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Full Length Research Paper

Overwintering physiology of the rice stem borer larvae, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae): Roles of glycerol, amino acids, low-molecular weight carbohydrates and antioxidant enzymes

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The rice stem borer, *Chilo suppressalis* (Walker), is a major rice pest around the world. A strong ability of the rice stem borer to adapt/resist cold temperature (cold hardiness) contributes to its survival through winter. However, the physiological mechanism of its cold hardiness is poorly understood. In this study, we determined the supercooling points (SCPs), the contents of amino acids and low-molecular weight carbohydrates of overwintering rice stem borer larvae. We also assessed the levels of their superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) antioxidative enzymes in the overwintering larvae. Our results revealed an intimate relationship of larval SCP with environmental temperature; SCP change reflected proportionally to change of environmental temperature. Analysis of low molecular weight carbohydrates established that the concentrations of glycerol and trehalose change in a manner that is inversely proportional to that of the environmental temperatures. Changes or significant changes of some amino acids and antioxidative enzymes were also observed in overwintering larvae. Our data suggest that decrease of the SCP in overwintering larvae is due primarily to the increase of cryoprotective glycerol and trehalose and also to the increase of several amino acids to an extent. The decreased SCP in-turn enabled rice stem borer larvae to withstand low temperature. Our study therefore provides an overall picture regarding seasonal changes of the cryoprotective substances in relation to the ability of the rice stem borer to survive cold environmental conditions.

Key words: *Chilo suppressalis* (Walker), cold hardiness, supercooling points (SCPs), amino acids, low-molecular weight carbohydrates, antioxidant enzymes.

INTRODUCTION

Many overwintering insects must adapt to subzero temperatures by either becoming freeze-resistant or freeze-tolerant (Lee and Denlinger, 1991). High concentrations of polyols function as cryoprotectants (Li et al., 2000), ice nucleators (Duman, 2001), antifreeze proteins (Duman, 2001), heat shock proteins (Kelty and Lee, 2001) or ubiquitin (Qiang et al., 2011). Some polyols also act in dehydration (Worland and Block, 2003; Bennett et al., 2005). Sugars and low-molecular weight carbohydrates play a role in insect survival at subzero temperatures. They may provide colligative depression of freezing points (Storey and Storey, 1991), stabilize cell

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Abbreviations: SCPs, Supercooling points; **SOD,** superoxide dismutase; **POD,** peroxidase; **CAT,** catalase.

membrane structures (Williams, 1990) and preserve the native states of some proteins at low temperatures. Presently, different physiological and biochemical aspects of insect cold hardiness are being studied in detail (Sømme, 1999). In addition to studies which focused on a single facet of overwintering, some studies took a more holistic approach considering several factors simultaneously with the goal of understanding broader ecological and evolutionary consequences of insect cold hardiness (Sinclair, 1999; Koštál and Šimek, 2000). The rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), is an economically important pest of rice around the world (Zibaee et al., 2008). In China, it has a very broad distribution from Heilongjiang to Hainan Provinces, and overwinters as diapausing larvae (Shen and Xue, 1988). Previous studies elucidated physiological regulation mechanisms of cold hardiness of the rice stem borer larvae, including the supercooling point (SCP), survival rates after exposure to low temperature, water, lipids, proteins, nucleation-activation components, and the activities of some related enzymes (Tsumuki and Kanehisa, 1980; Hiral and Tsumuki, 1995; Qiang et al., 2008; Qiang et al., 2012). There also were reports about seasonal changes in glycerol, phospholipids, major cryoprotectants and relative enzymes activities in overwintering larvae (Li et al., 2002; Ishiguro et al., 2007; Izumi et al., 2009; Hou et al., 2009; Atapour et al., 2009). However, no study has integrated the information obtained by simultaneous recordings of physiological and biochemical aspects of cold hardiness with variations among amino acids, low-molecular carbohydrates and three antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in the rice stem borer larvae during overwintering. Such integration aims to give a better understanding of overwintering strategy in a complexity according to my individual viewpoint. Therefore, the main purpose of the present study was to examine the changes in supercooling capacity and physiological mechanism of cold hardiness of the diapausing larvae of the rice stem borer during overwintering.

MATERIALS AND METHODS

Insects

Overwintering larvae of the rice stem borer were collected from the paddy fields in suburbs of Yangzhou City ranging from 32°15'N to 32°25'N, China, on 10/15/2008, 12/15/2008, 01/15/2009, 03/01/2009 and 04/01/2009. A portion of the overwintering larvae were held in -70°C for subsequent determination of physiological and biochemical indices. The data of monthly mean (environment temperature) air temperature, maximum and the minimum air temperature of Yangzhou City from October 2009 to April 2010 were provided by the Meteorological Bureau of Yangzhou City.

Determination of SCPs

SCPs of the overwintering larvae were measured by the method of Dautel and Knülle (1997) using a copper-constantan thermocouple connected to a chart recorder and cooling the sample at a rate of 3~5°C per minute. Fifteen individuals were used to determine SCPs for each collection date.

