

Cold hardiness strategy in field collected larvae of *Scrobipalpa ocellatella* (Lepidoptera: Gelechiidae)

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Abstract

The beet moth, *Scrobipalpa ocellatella* is recognized as a widespread agricultural pest. Cold hardiness strategy of the beet moth larvae was investigated through monitoring seasonal changes at supercooling points and lower lethal temperatures. Furthermore, the role of microhabitat in winter survival was studied. The mean SCPs of the last instar larvae was not significantly different from November 2010 to April 2011. Mean inoculative freezing point (-8.0 ± 1.44 °C) of the last instar larvae was significantly higher than mean SCP (-14.9 ± 0.93 °C). The cold hardiness of the pest shows seasonal fluctuation in response to reduction of air temperature. A 50% mortality (LT50) occurred at -11 °C in November and -14 °C in January and reduced to -18 °C in February and finally increased to -14.5 °C in April. Glycerol, sorbitol, trehalose, and myo-inositol were identified components in whole body extracts of *S. ocellatella* larvae. However, total cryoprotectants could not have significant effects on the cold tolerance. Larvae of *S. ocellatella* could tolerate subzero temperatures near their SCPs. Our findings show that beet moth larvae utilize moderately chill tolerance strategy during winter.

Key words: cold hardiness, inoculative freezing point, lower lethal temperature, beet moth larvae, supercooling point, Iran

چکیده

استراتژی سرما سختی لاروهای بید چغندر قند (*Scrobipalpa ocellatella* (Lepidoptera: Gelechiidae) جمع آوری شده از مزرعه زهرا گنجی و سعید محرمی پور

بید چغندر قند *Scrobipalpa ocellatella* آفتی با پراکنش گسترده است. به منظور مطالعه ساز و کار سرماسختی لاروهای بید چغندر قند، تغییرات فصلی نقطه انجماد و حداقل دمای کشنده مورد بررسی قرار گرفت. همچنین نقش زیستگاه در زنده ماندن حشرات مطالعه شد. میانگین نقطه انجماد لاروهای سن آخر از آبان ماه ۱۳۸۹ تا فروردین ۱۳۹۰ تفاوت معنی دار نداشت. میانگین نقطه انجماد تلقیحی (-8.0 ± 1.44 °C) لاروهای سن آخر به طور معنی داری بیشتر از میانگین نقطه انجماد (-14.9 ± 0.93 °C) بود. با کاهش دمای هوا، در سرماسختی آفت نوسانات فصلی مشاهده شد. حداقل دمای کشنده برای مرگ و میر ۵۰ درصد از افراد جمعیت (LT50) در آبان ماه، -11 درجه سلسیوس بود و در دی ماه به -14 درجه رسید. سپس تا -18 درجه سلسیوس در بهمن کاهش یافت و پس از آن تا -14.5 درجه سلسیوس در فروردین افزایش مشاهده شد. ترکیبات شناسایی شده در عصاره کل بدن لاروهای بید چغندر قند، گلیسرول، سوربیتول، ترهالوز و میواینوزیتول بود. لاروهای بید چغندر قند می توانند دماهای زیر صفر درجه نزدیک به نقطه انجماد خود را تحمل نمایند. یافته ها نشان می دهد که لاروهای بید چغندر قند از سازوکار تحمل متوسط به سرما در طی زمستان بهره می برند.

واژه های کلیدی: سرماسختی، نقطه انجماد تلقیحی، حداقل دمای کشنده، لاروهای بید چغندر قند، نقطه انجماد، ایران

Introduction

Cold hardening of arthropods is an important physiological adaptation that is activated mainly by low temperatures and short photoperiod (Horwath & Duman, 1982). Cold hardiness can be determined by combination of indices such as supercooling points (SCPs) and survival capacity at low temperatures (Leather *et al.*, 1995). In order to cope with extreme cold, insects exhibit diverse strategies. Insects survive subzero temperatures by either maintaining their body fluids in a liquid state at

temperatures that they might otherwise be expected to freeze (freeze avoidance) or by withstanding the formation of internal ice (freeze tolerance) (Lee, 2010). Most insects are freeze avoidant and by contrast to freeze tolerant species, ice formation within their tissues is fatal (Morewood, 1992). Overwintering freeze avoidant insects must adapt behaviorally, physiologically and biochemically to survive freezing temperatures (Zachariassen, 1985; Block *et al.*, 1990; Duman *et al.*,

