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Physiological response of ectomycorrhizal fungi (*Lactarius delicious*) to microplastics stress

Zhang L.[1,*] and Gao B.[2,*]
[1]School of Chemistry and Bioengineering, Taizhou College, Nanjing Normal University, Taizhou 225300, China
[2]School of Pharmaceutical Sciences, Nanjing Tech University, Nanjing 211816, China
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*to whom all correspondence should be addressed: e-mail: liangzai0061@126.com, gaobb@njtech.edu.cn
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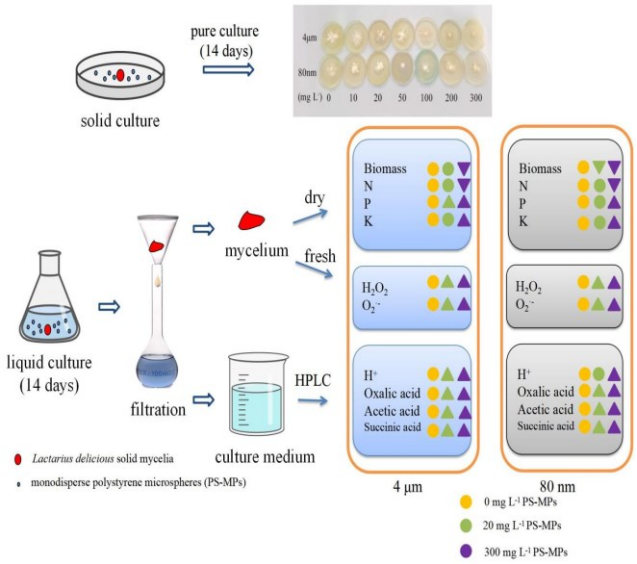
Graphical abstract



(△), (▽) and (○) represent up-regulation, down-regulation and no significant change in each parameter compared with the control group (0 mg L-1 PS-MPs), respectively.

Abstract

The pollution and toxicological effects of microplastics in soil have gradually attracted widespread attention, but studies on the ecological effects of microplastics on soil microorganisms and their mechanisms are still very scarce. To explore the physiological response of ectomycorrhizal fungi (*Lactarius delicious*, Ld) to microplastic stress, Ld was used as the test strain, and 80 nm and 4 μm monodisperse polystyrene microspheres (PS-MPs) were selected. The solid plate method and the liquid culture method were used to study the effect of PS-MPs with two particle sizes on the growth, nutrient content, reactive oxygen species (ROS) production, organic acid secretion and other indicators of the strain Ld. The results showed that with increasing PS-MPs concentration, the biomass of strain Ld decreased gradually. When the strain Ld was exposed to high concentrations of PS-MPs (200-300 mg L^{-1}), PS-MPs with particle size of 4 μm showed stronger inhibition of biomass than PS-MPs with particle size of 80 nm. After exposure to the PS-MPs environment of two sizes, the concentration of PS-MPs increased (0-300 mg L^{-1}), and the contents of phosphorus (P), potassium (K), H_2O_2 and superoxide anion radical ($O_2^{\cdot-}$) in Ld mycelia increased significantly ($p < 0.05$). This showed that the antioxidant substances in Ld could not remove excess ROS, but could reduce the stress of microplastics by increasing the absorption of P and K from the environment, which was beneficial to its own metabolic activities. In addition, the strain Ld could secrete oxalic acid, acetic acid and succinic acid into the culture medium, which the amount of oxalic acid secreted was the highest. We speculated that strain Ld responded to the stress of microplastics by secreting a large amount of organic acids. Therefore, the mechanism of the influence of microplastics on ectomycorrhizal fungi may involve oxidative stress, and the mycelia responded to the toxicity of microplastics by secreting more organic acids and increasing the absorption of P and K. The results of this study will provide a scientific basis for the study of the acute toxicity of microplastics to ectomycorrhizal fungi.

Keywords: Microplastics, *Lactarius delicious*, nutrients, reactive oxygen species, organic acids

1. Introduction

Microplastic refers to plastic particles with dimensions less than 5 mm or 1 mm (Thompson *et al*., 2004). The source of microplastics can be divided into two types: original microplastics, which mainly come from plastic industrial particles in cosmetics, paint and household wastewater, and secondary microplastics, which are formed by large particles (with a diameter of more than 5 mm) through long-term weathering, water flow, light and biological decomposition (Kumar *et al*., 2022; Zhao *et al*., 2018). Due to the small diameter of microplastics, they more easily migrate, and pollution has become ubiquitous. There is a large amount of microplastics in ecosystems such as lakes, oceans, and land, and even the human body develops microplastics due to the laminar flow of the food chain (Li *et al*., 2019). In addition, microplastics can adsorb chemical pesticides, antibiotics,

heavy metals, nonylphenol and other pollutants, thus posing a serious threat to the ecosystem and human health (Zhang *et al*., 2022). Therefore, as a new type of persistent environmental pollutant, microplastics have gradually become a research focus at home and abroad.

