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Involutin Is an Fe^{3+} Reductant Secreted by the Ectomycorrhizal Fungus *Paxillus involutus* during Fenton-Based Decomposition of Organic Matter

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Ectomycorrhizal fungi play a key role in mobilizing nutrients embedded in recalcitrant organic matter complexes, thereby increasing nutrient accessibility to the host plant. Recent studies have shown that during the assimilation of nutrients, the ectomycorrhizal fungus *Paxillus involutus* decomposes organic matter using an oxidative mechanism involving Fenton chemistry ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\cdot}OH + H_2O$), similar to that of brown rot wood-decaying fungi. In such fungi, secreted metabolites are one of the components that drive one-electron reductions of Fe^{3+} and O_2, generating Fenton chemistry reagents. Here we investigated whether such a mechanism is also implemented by *P. involutus* during organic matter decomposition. Activity-guided purification was performed to isolate the Fe^{3+}-reducing principle secreted by *P. involutus* during growth on a maize compost extract. The Fe^{3+}-reducing activity correlated with the presence of one compound. Mass spectrometry and nuclear magnetic resonance (NMR) identified this compound as the diarylcyclopentenone involutin. A major part of the involutin produced by *P. involutus* during organic matter decomposition was secreted into the medium, and the metabolite was not detected when the fungus was grown on a mineral nutrient medium. We also demonstrated that in the presence of H_2O_2, involutin has the capacity to drive an *in vitro* Fenton reaction via Fe^{3+} reduction. Our results show that the mechanism for the reduction of Fe^{3+} and the generation of hydroxyl radicals via Fenton chemistry by ectomycorrhizal fungi during organic matter decomposition is similar to that employed by the evolutionarily related brown rot saprotrophs during wood decay.

Boreal forests in the Northern Hemisphere are one of the major global sinks for carbon (C) (1). Carbon enters this system as plant litter and through below-ground allocation of photosynthates (2). Fungal communities play an important role in the turnover of this C. Saprotrophic fungi are thought to be the key decomposers of plant litter material (3–6). They can efficiently degrade recalcitrant lignocellulose polymers, which are the main components of plant litter material (7, 8). In contrast, the ectomycorrhizal (ECM) fungi are symbionts that depend on their plant hosts for their C sources. In return, the ECM fungi provide plants with nutrients such as nitrogen (N) (9). The soil organic matter that ECM fungi encounter is a heterogeneous environment consisting of humus-rich organic materials (10). Under such conditions, N is present mostly in organic forms that are associated with polyphenols and other degradation products of plant and microbial biopolymers (11, 12). Studies using stable isotopes have shown that ECM fungi have the capacity to mobilize at least some of this organic N (10). However, the molecular mechanism by which ECM fungi mobilize organic nutrients from the complex soil organic matter has not been well studied.

Recently, we showed that the ECM fungus *Paxillus involutus* (Basidiomycetes; Boletales) can decompose polysaccharides and lignin fragments while acquiring N from organic matter using a brown rot mechanism (13). Spectroscopy and transcriptome analyses revealed the action of a radical-based oxidation mechanism that is similar to that of brown rot wood-decaying fungi. Hydroxyl radicals (${}^{\cdot}OH$) are important oxidants in brown rot fungi and are produced via the Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\cdot}OH + H_2O$) (7, 14–16). The production of hydroxyl radicals requires the reduction of Fe^{3+} to Fe^{2+} (16). We demonstrated that *P. involutus* produced iron-reducing activity during the decomposition of organic matter extracts (13); however, the mechanism for reducing Fe^{3+} was not characterized.

Three different mechanisms have been proposed for the reduction of Fe^{3+} in wood-decaying brown rot basidiomycetes: (i) iron-reducing enzymes, such as cellobiose dehydrogenase (17), (ii) low-molecular-weight glycopeptides (18), and (iii) secondary metabolites, including hydroquinones, such as 2,5-dimethoxyhydroquinone (2,5-DMHQ), and catechols, such as 4,5-dimethoxycatechols (DMC) (16, 19). No genes encoding cellobiose dehydrogenase are found in the genome of *P. involutus* (20). Although genes encoding putative low-molecular-weight iron-reducing glycopeptides are present in *P. involutus*, they were not upregulated during organic matter degradation (13). Taken together, these findings suggest that secondary metabolites may act

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as the Fe^{3+} reductant that drives the Fenton-based decomposition mechanism in *P. involutus*.

