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Autoclaved spent substrate of hatakeshimeji mushroom (*Lyophyllum decastes* Sing.) and its water extract protect cucumber from anthracnose

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ABSTRACT

The protective effect of fresh spent mushroom substrate (SMS) of hatakeshimeji (*Lyophyllum decastes* Sing.), a popular culinary-medicinal mushroom, and its water extract against anthracnose of cucumber was investigated. Plants were treated with water extract from SMS or autoclaved water extract by spraying the whole plant or by dipping the first true leaf, and inoculated with *Colletotrichum orbiculare* seven days later. Plants treated with either of the extracts showed a significant reduction of necrotic lesions. On the other hand, when plants were grown in a mixture (1:2, v/v) of SMS or autoclaved SMS and soil, a disease reduction of over 70% was observed in autoclaved SMS. The water extract showed no antifungal activity against spore germination and mycelial growth of the pathogen. Real-time PCR analyses of chitinase and β-1,3-glucanase genes revealed a significant increase of expressions after 24 h of pathogen inoculation in water extract-treated plants compared with the control plants. These results suggest that water-soluble and heat-stable compounds in SMS enhance the state of systemic acquired resistance and protect cucumbers from anthracnose. Thus, the use of SMS for disease control may offer a new technology for the recycling and management of waste from mushroom cultivation.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (2008), the global production of cultivated edible mushrooms had increased from 2.26 million tons in 1998 to 3.48 million tons in 2008. About 53% of cultivated edible mushrooms are produced in Asian countries, followed by European countries (32%) and the Americas (13%). In Japan, mushrooms are eaten and appreciated for their flavor, and used medicinally for their healing properties. In 2007, the production of edible mushrooms in Japan was estimated to be 423,224 t, and it is expected that this amount will increase in the future due to market demand.

Despite the evident benefits of mushrooms, the exponential increase in their consumption worldwide is also generating a high volume of spent mushroom substrate (SMS). It has been reported that about 5 kg of substrate are needed to produce 1 kg of mushroom (Williams et al., 2001; Uzun, 2004; Finney et al., 2009), and about 17 million tons of SMS are produced each year. Consequently, one of the main problems faced by mushroom production companies is finding a way to properly dispose of the SMS without contaminating the water and soil. In fact, the lack of a sustainable waste management solution for SMS is the most significant barrier to the future development of the mushroom industry (Finney et al., 2009). Several studies have been carried out to demonstrate the benefits of SMS application in mushroom re-cultivation, enrichment of soils, restoring areas that have been destroyed through development, deforestation or environmental contamination (Sánchez, 2004), cultivation of vegetables, fruits and flowers in greenhouses and fields (Medina et al., 2009; Polat et al., 2009; Ribas et al., 2009), and soil amendment and degradation of organopollutants (Semple et al., 2001; Lau et al., 2003). The SMS can also be used as a potential energy feedstock (Williams et al., 2001; Finney et al., 2009), and ethanol production (Hideno et al., 2007).

In plant-fungal interactions, carbohydrate and protein elicitors that induce defense mechanism in plants are released from the mycelia of fungal pathogens (Shibuya and Minami, 2001). Once the plants recognize elicitors, many plants develop an enhanced resistance to further pathogen attack also in the uninoculated organs. This type of induced resistance is called systemic acquired resistance (SAR) (Durrant and Dong, 2004; Vallad and Goodman, 2004; Da Rocha and Hammerschmidt, 2005; Walters et al., 2005; Conrath, 2006). Therefore, the mycelia of mushrooms that are prevalent in SMS are abundant sources of elicitors, and thus

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application of SMS to plants may be useful for the control of plant diseases. However, the study of the potential role of SMS in disease control has not received adequate attention. In the few studies that have addressed this topic, the emphasis has been on exploiting the antibiotic-producing microorganisms in SMS by applying SMS as compost (Yohalem et al., 1994; Cronin et al., 1996; Viji et al., 2003; Choi et al., 2007).

Hatakeshimeji (*Lyophyllum decastes* Sing.), a gray basidiomycete family Tricholomataceae, belongs to the same genus as honshimeji (*Lyophyllum shimeji* Hongo), which is well known as the most delicious mushroom (Ukawa et al., 2000; Pokhrel et al., 2006). In addition to its delicious taste and excellent texture, hatakeshimeji has been well studied for its medical properties. Recently, powder from this mushroom has been made commercially available and has been used as a health-promoting supplement for prevention and treatment of various diseases (Ukawa et al., 2007).

The main objective of this work was to determine whether extracts from hatakeshimeji SMS can protect cucumber (*Cucumis sativus* L.) from anthracnose caused by *Colletotrichum orbiculare* (Berkeley & Montagne) Arx. In addition, SAR-related gene expression in water extract-treated cucumber was examined for analyzing the mechanism of disease resistance.

2. Materials and methods

2.1. Preparation of SMS

The hatakeshimeji strain TMIC30940 was inoculated in polypropylene bags containing 2.5 kg mixtures of Japanese cedar, sorghum, soybean pulp, rice bran and wheat bran (8:0.5:1:0.9:0.3, v/v). The bags were incubated at 20–22 °C for 65 days, and then incubated at 17–19 °C for 30 days for fruit body formation. After the fruit bodies were harvested, SMS was used immediately for water extract preparation.

