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The effects of fluctuating culture temperature on stress tolerance and antioxidase expression in *Esteya vermicola*

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The endoparasitic nematophagous fungus, *Esteya vermicola*, has shown great potential as a biological control agent against the pine wood nematode, *Bursaphelenchus xylophilus*. Fluctuating culture temperatures can affect fungal yields and fungal tolerance to desiccation, UV radiation, H_2O_2, and heat stress, as well as antioxidase expression. To explore these effects, *E. vermicola* cultured under five temperature ranges, 26°C, 15–26°C, 26–35°C, 20–30°C, and 15–35°C, were compared. The cultures grown at lower temperatures showed better growth, stronger tolerance to desiccation, UV, and H_2O_2 stresses, and increased catalase expression, However, these cultures also showed weaker heat stress tolerance and lower superoxide dismutase expression than the higher-temperature cultures. In particular, the *E. vermicola* cultured at 20–30°C, i.e., fluctuating in a narrow range around the optimal temperature, showed the best performance. Therefore, for production in practical applications, this narrowly fluctuating, moderate temperature appears to be optimal for yield and stress tolerance in *E. vermicola*.

Keywords: *Esteya vermicola*, fluctuating temperature, stress tolerance, antioxidase expression

Introduction

The endoparasitic nematophagous fungus, *Esteya vermicola*, has great potential as a biological control agent against the pine wood nematode (*Bursaphelenchus xylophilus*) and, consequently, against pine wilt disease (Liou *et al*., 1999; Kubátová *et al*., 2000; Wang *et al*., 2008, 2009). Some methods used to control pine wilt disease, including trunk injection of nematicides, aerial spraying of insecticides, felling and fumigation, felling and crushing, and felling and burning of pine wilt nematode-infested pine trees, can be harmful to the environment. Environmentally friendly control methods are needed. Previous studies have already shown that *E. vermicola* does not exert any pathogenic effect on pine (Wang *et al*., 2011b), and the survival index of four-year-old pine seedlings infected with pine wood nematode was increased from 0.067 to 0.67 (Wang *et al*., 2011a). However, *E. vermicola* was unable to resist lengthy exposure to ultraviolet light (UV), heat, or dry conditions (Wang *et al*., 2012).

It is well-known that temperature is an important factor influencing growth, reproduction, development, and metabolism in almost all life forms (Avilla and Copland, 1988; Montagnes and Weisse, 2000). In addition, fluctuating temperature can affect gene expression (Podrabsky and Somero, 2004) and biochemical parameters (Wang *et al*., 2007). Such effects can be especially strong with regard to heat shock protein, which is related to thermal tolerance (Nakano and Iwama, 2002; Fangue *et al*., 2006); and reactive oxygen species (Becker *et al*., 2011), which correlate with antioxidant enzyme levels. Some researchers addressed the hypothesis that temperature variability may affect climatic stress resistance of insect, and suggested that large temperature fluctuations could reduce its fitness of environment (Terblanche *et al*., 2010). Furthermore, in plants, stress tolerance (oxidation, drought, freeze, salt, heat, UV, and so on) can be affected by levels of the antioxidase enzymes superoxide dismutase (SOD) and catalase (CAT) (Bowler *et al*., 1992; Gupta *et al*., 1993; Zhang and Kirkham, 1994; Willekens *et al*., 1997; Zhang *et al*., 2005; Gill *et al*., 2010; Jalali-e-Emam *et al*., 2011). However, until now, few studies have explored the effects of fluctuating culture temperature on subsequent stress tolerance and antioxidase expression levels in microbes, and none in biocontrol fungus, *E. vermicola*.

