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Original Research

Different Responses of Two Ectomycorrhizal Fungi to Cd Treatment

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Abstract

Ectomycorrhizal fungi can absorb heavy metals and allow host plants to develop resistance to heavy metal stress, but their heavy metal tolerance is not fully understood. In this study, the ability of *Suillus bovinus* (Sb) and *Suillus placidus* (Sp) to tolerate cadmium (Cd) was evaluated. Sb and Sp were treated with different Cd concentrations (0 (control), 0.2, 0.4 and 0.8 mg/L) under pure culture conditions, and the biomass, osmotic adjustment substance contents (soluble sugar and soluble protein), antioxidant enzyme activities (superoxide dismutase (SOD) and catalase (CAT)), pH and conductivity were examined. Sb biomass, soluble protein content and SOD activity first increased (0.2 mg/L and 0.4 mg/L Cd) and then decreased (0.8 mg/L Cd). Sp showed a similar change rule but a smaller range. Cd treatment increased the CAT activity of Sb and Sp but significantly reduced soluble sugars in Sb mycelium, whereas 0.2 mg/L Cd significantly increased soluble sugars in Sp mycelium. The pH and conductivity in the medium of Sb treated with different Cd concentrations showed more significant changes than those of Sp. The pH of Sp at 0 mg/L Cd and conductivity at 0.8 mg/L Cd were significantly lower than those of Sb. Correlation analysis suggested that Cd affected Sb more than Sp. In general, Cd treatment had less effect on the indexes of Sp, and Sp tolerated Cd better than Sb.

Keywords: ECMF, heavy metal, pure culture, tolerance

Introduction

With the rapid development of modern industry, heavy metal pollution in soil has become increasingly prominent worldwide [1]. As one of the most common heavy metal pollutants [2], cadmium (Cd) has polluted most provinces in China [3]. It has been reported that Cd contamination exceeds the standard by 7.0%, ranking first among all inorganic pollutants [4]. This greatly threatens the balance of the ecological system and harms human health [5]. In this context, remediating soil contaminated by heavy metals (especially Cd) is of prime importance.

Physical remediation, chemical remediation and bioremediation are commonly used soil pollution control methods [6]. Among them, bioremediation technologies, including phytoremediation and microbial remediation, have received great attention due to their good ecological effects and economic development value [7]. Ectomycorrhizal fungi (ECMF) are widely distributed in soil and can coexist with most trees [8]. Numerous studies have shown that ECMF can improve the growth

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and heavy metal resistance of plants on heavy metal-contaminated soil [9-11], which is conducive to the sustainable development of phytoremediation of heavy metal-contaminated soil. Furthermore, ECMF can accumulate heavy metals by themselves [12]. Several Cd-tolerant fungi have been identified from the soil, and they had excellent Cd adsorption capacity while maintaining their own growth [13-15], which reflects their tolerance to Cd and helps to reduce heavy metal concentrations in the soil. Hence, ECMF have great potential to remediate heavy metal-contaminated sites, and screening of heavy metal-tolerant strains is vital for their application to heavy metal-contaminated environments.

Suillus bovinus (Sb) and *Suillus placidus* (Sp) are typical ECMF with excellent stress resistance. Studies have reported that Sb significantly increased the secretion of phenolic acids and organic acids under the combined stresses of low phosphate and acidic aluminium [16] and enhanced the adaptability of *Pinus massoniana* to low phosphorus by increasing the antioxidant enzyme activity and potassium content in leaves [17]. Moreover, Li et al., (2021) found that Sp could remove reactive oxygen species in *Pinus massoniana* seedlings by improving peroxidase activities and osmotic adjustment ability after inoculation [18]. These results show that Sb and Sp can help themselves or the host resist adverse environments by promoting their own or host physiological and biochemical reactions. However, little is known about the tolerance of Sb and Sp to various concentrations of Cd and how Sb and Sp respond to Cd stress physiologically and biochemically. In previous studies, the increase in the activities of antioxidant enzymes [19-21] and the production of organic acids and H^+ [22-24] were frequently reported as mechanisms by which fungi resist heavy metal stress. In addition, osmotic adjustment substances are secreted to chelate heavy metal ions and maintain osmotic balance in cells [25].