1991). Behavioral compatibility includes avoidance of lethal temperatures. Many overwintering insects avoided subzero temperatures by seeking sheltered microhabitats (Lee, 1989). Freeze tolerant species employ exogenous or endogenous ice nucleating agents (INAs) to stimulate ice growth (Lee *et al.*, 1993). Seasonal accumulation of low molecular weight carbohydrates (sugars and polyols) plays a principal role in overwintering abilities which may affect freezing and SCPs of insects (Lee, 2010).

The beet moth, *Scrobipalpa ocellatella* (Boyd) (Lepidoptera: Gelechiidae) is one of the most important pests of beet. This pest has three to six generations per year in Iran where is first reported from the city of Karaj in 1936 (Kheyri *et al.*, 1980). *S. ocellatella* has five larval instars and overwinters at different larval stages within non-harvested beet roots and beets left in the fields. The overwintering sites of *S. ocellatella* expose this pest in close contact with high humidity and frost at subzero temperatures which could be lethal. In Iran, sugar beet sowing is carried out in May and harvesting usually starts late October and early November. Most population densities of larvae can be observed at harvest time. Previous studies provided data on the biology (Kheyri *et al.*, 1980; Valich *et al.*, 2005; Timus & Croitoru, 2006), control (Saad & El-Abhrawi, 1977; Robert & Blaisinger, 1978; Marie, 2004; Sabry *et al.*, 2011; Arnaudov *et al.*, 2012) and supercooling points and cold tolerance trends of the beet moth (Ganji & Moharrampour, 2015a,b).

This study is intended to describe cold hardiness strategy and discuss the overwintering success of the beet moth larvae by measuring SCP, cold tolerance, and cryoprotectant contents during winter by emphasis on the type of microhabitat.

Materials and Methods

Collection of the test specimens

The sugar beet root heads were collected monthly through winters 2010 and 2011 in Karaj, Iran (35°83'96"N, 50°86'63"E; 1293 m above sea level). About 80-100 Kg root heads were randomly picked at each sampling site and transferred to the laboratory. The plants were cut open and last instar overwintering caterpillars carefully removed. In order to avoid inoculative freezing, the surface of larval bodies were dried using paper towel. Large, active larvae were collected to ensure their healthiness. In order to preserve

natural condition during the experiments, the sugar beets containing larvae held outside in ambient condition.

Weather Data

Weather data including daily mean, maximum and minimum temperature were obtained from the nearest meteorological station, Karaj Weather Station (35°48'23"N, 50°57'14"E).

Measurement of SCPs

The full protocol is provided by Khani *et al.* (2007). The SCPs were measured monthly from November 2010 to April 2011. To determine SCPs, about 20 larvae within a month were attached, in close contact with the thin thermocouple probe (NiCr-Ni probe), on ventral side of the body using tanglefoot (a substance used on insect adhesive traps) and sticky tape. Larvae were placed in the programmable thermal chamber (MK53, Binder, Germany) with the cooling rate of 0.5 °C per minute. The supercooling temperature was recorded at the moment of heat release during crystallization of supercooled liquid and indicated by the sudden increase in temperature (Lee, 2010). The lowest temperature prior to the temperature increase was recorded as the SCP.

Measuring the inoculative freezing points (IFP)

Ice inoculation was achieved by cooling the caterpillars wrapped in the cotton soaked in distilled water (see: Zachariassen & Kristiansen, 2000, for definition). A cooling rate of 0.5 °C per minute was used and the minimum temperature set at -30 °C. This experiment was performed in January 2010. The results were compared with SCPs in January 2010 as control.

Exposure to low temperature

To evaluate survival at low temperatures ranging from -5 to -21 °C for 2 h, larvae were confined in 15 ml test tubes sealed with parafilm. A dry strip of paper towel was placed into each test tube to absorb condensation. Test tubes were put inside the programmable thermal chamber where the temperature lowered at a cooling rate of 0.5 °C/min. After 2 h exposure, the larvae were removed and left to recover in the laboratory for 24 h at room temperature (about 25 °C) during which the larvae fed on sugar beet leaves. After 24 h, the insects that did not respond to stimulation, were considered dead. There

were four replicates for each treatment and each treatment contained at least 16 individuals. On the average 127 larvae was used to measure several parameters such as SCP, cold tolerance a month from November to April.