During the process of mass production of *E. vermicola*, the temperature inside the incubator usually fluctuates along with the environment, such that it is sometimes higher than the setting temperature and sometimes lower. If culture temperature during the production process does affect the stress tolerance ability of *E. vermicola*, optimizing culture temperature could improve the efficacy of *E. vermicola* as an anti-pine wood nematode biocontrol agent. In the present study, we therefore set out to investigate the effects of culture temperature on yields, stress tolerance, and antioxidase expression in this biocontrol fungus. We evaluated the effects of culture temperature fluctuation and compared high, low, and moderate culture temperatures. In addition, we investigated the roles of SOD and CAT in *E. vermicola* resistance to desiccation, oxidative stress, UV, and heat stress. Not only is this helpful in understanding the impact of temperature fluctuation on the incubation process for *E. vermicola*, but it will also contribute to the ability to forecast and analyze

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growth and stress tolerance status in the field, an environment characterized by fluctuating temperatures.

Materials and Methods

Spore preparation and mycelium dry weight

The *E. vermicola* CBS 115803 fungal strain was obtained from the CBS-KNAW Fungal Biodiversity Center. A total of 1×10^6 blastospores were inoculated into 10 ml potato dextrose broth (PDB) in a Petri dish, and incubated at 26°C, 15–26°C, 26–35°C, 20–30°C, and 15–35°C for 7 days. Because 26°C is the optimum culture temperature for *E. vermicola*, and since previous studies have shown that *E. vermicola* can still germinate and grow at 15°C, 30°C, and 35°C (Xue *et al.*, 2013), we set up five temperature groups for culturing: 26°C (constant temperature) as the control condition, 15–26°C (alternating every 12 h) as the fluctuating lower temperature condition, 26–35°C (alternating every 12 h) as the fluctuating higher temperature condition, 20–30°C (alternating every 12 h) as the fluctuating moderate temperature condition, and 15–35°C (alternating every 12 h) as the condition incorporating a wide temperature range. Then, the spore suspension and mycelium were harvested by filtering through four-layer sterilized gauze, the spore suspension was prepared for use in the next step, and the mycelium in each Petri dish was dried in a 60°C drying oven until it was completely dried. The dried mycelium was then weighed.

Tolerance to desiccation, UV, H_2O_2, and heat

Each spore suspension described above, at a concentration of 10^7 spores/ml, from *E. vermicola* cultured at different temperatures, was sprayed on a Petri dish and dried at a clean bench with gentle blown air at room temperature for 10 min. Then, the spores were washed off with triple-distilled water and dropped on a water agar (WA) plate.

For the UV stress condition, five 20-µl droplets of each 10^7 spore/ml spore suspension were placed on a Petri dish and irradiated by UV light on a clean bench for 60 sec. The spores were then collected, transferred to the WA plate, and wrapped with aluminum foil. For the hydrogen peroxide stress condition, the spore suspension was kept in a 0.05% H_2O_2 solution for 4 h, then the spores was transferred to the WA plate. For the heat stress condition, 100 µl of the spore suspension was heated in a 45°C water bath for 5 min, and then spread onto the WA plate. All of the WA plates described above were then cultured at 26°C for 36 h, and the germination rates were quantified.

Antioxidase expression measured on native polyacrylamide gel electrophoresis (PAGE)

E. vermicola was collected after culturing for 7 d, washed three times with PBS (10 mM, pH 7.0), and then ground in liquid nitrogen and sonicated in an ice bath for 5 min. After centrifugation at 7,000 rpm at 4°C for 10 min, the supernatant was stored at -70°C for use, avoiding repeated freeze thaws. The protein concentration was determined by Bradford assay.

Following the protocol of Weydert with a minor modification (Weydert and Cullen, 2010), 10% separating gels and 5% stacking gels were prepared for SOD and CAT. For SOD, after electrophoresis, the gels was washed three times with ddH_2O, stained with 0.1% nitro blue tetrazolium (NBT) for 15 min in the darkroom, and then transferred to a dye liquor consisting of the following mixture: 28 µM riboflavin and 28 mM tetramethylethylenediamine (TEMED) in 0.1 M potassium phosphate buffer (PPB) (pH=7) for 15 min in the darkroom. After washing three times with ddH_2O, the gel was exposed under a fluorescent lamp for 10–15 min until the appearance of an SOD transparent band. The gel for detecting CAT was soaked in 3.27 mM H_2O_2 for 30 min, washed twice with ddH_2O, and then placed in the same volume of 2% ferric chloride and 2% potassium ferricyanide. When achromatic bands began to form, the stain was poured off and the gel was rinsed extensively with ddH_2O. The SOD and CAT expression levels were analyzed by Gel-Pro analyzer (Media Cybernetic int.).

