agronomy

MDPI

Article

The Production of Oxalate by *Aspergillus niger* under Different Lead Concentrations

Yijun Huang [1,2,3,†], Liangliang Zhang [1,2,3,†], Shijia Yuan [1,2,3], Wenpei Liu [1,2,3], Chaochun Zhang [1,2,3], Da Tian [1,2,3,*] and Xinxin Ye [1,2,3,*]

1 Anhui Province Key Laboratory of Farmland Conservation and Pollution Prevention, College of Resources and Environment, Anhui Agricultural University, Hefei 230036, China
2 Anhui Province Engineering and Technology Research Center of Intelligent Manufacture and Efficient Utilization of Green Phosphorus Fertilizer, Anhui Agricultural University, Hefei 230036, China
3 Key Laboratory of JiangHuai Arable Land Resources Protection and Eco-Restoration, Ministry of Natural Resources, Anhui Agricultural University, Hefei 230036, China
* Correspondence: tianda@ahau.edu.cn (D.T.); yexx@ahau.edu.cn (X.Y.)
† These authors contributed equally to this work.

Abstract: In this study, using a typical acid-producing fungi, *Aspergillus niger* (*A. niger*, CGMCC 23272), we investigated the capacity of organic acid production under different lead (Pb) concentrations. *A. niger* has a high Pb tolerance, which can maintain the growth of hypha at 1500 mg/L Pb concentration. Oxalic acid is the primary organic acid produced by *A. niger*. *A. niger* was shown to maintain the ability to produce oxalic acid under different Pb concentrations, which ranged from 522.8 to 1130.5 mg/L. The formed lead oxalate also confirmed the production of oxalic acid by *A. niger*. Meanwhile, the formation of lead oxalate minerals dominated the resistance of Pb toxicity by *A. niger*. More than 95% of Pb cations were removed by *A. niger* under different Pb concentrations. The high Pb toxicity (1500 mg/L) could stimulate pyruvate dehydrogenase enzyme activities, which increased from 0.05 to 0.13 nmol/min/g after three days of incubation. The low Pb toxicity (500 and 1000 mg/L) could improve the production of oxalic acid by *A. niger*. This indicates that the metabolism of organic acid by *A. niger* can be improved by a high Pb concentration via the tricarboxylic acid cycle.

Keywords: *Aspergillus niger*; organic acids production; oxalic acid; lead precipitation; TCA cycle

Citation: Huang, Y.; Zhang, L.; Yuan, S.; Liu, W.; Zhang, C.; Tian, D.; Ye, X. The Production of Oxalate by *Aspergillus niger* under Different Lead Concentrations. *Agronomy* **2023**, *13*, 1182. https://doi.org/10.3390/agronomy13041182

Academic Editors: Marzia Vergine and Cornelia Rumpel

Received: 31 January 2023
Revised: 7 March 2023
Accepted: 20 April 2023
Published: 21 April 2023

1. Introduction

Lead (Pb) is one of the most common heavy metal pollutants in the environment [1,2]. As a cumulative contaminant, Pb can cause high toxicity and has long-term persistence in the environment [3,4]. The Pb pollutants in the environment are usually inputted by way of exhaust emissions, industrial wastewater, and waste residue discharge [5,6]. In soil, the high toxicity and persistence of Pb endangers soil organisms and reduces crop yield and quality [7]. In addition, Pb pollutants can also be easily transferred into groundwater and enriched through the food chain, which is a threat to the ecological environment, food safety and human health [8,9]. Especially in soil, the transfer of Pb cations into living organisms would be more serious due to the high concealment via the food chain pathway [10]. Therefore, the prevention of Pb pollutants' transmission in the food chain is necessary.

Bioremediation has been recognized as an effective pathway in Pb contamination remediation [11–14]. Compared with the typical physical or chemical methods, bioremediation has the characteristics of low cost and no secondary pollution [15–17]. For example, the combination of *Aspergillus niger* (*A. niger*) and apatite can remove 99% of lead cations in an aqueous solution [11]. On the one hand, the organic acids produced by *A. niger* can promote the release of phosphorus (P) from apatite and accelerate the formation of pyromorphite [18]. On the other hand, the organic acids produced by *A. niger* such as oxalic acid can also react with lead to form insoluble lead oxalate minerals [19]. Lead oxalate

has a low *Ksp* value (10^{-9}–10^{-11}), which is stable in the environment and can significantly reduce the Pb cations in the soil [19,20].