In view of the above situation, the purpose of this study was to (i) determine the fungal biomass, osmotic adjustment substance contents (soluble sugar and soluble protein) and antioxidant enzyme (superoxide dismutase (SOD) and catalase (CAT) activities as well as the pH and conductivity in the liquid medium; (ii) reveal the abilities of Sb and Sp to tolerate different concentrations of Cd. The results of this experiment can provide a theoretical basis for the further use of these two fungi to treat contaminated soils.

Materials and Methods

Experimental Materials and Stress Treatments

The two ECMF, Sb (GenBank ID: MT994628) and Sp (GenBank ID: MT994624), were provided by the Institute for Forest Resources and Environment of Guizhou Province, Guizhou University. They were first subcultured on modified Melin-Norkrans (MMN) agar medium for 20 days in the dark at 25ºC [16], with twenty replicates of each strain. Then, three agar mycelia discs from actively growing fungal colonies were cut with a sterile puncher (ϕ = 5 mm) and inoculated into 500 mL flasks filled with 200 mL of MMN liquid medium (without agar) at a pH of 7.2 and a conductivity of 1.29. Each strain was cultured in thirty flasks, and the cultures were stored under dark conditions at 25ºC and agitated at 150 r/min.

Cd stress treatments started 7 days later [26]. Fungi with consistent growth and no contamination were selected for exposure to different Cd concentrations for 45 days. First, 1 g/L $CdCl_2 \cdot 2.5\ H_2O$ (analytical grade) solution was obtained by mixing 2.04 g of $CdCl_2 \cdot 2.5\ H_2O$ with 1 L of distilled water. Subsequently, 40 ml, 80 ml and 160 ml of 1 g/L $CdCl_2 \cdot 2.5\ H_2O$ solution were added to the above flasks containing 200 ml of culture medium. That is, metal was introduced into the medium as $CdCl_2 \cdot 2.5\ H_2O$ (analytical grade) solutions at concentrations of 0.2, 0.4, and 0.8 mg/L. In the control medium, no Cd was added (0 mg/L). Three replicates of each strain were conducted for each treatment.

Determination Method

After 45 d of Cd treatment (25ºC and 150 rpm), mycelia were separated from the liquid medium, washed five times with distilled water to remove the culture medium, and then divided into two parts. One was dried at 80ºC until the absolute dry weight for determining the biomass of mycelium [23]. The other was stored at −80ºC for determining antioxidant enzyme activities and the contents of soluble sugars and soluble proteins in the mycelia of Sb and Sp. After the mycelium was filtered, the acidity and electrical conductivity of the culture fluid were determined with a pH meter (pHS-3C) and a conductivity meter (DDS-307), respectively.

Fresh hyphal material (0.5 g) was ground in liquid N2 and mixed with extraction buffer (5 mL of 0.05 mmol/L phosphoric acid buffer solution, pH 7.8, 0.2% (w/v) polyvinylpolypyrrolidone and 5 mmol/L ethylene diamine tetraacetic acid). The mixture was centrifuged at 10,000 rpm for 20 min at 4ºC; subsequently, the contents of soluble sugars and soluble proteins were measured using anthrone colorimetry and Coomassie brilliant blue G-250 [27]. The activities of SOD and CAT in the supernatant were measured by nitroblue tetrazolium and guaiacol [20], respectively. The contents of soluble sugars and soluble protein were expressed as mg/g FW, and the activities of SOD and CAT were expressed as U/g FW.

Statistical Analysis

Data were processed using Microsoft Excel 2019. Furthermore, data analysis was carried out in SPSS 26.0. The charts were presented using Origin 2021. The normality test was performed; then, two-way analysis of variance (two-way ANOVA) (multiple comparisons with the least significant difference (LSD) test, *P<0.05*) and principal component analysis were conducted using SPSS 26.0. Moreover, correlation analysis was performed using the corrplot package of the R4.2.3.