Determination of the contents of glycerol

Wet weight of the overwintering larvae were determined individually to the nearest 0.1 mg (M124, BEL Engineering, Italy), and homogenized in 20 µl dH_2O and then 480 µl dH_2O was added. The homogenate was centrifuged at 5000×g for 10 min under room temperature, after which the supernatant was collected and sediment was diluted with 500 µl dH_2O and gently mixed by inverting. After a second centrifugation step under the same conditions, the supernatants were combined. Glycerol content in the combined supernatant was determined using Fletcher method (Feltcher, 1968) and expressed as µg per milligram wet weight (µg/mg WW). We conducted 5 independent biological replicates.

Determination of the contents of amino acids

The overwintering larvae were weighed and homogenized in 2 ml absolute alcohol. The following extraction was carried out according to Izumi et al. (2005). Amino acids in the larval extract were derivatized using 6-aminoquinolyl-N-hydroxysuccinimi-dylcarbamate (AQC). The derivatized amino acids in the supernatant of larval extract were separated by reverse-phase column chromatography and detected by fluorescent detection(Bouchereau et al., 1999). The amounts of amino acids in the sample were calculated based on standard curves of derivatized individual authentic amino acid standards and expressed as mg per 100 mg wet weight (mg/100 mg WW). Each analyzed sample contained 10 larvae and treatment had three replicates.

Determination of the contents of low-molecular weight carbohydrates

Over 100 overwintering larvae were weighed and dried for 24 h at 60°C. Dry individuals, after the assessment of the dry weight were incubated for 15 min at 20°C in 20 ml of 80% ethanol. After centrifugated at 15000 g for 10 min, the supernatant was collected and the procedure was repeated twice. The following extraction was carried out Liang et al. (2005). Low-molecular carbohydrates in the overwintering larvae were determined by high performance liquid chromatography (HPLC; Agilent 100) with Sugarpak column (6.5 × 300 mm). The mobile phase was pure water within 60 min at the flow rate of 0.4 ml/min, and the column temperature was maintained at 85°C. Identity of the revealed components was established against authentic standard. The content of low-molecular weight carbohydrate was expressed as µg per milligram wet weight (µg/mg WW). Three independent biological replicates were performed.

Determination of the activity of antioxidant enzymes

The activities of three antioxidant enzymes, including SOD, CAT and POD, in the overwintering larvae were assayed as described in the kit protocols of Najing Jiancheng Bioengineering Institute (China). The whole process was performed on ice. About 100 larvae were homogenized and centrifuged, and then the supernatants were used immediately as the enzyme sources to assay SOD, CAT and POD activities. Specific enzyme activity was expressed as unit per g wet weight (U/g WW). All assays were performed in duplicate and each assay was repeated at least 3 times.

