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Research Article

Effects of soil water content on the survival, antioxidant status, and immune responses of leech (*Whitmania pigra*) during the overwintering

Qiufeng Y.[1]; Zhengrong W.[1]; Kun G.[1]; Yi C.[1]; Wei W.[2]; Mingzhong L.[1,2*]

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Abstract

Leech is an important aquatic animal parasite that poses a great threat to the aquaculture industry. However, leech (*Whitmania pigra*) is an important traditional Chinese medicine and has been widely used to treat cardiovascular or other chronic diseases. Although there has been a growing interest in *W. pigra* rearing in the South and East of China, the technology of artificial breeding of *W. pigra* during overwintering is still imperfect. In this study, various soil water contents (30%, 40%, 50%, 60%, and 70%) were tested on *W. pigra* during a 104-day overwintering period, and their physiological responses were evaluated. During the trial period, the survival rate of *W. pigra* varied under different soil water contents and the highest and lowest survival rates were 86.67±5.77 and 23.33±11.55 % at the soil water content of 50 and 70%, respectively. The weight-loss rate of *W. pigra* decreased when the soil water content increased. The activities of antioxidant enzymes, superoxide dismutase and catalase of the leech at 50% soil water content were lower than those in other groups, however, the highest activities of the immune enzyme, alkaline phosphatase, and lysozyme were obtained in the five groups. These results suggest that soil water content is a key environmental factor that affects *W. pigra* during overwintering and 50% soil water content is the optimal level for *W. pigra* during the overwintering period.

Keywords: *Whitmania pigra*, Soil water content, Overwintering, Survival, Antioxidant status, Lysozyme

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1-Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education, Hubei Key Laboratory of Waterlogging Disaster and Agricultural Use of Wetland, College of Animal Science, Yangtze University, Jingzhou 434025, China
2-State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China
*****Corresponding author's Email: kklmz413@yangtzeu.edu.cn

Introduction

Leeches are segmented parasitic or predatory worms, and they are distributed on all continents except Antarctica. Approximately 800 species of leeches have been described (Kvistet *et al*., 2013), of which approximately 30% are marine and terrestrial life, with the rest are freshwater (Sket and Trontelj, 2008). Leech is an important aquatic animal parasite and poses a great threat to the culture of many aquatic animals, such as *Epinephelus coioides*, *Monopterus albus*, and *Lates calcarifer* (Cruz-Lacierda *et al*., 2000; Kua *et al.,* 2014; Xiang *et al.,* 2021). However, according to the description in traditional Chinese literature, some species like *Whitmania pigra* have possible medical applications (Whitaker *et al*., 2004). *W. pigra* is an important traditional Chinese medicinal resource that is widely used against cardiovascular and other chronic diseases, and it demonstrates broad pharmacological effects such as the inhibition of platelet agglomeration and decrease blood lipids (Chen *et al*., 2011; Wang *et al*., 2014). Hirudin extracted from *W. pigra* not only has the effect of promoting blood circulation and removing blood stasis, but also it has anti-venous thrombosis effect and is currently one of the important drugs used for treating cardiovascular and cerebrovascular diseases (Wüstenhagen *et al.,* 2020). *W. pigra* is categorized as an endangered species in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Elliott and Tullett, 1984; Elliott and Kutschera, 2011) due to its over-exploitation and habitat pollution (Petrauskiene, 2003; Trontelj and Utevsky, 2012). To meet the demand for medicinal use, there is a great growing interest in culturing *W. pigra* in the South and East of China. The reproduction of parental leech, culturing of larvae, breeding technology, pharmacology, and physiology of *W. pigra* has been studied for several years (Zhang *et al*., 2008; Wang *et al*., 2016; Wang *et al*., 2018). However, the artificial breeding technology of the leech is still imperfect, and many problems, such as seed rearing, artificial reproduction, overwintering, etc. have not been solved yet.

W. pigra is an ectotherm and its hibernation is commonly observed when the temperature is below 4℃ and renews itself at the following spring when the environmental temperature rises above 20°C. Hibernation is an energy-saving and adaptive strategy adopted by leeches to survive at low temperatures. This phenomenon attracted attention from researchers because hibernation seriously affects the growth, feeding, and reproduction of leeches (Shi *et al*., 2020). Until now, the ecological simulation models under rearing conditions have mainly been adopted for the overwintering of *W. pigra*. For instance, ponds are drained and banks are covered with straw and other insulation materials in autumn and winter, and water is added to the ponds when *W. pigra* renews itself in the following spring. Generally, the survival rate of *W. pigra* is below 60% and the weight-loss rate is approximately 40% after hibernation (Guo *et al*., 2019). Thus, the high mortality rate of *W. pigra* during overwintering period has been identified as one of the most serious bottlenecks for the large-scale development of outdoor artificial production. Therefore, increasing survival rate and reducing the weight-loss rate during overwintering is important for culturing *W. pigra* in the aquaculture industry in Asia.

