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Geographic differences on accumulation of sugars and polyols in locust eggs in response to cold acclimation

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ABSTRACT

The accumulation of low molecular weight sugars and polyols is one of major mechanisms hypothesized to increase cold tolerance in overwintering insects. But little is known about whether these sugars and polyols are involved in geographic variation of cold tolerance. In this study, we investigated accumulation patterns of eight low molecular weight sugars and polyols of eggs in tropical and temperate populations of the migratory locust, which exhibits between-population variation in cold tolerance, in response to cold acclimation (5, 0 and −5 °C). Excluding erythritol, the other seven carbohydrates were identified as possible cryoprotectants in locust eggs. Basal maximal and minimal concentrations were 45 μg/g wet weight for trehalose and 0.59 μg/g wet weight for glycerol. Most sugars and polyols were elevated after a −5 °C exposure. In a tropical population, fructose, glucose, sorbitol and *myo*-inositol were significantly accumulated by low temperature treatments, but glycerol was not. In the temperate population, glycerol, glucose, mannitol, sorbitol, *myo*-inositol were significantly accumulated but trehalose did not increase. Our results suggest different accumulation patterns of these carbohydrates of locust eggs between tropical and temperate populations and highlighted possible roles for them in geographic variation of cold tolerance in the migratory locust.

1. Introduction

Low temperature during winter is a major environmental constraint influencing the geographic distribution of invertebrates (Lee, 1991; Bale, 1996). It is well documented that many insect species exhibit geographic variation of cold tolerance to adapt to local thermal environments (Lee, 1991; Bale, 2002; Kang et al., 2009). Although various biochemical, physiological and ecological aspects of insect cold tolerance have been revealed (Lee and Denlinger, 1991; Bale, 2002; Chown et al., 2002; Denlinger and Lee, 2010), the precise mechanisms of such geographic variation remain a subject of vigorous discussion (Hoffmann and Blows, 1994; Case and Taper, 2000; Holt and Keitt, 2000).

Low molecular weight sugars and polyols have been demonstrated as cryoprotective agents in a wide variety of insect species to endure cold stress (Lee, 1991; Storey and Storey, 1991). These compounds are believed to protect organisms during both freezing and thawing and prolonged exposures to low non-freezing temperatures in various ways (Salt, 1961; Storey and Storey, 1988). Temperatures triggering polyol synthesis are found typically from 0 to 5 °C, with maximal rates of synthesis in the lower range of 0 to −5 °C (Storey and Storey, 1991). Some studies have indicated that insects at different developmental stages may respond differently to low temperature cues (Denlinger and Lee, 2010) and it is suggested that there exists an underlying developmental component to sugar and polyol synthesis which could be further enhanced by low temperature (Lee and Denlinger, 1991).

The migratory locust (*Locusta migratoria* L.), which is distributed throughout the world, overwinters as eggs and exhibits geographic and seasonal variation in cold tolerance (Jing and Kang, 2003, 2004). Mortality of the overwintering eggs of the migratory locust is attributed to chill injury because of its occurrence well above the supercooling point of the eggs (Jing et al., 2005). Cold tolerance of locust eggs and the first instar hoppers are influenced by several environmental factors, such as exposure to acclimation temperatures, cooling rate, thermoperiod and soil moisture (Wang and Kang, 2003, 2005b; Wang et al., 2006; Qi et al., 2007). Several low molecular weight sugars and polyols and heat shock proteins are involved in cold tolerance of locust eggs (Wang and Kang, 2005b; Wang et al., 2006). Temperate populations of migratory locusts in China have higher cold tolerance than southern populations and this geographic variation has a genetic basis. And, different cold hardening responses were found to a cold acclimation regime of 0 or 5 °C among various geographic populations (Jing and Kang, 2003; Wang and Kang, 2005a,b).

