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# Hydrogen-rich water mediates redox regulation of the antioxidant system, mycelial regeneration and fruiting body development in *Hypsizygus marmoreus*

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ABSTRACT

*Hypsizygus marmoreus* is an important industrialized mushroom, yet the lack of basic research on this fungus has hindered further development of its economic value. In this study, mycelia injured by scratching were treated with hydrogen-rich water (HRW) to investigate the involvement of the redox system in fruiting body development. Compared to the control group, damaged mycelia treated with HRW regenerated earlier and showed significantly enhanced fruiting body production. Antioxidant capacity increased significantly in damaged mycelia after HRW treatment, as indicated by higher antioxidant enzyme activities and antioxidant contents; the levels of reactive oxygen species (ROS) and malondialdehyde (MDA) were also reduced at the mycelial regeneration stage after treatment with HRW. Furthermore, genes involved in ROS, $Ca^{2+}$, MAPK and oxylipin signaling pathways were up-regulated by HRW treatment. In addition, laccase and manganese peroxidase activities and mycelial biomass were higher after HRW treatment, suggesting that HRW might enhance the substrate-degradation rate to provide more carbon sources for fruiting body production.



## 1. Introduction

*Hypsizygus marmoreus* (Peck) H.E. Bigelow, also known as shimeji or beech mushroom, is a popular edible mushroom with a small, light-brown or white umbrella-shaped cap (Akavia et al., 2006). Cultivated worldwide, *H. marmoreus* has exceptional medicinal applications, including as an antitumor agent, in addition to its popular/exotic culinary status (Akavia et al., 2009; Matsuzawa, 1998). *H. marmoreus* is an important industrialized mushroom in Asia. According to data from the Chinese Edible Fungi Association, the total production of *H. marmoreus* in China in 2016 was 360,348.78 tons, ranking third among industrialized mushrooms; this production suggests a rapidly increasing demand for this mushroom (Zhang et al., 2016).

For cultivation, mechanical injury such as scratching is needed to stimulate fruiting body production in mushrooms, such as *H. marmoreus*. In both plants and fungi, mechanical damage-induced changes are mediated by elaborate signaling networks, including receptors/sensors, calcium ($Ca^{2+}$) influx, ATP release, kinase cascades, reactive oxygen species (ROS), and oxylipin signaling (Hernández_Oñate et al., 2012). For example, Zhang et al. (2015a) studied expression of different genes at four developmental stages of *H. marmoreus* and found that ROS had important roles in

*Abbreviations:* HRW, hydrogen-rich water; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; CAT, catalase; GSH, glutathione; AsA, ascorbic acid; MDA, malonaldehyde; $O_2^-$, superoxide radical; $H_2O_2$, hydrogen peroxide.

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fruiting body development. Moreover, enhancing laccase activity induced *H. marmoreus* primordium initiation and increased fruiting body production (Zhang et al., 2016, 2015a). However, the developmental mechanism of *H. marmoreus* has remained unclear, and the defects of basic biological studies have hindered further development of the commercial value of *H. marmoreus*.

ROS are particularly important for maintaining the balance between cell proliferation and differentiation. In fungi, important biological roles of ROS in growth and development have been demonstrated (Brun et al., 2009; Takemoto et al., 2007; Zheng et al., 2015). The link between ROS and differentiation in fungi was first established in *Aspergillus nidulans*, whereby NADPH oxidase A (NoxA) was shown to be necessary for cleistothecium development (Lara-Ortiz et al., 2003). Furthermore, ROS balance regulates *Epichloë festucae* hyphal morphogenesis and growth in culture and in plants (Takemoto et al., 2011). During the sclerotia formation of *Botrytis cinerea*, ROS play important roles, which are the basis for fruiting body development (Kim et al., 2011; Segmueller et al., 2008). Regarding mushrooms, ROS have also been shown to act as important factors in the mycelial growth and fruiting body development of *Ganoderma lucidum* (Mu et al., 2014; Ren et al., 2016; Zhang et al., 2017). Although ROS production is clearly crucial for signaling, other than a general association with mitogen-activated protein kinase (MAPK) cascades, the pathways involved have yet to be elucidated. Regardless, the scarcity of studies on the ROS system has hindered a deeper understanding of how the redox balance regulates the growth and development of *H. marmoreus*.

Research on gaseous hydrogen has progressed from focusing on its role as a fuel to its role as matter that is able to regulate biological and pharmacological functions. For instance, hydrogen-rich water (HRW) inhibited glucose- and α, β-dicarbonyl compound-induced ROS production in rat kidney homogenates *in vitro* (Katakura et al., 2012). HRW re-establishes the ROS homeostasis of radish sprouts by blocking the UV-A-induced increase in hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^-$) accumulation and by enhancing the UV-A-induced increase in superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities (Su et al., 2014). HRW also significantly decreased the ROS content, maintained the biomass and polar growth morphology of mycelium, and decreased secondary metabolism in *G. lucidum* under HAc-induced oxidative stress (Ren et al., 2016). By regulating ROS levels under oxidative stress, HRW increased the antioxidant activity and mycelial growth of *H. marmoreus* (Zhang et al., 2015a, b). However, there is no research to date on the effects of HRW on *H. marmoreus* fruiting body development.