Quinone-based redox cycles occur through the reduction of Fe^{3+} through a one-electron transfer to Fe^{3+} by secreted fungal hydroquinones and/or catechols (16, 19, 21, 22). Based on the Fe^{3+}-reducing activity *in vitro*, Eastwood et al. (23) proposed that variegatic acid is the metabolite driving Fenton chemistry in the brown rot fungus *Serpula lacrymans*, a member of the order Boletales. Variegatic and xerocomic acids are hydroxylated derivatives of pulvinic acid (24). However, results from experiments performed more recently challenge the view that variegatic acid is the key reducing agent driving Fenton chemistry in *S. lacrymans* (25): variegatic acid was not detected in wood undergoing decay. It was demonstrated that the well-characterized Fe^{3+} reductant 2,5-DMHQ was produced by *S. lacrymans* under the growth conditions described.

The aim of this study was to isolate and characterize the iron-reducing compound(s) produced by the ECM fungus *P. involutus* during the decomposition of organic matter. Our results showed that the Fe^{3+}-reducing activity was caused by one major compound that was secreted into the medium during organic matter decomposition. Mass spectrometry and nuclear magnetic resonance (NMR) analyses identified this compound as the secondary metabolite involutin, a diarylcyclopentenone. This pigment was described in 1967 (26), but its biological function has remained unknown. Our study demonstrates that a low-molecular-weight metabolite can function as the Fe^{3+} reductant in an ECM fungus during Fenton-based decomposition of organic matter.

MATERIALS AND METHODS

Reagents. 2,6-Dimethoxyhydroquinone (2,6-DMHQ), ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], and 2-hydroxyterephthalic acid (h-TPA) were acquired from Sigma-Aldrich, Sweden. Disodium terephthalate (TPA) was purchased from Alfa Aesar, Sweden.

Fungal strain and culture conditions. Cultures of *P. involutus* (Batsch) Fr. (strain ATCC 200175) were maintained aseptically on minimum Melin-Norkrans (MMN) medium containing 1.5% agar. MMN medium consists of 2.5 g $liter^{-1}$ D-glucose, 500 mg $liter^{-1}$ KH_2PO_4, 200 mg $liter^{-1}$ NH_4Cl, 150 mg $liter^{-1}$ $MgSO_4 \cdot 7H_2O$, 25 mg $liter^{-1}$ NaCl, 50 mg $liter^{-1}$ $CaCl_2$, 12 mg $liter^{-1}$ $FeCl_3 \cdot 6H_2O$, and 1 mg $liter^{-1}$ thiamine-HCl at pH 4.0. The fungus was grown in petri dishes on a layer of glass beads immersed in liquid medium (13, 27). After 9 days of incubation at 18°C in the dark, the MMN medium was removed with a sterile pipette. The glass beads and the mycelium were washed with 10 ml of sterile Milli-Q (MQ) water, and 10 ml of MMN medium without N was added to induce an N-deprived mycelium (28). After 24 h, the mycelium was again washed in MQ water, and 10 ml of an organic matter extract was added (13).

The organic matter consisted of a maize compost material (designated MH) and was extracted with hot water, as described previously (13). The organic C content of this extract was 225 mg $liter^{-1}$, and the iron concentration was 0.023 mg $liter^{-1}$ (13). Previous experiments showed that the addition of glucose was required for Fenton-based decomposition of organic matter by *P. involutus* (29). Hence, the extracts were supplemented with glucose (final concentration, 2.5 g $liter^{-1}$). The C/N ratio after the addition of glucose was ca 7.5 (13). The extracts were then sterilized by filtration with a 0.2-μm-pore-size filter. The cultures were incubated for 7 days at 18°C in the dark. Samples incubated for 7 days are designated MH7. Organic matter extracts that were not inoculated with fungi are designated MH0. The culture filtrate and mycelium were used for further analyses.

Ferrozine assay. Fe^{3+}-reducing activities were analyzed using the ferrozine assay (30). A 100-μl aliquot of the sample was mixed with 1.0 ml of 0.1 M acetate buffer (pH 4.4), 100 μl of freshly prepared 1.0 mM $FeCl_3$, and 100 μl of 1% (wt/vol) aqueous ferrozine. The reaction mixture was incubated for 5 min. Fe^{3+} reduction was assayed spectrophotometrically at 562 nm. A standard curve was constructed using $FeSO_4$ (0.0 to 3.0 mM).

