Induction of defense responses by extracts of spent mushroom substrates in rice

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We investigated the effect of treatment with hot water extracts from the spent mushroom substrates (SMSs) of Lentinula edodes and Hypsizygus marmoreus on the resistance of rice leaves to Pyricularia oryzae infection. The spraying of the SMS extracts clearly suppressed the development of lesions caused by Py. oryzae infection. The accumulation of phytoalexins monilactones A and B, oryzalexin A, and sakuranetin was markedly induced by the spraying of extracts. The enhanced expression of defense related genes PR1b and PBZ was also found in leaves sprayed with the extracts. Treatments with the extracts also affected phytohormone levels. The levels of N-Δ2-isopentenyl)adenine and trans-zeatin markedly increased in response to treatment, whereas the levels of salicylic and jasmonic acids were largely unchanged. © Pesticide Science Society of Japan

Keywords: Spent mushroom substrate, defense response, rice blast, phytoalexin, pathogenesis related protein, cytokinin.

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Introduction

Currently a large proportion of mushrooms on the market are produced using the block cultivation method. This method has enabled the stable and safe production of mushrooms, which is a market requirement. The mushroom substrate used for the mycelial block is composed of 80–90 wt% of sawdust and woodchips, supplemented with 10–20 wt% of wheat or rice bran. The spent mushroom substrate (SMS) is disposed of as waste after the fruiting bodies are harvested. The amount of mushroom substrate used for 350g of Lentinula edodes (shiitake) is approximately 1.2kg.3 Approximately 61,000 tons of Le. edodes mushrooms were produced by mycelial block cultivation in 2016;4 thus, 210,000 tons of SMSs are disposed of each year in Japan. A similar calculation estimated that the amount of SMS from Hypsizygus marmoreus (bunashimeji) production is 300,000 tons per year. As other mushrooms, such as Pholiota microspora (namsako) and Pleurotus eryngii (king oyster mushroom), are also produced by mycelial block cultivation, the total amount of SMS generated is considerable; this presents a serious problem for the mushroom industry, due to the land area required for disposal.

The utilization of SMS for other purposes has been attempted. Researchers, for instance, have tested the feasibility of using SMS as a material to improve soil quality5 and as an ingredient of silage for cows.6 However, these approaches are not yet commercially viable. In addition, the potential use of SMSs as agricultural material for crop protection has been examined. Inagaki and Yamaguchi (2009) found that the application of compost made from the SMS of Le. edodes effectively reduced the severity of anthracnose in cucumber plants.7 Similarly, Parada et al. (2012) found that spraying a water extract of the SMS of Lyophyllum dozens (hatakeshimeji) suppressed the development of lesions caused by infection with rice blast fungus6 (Pyricularia oryzae; syn: Magnaporthe oryzae).8) Similarly, Parada et al. (2012) found that the treating cucumber seedlings with SMSs of Ly. dozens and Pl. eryngii significantly reduced powdery mildew caused by Podosphaera xanthii and angular leaf spot caused by Pseudomonas syringae pv. lachrymans.9) Furthermore, it is of interest to note that volatile compounds from the SMSs of Le. edodes and H. marmoreus induced resistance to Alternaria sooty spot caused by Alternaria brassicola in cabbage.10) These protective effects...
of SMS treatment were attributed to the induction of systemic acquired resistance (SAR) because of the lack of antifungal activity in the extracts. Indeed, the resistance of pepper (Capsicum annuum) against Phytophthora blight disease has been induced by treatment with a water extract of the SMS of Le. edodes; this was accompanied by the accumulation of transcripts of pathogenesis-related genes (CaPR1, CaBGLU, CaPR-4, and CaPR-10) and an increase in the salicylic acid (SA) content.

Plant defense activators are chemicals that render crops resistant to pathogen infection. These compounds include probenazole, thiadiazin, and isothianil. Probenazole was the first plant defense activator used in practice; subsequently, thiadiazin and isothianil were developed. The resistance induced by the chemicals was quite similar to SAR. The toxicity of these chemicals to humans is low, and pathogens have not yet acquired resistance to them. These plant defense activators are extensively used in rice cultivation in East Asia. However, because their repertoire is limited, developing new compounds with different spectra effective for other crops is required.