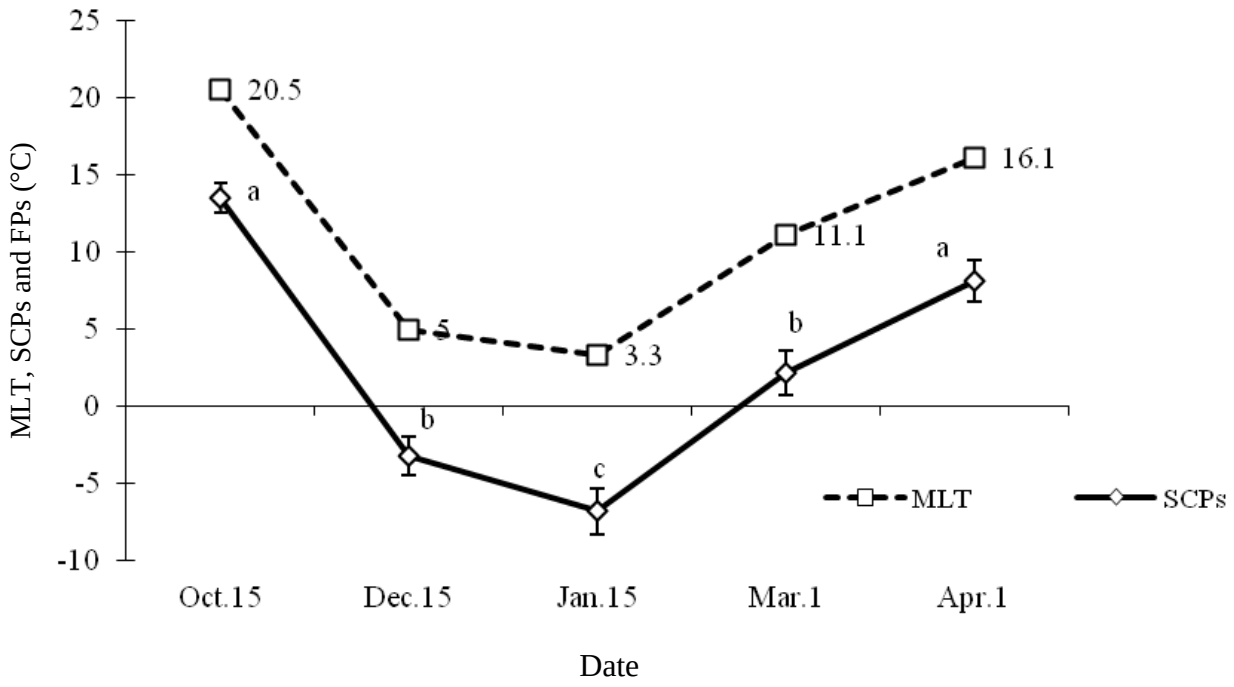

Figure 1. Dynamic changes of mean monthly temperature (MLT) in relation to SCP of the rice stem borer larvae during overwintering (mean ± SE). Different letters show significant difference at 0.05 level of the same index among different months.

Statistical analysis

Data were compared by multiple comparison for proportions at the five percent probability level with Duncan's test for the comparison through DPS2000 (Data processing system) software (version 6.55). The experimental results were reported as means ± standard error. Principal component analysis (PCA) and systemic cluster analysis of the DPS2000 software were used to evaluate the roles that some cold-hardiness substances might play in the rice stem borer larvae during overwintering.

RESULTS

Ambient temperature and SCPs of the overwintering larvae

The ambient temperature in Yangzhou City as provided by Meteorological Bureau was 9.4°C in October. This reached its lowest point in January (-4.8°C), and came back to 2.9°C in April. The monthly temperature dataset of the same period is given in Figure 1. The mean SCP of the overwintering larvae of the rice stem borer was the highest on October 15 (mean, -7.01°C), gradually decreased to -10.09°C on January 15, and then increased to -7.93°C on April 1. Thus, it could be seen that the SCP of the overwintering larvae from October 15 to April 1 first decreased and then increased. These changes were statistically significant different at the 0.05 level. The same patterns were also observed in the change of simultaneous ambient temperatures.

The contents of glycerol in the overwintering larvae

The contents of glycerol in whole bodies of the rice stem borer larvae varied greatly at different times in overwintering larvae ($P<0.05$), increasing linearly from about 4.15 μg mg^{-1} on October 15 to 16.33 μg mg^{-1} on January 15, and then dropping to 4.68 μg mg^{-1} on April 1, respectively (Figure 2). Simultaneously, there is a remarkably negative linear correlation between glycerol contents and its corresponding SCPs ($R=0.95$, $F=27.46$, $P<0.05$). These results suggest that glycerol, a primary colligative cryoprotectant, might directly regulate cold hardiness of the overwintering larvae.

The contents of amino acids in the overwintering larvae

Seventeen amino acids were detected in whole bodies of the rice stem borer larvae during overwintering (Table 1). The total contents of amino acids during the period from October 15 to January 15 increased significantly, but no apparent difference was observed during subsequent months ($P<0.05$). Other than cysteine, leucine and histidine, the changes of fourteen amino acids in overwintering larvae were first increased and then decreased ($P<0.05$). Only methionine was less than 0.35 mg·100 mg^{-1}. The amounts of serine, arginine, glycine valine, proline and tyrosine were higher on January 15 than on October 15 by 40%, whereas the amount of leucine,