According to Saeidi *et al.* (2012), we determined the lower lethal temperature at which 50 and 80 percent of the larvae are dead after 2 h exposure to subzero temperatures (LT₅₀ and LT₈₀, respectively).

Microhabitat importance

In order to study the role of microhabitat for winter survival, last instar larvae were exposed to five different temperatures from -5 to -15 °C for 2 hours with a cooling rate of 0.5 °C/min. Cold exposures were done in two ways: directly (larvae removed from sugar beet root head) and indirectly (without any manipulation in natural habitat). We separated 149 larvae, at their latest stage, from plants and exposed them to subzero temperatures. In the second experiment, infested sugar beets containing larvae were exposed to subzero temperatures for 2 h and then put in room temperature. After 24h the sugar beets were cut open and mortality rate of the larvae (n = 263) were determined.

In order to study the rate of temperature drop in microhabitat at subzero temperatures, sugar beets were directly placed in thermal chamber and the temperature reduced to -30 °C at a cooling rate of 0.5 °C/min. Sensors of temperature recorder were placed inside the feeding canals (root heads and petioles) and outside of the fresh sugar beet plants using four sensors; three inside and one outside the plant. The experiments were established in four replications in November 2010.

Measurement of sugars and polyols

Following Atapour & Moharramipour (2009), we studied the type and amount of sugars and polyols of last instar larvae during November, February and April. Three replicates for each month and three larvae for each sample were used. Each sample was homogenized in 2 ml of ethanol 80% and centrifuged at 12,000 g for 15 min at 20 °C. Then supernatant was evaporated at 30 °C in a vacuum drying oven, model VO 400 (Memmert, Schwabach, Germany) and later dissolved in 200 µl of HPLC grade water. Sugars and polyols were analyzed by HPLC (Waters, Milford, MA) equipped with a

supelcogel carbohydrate column (Supelco, Bellefonte, PA). Each HPLC run was stopped after 40 min. Several standards of sugars and polyols such as glucose, trehalose, glycerol, sorbitol and *myo*-inositol were injected to HPLC. Sugars and polyols were identified according to the retention time of standards and the retention time of peaks appeared in the injected samples.

Statistical analysis

The distribution of individual insects was studied by median SCP (-13.5 °C) as a break point (See Sinclair *et al.*, 2003; Chen & Kang, 2005; Cannon & Block, 1988). Therefore, insects with a value of -13.5 °C at the midpoint of the frequency distribution were placed above or below with equal probability. Individuals were divided into a lower group (LG, SCP < -13.5 °C) and higher group (HG, SCP > -13.5 °C). Data were checked for normality and, if needed, an arcsine square-root transformation was used. A One-way analysis of variance (ANOVA) and independent-samples t-test was used to test for differences between groups using SPSS version 18.0 (SPSS Inc., 2009). Once significant differences among treatments were detected, the means were separated by Tukey's post hoc honestly significant difference (HSD) test. A Pearson correlation test was used to investigate the relationship between two variables. LT₅₀ and LT₈₀ were determined using binary logistic model. Logistic regressions were calculated from the binary (alive or dead) mortality data.

Results

Weather data

Seasonal change in maximum, average and minimum temperatures from September 2010 to April 2011 are shown in Fig. 1. The average air temperature ranged from 11.1 °C in November to 0.6 °C in January and increased to 15.6 °C in April. The lowest minimum temperature varied from 1.4 °C in November to -12.6 °C in January and increased to 3.6 °C in April.

Supercooling points (SCPs)

Field collected larvae displayed relatively high supercooling capacity. SCPs of individuals varied from -6 to -25 °C. Mean monthly SCPs varied from -18.3 ± 0.92 in November to -14.9 ± 0.93 °C in January (Table 2). Variation of SCP among sampling dates (from

November 2010 to April 2011) was not significant ($F=1.552$; $df= 5,128$; $P= 0.178$). There was no significant correlation between monthly SCP and ambient temperature (maximum, mean and minimum).