We recently verified that the water extract of the SMS of Le. edodes suppressed the development of lesion caused by Py. oryzae infection and found that the extract strongly inhibited the conidial germination of Py. oryzae. The activity-guided fractionation of the extract resulted in the identification of three phenolic dicarboxylic acids, which were considered to be degradation products of lignin. However, the conidial germination rate of Py. oryzae remained high in the presence of the hot water extract of H. marmoreus SMS, but the extract effectively suppressed the development of lesions on rice leaves. In our study, we examined the working hypothesis that the suppression of lesion development was due to the activation of defense responses by treatments with SMS extracts.

Materials and Methods

1. Plant material and pathogenic fungi
Rice (Orzsa sativa ‘Nipponbare’) seeds were placed on moist paper and incubated at 28°C for 4 days under a 16hr light/8hr dark cycle. Five germinated seedlings were transplanted into a 1:1 mixture of vermiculite and artificial compost, Green soil (Shoii Sangyo, Okayama, Japan), in a pot (4.5 cm in diameter, 4 cm in depth). Rice blast fungus (Py. oryzae, race 007, strain: Naga 69–150) was the stock culture of the Laboratory of Plant Pathology, Shimane University.

2. Treatment of rice seedlings with SMS extracts
The SMSs of Le. edodes and H. marmoreus were kindly provided by JA Tottori Inaba, Hatto branch, Yazu Cho, Tottori, Japan, and Fukuda Noen, Tottori City, Japan, respectively. The preparation of hot water extracts of SMSs has been described previously. The SMS extract (5 mL) was sprayed on five 16-day-old seedlings in a pot. As a negative control, leaves were sprayed with distilled water, whereas, as a positive control, a BIT (1,1-benzisothiazol-3(H)-one-1,1-dioxide, Wako Pure Chemical Industries, Osaka, Japan) solution (56 mg/L, 20 mL) was added to a pot (50 mL) containing 13-day-old rice seedlings, as described by Nakashita et al. (2002). Then, droplets (5 µL) of the conidial suspension (5×10^5 conidia/mL) in 0.25% Tween 20 were placed at five points at 1 cm intervals on the third leaves of 18-day-old seedlings that were horizontally positioned on plastic plates. As a control, droplets of 0.25% Tween 20 were placed on the leaves. The inoculated seedlings were kept in a moist chamber for 12 hr. The leaves were observed five days after inoculation.

3. Analysis of rice phytoalexins
Rice phytoalexins were purified from rice leaves irradiated with UV light (GL-15 germicidal lamp, Hitachi, Tokyo, Japan) for 10 min. Ex extractions were made with methanol. Sakuranetin, momilactone A, momilactone B, and oryzalexin A were purified by silica gel column chromatography and reverse-phase HPLC in accordance with reported methods. The identities of compounds were confirmed by 1H NMR spectra and ESI-MS.

We sprayed hot water extracts of SMS on the 16-day-old rice seedlings. Third leaves were excised from the seedlings and extracted by immersing in 80% methanol for 24 hr. The extracts were subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis in multiple reaction monitoring (MRM) mode. Analysis was performed with a Quattro Micro API mass spectrometer (Waters, Milford, MA, USA) connected to an Acquity UPLC (Waters). LC conditions were as follows: column: AQUITY UPLC BEH C18 2.1×50 mm (1.7 mm) (Waters); column temperature: 40°C; solvents: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); gradient: 30–70% B/(A+B) within 10 min; flow rate 0.2 mL/min. MRM conditions for phytoalexin analysis were optimized by using purified phytoalexins and are summarized in Supplementary Table S1.