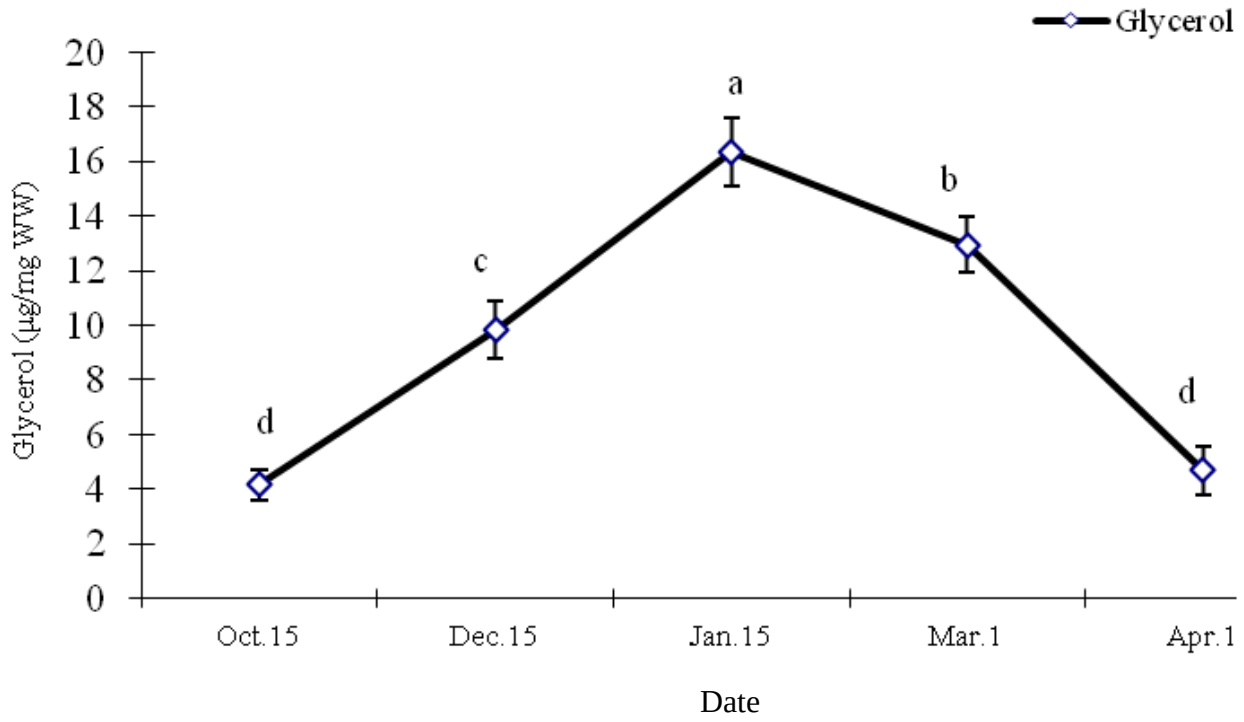

Figure 2. Dynamic changes of the contents of whole body glycerol in the rice stem borer larvae during overwintering. Values labeled with the same letters on the line are not significantly different at the 5% level by Duncan's test after DPS2000 software.

Table 1. Changes in the contents of amino acids in whole bodies of the rice stem borer larvae during overwintering.

Amino acid	Content (mg/100 mg WW)				
	October 15	December 15	January 15	March 1	April 1
ASP	1.13 ± 0.89^{c}	1.22 ± 0.94^{b}	1.38 ± 0.49^{a}	1.23 ± 0.73^{b}	1.15 ± 0.28^{c}
THR	0.42 ± 0.09^{b}	0.46 ± 0.08^{b}	0.56 ± 0.10^{a}	0.53 ± 0.07^{a}	0.447 ± 0.17^{b}
SER	0.52 ± 0.03^{e}	0.79 ± 0.14^{b}	1.00 ± 0.17^{a}	0.75 ± 0.21^{b}	0.72 ± 0.25^{c}
GLU	1.46 ± 0.52^{c}	1.44 ± 0.87^{c}	1.92 ± 0.51^{a}	1.52 ± 0.97^{b}	1.53 ± 0.59^{b}
GLY	0.45 ± 0.08^{d}	0.59 ± 0.04^{b}	0.67 ± 0.04^{a}	0.60 ± 0.05^{b}	0.54 ± 0.01^{c}
ALA	0.54 ± 0.01^{d}	0.63 ± 0.03^{b}	0.72 ± 0.01^{a}	0.59 ± 0.04^{c}	0.54 ± 0.01^{d}
CYS	0.07 ± 0.00^{ab}	0.06 ± 0.01^{b}	0.07 ± 0.00^{ab}	0.07 ± 0.01^{ab}	0.08 ± 0.02^{b}
VAL	0.54 ± 0.11^{c}	0.66 ± 0.20^{b}	0.78 ± 0.09^{a}	0.63 ± 0.10^{b}	0.58 ± 0.08^{c}
MET	0.21 ± 0.00^{c}	0.25 ± 0.05^{b}	0.28 ± 0.04^{a}	0.27 ± 0.01^{a}	0.23 ± 0.02^{bc}
ILE	0.47 ± 0.01^{c}	0.55 ± 0.06^{b}	0.63 ± 0.07^{a}	0.53 ± 0.10^{b}	0.47 ± 0.08^{c}
LEU	0.86 ± 0.12^{b}	0.73 ± 0.09^{c}	1.08 ± 0.10^{a}	0.85 ± 0.08^{b}	0.74 ± 0.05^{c}
TYR	0.65 ± 0.05^{d}	0.79 ± 0.04^{b}	0.92 ± 0.07^{a}	0.74 ± 0.01^{c}	0.78 ± 0.03^{b}
PHE	0.53 ± 0.01^{c}	0.68 ± 0.00^{a}	0.68 ± 0.04^{a}	0.60 ± 0.08^{b}	0.53 ± 0.05^{c}
HIS	0.37 ± 0.02^{d}	0.46 ± 0.01^{bc}	0.49 ± 0.03^{b}	0.45 ± 0.08^{c}	0.83 ± 0.07^{a}
LYS	0.73 ± 0.08^{c}	0.92 ± 0.01^{ab}	1.00 ± 0.09^{a}	0.86 ± 0.04^{b}	0.80 ± 0.05^{c}
ARG	0.56 ± 0.02^{d}	0.67 ± 0.05^{c}	0.88 ± 0.09^{a}	0.76 ± 0.04^{b}	0.72 ± 0.06^{bc}
PRO	0.48 ± 0.01^{c}	0.66 ± 0.08^{a}	0.69 ± 0.04^{a}	0.66 ± 0.05^{a}	0.55 ± 0.01^{b}
Total	9.98 ± 2.42^{b}	11.57 ± 1.61^{ab}	13.75 ± 2.43^{a}	11.73 ± 1.69^{ab}	11.22 ± 0.81^{ab}

serine, valine and isoleucine decreased from January 15 to April 1 by more than 20%. Serine and valine (major amino acids in whole bodies) showed a significant change over the winter season, increasing linearly from 0.52 and 0.54 mg·100 mg^{-1} on October 15 to 1.00 and 0.78 mg·100 mg^{-1} on January 15, and then dropped to