Currently, research on microplastics is mostly concentrated in marine environmental ecosystems and terrestrial aquatic environments (Schür *et al*., 2021; Jemec *et al*., 2016). For example, the abundance, source, distribution and migration of microplastics in oceans, estuaries, shoals, rivers and freshwater lakes have been researched (Jian *et al*., 2020; Islam *et al*., 2022; González-Pleiter *et al*., 2021). Toxicological studies of microplastics on aquatic organisms, such as algae, aquatic microorganisms and shellfish, have also been conducted (Neelavannan *et al*., 2022; Cauwenberghe *et al*., 2015; Abbasi *et al*., 2018). According to relevant research, soil ecosystems can store more microplastics, which are much higher than those in the ocean. Microplastics entering the soil ecosystem not only directly affect the physical and chemical properties of soil, but also have negative effects on the growth of soil animals and plants, and microbial diversity and functions (Xu *et al*., 2020; Allen *et al*., 2019; Zhang and Liu, 2018). Zhang *et al*. (2020) found that the accumulation of film residues in the field has a negative effect on plant growth and soil physical and chemical indicators, including affecting plant yield, plant height and soil physical and chemical parameters. De Souza Machado *et al*. (2019) found that microplastics can change the bulk density, structure and other physical parameters of soil, thereby affecting water flow and microbial activity and negatively affecting plant growth. In addition, relevant scholars have also explored the effects of microplastics on the seed germination and seedling growth of higher plants such as ryegrass, wheat and lentil. Among them, some scholars showed that the negative effect of biodegradable polylactic acid (PLA) microplastics on the germination rate and stem length of ryegrass was stronger than that of conventional high-density polyethylene (HDPE) microplastics (Boots *et al*., 2019). Lian *et al*. (2019) found that ethylene vinyl acetate copolymer, linear low-density polyethylene and polymethyl methacrylate had no significant effect on the root length, seedling length and dry weight of wheat in the range of 0-1000 mg L^{-1}. Liu *et al*. (2022) found that the nutrient-acquisition potential of crops and microbes was investigated under the treatment of two common microplastics (polyethylene [PE] and polyvinyl chloride [PVC]), 1,4-N-acetyl-glucosaminidase (NAG) and phosphomonoesterase (PHOS) hotspots in wheat roots expanding with the reduction in rhizosphere expansion, which indicates that PVC restricts the use of available nitrogen (N) and phosphorus (P), forcing crops to obtain nutrients from narrow root zones. De Silva *et al*. (2022) found that the superoxide dismutase (SOD) and catalase (CAT) activities of lentil (*Lens culinaris*) seedlings exposed to low concentrations of polyethylene microplastics (740-4990 nm PEMPs) significantly increased. However, with the increase in the concentration of PEMPs and the extension of the exposure time, the activity of antioxidant enzymes was significantly reduced, thereby inhibiting the seed germination and seedling growth of *Lens culinaris*. Li *et al*. (2022) showed that microplastics could induce time-dependent oxidative stress responses in bivalves. After short-term exposure to microplastics, the activities of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione transferase (GST) and SOD were activated. With increasing exposure time, the toxic effect of microplastics on the antioxidative system gradually increased, and the activities of these enzymes were inhibited after long-term exposure.

Mycorrhizae, as a symbiotic system of mutual benefit between microorganisms and plants, are an organic combination formed by fungi and plant roots, that affects the absorption of nutrients by host plants and the stability of soil structure, and has been studied for a long time. Plants provide carbon sources for mycorrhizal fungi. Correspondingly, after mycorrhizal fungi infected plants, they could increase the absorption of P, potassium (K), calcium (Ca), magnesium (Mg) and other nutrients, which was beneficial to improving their stress resistance (Lehmann *et al*., 2022). However, some microplastics can be further degraded to the micron or even nanometer level (Yang *et al*., 2021). Due to the strong adhesion of the microplastic particles, they are easily captured by exudates such as polysaccharide mucilage and organic acids produced by the plant root-exomycorrhizal fungal symbiont, so there is a higher risk of entering cells or organisms (Sun *et al*., 2020; Zhou *et al*., 2022). In addition, plastic particles have a certain degree of flexibility. They may enter the narrow apoplastic space of the root under the action of extrusion, further penetrate into the root cortex and fungal mycelium tissues, and even enter the interior of the cells (Li *et al*., 2020). At present, research on the mechanism by which ectomycorrhizal fungi respond to microplastic stress is still unclear and has not been reported. Therefore, in this study, *Lactarius delicious* (Ld) was selected as the test strain, and the solid plate method and the liquid culture method were used to explore the effects of microplastics with different particle sizes and concentrations on the growth, production of reactive oxygen species (ROS), organic acid secretion and nutrient absorption of Ld. This study is expected to provide a theoretical basis for evaluating the ecological effect of microplastics on soil microorganisms and the mechanism of action.

2. Materials and methods

2.1. Test materials

Ld was selected as the test strain. It was collected from strongly acidic soil (pH 4.0-4.2) under a *Pinus massoniana* forest in Jinyun Mountain of Chongqing, which is located in the northern subtropical zone. They were then inoculated into Pachlewsk solid medium and cultured at 25 °C for 14 d. The solid medium was formulated as follows: glucose 2 g, KH_2PO_4 0.1 g, ammonium tartrate 0.05 g, $MgSO_4$ 0.05 g, vitamin B_1 0.01 g, agar 2 g, trace element solution 0.1 mL, distilled water 100 mL, and the pH was adjusted to 5.5, autoclave steam sterilization (121 °C, 30 min). The formula of the trace element

solution was as follows: 8.45 mg boric acid, 6 mg ferrous sulfate, 5 mg manganese sulfate, 2.77 mg zinc chloride, 0.625 mg copper sulfate, and 0.27 mg ammonium molybdate were weighed and dissolved in 1 L of distilled water and sterilized.