4. Analysis of transcript amounts of defense-related genes
Rice leaves were frozen by liquid N2 and homogenized using a mortar and pestle. Total RNA was isolated by using an ISOGEN II kit (Nippon Gene, Tokyo, Japan). cDNA was prepared using a Prime Script RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Kusatsu, Japan). Real-time PCR analysis was performed with a KOD SYBR qPCR Mix (Toyobo, Osaka, Japan). We carried out these procedures in accordance with the manufacturers’ instructions. The PCR reactions were performed with a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Target genes were PBZ1 and PBZ2. The following specific primers were used to amplify the transcripts: PBZ1 forward: 5′-ACG GGC GGT GTA CGT ACT GGC TA-3′; reverse: 5′-CTC GGT ATG GAC CGT GAA G-3′; PBZ2 forward: 5′-GGC TGT TTC GTC GTG AGA GT-3′; reverse: 5′-CCA CCC ATT GAT GAA GCA AA-3′. The expression of these genes was normalized using the transcript amount of ubiquitin 1 (forward: 5′-GGA CTG TTG AAA ATC ATC ATA-3′; reverse: 5′-CCA TAT ACC AGC ACC GTCA AAA A-3′).
5. Analysis of phytohormones

N\textsuperscript{6}-\textit{Δ2-isopentenyl}adenine (IP), trans-zeatin (tZ) and salicylic acid (SA) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and jasmionic acid (JA) was from Tokyo Chemical Industry (Tokyo, Japan). For the analysis of IP and tZ, rice leaves were subjected to extraction with 10 volumes of 80% methanol for 24 hr. For the analysis of SA and JA, rice leaves were frozen in liquid nitrogen and well powdered using a mortar and pestle. Twenty volumes of methanol were added to the powder, and the powder was further homogenized. The resulting methanol extract was passed through a filter (TORAST Disc Syringe Filter GLCTD-HPTFE1322, Shimadzu GLC, Tokyo, Japan). The extracts were subjected to LC-MS/MS analysis in MRM mode with a Shimadzu LCMS-8040 system (Shimadzu, Kyoto, Japan). HPLC conditions were as follows: column: Inertsil ODS-4 5\textmu m, 4.6×150 mm (GL Sciences, Tokyo, Japan); column temperature: 40°C; solvents: water (A) and acetonitrile (B); gradient: 10% B/(A+B) (0–2 min), 10–100% (2–12 min), 100% (12–13 min); flow rate: 0.4 mL/min. ESI-MS/MS analysis in the MRM mode was performed in the positive mode for IP and tZ and in the negative mode for SA and JA. For quantitative analysis of each phytohormone, the precursor ion (m/z), product ion (m/z), Q1 pre-rod bias (V), Q3 pre-rod bias (V), and collision energy (V) were optimized as shown in Supplementary Table S2.

Results

1. Suppression of lesion development by treating rice leaves with water extracts of Le. edodes and H. marmoreus SMSs

The hot water extracts of the SMSs of Le. edodes and H. marmoreus were sprayed onto rice (‘Nipponbare’) leaves. After incubation for two days, the leaves were inoculated with the conidia of Py. oryzae (race 007), which is compatible with ‘Nipponbare.’ Large necrotic lesions were formed on the control leaves at the inoculation site, as shown in Fig. 1A and B. In contrast, pale brown lesions formed on leaves sprayed with the extracts of Le. edodes and H. marmoreus SMSs. We also treated rice seedlings with BIT that is the active metabolite of the plant defense activator probenazole as a positive control. The leaves of the seedlings treated with BIT showed dark brown lesions. The size of the lesions formed on the leaves treated with BIT and SMS extracts remained unchanged thereafter. The severity of lesions was evaluated by appearance using four grades: 0, no visible symptoms; 1, pale brown lesions; 2, brown lesions; 3, lesions with yellow necrotic zones. The average scores of lesions treated with the extracts of SMSs of Le. edodes and H. marmoreus were about 1.1 and 1.3, respectively, which were significantly lower than those of the control (score: 2.4) and BIT-treated (score: 1.8) leaves (Fig. 1C), indicating that the extracts of SMSs effectively suppressed lesion development.