Despite being one of the most important industrial mushrooms in Asia, *H. marmoreus* development is a long process. Our major research objective is to identify effective and safe methods to increase fruiting body production of *H. marmoreus*. As a reducer gas, it is unknown whether $H_2$ has a function in *H. marmoreus* fruiting body regulation. Thus, the aim of the current study was to examine the effect of HRW on fruiting body development and mycelial regeneration after mechanical damage. We also investigated the possible mechanism by which HRW affects ROS levels, the activities of the key enzymes involved in oxidative metabolism and the expression levels of ROS, $Ca^{2+}$, MAPK and oxylipin signaling pathway-related genes.

## 2. Materials and methods

### 2.1. Sample preparation and collection

*H. marmoreus* samples were obtained from the China General Microbiological Culture Collection Center (Beijing) (No. CGMCC5.01974) and grown at 25 °C on potato dextrose agar (PDA) medium for 14 d. The fungus was transferred once to a new PDA plate for 14 d, and HRW or distilled water was added for 12 h. HRW or the distilled water was removed, and the samples were cut into a grid pattern with a scalpel to detect ROS levels.

For cultivation experiments, primary cultures were grown in potato dextrose broth (PDB) on a rotary shaker incubator at 150 rpm at 25 °C for 7 d. The components of the basswood medium and the cultivated conditions were according to Zhang et al. (2015a). After scratching, the injured mycelia were mixed with 15 mL HRW or distilled water. During cultivation, mycelia were collected on the 4th d at the mycelial regeneration stage (H-M), the 8th d at the mycelial pigmentation stage (H–V), the 12th d at the primordium stage (H–P), the 16th d at the nailing stage (H–N) and the 20th d at the fruiting body stage (H–F). Samples from three randomly selected trays at these five developmental stages were frozen at −80 °C for later experiments. In addition, the numbers of fruiting bodies were counted and analyzed.

**Table 1**
Oligonucleotide primers used in this study.

| Primer name | Sequence (5′to 3′) |
|---|---|
| SOD-F | CGCTAATGGTGAAGGCGGAAAG |
| SOD-R | TTCGGAGTGGTCACAATCTCAAGC |
| CAT-F | CAACCGTCCGTTTCTCCACT |
| CAT-R | CCAATCCCAATTACCTTCCTC |
| GR-F | TGACATTCCCACTGTTGTATTCT |
| GR-R | CGGAACGACGACTTGTAGATT |
| GPX-F | CCATTGCCGAGTTCTGT |
| GPX-R | GCCTTCTTTGTTGACGAG |
| CAC-F | AGGACAAACAAGGATGGAGGAC |
| CAC-R | TATGGCAGACCGAAGACGAG |
| CAMK-F | ACCGCCTCCTCAAGACAACA |
| CAMK-R | CCACGCCTAAGCCAACTCCC |
| CPE-F | TTCATCGGACTCTGAGGTGG |
| CPE-R | CGAGATAATCGGCTGTAACGT |
| MAPK-F | ACTTGAAGCCTGCCAACCTC |
| MAPK-R | TCCTTGCCTCCTGGAACACT |
| PKC-F | TAAATGATTTGGTGGAGGC |
| PKC-R | CATCGGTGAAACTGAGGC |
| PKA-F | CGTCGCTCGTTCGCAGTA |
| PKA-R | TGGGCTCTGGGCTATGCT |
| STE7-F | ACTTCTTCGTGAGGTATCCA |
| STE7-R | GTTTGCCAACGGTTTCTA |
| STE20-F | TCTAGTGCTGAGTTGAGTGCG |
| STE20-R | TGTGCCTGGGATGTCTGG |
| NOXA-F | ATCCTTTGACGCTCCTCC |
| NOXA-R | CGCCTCCTTGATTGATTT |
| NOXB-F | TGTCCCGAGGTCTCCTATTT |
| NOXB-R | GTTCCGATAACCTTTCCACC |
| NOXR-F | AGAAATGCCAACCCAAATCC |
| NOXR-R | AGTCTCCGCTCGCATAACCC |
| DOX-F | AAAGCGAAATACGCACCG |
| DOX-R | TGATCTTGGCCTCCTCCC |
| LOX-F | GCGGAGGAGATTATTGG |
| LOX-R | ACGGAGCGAGATCACAG |
| PSA-F | TGGACATTCGGTGGTGA |
| PSA-R | TATTGCTGGGTGGACGA |
| PSD-F | AGGAGCCGAGACCTAAAC |
| PSD-R | GTTGGCAATGTCTTCACC |
| 18S–F | GAGGGACCTGAGAAACG |
| 18S-R | ATAAGACCCGAAAGAGCC |

### 2.2. Determination of $H_2$ concentrations

HRW was kindly supplied by Beijing Hydrovita Beverage Co., Ltd. (Beijing, China); the $H_2$ concentration of fresh HRW, obtained in a hermetically sealed canister, was 0.80 mM; the freshly prepared HRW was maintained at a relatively constant level at 25 °C for at least 12 h. After opening the canister, 25 or 50 mL of 100 % HRW was added to 75 or 50 mL of distilled water to obtain 25 % or 50 % HRW.