Inoculative freezing point (IFP)

The IFPs of last instar larvae ranged from -2 to -20 °C during inoculative freezing. Unlike dry condition (SCP = -14.9 ± 0.93 °C), wet caterpillars had limited ability to supercool. Caterpillars froze easily at -8.0 ± 1.44 °C when body was in contact with water. Only 37% of the individuals were in HG in dry condition; while about 90% of the wet caterpillars placed in LG group and may froze at temperatures higher than break point (Fig. 2). Frequency of distribution of SCPs indicated that the

Lower lethal temperatures

Lethal temperature for 50% mortality (LT_{50}) decreased from -11.4 °C in November to -18.0 °C in February, and then increased to -14.6 °C in April. LT_{80} value decreased from -14.0 °C in November to -15.5 and -16.7 °C in December and January, respectively. The minimum LT_{80} (-22.4 °C) was observed in February. Therefore, 20% of individuals tolerated -22.4 °C (5 °C below their mean SCP) for 2 hours (Table 2).

Microhabitat importance

No significant differences were observed between direct and indirect exposure to -5, -7 and -10 °C. Most of the specimens tolerated -5 °C for 2 h. Survival rate of larvae inside the plant were 95% and 94% at -7 and -10 °C, respectively. There were significant differences between direct and indirect exposure to -12 °C ($t = -3.483$; $df = 4$; $P < 0.05$) and -15 °C ($t = -16.761$; $df = 5$; $P < 0.001$) for 2 h. During direct cold exposure only $36 \pm 7.63\%$ and $12 \pm 2.05\%$ of caterpillars from sugar beet root heads successfully tolerated -12 and -15 °C for 2 h, respectively; while, 89 ± 11.11 and $82 \pm 2.86\%$ of larvae survived when they were left in their natural habitat inside the plants (Fig. 3).

Significant differences were observed between temperatures inside and outside plants at subzero temperatures ($t = -4.167$; $df = 30$; $P < 0.001$). When the

larvae had non-normal distributions that could be considered bimodal.

Cold tolerance

The lowest tolerance at subzero temperatures was observed in November and the highest was found in February. All caterpillars survived at -5 °C for 2 h from November 2010 to April 2011 and showed low tolerance at -15 °C for 2 h (about 90% mortality) in November. At the same condition, the mortality decreased to 60% in January. This value was 15% in March and 25% in April. Caterpillars died at -19 °C for 2 h in January. At the same condition, the mortality rate was 41% in February and about 70% and 100% in March and April, respectively (Table 1).

chamber temperature reached -30 °C, mean temperature inside sugar beet plants was about -10 °C (Fig. 4).

Sugars and Polyols

Myo-inositol, trehalose, and glycerol were identified components in whole body extracts of *S. ocellatella* larvae. No significant differences were observed in *myo*-inositol level ($F = 1.091$; $df = 2, 6$; $P = 0.394$). It ranged from 1.29 ± 0.03 $\mu\text{mol/g}$ fresh weights (fw) in November to a peak of 1.90 ± 0.76 $\mu\text{mol/g}$ fw in February and decreased to 0.85 ± 0.43 $\mu\text{mol/g}$ fw in April.

The level of trehalose differed significantly during three months of sampling ($F = 6.677$; $df = 2, 6$; $P < 0.05$). The lowest trehalose content was 0.68 ± 0.25 $\mu\text{mol/g}$ fw in November and the highest amount (3.31 ± 0.68 $\mu\text{mol/g}$ fw) was measured in April. There was no difference in glycerol levels ($F = 4.484$; $df = 2, 6$; $P = 0.064$). The highest level of glycerol (2.49 ± 0.72 $\mu\text{mol/g}$ fw) was found in February.

There were no significant differences in total cryoprotectants from November to April. The lowest amount (3.45 $\mu\text{mol/g}$ fw) was found in November and the highest amount (5.7 $\mu\text{mol/g}$ fw) in February and decreased to 4.89 $\mu\text{mol/g}$ fw in April. Sorbitol was found at trace amounts (Table 3).

Table 1. Mortality rate of field collected larvae of *Scrobipalpa ocellatella* exposed to cold temperature (°C for 2 h) from November 2010 to April 2011. Mortality was checked after 24 h.

