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Low temperature induces two growth-arrested stages and change of secondary metabolites in Bursaphelenchus xylophilus

L. L. Zhao

W. Wei

David L. Kulhavy
dkulhavy@sfasu.edu

Xing Yao Zhang

Jiang Hua Sun

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Low temperature induces two growth-arrested stages and change of secondary metabolites in *Bursaphelenchus xylophilus*

Li Lin ZHAO [1], Wei WEI [1], David L. KULHAVY [2], Xing Yao ZHANG [3] and Jiang Hua SUN [1,*]

[1] *State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, P.R. China*

[2] *Arthur Temple College of Forestry and Agriculture, Stephen F. Austin State University, Nacogdcohes, TX 75962, USA*

[3] *Research Institute of Forest Ecology, Environment and Protection, Chinese Academy of Forestry, Beijing 100091, P.R. China*

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Summary – The third-stage dispersal juvenile (JIII) is the stage for survival and dispersal in the winter of the pine wood nematode, *Bursaphelenchus xylophilus*. Through investigations at different temperatures, we found two kinds of growth-arrested development, including the adult longevity extension and JIII formation induced by low temperature. They showed similar characters: densely packed lipid droplets and extended longevity. We considered that there were four stages in the formation of growth-arrested stages: induction, growth-arrested pathway, growth-arrested development and cold-tolerance duration. Moreover, at 4°C there were significant changes in secondary metabolites, which may be related to signal communication and metabolism associated with the formation of growth-arrested stages. The results suggested that low temperature was necessary for the dispersal of pine wood nematode and influenced distribution and intensity of pine wilt.

Keywords – pine wood nematode, secondary metabolism, survival, third-stage dispersal juveniles.

Postembryonic development of nematodes proceeds through four juvenile stages to the adult during favourable environmental conditions. In response to a less favourable environment, nematodes cease development and enter a growth-arrested stage, specialised for long-term survival or dispersal to new hosts (Riddle & Georgi, 1990; Sommerville & Davey, 2002). There are at least two types of growth-arrested stages: dauer diapause stage and temporal tolerant stage (Vanfleteren & Braeckman, 1999).

Dauer diapause probably results from the activation of an enhanced life-maintenance programme, which is normally operative during dauer diapause. Diapause in parasitic nematodes is a mechanism that ultimately enables development of the parasite to be synchronised with the seasonal abundance of suitable hosts (Michel, 1974). Sommerville and Davey (2002) considered that the division of developmental cycles into diapausing and non-diapausing stages confers flexibility on the organism that enhances the prospect of survival. Furthermore, these authors postulated that there are four stages in diapause. The first is induction, typically brought about by environmental signals. In the second phase, termed 'diapause pathway', nematodes have been induced to enter diapause at a later developmental stage. Surprisingly, entry into the diapause pathway may be reversible under some circumstances. The third stage is diapause development, an incompletely understood process that must be completed prior to the fourth stage, emergence from diapause. After diapause development is complete, resumption of development may be further delayed by host or environmental conditions.

The basic mechanism of dauer diapause is common among species but different species adopt different survival strategies and have different diapausing stages. The dauer juvenile of *Caenorhabditis elegans* is JIII, and its formation is induced by crowding, a low food supply and high temperature. However, the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, has a different survival diapause strategy (Riddle & Georgi, 1990). The pine wood nematode is dispersed worldwide and has destroyed large areas of pine forests in Japan. It has become a global invasive alien

* Corresponding author, e-mail: sunjh@ioz.ac.cn

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species (Rautapaa, 1986; Dwinell, 1997; Mota *et al*., 1999; Schrader & Unger, 2003; Yang *et al*., 2003).

The pine wood nematode is a destructive pest of pines that develops through two pathways: propagative and dispersal. Propagative stages (J*n*) reproduce rapidly and increase the number of nematodes in infected pines and cause severe damage to healthy pines. In the autumn, with the tree weakening, the pine wood nematode switches to the dispersal pathway. JIII dispersal juveniles develop from second-stage propagative juveniles (J2) and accumulate around *Monochamus* pupal chambers (Mamiya, 1972, 1983). As adult beetles emerge the following spring, developmentally-arrested dauer fourth-stage juvenile JIV enter the tracheae of the beetle to be vectored to another pine tree (Ishibashi & Kondo, 1977; Dwinell, 1997).