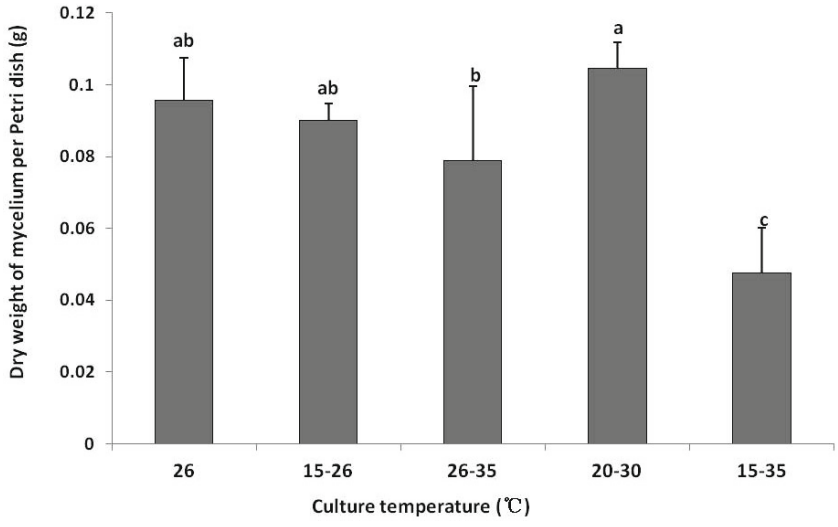

Fig. 1. Dry weight of *Esteya vermicola* cultures. *The E. vermicola* cultures were grown for seven days at a constant temperature of 26°C and four fluctuating temperatures ranges (with an alteration every 12h): 15–26°C, 26–35°C, 20–30°C, and 15–35°C. Error bars represent the standard deviation of triplicate determinations. Letters on the same shaped bars indicate differences that are significant at $P < 0.05$ according to the Duncan multiple comparison test.