The acid-producing fungi of *A. niger* have been successfully applied in Pb remediation via the combination of phosphate [10,11]. Due to its high ability to produce organic acid, *A. niger* also showed a high Pb tolerance [19]. Compared with other phosphate-solubilizing fungi, e.g., *Penicillium oxalicum*, *A. niger* can also maintain the ability of organic acid production at 1500 mg/L Pb concentration. Moreover, the low Pb concentration ($\leq$1000 mg/L) can stimulate the activity of *A. niger* and increase the production of organic acid, especially for oxalic acid [19].

The tricarboxylic acid (TCA) cycle is the main metabolic pathway that regulates the production of organic acids by phosphate-solubilizing fungi [21,22]. The organic acids produced by the TCA cycle include oxalic acid, citric acid, tartaric acid, gluconic acid, formic acid, and malic acid [23–25]. These organic acids are mostly low-molecular-weight organic acids, with molecular weights close to 300 g/mol [21,26]. The synthesis of organic acids via the TCA cycle is usually regulated by different enzymes [27]. The pyruvate dehydrogenase (PDH) enzyme is important to support the production of organic acids in the TCA cycle [28,29]. Mitochondrial isocitrate dehydrogenase (ICDHm) is also one of the key rate-limiting enzymes in the TCA cycle for organic acid production [30]. However, the production of organic acid by *A. niger* under different Pb concentrations via the TCA cycle is unclear. Therefore, the production of organic acid by a typical acid-producing fungi, *A. niger*, via the TCA cycle was investigated in this study under different Pb concentrations.

2. Materials and Methods

2.1. Reagents and Media

A potato dextrose agar medium (PDA) was prepared for the fungal incubation. Before the incubation, 200 g of potato was cut into small pieces and boiled with double-deionized water for 20 min. After filtration with sterile gauze, the filtrate was collected and made up to 1000 mL with deionized water. Then, 20 g of dextrose and 20 g of agar were added to the above 1000 mL solution (China National Pharmaceutical Group Chemical Reagent Co., Ltd., Shanghai, China). The preparation of potato dextrose broth medium (PDB) followed the same process as PDA without agar. Solid $Pb(NO_3)_2$ powder (Xilong Scientific Ltd., Shantou, China) was supplemented for the different Pb concentrations in this study.

2.2. Strain Preparation

Aspergillus niger (*A. niger*, AH-F-1-2) was received from the China General Microbiological Culture Collection Center (CGMCC No. 23272). *A. niger* was isolated from maize rhizosphere soil in the Northern Anhui Experimental Station, Suzhou City, Anhui Province [31]. Before the experiment, *A. niger* was cultivated on the PDA medium for five days at 28 °C. Then, the spores were gently scraped from the surface of the medium with sterile water on an ultra-clean bench. The liquid was filtered and scraped using three layers of sterile gauze to obtain an *A. niger* spore suspension [31]. The spore suspension was diluted to a concentration of 10^7 CFU/mL using 0.85% sterile saline and measured using a blood counting chamber [31].

2.3. Experimental Design

In this experiment, four Pb concentrations were used, i.e., 0, 500, 1000, and 1500 mg/L. Before the incubation, a 250 mL conical flask was filled with 100 mL of PDB medium and sterilized at 121 °C for 20 min. After being cooled to room temperature, 0, 0.08, 0.16, and 0.24 g of $Pb(NO_3)_2$ powder (Xilong Technology Co., Ltd., Shenzhen, China) were added to the 250 mL conical flask to simulate the Pb toxicity environment at 0, 500, 1000, and 1500 mg/L. Then, 1 mL of *A. niger* spore suspension was added to each treatment on a sterile bench. The medium was adjusted to pH 6. 5 with KH_2PO_4. Conical flasks were sealed with sealing film (BS-QM-003, Biosharp, Hefei, China) and cultivated at 28 °C and 180 rpm/min for one, three, and five days to collect *A. niger* hypha. The filtrate was

collected by filtration through a 0.22 μm polyether sulfone membrane (PES). The collected filtrate was used for the determination of pH, organic acid, and lead concentration in the solution. The filtered mycelium was freeze-dried for biomass and scanning electron microscopy analyses.

