

Implication of pupal cold tolerance for the northern over-wintering range limit of the leafminer *Liriomyza sativae* (Diptera: Agromyzidae) in China

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Abstract

This study investigated the cold tolerance of a laboratory-reared population and latitude-separated populations of the leafminer *Liriomyza sativae* in China in terms of low-temperature survival rate and supercooling capacity. Pupae of the laboratory-reared population are susceptible to freezing temperatures. The supercooling point of the pupae varied at a maximum of up to 10°C among the geographic populations. Both acclimation of the pupae at 5 and 10°C significantly increased the cold survival rate of laboratory-reared and field-collected populations. But the field population had a more active response to the same acclimation regime. Combining the experimental data with previous field investigations, the -2°C isotherm of the minimum mean temperature of January was proposed as the leafminer's over-wintering range limit. Meanwhile, with the widespread availability of greenhouses as a source of re-infestation in northern China, the leafminer's natural border of distribution should be determined by the minimum temperature in warm seasons. Our results suggest that biologically similar *Liriomyza* species may cope with the intensifying cold stress along the latitude by adopting a mixed cold-tolerance strategy, which is closely associated with the greenhouse microhabitats. The physiologically based modeling of the over-wintering limit provides a tool for guiding the management for greenhouse pests and predicting the source of pest infestation.

Key words: Cold tolerance; range limit; geographic variation; leafminer; *Liriomyza sativae*

INTRODUCTION

The vegetable leafminer *Liriomyza sativae* Blanchard is a worldwide pest that inflicts severe damage to agricultural products (Parrella, 1987). This pest species is polyphagous, and infests numerous plants including agricultural and ornamental plants in more than 14 families (Spencer, 1992). Female adults of the leafminer feed by puncturing the leaf mesophyll cells and deposit eggs through the adaxial or abaxial leaf surface. Larvae begin feeding immediately after eclosion and form tunnels in the leaf until they are ready to emerge from the leaf (Parrella, 1987). Thus global dissemination of this pest is often associated with international trade in plant produce or growing plants. In 1993, the vegetable leafminer was found for the first time on Hainan Island, in southernmost China (Kang, 1996). Thereafter, outbreaks of the leafminer as a local pest were reported in several other provinces

almost simultaneously (Kang, 1996; Zhang, 1997), implying an earlier establishment of this species in China.

Several studies reported the coexistence or displacement of *L. sativae* with other vegetable leafminers such as *L. huidobrensis*, *L. trifolii* or *L. bryoniae* in Asia and South America (Rauf et al., 2000; Abe and Kawahara, 2001; Andersen et al., 2002; Chen and Kang, 2002a; Reitz and Trumble, 2002). This leafminer, together with another biologically and systematically related leafminer, *L. huidobrensis*, are today two of the most economically important pests on vegetable and ornamental plants throughout China (Chen and Kang, 2002a). Previous studies suggest that the widespread infestation and severe outbreaks of the leafminers are attributed to many factors, including their rapidly developed insecticide resistance, multivoltine nature, polyphagous feeding, and lack of natural enemies in area invaded (Kang, 1996; Abe and Kawa-

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hara, 2001; Reitz and Trumble, 2002). However, all of these factors fail to account for the observed interspecific displacement or divergence of population dynamics over a large spatial and temporal scale between *L. sativae* and *L. huidobrensis* (Rauf et al., 2000; Weintraub, 2001; Chen and Kang, 2002a).

The range of species' distribution is usually closely associated with its ecophysiological adaptation to climatic stresses (Hoffmann and Blows, 1994; MacDonald et al., 1999). *L. sativae* is considered to be native to the southern United States and South America, but has become adapted to localized environments that range from holarctic to tropical (Spencer, 1973; Reitz and Trumble, 2002). The leafminer has 15–18 generations throughout the year in open fields in the sub-tropical regions of southern China (Zhang, 1997). While it expands to more northerly temperate regions, low temperatures become a critical determinant of the viability of an insect population (MacDonald et al., 1999; Bale et al., 2002). Hodkinson (1999) also recognized that the distribution of such polyphagous species appears to be directly restricted by its own ecophysiological tolerances, largely independent of its host plant. Thus cold stress tolerance is commonly essential for the completion of the lifecycle and successful over-wintering. For instance, although the species occurs on vegetable and ornamental plants throughout the southern United States, it does not normally over-winter as far north as Maryland (Webb and Smith, 1970). Accordingly, it is speculated that this leafminer should have a northern over-wintering range limit closely related to the species' cold tolerance.