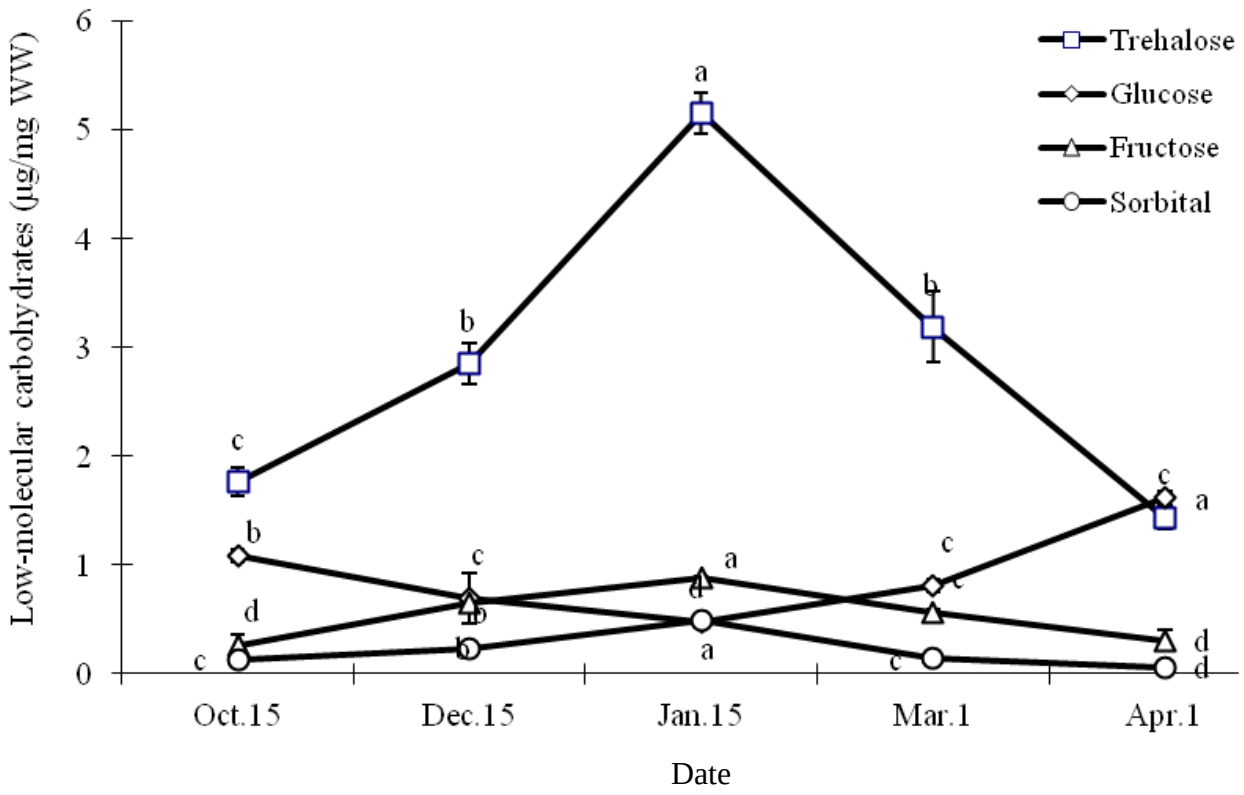

Figure 3. Dynamic changes of the contents of low-molecular weight carbohydrates in whole bodies of the rice stem borer larvae during overwintering. Values labeled with the same letters on the same line are not significantly different at the 5% level by Duncan's test after DPS2000.

0.72 and 0.58 mg· 100 mg^{-1} on April 1, respectively.

The contents of low-molecular weight carbohydrates in the overwintering larvae

The chromatograms of supernatant of the overwintering larval extracts contained four peaks corresponding to trehalose, glucose, fructose and sorbitol, with elution times at 10.6, 13.2, 16.3 and 23.6 min, respectively during high performance liquid chromatography (HPLC) separation. The amount of trehalose, fructose and sorbitol increased remarkably to 190.82, 241.17 and 270.28% on January 15 over those on October 15, and then decreased again to 71.96, 64.80 and 81.27% on April 1 over those on October 15, respectively (P<0.05) (Figure 3). In short, these three carbohydrates first decreased and then decreased during overwintering. However, glucose change was different from that of trehalose, fructose and sorbitol. Glucose content on January 15 was about 55.25% of that on October 15, but it increased again on April 1 to about 234.51% over January 15 (P<0.05). A remarkably negative linear correlation between the contents of trehalose, fructose and their corresponding SCPs (*R*=0.91, *F*=17.50, *P*<0.05; *R*=0.92, *F*=15.69, *P*<0.05) can be obtained, respectively. There is no apparent correlation between the contents of glucose and sorbitol and the level of larval SCPs (*P*<0.05).