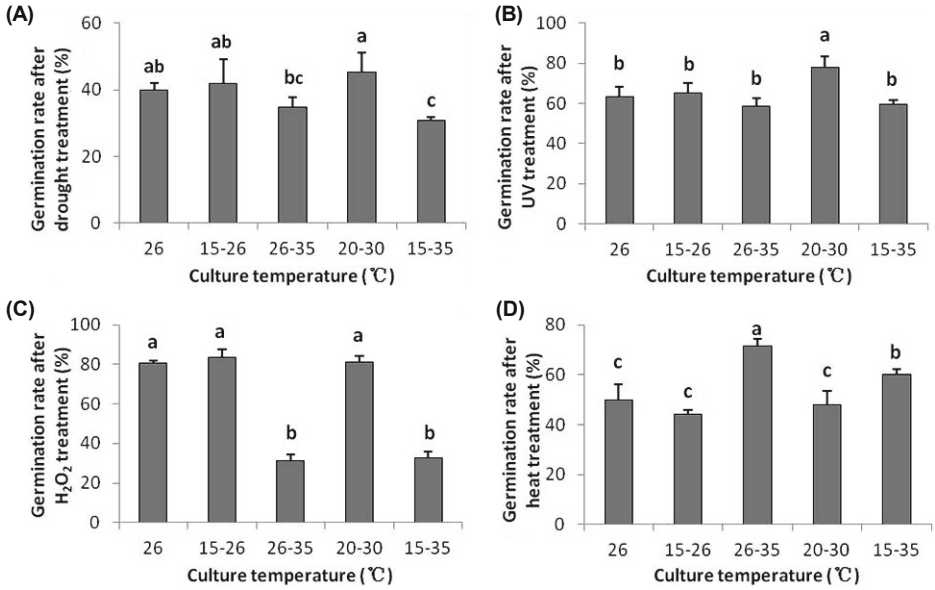

Fig. 2. The germination rates of *E. vermicola* cultures. The *E. vermicola* cultures were grown for seven days at a constant temperature of 26°C and fluctuating temperatures ranges (with an alteration every 12 h): 15–26°C, 26–35°C, 20–30°C, and 15–35°C. After growth for seven days, the cultures were subjected to each of the following treatment conditions: dry treatment for 10 min at room temperature (A), UV treatment for 60 sec at room temperature (B), H_2O_2 treatment for 4 h at room temperature (C), heat treatment for 5 min at 45°C (D). Error bars represent the standard deviation of triplicate determinations. Letters on the same shaped bars indicate differences that are significant at $P < 0.05$ according to the Duncan multiple comparison test.

Data analysis

All experiments were conducted in three replications and repeated two times. The data are presented as means and standard deviations and any significant differences were determined by one-way analysis of variance with Duncan multiple comparison module, using Minitab statistical software, version 13.0 (Minitab Inc.). Differences with *P*-values of < 0.05 were considered statistically significant.

Results

Mycelium dry weight

The dry weight values of *E. vermicola* grown at each of the tested temperature conditions are shown in Fig. 1. *E. vermicola* cultured under 20–30°C showed the highest yield, as measured by dry weight. Cultures grown at 26°C and 15–26°C showed lower yields than cultures grown at 20–30°C, but higher yields than cultures grown at 26–35°C. The lightest dry weight of *E. vermicola* resulted from culturing under the widest temperature range, 15–35°C.

Tolerance to desiccation, UV, H_2O_2, and heat stress

The germination rates of *E. vermicola* after dry treatment for 10 min at room temperature are shown in Fig. 2A. The spores grown at 20–30°C had the highest desiccation resistance; those grown at 26°C and 15–26°C were less desiccation- resistant than those grown at 20–30°C, but were more desiccation- resistant than those grown at 26–35°C; *E. vermicola* cultured at 15–35°C showed the poorest ability to tolerate desiccation stress. The germination rates of *E. vermicola* after UV treatment for 60 sec at room temperature are shown in Fig. 2B. The *E. vermicola* cultured at 20–30°C was much more UV tolerant than that in the other four temperature groups, which displayed no significant difference among each other, with regard to UV stress. The germination rates of *E. vermicola* after H_2O_2 treatment for 4 h at room temperature are shown in Fig. 2C. The *E. vermicola* cultures grown at 26°C, 15–26°C, and 20–30°C were significantly more resistant to oxidative stress than the cultures grown at 26–35°C and 15–35°C. The germination rates of *E. vermicola* after heat treatment for 5 min at 45°C are shown in Fig. 2D. *E. vermicola* cultured at the high temperature range of 26–35°C had the strongest resistance to heat. Cultures grown at 15–35°C showed the second strongest heat tolerance, and those grown at the lower temperatures of 26°C, 15–26°C, and 20–30°C had the poorest performance with respect to heat tolerance.

Antioxidase expression on native PAGE

SOD expression on native PAGE is shown in Fig. 3A. The SOD expression levels of *E. vermicola* cultured at 26–35°C (band 3), 20–30°C (band 4), and 15–35°C (band 5) were higher than those *of E. vermicola* cultured at 26°C (band 1) and 15–26°C (band 2). CAT expression on native PAGE is shown in Fig. 3B. CAT expression levels of *E. vermicola* cultured at 26°C (band 1), 15–26°C (band 2), and 20–30°C (band 4) were higher than those *of E. vermicola* cultured at 26–35°C (band 3) and 15–35°C (band 5).