Results and Discussion

Effects of Cd on the Biomass and pH of the Two ECMF

The Biomass

The biomass of Sb and Sp treated with the same concentration of Cd was significantly different between except at 0.2 mg/L Cd (Fig. 1). Under 0.4 mg/L and 0.8 mg/L Cd, the biomass of Sp was significantly higher than that of Sb (*P<0.05*). As the Cd concentration gradually increased, the biomass of Sb and Sp showed a "rising-falling" trend. Maximum values of 0.21 g and 0.23 g were observed after exposure to 0.4 mg/L, respectively, and these values were significantly higher than that of the control group (0 mg/L Cd treatment) by 25.18% and 128.36% (*P<0.05*). In contrast, the biomass of Sb was significantly lower than that of the control by 53.88% (*P<0.05*) under the 0.8 mg/L Cd treatment, while the biomass of Sp was still higher by 7.56% than that of the control.

Under heavy metal stress, the biomass of fungi can reflect the degree of heavy metal damage. The greater the biomass of fungi, the stronger the tolerance to heavy metals [12]. In this study, 0.2 mg/L and 0.4 mg/L Cd treatments significantly stimulated the biomass of Sb and Sp (*P<0.05*). However, it was found that the biomass of fungi was inhibited by Cd in the culture medium [4, 28]. We also found that high Cd concentration treatment (0.8 mg/L) had a significant inhibitory effect on the biomass of Sb (*P<0.05*), but had no significant effect on the biomass of Sp. This is related to the different tolerance thresholds of different fungi to Cd. When the concentration of heavy metals reaches the tolerance threshold of fungi, it will inhibit their normal growth [29]. Li et al., (2017) found that low concentration of Cd had no significant effect on the biomass of *Pleurotus ostreatus*, while high concentration of Cd significantly inhibited its biomass (*P<0.05*) [26]. Shi et al., (2022) found that Cd-tolerant strains have higher biomass than Cd-sensitive strains [30]. It indicated that both Sb and Sp could tolerate 0.2 mg/L and 0.4 mg/L Cd, which might act as a promoter to activate cytokinin synthase [31] to stimulate the growth of the two fungi. However, 0.8 mg/L Cd had a certain degree of toxicity to Sb, resulting in a significant decrease in its biomass (*P<0.05*), while Sp had a strong tolerance to Cd, so it had no significant effect on its biomass. In the future, the effect of higher concentrations of Cd on Sp can be explored to apply it to the treatment of heavy metal contaminated soil.

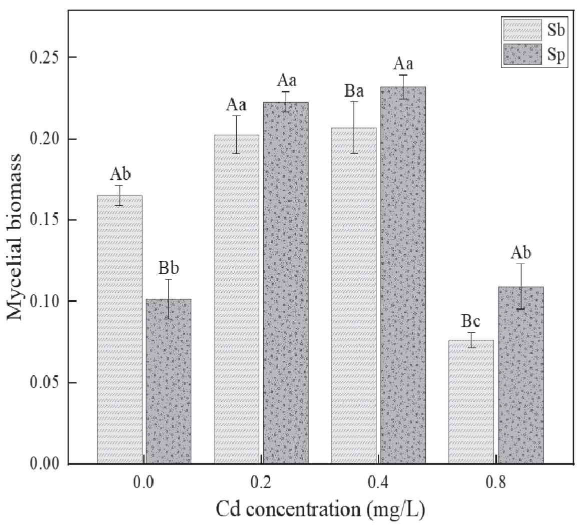

Fig. 1. The mycelial biomass of Sb and Sp under different Cd concentrations. Note: Different lowercase letters indicate a significant difference between the same strain under different Cd concentrations (*P<0.05*), while different uppercase letters indicate a significant difference between the two strains under the same Cd concentration (*P<0.05*). All values are expressed as the means ± standard deviations, the same as below.