2.4. Instrumentation

The pH of the filtrate was determined using an FE20 pH meter (Mettler Toledo, Columbus, OH, USA). High-performance liquid chromatography (Agilent 1200, Santa Clara, CA, USA) was used to determine the content of organic acids in the filtrate. The concentrations of the organic acid standard solutions (i.e., oxalic, tartaric, formic, malic, and citric acids) were 1000, 500, 200, 100, 50, 10, and 0 mg/L, and the R-squared value of the standard curve was 0. 9999. The liquid phase was an Agilent Zorbax SB-Aq (4.6 mm × 250 mm, 5 μm) column with mobile phase A at 2.5 wt‰ of potassium dihydrogen phosphate, with the pH adjusted to 2.8 with phosphoric acid. The mobile phase B was methanol, and the elution gradient program was 0–20 min with 99% A and 1% B phases, followed by a 5 min equilibration run. The test flow rate was 1 mL/min, the column temperature was 30 °C, the detection wavelength was 214 nm, and the injection volume was 20 μL. The compounds were identified by a comparison of retention times with standards and quantified by an external standard method.

The Pb remove ratio was analyzed by inductively coupled plasma optical emission spectrum (ICP-OES, PerkinElmer, Avio 200, Waltham, MA, USA). The morphologies of *A. niger* and Pb minerals were observed by scanning electron microscope–energy dispersive spectrometer (SEM-EDS, S-4800, Hitachi, Tokyo, Japan). The SEM-EDS analyses of the samples were gold-plated on a sputtering machine (Hitachi E-1010, Hitachi, Tokyo, Japan) for 1 min before testing. The surface morphology was analyzed under a scanning electron microscope (S4800-Hitachi) with an accelerating voltage of 5 kv, and an energy spectrometer (EDS, X-Max-n150) was used for the elemental analysis [31].

2.5. Enzyme Activity Determination

Pyruvate dehydrogenase (PDH) and mitochondrial isocitrate dehydrogenase (ICDHm) kits (Hefei Lyle Biotechnology Co., Hefei, China) were used to determine the enzyme activity. The specific assay procedure was carried out according to the kit instructions, and the Shimadzu UV 1800 spectrophotometer was used for the determination. Before the enzyme activity test, the collected mycelium was ground into a homogenate using agate mortar with liquid nitrogen to rupture the cell wall before subsequent determination. The enzyme activity of PDH (nmol/min/g) was calculated by consuming 1 nmol of 2, 6-dichlorophenol indophenol sodium per minute in 1 g of tissue, detected at 605 nm. ICDHm enzyme activity (nmol/min/g) was calculated by consuming 1 nmol nicotinamide adenine dinucleotide (NADH) per minute in 1 g of tissue, detected at 340 nm.

2.6. Data Analysis

Statistical analyses were performed using OriginPro 8.5 (OriginLab, Northampton, MA, USA). A one-way analysis of variance (ANOVA) was used to analyze biomass, Pb^{2+}, pH, organic acid, and enzyme activity for each treatment.

3. Results

3.1. Fungal Dry Biomass and pH Value

The fungal dry biomass was collected from a 250 mL medium after incubation. In the control experiments without Pb, the dry biomass of *A. niger* was 0.53, 0.56, and 0.52 g after incubation for one, three, and five days, respectively (Figure 1A). The dry biomass of *A. niger* in the 500 mg/L Pb concentration increased from 0.49 g to 0.60 g during the incubation (Figure 1A). In the 1000 mg/L Pb treatment, the biomass of *A. niger* was 0.47, 0.47, and 0.46 g after one, three, and five days of incubation (Figure 1A). However, the dry biomass of *A. niger* significantly decreased to 0.27, 0.34, and 0.33 g after one, three,

and five days in the 1500 mg/L Pb concentration (Figure 1A). The growth of *A. niger* was significantly inhibited at the 1500 mg/L Pb concentration.