2. Antifungal activity of water extracts of SMS

To detect the antifungal activity of the SMS extracts, we performed an assay to detect the inhibitory activity on conidial germination. As reported previously, the extract of SMS of Le.
edodes strongly inhibited conidial germination, with the germination rate of conidia in the presence of the extract being 4.1%. However, the extract of SMS of H. marmoreus did not show antifungal activity in this assay. The germination rate of conidia in distilled water was 91.2%, and in the H. marmoreus SMS extract it was 90.5%. This lack of antifungal activity in the H. marmoreus SMS extract prompted us to analyze the induction of defense responses in rice leaves treated with SMS extracts.

3. Phytoalexin accumulation in leaves treated with water extracts of Le. edodes and H. marmoreus SMSs

Representative phytoalexins of rice, momilactones A and B, oryzalexin A, and sakuranetin, extracted from leaves treated with water extracts of Le. edodes and H. marmoreus SMSs were analyzed. In the leaves treated with both extracts, a marked accumulation of phytoalexins was detected, as shown in Fig. 2. The accumulation of phytoalexins started 24–72 hr after treatment. Momilactone A reached its peak 72 hr after treatment, but the other phytoalexins continued to increase until 96 hr, except for momilactone B in the leaves treated with H. marmoreus SMS extract. The extract of H. marmoreus SMS showed somewhat strong activity as compared with the extract of Le. edodes SMS. Among the analyzed phytoalexins, momilactone A accumulated at the highest concentration, with the maximum being 237 nmol/gFW (74.4 µg/gFW).

We also prepared a hot water extract of the mushroom substrate for Le. edodes before culturing the mushroom, and examined its activity on phytoalexin accumulation by spraying it on rice leaves. As shown in Fig. 3, spraying with the extract induced the accumulation of phytoalexins although the amounts were 13.7–5.7% of those in leaves sprayed with the SMS extract.

4. Induced accumulation of defense related gene transcripts

To examine whether defense responses other than phytoalexin accumulation are activated simultaneously by treatment with SMS extracts, we determined the amounts of transcripts of PR1b and PBZ1 by qRT-PCR (Fig. 4). Both of these genes are known to be responsive to P. oryzae infection and have been implicated in the SA-mediated signaling branch of the rice defense response. PBZ1 was first found to be a gene inducible by treating rice plants with probenazol. The amount of PR1b transcript increased in response to treatment with SMS extracts, reaching its maximum 48 hr after treatment (Fig. 4A). The amount of PBZ1 also increased in leaves treated with both extracts (Fig. 4B). Both transcripts increased slightly in control leaves, probably due to the aging of the leaves.

5. Changes in phytohormone levels

It has been suggested that phytohormones are involved in the

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Fig. 2. Accumulation of momilactones A (A) and B (B), oryzalexin A (C), and sakuranetin (D) in leaves treated with the extracts of SMSs of H. marmoreus and Le. edodes. The leaves of 16-day-old seedlings were sprayed with the extracts of SMSs of H. marmoreus (black circles) and Le. edodes (gray triangles). As a control, leaves were sprayed with distilled water (white squares). Data are presented as the mean of three replicates. Error bars indicate standard deviations. Asterisks indicate statistical differences from the control (*p<0.05, **p<0.01, Dunnett’s test).
Induction of defense responses by SMS extracts in rice

We analyzed changes in the phytohormone levels in leaves treated with SMS extracts. The analysis clearly indicated an increase in the levels of IP and tZ in leaves treated with SMS extracts (Fig. 5A and 5B). The IP started to increase 24 hr after treatment with Le. edodes and H. marmoreus extracts, and the concentrations

Fig. 3. Accumulation of momilactones A (A) and B (B), oryzalexin A (C), and sakuranetin (D) by the treatment of rice leaves with the hot water extract of Le. edodes substrate without culturing of the mushroom and SMS. These extracts were sprayed onto the leaves of 16-day-old seedlings. After a 72-hr incubation, the third leaves of seedlings were extracted. The amounts of phytoalexins were analyzed by LC-MS/MS in the MRM mode. Error bars indicate standard deviations. Asterisks indicate statistical differences from the control (*p<0.05, **p<0.01, Dunnett's test).