pH

As shown in Fig. 4a), the pH of Sb did not change significantly at 0.2 mg/L Cd (*P>0.05*). Under 0.4 mg/L and 0.8 mg/L Cd, the pH of Sb was significantly lower than that of the control group, and reached the minimum value at 0.8 mg/L (pH = 4.26), which was 28.19% lower than that of the control group (*P<0.05*). At 0.2 mg/L Cd, the pH of Sp was significantly higher than that of the control group (*P<0.05*). At 0.4 mg/L and 0.8 mg/L Cd, the pH of Sp did not change significantly. There was no significant difference in pH between Sp and Sb under 0.8 mg/L Cd (*P>0.05*). However, the pH of Sp was significantly lower than that of Sb (*P<0.05*) at 0 mg/L, 0.2 mg/L and 0.4 mg/L Cd, especially at 0 mg/L Cd, the pH of Sp was lower than that of Sb 29.17%, indicating that the pH of Sp was less affected by Cd than Sb.

Fungi can secrete organic acids into the surrounding environment, which is more obvious under heavy metal stress [32]. Organic acids play an important role in the change of pH. It was found that the pH in the culture medium of *Aspergillus* sp. under copper stress was significantly negatively correlated with the content of oxalic acid and succinic acid (*P<0.001*) [6]. The organic acid secretion ability of different fungi is quite different. In this study, the organic acid secretion ability of Sp is stronger than that of Sb, which leads to the pH of Sp in the control group is significantly lower than that of Sb. The pH of Sb showed a decreasing trend with the increase of Cd concentration, which is consistent with the results of Wang et al., (2020) [33]. Further analysis showed that the pH of Sp increased significantly only at 0.2 mg/L Cd, and the pH was small and relatively stable at other Cd concentrations, indicating that Sp secreted more organic acids under 0.4 mg/L and 0.8 mg/L Cd. There was no significant difference in pH between Sp and Sb at 0.8 mg/L Cd. However, under 0 mg/L, 0.2 mg/L and 0.4 mg/L Cd, the pH of Sp was significantly lower than that of Sb (*P<0.05*). It is further proved that Sp itself can secrete a large number of organic acids and reduce the Cd stress. Therefore, Cd has a weak stimulating effect on the secretion of organic acids of Sp. However, the acid production capacity of Sb itself is weaker than that of Sp. Only when subjected to high concentration of Cd stress (0.8 mg/L), Sb will secrete organic acids equivalent to the level of Sp to chelate Cd [34], which is beneficial to improve the tolerance of Sb to Cd. It was also found that the pH value of *S. capillatus* with strong tolerance was significantly lower than that of *C. graminis* under 0-600 mg/L lead treatment, but the organic acid content showed the opposite change [35]. Therefore, the ability of organic acid secretion is closely related to the heavy metal tolerance of fungi. Combined with the biomass changes of Sb and Sp, it is further explained that Sp has stronger Cd tolerance. Based on this, it is recommended to continue to study the differences in organic acid secretion of two fungi under Cd stress, which is beneficial to their prOsmotic and oxidative regulation of the two ECMF upon exposure to Cd stress, which is beneficial to their practical application.

Osmotic and Oxidative Regulation of the Two ECMF upon Exposure to Cd

As shown in Fig. 4b), the conductivity of Sb under 0.2 mg/L and 0.4 mg/L Cd treatment was lower than that of the control group; when the Cd concentration increased to 0.8 mg/L, the conductivity of Sb was significantly higher than that of the control group by 6.75% (*P<0.05*). The conductivity of Sp showed a downward trend as a whole, and the minimum value of 1.54 was obtained under 0.4 mg/L Cd treatment, which was significantly lower than that of the control group by 3.75% (*P<0.05*). Among them, there was no significant difference in the electrical conductivity of Sp and Sb when treated with 0 mg/L and 0.4 mg/L Cd. However, when treated with 0.2 mg/L and 0.8 mg/L Cd, the difference between the two was significant. In particular, the conductivity of Sb was significantly higher than that of Sp 12.26% at 0.8 mg/L Cd treatment.