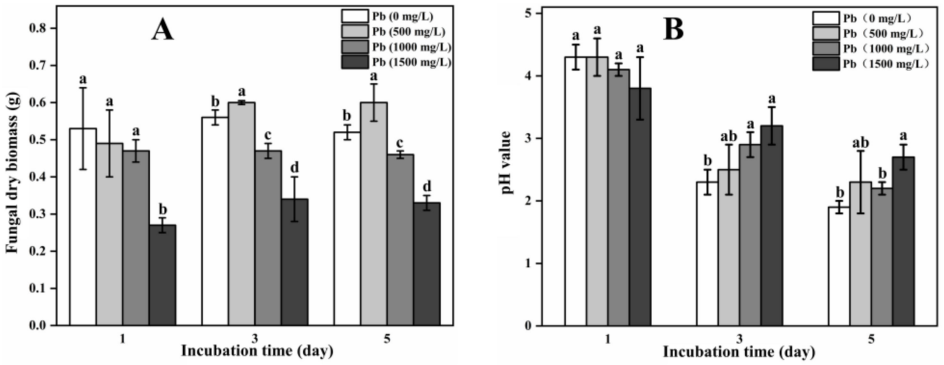

Figure 1. Fungal dry biomass (**A**) and pH value (**B**) in different Pb concentrations during the incubation time (1, 3, and 5 days). The error bars represent the standard deviations of three replicates for each treatment. The different lower-case letters indicate a significant difference between the treatments ($p < 0.05$).

The initial pH of the PDA medium was 6.5. In the control experiments without Pb, the pH value decreased from 4.3 to 1.9 during the incubation (Figure 1B). In the 500 mg/L and 1000 mg/L Pb treatments, the pH values showed similar values during the incubation, i.e., decreased from 4.3 and 4.1 to 2.3 and 2.2 (Figure 1B). In the 1500 mg/L Pb treatment, the pH value was 3.8, 3.2, and 2.7 after incubation for one, three, and five days, respectively (Figure 1B).

3.2. Organic Acid Production and Pb Remove by A. niger

Oxalic acid was the primary organic acid produced by *A. niger* (Table 1). In the control experiments without Pb, the oxalic acid concentration was only 73.9 mg/L on day one, and then, it significantly increased to 1147.1 and 1133.9 mg/L on days three and five, respectively (Table 1). In the 500 and 1000 mg/L Pb treatments, the production of oxalic acid ranged from 1101.7 to 1002.3 mg/L and 959.9 to 1024.3 mg/L during the incubation, respectively (Table 1). However, the oxalic acid in the 1500 mg/L Pb treatment showed a decreased trend, i.e., 753.2, 705.1, and 632.5 mg/L in one, three, and five days (Table 1). *A. niger* can maintain the ability of malic and citric acid production under 500 and 1000 mg/L Pb concentrations (Table 1). Compared with the 0 mg/L Pb concentration, the 500 and 1000 mg/L Pb concentrations increased the production of malic acid by *A. niger*, and the highest malic acid concentration reached 283.4 mg/L in the 1000 mg/L Pb concentration after three days of incubation (Table 1). The production of citric acid was limited with the increased Pb concentration, decreasing from 365.4 mg/L (in 0 mg/L Pb concentration on day one) to 14.5 mg/L (in 1500 mg/L Pb concentration on day three) (Table 1). The production of tartaric acid by *A. niger* had the highest value of 251.2 mg/L in the 1000 mg/L Pb concentration after one day of incubation (Table 1). The formic acid concentration had the highest value of 591.2 mg/L in the 1500 mg/L Pb concentration after five days of incubation (Table 1). Compared with the 1500 mg/L Pb concentration, the 500 and 1000 mg/L Pb concentrations could promote the production of malic and citric acids by *A. niger*. After three days of incubation, the citric acid concentrations in the 500 and 1000 mg/L Pb concentrations were significantly higher than that in the 1500 mg/L Pb concentration (Table 1).

Table 1. The oxalic, tartaric, formic, malic, and citric acids produced by *A. niger* under different Pb levels after incubation for one, three, and five days.