The JIII of the pine wood nematode corresponds to the pre-dauer (J2) stage of *C. elegans*, which contains densely packed lipid droplets. However, instead of rapidly moulting to the dauer stage equivalent of *C. elegans*, it can survive as a special sustained diapause stage, and moults to JIV only when vector beetles are present. The formation of JIII is not only important in ensuring within-tree survival of the nematode, it is also the precursor to formation of JIV which ensures transmission to a new host tree by *Monochamus* beetles (Linit, 1988). However, the mechanisms that mediate the switch in *B. xylophilus* development from the reproductive to the dispersal pathway are poorly understood.

Sometimes the cues that induce diapause are intrinsic, as genetic and signal pathways (Riddle & Georgi, 1990; Wolkow, 2002; Braeckman & Vanfleteren, 2007), but diapause in the parasite is a seasonal phenomenon and external signals, such as pheromones, dehydration and temperature, can also induce diapause (Sommerville & Davey, 2002; Jeong *et al*., 2005; Olsen *et al*., 2006). Many laboratory experiments confirmed the importance of low temperature as an environmental cue, signalling the onset of autumnal conditions and preparing the nematode to enter winter diapause in the host (Sommerville & Davey, 2002). At the same time, the longevity of adults of plant-parasitic nematodes can be extended during low temperatures as a type of temporal growth-arrested stage (Ishibashi & Kondo, 1977). However, the effect of low temperature on the adult of the pine wood nematode has not been investigated.

Two different protective mechanisms that mediate the formation of diapause stage JIII and extension of adult longevity in *B. xylophilus* have not been reported previously. In the present work we chose low temperatures to treat J2 and adults of the pine wood nematode to characterise and compare the changes in morphology and secondary metabolites during the formation of diapause stage JIII and adult longevity. We discuss the cold tolerance metabolic mechanisms of the pine wood nematode.

Knowledge of development and diapause in the pine wood nematode will provide a reference point for predicting the potential global distribution, which is an important factor in relation to the management of the pine wood nematode.

Materials and methods

NEMATODES

In April 2006, pine wood nematodes *B. xylophilus* were obtained from Zhejiang, China. The J*n* were cultured with the fungus *Diplodia* sp. Nematodes for experimental purposes were rinsed from the culture dish lids with distilled water.

EFFECTS OF DIFFERENT TEMPERATURES ON THE GROWTH-ARRESTED DEVELOPMENT OF PINE WOOD NEMATODE

The longevities of nematodes were determined at −4, 0, 4, 10, 15, 20, 25 and 30°C. About 0.3 million nematodes in 80 ml distilled water were mixed evenly by a magnetic stirrer. A suspension (1 ml) containing about 3000 nematodes was transferred to each Axygen 2 ml screw cap tubes (Beijing Zohonice Science & Technology Development, Beijing, China). Ten replicate tubes were included for each temperature. The number of surviving nematodes was counted by assessing movement response to mechanical prodding every 5 days over 6 months by direct observation through a dissecting microscope.

EFFECTS OF LOW TEMPERATURE ON THE MORTALITIES OF J2 AND ADULT

For life-span analysis nematodes were reared at 4°C. About 2000 young adults and J2 were select out of a mixed population including third-stage (J3) and fourth-stage (J4) juveniles and transferred into separate 2 ml tubes. Ten replicates were included for each time. The numbers of surviving nematodes were counted as above every 5 days for over 2 months by direct observation through a dissecting microscope.

Effects of low temperature on the body lengths of J2 and adult

Fifteen each of live female adults, male adults and J2 were chosen from 2 ml tube suspensions to measure body lengths every 5 days over 2 months at 4°C. The nematodes were measured after killing by gentle heat.