In recent years, limited research has been directed toward the influence of environmental factors on overwintering of *W. pigra*, and it is unknown whether soil water content is the key factor during hibernation. The aim of the present study was to evaluate the effects of various soil water contents on the survival rate, antioxidant status, and immune response of *W. pigra* during overwintering period. The results will help to understand the regulatory mechanism underlying the remarkable physiological phenotype of leech during hibernation and provide information on management technologies for *W. pigra* during overwintering period.

Materials and methods

Animals and acclimation

The leeches (*W. pigra*) were obtained from a local farm (Jingzhou, China), then transported to a laboratory, and acclimated in round GFRP (glass fibre reinforced plastics) tanks as a holding tank (200cm, diameter; 60cm, height; volume, 1800 L). GFRP tanks were filled with rice soil (30cm in depth, volume: 900 L) for 2 weeks with the natural light of 12-h light and 12-h darkness and temperature (13.2±5.3°C). All experiments were conducted in the Aquatic Animal Research Center of Yangtze University. During the acclimation period, the leeches were fed freshwater snails (*Cipangopaludina cahayensis*), which is the main food of leeches in the artificial culture conditions (Wang and Wang, 2005).

Experimental design

After acclimation for 2 weeks, 300 leeches (average weight 13.47±1.14 g) were randomly divided into 30 plastic tanks (25 cm, width; 40 cm, length; and 30 cm, height; volume 30 L) of five groups (6 tanks for each group). Rice soil (20 cm in depth) was used as substrate in each tank. Groups 1-5 were under soil water contents of 30%, 40%, 50%, 60%, and 70%, respectively, for 104 days (2020.11.20 to 2021.3.3). During the experiment, the soil water content in each plastic tank was measured once a day by using a hygrometer (SIN-PH182, Hangzhou Joint Automatic Biochemical Technology, Hangzhou, China.), and freshwater was sprayed when the soil water content was decreased. The leeches in all groups were cultured under a temperature of 11.5±7.6°C and natural photoperiod of 12-h light and 12-h darkness during the experiment period.

Survival and weight-loss rates analyses

The body weight of the leech was recorded at the beginning and the end of the experiment. The numbers were counted for survival analysis at the end of the experiment. The survival rates (SR) and weight-loss rates (WLR) in each group were calculated using the following formulas:

SR, % = Final leech number /Initial leech number × 100%

WLR, % = (Initial body weight - Final body weight) / Initial body weight × 100%

Sample collection and physiological tests

The leeches were dissected on ice and the digestive tract samples were collected at 0, 26, 52, 78, and 104 days after the experiment (DAR) from each group for physiological tests. The samples were homogenized in 5 volumes (v/w) of ice-cold water and the supernatants were collected via centrifugation at 10,000 × *g* for 20 min and stored at –20°C until analysis. The activities of superoxide dismutase (SOD) and catalase (CAT) in the tissue supernatants were used to determine the antioxidant responses (Peskin and Winterbourn, 2000) using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Biological Engineering Research Institute, Nanjing, China). Briefly, the SOD activity was determined using hydroxylamine method according to Ji *et al*. (2008). The CAT activity was determined using the visible light method according to Zang *et al*. (2012). Briefly, it was calculated at 240 nm by measuring the initial rate of H_2O_2 decomposition. The activities of lysozyme (LYZ) and alkaline phosphatase (AKP) in the supernatants were used as immune parameters (Dai et al., 2018). LYZ activity was determined using the turbidimetry method according to Helal and Melzig (2008), whereas AKP activity was determined using the visible light colorimetric method according to Zang *et al*. (2012). The protein content was determined according to the Coomassie Brilliant Blue method (CBBG - 250).

Statistical analysis

Statistical analyses were performed using Duncan's test. The data are presented as mean±standard deviation (SD). The level of significant difference was set at *p*-value<0.05. A least significant difference (LSD) multiple comparisons were used to conduct statistical tests when differences were detected. Statistical analysis was performed using the SPSS version 19.0 for Windows (SPSS Inc., Chicago, USA).

Results

Effect of soil water content on survival rate

At the end of the experiment, we investigated the effects of different soil water contents on the SR of *W. pigra* during the overwintering period (Fig.

