RESEARCH ARTICLE

The cold tolerance of the northern root-knot nematode, *Meloidogyne hapla*

Xiaojing Wu¹, Xiaofeng Zhu¹, Yuanyuan Wang², Xiaoyu Liu³, Lijie Chen¹, Yuxi Duan¹*

¹ College of Plant Protection, Shenyang Agricultural University, Shenyang, Liaoning, China, ² College of Biology science and technology, Shenyang Agricultural University, Shenyang, Liaoning, China, ³ College of Science, Shenyang Agricultural University, Shenyang, Liaoning, China

* duanyx6407@163.com

Abstract

The northern root-knot nematode, *Meloidogyne hapla*, is one of the most important nematode pathogens occurring in cold regions. It is a sedentary, biotrophic parasite of plants and overwinters in the soil or in diseased roots. This study showed that the cold tolerance for the second-stage juveniles (J2) of *M. hapla* was moderate with the 50% survival temperature (S₅₀) of -2.22°C and the fatal temperature was -6°C when cooling at 0.5°C min⁻¹. Cryoprotective dehydration significantly enhance cold tolerance of *M. hapla* J2 with the lowest S₅₀ of -3.28°C after held being at -1°C for 6 h. Moreover, cold shock and cold acclimation had significant effects on the freezing survival of *M. hapla* J2. The lethal temperature of eggs was -18°C. Therefore, the cold tolerance of *M. hapla* is sufficiently favorable to withstand winters in cold temperature environments.

Introduction

The northern root-knot nematode, *Meloidogyne hapla*, are sedentary, biotrophic parasites of plants with wide host ranges [1]. The second-stage juvenile (J2) of *M. hapla* infect plant roots and induce root-knots, which affect water and nutrient absorption and translocation of root systems. Infection results in reduced crop yield or quality [2], and consequently causes severe economic losses [3, 4]. *M. hapla* occurs in cold regions of crop production [4, 5]. According to a previous report, *M. hapla* is mainly distributed in cool areas, where the mean temperature is -15°C in the coldest month and approximately 27°C in the warmest month or in high altitude mountainous areas [5]. The only infective stage of *M. hapla* is the J2, which must overcome adversely low-temperature environmental conditions before reaching plant roots. It was shown that *M. hapla* J2 survived at freezing temperatures [6, 7], and the minimal temperature for development was 8.8°C [8]. Meanwhile, the egg masses also played a key role in overwintering in the soil. *M. hapla* eggs survived sub-zero temperatures in the field [9, 10] and developed at the low temperature of 6.74°C [11]. This indicates the ability of *M. hapla* to survive at low temperatures.

Cold tolerance is the ability of nematodes to survive low temperatures in their living environment [12]. There are three main cold tolerance strategies in nematodes [13]. Freezing avoidance is where a nematode body fluid remains a liquid below 0°C to avoid freezing.
Freezing tolerance enables nematode survival when their bodies undergo ice formation while showing supercooling ability. Cryoprotective dehydration protects nematodes from low temperatures by dehydration caused by surrounding ice. Cold tolerance has been studied in other nematodes, including entomopathogenic nematodes, Antarctic nematodes, and stem nematodes [14–18]. 

Steinernema feltiae and Heterorhabditis bacteriophora survived the low temperature of -13˚C by cryoprotective dehydration, and the S_{50} of S. feltiae was -3.73˚C [19]. Panagrellus redivivus survived at low temperature by freezing tolerance and cryoprotective dehydration [17]. Marshallagia marshalli survived rapid exposure to temperature below -30˚C [20]. One study determined the cold tolerance of six nematodes with cold acclimation (Ditylenchus dipsaci, P. redivivus, Steinernema carpocapsae, Panagrolaimus rigidus, Rhabditophanes sp. and Panagrolaimus davidi), while the S_{50} of P. davidi was -43.6˚C lower than the others [12].

Cold acclimation is an adaptive response of organisms to low temperature that increases their capacity to tolerate freezing, and this response has been observed in P. redivivus, P. davidi [21, 22], S. feltiae, S. riobrave, S. carpocapsae, S. anomaly and H. bacteriophora [18, 23]. In a variety of prokaryotes and eukaryotes, cold shock improved cold tolerance by inducing cold shock proteins [24]. The CspA was a major cold-shock protein induced by Escherichia coli when E. coli was subjected to cold shock [25, 26]. Nematodes H. bacteriophora and Trichinella nativa produced proteins to respond to cold shock [27, 28]. However, the effects of cold shock and cold acclimation on the M. hapla J2 are unknown, although they can survive at low temperatures.

