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Hydrogen ions and organic acids secreted by ectomycorrhizal fungi, *Pisolithus* sp1, are involved in the efficient removal of hexavalent chromium from waste water

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ABSTRACT

Pisolithus sp1 is an ectomycorrhizal (ECM) fungi that was chosen during a screening test of six strains of ECM fungi due to its ability to tolerate and remove hexavalent chromium (Cr(VI)). The physiological responses of *Pisolithus* sp1 to Cr(VI) exposure, the relationship between *Pisolithus* sp1 and exogenously added organic acids (EAOAs) or Na_3VO_4 (H^+-ATPase inhibitor) and the ability of *Pisolithus* sp1 to reduce Cr(VI) in liquid culture were also investigated. Hydrogen ions (H^+), which were produced directly by *Pisolithus* sp1, reduced the pH of the medium and played an important role in Cr(VI) reduction; however, Na_3VO_4 significantly inhibited this process and resulted in a decrease in the Cr(VI) reduction rates. Organic acids were secreted after the reduction in Cr(VI) by *Pisolithus* sp1, and EAOAs did not significantly affect Cr(VI) reduction; those results revealed the secondary role of organic acids in Cr(VI) reduction. The Cr(VI) removal rate of *Pisolithus* sp1 approached 99% after Cr(VI) treatment for 12 days. Overall, 75% of the Cr(VI) removal was due to extracellular reduction and 24% was due to adsorption. The results of this study provide a strong basis for using Cr(VI)-tolerant and Cr(VI)-reducing fungi, as well as ectomycorrhiza, in the remediation of Cr(VI)-contaminated sites.

1. Introduction

Chromium (Cr) is a poisonous and harmful environmental pollutant. The intensification of industrial activities and urbanisation has led to Cr pollution in the ecosystem (Gil-Cardeza et al., 2014; Shanker et al., 2005). Hexavalent chromium (Cr(VI)) is the longest-lasting form of Cr in the soil, and it acts as a strong poison (Gill et al., 2015). Cr (VI) can cause cell mutation and carcinogenesis, induce DNA damage and affect gene expression, which is of concern in Cr(VI)-contaminated areas (Das et al., 2014). Traditional methods for removing Cr(VI) from the environment include ion exchange, chemical precipitation, evaporation and membrane separation. However, these methods are costly and produce secondary contamination (Bertagnolli et al., 2014; De Sotto et al., 2015).

Bioremediation of Cr(VI) pollution is an economic and environment-friendly method (Bennett et al., 2013). Many types of mechanisms for Cr(VI) tolerance or detoxification have been reported in bacteria. For example, Cr(VI) can be reduced to Cr(III) by bacteria such as *Staphylococcus*, *Escherichia*, *Achromobacter*, *Pantoea*, *Cellulomonas* and *Micrococcus*, which isolate Cr from polluted (Das et al., 2014). *Mycobacterium* sp, *Bacillus firmus* and *Klebsiella pneumoniae* have the ability to absorb Cr(VI) (Masood and Malik, 2011).

Few studies have investigated mechanisms of reducing Cr(VI) by fungi. Carbon metabolism can induce some filamentous fungi to reduce Cr(VI) to Cr(III), such as *Trichoderma inhamatum* and *Penicillium* spp (Acevedo-Aguilar et al., 2006; Morales-Barrera and Cristiani-Urbina, 2008). Mechanisms of Cr(VI) reduction mediated by cell wall binding characteristics have been explored in *Aspergillus flavus* and *Aspergillus niger* (Masood and Malik, 2011). Some arbuscular mycorrhizal fungi can also reduce Cr(VI) (Juwarkar and Jambhulkar, 2008; Singh et al., 2014). Fungi have high biomass and are easy to produce, making them good candidates for the removal of heavy metals (Park et al., 2005; De Sotto et al., 2015). However, the physiological mechanisms of Cr(VI) reduction by ectomycorrhizal (ECM) fungi require further investigation.

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Table 1
Source and information of ectomycorrhizal fungal strains in this study.

Strains	Sites	Isolation source	Latitude and Longitude	Sequence ID
Cenococcum geophilum	Mountain Sanqing	Mycorrhizal root tips	29.03N 118.26E	KY075873
Pisolithus. sp1	Mountain Sanqing	Sporocarps	28.54N 118.03E	KY075875
Laccaria. sp	Mountain Sanqing	Sporocarps	28.53N 118.32E	KY075876
Pisolithus. sp2	Mountain Tang	Sporocarps	32.07N 119.50E	KY075877
Laccaria amethystina	Mountain Sanqing	Sporocarps	28.55N 118.09E	KY075878
Hebeloma vinosophyllum	Mountain Sanqing	Sporocarps	28.54N 118.03E	KY075879

The direct mechanisms of Cr(VI) reduction include: (1) reduction by soluble chromate reductase under aerobic conditions, which requires NADH/NADPH as cofactors, and (2) as an electron acceptor that performs reduction under anaerobic conditions (Viti et al., 2014). The first chromate reductase that was discovered, a membrane-associated enzyme, came from *Enterobacter cloacae* HO1 (Ohtake et al., 1990). The Cr (VI) reductases NfsA/NfsB and the ferric reductases FerB and NemA have been identified in *Vibrio harveyi*, *Pseudomonas denitrificans* and *Escherichia coli*, respectively. (Mazoch et al., 2004; Robins et al., 2013).

The proton pump is an important part of the plasma membrane in the cytoplasm of eukaryotic cells, and functions in the growth and development of eukaryotic cells (Kühlbrandt, 2004). One function of the proton pump is for hydrolysis to drive the transport of solutes, which results in a pH and H^+ transmembrane potential gradient (Sondergaard et al., 2004). The transmembrane transport of various ions and nutrients driven by an electrochemical gradient occurs during this process (Alves et al., 2003). Another function of the proton pump is to promote cell growth and regulate intracellular pH. On the plasma membrane, transmembrane transport of H^+–ATPase to protons leads to H^+ secretion and the acidification of the cytosol, which promotes the release of protons (Alves et al., 2003).