Extraction of Fe^{3+}-reducing compounds. Fe^{3+}-reducing compounds were extracted from the culture filtrates by adopting a procedure used for isolating such compounds from *S. lacrymans* (23). Briefly, equal volumes of culture filtrate and ethyl acetate (EtOAc) were mixed and vortexed. The EtOAc phase was recovered and was dried under a stream of N_2. The dried EtOAc phase was redissolved in EtOAc. The extract was then adsorbed onto a 10-ml Bond Elut SI solid-phase (SP) fractionation cartridge (Agilent Technologies, Sweden), followed by sequential elution with cyclohexane, EtOAc, and methanol (MeOH). Fractions were designated $SP_{Cyclohex}$ (the fraction resulting from SP extraction with cyclohexane), SP_{EtOAc}, and SP_{MeOH}. Fe^{3+}-reducing activity was measured by using the ferrozine assay at every step of purification.

An intracellular mycelial extract was prepared by sonication followed by centrifugation, as described by Shah et al. (28). The supernatant was analyzed for Fe^{3+}-reducing activity.

Metabolites were also extracted directly from the mycelium according to the procedure of Feling et al. (31). Briefly, mycelia from 20 petri dishes (~0.5 g dry weight) were shaken with a mixture of acetone (0.5 liter), 2 M HCl (5 ml), and ascorbic acid (0.3 g) for 20 h. After filtration, the filtrate was extracted three times using 200 ml of EtOAc each time. The combined EtOAc phase was dried under N_2 and was directly analyzed by mass spectrometry.

Mass spectrometry. To identify secreted fungal metabolites, the MH0 SP_{EtOAc}, MH7 SP_{EtOAc}, and MH7 SP_{MeOH} samples and the EtOAc phase of the MH7 mycelial extract were analyzed by liquid chromatography-mass spectrometry (LC-MS). The instrument used was an Accela high-performance LC (HPLC) system with an Exactive Orbitrap mass spectrometer equipped with a BetaSil C_{18} column (length, 150 mm; inside diameter, 2.1 mm; particle size, 3 μm) (Thermo Fisher Scientific, Germany). Samples were dissolved in 1 ml MeOH and were filtered (0.45-μm polytetrafluoroethylene [PTFE] filter; VWR, Germany). A gradient consisting of 0.1% (vol/vol) formic acid in water (solvent A) and 0.1% (vol/vol) formic acid in acetonitrile (solvent B) was applied at a flow rate of 0.2 ml min^{-1}, with an initial hold for 1 min at 5% solvent B, followed by a linear increase to 98% solvent B within 16 min. These conditions were held for an additional 3 min. High-resolution electrospray ionization MS (HRESIMS) data were acquired in both positive and negative ionization modes. Data were analyzed using Xcalibur software (Thermo Fisher Scientific, USA) and the Reaxys database.

HPLC purification. To purify secreted fungal metabolites, reverse-phase semipreparative HPLC was performed on the MH7 SP_{EtOAc} fraction by using an Agilent 1200 HPLC system (Agilent Technologies, Germany) with a diode array detector and a Zorbax XDB C_{18} column (length, 250 mm; inside diameter, 9.4 mm; particle size, 5 μm). The sample was dissolved in MeOH. An isocratic run was applied using a solvent system with 92% solvent A (0.1% trifluoroacetic acid in water) plus 8% solvent B (acetonitrile) at a flow rate of 1 ml min^{-1}. The chromatograms were recorded at 254, 260, 285, and 295 nm with peak-actuated scanning from 200 to 400 nm. Fractions collected according to peaks were analyzed for Fe^{3+}-reducing activity. Fractions containing Fe^{3+}-reducing activity were pooled, dried in a rotary evaporator, and freeze-dried to remove traces of trifluoroacetic acid. The fractions were analyzed by mass spectrometry and NMR.

NMR analysis. ^1H and ^{13}C NMR spectroscopy of the isolated Fe^{3+}-reducing activity was carried out on a Bruker Avance III instrument (Bruker, USA). The samples were dissolved in CD_3OD (99.8%; Deutero, Germany). Signals were referenced to residual MeOH at 3.31 ppm. Spectra were analyzed using TopSpin software, version 3.1 (Bruker, USA). Involutin ^1H NMR (600 MHz, CD_3OD): δ_H 3.96 (d, ^3J = 6.8 Hz, 1H, 5-H), 4.68 (d, ^3J = 6.8 Hz, 1H, 4-H), 6.5 (dd, ^3J = 8.1 Hz, ^4J = 2.0 Hz, 1H), 6.61 (d, ^4J = 2.0 Hz, 1H), 6.77 (d, ^3J = 9.0 Hz, 2H), 6.72 (d, ^3J = 8.1 Hz,

1H), 7.71 (d, $^3J = 9.0$ Hz, 2H). Involutin ^{13}C NMR (125 MHz, CD_3OD): δ 58.3 (C-5), 72.7 (C-4), 115.6 (CH), 116.1 (CH), 117.9 (CH), 122.8 (CH), 123.8 (C), 128.7 (C), 130.5 (CH), 145.6, 146.1, 157.2 (each C); signals for C-1, C-2, and C-3 not visible. HRESIMS (negative mode): *m/z* found 313.0719; *m/z* calculated 313.0718.