Electrical conductivity is one of the indicators reflecting the degree of stress on plants [36]. Pei et al., (2020) have shown that the leaf conductivity of ‘Fengdan’ peony continues to rise with the increase of stress time and stress degree [37], indicating that stress has caused damage to its cell membrane, and the stronger the stress, the greater the damage. In this study, the conductivity of Sb was significantly increased at 0.8 mg/L Cd (*P<0.05*), which was consistent with the results of Lin et al., (2010) [38], indicating that the cells were damaged at 0.8 mg/L Cd, which led to the outflow of intracellular substances, resulting in an increase in conductivity. However, the conductivity of Sp remained relatively stable and did not increase due to the addition of Cd. It further shows that Sp has strong tolerance to Cd and is less stressed by Cd.

Soluble Sugars and Soluble Proteins Levels after Cd Treatment

Upon exposure to 0.2 mg/L Cd, the soluble sugar content varied significantly between Sb and Sp mycelia (*P<0.05*, Fig. 2a). On the other hand, the content of soluble sugar in Sb mycelia displayed a significant decrease due to the addition of Cd. A minimum value of 7.48 mg/g was observed under 0.4 mg/L Cd, with a significant decrease of 50.66% compared with the control (*P<0.05*). In contrast, the soluble sugar content in Sp mycelium showed a significant increase of 51.36% at 0.2 mg/L Cd (*P<0.05*) and then significantly decreased by 36.59% under 0.4 mg/L Cd compared with the control.

With the increase of Cd concentration, the content of Sb soluble protein increased first and then decreased, and reached the maximum value of 20.71 mg/g under 0.4 mg/L Cd treatment, which was significantly higher than that of the control group 63.46 % (*P<0.05*, Fig. 2b). Sp also showed similar changes, but there was no significant difference compared with the control group (*P>0.05*, Fig. 2b).

Adverse factors induce the accumulation of osmotic adjustment substances in organisms to resist the stress of the external environment [39], where soluble sugar and soluble protein are important osmotic regulators in organisms. This study found that the content of soluble sugar and soluble protein of Sp increased first and then decreased with the increase of Cd concentration, and the soluble protein content of Sb also showed similar changes. It is indicated that low concentration of Cd can promote the increase of soluble sugar and soluble protein, so as to chelate Cd entering the cell [40] and maintain the osmotic potential of the cell [41]. However, with the increase of Cd concentration, the soluble sugar and soluble protein produced are not enough to offset the Cd entering the cell, resulting in a significant decrease in their content. At the same time, the soluble sugar content of Sb decreased significantly with the increase of Cd concentration (*P<0.05*), which was consistent with previous studies [42], indicating that different fungi may take different measures to resist heavy metal stress. In this study, the soluble sugar in Sb was not sensitive to Cd stress, and its role in resisting Cd stress was not obvious.

Responses of SOD and CAT to Cd Exposure

With the increase of Cd concentration, the SOD activity of Sb and Sp showed different trends, but there was no significant change compared with the control group ($P>0.05$, Fig. 3a). The CAT activity of the two fungi showed an overall upward trend. The CAT activity of Sb did not change significantly at 0.2 mg/L and 0.4 mg/L Cd ($P>0.05$, Fig. 3b), and was significantly higher than that of the control group at 0.8 mg/L Cd, while the CAT activity of Sp did not change significantly at 0.2 mg/L Cd, and was significantly higher than that of the control group at 0.4 and 0.8 mg/L Cd ($P<0.05$, Fig. 3b).

Under heavy metal stress, fungi will produce excessive reactive oxygen species, which will cause oxidative stress if not removed in time [11]. Fungi form a set of active oxygen scavenging system, in which SOD, POD and CAT are important protective enzymes, which can remove the active oxygen produced in the body [26,43]. In this study, there was no significant difference in the SOD activity of Sb and Sp between different Cd concentrations, and the CAT activity of Sb and Sp did