2.2. Water extract from SMS

Spent mushroom substrate (200 g) was homogenized in a Waring blender (Nissei, Tokyo, Japan) with 500 ml of distilled water (DW) for 2 min at 1500 rpm. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and then centrifuged for 5 min at 800 *g*. The supernatant was used immediately for leaf treatment or stored at −80 °C until use as the water extract from SMS (hereinafter WESMS). WESMS was autoclaved at 121 °C for 30 min (hereinafter AWESMS), and AWESMS was also used for leaf treatment.

2.3. Treatments with WESMS and SMS on cucumber

2.3.1. WESMS

The cucumber (*C. sativus*) cultivar Natsusuzumi was grown in plastic pots (9 cm) containing soil mixed with Tanemaki soil (JA, Tokyo, Japan) and Nippi No. 1 soil (JA, Tokyo, Japan) in a climate-controlled room at 25 °C with a 14-h light and 10-h dark period. Cucumber plants (16–20 days old) with the first true leaf (next to cotyledon) and second true leaf fully expanded and the third true leaf one-third-one half of its full size were used for foliar application of WESMS. The first true leaf was dipped for 10 s in WESMS or the whole plants were sprayed with WESMS. The first true leaf or the whole plants of cucumber were also treated with AWESMS. DW was used as a control.

2.3.2. SMS

Cucumber seeds were germinated in a cell tray containing Tanemaki soil. To examine the effects of SMS in soil on cucumber plants, the seedlings were transplanted after seven days to plastic pots (9 cm) containing a mixture of SMS and Nippi No. 1 soil (1:2, v/v). The same experiment was conducted using SMS autoclaved at 121 °C for 30 min (hereinafter ASMS). Seedlings were also grown in pots containing only Nippi No. 1 soil as a control.

2.4. Conidium inoculation and disease assessment

The strain C-14 of *C. orbiculare*, which causes anthracnose of cucumber, was obtained from the ZEN-NOH Agricultural R & D Center (Hiratsuka, Japan). The culture was maintained on a potato dextrose agar (PDA) (Difco) plate (9 cm) at 25 °C and sub-cultured on a PDY (PDA amended with 0.5% yeast) plate to obtain abundant conidia for the inoculation test. The conidial suspensions were prepared from 7–12 day-old cultures. For inoculation, conidia of C-14 were collected from the plate with DW, filtered through Kimwipes tissue S-200 (Nippon Paper Crecia, Tokyo, Japan) and washed three times by centrifugation (5 min at 800 *g*). The concentration was adjusted to 5×10^5 or 1×10^5 conidia/ml. The conidial suspensions (5×10^5 conidia/ml) were sprayed onto whole plants after seven days of treatment with WESMS or AWESMS. In the plants treated with SMS or ASMS, spore suspensions (1×10^5 conidia/ml) were applied as 5–10 drops (30 μl each) on the first and second true leaves after approximately 15 days of treatment. Inoculated plants were kept in the dark at 90% RH in a growth cabinet (MLR-351H) (Sanyo, Tokyo, Japan) for 24 h at 20 °C, and then transferred to an incubator room at 25 °C. The number of lesions on each examined leaf in the spray inoculation experiment and the lesion area on each leaf in the drop inoculation experiment were recorded 5–7 days later.

2.5. Antifungal activity of WESMS

Conidia of C-14 were washed three times with DW by centrifugation. The spore pellets after centrifugation were mixed with WESMS and AWESMS passed through a 0.22-μm-pore filter unit (Millipore, Bedford, MA) and the concentration was adjusted to 5×10^5 conidia/ml. DW was used as a control. Conidial suspensions (3 ml) were sprayed on glass slides and incubated in a moist chamber at 25 °C in the dark for 24 h. Conidial germination and germ-tube length were measured microscopically. For mycelial growth tests, mycelia discs (5 mm in diameter) were transferred onto potato dextrose yeast (PDY) or Czapek solution agar (Difco) (10 ml) plate (9 cm), supplemented with WESMS or AWESMS (1 ml/plate) sterilized by filtration. Sterile DW was added to each medium as a control. The plates were incubated at 25 °C in the dark and radial colony growth of the fungus was recorded after three weeks.

2.6. Gene expression in the cucumber

We selected six genes which have been reported to be involved in the defense response against biotic or abiotic stress in plants: callose synthase, lignin peroxidase, chitinase, β-1,3-glucanase, pathogenesis-related protein-1 and phenylalanine ammonia-lyase (Table 1). For RNA extraction, samples were harvested at 7 days after treatment and 1 day after the pathogen inoculation from the leaves of whole plants treated by spraying with WESMS. Cucumber leaves treated by spraying the whole plants with DW was used as a control.

Samples were snap frozen in liquid nitrogen, and stored at −80 °C until required. Total RNA was isolated using TRIzol-Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and treated with DNase (DNA-free; Ambion, Austin, TX). One microgram of RNA was reverse-transcribed with an RT reagent kit (Takara Bio, Tokyo, Japan) using a random 6-mer primer, according to the manufacturer's instructions. Real-time PCR was performed on