Detection of hydroxyl radicals. TPA was used as a probe for measuring the generation of hydroxyl radicals during the Fenton reaction with involutin. TPA is selective for hydroxyl radicals and produces a fluorescent product, h-TPA, upon oxidation (32, 33). Briefly, a 100-μl aliquot of HPLC-purified involutin (~0.6 mM) was allowed to react with TPA in a mixture containing 2.0 ml of 100 μM TPA, 1.0 ml of 3 μM H_2O_2, and 2.0 ml of 30 μM $FeCl_3$ (in 0.1 M acetate buffer, pH 4.0). The increase in fluorescence intensity due to h-TPA production was measured at an excitation wavelength of 315 nm and an emission wavelength of 425 nm. The reaction mixture was incubated in the dark, and the increase in fluorescence intensity was measured after 0, 1, 2, and 3 h. 2,6-DMHQ (0.6 mM) was used as a positive control for hydroxyl radical production. The 2,6-DMHQ isomer is more stable than 2,5-DMHQ and has similar Fe^{3+}-reducing properties; thus, it is the preferred isomer for this assay (25, 34). An assay mixture without involutin or 2,6-DMHQ was used as a negative control. An h-TPA standard curve (0.0 to 100.0 nM) was used for comparison (see Fig. S1 in the supplemental material).

RESULTS

Purification of secreted Fe^{3+}-reducing compounds. *P. involutus* was grown on a maize compost (MH) substrate. Previous studies using spectroscopic analyses showed that this substrate is decomposed by the fungus using a brown rot mechanism involving Fenton chemistry (13). The production of Fe^{3+}-reducing activity was analyzed both in the mycelium and in the culture filtrate (Fig. 1). At the start of the experiment, trace amounts of Fe^{3+}-reducing activity were detected in the culture filtrate (MH0). After 7 days of incubation, high levels of Fe^{3+}-reducing activity were detected in the culture filtrate (97% of the total activity) but only minute amounts in the mycelium (3% of the total activity). Fe^{3+}-reducing activity was not detected in the mineral nutrient medium (MMN) incubated with the fungus for 7 days.

To purify the Fe^{3+}-reducing activity secreted by *P. involutus* after 7 days of growth in the MH substrate, the activity was extracted with EtOAc, fractionated on solid-phase silica gel cartridges, and purified by HPLC. Approximately 87% of the Fe^{3+}-reducing activity present in the culture filtrate was extracted with EtOAc (Fig. 1). This activity was further separated into two fractions by solid-phase fractionation, 51% in the SP_{EtOAc} fraction and 32% in the SP_{MeOH} fraction. No activity was detected in the $SP_{Cyclohex}$ fraction (Fig. 1).

Identification of involutin. LC-MS analysis of the MH0 SP_{EtOAc}, MH7 SP_{EtOAc}, and MH7 SP_{MeOH} fractions revealed that one major compound was formed during the decomposition of the organic material (Fig. 2). Based on the HPLC and HRESIMS total-ion chromatogram, the signal corresponded to a single compound with *m/z* 313.0719 and thus was tentatively identified as involutin (Fig. 2B, inset). The total ion current profiles for the MH7 SP_{EtOAc} and MH7 SP_{MeOH} fractions were similar except that the peak associated with involutin was considerably lower in the MH7 SP_{MeOH} fraction than in the MH7 SP_{EtOAc} fraction (Fig. 2B and C).

