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Effect of cold acclimation and rapid cold hardiness on cold tolerance and cryoprotectants of the greenbug *Schizaphis graminum* (Hemiptera: Aphididae)

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Abstract
Insects can increase their survival at subzero temperatures, prior to long or short term exposure, to non-lethal cold temperatures by cold acclimation (ACC) or rapid cold hardiness (RCH). In this research, the effect of rapid or gradual decrease in temperatureon cold tolerance of adults of the greenbug, *Schizaphis graminum* (Rondani) was investigated. LT_{50} (lower lethal temperature for 50% mortality) of aphids acclimated at 10 °C for one week showed no significant differences with control (aphids reared at 20 °C). In addition to the cold acclimation, adults of *S. graminum* showed RCH response too.When the rearing aphids at 20 °C were transferred directlyto a range of sub-zero temperatures for 2 h, LT_{80} (lower lethal temperature for 80% mortality) was -11.6 °C, but acclimation at 0 °C for 5 h before transfer to -11.6 °C, induced maximum RCH, led to increase of survival to 73%. RCH was induced by cooling of the insects at 0 °C for different rates.Maximum survival (66%) was achieved by cooling at 0.05 °C/min. Accumulationof sugars and polyols is one of the major mechanismsunderlying ACC and RCH. In this study, trehalose and glucose increased considerably through ACC and RCH treatments, suggesting the role of these compounds in increasing cold tolerance of *S. graminum.*
Key words: *Schizaphis graminum*, cold acclimation, rapid cold hardiness, cryoprotectants, trehalose

اثر سازگاری سرمایی و سرماسختی سریع روی تحمل سرما و ترکیبات ضد یخ در شته معمولی گندم، *Schizaphis graminum* (Hemiptera: Aphididae)

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چکیده
حشرات بقای خود را در دماهای زیر صفر با قرار گرفتن به مدت طولانی یا کوتاه در دماهای پایین اما غیرکشنده افزایش می‌دهند که به ترتیب سازگاری سرمایی(ACC) و سرماسختی سریع(RCH) نامیده می‌شود. در این تحقیق اثر کاهش تدریجی یا سریع دما روی تحمل به سرما در افراد بالغ شته معمولی گندم، *Schizaphis graminum* (Rondani) مورد بررسی قرار گرفت. LT_{50} (دمایی که موجب مرگ و میر ۵۰ درصد افراد جمعیت می‌شود) با قرار گرفتن در دمای ۱۰ درجه سلسیوس به مدت یک هفته اختلاف معنی‌داری با شاهد (افراد پرورش یافته در دمای ۲۰ درجه سلسیوس) نشان داد. علاوه بر سازگاری تدریجی، افراد بالغ شته *S. graminum* سرماسختی سریع نیز نشان دادند؛ زمانی‌که شته‌های پرورش یافته در دمای ۲۰ درجه سلسیوس به طور مستقیم در معرض دماهای زیر صفر به مدت دو ساعت قرار گرفتند LT_{80} (دمای ۸۰ درصد مرگ و میر) برابر با ۱۱/٦- درجه سلسیوس به دست آمد. اما قرار گرفتن در دمای صفر به مدت پنج ساعت قبل از انتقال به دمای ۱۱/٦- درجه سلسیوس منجر به ایجاد بیشینه سرماسختی سریع شد، به این معنی که بقا به بیشترین مقدار (۷۳ درصد) رسید. همچنین RCH در اثر سرمادهی افراد بالغ با نرخ‌های کاهش دمایی متفاوت ایجاد شد. به طوری‌که بیشترین میزان بقا در اثر سرمادهی بانرخ ۰/۰۵°C/min به دست آمد. افزایش قندها و پلی‌الها یکی از عوامل موثردر ACC و RCH می‌باشند. در این مطالعه ترهالوز و گلوکز در ACC و RCH به میزان قابل توجهی افزایش یافتند که نقش این ترکیبات در افزایش تحمل به سرما در شته *S. graminum* را نشان می‌دهد.
واژگان کلیدی: *Schizaphis graminum*، سازگاری سرمایی، سرماسختی سریع، ترکیبات ضد یخ، ترهالوز