Incubation Time (Day)	Organic Acid (mg/L)	Pb Concentration (mg/L) 0	500	1000	1500
One day	Oxalic acid	73.9 ± 5.4 c	1101.7 ± 22.6 a	959.9 ± 163.6 ab	753.2 ± 30.7 b
	Tartaric acid	2.51 ± 0.2 c	117.9 ± 11.4 b	251.2 ± 44.7 a	193.1 ± 10.5 a
	Formic acid	63.9 ± 7.2 b	214.1 ± 14.3 a	N.A.	N.A.
	Malic acid	N.A.	33.5 ± 2.7 a	32.9 ± 4.6 a	25.4 ± 3.8 b
	Citric acid	365.4 ± 33.7 a	205.4 ± 12.4 b	79.2 ± 4.6 c	40.2 ± 4.8 d
Three days	Oxalic acid	1147.1 ± 56.8 a	1130.5 ± 33.7 a	1059.1 ± 35.5 a	705.1 ± 163.6 b
	Tartaric acid	N.A.	53.6 ± 2.7 a	N.A.	N.A.
	Formic acid	19.4 ± 1.9 c	14.1 ± 0.9 d	71 ± 5.9 b	522.8 ± 36.6 a
	Malic acid	N.A.	71.2 ± 9.6 b	283.4 ± 16.5 a	66.4 ± 7.9 b
	Citric acid	233 ± 1.33 a	159.7 ± 5.5 b	65.2 ± 7.8 c	14.5 ± 1.4 d
Five days	Oxalic acid	1133.9 ± 67.9 a	1002.3 ± 31.9 b	1024.3 ± 165.3 ab	632.5 ± 56.6 c
	Tartaric acid	N.A.	N.A.	N.A.	N.A.
	Formic acid	N.A.	N.A.	N.A.	591.2 ± 22.2 a
	Malic acid	N.A.	N.A.	N.A.	143.4 ± 15.7 a
	Citric acid	N.A.	N.A.	N.A.	182.4 ± 11.8 a

Note: The significant differences among the treatments were identified by Tukey's honestly significant difference test ($p < 0.05$) via one-way ANOVA. The different lower-case letters indicate a significant difference between the treatments ($p < 0.05$). N.A. is not available.

The Pb concentration in each treatment was lower than 80 mg/L (Figure 2A). In the 500 and 1000 mg/L Pb treatments, the Pb concentration was 2.45 and 11.68 mg/L in one day (Figure 2A). After three and five days of incubation, the Pb concentration in these two treatments decreased to 0.34 and 0.47 mg/L, respectively (Figure 2A). In the 1500 mg/L Pb treatment, the Pb concentration was 24.5 mg/L on day one and then increased to 28.45 and 79.33 mg/L on days three and five, respectively (Figure 2A). In addition, *A. niger* also showed a high Pb remove ratio in each treatment. During the incubation, the Pb remove ratio in the 500 and 1000 mg/L Pb treatments increased from 99.5% and 98.8% to 99.9% and 99.9% during the incubation, respectively (Figure 2B). In the 1500 mg/L Pb treatment, the Pb remove ratio was 98.3% on day one, and it then decreased to 98.1% and 94.7% on days three and five (Figure 2B).

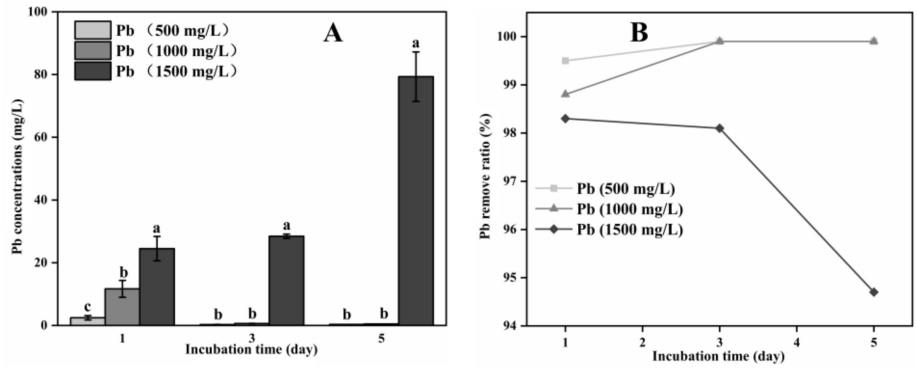

Figure 2. Pb concentrations in the medium (**A**) and Pb remove ratio (**B**) in different lead concentrations during the incubation time (1, 3, and 5 days). The error bars represent the standard deviations of three replicates for each treatment. The different lower-case letters indicate a significant difference between the treatments ($p < 0.05$).