The ability of the eggs of *L. sativae* to survive in chrysanthemum was examined during cold storage (Webb and Smith, 1970). However, the leafminer usually over-winters at the stage of pupa (Parrella, 1987). Zhao and Kang (2000) reported the cold tolerance of leafminer pupae from a laboratory-reared population that has an average supercooling point of about -11°C , and the lethal time leading to 50% mortality of 4-d-old pupae at 0°C was 4.5 d. However, it has not been determined how the pupae of *L. sativae* respond to subzero temperatures that may be frequently experienced by the insects in northern China in the late autumn or winter. For example, the mean temperature in January in WuHan ($30^{\circ}38'\text{N}$), where the leafminer can suc-

cessfully over-winter, is -5.2°C (Wang et al., 1999). Therefore, survival response at subzero or freezing temperatures is a factor important for subsequent population levels in the coming spring.

However, with the availability of the widespread greenhouses in northern China, the leafminer has dispersed far beyond its over-wintering range limit in northern China where they inhabit and develop using the greenhouses as thermally-buffered microhabitats most of the year. For instance, there are only 3–4 generations in fields, but 8–11 generations each year in the greenhouses in Chifeng ($42^{\circ}16'\text{N}$). The fly can't be found in fields until mid-May when the mean ambient temperature rises to 15.5°C (Guo et al., 2000). Accordingly, the population dynamics may be changed when the leafminers explore different habitats on the two sides of the over-wintering range limit, which in turn influences the management strategy of the pest (Kirk and Terry, 2003; Kimura, 2004). Unfortunately, the ecological importance of the over-wintering range limit is seldom recognized, and thus not discriminated from that of the species' border of distribution in the past studies.

On the other hand, populations in the field may have developed a differential response to cold stress as compared to laboratory-reared populations (Lee, 1991). For western flower thrips, *Frankliniella occidentalis*, it appeared that they were able to over-winter in colder areas than laboratory experiments could predict because of the protected microclimates. Thus the effect of greenhouses on the development of cold tolerance needs to be taken into account (MacDonald et al., 1997). Linden (1993) investigated the ability of *Liriomyza bryoniae* and *L. huidobrensis* pupae to over-winter outdoors, and found that the survival of over-wintering pupae was lower when compared with directly reared pupae. Cold acclimation is an important strategy adopted to enhance the ability of many insects to survive (Lee, 1991; Danks, 1996). *L. sativae* doesn't enter diapause (Parrella, 1987), but may be able to acclimate in response to low temperatures in the early spring and late autumn. However, we don't know to what degree *L. sativae* can adapt to harshening cold by enhancing cold tolerance except by shelter exploitation.

To clarify these questions, we investigated the survival response of *L. sativae* pupae to subzero temperatures down to their supercooling point

(SCP). The cold tolerance of pupae from different origins was also compared, in association with that of the pea leafminer, *L. huidobrensis*, since the two leafminers species are closely related in population dynamics, history of establishment in China and potentially compete on the same host plant (Bird and Hodkinson, 1999; Chen and Kang, 2002a). The effects of acclimation on the cold tolerance of different types of *L. sativae* populations were investigated, so that we can precisely predict their northern over-wintering limit and border of distribution.

MATERIALS AND METHODS

Insect colony. The laboratory-reared population of *L. sativae* was originally collected from vegetable greenhouses in Beijing in 1997, and had been reared in the laboratory for more than 90 generations. The rearing conditions were kept at $25 \pm 1^\circ\text{C}$ with 14L : 8D, 6,000 lx and 70% RH in an environmental chamber (Conviron Co., Winnipeg, Manitoba, Canada). The host plant was a vegetable bean, *Phaseolus vulgaris* L. Details of the nursing method were described by Zhao and Kang (2000).

Natural populations of *L. sativae* were collected from six places to investigate their variation in super-cooling capacity along a latitudinal gradient: SanYa (18°10'N, 109°32'E), GuangDong (23°00'N, 113°13'E), WuHan (30°38'N, 114°04'E), Xi'an (34°15'N, 108°55'E), Beijing (39°57'N, 116°19'E) and ChiFeng (42°16'N, 118°54'E) in 2001 (Fig. 1). Three other collections of natural populations were made to investigate the difference in acclimation effect and seasonal variation in the SCP: (1) a greenhouse population collected in Beijing, October 15, 2002, when *L. sativae* were not available in fields but could be obtained in greenhouses; (2) a field population collected in Beijing, June 8, 2004; and (3) a field population collected in SanYa, March 15, 2002. The 1-d-old pupae of each sample were subject to measurements as soon as they were transferred to the laboratory of the Institute of Zoology in Beijing.