The activities of antioxidant enzymes in the overwintering larvae

The activities of three antioxidant enzymes including SOD, POD and CAT in the rice stem borer larvae during overwintering were analyzed (Figure 4). SOD activity increased from October 15 to March 1 by 66.62% and then decreased again on April 1 by 4.75% over March 1 (P<0.05). CAT activity also increased consistently during overwintering.

Meanwhile, POD activity on January 15 decreased remarkably to about 34.69% of that on October 15, and then increased to about 63.58% of that on April 1 during overwintering (*P*<0.05).

Principal component analysis and systemic cluster analysis

Twenty-seven physiological indices in Table 3 of the rice stem larvae borer during overwintering were transformed

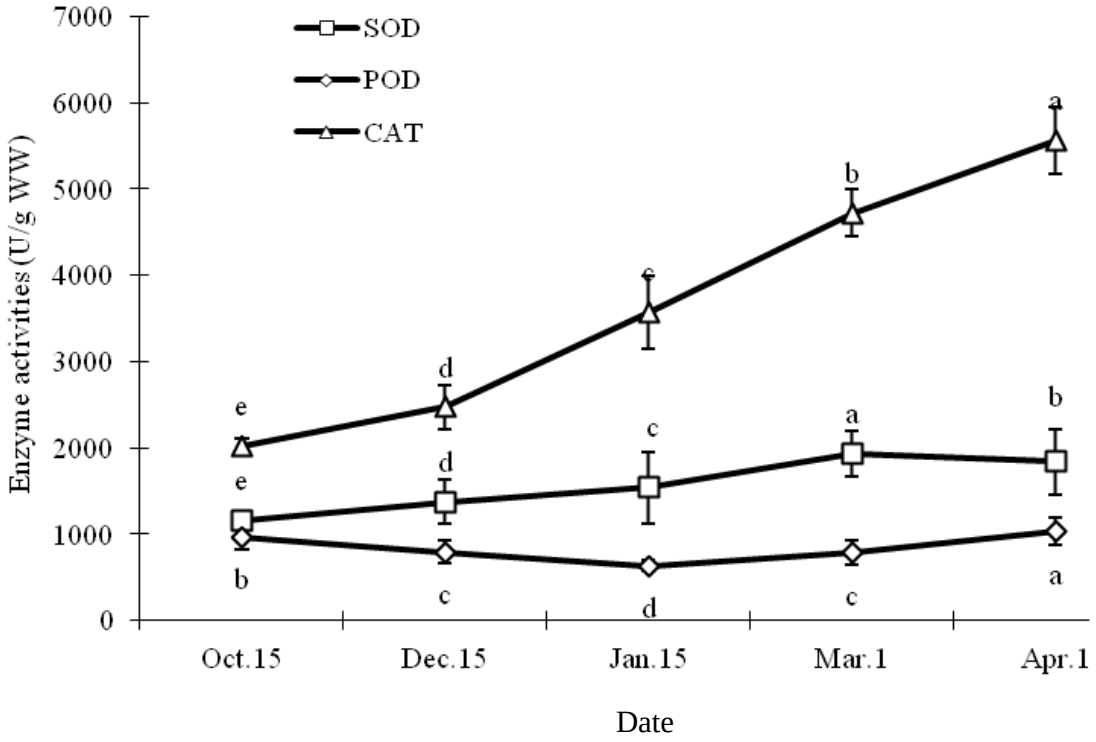

Figure 4. Dynamic changes of the activities of antioxidant enzymes in whole bodies of the rice stem borer larvae during overwintering. Values labeled with the same letters on the same line are not significantly different at the 5% level by Duncan's test after DPS2000.

Table 2. Characteristic root, contribution rate and cumulative contribution rate (%).

Number	Characteristic root	Contribution rate	Cumulative contribution rate
1	19.7052	72.9824	72.9824
2	4.3608	16.1509	89.1333
3	1.8575	6.8797	96.013
4	1.0765	3.987	100

Two PCA equations of cold-hardiness substances of the overwintering larvae were established by the Eigenvectors from the first two characteristic roots (Table 3). Contribution rate of the first characteristic root reached about 72.98%. The coefficients of glycerol, aspartic acid, valine, isoleucine, trehalose and fructose are higher than other indexes; thus they composed the main cold-hardiness substances of the overwintering larvae. Contribution rate of the second characteristic root reached about 16.15%. The coefficients of cysteine, histidine, SOD and CAT were relatively higher than others, indicating that their accumulative effects are the second cold-hardiness substances of the overwintering larvae.

into 27 Eigenvectors using the DPS2000 software. The calculated results of characteristic roots, contribution rates and cumulative contribution rates were shown in Table 2. Contribution rates of the first two characteristic roots accounted for 72.98 and 16.15% of the total variance, respectively and their cumulative contribution rates reached about 89.13%, involving cold-hardiness substances of the overwintering larvae.The systemic cluster graph was generated based on the general scores of twenty-seven physiological indices using the cluster-average method of DPS2000 software (Figure 4). According to the cluster graph and 5.69 distance modulus, cold-hardiness roles of glycerol, amino acids, low-molecular carbohydrates and antioxidant enzymes in the larvae during overwintering was divided into three groups: October 15 and April 1, December 15 and March 1, and January 15. The sequence was: January 15>December 15 and March 1>October 15 and April 1. Cold-hardiness roles of these substances on October 15 might be the same as that on April 1, and the roles between December 15 and of March 1 were also uniform.