Exposure Temperature (°C)	Mortality (%)					
	Nov	Dec	Jan	Feb	Mar	Apr
-5	0	0	0	0		0
-7	25.0	0	0	0		25.0
-10	22.5	12.5	0	19.0	25.0	21.4
-12	62.2	55.0	45.0	19.0	12.5	25.0
-15	87.8	79.2	57.1	31.3	12.5	25.0
-17			66.7	27.3	44.0	68.8
-19			100	41.2	71.0	100
-21				100	100	

Table 2. Mean SCP and LT50 values of field collected larvae of *Scrobipalpa ocellatella* during November 2010 to April 2011.

Month	SCP ± SE (°C)	LT ₅₀ (Lower, Upper)* (°C)	LT ₈₀ (Lower, Upper) (°C)
Nov	-18.3 ± 0.92	-11.4 (-11.5, -11.3) a	-14.0 (-15.2, -13.4) a
Dec	-15.6 ± 1.01	-12.4 (-12.8, -12.2) b	-15.5 (-18.1, -14.4) b
Jan	-14.9 ± 0.93	-14.2 (-14.3, -14.1) c	-16.7 (-18.1, -16.0) b
Feb	-17.4 ± 1.09	-18.0 (-18.7, -17.6) f	-22.4 (-25.6, -20.9) d
Mar	-15.5 ± 1.22	-17.1 (-17.2, -17.0) e	-19.4 (-20.9, -18.8) c
Apr	-16.7 ± 1.03	-14.6 (-14.7, -14.5) d	-19.1 (-21.7, -17.7) c

* Different letters in a column are considered significantly different if their 95% confidence interval (CI) did not overlap.

Table 3. Changes in the identified carbohydrates in whole bodies of *Scrobipalpa ocellatella* larvae from November to April.

Month	Mean ± SE (µmol/g f.w) ^a				
	Trehalose	Glucose	Myo-inositol	Glycerol	Sorbitol
Nov	0.68 ± 0.25 b	ND	1.29 ± 0.03a	1.48 ± 0.06 a	Trace
Feb	1.31 ± 0.57 ab	ND	1.90 ± 0.76 a	2.49 ± 0.72 a	Trace
Apr	3.31 ± 0.68 a	ND	0.85 ± 0.43 a	0.73 ± 0.05 a	Trace

^a Means with the same letters are not significantly different (One-way ANOVA followed by Tukey's test, $P < 0.05$).

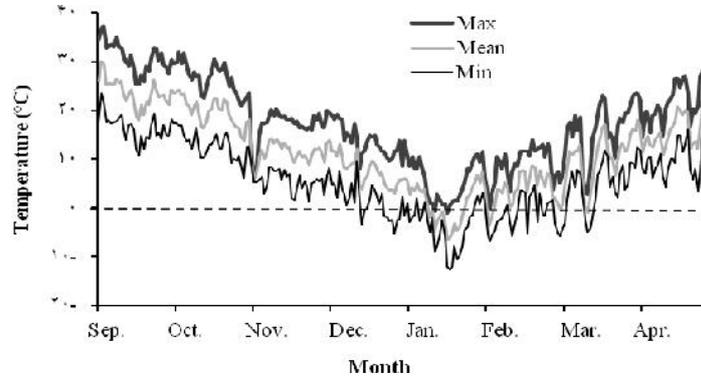


Fig. 1. Seasonal changes air temperature at the nearest weather station to collection site. Data are based on means calculated from data collected by the Karaj meteorological station from November 2010 to April 2011.