At the collection sites, the mean temperature is lowest in January and highest in July. The mean temperature in January decreases as latitude increases, but the annual air-temperature average increases as latitude increases. *L. sativae* develops year-round in the local fields of SanYa, on the

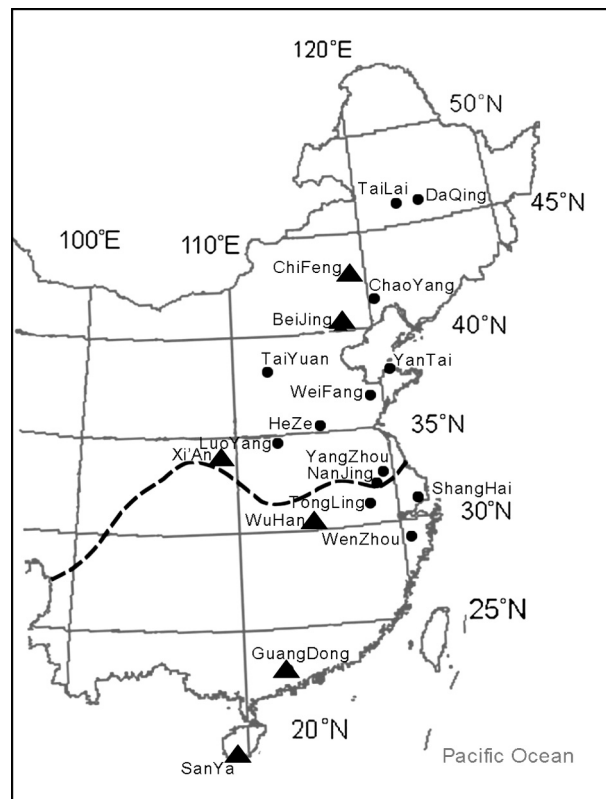


Fig. 1. Distribution of *L. sativae* in east China. Triangles (▲) indicate the locations of the collection sites in the present study; Dots (●) indicate some of the locations near the southern border, northern border and over-wintering limit of distribution where *L. sativae* were recorded to appear each year. The broken curved line between 31°N and 34°N is the northern over-wintering limit of *L. sativae*, beyond which the fly can't over-winter outdoors. The line was simulated based on the -2 isotherm of monthly minimum mean temperature in January (Meteorological Documentation Department of National Weather Bureau of China, 1990; Also see text).

southernmost Hainan Island. In contrast, the pest in northern China mainly thrived in greenhouses. For example, in Beijing, the pest spends the winter in greenhouses when the minimum mean temperature in January drops to -9.9°C , and cannot over-winter outdoors (Table 1).

Low-temperature survival of 1-d-old pupae. The pupae were dried on the surface and enclosed with pin-holed parafilm when exposed to low temperature in an incubator ($\pm 0.3^\circ\text{C}$ temperature fluctuation). The temperature was lowered at a rate of 1°C per min from the start (25°C) to the scheduled temperatures. The pupae were held at those temperatures for various periods. After exposure treatment, the temperature was increased at a rate of 1°C per min to 10°C . Then the pupae were re-

Table 1. Monthly and yearly averages of mean, maximum mean (abbreviated as max) and minimum mean (abbreviated as min) temperatures at six sample sites (2004) in China. The data were recorded during 1970–1990 and obtained from the Meteorological Documentation Department of National Weather Bureau of China (1990). See Fig. 1 for the location of sample sites

	February (°C)		July (°C)		Yearly (°C)		
	Mean	Min	Mean	Max	Mean	Min	Max
SanYa	21.4	18.1	28.5	31.7	25.5	22.5	29.4
GuangDong	13.6	9.2	28.3	32.7	21.8	18.1	26.2
WuHan	13.6	9.2	28.3	32.7	21.8	18.1	26.2
XiAn	-2.9	-6.2	26.4	34.3	12.8	9.0	20.9
Beijing	-4.7	-9.9	26.1	31.8	10.8	6.1	18.2
ChiFeng	-12.9	-20.1	23.8	30.2	6.6	-0.6	13.8

moved from the incubator, unwrapped from the parafilm and kept in an environmental chamber at $26 \pm 0.5^\circ\text{C}$. The number of adults emerged and the number of dead pupae were checked after 20 d.