In this study, we investigated the effect of low temperature on the survival of M. hapla J2 and the in vitro hatch rate of egg masses. We determined the influence of cryoprotective dehydration, cold acclimation and cold shock, on M. hapla cold tolerance.

**Methods and materials**

**Nematode culture**

The M. hapla were generous gift by Congli Wang (Northeast Institute of Geography and Agro-ecology, Chinese Academy of Sciences), and maintained on a nematode-susceptible tomato (L-402) in the greenhouse according to the described by Forge and MacGuidwin[6]. The eggs were collected by root bleaching and centrifugation with 36% (wt/vol) sucrose [29, 30]. Eggs were hatched in sterile distilled water at 25˚C under dark conditions. J2 were collected 24 h after hatching and used for the experiment.

**Freezing regime**

A 50-μl suspension containing approximately 20 M. hapla J2 nematodes were transferred to a 0.5-ml Eppendorf tube and placed in a cooling block. The temperature of cooling block was controlled by a programmable cooling device (Temperature chamber: TEMI990). The samples were cooled from 1˚C to various minimum temperatures (T_{min}: -2, -3, -4, -5, -6˚C) at 0.5˚C min^{-1} and frozen by adding ice crystals (made by AWT) at T_{min} and held for 30 min, then rewarmed to 1˚C at 0.5˚C min^{-1}. The samples were then removed from the cooling block. After thawing, 300 μl AWT was added to the samples, and placed at room temperature for 24 h. Survival was determined by counting the proportion of moving nematodes after a mechanical stimulus by touching nematodes with a homemade eyelash-needle. Control samples were unfrozen at -1˚C. Two runs of this regime were used with 5 replicates per run. The temperature at which 50% of the J2 were killed (S_{50}) was determined using a Probit analysis [12, 17, 19].
Cryoprotective dehydration regime
To test whether the cold tolerant mechanism of *M. hapla* J2 was a cryoprotective dehydration strategy, samples were cooled from 1˚C to -1˚C at 0.5˚C min\(^{-1}\), frozen by inoculating with ice crystals at -1˚C and held for a specific period time (2, 6, 12 h) before cooling to T\(_{\text{min}}\) (-3, -4, -5˚C) at 0.5˚C min\(^{-1}\). They were kept at T\(_{\text{min}}\) for 30 min and finally rewarmed to 1˚C at 0.5˚C min\(^{-1}\). Two runs of this regime were used with 5 replicates per run. Survival was determined as previously described.

Cold shock
To test the effect of cold shock on the survival of *M. hapla* J2, samples were cooled from 1˚C to -1˚C at 0.5˚C min\(^{-1}\) and held for 1 h at -1˚C. They were rewarmed to 1˚C at 0.5˚C min\(^{-1}\) in cool block, and then samples were taken from cool block and maintained at room temperature for 1 h [17] before exposed to T\(_{\text{min}}\) (-3, -4, -5˚C) using similar methods as in the ‘freezing regime’. Survival was detected as before.

Cold acclimation
To test the effect of cold acclimation on survival of *M. hapla* J2, samples were acclimated at 4˚C for 12 h before cooling to T\(_{\text{min}}\) (-3, -4, -5˚C) at 0.5˚C min\(^{-1}\), the cold exposure was using the ‘freezing regime’. Survival was detected as before.

Effect of low-temperature on egg mass hatching rates
To test the hatch rate of the egg mass of *M. hapla*, which was exposed to low temperature, seven temperature treatments (T\(_{\text{min}}\): -2, -6, -10, -14, -15, -16, and -18˚C) were used. Similar sizes of fresh egg masses were chosen and sterilized by 0.4% NaOCl solution, then placed in the homemade hatching pond. Samples cooled from 1˚C to T\(_{\text{min}}\) at 0.5˚C min\(^{-1}\), kept at T\(_{\text{min}}\) for 30 min and then warmed to 1˚C at 0.5˚C min\(^{-1}\), finally the samples were removed from the cooling block. Control samples were kept at 25˚C. All treatments were hatched in sterile distilled water at 25˚C. Each treatment had 3 replicates per run, and two runs of this regime were used. The number of eggs in each egg-mass were 672 ± 5.5 (mean ± SE) on average. After 10 days, the number of hatching nematodes compared to the proportion of eggs were examined according to the formula as below:

\[
\text{Hatching Percentage (\%)} = \left( \frac{\text{the number of hatching J2}}{\text{the number of eggs in egg mass}} \right) \times 100
\]

Statistical analysis
All statistical analyses were calculated by using SPSS v. 17.0 [31]. Probit analysis models were used to determine the temperature at which 50% of nematodes were killed (S\(_{50}\)). The minimum temperatures (T\(_{\text{min}}\)) were log\(_{10}\) transformed to linearize the data. The relative median potency (RMP) estimated the difference of the S\(_{50}\) between two groups. Significant differences were defined between groups, if the 95% confidence limits (CL) of RMP estimation does not encompass the value 1. The effect of treatments on survival were tested using a factorial ANOVA [12, 17, 19].