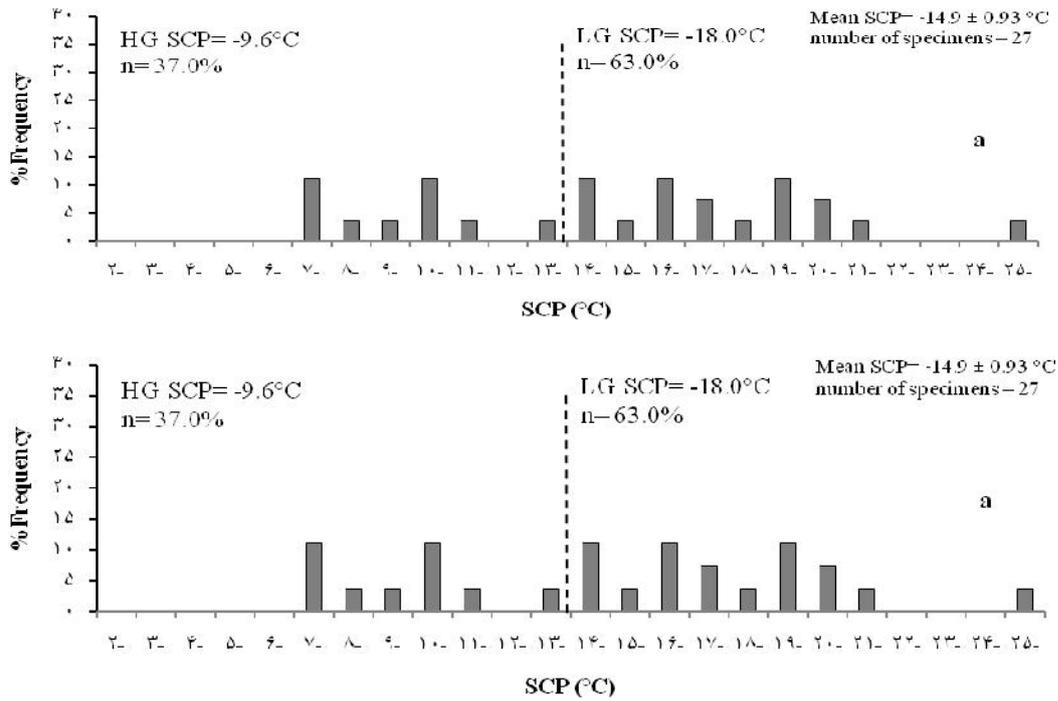


Fig. 2. Bimodal frequency distribution of SCP in larvae of *Scrobipalpa ocellatella* showing difference between dry (a) and moist (b) condition. The break point is designated at -13.5 °C between higher group (HG; SCP > -13.5 °C) and lower group (LG; SCP < -13.5 °C). Mean SCP, the percentage in LG or HG and mean SCPs in each group are indicated in the figure.

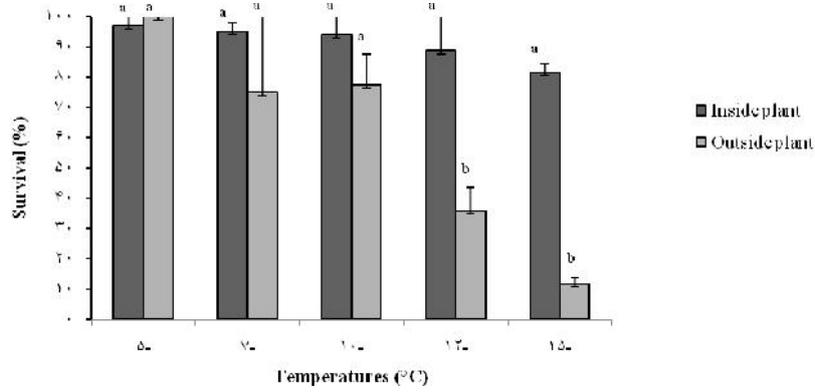


Fig. 3. Survival rate of last instar larvae of *Scrobipalpa ocellatella* after 2 h exposure at subzero temperatures in direct (larvae separated from sugar beet plants) and indirect (larvae left in natural microhabitat) exposure. Vertical lines in the graph indicate standard error of mean. Means followed by the same letters are not significantly different (t - test, $P < 0.05$).

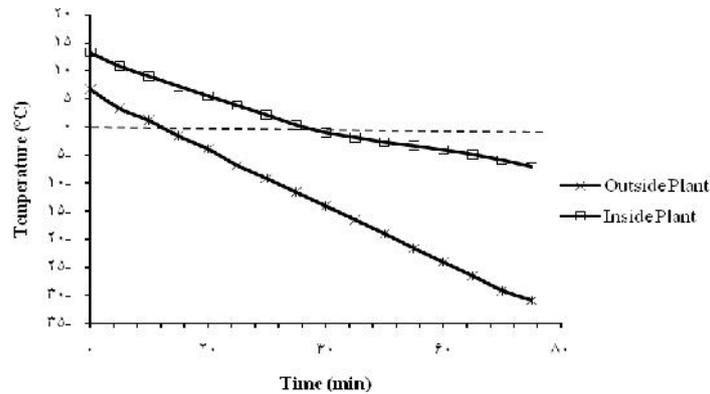


Fig. 4. Mean temperatures inside and outside of sugar beets. A cooling rate of 0.5°/min was used and the minimum temperature was set to -30 °C (t - test, $P < 0.001$).