3.3. TCA Cycle Enzyme Activity in A. niger

In the control experiments without Pb, the PDH enzyme activity of A. niger was 0.05, 0.05, and 0.03 nmol/min/g during the incubation (Figure 3A). Similarly, the PDH enzyme

activity in the 500 mg/L Pb treatment also decreased from 0.05 to 0.01 nmol/min/g during the incubation (Figure 3A). In the 1000 mg/L Pb treatment, the PDH enzyme activity was 0.11, 0.07, and 0.02 nmol/min/g during the incubation (Figure 3A). The PDH enzyme activity in the 1500 mg/L Pb treatment was not detected on day one, but it significantly increased to 0.13 and 0.08 nmol/min/g on days three and five (Figure 3A). In addition, the ICDHm enzyme activity in each treatment showed a decreasing trend (Figure 3B). In the 0, 500, 1000, and 1500 mg/L Pb treatments, the ICDHm enzyme activities, respectively, decreased from 33.4, 36.3, 32.5, and 13.1 to 10.3, 13.9, 13.6, and 1.2 nmol/min/g in the three- and five-day incubation periods (Figure 3B).

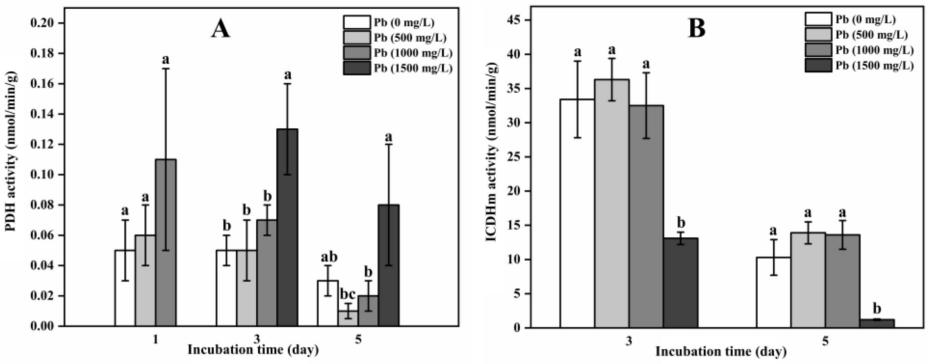

Figure 3. The enzyme of PDH (**A**) and ICDHm activity (**B**) in different lead concentrations during the incubation time (1, 3, and 5 days). The error bars represent the standard deviations of three replicates for each treatment. The different lower-case letters indicate a significant difference between the treatments ($p < 0.05$).

3.4. Analysis of SEM and EDS

Figure 4 shows the morphologies of fungal hypha and Pb minerals in 0, 500, 1000, and 1500 mg/L Pb concentrations after three days of incubation. In the 0 mg/L Pb concentration, a large amount of fungal hypha and spores were observed (Figure 4A). In the 500, 1000, and 1500 mg/L Pb concentrations, the large amounts of fungal hypha showed that *A. niger* can survive under different Pb concentrations (Figure 4). The point scanning by EDS indicated that the formed minerals surrounded by hypha were lead oxalate (LO) (Figure 4).

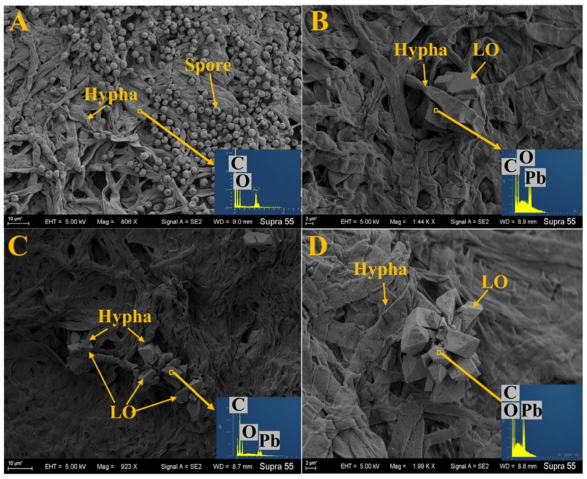

Figure 4. SEM image and EDS point data in 0 mg/L (**A**), 500 mg/L (**B**), 1000 mg/L (**C**), and 1000 mg/L Pb concentrations (**D**) after three days of incubation. LO: lead oxalate.