One-day-old pupae of the leafminer were exposed to 0, -2.5 and -5.0°C for 0.5, 1, 2, 4 and 8 d, respectively. Survival response to freezing temperature near the SCP of the pupae was also investigated by exposing pupae to -10.0 and -11.0°C for 2, 4, 8, 12 and 16 h, respectively. Twenty to 30 specimens were used in each of three to five replications.

SCP. Each pupa of the leafminer was affixed with plastic tape to the tip of a thermocouple that was linked to an automatic recorder (uR100, Model 4152, Yologama Elect. Co., Seoul, Korea). The sensor and pupa were put inside an insulated Styrofoam box in a freezer chest. The temperature was kept constant at -26°C to ensure that cold exposure temperature was lowered at an averaged rate of 1°C per min. The temperature at which an abrupt rebound occurred was taken to indicate the crystallization temperature (e.g., SCP). Details of the methods were described in Zhao and Kang (2000) and Chen and Kang (2002b).

Cold acclimation. To investigate the effect of cold acclimation on low-temperature survival, pupae were pre-chilled at temperatures of 5 and 10°C for a few days, respectively. The pre-chilled pupae were exposed to -5°C for 2 d as a discriminating regime. Control groups were exposed to -5°C for 2 d without pre-chilling. All other handling was similar to the previously described methodology. Twenty to 30 specimens were used in each of three to five replications.

Statistical analysis. The survival and corrected

mortality of pupae were calculated as described by Zhao and Kang (2000). GLM ANOVA (SPSS) was used where appropriate to test for the effects of sampling site, temperature, exposure time and main interactions on pupal mortality. Where significant differences were observed, the effect of treatment was determined by Least Significant Difference (LSD). $L_{\text{time}_{50}}$ and $L_{\text{time}_{90}}$, estimated by Probit analysis, indicate the time required to achieve 50 and 90% mortality, respectively, at a specific temperature. Differences among SCPs were compared using LSD one-way ANOVA.

RESULTS

Low-temperature survival of 1-d-old pupae

Survival of the leafminer pupae decreased with the extended cold exposure time (Fig. 2). ANOVA of the data set at 0, -2.5 , -5.0°C showed a strong significant effect of exposure temperature and time on survival (temperature: $F_{2,55}=48.464$, $p<0.001$; time: $F_{4,55}=35.484$, $p<0.001$); the effect of interaction between the exposure temperature and time of survival was also significant ($F_{7,55}=4.668$, $p=0.001$). Little mortality of the pupae occurred at 0°C , with an average survival rate of 78% after 8-d exposure. In contrast, exposure to -2.5°C led to a 56% mortality rate after 8-d exposure. There was a sharp decrease in the survival rate from 71% after 12 h to 6% after 4-d cold exposure to -5°C . However, no pupae could survive more than 1 d when exposed to low temperatures near their SCP. All pupae were frozen to death after 14-h exposure at -10°C . The exposure time of a few hours had a significant effect on the survival of pupae ($F_{4,20}=20.406$, $p<0.001$). No pupae could survive