Effects of low temperature on the lipid droplets of J2 and adult

The male adults and J2 chosen from separate 2 ml tubes were fixed in 70% ethanol and the droplets of unbound neutral lipid within the nematodes were then stained with Oil Red O (Croll, 1972). Individually stained nematodes were recorded with a high resolution video camera mounted on a brightfield optical microscope, and images were videotaped. Image analysis software (Mocha, SPSS, Chicago, IL, USA) was used to determine total body area and lipid droplet area of each videotaped nematode. The percentage of body area occupied by droplets of lipid (percent lipid area) was measured on 15 surviving adults and J2 every 5 days over 2 months at 4°C (Stamps & Linit, 2001).

Effects of low temperature on the secondary metabolic materials of the pine wood nematode

Chemicals

The standards in GC-MS and materials in bioassays included isobutyl propylketane, pentadecane, 2-methyl-4-heptanol, 1-dodecano, 2,6-di-*tert*-butylphenol and ethyl-arachidonate (all from Acros Organics, NJ, USA; purity >95%).

Collection and analyses of volatiles

Batches of 5000 propagative nematodes at 25°C and 5000 of the diapause nematodes treated for 60 days at 4°C were each rinsed, centrifuged and concentrated in 1 ml distilled water. Concentrated nematodes were extracted with 2 ml n-hexane for 5 min, and the resulting extracts were dehydrated through anhydrous sodium sulphate and stored at −20°C until needed.

Extracts were analysed by GC-MS. Extracts were concentrated to 10 μl, in which 1 μl extracts were analysed with an Agilent 6890N Network GC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 5973 network mass selective detector. The GC was fitted with a DB-WAS capillary column (0.25 mm i.d. 30 m; Agilent Technologies), and the column temperature programme was 50°C for 2 min, then 5°C/min to 220°C and then held for 20 min. Injections were run in the spitless mode. Components were identified by comparing their retention times and mass spectra with those of known standards (Lacey *et al*., 2004).

Statistical analysis

Data analyses were analysed using statistical software SPSS 11.0 for Windows (SPSS, 2001). Differences among different times were compared by ANOVA, with significance at $P \leqslant 0.05$.

Results

Effects of different temperatures on the growth-arrested development of pine wood nematode

Temperature-response curves showed the number of surviving nematodes after the pine wood nematode was exposed to a variety of temperatures for different times. Results indicated that temperature had a marked influence on the survival of the pine wood nematode. Between 15 and 30°C, the nematodes were in a propagative pathway and did not enter growth-arrested stages. At 25 and 30°C, there was 88.9-98.1% mortality before 15 days (Fig. 1) and 100% mortality after 80 days. At −4, 0, 4 and 10°C, the pine wood nematode formed lipid droplets in the body. At 0 and 4°C the nematodes entered growth-arrested stages for a long-term survival and 25.5% at 0°C and 25.6% at 4°C remained alive after the base level of 200 days (Fig. 1). The growth-arrested nematodes formed after 60 days and survived until the end of the study. Low temperatures (0-4°C) induced the development of growth-arrested J2 and adult (Fig. 1) and lengthened the life span of the pine wood nematode. However, the nematodes at −4 and 10°C with lipid droplets died by day 60 (Fig. 1).

Effects of low temperature on the formation of growth-arrested stages

J2 and adult nematodes were treated at 4°C to compare their different developing processes of cold tolerance. This temperature was used instead of 0°C because it was conducive to a more rapid development of the life stages.