1). During the trial period, the SR increased at first when the soil water content increased to 50% and then decreased. The highest and lowest SR were 86.67±5.77 and 23.33±11.55% at a soil water content of 50 and 70%, respectively. The results indicated that 50% soil water content was the optimal level for *W. pigra* to have the highest survival rate during the overwintering period.

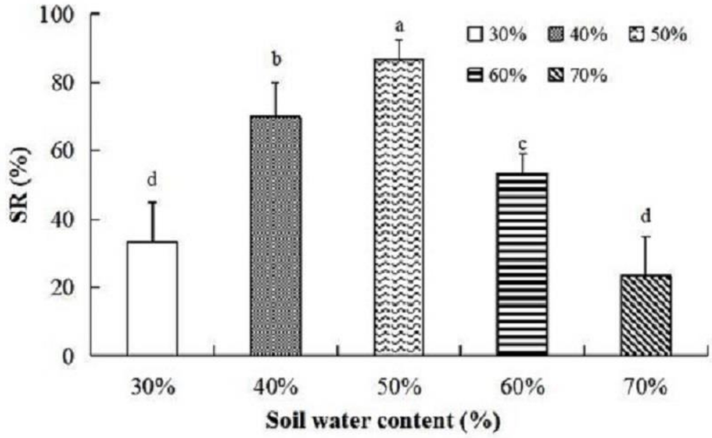

Figure 1: Effect of soil water content on survival rates of *Whitmania pigra.* Values with different letters indicate significant differences among different groups (p<0.05). Data are expressed as mean±standard deviation (n=35).

Effect of soil water content on weight-loss rate

Soil water content had significant effects on the WLR of *W. pigra* in the five groups during the 104-day overwintering period (Fig. 2). The WLR decreased from 15.96±1.03% to 2.37±0.49% when the soil water content increased, and the highest and lowest WLR were 30 and 70%, respectively.

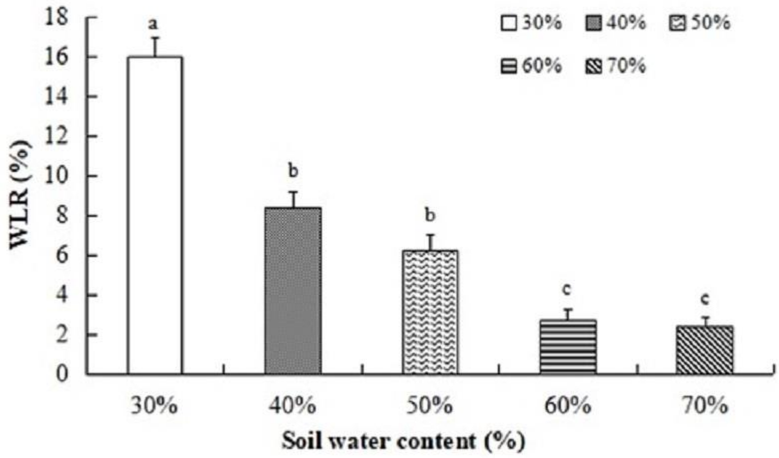

Figure 2: Changes in the weight-loss rates of *Whitmania pigra* under five soil water contents during overwintering period. Values with different letters indicate significant differences among different groups (p<0.05). Data are expressed as mean±standard deviation (n=35).

Effect of soil water content on antioxidant status

We examined the effects of different soil water content on the antioxidant status of *W.pigra*. The results of antioxidant enzyme activities (SOD and CAT) at each time are presented in Figure 3. The soil water content had significant effects on the antioxidant enzymes (SOD and CAT activities). The activities of SOD and CAT increased and showed the highest activities at 26 DAR, and then decreased during the trial period (Fig. 3). The activity of SOD was significantly lower at 50% water content than that of other four water contents at 52 DAR (Fig. 3a). In addition, the activity of CAT was significantly decreased at 50% water content compared to other water contents at 52, 78, and 104 DAR (Fig. 3b). These comparison results indicated that the antioxidant status of *W. pigra* was sensitive to soil water content and the antioxidant level in *W. pigra* from 50% soil water content group was lower than other groups.