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Introduction

Activity and survival of insects as poikilothermic organisms are directly affected by changes in their environmental temperatures. Insects increase their cold tolerance via some behavioral and physiological mechanisms to prepare for overwintering (Lee & Denlinger, 1991). Cold tolerance of an insect is influenced by thermal history within its lifetime like seasonal or diurnal temperature cycles (Hoffmann *et al*., 2003). Cold tolerance can be increased either gradually through process of cold acclimation (ACC) or rapidly through rapid cold hardiness (RCH) (Chen *et al*., 1987; Lee *et al*., 1987, 2006).

Cold acclimation occurs gradually over several days, weeks or months. In contrast, RCH is induced by a short term exposure (minute or hours) to low but non-lethal temperatures usually from 5 °C to 0 °C or by slow cooling over a range of temperatures (Lee *et al*., 1987; Kelty & Lee, 1999). RCH helps insects to increase their cold tolerance rapidly in response to sudden decrease in environment temperature (Chen *et al*., 1987; Lee *et al*., 1987). Both ACC and RCH are well-adapted mechanisms for tolerating low temperatures in insects.

It is hypothesized that in anholocyclic aphids, RCH is likely more important because they do not enter into diapause during cold season and continue to feed and reproduce (Powell and Bale, 2004). Therefore, their ability to rapid response to unexpected changes of environmental temperature plays an important role in aphids' survivorship.

The greenbug, *Schizaphis graminum* (Rondni) is one of the important aphid pests of Poaceae. It damages to host plant by phloem feeding (Al-Mousawi *et al*., 1983) and transmitting plant viruses including barley yellow dwarf (Murphy, 1959).This pest overwinters as asexual stages (anholocyclic) in warm or mild climates, but in cold regions overwinters as egg (holocyclic) (Blackman & Eastop, 2000; Gorena, 2004). Based on our observations, in Tehran and Karaj, Iran, this aphid is anholocyclic, overwintering as active nymphs and adults on grasses and weeds in Poaceaefamily. Cold temperatures are the key mortality factor of aphids including *S. graminum* in temperate regions (Knight & Bale, 1986). Therefore, increasing cold tolerance gradually through ACC and rapidly through RCH have important role in maintaining aphids population survival in unfavorable conditions.

Synthesis of low molecular weight sugars and polyolsas cryoprotectants are considered as the main biochemical mechanisms surviving low temperature in insects (Storey & Storey, 1991).Trehalose, myo-inositol, and glycerol have been reported as major cryoprotectants in most insect species (Kostal *et al*., 2001; Khani *et al*, 2007; Atapour & Moharramipour, 2009). In many studied insects, concentration of sugars and polyols increased during acclimation period (Lee & Denlinger, 1991) but the relation between accumulation of these compounds and RCH remained poorly understood especially in aphids (Kelty & Lee, 1999). In this study synthesis of sugars and polyols during processes

of ACC and RCH and the relation with increasing cold tolerance in *S. graminum* is discussed.

Many studies have been done on biology, ecology, chemical and biological control of *S. graminum* in Iran and other countries; but there was a lack of sufficient work on cold tolerance of this aphid. Previous studies have just been focused on measuring supercooling point (SCP) (the temperature at which insect's body fluids begin to freeze) (Jones *et al*., 2008). Studies have shown that SCP is not an appropriate index for evaluating aphids' cold tolerance (Knight & Bale, 1986). In this research lethal temperatures like LT_{50} (lethal temperature for 50% mortality) or LT_{80} (lethal temperature for 80% mortality) are used to evaluate cold tolerance potential of *S. graminum*. So far, there is no study about the effects of ACC and RCH, two main mechanisms of cold tolerance in aphids, on the cold tolerance of *S. graminum.* So, our main objective during current study was to determine the low temperature tolerance of *S. graminum* during ACC and RCH as well as to investigate the changes in concentration of sugars and polyols during ACC and RCH.