The number of surviving young-adult nematodes declined rapidly before 10 days, followed by a stable period, then 16.0% entered into diapause. The numbers of J2 that

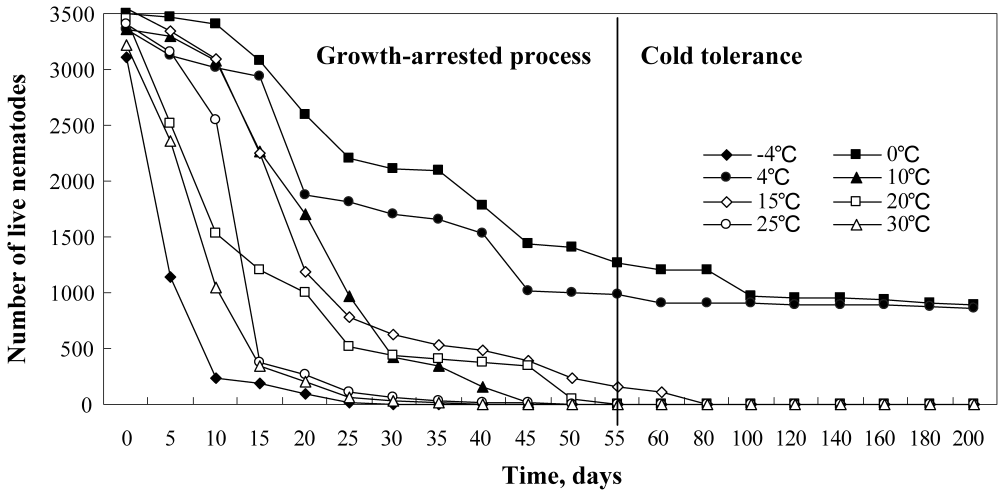

Fig. 1. *Effects of different temperatures on* Bursaphelenchus xylophilus. *Solid symbols indicate temperatures at which the lipid droplets filled the body of nematodes: open symbols stand for temperatures at which no lipid droplets occurred in the body of nematodes.*

survived were nearly constant over time and the proportion of worms that moulted into JIII and entered diapause was up to 53.7%, a significantly greater percentage than adults (Fig. 2A).

Low temperature influenced the body length of the pine wood nematode, which showed different trends between adults and J2. The body length of live adults decreased gradually but the length of J2 increased and after 35 days, the change in range was as great as 39.05 μm per 5 days. Eventually, after 50-60 days, the length of adults and JIII were uniform at 492.66-495.97 μm (Fig. 2B).

The lipid droplets developed more slowly in J2 than in adults. Lipid droplets appeared in the adults at 5 days. Lipid droplets appeared in J2 at 15 days. At that time, lipid droplets occupied 58% of the adult's body. After 45 days, both adults and J2 were full of lipid droplets and J2 were believed to moult into JIII (Fig. 2C). The lipid droplets appeared from the ventral side of adult nematodes, then appeared in the dorsal side, and then gradually increased from hypodermis to intestine, eventually filling the whole body.

EFFECTS OF LOW TEMPERATURE ON THE SECONDARY METABOLIC MATERIALS OF THE PINE WOOD NEMATODE

After treatment at 4°C, there were specific components, such as isobutyl propylketane, pentadecane and ethyl-arachidonate, in the growth-arrested stages. 2-methyl-4-heptanol and 2,6-di-*tert*-butylphenol increased greatly. By contrast, 1-dodecanol disappeared (Fig. 3).

Discussion

Nematodes cease development and enter a growth-arrested stage in a less favourable environment (Riddle & Georgi, 1990; Sommerville & Davey, 2002). We found that the pine wood nematode had two growth-arrested stages, JIII and adult, at low temperature at 0 and 4°C. We found that growth-arrested adults had similar characters compared to JIII: lipid droplets were densely packed and longevity was extended. There were four stages during the formation of the two growth-arrested stages. The first is induction (0-5 days), typically brought about by low temperature. The nematodes were not active until placed at room temperature for 2-3 h. Many nematodes froze during this phase. In the second phase (5-35 days), the growth-arrested pathway, the amount of neural lipids increased rapidly. Entry into the diapause pathway may be reversible at room temperature. The third stage is growth-arrested development (35-60 days). Nematodes completed the main body length changing process and established a uniform morphology and J2 moult into JIII. The last stage was cold tolerance duration (60+ days). Although growth-arrested development is complete, resumption of development may be further delayed at low temperature. The results correspond to four stages in diapause summarised by Sommerville and Davey (2002).