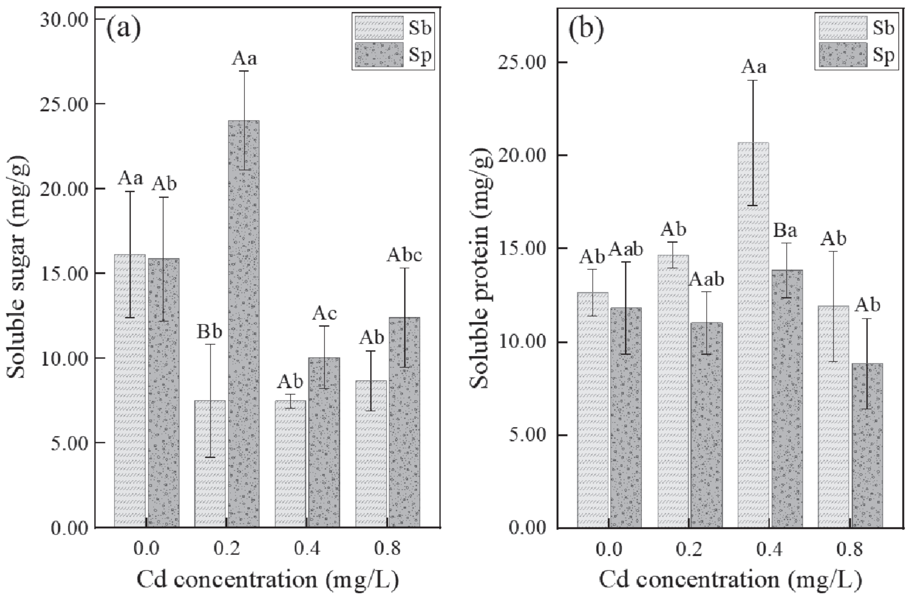

Fig. 2. Soluble sugar a) and soluble protein b) levels after Cd treatment. Note: Different lowercase letters indicate a significant difference between the same strain under different Cd concentrations ($P<0.05$), while different uppercase letters indicate a significant difference between the two strains under the same Cd concentration ($P<0.05$).

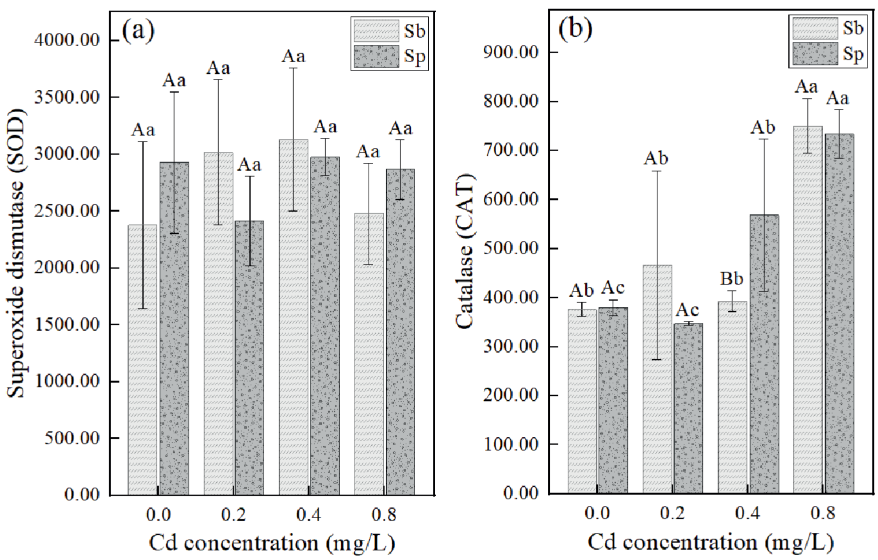

Fig. 3. Responses of SOD a) and CAT b) to Cd exposure. Note: Different lowercase letters indicate a significant difference between the same strain under different Cd concentrations ($P<0.05$), while different uppercase letters indicate a significant difference between the two strains under the same Cd concentration ($P<0.05$).