After five days of incubation, the fungal hypha and LO were observed in the 1000 mg/L Pb treatment (Figure 5A). However, in the 1500 mg/L Pb treatment, the formed minerals

were more concentrated on a single hypha (Figure 5B). The EDS mapping also demonstrated that the minerals surrounding the hypha were lead oxalate, and C, O, and Pb elements were recorded (Figure 5).

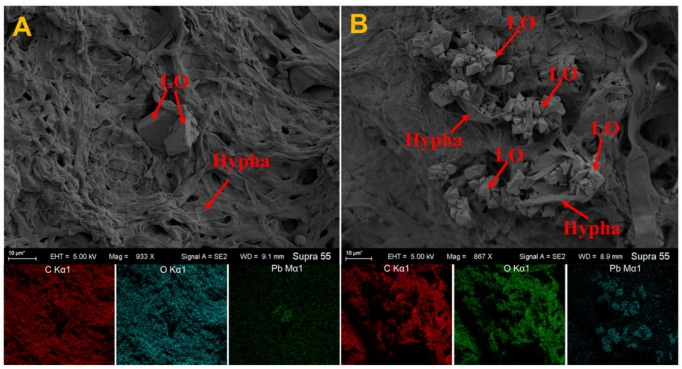

Figure 5. SEM image and EDS mapping data in 1000 mg/L (**A**) and 1500 mg/L (**B**) Pb concentrations after five days of incubation. LO: lead oxalate.

4. Discussion

A. niger has been successfully applied to Pb remediation, especially via the combination of apatite [11,32]. On the one hand, *A. niger* usually has a strong tolerance to Pb [19]. On the other hand, these fungi could also maintain the ability of organic acid production in the presence of Pb cations. This produced organic acid not only reacts with Pb to form lead oxalate but also promotes the release of phosphorus from apatite [33]. The released phosphorus can also react with Pb to form highly insoluble pyromorphite [34]. Compared with lead oxalate, pyromorphite is more stable, with low solubility [35]. Therefore, the production of organic acid by acid-producing fungi is important in Pb remediation and toxicity tolerance, especially for oxalic acid production.

Organic acids produced by *A. niger* usually include oxalic, citric, malic acids, etc. [26]. Our research indicated that oxalic acid is the primary organic acid produced by *A. niger* under different Pb concentrations. The large amounts of lead oxalate formed in different Pb concentrations also provided the production of oxalic acid (Figures 4 and 5). Oxalic acid is susceptible to micro-precipitation with heavy metal ions extracellularly, forming insoluble oxalate, which can reduce the mass concentration of heavy metal ions in the environment [36]. Although the production of oxalic acid does not change the absolute content of heavy metals in the environment, the toxicity of heavy metals is reduced. In Pb remediation, oxalic acid reacts with Pb cations to form lead oxalate minerals with the following reaction equation [37]:

$$Pb^{2+} + C_2O_4{}^{2-} = PbC_2O_4 \quad (1)$$

The formed lead oxalate not only reduces the Pb cations content but also promotes the survival of *A. niger* in Pb toxicity.

Oxalic acid production by *A. niger* is usually influenced by different environmental factors, such as heavy metal cations, phosphate types, pH, carbon, nitrogen resources, etc. [22,38–40]. A high Pb concentration (1500 mg/L) can inhibit the production of oxalic acid by *A. niger* [19]. Our results also indicate that the oxalic acid production by *A. niger* is inhibited at the 1500 mg/L Pb concentration (Table 1). In addition, the production of oxalic acid by *A. niger* is only weakly decreased in low Pb toxicities (500 and 1000 mg/L) compared with no Pb toxicity (0 mg/L). However, more than 94.7% of Pb cations were removed and formed the lead oxalate (Figures 2 and 5). This formed lead oxalate would also contribute to the consumption of produced oxalic acid by *A. niger* [15]. Therefore, although the oxalic acid concentration is lower than the no Pb treatment in different Pb concentrations, the existence of high Pb toxicity could stimulate the production of oxalic acid by *A. niger*. *A. niger* is also maintains the high production of oxalic acid in different