In *C. elegans*, during the formation of growth-arrested stages, the decision whether or not to enter the diapause pathway and arrest development is made primarily by

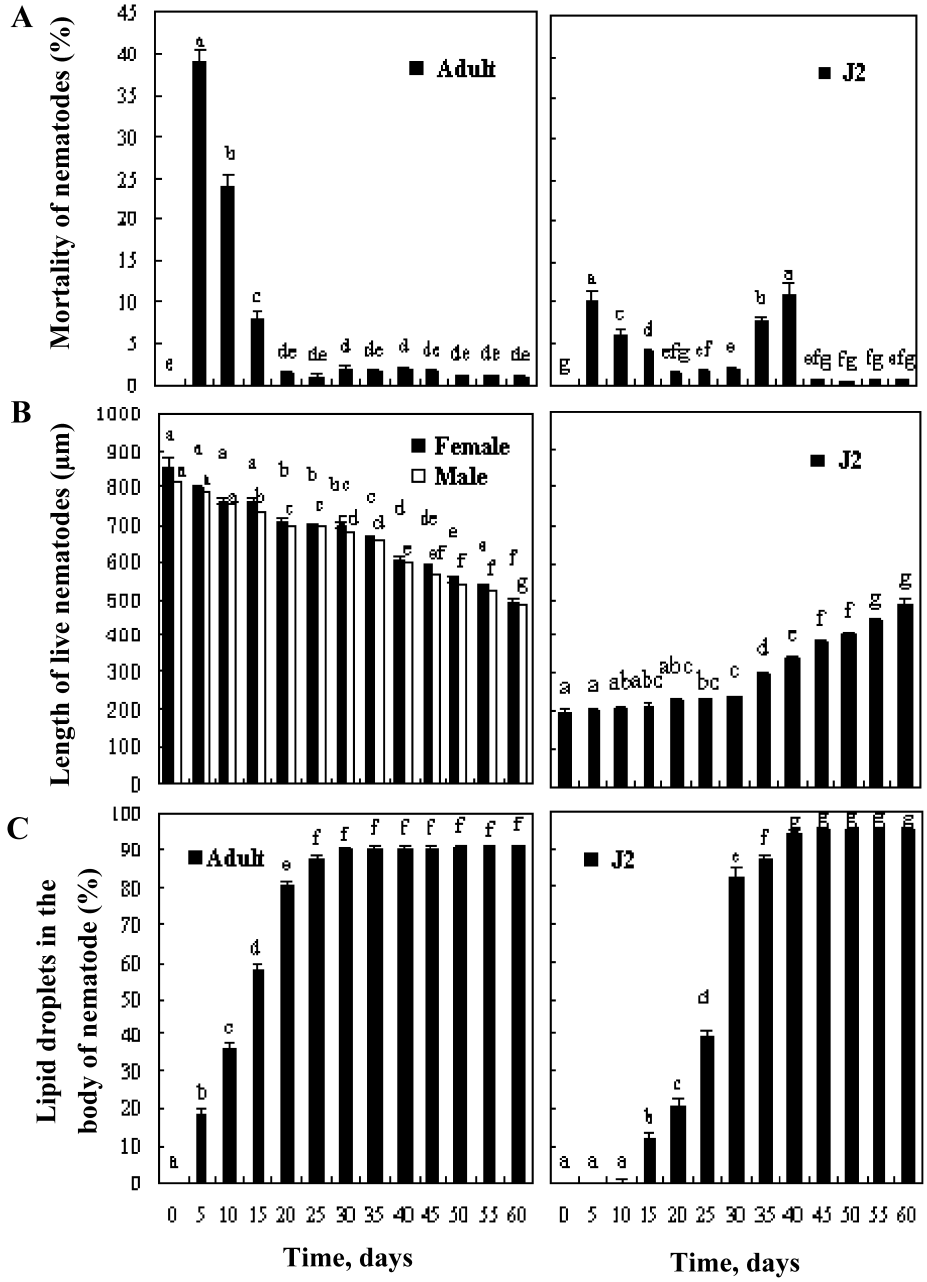

Fig. 2. *Effects of low temperature on the developments of second-stage juveniles (J2) and adults of* Bursaphelenchus xylophilus. *A: Effects of low temperature (4°C) on the longevity of J2 and adults; B: Effects of low temperature (4°C) on the body lengths of J2 and adults; C: Effects of low temperature (4°C) on the lipid droplets of J2 and adults.*