Discussion

The mean SCPs of field collected beet moth larvae ranged from -14.9 to -18.3 °C and showed no significant change over autumn and winter. Similarly, the SCPs of other gelechiid moth larvae, *Pectinophora gossypiella* (Saunders) show no significant changes (-14.6 to -17.1 °C) during winter (Kalsta *et al.*, 2006). The SCP of the cabbage maggot remained relatively unchanged throughout the year while the cold tolerance

changed through the season (Košťál & Šimek, 1995). According to our experiments, the caterpillars with dry body surface may have a greater capacity to supercool than moist ones because of higher frequency for ice inoculation and lethal freezing. The effect of surface moisture has been investigated on many insect species (Larsen & Lee, 1994; Klok & Chown, 2005; Coyle *et al.*, 2011; Boardman *et al.*, 2012). It has been reported that

external moisture induces the freezing of body fluid at subzero temperatures (Zachariassen, 1985). Beet moth larvae shelter in sugar beet plants where microenvironment is more stable than surrounding areas. Some authors have underscored the type of winter microhabitats for the survival (Danks & Jones, 1978; Bale, 1987). Freeze avoidant species usually avoid moist sites because any contact with water drops or ice triggers freezing that is usually fatal. A similar pattern have been observed with the larvae of *Ostrinia nubilalis* (Hübner) that overwinter in the stalks of host plant (Coll & Bottrell, 1991) where the fluctuation of temperature is lower than in the air.

Usually cold hardiness increases with decreasing ambient temperature as in beet moth larvae that occurs seasonally in response to subzero temperatures. The cold hardiness of an insect species is closely related to the environmental conditions and the minimum temperature that they experience in the long term. Larvae could tolerate subzero temperatures (for 2 h) near their SCPs. Interestingly, 2 and 24 h exposure to subzero temperatures showed almost identical results such as 35% and 38% mortality respectively after being exposed to -19 °C (Ganji & Moharramipour, 2015a). Most of beet moth larvae failed to tolerate temperatures below their SCP except those in February and March (Table 2). Therefore, it suggests that beet moth larva is almost a freeze avoidant insect, although a portion of population was able to tolerate temperatures below their SCP (SCP = -17.4 °C and LT₈₀ = -22.4 °C). Beet moth should be

classified as moderately chill tolerant (Bale 1996). It has been reported that many species in temperate region utilize freeze avoidance strategy (Chen & Kang, 2002; Khani & Moharramipour, 2010; Rozsypal *et al.*, 2013; Heydari & Izadi, 2014).

In the present study, we find no significant correlation between cold hardiness and cryoprotectant contents in overwintering larvae. The low rate of *myo*-inositol, trehalose, and glycerol remained during autumn and winter was indicative of the level of feeding in these months whenever the weather was favorable (Ganji & Moharramipour, 2015b). However, in *Hyphantria cunea* (Drury), trehalose, glycerol, and *myo*-inositol are the main carbohydrates contributed in protection against cold (Li *et al.*, 2001). In many insects glycerol, sorbitol and trehalose are accumulated in response to low temperatures (Pullin & Bale, 1989; Storey & Storey, 1991; Jo & Kim, 2001; Bale, 2002; Izumi *et al.*, 2005; Khani *et al.*, 2007; Atapour & Moharramipour, 2009; Andreadis *et al.*, 2011).

Our findings indicated that sugar beet root heads are suitable sites for winter survival of beet moth larvae, although they showed high supercooling and cold tolerance capacities. Subzero temperatures, during the winter, do not represent a major threat to the survival of the pest larvae in Karaj, Iran where large proportions of overwintering larvae are able to endure low winter temperatures and successfully enter the soil and pupate. However, overwintering physiology of the pupae is remained unknown.

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