To examine whether involutin was responsible for the Fe^{3+}-reducing activity produced during the decomposition of organic matter, the MH7 SP_{EtOAc} fraction was further analyzed by semipreparative HPLC using an isocratic gradient. Scanning of the chromatograms recorded at different wavelengths revealed that

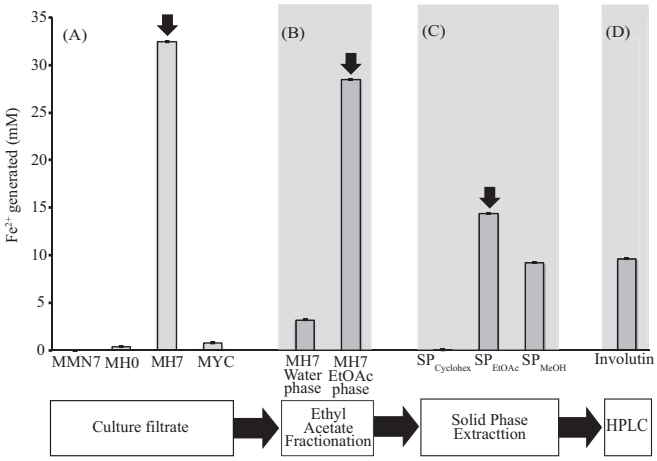

FIG 1 Purification of Fe^{3+}-reducing activity secreted by *P. involutus* during the decomposition of an organic matter (maize compost) extract. Shown are the concentrations of Fe^{2+} generated by the reduction of Fe^{3+} in samples collected at different steps of the purification procedure. The amounts of Fe^{2+} are normalized per milliliter of culture filtrate. Fe^{2+} generated by the mycelium extract is normalized per gram (dry weight) of biomass (1 g biomass is produced through ~40 ml culture extract). Sample details: MMN7, culture filtrate collected after 7 days of growth on a synthetic mineral nutrient medium (MMN) ($n = 3$); MH0, culture filtrate collected before the fungus was added to the maize compost substrate (MH) ($n = 3$); MH7, culture filtrate collected after 7 days of growth on the MH substrate ($n = 3$); MYC, extract of the mycelium grown for 7 days on the MH substrate ($n = 3$). Error bars indicate standard errors. Thick arrows indicate the samples that were transferred to the next purification step. (A) Activities in the crude culture filtrates and the mycelium extract. (B) Ethyl acetate (EtOAc) phase extraction of the MH7 culture filtrate. (C) Solid-phase fractionation of the EtOAc phase eluted with cyclohexane ($SP_{Cyclohex}$), EtOAc (SP_{EtOAc}), or methanol (SP_{MeOH}). (D) HPLC-purified involutin from the MH7 SP_{EtOAc} eluate.

only one peak detected at 254 nm had Fe^{3+}-reducing activity (Fig. 1 and 3A). HRESIMS showed that the peak contained a single compound with an *m/z* of 313.0718 calculated for $C_{17}H_{13}O_6$ (found 313.0719) and UV absorption maxima at 228 nm and 248 nm with the solvent mixture of acetonitrile and water; thus, the compound was tentatively identified as involutin (26, 31). The structure of involutin was finally confirmed by NMR analysis (Fig. 3B) (see Materials and Methods). Purification yielded 0.4 mg of involutin with >90% purity, which corresponds to a titer of ~7.5 μg ml^{-1} of organic matter extract.

Other metabolites. LC-MS analysis also detected traces of involutin in the mycelium grown on the organic matter extract (see Fig. S2A in the supplemental material). Other compounds detected in the mycelial extract were (i) $C_7H_{11}O_5N$, *m/z* 188.0553, and (ii) $C_{10}H_{16}O_3$, *m/z* 183.1024 (see Fig. S2B and C in the supplemental material). These compounds have not been reported previously in *P. involutus*.

The culture filtrate and mycelial extract were searched for the presence of hydroquinones, such as 2,5-DMHQ ($C_8H_{10}O_2$, *m/z* 138.1638), its oxidized form, 2,5-dimethoxybenzoquinone (DMBQ) ($C_8H_8O_4$, *m/z* 168.0423), and catechols, such as DMC ($C_8H_{10}O_4$, *m/z* 170.0579), by using HPLC-HRESIMS data from authentic compounds. These metabolites were not detected in the culture filtrate or in the mycelial extract of *P. involutus* grown in the organic matter. Similar techniques, as described above, were used to search for pulvinic acid-derived metabolites, such as