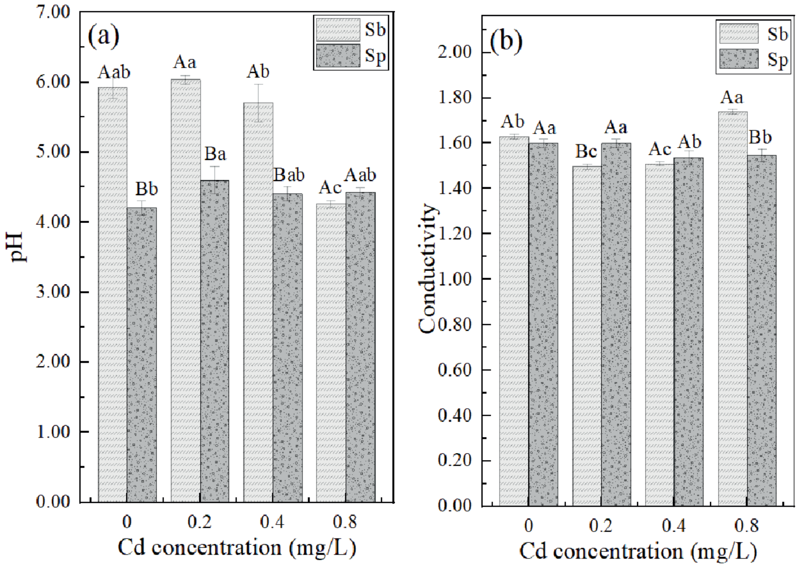

Fig. 4. Variations in pH a) and conductivity b) in the liquid culture medium of Sb and Sp. Note: Different lowercase letters indicate a significant difference between the same strain under different Cd concentrations (*P<0.05*), while different uppercase letters indicate a significant difference between the two strains under the same Cd concentration (*P<0.05*).

not change significantly at lower Cd concentrations. It was found that, although fungi can activate the activity of SOD and CAT in fungi under a certain concentration of heavy metal stress, the activity of SOD and CAT will be inhibited when the concentration of heavy metals reaches a certain value, which exceeds the tolerance range of fungi [4, 44, 45]. This indicates that Cd concentration does not exceed the tolerance range of Sb and Sp, and the production of superoxide radicals is not enough to activate SOD and CAT activities, so they are less oxidatively damaged. Further analysis showed that the CAT activity of Sb was significantly increased at 0.8 mg/L, while the CAT activity of Sp was significantly increased at 0.4 mg/L and reached the maximum at 0.8 mg/L. This further proves that the increased activity of SOD and CAT can help Sb and Sp to resist stress and improve their tolerance.

In addition, Sb and Sp may also develop them resist Cd stress by regulating other antioxidant enzymes. In the study of Zhang et al., (2021) [46] and Huang et al., (2022) [45], the increase of Cd concentration can increase the activity of SOD and CAT, and promote the activity of scorbate peroxidase, glutathione peroxidase or glutathione reductase to enhance the resistance of fungi to Cd. Therefore, future studies can continue to determine the activity of these enzymes to enrich the study of tolerance to the two fungi.