Monodisperse polystyrene microspheres (PS-MPs) with particle sizes of 80 nm and 4 μm were purchased from Tianjin Saierqun Technology Co., Ltd., and the PS-MPs were dispersed in sterile water (concentration: 25 g L^{-1}) and stored in a refrigerator at 4 °C.

2.2. Experimental design

Under ultraclean bench conditions, PS-MPs stock solutions with two particle sizes (concentration of 25 g L^{-1}) were diluted into 1000 mg L^{-1} stock solution with sterile water. Then, a certain volume of PS-MPs stock solution was added to the sterilized Pachlewsk solid and liquid medium, and ultrasonic dispersion treatment was performed before and after dilution to prepare PS-MPs concentrations of 0 (control group), 10, 20, 50, 100, 200, and 300 mg L^{-1} Pachlewsk solid and liquid medium. Solid plates and liquid culture medium were prepared from 15 mL and 20 mL of culture medium, respectively. Then, a piece of activated solid mycelia with a diameter of 6 mm was inoculated on each plate and liquid medium, and each treatment was repeated five times. After all inoculations, the solid plates were placed in a 25 ℃ incubator for 14 days of static culture. The liquid medium was cultured in a shaker at 25 ℃ and 120 r min^{-1} for 14 days to prepare for the measurement of relevant indicators.

2.3. Sampling and sample analysis

2.3.1. Characterization of PS-MPs

Stock solutions of PS-MPs with two particle sizes were diluted to a certain number of times, and ultrasonic dispersion treatment was performed for 10 min before and after dilution. The morphological characteristics of PS-MPs were observed using a scanning electron microscope (SEM, Gemini SEM500, ZEISS company, Germany) and transmission electron microscope (TEM, Tecnai G2 F20, FEI company, USA).

2.3.2. Measurement of mycelial biomass

Mycelial biomass was determined according to the method of Peng *et al*., 2021. After 14 days of culture, the growth of fungi in the solid plates was observed. After the liquid culture was completed, the mycelia and the culture medium were separated by filtration, and then the mycelium was dried in an oven at 105 °C to a constant weight, i.e., the biomass of each bottle of fungi.

2.3.3. Determination of total N, P and K contents

Mycelia of Ld were placed in an oven at 105 °C for 30 min and then dried to constant weight at 60 °C for 24 h. After grinding with a pulverizer, approximately 0.2 g of sample was placed into a digestion tube, and the concentrated sulfuric acid (70%) and hydrogen peroxide (30%) were added. Then, the N concentration was obtained by Kjeldahl methods, the P content was obtained by the molybdenumblue method, and the K content was obtained by a flame photometry (Page, 1982).

2.3.4. Determination of H_2O_2 and superoxide anion radical contents

The H_2O_2 content was determined according to the method of Velikova *et al*., 2000. An appropriate amount of mycelia was used to prepare a tissue homogenate with 1 mL of 5% (w/v) trichloroacetic acid. Then, the homogenate was centrifuged (9000× *g,* 15 min, 4 °C). The supernatant was mixed with the reaction mixture [the reaction mixture contained 2.5 mM potassium phosphate buffer (pH 7.0) and 500 mM potassium iodide], and the absorbance was measured at 390 nm.

The hydroxylamine hydrochloride oxidation reaction method was used to determine the superoxide anion radical ($O_2^{\cdot-}$) content (Yang *et al*., 2020). An appropriate amount of mycelia was used to prepare a tissue homogenate with 65 mM phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged (9000× *g*, 15 min, 4 °C). A total of 2 mL of the reaction mixture containing 1 mL of supernatant, 0.9 mL of 65 mM potassium phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride was reacted at 25 °C for 30 min. Finally, 1 mL of 17 mM sulfanilic acid and 1 mL of 7 mM α-naphthylamine were added, and the absorbance was measured at 530 nm.

2.3.5. Determination of hydrogen ion and organic acid content in liquid culture medium

The pH and organic acid content in liquid culture medium were determined according to the method of Wang *et al*., 2012. The pH of the collected culture medium was measured using a PHSJ-4A pH meter, and then the pH was converted into the hydrogen ion (H^+) concentration. The contents of oxalic acid, malic acid, acetic acid, and citric acid in liquid culture medium were determined using high-performance liquid chromatography (Model 1260, Agilent company, USA). The chromatographic conditions were as follows: Hi-Plex H organic acid analytical column (300 mm × 7.7 mm, 8 μm), the mobile phase was water (dilute sulfuric acid was adjusted to pH 2.5), the flow rate was 0.6 mL Min^{-1}, the sample injection volume was 20 μL, the column temperature was 60 °C, and the UV detection wavelength was 210 nm.

2.4. Data statistics and analysis

Treatment effects were evaluated through variance analysis, using of SAS statistical software package (version 9, SAS Institute, Cary, NC, USA). The differences in means were tested by Fisher's protected least significant difference (LSD), and the significance level was set at $p<0.05$.

3. Results

3.1. Characterization of PS-MPs

The PS-MPs were characterized by transmission electron microscopy, scanning electron microscopy, and laser particle size analysis (Figure 1). The 80 nm and 4 μm PS-MPs used in this study were spherical in aqueous solution, with average particle sizes of 80.05 nm and 3.94 μm, respectively.