Discussion

In general, compared with the control culture at a constant

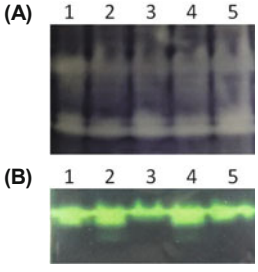

Fig. 3. Superoxide dismutase (A) and catalase (B) expression as visualized by native polyacrylamide gel electrophoresis (PAGE). The bands, left to right, show the proteins from *E. vermicola* cultures grown for seven days at a constant temperature of 26°C (band 1) and each of four fluctuating temperatures ranges (with an alteration every 12 h): 15–26°C (band 2), 26–35°C (band 3), 20–30°C (band 4), and 15–35°C (band 5).

temperature of 26°C, fluctuating culture temperatures do indeed influence growth, stress tolerance, and antioxidant enzyme expression in *E. vermicola*. In terms of growth, different ranges of fluctuating temperatures significantly affected *E. vermicola* dry weight yields. A moderate range of fluctuation around the optimum temperature led to much better growth (in terms of dry weight) than a wider range of fluctuating temperature, and lower temperatures yielded slightly better growth than higher temperatures. This is consistent with the observation of Burgess and Griffin (1968), the temperature fluctuation does not affect the linear growth rate of fungi, but the larger amplitude of fluctuation would have a noticeable reduction in growth of fungi, and the higher temperature may have the detrimental effects on the fungi subsequent growth at the lower temperature. So depending on the amplitude of temperature fluctuation, the growth of fungi at the fluctuation temperature may be greater or less than that at the constant median temperature. The results here indicate that, in the production process, a small range of temperature change is not bad for fungi production.

Under high temperatures, as well as under a wide temperature range, *E. vermicola* produced more SOD, but less CAT, than *E. vermicola* cultured at lower temperatures. This is consistent with other studies in plants; high temperature leads to the production of reactive oxygen species (ROS), which cause oxidative stress; SOD, an ROS-scavenging enzyme, will increase along with this increase in ROS (Tang *et al*., 2006). The increased SOD may play a part in heat stress tolerance, as Almeselmani (2006) reported that the wheat genotypes with greater increase in SOD activity, which have better scavenging capacity and higher tolerance to heat stress. At the same time, CAT activity is associated with the scavenging of H_2O_2 and an increase in its activity is related with increase in stress tolerance (Almeselmani *et al*., 2006), since high temperature can increase endogenous H_2O_2 and reduce CAT activity (Dat *et al*., 1998), and lower, moderate temperature (not chilling stress) can increase CAT activity (Prasad, 1996, 1997). This might explain why *E. vermicola* cultured at lower temperatures had a stronger tolerance to exogenous H_2O_2 stress. The overexpression of CAT in transgenic plants showed increased tolerance to the oxidative damage caused by desiccation stress (Mohamed, 2003), and *E. vermicola* with a higher CAT expression level was also better able to tolerate desiccation (e.g., *E. vermicola* cultured at 20–30°C). The relationship between UV tolerance and SOD and CAT expression was not very clear, but the highest CAT expression was seen in *E. vermicola* cultured at the moderate, narrowly fluctuating temperature range of 20–30°C, and this culture temperature also yielded the greatest UV tolerance. It has been reported that high catalase activity can play an important role in UV tolerance in microorganisms (Di Capua *et al*., 2011). In general, not only H_2O_2 stress, but also the heat (Heise *et al*., 2006), desiccation (Abedi and Pakniyat, 2010; Slade and Radman, 2011), and UV (Slade and Radman, 2011) stress are, indirectly, associated with oxidative stress, due to enhanced accumulation of ROS, particularly O_2^- and H_2O_2, in the cell (Abedi and Pakniyat, 2010). Furthermore, our results indicate that it is feasible to regulate antioxidase quantity by controlling the culture temperature of *E. vermicola*. This would help to adapt the fungi to an outdoor climate, so as to achieve a better insecticidal effect.

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