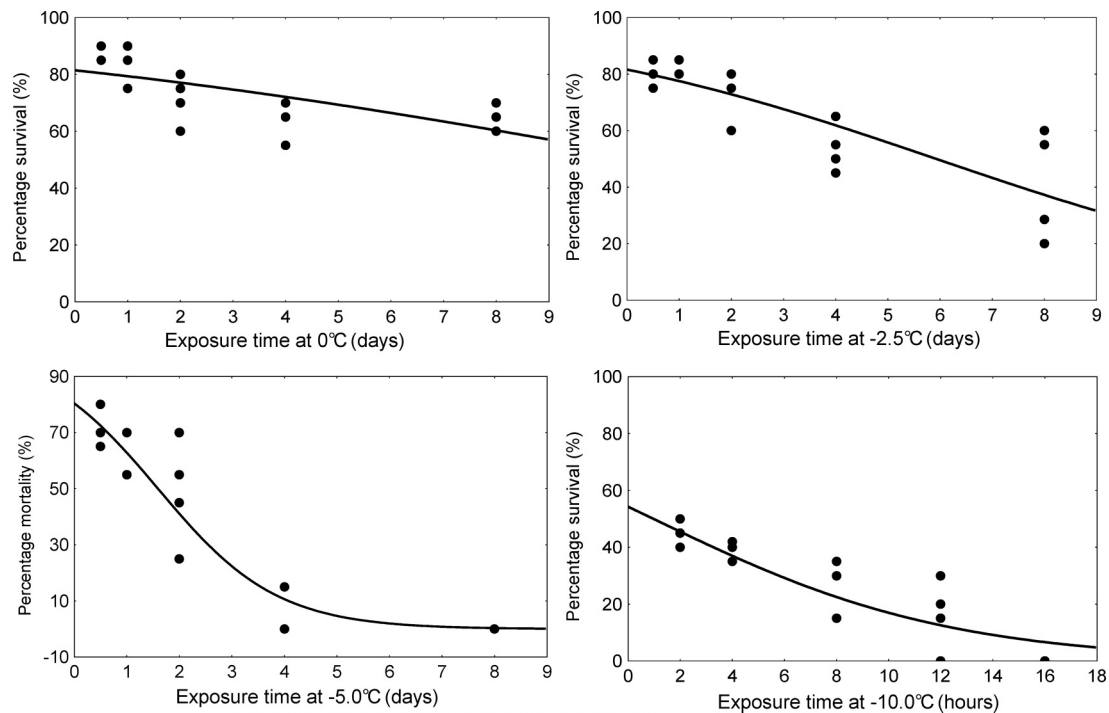


Fig. 2. Average corrected survival of *L. sativae* pupae as affected by time and low temperature. Dots are observed values; some are overlapped. Lines are expected values from logistic regression. The regression line uses the formula: $P_t = 100 / (1 + \exp(a + b \times t))$. P_t represents the corrected percentage mortality, and t represents the exposure time (days or hours). The survival line of pupae exposed to -12°C is not listed because no pupae could survive at that temperature.

Table 2. Parameters estimate for logistic regression models relating mortality of the pupae of *L. sativae* to exposure time at different low temperatures

Temperature ($^\circ\text{C}$)	a	b	r	Ltime ₅₀ (d) ($\pm 95\%$ FL)	Ltime ₉₀ (d) ($\pm 95\%$ FL)
0	-1.4795	0.1327	0.70	—	—
-2.5	-1.4888	0.2512	0.84	6.5 (4.7–11.8)	13.6 (9.6–29.4)
-5.0	-1.4108	0.8846	0.94	1.9 (0.6–4.5)	4.0 (2.7–16.1)
-10.0	-0.1182	0.1659	0.90	0.06 (0–0.13)	0.51 (0.45–0.67)

The model uses the formula: $P_t = 100 / (1 + \exp(a + b \times t))$, where P_t is the corrected percentage mortality at exposure time t (days or hours). $r^2 = (1 - \text{residual SS of regression}) / \text{corrected SS}$. FL indicates the fiducial limits.

cold exposure at -12°C for more than 2 h.

A nonlinear model fitting procedure showed that the response of the leafminer pupae to exposure time at different low-temperature regimes was best fitted by a logistic relationship ($p < 0.001$) (Fig. 2, Table 2). Ltime₅₀ at -2.5 , -5.0 and -10°C are significantly different from each other ($p < 0.05$). Ltime₅₀ at -2.5 and -5°C extended for a few days (1.9–6.5 d). However, most pupae only survived at -10°C for a few hours. For example, the lethal time Ltime₉₀ was 0.51 d (12.3 h) at that temperature. No Ltime₅₀ and Ltime₉₀ at 0°C were calcu-

lated because most pupae could survive at that temperature.

Supercooling capacity

The mean SCPs of *L. sativae* samples differed significantly among the six geographic populations collected in the year 2001 ($F_{5,71} = 53.749$, $p < 0.001$) (Table 3). The three southern populations (SanYa, GuangDong and WuHan) significantly depressed their SCPs as latitude increased ($p < 0.05$). The southern most population in SanYa had the highest value of SCP (-10.2°C), similar with that

Table 3. Collection data and supercooling points of *L. sativae* pupae from different geographic populations

Source (Latitude)	Collection date	Host plant	<i>n</i> ^a	SCP±SE (°C) ^b
SanYa (18°N)	May 26, 2001	<i>Phaseolus vulgaris</i>	11	-10.2±0.2 a
	May 15, 2002	<i>P. vulgaris</i>	50	-12.1±0.6
GuangDong (23°N)	Mar. 15, 2001	<i>P. vulgaris</i>	27	-14.6±0.8 b
WuHan (30°N)	May 20, 2001	<i>P. vulgaris</i>	24	-19.5±0.4 c
Xi'An (34°N)	Sep. 19, 2001	<i>Cucumis sativus</i>	13	-13.2±1.3 b
Beijing (40°N)	June 5, 2001	<i>C. sativus</i>	12	-20.3±0.3 c
	Oct. 15, 2002	<i>C. sativus</i>	15	-17.9±1.2
	June 8, 2004	<i>C. sativus</i>	40	-19.1±0.5
ChiFeng (42°N)	Sep. 8, 2001	<i>Lycopersicon esculentum</i>	12	-19.9±0.6 c
Lab ^c	—	<i>P. vulgaris</i>	35	-11.7±0.1

^aNumbers of individuals tested.