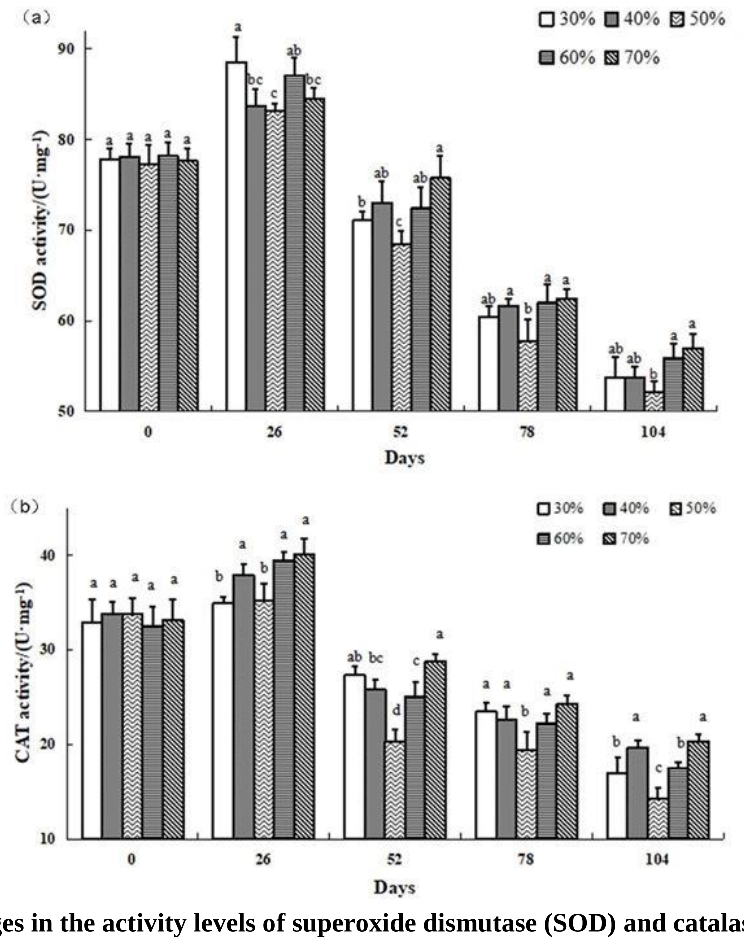

Figure 3: Changes in the activity levels of superoxide dismutase (SOD) and catalase (CAT) in tissue fluid under five soil water contents. Samples were collected from five groups of *Whitmania pigra* on days 26, 52, 78, and 104, and SOD (a) and CAT (b) were measured using a spectophotometer. Values with different letters indicate significant differences among different groups (p<0.05). Data are expressed as mean±standard deviation (n=5).

Effect of soil water content on immune response

We examined the effect of different soil water content on the immune response of *W. pigra*. The results for immune-related enzyme activities (AKP and LYZ) at each time point are presented in Fig. 4. The soil water content significantly influenced the immune-related enzyme activities (AKP and LYZ). Although the activities of AKP and LYZ decreased during the trial period, the activities of AKP and LYZ were significantly higher in 50% water content group than in other water content groups (Fig. 4). These comparison results indicated that the immune response of *W. pigra* showed a downward trend and the immune-related enzyme activities of *W. pigra* in 50% soil water content was higher than those in other groups.

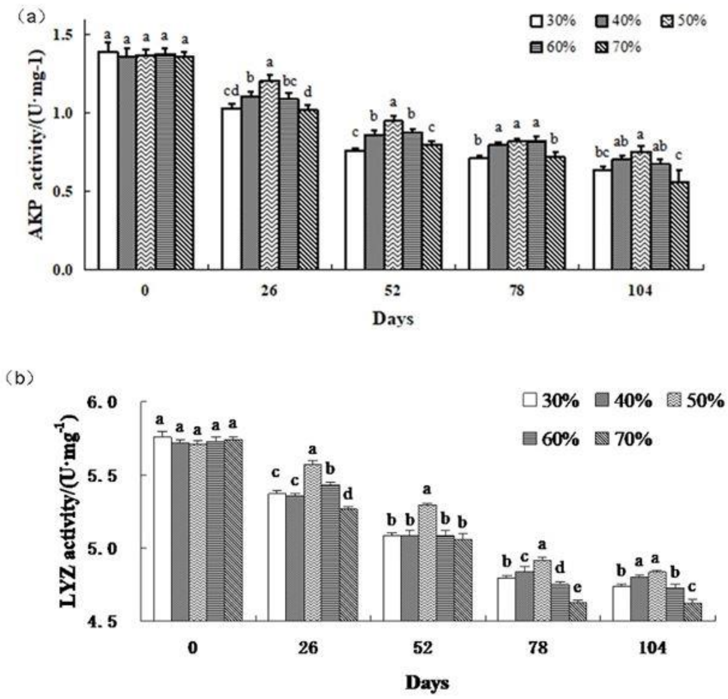

Figure 4: Changes in the activity levels of lysozyme (LYZ) and alkaline phosphatase (AKP) in tissue fluids under five soil water contents. Samples were collected from five groups of *Whitmania pigra* on days 26, 52, 78 and 104, and AKP (a) and LYZ (b) were measured using a spectophotometer. Values with different letters indicate significant differences among different groups (p<0.05). Data are expressed as mean ± standard deviation (n=5).