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In this study, we investigated changes in the levels of various low molecular weight sugars and polyols which have previously been associated with insect cold hardiness, including glycerol, fructose, glucose, mannitol, sorbitol, *myo*-inositol, trehalose and erythritol, in locust eggs of both tropical and temperate populations in response to cold acclimation. Our results suggested that there are different patterns of change for low molecular weight sugars and polyols between tropical and temperate populations after low temperature exposure and highlighted possible roles of these carbohydrates on geographical differences of cold hardiness in the migratory locust.

2. Materials and methods

2.1. Insects

Laboratory populations were established from individuals of *L. migratoria* that were collected in June 2002 from two extreme sites of the species range in China: in tropical Hainan Province (18°23′N, 109°30′E) where the mean monthly ambient temperature is 18 °C in winter and 29 °C in summer and in temperate Liaoning Province (41°10′N, 122°06′E) where the mean monthly ambient temperature is −10 °C in winter and 21 °C in summer (Wang and Kang, 2005a,b). The underground temperature which locust eggs experience at 5 cm depth is usually 0.5–1 °C less than the air temperature. All insects were reared in two-floor boxes (50 cm × 70 cm × 80 cm) with wheat bran and wheat seedlings at 30 °C (14 h daily photophase). Sterilized sand was provided as the oviposition medium. Eggs were collected daily to ensure an even stage of development. Egg pods were incubated at 30 °C in sterilized sand, and individual eggs were separated from egg pods when required. Water content of the sand was kept at 10%.

2.2. Cold acclimation treatments

To examine the effect of acclimation on the concentration of low molecular weight sugars and polyols of locust eggs in the tropical and temperate populations, we used one acclimation period of 72 h, which is optimal acclimation period for locust eggs according to our previous studies (Jing and Kang, 2003), at three different temperatures, 5, 0 and −5 °C, to carry out acclimation treatments. Eggs of two lab strains, which developed for 7 days at 30 °C, were used. The control groups were eggs incubated at 30 °C. Each group of eggs was transferred to plastic tubes, the temperature of which was controlled by a programmable refrigerated bath (Polyscience, USA). Eggs were cooled from 30 °C to 5, 0 and −5 °C at 0.1 °C min^{-1}, then held at these temperatures for 72 h. Three to nine replicates were used for each treatment. Each replicate contained 8 eggs. After acclimation treatments, all samples were immediately frozen in liquid nitrogen and stored at −70 °C until analysis.

2.3. Measurement of low molecular weight sugars and polyols

Low molecular weight cryoprotectants were measured by capillary gas chromatography as their *o*-methyloxime trimethylsilyl (TMS) derivatives (Wang and Kang, 2005b). Each group of eggs was weighed and homogenized with 0.4 ml of 70% (v/v) ethanol containing 25 μg dulcitol (an internal standard) in an Eppendorf tube that had been rinsed with 0.2 μl of 70% ethanol. After centrifugation at 10,000 × *g* for 5 min, the supernatant was removed and the process repeated. The pooled supernatants were evaporated until dry under a stream of nitrogen at 40 °C. Twenty-five microliters of dimethylformamide and 25 μl *o*-methylhydroxylamine in pyridine (200 mg ml^{-1}) were added to the residue for oximation, then heated at 70 °C for 15 min. Silylation was accomplished by adding 75 μl dimethylformamide and 30 μl trimethylsilylimidazol to the reaction mixture which was further heated to 80 °C for 15 min. After re-extraction of the desired derivatives into isooctane, using 2 × 75 μl of the solvent, a 1-μl aliquot was injected into an injection port of a gas chromatograph (Pye Unicam 204). Separation and quantification of sugars and polyols were achieved on a 25 m × 0.25 mm i.d. BP-5 silica capillary column. The temperature program was: 3 min at 120 °C then 12 °C min^{-1} to 280 °C for 40 min. Identity of the revealed components was established against authentic standards.

2.4. Statistical analysis

Differences between treatments were compared either by *t*-test (for comparison of two means), or by one way analysis of variance (ANOVA) followed by a Tukey's test for multiple comparisons. The relationship between cooling rate and survival rate was simulated by linear regression analysis. Treatment differences were considered significant at $P < 0.05$. Values were reported as means ± SE. Data were analyzed using SPSS 15.0 software.