DISCUSSION

In this study, overwintering larvae of the rice stem borer

Table 3. Eigenvectors of physiological indices of the larvae during overwintering.

Index	1st PC	2nd PC	Index	1st PC	2nd PC
SCP	-0.2168	-0.1195	TYR	0.1964	0.1422
FP	-0.2149	0.1234	PHE	0.1948	-0.1569
Glycerol	0.2173	-0.0103	HIS	-0.0424	0.4250
ASP	0.2236	0.0043	LYS	0.2174	-0.0007
THR	0.2072	0.091	ARG	0.1968	0.2298
SER	0.2127	0.1236	PRO	0.2033	0.0272
GLU	0.1818	0.1137	Trehalose	0.2202	-0.0541
GLY	0.2115	0.1115	Glucose	-0.182	0.2716
ALA	0.2180	-0.0695	Fructose	0.2223	-0.0503
CYS	0.0180	0.3945	Sorbitol	0.2032	-0.1007
VAL	0.2212	0.0139	SOD	0.0379	0.3963
MET	0.2152	0.0443	POD	-0.2155	0.1231
ILE	0.2221	-0.0743	CAT	0.0065	0.4606
LEU	0.1595	-0.025			

were collected from the paddy fields and seasonal change of cold hardiness was compared. The results show that seasonal change of SCPs of the larvae during overwintering took on V-shaped characteristic, the SCP being lowest (mean, -11°C) on January 15 when ambient temperatures reached the lowest point (-4.8°C) (Figure 1). Overall dynamic change of larval SCP is closely related to the change of environmental temperature. Based on our data, we concluded that the rice stem borer larvae cannot tolerate freezing of body liquids and are able to decrease their SCP in adapting cold temperature through increasing the concentrations of cryoprotectants in their body.

Our data also indicate that glycerol is a primary cryo-protectant or at least one of the primary ones. We believe that low temperature stimulates the shift of some metabolic pathways, leading to the accumulation of glycerol. Accumulation of low-molecular weight carbohydrates, polyols and sugars and so forth, as cryoprotectants to endure cold stress, has been observed in many overwintering insects, and their physiological and biochemical mechanisms had also been studied (Goto et al., 2001; Storey and Storey, 1991; Izumi et al., 2006). Similar to *Pyrrhocoris apterus* (Koštál and Šimek, 2000), *Ostrinia furnacalis* (Goto et al., 2001) and *Cucujus clavipes* (Bennet et al., 2005), glycerol accumulation in the rice stem borer larvae is closely related to ambient temperatures and its peak appeared in January (Figure 2). Apparently, the rice stem borer has more carbon sources devoted to glycerol synthesis pre-winter and during winter to endure lower-temperature stress as a prevailing cryoprotectant similar to some other insect species (Tsumuki and Kanehisa, 1978; Goto et al., 2001; Li et al., 2001b; Li et al., 2002; Ishiguro et al., 2007). In addition to the positive relationship between glycerol concentration and larval cold hardiness, our data suggest that trehalose also may contribute considerably to the ability of the rice stem borer larvae in resisting low temperature (Figure 3). Seasonal change of trehalose was also reported in *Cylindrocopturus adspersus* larvae during overwintering (Rojas et al., 1994). It has generally been considered that high concentrations of trehalose in the overwintering larvae might provide them with better protection against cold injury during prolonged sub-zero temperatures by stabilizing proteins and/or cell membranes (Rojas et al., 1994).

Sorbitol, fructose and glucose have been analyzed in some insects and their potential function in cryoprotection has also been discussed (Block and Zettel, 1980; Sømme, 1982). For example, sorbitol accumulated from the onset of diapause larvae of *Pieris brassicae* L. in October until December, reaching a concentration of 0.40 mM in both years, and then declined from January until adult eclosion in May. Therefore, it was argued that sorbitol accumulation might have no cryoprotective role as a result of metabolic suppression. The present study shows that trehalose and fructose, especially the former, may play an important role in cold hardiness of the larvae during overwintering (Figure 3).

