 0.4 (38)	86	43	4.9

[1]Mean ∓ SEM with n below.
[2]Mortality after 24 hrs at -10°C, 24 hrs at 5°C, and 4 hrs at 30°C, n = 100/treatment.

Pseudomonas syringae also increased the mortality of cold-acclimated *C. ferrugineus* adults. After 7 to 21 days at -10°C, insects held on *P. syringae* treated wheat had approximately 20% higher mortality than controls (Table III).

Table III. The mortality of cold acclimated adult *C. ferrugineus* that had been placed on wheat containing various concentrations of *P. syringae* (powder form), held at 30°C, 75% RH for 6 days, 10°C, 75% RH for 28 days, and then placed at -10°C for 0 to 21 days before assessing mortality.

Time at -10°C (days)	Mortality (%)[1]					
	Concentration of *P. syringae* (ppm)					
	0	2	10	50	100	1000
0	17	23	23	20	25	21
1	44	47	31	29	35	37
2	32	13	26	20	29	45.*
7	31	39	50 **	55 **	45 **	51 **
14	37	37	34	46 *	46 *	69 **
21	45	43	38	55	61 **	64 **

[1]Differences between 0 ppm concentration and diets treated with various concentrations of *P. syringae* for a given time at -10°C was tested using a single tailed confidence limit for percentages test (Sokal and Rohlf 1981), $p \leq 0.05$ indicated by *, $p \leq 0.01$ indicated by **.

Discussion

My ultimate goal is to provide guidelines for the use of low temperature to control insects in grain. The basic questions, of how long and how cold must it be to control stored-product insect pests, are complicated by changes in insect cold-hardiness due to cold acclimation, stage specific susceptibility, or grain moisture content. The preliminary results presented in this paper on the cold-hardiness of cold-acclimated *C. ferrugineus* indicate that if all the adults are killed, the larvae and eggs, being less cold hardy would also be killed. Work on cold-acclimated pupae is not complete, but if the trends in non-cold-acclimated individuals (Smith 1970) are an indication of the cold-hardiness of cold acclimated individuals, then adults will be more cold hardy than pupae in field situations.

Field cold acclimated *C. ferrugineus* adults have cold-hardiness similar to those acclimated in the laboratory (Smith 1970, this paper). These results indicate that the grain must be cooled to -10°C for approximately 75 days to kill 95% of the most cold hardy *C. ferrugineus*. However, this is only an extrapolation via the probit analysis, as insects were only held a maximum of 35 days at -10°C. This time could be reduced if the level of cold-acclimation was known for the insects in a particular grain mass. However, as grain

temperature varies greatly depending upon harvest conditions, type of granary and year-to-year variations in ambient temperatures, I doubt that a recommendation based on a calendar date would be sufficient. Data from several granaries over several years are needed to make sound recommendations.

Grain moisture content greatly affected overwintering survival, indicating that the cause of overwintering mortality may be desiccation rather than low temperature. Grain moisture content also affects cold-hardiness, but further studies are needed to determine if the effects are great enough to warrant including them in recommendations to grain managers.

In the Canadian Prairies, ventilating grain between December and February would lower grain temperatures enough to kill any insects infesting the grain. However this is not possible in many situations as only about 15% of granaries in the Prairies are equipped with ventilation (Madrid *et al.* 1990) and primary and terminal elevators generally do not have aeration. It is a common practice to turn the grain in the winter, but it is difficult to cool the grain a great deal using this method. Also, for areas with milder winters, even for granaries equipped with aeration, it is often difficult to lower the grain temperature enough to control stored-product insects. For these situations a cold synergist, such as ice nucleation - active bacteria, could greatly increase the effectiveness of low temperature as a control measure.

Theoretically, insects exposed to ice nucleation - active bacteria could be killed at -1°C in less than 1 hour, as there are strains of *P. syringae* that nucleate at -1°C (Lindow 1984) and stored-product insects are freeze intolerant. Realistically, insects would have to be exposed to -5 to -10°C for 1 week before substantial mortality occurred. To realize this potential more work has to be done on the formulation, the method and timing of application. Preliminary work with the *C. ferrugineus* on wheat at 14% MC and 20°C has shown a dramatic increase in the duration of activity as compared with grain held at 30°C, 16% MC. Further research is necessary to determine how the ice nucleating activity changes with grain temperature, moisture content, and type. For ice nucleating bacteria to be an effective grain protectant it must show activity against the major stored-product insect pests. Preliminary results indicate that *P. syringae* is active against other stored-product pests (Richard Lee, personal communication), however more tests are needed to verify activity against cold acclimated individuals.

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LA RESISTANCE AU FROID DE SOUCHES SAUVAGES OU D'ELEVAGE DE *CRYPTOLESTES FERRUGINEUS*

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RESUME

On a utilisé pendant de nombreuses années les basses températures pour éliminer les populations d'insectes des produits stockés. Cependant, il n'existe que peu d'informations décrivant comment ces déprédateurs régissent à ces températures en milieu naturel. L'endurance au froid est modifiée chez les insectes par de nombreux facteurs dont l'un des plus importants est le niveau d'acclimatation au froid. La résistance de *Cryptolestes ferrugineus*, l'insecte ravageur du grain le plus répandu et le plus tolérant au froid du Canada, a été étudiée afin d'utiliser les basses températures comme moyen de lutte. Dans les fermes des prairies canadiennes des alentours de Winnipeg (Manitoba) une importante proportion d'adultes et de stades immatures de *C. ferrugineus* peut survivre tout l'hiver à l'intérieur des cellules de stockage non chauffées et non aérées. Plus l'hiver progresse et plus les adultes sont difficiles à détruire aux basses températures. En automne, tous les adultes meurent après deux semaines d'exposition à -10° C, tandis qu'à la mi-hiver, les adultes récoltés dans les cellules présentent un taux de survie allant jusqu'à 60 % après 4 semaines d'exposition à -10° C. Cette résistance au froid disparait complètement chez les adultes maintenus à 30° C pendant une semaine mais elle se maintient chez ceux ayant été exposés à 10° C. Un grain plus sec a fait diminuer la survie hivernale et diminue la résistance au froid. Les basses températures provoquent également des effets sub-létaux. Une exposition à -10° C pendant 4 semaines a provoqué une réduction de la fécondité des femelles survivantes de 35 %. En laboratoire, plusieurs stades de *C. ferrugineus* ont été exposés à des chutes brutales ou progressives de températures avant la mesure d'endurance. C'est avec une diminution plus lente de température qu'il y a eu le plus d'individus résistants aux basses températures avec, dans l'ordre décroissant de tolérance aux basses température : stade larvaire > deuxième stade larvaire > oeufs.