3. Results

3.1. Content of low molecular weight sugars and polyols in locust eggs of both tropical and temperate populations incubated at 30 °C

Excluding erythritol, seven other low molecular weight sugars and polyols which have been reported to be related to cold tolerance in insects including glycerol, fructose, glucose, mannitol, sorbitol, *myo*-inositol and trehalose were detected in locust eggs of both tropical and temperate populations (Fig. 1). Trehalose content was the highest (45.52 and 34.12 μg/g, respectively) and glycerol showed the lowest content (3.91 and 0.54 μg/g, respectively) among the seven low molecular weight sugars and polyols in both populations. The content of four sugars and polyols, including glycerol, fructose, glucose and sorbitol were higher in the tropical eggs than in eggs from the temperate population ($P < 0.05$). There were no significant differences of the other three sugars and polyols including mannitol, *myo*-inositol and trehalose between the two populations ($P > 0.05$).

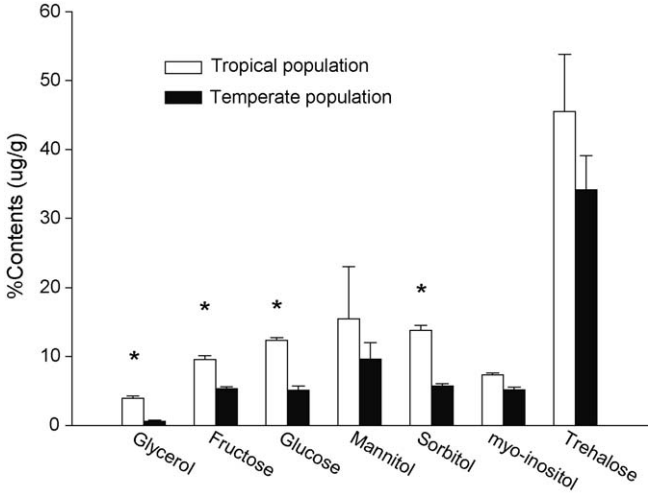

Fig. 1. Content of various low molecular weight sugars and polyols including glycerol, fructose, glucose, mannitol, sorbitol, *myo*-inositol and trehalose in locust eggs from tropical and temperate populations of the migratory locust which were incubated at 30 °C (mean ± SE μg/g wet weight). *P value < 0.05.

3.2. The effects of cold acclimation temperatures on low molecular sugars and polyols levels in locust eggs

To investigate a possible role for low molecular weight molecular sugars and polyols on geographical differences of cold hardiness, the levels of glycerol, fructose, glucose, mannitol, sorbitol, *myo*-inositol and trehalose were determined 72 h after exposure to 5, 0 and −5 °C. We found that there were different patterns of change in the various sugars and polyols in response to cold acclimation between tropical and temperate populations (Fig. 2).

Glycerol content of locust eggs significantly decreased after a 72 h exposure to all three low temperatures for the tropical population ($F_{3,24} = 13.023$, $P < 001$). Especially after the −5 °C treatment, glycerol content was not detected. However, for temperate population, glycerol content of the locust eggs increased significantly after low temperature exposure ($F_{3,28} = 4.73$, $P < 001$) (Fig. 2A).

The fructose content of locust eggs rose significantly as temperature decreased at −5 °C in the tropical population ($F_{3,22} = 13.63$, $P < 0.01$). No significant difference in fructose content was detected among the various low temperatures ($F_{3,27} = 2.39$, $P > 0.05$).

Significant increases in the levels of glucose were detected at all cold acclimation temperatures in the tropical population ($F_{3,21} = 11.2$, $P < 0.001$) and at 0 and −5 °C acclimation in the temperate population ($F_{3,27} = 15.77$, $P < 0.001$) when compared with locust eggs at 30 °C.