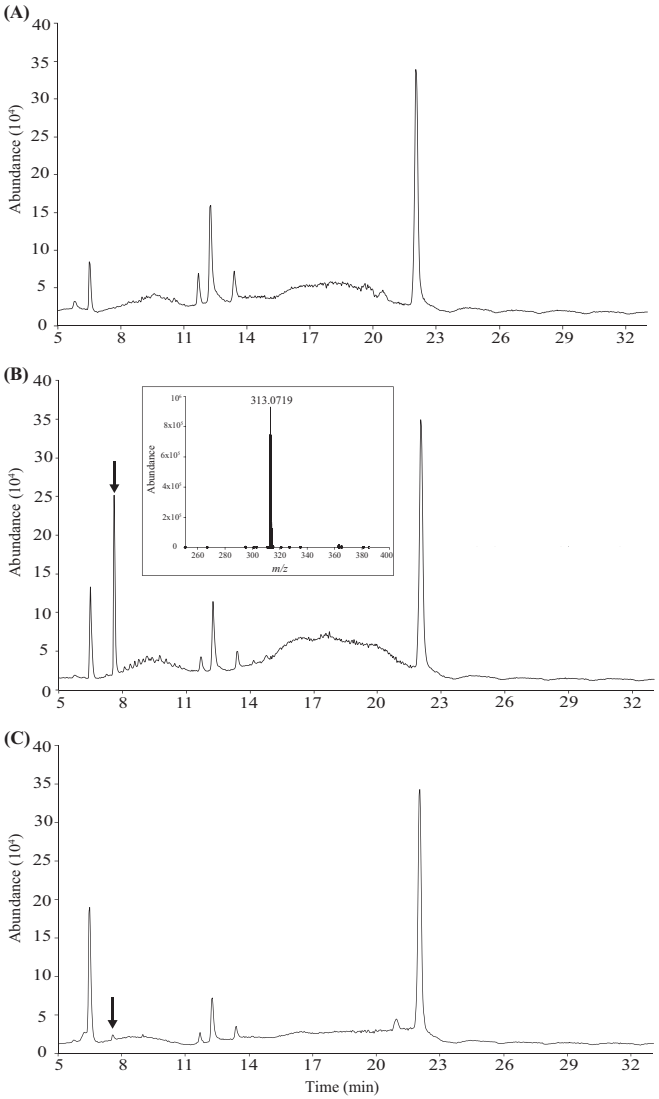

FIG 2 Analysis of secondary metabolites secreted by *P. involutus* during growth on organic matter. Shown are total ion current profiles from the HRESIMS (negative mode) spectra of the MH0 SP_{EtOAc} eluate, recovered from the initial organic material (A), and of the MH7 SP_{EtOAc} (B) and MH7 SP_{MeOH} (C) fractions, sampled after 7 days of growth. Note that the MH7 SP_{EtOAc} sample contains one major peak, indicated by the arrow (involutin), that is not present in the MH0 sample. Involutin is also present in MH7 SP_{MeOH}. (Inset) HRESIMS profile of involutin.

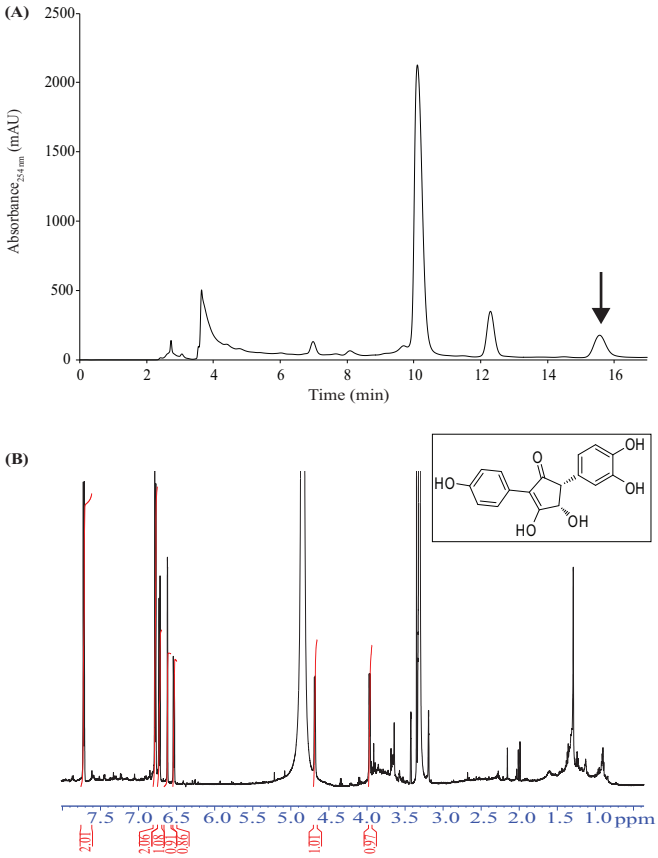

FIG 3 HPLC purification of the Fe^{3+}-reducing compound secreted by the ECM fungus *P. involutus* during the decomposition of organic matter. (A) Chromatogram of the MH7 SP_{EtOAc} eluate (see Fig. 1) separated by isocratic reverse-phase chromatography. Fractions were collected according to peaks (λ = 254 nm). Fe^{3+}-reducing activity was detected in one peak, indicated by the arrow, and the compound was identified as involutin by HRESIMS (see Fig. 2 inset). (B) ^{1}H NMR spectrum of HPLC-purified involutin (>90% pure). The red lines/numbers represent the integrator trace. (Inset) Chemical structure of involutin.

variegatic and xerocomic acids; however, these compounds were not detected.