Materials and methods

Aphid stock colony

The aphids were originally hand-collected from wheat fields in Karaj **(**35°48'N, 51°00'E) Iran, in the spring of 2014and were transferred to the laboratory. The aphids were reared on wheat seedlings cultivar "Pishtaz" grown in plastic pots (10.5 cm diameter and 9.5 cm height) and covered with transparent cylindrical plastic containers in a growth chamber at 20 ± 1°C, photoperiod of 12L:12D and 65±5% RH.

Effect of ACC on cold tolerance

For conducting ACC experiments some pots including large population of *S. graminum* were transferred to a growth chamber at 10±1°C, photoperiod of 8L: 16D and 65±5% RH. The survival rate was measured a week after treatment.

The LT_{50} was also determined for both rearing temperature 20 °C (control) and 10 °C (ACC). For this purpose, 100 adults (10 replicates of 10 adults) were transferred in to a programmable refrigerated test chamber whose temperature was lowered from 20 or 10 ºC to -5, -7, -10, and -13ºC (the temperatures were chosen based on our previous studies and knowledge of aphids cold tolerance) at the rate of 0.5 ºC /min, held at the target temperature for 2 h and 6 h separately, and then raised to 20 ºC or 10ºC at the rate of 0.5 ºC /min. Mortality was determined 24 h after treatment. Aphids were considered being dead by showing no movement when the stimulated by a fine brush. The LT_{50} was estimated by binary logistic model. Treatments are as illustrated in Fig. 1.

Effect of RCH on cold tolerance

The experiments were conducted in two series: 1) first determining $LT_{80}$by directly transferring samples of 100 adults from 20 °C to a range of temperatures between -5 °C and -13 °C (the temperatures were chosen based on our previous studies and knowledge of aphids cold tolerance) for 2 h. After 2h, samples were returned to 20 °C at the rate of 0.5 °C/min. Survival was determined after 24 h. The ability to move was defined as surviving index. The LT_{80} was calculated by binary logistic model. LT_{80} was chosen as discriminating temperature based on references (Powell and Bale, 2004 & 2005) and it seems that LT_{80} could make better comparison between acclimated and unacclimated aphids. 2) For induction of RCH, the adults were subjected to two following treatments: (i) they were transferred directly from 20 °C to 0 °C during different time intervals(0.5, 1, 2, 3, 4, 5 or 6 h),and subsequently exposed to the discriminating temperature for 2 h; and (ii) they were gradually cooled from 20 °C to 0 °C at different rates (1, 0.5, 0.1 and 0.05) immediately before exposure to the discriminating temperature for 2 h. In all cases, survival was determined 24 h after the adults were returned to 20 °C. All treatments are as illustrated in Fig. 2.

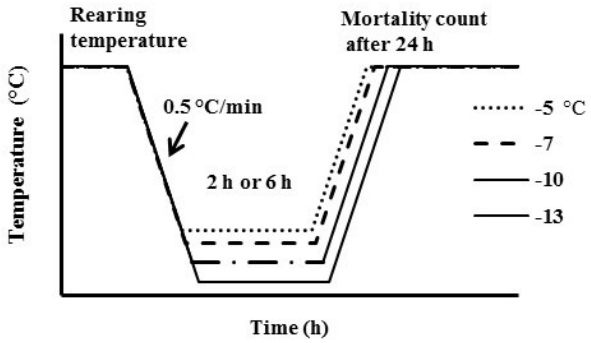

Fig. 1. Experimental protocol for determining LT_{50} of adults of *S. graminum* by transferring them from 20 °C and 10 °C to a range of subzero temperatures for 2 h or 6 h.