Mannitol content did not differ significantly among all cold acclimation temperatures for the tropical population ($F_{3,21} = 0.63$, $P > 0.05$), and only mannitol content increased significantly at −5 °C compared to other temperatures in the temperate population ($F_{3,24} = 22.64$, $P < 0.001$).

The levels of sorbitol of locust eggs rose significantly at 5 and −5 °C acclimations but no significant difference was detected at 0 °C in the tropical population ($F_{3,21} = 16.55$, $P < 0.001$). For the temperate population, only −5 °C exposure significantly enhanced sorbitol content ($F_{3,27} = 67.78$, $P < 0.001$).

After −5 °C treatment, *myo*-inositol was higher than after other temperature treatments in the tropical population ($F_{3,21} = 106.82$, $P < 0.001$). For the temperate population, all cold acclimation temperatures significantly increased *myo*-inositol contents of locust eggs while the acclimation effect at −5 °C was highest ($F_{3,27} = 27.32$, $P < 0.001$).

No significant differences of the levels of trehalose were detected among various temperatures in the tropical population ($F_{3,21} = 1.46$, $P > 0.05$), whereas, levels of trehalose decreased gradually as temperature declined in the temperate population ($F_{3,27} = 9.22$, $P < 0.003$).

4. Discussion

Cold acclimation can enhance survival of locust eggs at low temperatures during overwintering (Jing and Kang, 2003). Many studies have demonstrated that accumulation of low molecular weight sugars and polyols is associated with cold hardiness in many insects during overwintering (Storey and Storey, 1991). Sugars and polyols can alleviate osmotic stress during freezing and colligatively decrease the probability of water crystallization in the unfrozen cell contents and alter the melting point. However, their function on cold tolerance of insects remains debatable because not all insects accumulate high concentration of low molecular weight sugars and polyols during overwintering (Lee et al., 1987; Meier and Zettel, 1997). In the present study, we identified seven low molecular weight sugars and polyols including glycerol, fructose, glucose, mannitol, sorbitol, *myo*-inositol and trehalose as possible cryoprotectants in eggs of the migratory locust and suggest they are involved in geographical variation of locust cold tolerance.

Among the sugars and polyols that we detected, trehalose and glycerol exhibited the maximal and minimal concentration, respectively. There were no differences in sugar and polyol categories between different populations, but the tropical population had higher basal concentrations than the temperate population for most of sugars and polyols at the normal incubation temperature. Synthesis rate of sugars and polyols reflects to some extent metabolic rate, as a part of carbohydrate and lipid metabolic pathways in non-diapause eggs (Pullin et al., 1991). Therefore, we supposed that there is a lower basal metabolic rate of locust eggs in the temperate population. Consistently with this conclusion, we also found the eggs of tropical populations have a higher respiration rate than those of temperate population (Wang and Kang, unpublished data). Low metabolic demands of the tissues may be helpful at low temperatures when activity of enzymes is inhibited. This could partly explain the cause of high cold tolerance of locust eggs in temperate population.

Exposure to low temperatures (5 to −5 °C) triggers accumulation of most low molecular weight sugars and polyols in eggs of both tropical and temperate populations. Among the three acclimation temperatures tested, −5 °C exhibits the most obvious accumulation effect. Our previous studies have suggested that cold acclimation at 5 °C can induce accumulation of several low molecular weight sugars and polyols in various development-stages of locust eggs, and their accumulation is influenced by cooling rates and thermoperiod (Wang and Kang, 2005b; Wang et al., 2006). But we did not compare the accumulation effects between different cold acclimation temperatures. Usually, temperatures triggering sugar and polyol synthesis are from 0 to 5 °C, with maximal rates of synthesis in the lower range of 0 to −5 °C in various insects (Storey and Storey, 1991). These temperatures have significant acclimation effects on the increase of cold tolerance in locust eggs (Jing and Kang, 2003). A period of 2–5 days has an optimal acclimation effects on the survival of locust eggs although it usually takes several weeks in other insects (Lee and Denlinger, 1991). Apparently, this temperature regime may provide the signal for locust eggs to prepare for the coming of winter. However, the accumulation of low molecular weight sugars and polyols does not occur naturally in the tropical population because they have no chance of being exposed to such a low temperature (Wang and Kang, 2005a). Thus, there might be different mechanisms for sugar and polyol accumulation in locust eggs.