Discussion

It is necessary to determine optimal environmental conditions such as soil water content for successful overwintering of aquatic animals (Lambert and Dutil, 2001; Shan *et al.*, 2008). In the present study, the survival rate of *W. pigra* was positively affected by soil water content and 50% water content was the optimal level for *W. pigra* survival during overwintering period. In natural environments, when the water temperature is below 10℃, leeches hibernate on the shore of lakes or rivers with dead leaves and similar soil humidity, and the oxygen in the air is dissolved by the glands of the skin epidermis (Wang and Wang, 2005).

Soil as a substrate for leech inhabitation has better heat retaining and moisture retention properties during overwintering period. Moreover, leeches can escape low temperatures when the soil temperature is higher than the water temperature and the soil helps the growth of beneficial microorganisms, which enables the excreted waste of the leech to decompose faster (Kutschera and Wirtz, 2001). The thermal insulation and air permeability of the soil decrease when the soil water content increases, however, that is unfavorable for leech respiration when the soil water content decreases. Therefore, inappropriate soil water content may not be conducive to the respiration and survival of leech. In the present study, the highest survival rate of *W. pigra* was at 50% soil water content treatment during the 104-day overwintering period, probably due to appropriate humidity, temperature, and air permeability.

Leeches are ectotherms and their hibernation is an effective survival strategy adopted by them to adverse winter conditions. During an overwintering period without feeding, metabolic rates, and energy usage for both biochemical (e.g. transcription, translation, cell cycle, etc.) and physiological processes (e.g. body temperature and cardiac rhythm, etc.) have inhibiting effect on leeches (Geiser, 2004; Storey, 2010), consequently resulting in body weight loss. In this study, we found that the weight-loss rate of *W. pigra* decreased with increasing in the soil water content, and the highest and lowest weight-loss rates were at 30% and 70% soil water treatments, respectively. High soil water content probably made *W. pigra* to replenish the water easily.

Reactive oxygen species (ROS) can affect cell viability by causing membrane damages and enzyme inactivities. The increase in ROS induces the secretion of antioxidant enzymes such as SOD and CAT (Basha and Rani. 2003; Hegazi *et al*., 2010). The results showed that the activities of SOD and CAT increased first (at 26 DAR), and then decreased during the trial period. This result indicated that the temperature stress might results in the production of excessive ROS first, which damages cells and tissues and later decrease the activities of SOD and CAT. At 50% soil water content treatment, the activities of SOD and CAT were significantly lower than other four treatments, indicating that *W. pigra* at 50% soil water content group were subjected to the least environmental pressure. At 50% soil water content treatment, it has good properties of the thermal insulation for metabolic and air permeability for respiration.

The immune system is considered to be a mechanism of self-adaptation and help the animal cope with injuries (Bowden *et al*. 2007; Wang *et al*., 2018). LYZ is a very important immune parameter that can hydrolyze the B-1,4-glycoside bond between N-acetyl-paric acid in the cell wall of gram-positive bacteria and destroy peptidoglycan in cell walls and disintegrate bacterial cells (Zhang *et al*., 2019). AKP can catalyze the transformation of all mono phosphate esters and phosphate groups, which are directly involved in the metabolism of phosphorus in microorganisms (Muta and Iwanaga, 1996). Hibernation is an effective strategy to cope with cold environments and/or limited food availability, which by lowering metabolic rate of whole animal, allowing core body temperature to fall, and decreasing metabolic rate to a fraction of the basal metabolic rate. The results showed that the activities of AKP and LYZ decreased during the trial period, indicating that the immune response of *W. pigra* showed a downward trend during overwintering period. However, *W. pigra* in 50% soil water content treatment had a higher immune response than those in the other groups. A major decrease in body weight and a weakened immune system occurs during hibernation, but there have high metabolic efficiency and provide more energy for immunization in 50% soil water content treatment.

This study indicated that soil water content was one of the key environmental factors that affects *W. pigra* during overwintering and 50% soil water content is the optimal level for *W. pigra* with the highest survival rate. In addition, soil water content affected the antioxidant status and immune response of *W. pigra* during overwintering period.

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