Polyol and sugar analysis

To detect the effect of ACC on cryoprotectants, samples were frozen at the end of one week cold acclimation at 10°C. To detect the effect of RCH on cryoprotectants, aphids were frozen at the end of three treatment groups set at: 1) aphids exposed directly to the LT_{80} for 2 h (cold shocked), 2) aphids rapidly cold hardened by acclimating at 0 °C for 5 h before direct transfer to LT_{80} (RCH1), 3) aphids rapidly cold hardened by cooling at 0.05 °C/min to 0 °C before direct transfer to LT_{80} (RCH2).Samples of aphids maintained continuously at 20 °C were frozen as control.

Each sample (3 replicates for each treatment) was homogenized in 1.5 ml of 80% ethanol. After centrifugation at 12000 gr for 15 min, the supernatant was removed. The extract (ethanol 80%) was evaporated at 30 ºC in a vacuum drying oven (Memmert, VO 400) and then dissolved in 300 µl of HPLC grade water. Just before the sample injection, the samples were cleaned by cellulose acetate filter syringe. Sugars and polyols were

analyzed using high performance liquid chromatography (HPLC) (Waters, Milford, USA) equipped with Supelco carbohydrate column (300 × 7.8 mm, Supelco, USA). The eluent was HPLC grade water and elution speed was 0.5 ml/min. sugar and polyols were detected by RI (refractive index) detector (Pullin & Bale, 1989).

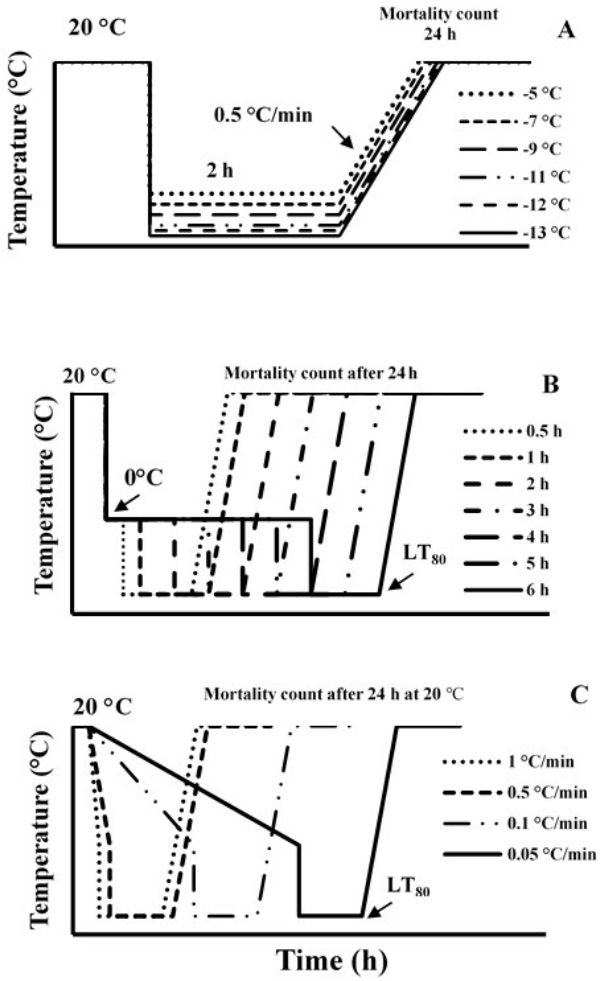

Fig. 2. Experimental protocols for rapid cold hardiness treatments of adults of *S. graminum* starting from 20°C (A) direct transfer to a series of subzero temperatures for 2 h to determine LT_{80} (discriminating temperature); (B) acclimation at 0°C for different durations prior transferring to LT_{80}; (C) cooling to 0°C at different cooling rates prior transferring to LT_{80}. The LT_{80} value was determined -11.6°C.

Statistical analysis

Differences among more than two treatments were tested by one-way analysis of variance (ANOVA) with a post-hoc turkey's test using SPSS version 16.0. The results were expressed as mean ± S.E. and considered significantly different at $P < 0.05$.A *t*-test was used for comparison of two means, including differences between cryoprotectants at 10 °C and 20 °C.