Different accumulation patterns of those cryoprotectants were found between locust tropical and temperate populations in response to cold acclimation. Among the sugars and polyols that increased the most, fructose and glucose are the two major compounds, especially in tropical populations, and another two polyols, mannitol and sorbitol, are the dominant compounds in the temperate population. *Myo*-inositol exhibited a large increase in both populations after −5 °C exposure. In addition, the levels of two carbohydrates, glycerol and trehalose, appeared to significantly decrease in both tropical and temperate populations. Our previous studies demonstrated that the temperate population displays high basal cold tolerance although cold acclimation (5 and 0 °C) induces an increase of cold tolerance in all geographic populations in the migratory locust. Exposure at −5 °C causes mortality of locust eggs in the tropical population but increases significantly the survival of locust eggs in the temperate population (Jing and Kang, 2003). In the tropical region of China, the temperature almost never falls below 0 °C; there is no opportunity for cold acclimation for locust eggs. So, several sugars and polyols which have large accumulation mainly in tropical

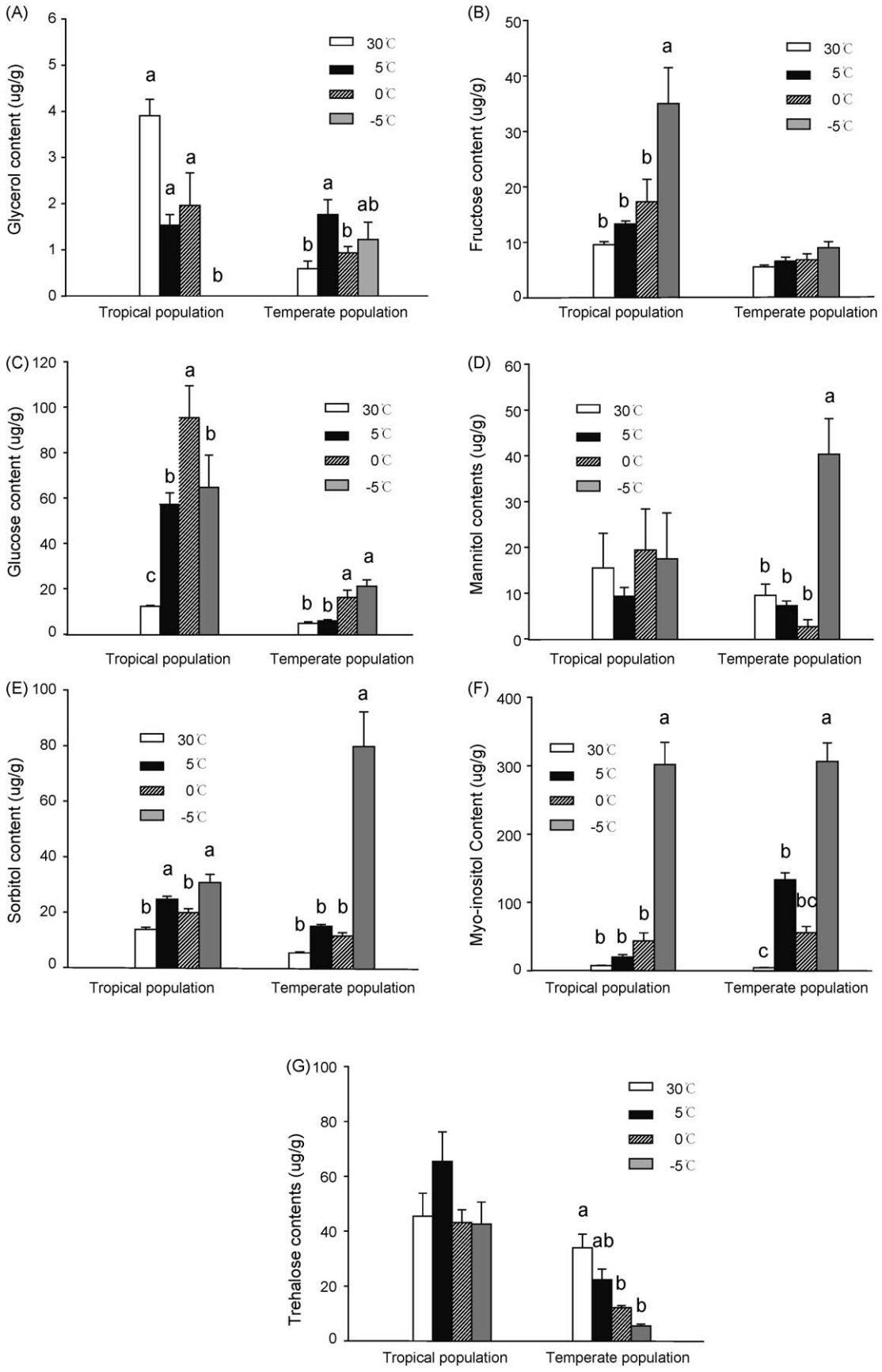

Fig. 2. Effects of cold acclimation temperatures (5, 0, −5 °C) on contents of various low molecular weight sugars and polyols including glycerol (A), fructose (B), glucose (C), mannitol (D), sorbitol (E), *myo*-inositol (F) and trehalose (G) in locust eggs of both tropical and temperate populations in the migratory locust, *Locusta migratoria* (mean ± SE μg/g wet weight). Eggs as the control were incubated at 30 °C.

population after −5 °C exposure, such as fructose and glucose, may be a 'by product' of metabolic suppression, which may have been unrelated to cold hardiness (Pullin et al., 1991). Recent studies in *Drosophila melanogaster* also suggested that accumulation of glucose is neither necessary nor sufficient for cold hardiness (MacMillan et al., 2009). However, in the temperate region, the minimum winter temperature frequently falls to −20 °C. So, overwintering eggs there have experienced a cold