By nonspecific reactions associated with organic acids, amino acids or glutathione, Cr(VI) can also be reduced indirectly (Laborda et al., 2007; Nancharaiah et al., 2015; Robins et al., 2013;). Oxalic acid (OA) and citric acid (CA), which have α-OH groups, can transform Cr(VI) into Cr(III) in soils (Hug and Laubscher, 1997; Tian et al., 2010). However, few studies have focused on this mechanism in ECM fungi. In addition, plant-associated microbes can secrete low-molecular-weight organic acids (LMWOAs), which may play a role in mineral nutrient mobilisation and the solubility of heavy metals in the rhizosphere (Rajkumar et al., 2012), as well as nutrient supplementation, mineral weathering and heavy metal detoxification (Ma et al., 2016). Chen et al. (2014) found that *Pseudomonas* sp. Lk9 improved the metal availability of iron (Fe) and phosphorus (Pi) in the soil by promoting LMWOA secretion in the host plant, and by significantly enhancing zinc (Zn), copper (Cu) and cadmium (Cd) accumulation in *Solanum nigrum*. Additionally, OA exuded by ECM fungi may change the bioavailability of toxic heavy metals in the rhizosphere (Bellion et al., 2006; Colpaert et al., 2011).

Heavy metal tolerance is critical for metal accumulation in plants and the phytoremediation that is assisted by microorganisms (Ma et al., 2016). LMWOAs can be used to reduce heavy metals in plants, such as As, Pb, Cd, and Cr, by chelation in the rhizosphere (Magdziak et al., 2011). ECM fungi can also secrete organic acids to chelate toxic metal ions, which act by detaching the heavy metal from soil, thereby reducing toxicity to the host (Rajkumar et al., 2012; Targhetta et al., 2013). Ray and Adholeya (2009) reported a correlation between organic acid exudation and metal uptake by ECM fungi grown on pond ash *in vitro*.

To date, research has focused on the effect of Cr on ECM fungi growth (Raman et al., 2002) or on the tolerance of ECM fungi and plants to toxic levels of Cr (Magdziak et al., 2011). However, H^+ and LMWOA production by ECM fungi in response to Cr(VI) stress has not been reported. Therefore, (1) to verify the H^+ and organic acids involved in the response of *Pisolithus* sp1 following exposure to Cr(VI) stress, and (2) to reveal the physiological mechanism of Cr(VI) removal by *Pisolithus* sp1, we investigated the effects of EAOAs and H^+-ATPase inhibitors on Cr(VI) removal by *Pisolithus* sp1 in liquid culture.

2. Materials and methods

2.1. Field sampling

Six ECM fungi isolates—*Cenococcum geophilum* (KY075873), *Laccaria amethystea* (KY075878), *Laccaria* sp (KY075876), *Hebeloma vinosophyllum* (KY075879), *Pisolithus* sp1 (KY075875) and *Pisolithus* sp2 (KY075877)—were obtained from Sanqing Mountain, Jiangxi Province, and Tang Mountain, Jiangsu Province, China (Table 1). The methods of soil samples collection were refered to Wen et al. (2018) and the molecular identification of fungal species in the established pure culture isolates were refered to Koizumi et al. (2018), respectively. The detailed methods of strains isolation and purification as follows:

2.2. Pure culture isolation of sporocarps and root-colonising fungi

Five ECM fungi (*Pisolithus*. sp1, *Pisolithus*. sp2, *Laccaria*. sp, *Laccaria amethystine* and *Hebeloma vinosophyllum*) were isolated from sporocarps and the surfaces of well-preserved fresh sporocarps were cleaned using cotton moistened with 70% ethanol. The inner tissues were removed in 5 × 5 mm pieces with a blade and were inoculated onto Kottke agar media (Kottke et al., 1987). Fungus-inoculated agar plate media were incubated at 25 ± 2 °C for 2 months (Endo et al., 2013). *Cenococcum geophilum* was isolated from root tips, and to isolate root-inhabiting fungal strains into pure culture, roots were first washed with water, sterilised in 35% hydrogen peroxide for 30 s, and rinsed three times in sterile deionised water. Root fragments of approximately 1 cm were incubated on Kottke medium. Ampicillin (100 mg/L) and chloramphenicol (100 mg/L) were added to the media to avoid bacterial contaminations. Dishes were incubated at 25 °C in the dark from 1 week up to 3 months (Lacercat-Didier et al., 2016).

Whether it were sporocarps samples or root tip samples, when the desired mycelial growth was observed on a given agar plate, it was subcultured to another fresh Kottke agar plate to establish an isolate by cutting out a 5 × 5-mm mycelial plug. Established pure culture isolates (Table 1) were subcultured once on Kottke agar plates, stored on Kottke agar as slant cultures at 4 °C in a refrigerator and used for the molecular identification (Endo et al., 2013).

2.3. Screening of Cr(VI)-tolerant strains

The six ECM fungi strains were treated with 0, 2, 5, 10 and 50 mg/L Cr(VI), with three replicates per concentration. Agar plugs (6.5 mm in diameter) from actively growing fungal colonies on Kottke agar (without Cr) were grown in a 25 °C incubator in the dark and used as inocula after 25 days culture. The medium was adjusted to pH 5.5 and autoclaved for 20 min at 121 °C. Mycelial growth on the agar and the diameter of strains were recorded using the crossroads method, and the concentrations of Cr that gave a half-maximal response (EC_{50}) in Kottke medium were calculated every three days.

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