Ability of involutin to generate hydroxyl radicals. To examine whether involutin may play a role in the production of hydroxyl radicals during organic matter decomposition by *P. involutus*, we analyzed the ability of purified involutin to drive Fenton chemistry *in vitro* via Fe^{3+} reduction. In the presence of involutin, $FeCl_3$, and H_2O_2, significant amounts of h-TPA were produced, suggesting the production of hydroxyl radicals (Fig. 4). Under these conditions, the generation of h-TPA by involutin was similar to that of the hydroquinone 2,6-DMHQ. In the absence of H_2O_2, no hydroxyl radicals were formed in the presence of either involutin or 2,6-DMHQ. It has been shown previously that hydroxyl radical production requires the reduction of Fe^{3+} in the presence of H_2O_2 (33). Thus, in our experimental system, the metabolite involutin drives a Fenton reaction via Fe^{3+} reduction.

DISCUSSION

Ectomycorrhizal fungi have evolved from saprotrophic ancestors multiple times (20). During evolution, ECM fungi have lost many of the genes encoding plant cell wall-degrading enzymes, suggesting a reduced decay capacity. Nevertheless, we recently showed that the ECM fungus *P. involutus* decomposes lignocellulosic material during the assimilation of organic N from organic matter extracted from forest soil or maize compost (13). Data from spectroscopic analyses and transcriptome profiling suggested the involvement of a Fenton-based oxidation mechanism similar to that used by brown rot fungi for decaying lignin in wood tissues (13). In the present study, the oxidation reaction was further examined by characterizing the mechanism involved in the production of the Fe^{2+} required for the Fenton reaction.

In agreement with the findings for several brown rot wood decayers (16, 19, 21–23, 25), we showed that in *P. involutus*, the Fe^{2+} involved in Fenton chemistry is formed by the activity of a

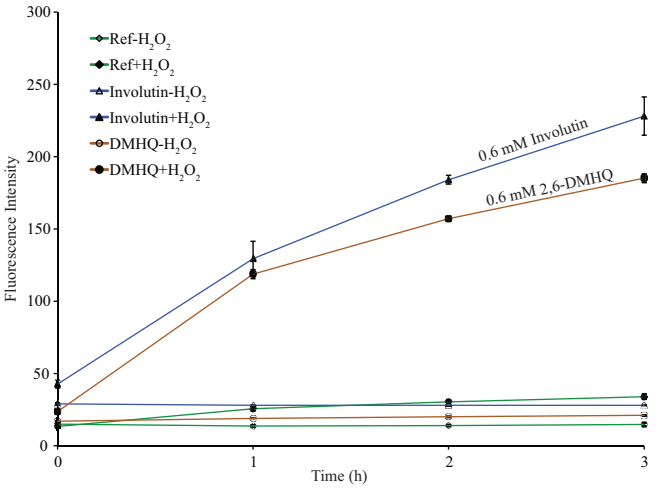

FIG 4 *In vitro* Fenton reaction with involutin. Shown is the generation of h-TPA, an indicator of hydroxyl radical production, by involutin and 2,6-DMHQ, as measured by fluorescence spectroscopy at an excitation wavelength of 315 nm and an emission wavelength of 425 nm. Error bars indicate standard errors ($n = 3$).

low-molecular-weight Fe^{3+} reductant. Four observations support the conclusion that this reductant is the previously known pigment involutin (26, 31). First, the Fe^{3+}-reducing activity that was secreted into the medium during organic matter decomposition was due largely to a single compound. The compound was identified by mass spectrometry and NMR as involutin. Second, the production of the Fe^{3+}-reducing activity was significantly enhanced during the decomposition of the organic matter extract. In contrast, only trace amounts of extracellular Fe^{3+}-reducing activity were detected when the fungus was grown on a mineral nutrient medium. Third, a large part of the involutin that was synthesized during organic matter decomposition was secreted into the medium. Fourth, a purified fraction of involutin had the ability to drive a Fenton reaction in the presence of H_2O_2 *in vitro*.