acclimation period in late autumn and early winter. Mannitol and sorbitol may play important roles for cold tolerance in temperate populations of the migratory locust, similar to other insect species (Kostál and Simek, 2000; Kostál et al., 2007; Michaud and Denlinger, 2007). Glycogen and trehalose may be used for conversion into other sugars and polyols in tropical and temperate populations, respectively (Worland et al., 1998; Ishiguro et al., 2007).

Our results showed that the concentrations of low molecular weight sugars and polyols that accumulated in locust eggs were very low. Even if the amount of *myo*-inositol in a temperate population after a −5 °C exposure, which is the maximal accumulation concentration in all samples, reaches only 307 μg/g, this concentration is not sufficient to bring about any substantial effect through depression of melting and supercooling points by colligative action (Zachariassen, 1985). Supposedly, they function in stabilizing the cell membrane structure (Williams, 1990) and the native state of proteins at low temperature through non-colligative action (Storey and Storey, 1988). The accumulation of these sugars and polyols is just one facet of cold hardening in overwintering locust eggs. Other mechanisms, such as the accumulation of free amino acids, restructuring of phospholipids, expression of freezing proteins and heat shock proteins are also involved in cold tolerance of locust eggs (Lee, 1991; Wang and Kang, 2005a).

In conclusion, the present results show that several sugars and polyols display different accumulation patterns between tropical and temperate populations of the migratory locust after cold exposures. But not all sugars and polyols may be related to cold tolerance in locust eggs. We highlighted that two polyols, mannitol and sorbitol, whose concentrations increase dramatically only in temperate population, may play a role on geographic variation of cold tolerance in the migratory locust. Further studies are needed to elucidate the regulating mechanisms underlying the differences in the synthesis of these sugars and polyols between different geographic populations of the migratory locust.

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