^bValues of SCPs followed by the same letters are not significantly different among the six natural populations collected in 2001 at the 5% level by Tukey's-b one-way ANOVA.

^cLaboratory-reared population kept in a laboratory at 25°C for more than 6 y (data from Zhao and Kang (2000)).

of the laboratory-reared population (-11.7°C). However, the central population in Xi'An had a sharply increased SCP. The two northern populations depressed their mean SCP further, with the lowest SCP dropping down to -20.3°C in the Beijing population. There was no significant difference in the SCPs between the two northern populations in Beijing and ChiFeng.

On the other hand, the two summer populations in SanYa had no significant differences in their mean SCPs ($F_{1,63}=2.789$, $p=0.100$). The SCPs of the three Beijing populations collected in different seasons also showed no significant difference ($F_{2,80}=1.481$, $p=0.232$).

Cold acclimation

A comparison of the survival rates of the control groups in the three populations showed that the natural population collected from greenhouses in Beijing was the least cold resistant, and the laboratory-reared population was the most cold resistant (Fig. 3). However, the laboratory-reared population only achieved a significantly enhanced survival rate when acclimated at 10°C for 4 d (Fig. 3A). In contrast, the survival ability of the greenhouse population in Beijing was significantly enhanced by pre-chilling at 10°C for 1, 4 and 8 d, as well as pre-chilling at 5°C for 4 d ($p<0.05$) (Fig. 3B). The survival rate of pupae increased more after acclimation at 10°C than at 5°C. For the southern most population in SanYa, pre-chilling at 5°C for various days and pre-chilling at 10°C for 1 and 2 d re-

sulted in a significant increase in the survival rate. Generally, the induced survival rate was higher for pupae acclimated at 5°C than at 10°C (Fig. 3C).

DISCUSSION

Survival data of the laboratory-reared population of *L. sativae* showed that pupae of the leafminer could survive long-term exposure to above-zero temperatures. Some pupae could develop normally even after exposure to sub-zero temperatures for a few days. For example, the $L_{time_{50}}$ of pupae is 7 d at -2.5°C and 2 d at -5°C. The mean SCP of the laboratory-reared population of pupae is -11.7°C (Zhao and Kang, 2000), indicating a low crystallization temperature for the pupae. Thus, the leafminer pupae may tolerate sub-zero temperatures by supercooling or other physiological mechanisms, so as to keep their body fluids liquid at temperatures well below their melting point (Bird and Hodkinson, 1999; Sinclair et al., 2003). However, the regulation ability of the leafminer pupae exposed to extremely low temperatures is limited, with a high mortality rate observed above the SCP. No pupae could survive temperatures below their SCPs. Therefore, it was concluded that the leafminer is a freeze-susceptible or freeze-avoiding insect (Bale, 1996). The cold-tolerance strategy adopted by *L. sativae* is similar to that of the pea leafminer, *L. huidobrensis*, though the latter is much more cold-tolerant, with an average pupal SCP of -20.9°C (Chen and Kang, 2002b). Inter-