Results
Freezing regime and cryoprotective dehydration
The effect of temperature on survival of *M. hapla* J2 in the freezing regime is significant. With the temperature reduced, survival decreased significantly (Welch test, Alpha = 0.05, df = 3; \(P < 0.001\)) (S1 Fig). The S\(_{50}\) values of freezing regime was -2.22˚C (95%CL = -2.01, -2.38˚C).
Moreover, the $S_{50}$ values were significantly decreased with an increased freezing time at -1˚C after 2–6 h, which was lower 1.06˚C after 6 h compared to the freezing regime (RMP = 1.47; 95%CL = 1.23, 2.10), but there was no significant difference between 6 h and 12 h (Fig 1).

**Cold shock and cold acclimation**

Survival of *M. hapla* J2 subjected to cold shock at -1˚C for 1 h was significantly increased (S2 Fig). The $S_{50}$ was -2.58˚C (95%CL = -2.34, -2.95˚C), lower than the $S_{50}$ in the freezing regime (RMP = 1.16; 95%CL = 1.05, 1.32). Survival of *M. hapla* J2 significantly improved by acclimated at 4˚C compared to the freezing regime (Fig 2). And that the $S_{50}$ was -2.79˚C (95%CL = -2.58, -3.08˚C), which was significantly different from the freezing regime (RMP = 1.26; 95% CL = 1.04, 1.49).

**Fig 1.** The $S_{50}$ values of *M. hapla* J2 exposed to freezing regime and cryoprotective dehydration. FR = freezing regime, CD-2 h = Cryoprotective dehydration regime that held for 2 h at -1˚C before cooled to $T_{\text{min}}$, CD-6 h = Cryoprotective dehydration regime that held for 6 h at -1˚C before cooled to $T_{\text{min}}$, CD-12 h = Cryoprotective dehydration regime that held for 12 h at -1˚C before cooled to $T_{\text{min}}$. The bars are the estimations in the 95% confidence limits. The different lowercase letters on the bars represent significantly different among various regimes, according to RMP estimates. N = 10.

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Moreover, the $S_{50}$ values were significantly decreased with an increased freezing time at -1˚C after 2–6 h, which was lower 1.06˚C after 6 h compared to the freezing regime (RMP = 1.47; 95%CL = 1.23, 2.10), but there was no significant difference between 6 h and 12 h (Fig 1).

**Fig 2.** The effect of cold acclimation on survival of *M. hapla* J2. Samples acclimated at 4˚C (open bars), and survival at freezing regime without acclimation (filled bars). The different lowercase letters on the bars represent significantly different ($P < 0.05$) between treatments at the same temperature. The bars are the mean ± SE in this figure. N = 10.

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Moreover, the $S_{50}$ values of various regimes were compared in Fig 3. The $S_{50}$ of cryoprotective dehydration for 6 h was lower than those achieved through cold shock ($RMP = 1.27; 95\% CL = 1.07, 1.75$) and acclimation ($RMP = 1.17; 95\% CL = 1.04, 1.46$), but there were no significant differences between cold shock and acclimation.

**Effect of low-temperature on egg mass hatching percentage**

The hatching percentage for egg mass of *M. hapla* declined with decreasing temperatures (Welch test, $\alpha = 0.05$, $df = 6$; $P < 0.001$) (Fig 4).

**Discussion**

*M. hapla* J2 indeed survived at low temperature. Previous studies showed that the *M. hapla* J2 were frozen spontaneously at -8˚C at the freezing rate of 1˚C min^{-1} [7]. However, in our experiment, the freezing rate was 0.5˚C min^{-1} because the physical damage of ice formation...
decreased using a slow freezing rate [32]. The results indicated the cold tolerance of *M. hapla* J2 was modest, with the *S*<sub>50</sub> of -2.22˚C and fatal temperature of -6˚C in the freezing regime. Comparatively, the *S*<sub>50</sub> of *P. davidi* was -43.6˚C [12].