3.2. The effect of PS-MPs on the growth of Ld

After 14 days of solid plate culture, the effects of different concentrations of PS-MPs with two particle sizes on the

growth of strain Ld were determined (Figure 2). After treatment with 4 μm PS-MPs, when the concentration of PS-MPs was 0-50 mg L^{-1}, there was no significant difference among the treatments. When the concentration was greater than 100 mg L^{-1}, compared with the control group (0 mg L^{-1}), the growth of Ld was gradually inhibited, and the higher the concentration of PS-MPs, the more obvious the inhibition of mycelial growth. However, after treatment with 80 nm PS-MPs, as the concentration of PS-MPs increased, the growth of Ld was gradually inhibited.

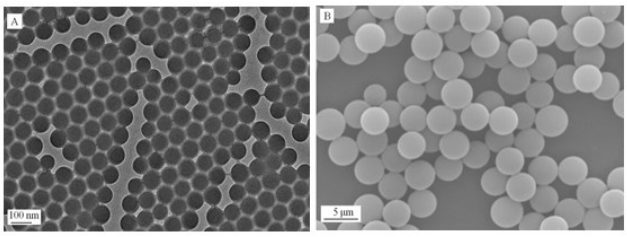


Figure 1. Morphology and particle size distribution of PS-MPs
PS-MPs: monodisperse polystyrene microspheres; Ld: *Lactarius delicious*; A: transmission electron microscope (TEM) images of 80 nm PS-MPs; B: scanning electron microscope (SEM) images of 4 μm PS-MPs.

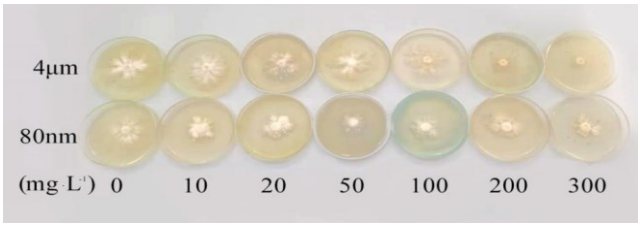


Figure 2. Effect of PS-MPs on the growth of Ld

PS-MPs: monodisperse polystyrene microspheres; Ld: *Lactarius delicious*.

3.3. Effect of PS-MPs on the mycelial biomass of Ld

After the liquid culture was completed, the changes in the mycelial biomass of Ld with the concentration of PS-MPs are shown in Figure 3. When the 4 μm PS-MPs were treated, the biomass of Ld was not significant between the 0-50 mg L^{-1} treatments. When the concentration of PS-MPs was greater than 100 mg L^{-1}, the biomass of Ld was significantly lower than that of the control group (0 mg L^{-1}) (p < 0.05). In the treatment with 80 nm PS-MPs, the biomass of Ld gradually decreased with increasing mass concentration of PS-MPs, and there were significant differences among the treatments (p < 0.05). When the concentration of PS-MPs was 300 mg L^{-1}, compared with the control group (0 mg L^{-1}), the biomass of Ld decreased significantly (p < 0.05) by 65.6% and 54.4% under PS-MPs treatment with particle sizes of 4 μm and 80 nm, respectively. However, when treated with high concentrations of PS-MPs (200-300 mg L^{-1}), 4 μm PS-MPs exhibited stronger growth inhibition of mycelial biomass than 80 nm PS-MPs.

3.4. Effect of PS-MPs on N, P and K contents of Ld

After the culture, the changes of N, P and K contents in Ld mycelia with the concentration of PS-MPs are shown in Table 1. The N content in Ld mycelia decreased with increasing PS-MPs concentration when treated with PS-MPs of two particle sizes. When the concentration of PS-MPs was 300 mg L^{-1}, compared with the control group (0 mg L^{-1}), under the treatment of PS-MPs with two particle sizes of 4 μm and 80 nm, the N content in Ld mycelia decreased by 21.8% and 18.0%, respectively (p <0.05). The contents of P and K in Ld mycelia increased with increasing PS-MPs concentration when treated with two particle sizes. When the concentration of PS-MPs was 300 mg L^{-1}, compared with the control group (0 mg L^{-1}), under the treatment of PS-MPs with two particle sizes of 4 μm and 80 nm, the P content in Ld mycelia increased significantly (p <0.05) by 52.2% and 49.3%, while the K content in Ld mycelia increased significantly (p <0.05) by 40.5% and 35.7%, respectively.

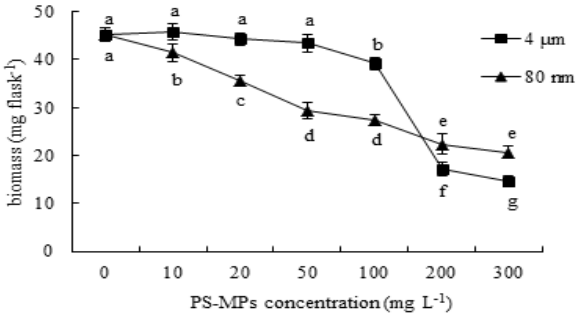


Figure 3. Effect of PS-MPs on Ld mycelial biomass.
PS-MPs: monodisperse polystyrene microspheres; Ld: *Lactarius delicious*; Data are presented in the form of mean ± S.D. (n = 5 in each group); there is a significant difference in different letters between different treatments (p < 0.05).