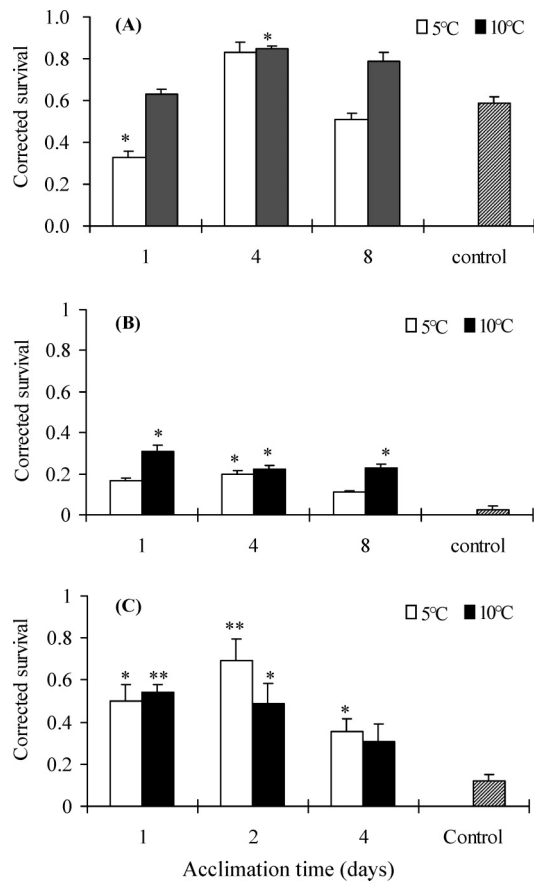


Fig. 3. Corrected mean survival rate (\pm SE) of *L. sativae* pupae acclimated for the indicated number of days at 5 and 10°C before exposure to -5°C for 2 d as a discriminating regimen. (A) Laboratory-reared population; (B) Natural population collected from greenhouses in Beijing on October 15, 2002; (C) Natural population collected in SanYa, southernmost China, on March 15, 2002. Control group: Pupae were exposed to -5°C for 2 d without prechilling. * Mean significant difference from control with *t* test at $p=0.05$ and ** at $p=0.01$.

specific differences in cold tolerance between the two leafminers are consistent with the field observation that *L. huidobrensis* is more adaptable to cool climate than *L. sativae* in China (Chen and Kang, 2002a). Thus the results confirm the hypothesis that *L. huidobrensis* tends to be a more temperate species than *L. sativae* (Spencer, 1973).

On the other hand, the interaction between exposure temperatures and periods was also shown to be an important determinant of pupae mortality. Meanwhile, the leafminer had a high mortality when exposed to above-zero temperatures. These results suggest that chilling injury might be a more important cause of low-temperature mortality than

the freezing injury for this leafminer species in nature.

Physiological measurements made for the laboratory population may be different from those in the field populations, where the modes and periods of exposure, particularly the thermo-period, become important influential factors (Lee, 1991; Bird and Hodkinson, 1999; Kim and Song, 2000). While glasshouse is daily unveiled to volatile, the leafminers in greenhouses are frequently exposed to cold air in the spring or fall in northern China, which may have a lethal effect on the leafminer. At this moment, the insect could acquire enhanced resistance to cold exposure by rapid cold hardening (Chen et al., 1987) or cold acclimation (Danks, 1996). Our experiment showed that both the populations reared in the laboratory and in the field had significantly enhanced their cold tolerance in response to pre-chilling at 5 and 10°C. Though the natural population was less cold-resistant than the laboratory-reared population, it has developed a more active response to the same pre-chilling. On the other hand, the mechanism underlying the survival response of the southernmost population can be triggered at a relatively high temperature compared with that of the greenhouse population. Therefore, although insect populations inhabiting warm climates are less cold-resistant than the temperate populations, they are more sensitive to pre-chilling; a characteristic that may function as an important mechanism to ensure their maximum inducible survival under severe cold stress in the field.

A recent study found that the difference in the cold tolerance of *L. huidobrensis* and *L. sativae* well explains their differential geographic distribution and phenology in China (Chen and Kang, 2002b). It suggests that the ability to survive at low temperatures is a critical factor determining the range of distribution of *L. sativae*. Meteorological data showed that the minimum low temperature of each locality generally decreased as the latitude increased in east China (Meteorological Documentation Department of National Weather Bureau of China, 1990). However, the isotherms in these locations are not always parallel to the latitudinal line because of the diverse topography across China. Therefore, it is not exactly the case if the species' border of distribution is estimated in terms of latitudinal line (Ungerer et al., 1999). The temperature

in each locality in January is the lowest of the year, and is therefore critical for the successful winter survival of the species (Lee, 1991; Bale et al., 2002; Chen and Kang, 2004). Accordingly, the isotherm in January instead of latitudinal line was selected for predicting the leafminer's northern over-wintering range limit.