LT_{50} and LT_{80} were estimated from Binary logistic model using the equation below:

$$y = \frac{e^{a+bx}}{1+e^{a+bx}}$$

Where, *a* is intercept, *b* characterizes the slope of the line, *x* describes the temperature, and *y* is survival percent (Vittingh off *et al*., 2005)

Results

Effect of ACC on cold tolerance

LT_{50} values in 2h exposure to subzero temperatures decreased significantly following acclimation at 10 °C compared to 20 °C (control). However, there was no significant difference in LT_{50} values of 6 h exposure to subzero temperatures (Table 1).

Effect of RCH on cold tolerance

Mean mortality of adults exposed directly from 20°C to a range of subzero temperatures showed significant difference ($F = 57.608$; $df = 5, 54$; $P < 0.0001$). Mortality increased from 22% at -5 significantly to 68% at -11 and reached to 100% at -13°C (Fig. 3.). According to these findings, LT_{80} (discriminating temperature) were determined -11.6 °C bases on binary logistic analysis. Pretreatment(acclimating) at 0 °C for various durations before direct transfer to -11.6°C, increased survival significantly ($F = 9.205$; $df = 7, 72$; $P < 0.0001$). Significant survival increase was observed by acclimating aphids at 0 °C for 1 h (46%) and maximum survival was 73% in 5h pretreatment at 0°C (Fig. 4.). Decreasing cooling rate from 20 °C to 0 °Cat the rates of 1, 0.5, 0.1 and 0.05 °C /min before direct transfer to -11.6 °C increased survival significantly($F = 15.128$; $df = 4, 45$; $P < 0.0001$). The highest survival was achieved 66% at the cooling rate of 0.05 °C /min (Fig. 5.).

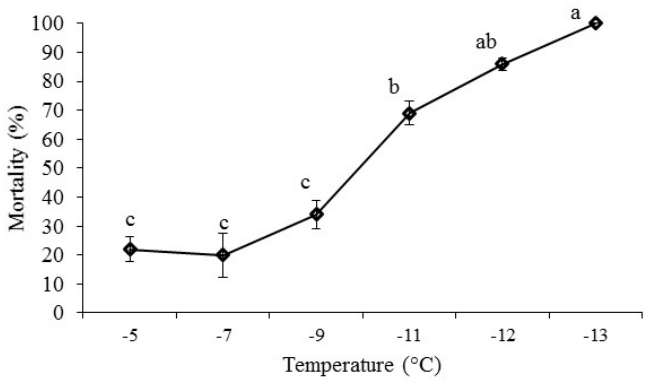

Fig. 3. Mean (± SE) percent mortality of adults of *S. graminum* by direct exposure from 20°C to a range of sub-zero temperatures for 2 h for determining LT_{80}. Means with the same letters are not significantly different (Tukey's test after ANOVA, $P < 0.05$). For the method refer to Fig. 2- A.

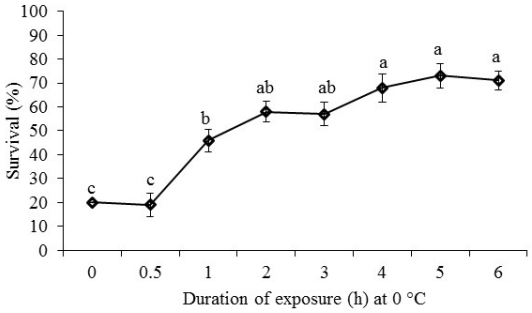

Fig. 4. Mean (± SE) percent survival of adults of *S. graminum*that were transferred from 20°C to 0°C for 0.5 to 6 h before direct transfer to LT_{80} in order to investigate RCH response. Means with the same letters are not significantly different (Tukey's test after ANOVA, $P < 0.05$). For the method refer to Fig. 2- B.