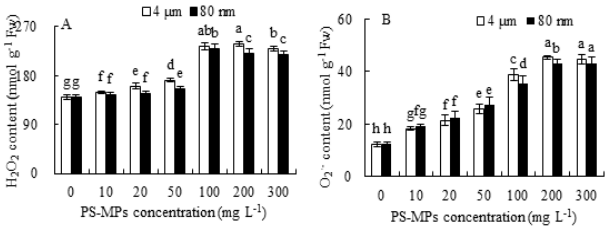


Figure 4. H_2O_2 (A) and O_2^- (B) contents in Ld mycelia
PS-MPs: monodisperse polystyrene microspheres; Ld: *Lactarius delicious*; $O_2^{\cdot-}$: superoxide anion radical; Fw: fresh weight; Data are presented in the form of mean ± S.D. (n = 5 in each group); there is a significant difference in different letters between different treatments (p < 0.05).

3.5. Effect of PS-MPs on the H_2O_2 and $O_2^{\cdot-}$ of Ld

When fungal cells are subjected to abiotic stress, the production of ROS in the body is greatly increased. When the antioxidant defense capacity of the body is exceeded, it will accumulate in the body and cause oxidative damage to the fungal cells (Gessler *et al*., 2007; Kalsotra *et al*., 2018). Figure 4A shows that the H_2O_2 content in the mycelia of Ld showed a significant increasing trend with increasing PS-MPs concentration (p < 0.05). The results showed that PS-MPs in the culture medium had an obvious inducing effect on H_2O_2 production, and Ld mycelia were stressed by PS-MPs. Among them, the H_2O_2 content in the mycelia treated with 4 μm PS-MPs (concentrations of 20-300 mg L^{-1}) was significantly higher

than that in the 80 nm PS-MPs treatment group. This indicates that 4 μm PS-MPs were more toxic to the mycelia of Ld, and it was also possible that more PS-MPs were enriched in Ld, resulting in the inability of antioxidant enzymes to clear excess H_2O_2.

Figure 4B shows that the content of $O_2^{\cdot-}$ in Ld mycelia increased significantly with increasing PS-MPs concentration ($p < 0.05$), indicating that the PS-MPs in the culture medium had an obvious inducing effect on $O_2^{\cdot-}$ production and that Ld mycelia were stressed by PS-MPs. Among them, the $O_2^{\cdot-}$ content in the mycelia treated with 4 μm PS-MPs (concentrations of 100-200 mg L^{-1}) was significantly higher than that in the 80 nm PS-MPs treatment group at the same concentration, indicating that when PS-MPs were treated at 100-200 mg L^{-1}, 4 μm PS-MPs were more toxic to the mycelia of Ld, resulting in the accumulation of excessive $O_2^{\cdot-}$ in the mycelia. In addition, this also reflects that 4 μm PS-MPs were more toxic to the mycelia, leading to the inability of antioxidant substances in the body to remove excessive $O_2^{\cdot-}$.

3.6. Changes of H^+ concentration in liquid medium

After Ld was cultured for 14 days, the H^+ concentration in liquid medium changed significantly with increasing PS-MPs concentration (Figure 5). The results showed that the H^+ concentration in liquid culture medium showed a trend of "first rise, then decrease" under the two particle sizes of PS-MPs treatments. The H^+ concentration in liquid culture medium was increased most significantly when the concentration of PS-MPs was 200 mg L^{-1} (p <0.05), which was increased by 8.8 times and 3.9 times respectively, compared with that in the control group (0 mg L^{-1}).

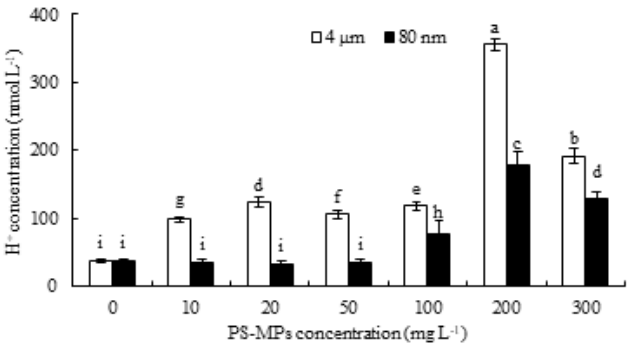


Figure 5. The change of hydrogen ion concentration in liquid medium

Table 1. Effect of PS-MPs on the contents of N, P and K in Ld (mg g^{-1} dry weight)

PS-MPs concentration (mg L^{-1})	4 μm			80 nm		
	N	P	K	N	P	K
0	28.4±0.7a	6.7±0.3c	4.2±0.4b	28.4±0.7a	6.7±0.3b	4.2±0.4b
10	28.5±1.2a	7.5±0.7bc	4.3±0.6b	28.2±1.2ab	7.3±0.2b	4.7±0.4ab
20	28.2±1.1a	7.9±0.3b	4.6±0.3ab	29.1±1.1a	8.3±0.5ab	4.2±0.2b
50	27.3±1.3ab	7.5±0.6bc	4.7±0.2ab	26.5±0.9b	8.4±0.7ab	4.5±0.1ab
100	26.8±0.8ab	8.7±0.4b	5.8±0.4a	26.4±0.5b	9.2±0.6a	4.8±0.3ab
200	24.4±0.9b	8.8±0.2b	5.7±0.5a	25.2±0.8bc	9.8±0.3a	5.6±0.2a
300	22.2±0.7bc	10.2±0.4a	5.9±0.1a	23.3±0.9c	10.0±0.6a	5.7±0.2a

PS-MPs: monodisperse polystyrene microspheres; Ld: Lactarius delicious; N: nitrogen; P: phosphorus; K: potassium; Data are displayed in the form of mean ± S.D. (n = 5 in each group). Mean values with a same letter presented in the column represent the absence of significant difference (p < 0.05) upon Fisher's least significant difference test.