MacDonald et al. (1997) suggested that $L_{time_{90}}$ is more indicative of the level of cold exposure that may represent a serious threat to over-wintering success at the population level. The leafminer has an estimated $L_{time_{90}}$ of 13.6 d at -2.5°C , but a lower mortality occurred at above-zero temperatures. Integrating the effect of cold acclimation on survival, we postulated that the isotherm of minimum mean temperature at -2°C in January is the range limit for *L. sativae* over-wintering in the field (Fig. 1). The -2°C isotherm lies mostly to the south of 34°N latitude, which is the proposed northern latitudinal limit of *L. sativae* based on the chilling survival ability of the laboratory-reared population by Zhao and Kang (2000). Populations of this leafminer will risk freezing to death if moving further north beyond this over-wintering thermal limit. A field investigation found that *L. sativae* could not successfully over-winter in open fields in NanJing ($32^{\circ}04'$), YangZhou ($33^{\circ}32'$), Hezhe ($35^{\circ}18'\text{N}$), LuoYang ($34^{\circ}40'$) and Xi'An ($34^{\circ}15'\text{N}$), which are located just beyond the over-wintering limit, but it could over-winter in Tong Ling ($30^{\circ}57'$), WuHan ($30^{\circ}38'$) and WenZhou ($28^{\circ}01'$), within the over-wintering limit (Dong et al., 2001) (Fig. 1). These reports verified our proposal of the over-wintering range limit for *L. sativae*.

Our study showed that a difference in the mean SCPs among the different seasons/years was not significant for each geographic population. A stable latitudinal pattern for variation in SCP was also recorded in another biologically closely related leafminer, *L. huidobrensis* (Chen and Kang, 2004), which suggests selection for an adaptive trait under stressful conditions. Therefore, the SCP has been used as a measurement of low lethal temperature for many insects (Worland and Convey, 2001; Sinclair et al., 2003). A maximum variation in SCPs of 10°C was observed between geographic populations of the leafminer pupae, which is a rare record for insects (Turnock et al., 1998; Shintani and Ishikawa, 1999; Klok et al., 2003; Chen and Kang,

2004). The northern populations are commonly more cold-tolerant than southern populations, so as to adapt to local environmental stress through evolutionary pathways or natural selection (Masaki, 1996; Jing and Kang, 2003; Chen and Kang, 2004; Kimura, 2004). For *L. sativae*, the southernmost population had the highest mean SCP value. The SCP of the pupae decreased with the increase in latitude for the three southern populations, but not for the three northern populations, which was interrupted by the over-wintering limit. It is speculated that the populations within the over-wintering limit might cope with the ever-intensifying cold stress along the latitude by gradually enhancing cold tolerance (Chen and Kang, 2004). In contrast, each stage of the leafminer can't successfully over-winter in the field in northern China, and opportunistically explore shelters beyond the over-wintering limit (Zhao and Kang, 2000). The mixed cold-tolerance strategies are thereby adopted as the best adaptation for insects to endure varied severe winter temperatures (Voituron et al., 2002).

It is commonly believed that extremely low winter temperatures might also prevent movement of such species northward because the freeze-intolerant insect cannot survive extremely harsh winters (MacDonald et al., 1997; Chen and Kang, 2002a). Resistance to thermal stresses is believed to be associated with the borders of many groups (Hoffmann and Blows, 1994). However, the leafminer has become established in northern China as a result of the widespread use of greenhouses, which is an important factor usually ignored in the past prediction for species' range borders. With the large-scale availability of greenhouses at high latitudes, the northern boundaries of distributions are not solely limited by the insects' physiological tolerance. Kimura (2004) also recognized that the use of human-protected warm places for over-wintering, competition or predation would also affect insect distributions. Considering the wide choice of host plants nearly everywhere, and the nationwide dissemination of *L. sativae* in association with international trade in plant produce and growing plants, we speculate that the natural distribution border of this species is determined by the mean minimum temperature in the warm season when it can survive outdoors. For example, *L. sativae* could infest plants in northern China at 47°N in Daqing (Guo et al., 2000), where the annual mean

minimum temperature is -3°C and the mean minimum temperature in July is 17.5°C (Meteorological Documentation Department of National Weather Bureau of China, 1990). Thus, the rapid rise in minimum winter temperature, milder winters due to regional warming or increased resistance to cold, along with the availability of the microhabitats, could have allowed this leafminer to expand its range (Chen and Kang, 2002a, 2005; Kirk and Terry, 2003).

In summary, we combined the physiological measurement of the leafminer's low-temperature ability to survive with microclimatic change to propose the northern over-wintering range limit and border of distribution. The results suggest that pest management would be different for populations on both sides of the over-wintering limit. The source for further infestation in northern China is mainly in greenhouses. Thus, beyond the over-wintering limit, management strategies can be directly specifically against greenhouse populations that act as population reservoirs (MacDonald et al., 1997). Our results may facilitate future predictions of range expansion and integrated pest management associated with pest source blocking.

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