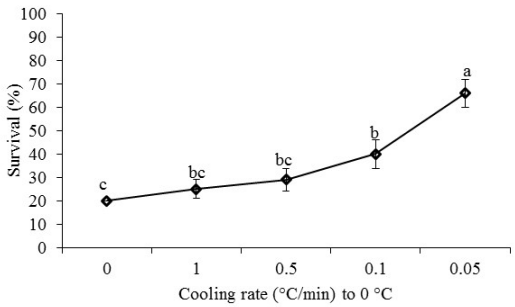

Fig. 5. Mean (± SE) percent survivals of adults of *S. graminum* by various cooling rates (°C/min) from 20°C to 0°C before direct transfer to LT_{80} in order to investigate RCH response. Means with the same letters are not significantly different (Tukey's test after ANOVA, $P < 0.05$). For the method refer to Fig. 2- C.

Polyols and sugar analysis

Trehalose, glucose and mannitol were major identified sugars and polyols in both ACC and RCH. Glucose had the highest amount among two other compounds. Concentration of three identified compounds increased by cold acclimation at 10 °C for 1 week compared to those in control (20°C) (Fig. 6.). However, just trehalose showed significant difference ($F = 0.051$; df = 4, 3.902 ; $P < 0.0001$) whose concentration increased about 3 fold from 5.95 µmol/g f. w. in control to 14.56µmol/g f. w. in ACC. Concentration of glucose increased from 36.89 µmol/g F. W.in control to 57.15µmol/g f. w. in ACC. Mannitol did not show remarkable changes.

Table 1. LT_{50} values in adults of *S. graminum* which were acclimated at 10 °C for 1 week and reared at 20 °C (control).

Treatments (°C)	LT_{50} (°C)	2 h exposure to cold 95% CI (°C)		LT_{50} (°C)	6 h exposure to cold 95% CI (°C)	
		lower	upper		lower	upper
20	-9.59	-9.65	-9.56	-7.28	-7.14	-7.38
10	-9.91	-10.05	-9.83	-7.37	-7.03	-7.59

[1] LT_{50}s with same letters in a column are considered not significantly different if their 95% confidence intervals (CI) overlap.

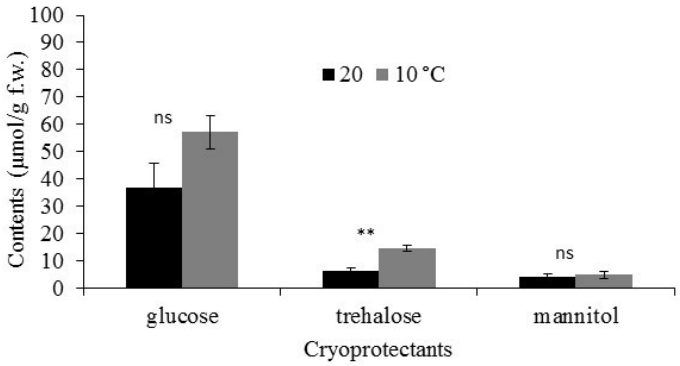

Fig. 6. Mean cryoprotectants concentration in adults of *S. Graminum* which were acclimated at 10 °C (1 week) and reared at 20 °C (control). Means were compared pairwise for each cryoprotectant between control and cold acclimated aphids by t-test. Statistically significant differences are denoted with *($p < 0.05$) or ** ($p < 0.01$). ns: no significant difference.

The amount of trehalose and glucose showed significant difference in RCH treatments ($F = 26.123$; $df = 3, 8$; $P < 0.0001$ and $F = 4.945$; $df = 3, 8$; $P = 0.031$respectively). The most remarkable changes were observed in trehalose whose concentration increased from 5.9 µmol/g f. w. in control and 9.4 µmol/g f. w. in cold shocked to the highest amount of 16.5 µmol/g f. w. in RCH1. The highest amount of glucose was58.10µmol/g f. w. in RCH1 which showed significant difference with the control. No significant differences in the levels of mannitol were detected (Fig. 7).