Fig. 4. Accumulation of transcripts of PR1b (A) and PBZ1 (B) in leaves treated with the extracts of SMSs of H. marmoreus and Le. edodes. The leaves were sprayed with the extracts of SMSs of H. marmoreus (black circles) and Le. edodes (gray triangles). As a control, leaves were sprayed with distilled water (white squares). Data are presented as the mean of three replicates. Error bars indicate standard deviations. Asterisks indicate statistical differences from the control (*p<0.05, **p<0.01, Dunnett's test).
were 2.1- and 6.7-fold larger, respectively, than that in the control. IP in the leaves treated with SMS extracts increased up to 96 hr after the treatment. The maximal concentrations (274 and 583 pmol/gFW) in leaves treated with the extracts of *Le. edodes* and *H. marmoreus* SMSs were 110- and 230-fold larger than the control. On the other hand, the significant increase in the tZ concentration was detected only 96 hr after treatment with the SMSs extracts. The tZ concentrations in the leaves treated with extracts of *Le. edodes* and *H. marmoreus* SMSs were 1.9- and 2.8-fold larger, respectively, than that in the control. The concentrations of SA started to increase from 48 to 72 hr after treatment, even in the control leaves (Fig. 5C). At 96 hr after treatment, the amounts of SA in the leaves treated with extracts from *Le. edodes* and *H. marmoreus* SMSs were 1.9- and 2.8-fold larger, respectively, than that in the control. The concentrations of SA started to increase from 48 to 72 hr after treatment, even in the control leaves (Fig. 5C). At 96 hr after treatment, the amounts of SA in the leaves treated with extracts from *Le. edodes* and *H. marmoreus* SMSs were 1.9- and 2.8-fold larger, respectively, than that in the control. Levels of JA were largely unaffected by the treatment, being approximately 5 nmol/gFW both in both control and treated leaves (Fig. 5D).

**Discussion**

We found that spraying SMS extracts onto rice seedlings elicited the accumulation of phytoalexins. The phytoalexin concentrations were at levels sufficient for the inhibition of *Py. oryzae*. The respective concentrations of momilactone A in leaves treated with extracts of *Le. edodes* and *H. marmoreus* SMSs were 57 and 76 nmol/gFW (17.8 and 23.8 µg/gFW) 48 hr after spraying and increased to 159 and 226 nmol/gFW (49.0 and 71.0 µg/gFW) by 72 hr. The concentration of momilactone A giving 50% inhibition of germ tube growth was 4.8 µg/g.15) The application of 0.2 mM momilactone A was shown to enhance the rice blast resistance of rice plants.24) The respective concentrations of momilactone B, oryzalexin A, and sakuranetin were 6.7, 2.5, and 0.56 µg/gFW in the leaves treated with the extract with *Le. edodes* SMS, and 5.4, 1.1, and 1.0 µg/gFW in the leaves treated with the extract of *H. marmoreus* 72 hr after treatment. The respective ED$_{50}$ values of momilactone B, oryzalexin A, and sakuranetin on the inhibition of the germ tube elongation of *Py. oryzae* were 1 µg/mL,25) 35 µg/mL,25) and 5 µg/mL18) Because the concentrations of momilactones A and B were higher than these reported ED$_{50}$ values for *Py. oryzae*, among the analyzed phytoalexins, they were considered to play the most prominent roles in enhancing resistance to *Py. oryzae* infection in rice leaves treated with SMS extracts.

The accumulation of phytoalexins was also induced by treatment with extract of mushroom substrate before the inoculation of *Le. edodes*. Mushroom substrates are mainly made of sawdust supplemented with rice or wheat bran; thus, the active compounds are present in these materials. However, the induced amounts of phytoalexins in leaves treated with mushroom substrate before use were much smaller than those in leaves treated with the SMS extract; thus, a large part of active compound(s) is
considered to be formed during the cultivation of Le. edodes in the substrate. To date, several plant defense activators have been used in practice for crop protection. Plants treated with these chemicals showed rapid and enhanced responses to pathogenic infection.\textsuperscript{26,27} This effect is referred to as “priming,” and the treatment itself does not induce defense responses such as phytoalexin production. Thus, the action of SMS extracts is different from plant defense activators in the respect that treatment with SMSs involves the activation of defense responses, including phytoalexin accumulation and the expression of defense-related genes.