assessing the concentration of a constitutively secreted pheromone in the environment (Riddle & Georgi, 1990). Under continuing detection of pheromone signals by sensory receptors, the genetic pathway would be induced; neurosecretion would then alter the hormonal balance in the nematode to initiate dauer morphogenesis, and finally nematodes store nutrients within intestinal and hypodermal granules (Riddle & Georgi, 1990). During development, the basic metabolism of the nematode decreased (Rea, 2005). However, the secondary metabo-

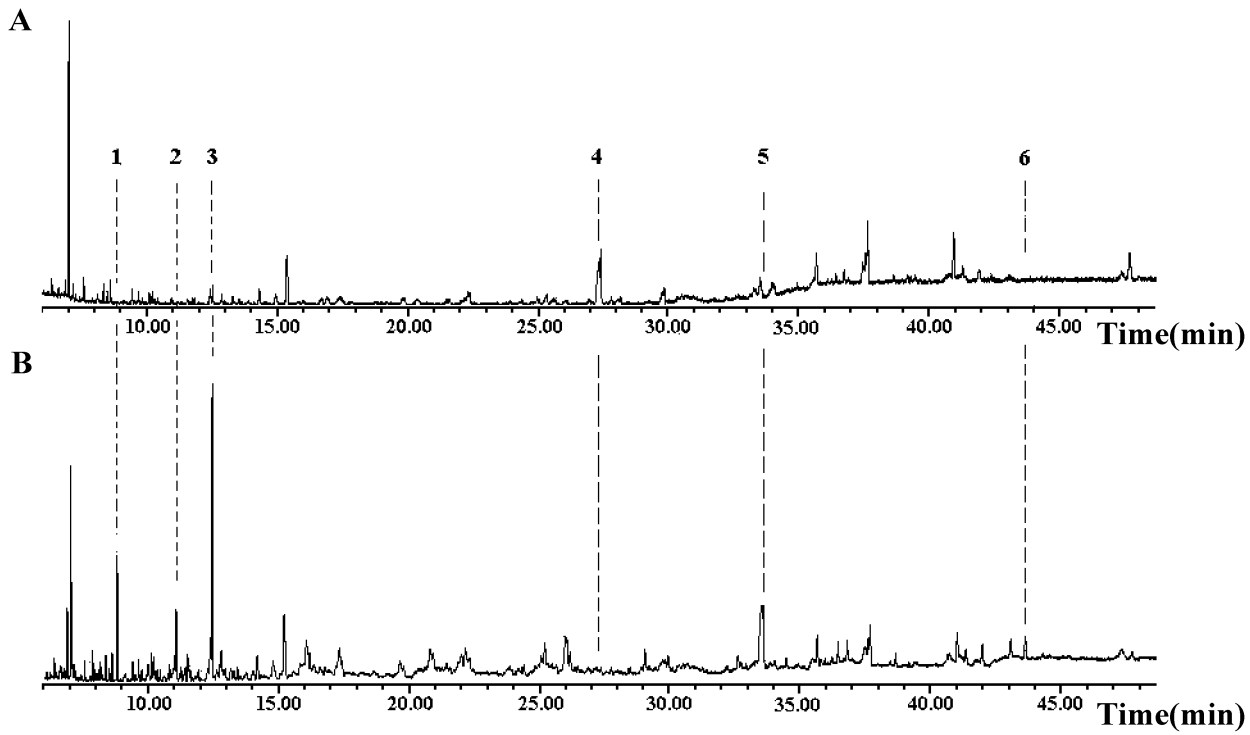

Fig. 3. *Effects of low temperature (4° C) on the metabolic materials of J2 and adults of* Bursaphelenchus xylophilus. *A: GC-MS figure of extracts of propagative nematodes; B: GC-MS figure of extracts of the growth-arrested stages. 1. Isobutyl propylketane; 2. Pentadecane; 3. 2-Methyl-4-heptanol; 4. 1-Dodecanol; 5. 2,6-Di-*tert*-butylphenol; 6. Ethylarachidonate.*

lites that may relate to cold tolerance have not been reported.