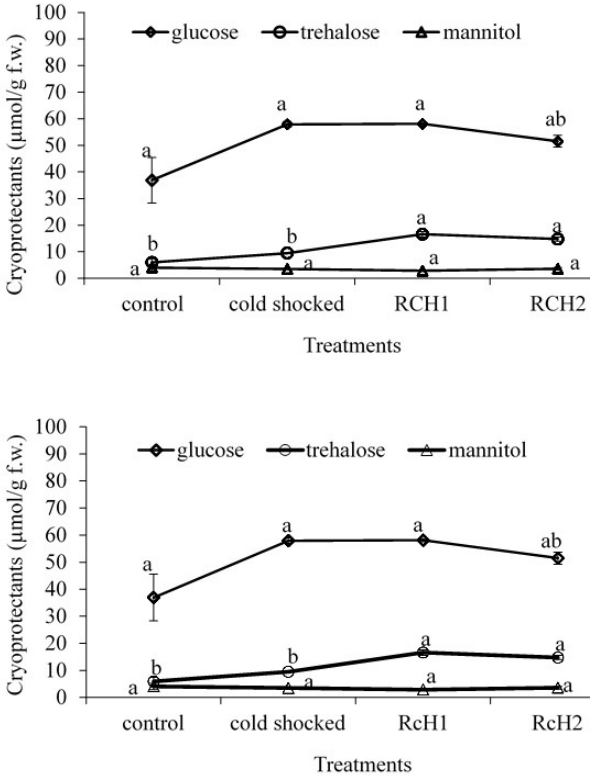

Fig. 7. Mean cryoprotectants concentration in adults of *S. graminum* after RCH treatments compared adults reared at 20 °C (control). Means with the same letters are not significantly different (Tukey's test after ANOVA, $P < 0.05$).

Discussion

All organisms including insects can increase their survival at sub-zero temperatures by long or short term acclimation at low but non-lethal temperatures (Rako *et al*., 2006). In the current study, cold acclimation at 10 °C for one week only decreased LT_{50} by approximately 0.3 °C compared to 20°C, which is considerably less than earlier reports from other aphid species. In studies on *Myzus persicae* (Sulzer) and *Sitobion avenae* (Fabricius), rearing for one generation at 10°C,$LT_{50}$was decreased for about 2.5 °C and 4°Crelative to 20 °C, respectively (Powell & Bale, 2008; Hazell *et al*., 2010). In these studies adult aphids were kept at 10 °C for longer time than in the present study. Most studies on other insect species showed that acclimating insects for several days at 10 °C or even 15 °C decreased mortality at low temperatures. For example acclimation of *Corythucha ciliate* (Say) at 15 °C for 5 days increased survival at -12 °C compared to unac climated insects (Ju *et al*., 2011).In addition to acclimation time, the temperature of acclimation affects cold tolerance and it may differ in insect species. In most insect species, acclimation temperatures about 5 °C or 6 °C below their optimal temperature for development, usually 15 °C could increase survival at extreme low temperatures. For example acclimation at 15.5 °C for 9 days enhanced egg cold tolerance of *Psacothea hilaris* (Pascoe) (Shintani & Ishikawa, 2007). In diapausing adults of *Hippodamia variegate* (Goeze) acclimation at 0 °C for 30 days decreased mortality to zero at subzero temperatures, but more than 80% mortality was observed in acclimation at 10°C (Hamedi & Moharramipour, 2013). One week acclimation at 0 °C or 5 °C resulted in a significant decrease in mortality of 1st in star nymphs and pupae of potato tuber moth, *Phthorimaea operculella* Zeller (Hemmati *et al*., 2014).Results of the current study showed that longer acclimation time at 10 °C or lower acclimation temperatures such as 5 °C or 0 °C are required to induce significant cold tolerance.