3.7. Effect of PS-MPs on the organic acid secretion of Ld

In this study, the contents of oxalic acid, malic acid, acetic acid and succinic acid in the culture medium were detected, and the secretion of oxalic acid was the highest. The results are shown in Table 2. Under the two PS-MPs particle size treatments, the oxalic acid content in the liquid medium showed a trend of "first increase-then decrease". The oxalic acid content increased most significantly when the concentration of PS-MPs was 100 mg L^{-1} (4 μm) and 200 mg L^{-1} (80 nm) (p <0.05). Compared with the control group (0 mg L^{-1}), it increased by 125.1% and 80.1%, respectively. Under the treatment of PS-MPs with two particle sizes, the contents of acetic acid and succinic acid in the liquid medium showed a trend of gradually increasing with increasing PS-MPs concentration. Except for the succinic acid content in the 80 nm PS-MPs treatment group (the highest content was at 200 mg L^{-1}), the content of the two organic acids in the other treatment groups was the highest when the concentration of PS-MPs was 300 mg L^{-1}, which was 1.4, 1.0, 1.1, and 0.7 times higher than that of the control group (0 mg L^{-1}), respectively.

3.8. Principal component analysis

Principal component analysis (PCA) was used to evaluate the 10 indicators of the Ld physiological response under microplastics stress. The results showed that the cumulative contribution rate of PCA1 and PCA2 was 93.29% when the mycelium was exposed to PS-MPs at 4 μm (Figure 6A), which reflects the influence of microplastic stress on the physiological indexes of ectomycorrhizal fungi. The characteristic value of PCA1 was 8.64, and the contribution rate was 86.40%. Among them, the indices with large positive characteristic values included succinic acid content, P content, $O_2^{\cdot-}$ content and acetic acid content. The indices with a large absolute value of negative characteristics included N content and biomass. It showed that when the PCA1 value increased, the values of organic acid secretion and ROS content also increased, while the values of biomass and N content index conducive to the growth of Ld decreased accordingly. The characteristic value of PCA2 was 0.69 and the contribution rate was 6.89%. The indices with large positive eigenvalues included biomass and oxalic acid content, in which the H^+ concentration was highly negatively correlated. When the mycelium was exposed to PS-MPs at 80 nm, the results of PCA were similar to those of the 4 μm PS-MPs treatment (Figure 6B).

4. Discussion

4.1. Effect of PS-MPs on the growth and N, P, K contents of Ld mycelia

At present, studies on the ecotoxicological effects of microplastics are mostly concentrated on marine fish, algae, soil plants, and soil protozoa, while the toxicity of microplastics to soil ectomycorrhizal fungi is relatively rare (Jian *et al*., 2020; Islam *et al*., 2022; González-Pleiter *et al*., 2021).

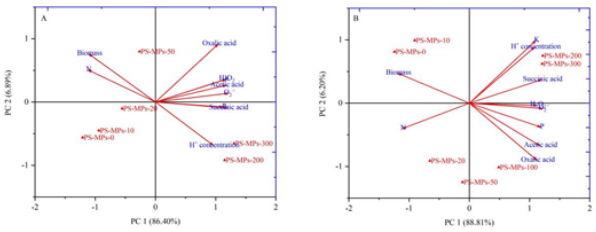


Figure 6. PCA analysis of physiological response indices of ectomycorrhizal fungi (Lactarius delicious) under microplastics stress

A: 4 μm PS-MPs treatment; B: 80 nm PS-MPs treatment; PS-MPs: monodisperse polystyrene microspheres;

K: potassium; N: nitrogen; P: phosphorus; H^+: hydrogen ion; $O_2^{\cdot-}$: superoxide anion radical.

Table 2. Concentration of organic acid released by Ld in liquid medium (mg L^{-1})

PS-MPs concentration (mg L^{-1})	4 μm				80 nm			
	Oxalic acid	Malic acid	Acetic acid	Succinic acid	Oxalic acid	Malic acid	Acetic acid	Succinic acid
0	83.2±1.3f	ND	9.3±0.3de	3.5±0.1d	83.2±1.3f	ND	9.3±0.3d	3.5±0.1d
10	98.3±1.6e	ND	10.2±0.5d	3.6±0.2d	89.3±2.1e	ND	9.8±0.4d	3.8±0.1cd
20	112.4±2.1d	ND	15.3±0.8c	4.2±0.2c	109.2±2.7d	ND	13.7±0.6c	3.9±0.2c
50	157.2±2.5c	ND	17.5±0.9bc	4.9±0.3bc	134.7±1.9c	ND	16.6±0.5b	4.2±0.3bc
100	187.3±2.2a	ND	19.5±0.7b	5.3±0.4b	146.8±2.5a	ND	17.4±0.7b	4.7±0.4b
200	168.3±1.8b	ND	21.9±0.5a	6.3±0.3a	149.9±3.1a	ND	19.3±0.9a	5.9±0.7a
300	163.5±2.4bc	ND	22.8±0.4a	7.0±0.5a	139.6±2.9b	ND	19.6±0.8a	5.6±06a

PS-MPs: monodisperse polystyrene microspheres; H^+: hydrogen ion; Data are presented in the form of mean ± S.D. (n = 5 in each group); there is a significant difference in different letters between different treatments (p<0.05).

