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

Yun-bo Wang¹, Wen-xing Pang², Xiao-na Yu², Jing-jie Li¹, Yong-an Zhang³, and Chang-keun Sung¹*

¹Department of Food Science and Technology, College of Agriculture and Biotechnology, Chungnam National University, Daejeon 305-764, Republic of Korea
²Molecular Genetics and Genomics Lab, Department of Horticulture, Chungnam National University, Daejeon 305-764, Republic of Korea
³Chinese Academy of Forestry, Beijing 100091, P. R. China

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, H₂O₂, 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 H₂O₂ 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; Kubáтовá 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 0.67 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; Willemens 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 ant 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...
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 $1 \times 10^6$ 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$_2$O$_2$, 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$_2$O$_2$ 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$_2$O, 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$_2$O, 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$_2$O$_2$ for 30 min, washed twice with ddH$_2$O, 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$_2$O. 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.