In this study, *S. graminum* has the ability to show RCH response. RCH was induced by 1 h acclimating at 0 °C before transferring to LT_{80}, but maximum survival was achieved when the induction period was 5 h. Also, temperature decrease to 0 °C at the rate of 0.05 °C/min resulted in the highest RCH response (Chen *et al*., 1987; Worland & Convey, 2001; Shintani & Ishikawa, 2007; Qiang *et al*.,2008). Increasing cold tolerance through RCH has been shown in many insect species. Despite the ecological and physiological importance of this response in aphids, only has been studied in *S. avenae* (Powell & Bale, 2004). The study showed that 3 h preconditioning of adults of *S. avenae* at 0 °C resulted in 68% survival or cooling them to 0 °C at the rate of 0.05 caused 58% survival prior transferring to LT_{80}. In nature, RCH helps *S. graminum* to response rapidly to unexpected decreasing of environmental temperature, especially occurs in early autumn or early spring where the physiological adaptations are incomplete or diminishing or response to daily changes of environmental temperatures. But ACC is a response to seasonal changes of temperature. In

many studies has been shown that capability of ACC and RCH to enhance cold tolerance is different. For example, in *Chilo suppressalis* Walker (Qiang *et al*., 2008) and *Corythuca ciliate* (Say) (Ju *et al*., 2011) RCH increases cold tolerance more than acclimation. But in *Sarcophaga crassipalpis* Macquart (Chen *et al*., 1987), *Danaux plexippus* (Linnaeus) (Larsen & Lee, 1994), and *Frankliniella occidentalis* (Pergande) (McDonald *et al*., 1997) ACC showed more protection against cold. In our study, it seems that RCH increases cold tolerance more than ACC. However further investigations are needed to judge the beneficial effects of ACC and RCH in aphids.

The accumulation of low molecular sugars and polyols (sugar alcohols) is one of the major physiological mechanisms hypothesized to increase cold tolerance via ACC but little is known about these cryoprotectants involved in RCH especially in aphids. In many studies have been mentioned that ACC and RCH may have (undergo) different physiological mechanisms (McDonald *et al*., 1997). Our study is the first which investigated the effect of ACC and RCH on accumulation of sugars and polyols in aphids. Glucose, trehalose and mannitol were identified as major compounds in this research. In both ACC and RCH glucose had the highest amount. The concentration of glucose and trehalose increased considerably via ACC and RCH compared to control (aphids reared at 20 °C). Accumulation of glucose and trehalose in adults of *S. graminum* due to ACC and RCH processes could play important role in increasing their survival by stabilizing proteins and membrane lipids.

Trehalose the major blood sugar of insects has been previously considered as major cryoprotectants in insects like overwintering larvae of *Cydia pomonella* (L.)(Khani *et al*., 2007) and pupae of *Hyphantria cunea* (Drury)(Li *et al*., 2001). Glucose is known to be as cryoprotectant in cold acclimated eggs of *Locusta migratoria*L. (Wang *et al*., 2010) and overwintering pupae of *H. cunea* (Li *et al*., 2001). In our previous study on *Brevicorryne brassicae* (L.) mannitol along with glucose was detected as major cryoprotectants in overwintering adults (Saeidi *et al*., 2012). In the present study trace amount of mannitol was detected. It is likely that lower acclimation temperatures are needed to trigger accumulation of this polyol. Because most polyols and sugars are triggered to accumulate at 5°C or 0°C (Storey & Storey, 1991). The role of sugars and polyols in RCH is a controversial issue. Kelty & lee (1999) found no evidence for RCH induced accumulation of cryoprotectants in *Drosophila melanogaster* Meigen. Although, Overgaard *et al*.,(2007) found concentration of trehalose and glucose increase by RCH. In another study metabolomic analysis of RCH in *S. crassipalpis* showed increased level of glycerol, glucose and sorbitol, however trehalose and mannose decreased (Michaud & Denlinger, 2007). During our study glucose and trehalose level increased through RCH but it is not yet clear whether this amount of increase can enhance survival. It is suggested that RCH may involve additional mechanisms such as synthesis of heat shock proteins (HSPs),

aminoacids, changes of membrane phospholipids which should be investigated in future researches.

Refrences

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