Inoculating ice could make the surrounding medium freeze rapidly at high subzero temperatures and provide the basic environment of cryoprotective dehydration of nematodes [33]. Cryoprotective dehydration [13, 33–36] had a significant effect on cold tolerance of some nematodes, such as *P. davidi*, *S. feltiae*, and *H. bacteriophora* [16, 19]. In our freezing regime, the *S*<sub>50</sub> of *M. hapla* J2 was -2.22˚C, and the value significantly decreased with increased time at -1˚C in the cryoprotective dehydration regime. This result might explain that cryoprotective dehydration had an effect on the cold tolerance of *M. hapla* J2. Moreover, a previous study showed that following exposure to -15˚C for 10 min, the cuticle of *M. incognita* J2 had been torn away from the body by freezing but not in the *M. hapla* J2 [7]. Thus, the cold tolerance of this species may have been aided by cryoprotective dehydration and freezing tolerance.

Survival of *M. hapla* J2 was improved significantly by cold shock at -1˚C for 1 h, and the *S*<sub>50</sub> was -2.58˚C, which was lower than the freezing regime. Cold shock occurs in a variety of organisms [24]. *H. bacteriophora* induces the trehalose-6-phosphate synthase by cold shock [27], and Hsp70 was markedly increased in response to cold shock in *Trichinella* native [28], while, cold shock had no significant effect on survival of *P. redivivus* [17] and *S. feltiae* [19].

*M. hapla* J2 that were acclimated at 4˚C for 12 h showed a significant enhancement in survival. These were similar results to those found by Forge and MacGuidwin [6]. Cold acclimation response has been studied on a variety of nematode species [33]. The supercooling points of *P. redivivus* were decreased by cold acclimation, enabling survival at lower temperatures [21]. The freezing tolerance of *S. feltiae*, *S. anomaly* and *H. bacteriophora* was increased after cold acclimation [18]. Moreover, cold acclimation response induced trehalose accumulation in entomopathogenic nematodes (*S. feltiae*, *S. riobrave*, *S. carpocapsae*) [23] and *P. davidi* [22]. Meanwhile, calcium or calmodulin-mediated signaling played a pivotal role in response to cold acclimation in plants [37]. However, the mechanism of cold acclimation and cold shock effects on the survival of *M. hapla* J2 needs to be further investigated.

In nature, nematodes usually overwinter by egg masses in the plant debris or soil when the soil temperature is subzero. In this study, the percentage of egg-hatching for *M. hapla* within 10 days was 65.30% at -6˚C and 4.27% at -14˚C, indicating that *M. hapla* survived at low temperatures, which was similar to the results by Daulton et al. [9]. However, pre-exposure to 4, 12 and 18˚C for two weeks did not significantly affect the hatch rate of the *M. hapla* egg masses [7].

We found that the lethal temperature of *M. hapla* J2 was -6˚C with freezing by adding ice, and cryoprotective dehydration improved the cold tolerance of *M. hapla* J2. The lethal temperature of *M. hapla* egg mass in our experiments was -18˚C after freezing spontaneously. Moreover, cold acclimation and cold shock significantly improved the cold tolerance of *M. hapla* J2, which is advantageous for withstanding the winter in cold environments.

**Supporting information**

**S1 Fig. Survival of *M. hapla* J2 after cooling to various minimum temperatures.** Treatments were frozen by adding ice (open circle), and unfrozen (closed circle). The values are the mean ± SE in this figure. N = 10. (TIFF)

**S2 Fig. The effect of cold shock on survival of *M. hapla* J2.** Samples cold shocked at -1˚C for 1 h and then kept at room temperature for 1 h before being cooled to *T*<sub>min</sub> (filled circles), and survival at freezing regime at the corresponding test temperature (-3, -4, -5˚C) without cold
shock (open circles). The values are the mean ± SE in this figure. N = 10.

(TIFF)

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Author Contributions

Conceptualization: Xiaojing Wu, Lijie Chen, Yuxi Duan.

Data curation: Xiaojing Wu.

Formal analysis: Xiaojing Wu, Xiaofeng Zhu.

Funding acquisition: Lijie Chen.

Investigation: Xiaojing Wu.

Methodology: Xiaojing Wu.

Project administration: Xiaofeng Zhu, Yuanyuan Wang, Xiaoyu Liu, Lijie Chen, Yuxi Duan.

Resources: Xiaojing Wu.

Software: Xiaojing Wu, Xiaofeng Zhu, Yuanyuan Wang, Xiaoyu Liu.

Supervision: Lijie Chen, Yuxi Duan.

Validation: Xiaojing Wu, Yuxi Duan.

Visualization: Xiaojing Wu, Xiaofeng Zhu, Yuanyuan Wang, Xiaoyu Liu.

Writing – original draft: Xiaojing Wu.

Writing – review & editing: Lijie Chen, Yuxi Duan.

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