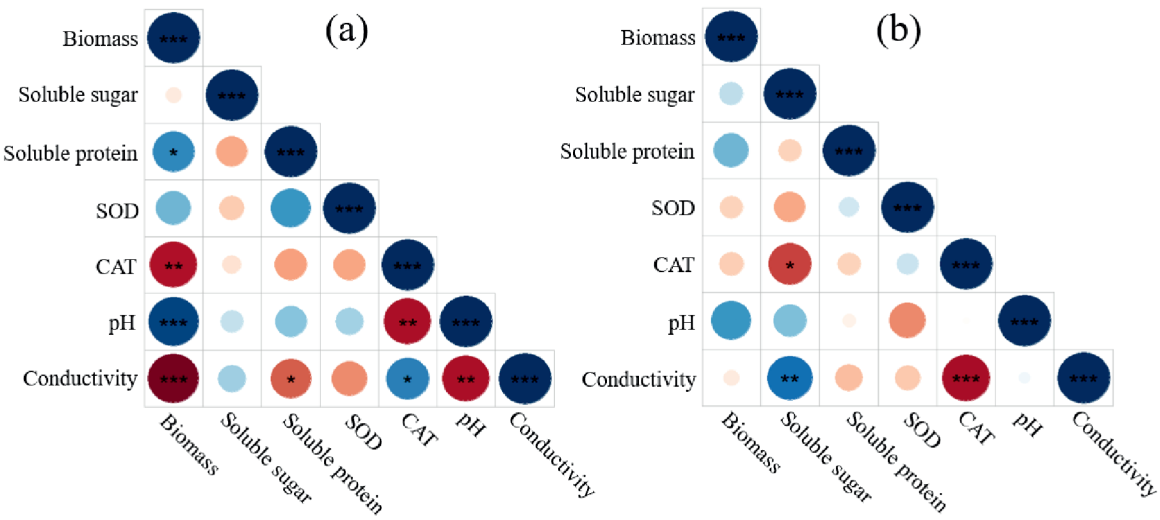

Fig. 5. Pearson correlation analysis of biomass, osmotic adjustment substances, enzyme activities, pH and conductivity in Sb a) and Sp b). Note: *** *P*<0.001, ** *P*<0.01, * *P*<0.05, the same as below.

Comparison of Cd Tolerance between the Two ECMF

Fig. 5 show the various correlations. For Sb, mycelial biomass was significantly positively correlated with soluble protein content and pH (correlation coefficient: 0.65, $P<0.05$; 0.91, $P<0.001$, Fig. 5a) and negatively correlated with CAT activity and conductivity (correlation coefficient: -0.81, $P<0.01$; -0.94, $P<0.001$, Fig. 5a). CAT activity was closely negatively correlated with pH (correlation coefficient: -0.82, $P<0.01$, Fig. 5a) and positively correlated with conductivity (correlation coefficient: 0.67, $P<0.05$, Fig. 5a). Moreover, conductivity was closely negatively correlated with soluble protein content and pH (correlation coefficient: -0.60, $P<0.05$; -0.81, $P<0.01$, Fig. 5a). For Sp, the correlation coefficient between soluble sugar content and CAT activity was -0.68, showing an extremely significant negative correlation ($P<0.05$, Fig. 5b). Conductivity was closely positively correlated with soluble sugar (correlation coefficient: 0.75, $P<0.01$, Fig. 5b) and negatively correlated with CAT activity (correlation coefficient: -0.82, $P<0.001$, Fig. 5b).

Various physiological and biochemical processes in plants are affected by the external environment. It was found that the enrichment coefficient of Cd in brown rice is small, so the correlation between Cd content in brown rice and various indicators is not high [47]. Chen et al., (2022) showed that there is a significant correlation between most indicators of millet under drought stress ($P<0.05$), and drought stress affects the growth of millet [48]. This indicates that the correlation between the indicators of plants reflects the extent to which plants are affected by the environment to some extent. In this study, the biomass, soluble protein, CAT, pH, and electrical conductivity of Sb were significantly correlated ($P<0.05$), while the correlation between soluble sugar, SOD, and various indicators was not significant. The correlation between soluble sugar, CAT, and conductivity of Sp was significant ($P<0.05$), while the correlation between other indicators was weak. The correlation between Sb indicators is closer than Sp, which is related to the degree of stress that the two fungi are subjected to. Sb was more affected by Cd stress, so the close relationship between most of its indicators is beneficial for reducing Cd stress. Sp has a strong tolerance to Cd, so it is less affected by Cd and the correlation between its indicators is weak.

Conclusions

Cd addition caused no obvious inhibition of the increase in biomass of Sb and Sp (except for Sb under 0.8 mg/L Cd) and promoted increases to different extents in the soluble protein content and antioxidant enzyme activity of the two strains. However, the two strains showed different trends in soluble sugar, pH and conductivity. The pH under 0 mg/L Cd and the conductivity under 0.8 mg/L Cd were significantly lower for Sp than for Sb. In addition, the soluble sugar in Sp mycelium was more sensitive to Cd stress than that in Sb mycelium.

In general, the effects of different concentrations of Cd on the indexes of Sb were greater than the effects on the indexes of Sp, and the regulation of each index was inhibited under 0.8 mg/L Cd. These results reflect that both Sb and Sp were subjected to a low level of Cd stress, and Sp was more tolerant than Sb.

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Conflict of Interest

The authors declare no conflict of interest.

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