Studies have shown that a high amount of microplastic mulch (15000 mg kg^{-1}) significantly inhibited the elongation of wheat germ and roots; At the same time, it affected the photosynthetic efficiency of leaves and hindered the synthesis of proteins (Zhang *et al.,* 2021). In addition, microplastics produced by high-density polyethylene mulches could be used as unique habitats for bacteria in agricultural soils (Zhang *et al.,* 2019). The presence of PS-MPs could have a negative impact on the richness and diversity of bacterial communities (Fei *et al*., 2020). Tao *et al*. (2022) found that when 1 μM PS-MPs at concentrations of 0.1-30 mg L^{-1} increased the biofilm biomass of *Proteus* spp. at all stages. When the PS-MPs concentration was ≥ 40 mg L^{-1}, the development of biofilm was inhibited, which might be due to the imbalance of ROS in the biofilm or the impact of nutrient circulation. In this study, it was found that with increasing PS-MPs concentration, the biomass of Ld decreased significantly (p < 0.05) under the treatment of PS-MPs with two particle sizes. Li *et al*. (2019) found that microplastics not only adhered to the surface of mussel foot threads but also combined with each other in newly formed mussel foot threads. In addition, studies have shown that the uptake of *E. coli*-coated microplastics by *Ostrea edulis* was significantly higher than that of the original microplastics. This was because after entering the ocean, microplastics were colonized by various microorganisms to form multiple species. Biofilm-covered microplastics were more likely to be ingested by marine organisms (Fabra *et al*., 2021). This study found that at high concentrations of PS-MPs (200-300 mg L^{-1}), treatment with 4 μm PS-MPs exhibited stronger growth inhibition of Ld mycelial biomass than treatment with 80 nm PS-MPs. This might be because after the 4 μm PS-MPs migrated to the surface of the mycelia, due to the large diameter of the particles, they could not penetrate into the fungal tissues and cells, aggregate with extracellular secretion of mycelia, adhere to the surface of mycelia, and disturb the normal absorption and operation of water and nutrients by ectomycorrhizal fungi.

After symbiosis between mycorrhizal fungi and plant roots, the mycorrhizal fungi that form can promote the absorption of plant nutrients, including N, P, K and Mg (Yin *et al*., 2021; Gao *et al*., 2021; Khullar and Reddy, 2020). For example, in nutrient-poor soil, microplastics affect the absorption of K, P, Mg and sulfur (S) by plants. After inoculation with arbuscular mycorrhizal fungi, the impact of microplastics could be buffered by balancing nutrient utilization and plant absorption. Mineral elements such as K, Mg and S in onion shoots increased significantly (Garcia *et al*., 2014; Moreno-Jiménez *et al*., 2022). Under pure culture conditions, after being stressed by heavy metals such as cadmium (Cd), plumbum (Pb) and cuprum (Cu), ectomycorrhizal fungi undergo a series of biochemical reactions beneficial to resistance to heavy metal stress, including an increase in the content of P and K in mycelia (Khullar and Sudhakara, 2019; Chen *et al*., 2015). In this study, the contents of P and K in Ld mycelia increased with increasing PS-MPs concentration after treatment with PS-MPs of two particle sizes. P is the basic element for synthesizing genetic materials and other key organic molecules. K is the activator of a variety of

enzymes in the organism. Ld was beneficial to its metabolic activities by increasing the absorption of P and K, reducing the stress of microplastics.

4.2. Effect of PS-MPs on the production of ROS in Ld mycelia

Microorganisms continuously produce ROS in the process of life, and at the same time, they form a defense system for ROS removal, including enzymatic and nonenzymatic systems, so that the production and removal of ROS in biofilms can be maintained in a dynamic equilibrium state. Enzymatic systems include SOD, CAT and ascorbate peroxidase (APX) (Tang *et al*., 2018; Das and Roychoudhury, 2014). The main function of antioxidant enzymes such as SOD, CAT and peroxidase (POD) in ectomycorrhizal fungi is to remove excess ROS free radicals and maintain their dynamic balance (Yin *et al*., 2021). Studies have shown that *Daphnia magna* were treated with PS-MPs with three particle sizes (100 nm, 5 μm, and 50 μm), which could promote an increase in SOD and CAT activities in *Daphnia magna* and induce malondialdehyde (MDA) production. The increase in the content results in oxidative damage to *Daphnia magna* (Gao *et al*., 2021). In other articles, it was found that the toxicity of nanoparticles [such as polystyrene nanoplastics (PS-NPs), TiO_2 and Fe_2O_3] to microorganisms was mainly attributed to oxidative damage of cells, resulting in excessive generation of ROS in the body that could not be removed (Tang *et al*., 2017). De Silva *et al*. (2022) found that compared with the control group (0 mg L^{-1}), exposure to 50 mg L^{-1} and 100 mg L^{-1} PEMPs (740-4990 nm) significantly increased the H_2O_2 content in the leaves of *lens culinaris* seedlings. In addition, the MDA activity of all PEMPs treatments also increased, and the increase was the highest at a concentration of 100 mg L^{-1}. This indicates that PEMPs induce the most severe damage to the cell membrane at the highest concentration. In this study, it was found that the H_2O_2 and $O_2^{\cdot-}$ contents in Ld mycelia increased significantly with increasing PS-MPs concentration ($p < 0.05$), indicating that PS-MPs in the culture medium had an obvious inducing effect on the production of ROS. When the antioxidant enzymes in the body cannot remove excess ROS, Ld mycelia undergo oxidative damage. In addition, when Ld mycelia were exposed to medium-to-high concentrations (100-300 mg L^{-1}) of 4 μm PS-MPs, the accumulated ROS content in the mycelia was significantly higher than that in the mycelia treated with 80 nm PS-MPs at the same concentration. This indicates that 4 μm PS-MPs were more toxic to Ld mycelia, and the oxidative damage caused by PS-MPs to Ld mycelia exhibits a difference in particle size. The results of these enzyme activities and oxidative damage are basically consistent with previous results (Guo *et al.*, 2022; Jiang *et al*., 2019).