The order Boletales is one of the major groups of the Agaricomycetes, containing ca. 1,300 described species (35). The ancestor of the Boletales was likely a brown rot saprotroph; however, lineages with ECM species have evolved several times within the order (36). Fungi within the Boletales have been extensively examined for secondary metabolites (24, 37). So far, involutin has been detected from the fruiting bodies in only three species: *P. involutus*, *Gyrodon lividus*, and *Melanogaster broomeianus* (24, 38). These species belong to the family Paxillaceae, which was recently characterized as a well-resolved lineage of the suborder Boletineae (39). At present, only ECM species are known in Paxillaceae. Involutin is probably synthesized from the terphenylquinone atromentin, which is a key intermediate in the biosynthetic pathways of many basidiomycete secondary metabolites (24, 40). Among them are the pulvinic acid-derived pigments variegatic acid and xerocomic acid, which are commonly found in species of the Boletales but not in species of the Paxillaceae (24, 37). The lack of these pigments in *P. involutus* was confirmed in this study. These observations suggest that the gain of a biosynthetic pathway for involutin in the family Paxillaceae has been accompanied by a loss of the pathways leading to variegatic acid and xerocomic acid.

The ecological function of the terphenylquinones and related derivatives is not well known. It has been proposed that variegatic acid can function as a Fe^{3+} reductant during Fenton-based wood decay in the bolete *S. lacrymans* (23). A more recent experiment has provided evidence that the hydroquinone 2,5-DMHQ is a primary Fe^{3+}-reducing agent expressed by *S. lacrymans* during brown rot decay of aspen wood (25). There are also studies showing that 2,5-DMHQ is the reductant involved in Fenton-based wood decay in species from two other divergent agaricomycete lineages, the Gloeophyllales and the Polyporales (41, 42). Although the biosynthetic pathway for 2,5-DMHQ is not known, the data imply that this pathway was present in the ancestor of the Agaricomycetes (25). Neither 2,5-DMHQ nor the more stable isomer 2,5-DMBQ was detected in the mycelium or the culture filtrates during organic matter decomposition by *P. involutus*. Since studies of brown rot fungi have shown that the production of iron-reducing compounds and iron chelators can be affected by the composition of the medium, including the concentrations of C, N, and iron (43, 44), the possibility that *P. involutus* has the capacity to synthesize other Fe^{3+}-reducing metabolites and iron chelators in addition to involutin cannot be ruled out. However, considering the fact that *P. involutus* was grown under conditions promoting oxidative decomposition, our results suggest that involutin is a key Fe^{3+} reductant secreted during Fenton-based decomposition of organic matter by *P. involutus*.

It has been shown that in the brown rot wood-decaying system, 2,5-DMHQ has a dual role and drives Fenton chemistry by reducing both Fe^{3+} and O_2 (to produce H_2O_2), generating hydroxyl radicals (16). In our experiments, involutin produces hydroxyl radicals only in the presence of H_2O_2, suggesting that involutin functions as a Fe^{3+} reductant. H_2O_2 can also be generated by the activity of a number of oxidases, including laccases, glucose-methanol-choline (GMC) oxidoreductases, and copper radical oxidases, and genes encoding such enzymes are expressed by *P. involutus* during the decomposition of organic matter (13).

The formation of the Fe^{2+} that is involved in Fenton-based decomposition of lignocellulose has been extensively studied in brown rot wood-decaying fungi (45). Apart from a mechanism to reduce Fe^{3+} to Fe^{2+}, an additional mechanism is needed for solubilizing the iron from the insoluble Fe (oxyhydr)oxide complexes found in plant tissues. Brown rot fungi are thought to retrieve such iron by secreting oxalic acid, which binds to and solubilizes Fe^{3+} from Fe (oxyhydr)oxide complexes. The oxalate tightly chelates Fe^{3+}, making it unreactive with hydroquinones (46). Studies with *Postia placenta* have shown that the hydroquinone 2,5-DMHQ can be oxidized to a semiquinone by the activity of an extracellular laccase and that this reaction could generate a complete Fenton reaction system (42). The mycelia of ECM fungi are present mainly in the humus-rich region of soil horizons, where Fe^{3+} is found in iron oxide crystals with very low solubility, such as hematite, goethite, and ferrihydrite (47). Fe^{3+} is solubilized from such minerals only when it is reduced to Fe^{2+} or when it forms complexes with ligands such as citrate or humic acids (47). It remains to be demonstrated whether involutin is capable of releasing and reducing Fe^{3+} from these solid phases or whether cooperative processes, including the action of other metabolites and enzymes, such as laccases, are involved.

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