Our results showed that the secondary metabolites of growth-arrested stages at low temperature were different to propagative stages of pine wood nematodes. There were specific secondary metabolites induced by the pine wood nematode at 4°C: isobutyl propylketane, pentadecane, 2-methyl-4-heptanol, ethylarachidonate and 2,6-di-*tert*-butylphenol. Isobutyl propylketane, pentadecane and 2-methyl-4-heptanol, which are pheromones found in some insects (Ali *et al*., 1989; Do Nascimento *et al*., 1998; Cho & Kim, 2003), might also act as chemical signals secreted by the pine wood nematode to induce the growth-arrested genetic pathway. Ethylarachidonate could increase the synthesis of arachidonic acid (Pantaleo *et al*., 2004), which plays an important role in the formation of phospholipids, decreases penetration and stabilizes membranes (Tanaka *et al*., 1996), and may be involved in metabolism, protecting nematodes against the effects of low temperature. 2,6-di-*tert*-butylphenol could regulate biological development (Chu *et al*., 1999), and may regulate the formation of growth-arrested stages and extend the longevities of both J2 and adult nematodes. 1-dodecanol may disturb the darkening process of the cuticle of *Rhodnius prolixus* and *Triatoma infestans*, and has the role of a penetration enhancer on epithelial membrane lipid domains (Turunen *et al.*, 1994). The disappearance of 1-dodecanol indicated that 1-dodecanol might participate in some depressed metabolism at low temperature. In conclusion, we speculate that low temperature induced the pheromone secretion and special secondary metabolism of pine wood nematode. In the future, we will investigate the functions of secondary metabolites in the formation of growth-arrested stages of pine wood nematode.

Acquired tolerance metabolism to low temperature stress is a major protective strategy of nematodes. The life span-controlling mechanisms involved in growth-arrested development remain complicated. In *C. elegans* at least two life span-controlling pathways have been discovered. An insulin-like signalling regulates dauer diapause. A different mechanism regulates many temporal tolerant processes (Vanfleteren & Braeckman, 1999).

We found development differences between J2 and young-adults of pine wood nematode at low tempera-

ture, which suggested that the formation of JIII and adult longevity extension may be regulated by different genetic pathways. The development of J2 was stable and a significantly greater proportion entered diapause compared with the adult stage. Therefore, the formation of JIII was activated by an enhanced life maintenance programme in J2 rather than the adult, which is normally operative during diapause. However, the adult entered the growth-arrested development more rapidly, was more unstable, and had greater mortality and change of body characters, which may belong to the temporal tolerant process.

Environmental factors including temperature are important to determine whether the invasive species will succeed in establishing a persistent population at any potential sites of establishment (Bartell & Nair, 2003). Temperature may directly influence distribution and intensity of pine wilt. The natural life cycle of pine wood nematode is completed by propagative and dispersal stages together. Propagative pine wood nematodes multiply rapidly and cause epidemic pine wilt disease in regions of Europe, North America and Japan, where mean summer air temperatures of more than 20°C occur (Sikora & Malek, 1991; Fielding & Evans, 1996). At the same time, low temperatures had significant influence on the successful dispersion of the pine wood nematode to healthy pine hosts. The −10°C January mean air temperature isotherm was suggested as the northern limit of vector sawyer, *Monochamus alternatus* (Ma *et al*., 2006a, b). This limits the potential distribution in China because *M. alternatus* did not survive to vector the pine wood nematode.

Our research showed that low temperatures are necessary for the formation of dispersal stages of *B. xylophilus*, which suggested that there is a southern limit to the potential distribution of pine wood nematode.

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