In this context, the extracts of SMSs appear to serve as microbe-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), and the response of rice plants induced by the extract is categorized as plant pattern-triggered immunity (PTI). Rice cells sense N-acetylchitooligosaccharides,\textsuperscript{28} cerebrosides,\textsuperscript{29} and flagellin\textsuperscript{30} as PAMPs that induce defense responses. Because mushrooms belong to the kingdom Fungi, in common with fungal phytopathogens, it is reasonable to assume that the extracts of SMSs contain compounds that are recognized as PAMPs by rice cells. On the other hand, extracts may also contain damage-associated molecular patterns (DAMPs). In rice, the prior treatment of leaves with lipase (LipA) secreted by Xanthomonas oryzae pv. oryzae can confer enhanced resistance to subsequent X. oryzae pv. oryzae infection. The degradation product of rice tissue by the action of lipase was suggested to serve as a DAMP.\textsuperscript{31} In addition, we recently determined that rare oxylipins, 9- and 13-oxooctadeca-9,11-dienoic acid (9- and 13-KODEs), are accumulated in leaves infected with Bipolaris oryzae, and the exogenous accumulation of these compounds can induce the accumulation of defensive secondary metabolites, including sakuranetin, naringenin, and serotonin. Thus, these oxylipins could also be regarded as DAMPs.\textsuperscript{32} Mushroom substrates are mainly made of sawdust and the bran of rice or wheat. Mushrooms degrade these components by secreting degradation enzymes, such as peroxidase, laccase, cellulase, and glucanase during cultivation. Indeed, we detected phenolic acids that are formed by the degradation of lignin in the water extract of SMS of Le. edodes.\textsuperscript{33} It is reasonable that degradation products of mushroom substrates are recognized as DAMPs by rice cells. The identification of active compounds in the SMS extracts is currently on going.

It is of interest to note that the treatment with hot water extracts of SMSs did not induce marked increases in the concentrations of SA and JA, which have been considered central players in signal transduction leading to the activation of defense responses in plants. In rice, SA has not been shown to directly induce phytoalexin accumulation. By contrast, JA and its active form jasmonoyl-L-isoleucine have been implicated in the accumulation of flavonoid phytoalexins, but not in the accumulation of diterpenoid phytoalexins.\textsuperscript{34} The major phytoalexin in leaves treated with SMS extracts was momilactone A, and the accumulation of sakuranetin was relatively late and small. This may be a reflection of the limited contribution of the JA pathway in phytoalexin induction in leaves treated with SMS extracts.

The treatment of rice leaves with SMS extracts induced the accumulation of IP, one of active cytokinins in plants at high concentrations. Cytokinins have attracted attention because the exogenous application of cytokinins elicits phytoalexin biosynthesis and the expression of defense-related genes in rice.\textsuperscript{34,35} In addition, the concentrations of IP, IP riboside, and IP riboside phosphates increased dramatically in leaves infected with Py. oryzae.\textsuperscript{36} In our experiments, the accumulation of mimosalactone A and the transcripts of PR1b and PBZ1 started as early as 24 hr after spraying with the SMS extract. The concentration of IP 24 hr after treatment was already significantly higher than those in control leaves. Thus, IP may function as a signal mediator in leaves treated with SMS extracts. IP and SA have been shown to exert a synergistic effect on the induction of defense responses in rice.\textsuperscript{34,36} Thus, the involvement of IP in the SA signal transduction pathway has been proposed. However, in our experiments, the SA level remained unchanged after treatment with SMS extracts, suggesting that cytokinin may serve as a signal leading to the activation of defense responses independent of the SA signaling pathway.

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