4.3. Effect of PS-MPs on the secretion of H^+ and organic acids from Ld mycelia

Studies have shown that PS-NPs could induce *Arabidopsis thaliana* roots to produce more organic acids. When the concentration of PS-NPs was 50 μg mL^{-1}, the content of oxalic acid secreted by *Arabidopsis thaliana* roots was approximately 2.6 times that of the control group (0 μg mL^{-1}) (Sun *et al*., 2020). Low-molecular-weight organic acids are the most common and important plant root exudates. On the one hand, they can mobilize mineral elements such as Ca, K, P, and Mg in the soil to provide nutrients for plant growth. On the other hand, they can form stable metal coordination with metal ions such as Pb, Cu, and Cd. It can change the mobility and bioavailability of heavy metals and prevent metal ions from entering the plant body or avoiding their accumulation in the sensitive spots of the roots. In the abiotic stress environment, the formation of a symbiotic with the strain Ld was conducive to enhancing the secretion of plant root exudates (Balogh-Brunstad *et al*., 2017; Remiszewski *et al*., 2016; Tang *et al*., 2019; Zheng *et al*., 2009; Wu *et al*., 2021). Studies have shown that strain Ld could secrete a large amount of organic acids, such as oxalic acid, citric acid, and acetic acid, etc. to the surrounding environment under the condition of P deficiency, and this organic acid could activate insoluble phosphate and provide P nutrition for plants (Montiel-Rozas and Madejón, 2016; Zhang *et al*., 2014). In this study, it was found that strain Ld could secrete oxalic acid, acetic acid and succinic acid into the culture medium under the two particle sizes of PS-MPs treatment, and the secretion of oxalic acid was the highest. As the concentration of PS-MPs increased, the contents of acetic acid and succinic acid in the liquid medium showed a gradual increasing trend, while the contents of oxalic acid showed a trend of “first rise-then decrease”. Boots *et al*. (2019) found that HDPE microplastics could reduce soil pH, which indicated that microplastics could stimulate *Lolium perenne* (perennial ryegrass) roots to secrete more hydrogen ions in this study, it was found that when PS-MPs were 200 mg L^{-1}, the concentration of H^+ in the culture medium could be increased by 8.8 times and 3.9 times respectively. This indicates that ectomycorrhizal fungi could secrete organic acids, H^+ and other secretions into the environment. The presence of secretions would affect the fluidity, bioavailability and reactivity of microplastics in mycelia and reduce the toxic effect of microplastics on ectomycorrhizal fungi.

In addition, PCA results showed that under the treatment of PS-MPs with two particle sizes, with the increase in the concentration of PS-MPs, the amount of organic acid secretion and ROS accumulation of strain Ld increased significantly, while indicators conducive to the growth of Ld, such as biomass and N content, decreased accordingly.

5. Conclusion

The results of this study showed that exposure to micron- and nanoscale PS-MPs had certain effects on the growth, nutrient content, ROS production and organic acid secretion of ectomycorrhizal fungi, and different particle sizes and concentrations had different ecological effects on ectomycorrhizal fungi. Among them, 4 μm PS-MPs showed a stronger inhibitory effect on the biomass of strain Ld than 80 nm PS-MPs. After exposure to the two particle sizes of PS-MPs, the contents of P, K H_2O_2 and $O_2^{\cdot-}$ in Ld mycelia increased significantly with increasing PS-MPs concentration. This showed that the antioxidant

substances in Ld could not remove excess ROS, but could reduce the stress of microplastics by increasing the absorption of P and K from the environment, which was beneficial to its own metabolic activities. In addition, strain Ld could secrete oxalic acid, acetic acid and succinic acid into the culture medium, and the amount of oxalic acid secreted was the highest. We speculate that strain Ld responds to the stress of microplastics by secreting organic acids. The results of this study will provide a scientific basis for the study of the acute toxicity of microplastics to soil ectomycorrhizal fungi.

Author Contributions

Conceptualization, L.Z. and B.G.; methodology, L.Z. and B.G.; software, L.Z. and B.G.; validation, L.Z. and B.G.; formal analysis, L.Z. and B.G.; resources, L.Z. and B.G.; data curation, L.Z.; writing—original draft L.Z.; preparation, L.Z. and B.G.; writing—review and editing, L.Z. and B.G.; supervision, B.G.; project administration, L.Z. and B.G.; All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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