However, the change of glucose may suggest that the sugar is converted to glycerol to low temperature stress during cold seasons (Atapour and Moharramipour, 2009). Temperature-dependent modulation of polyol content also occurred in insect species that seasonally synthe-sized polyols as colligative cryoprotectants (Denlinger et al., 1991; Wolfe et al., 1998). Although seasonal changes of these sugars were observed in rice stem borer, their relatively low concentrations suggest that they have very limited (if any) contribution to the cold hardiness of this species. Under field conditions, the level of total amino acids often increase by 1.5 to 2.0 folds during insect cold acclimation, but individual amino acids increase or decrease depending upon their roles in low-temperature stress (Hanzal and Jegorov, 1991; Goto et al., 2001). The

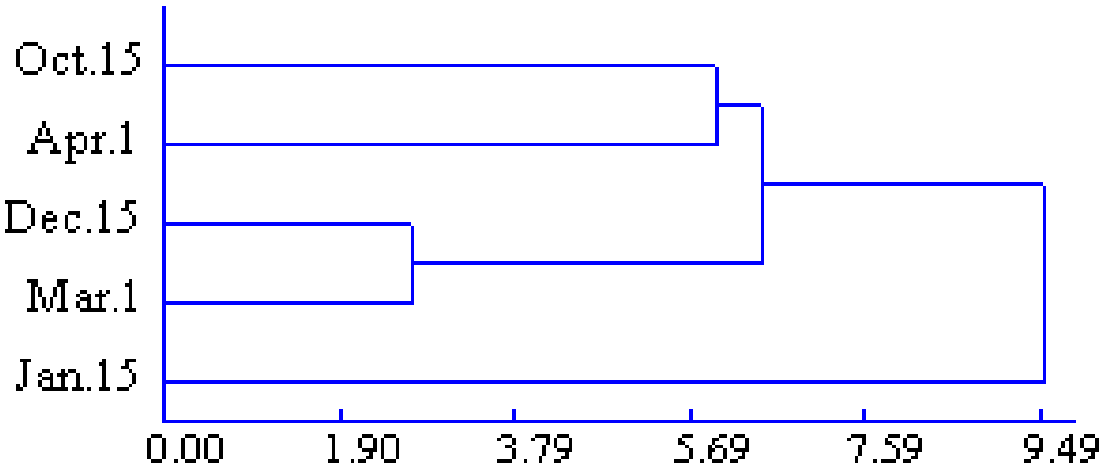

Figure 5. Systemic cluster graph of the general score of the cold-hardiness substances in the rice stem borer larvae during overwintering.

present result was consistent with those reports (Table 1). For example, the increased concentration of alanine in the rice stem borer larvae under low temperatures or during overwintering was also reported in *Enosima leucotaeniella* (Goto et al., 1998), *Cryptolestes ferrugineus* (Fields et al., 1998), and *O. furnacalis* (Goto et al., 2001). The methyl group of alanine was indicated to be related to insect cold hardiness, but there was no direct evidence supporting this prediction. The biggest change of serine content in all amino acids might be cause of certain low-temperature environment dominating glycogen transformation by glycolytic pathways in insects (Mitsuhashi, 1978; Morgan and Chippendale, 1983; Goto, et al., 2001). Therefore, these amino acids in insects were produced and accumulated during low temperature stress to decrease theirs SCPs and freezing points by the effects of the solution (Huang et al.,1990).

An eco-physiological response pattern was found in the rice stem borer larvae during overwintering according to SOD, CAT and POD activity (Figure 4). We speculated that the increased contents of $O_2^{\bullet-}$ and $OH^{\bullet}$ in the overwintering larvae occurred at the same time as the increased activities of SOD and CAT to clean out free radicals. SOD activity began to decrease along with ambient temperature, whereas CAT activity increased consistently during overwintering. CAT is the downstream enzyme of an antioxidant system in many insects (Li et al., 1999). Many superfluous H_2O_2 were decomposed by CAT- and POD-mediated reactions. Interestingly, there was a transferable phenomenon among the functions of antioxidant enzyme from SOD and CAT to CAT and POD to mitigate freezing injury to the larvae under low temperature. Therefore, the antioxidant system might provide some protection against oxidative stress for chill tolerance through coordination among SOD, POD and CAT (Felton and Summers, 1995).

As far as we know, PCA was first used to evaluate physiological roles of some relative cold-hardiness substances of overwintering insects. Our results not only suggested that glycerol, aspartic acid, valine, isoleucine, trehalose and fructose were the main cold-hardiness substances of overwintering larvae (Tables 2 and 3), but also showed the direction for relative research into the future. Systemic cluster graph of the general score of glycerol, amino acids, low-molecular carbohydrates and antioxidant enzymes in the overwintering larvae showed that the sequence of their cold-hardiness roles was January 15>December 15 and March 1>October 15 and April 1 (Figure 5). This result was similar to seasonal changes in SCPs of the overwintering larvae (Figure 1).

In summary, our data provide an overall picture regarding the seasonal changes of the cryoprotective species in the rice tem borer and a reasonable basis to suggest their physiological roles in the survival of the species in a cold environment. *C. suppressalis* Walker is a major pest of rice, the most important food resource for hundreds of thousands people in the world. Hence, a comprehensive understanding of the biochemical mechanisms and overwintering behaviors that promote its mortality during cold season may provide insight for the development of integrated pest management of the rice stem borer in paddy field.

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