Pb concentrations. In addition, the different *A. niger* species also have different capacities in oxalic acid production under the same Pb toxicity. *A. niger* CGMCC No.11544 shows lower production of oxalic acid (457.9 mg/L) under 1000 mg/L Pb toxicity after five days of incubation [41]. In our research, *A. niger* CGMCC 23272 showed higher production of oxalic acid, i.e., 1024 mg/L after five days of incubation under 1000 mg/L Pb toxicity (Table 1). This result indicated that *A. niger* CGMCC 23272 could be more efficient in aqueous-solution Pb cation removal.

The TCA cycle is the most important pathway for the biosynthesis of oxalic acid by acid-producing fungi, which would be influenced by different enzyme activities [21,42,43]. For example, the biosynthesis of both citric and oxalic acids is influenced by the enzyme of PDH and ICDHm in the TCA cycle [21]. The enzyme of PDH oxidizes pyruvate to acetyl coenzyme A and promotes the production of organic acids in the TCA cycle [28,29]. Environmental factors such as $FePO_4$ and $Ca_3(PO_4)_2$ can influence the enzymes of PDH and ICDHm activities to change the production of organic acid [22]. In our study, we found that the high Pb concentration (1500 mg/L Pb) significantly increased PDH activities in three and five days (Figure 3A). However, the oxalic acid concentration in the 1500 mg/L Pb treatment was significantly lower than other treatments, i.e., ~700 mg/L vs. 1000 mg/L. This could be because the high Pb concentration (1500 mg/L) consumed more oxalic acid than that secreted by *A. niger*. According to Le Chatelier's principle, the increased Pb concentrations would cause the shift of equation 1 to lead oxalate and gradually decrease the concentration of Pb [44]. In addition, due to the perception of oxalic acid by Pb cations in different Pb concentrations, the actual production of oxalic acid by *A. niger* in a high Pb concentration (1500 mg/L) would be much higher than the content in a low Pb concentration (500 and 1000 mg/L). Therefore, the PDH result also partly proves that the high concentration of Pb can stimulate *A. niger* to secrete more oxalic acid to resist lead toxicity. In contrast, the activity of ICDHm decreased in all treatments after five days of incubation (Figure 3B). However, compared with three days, the ICDHm activity in the no Pb treatment decreased by 23.1 nmol/min/g after five days of incubation (Figure 3B). Meanwhile, the 1500mg/L Pb treatment only decreased 11.9 nmol/min/g (Figure 3B). The enzyme of ICDHm is one of the key rate-limiting enzymes in the production of organic acids in the TCA cycle. Although high Pb toxicity significantly decreased the ICDHm activities, the decline rate of this activity slowed down to maintain the production of oxalic acid. In addition, *A. niger* also has a capacity in the production of mycotoxins such as ochratoxin A, etc., which can elevate the risk to food security and human health [45–48]. Hence, the use of *A. niger* needs to consider its function and scope in the future.

5. Conclusions

A. niger CGMCC 23272 can survive under different Pb concentrations and maintain the ability to secrete organic acids. Oxalic acid is the primary organic acid produced by *A. niger* in Pb toxicity resistance. High Pb toxicity can stimulate the activity of the PDH enzyme in the TCA cycle. The production of oxalic acid by *A. niger* was improved under 500 and 1000 mg/L Pb concentrations. The formation of lead oxalate minerals dominates the resistance of Pb toxicity by *A. niger*. This research suggests that the use of *A. niger* in Pb remediation should increase the production of oxalic acid and improve the TCA cycle enzyme activity.

Author Contributions: Conceptualization, D.T. and X.Y.; methodology, Y.H. and L.Z.; validation, Y.H., L.Z. and S.Y.; formal analysis, Y.H., L.Z., S.Y. and W.L.; investigation, Y.H., L.Z., S.Y. and W.L.; resources, D.T. and C.Z.; data curation, D.T. and Y.H.; writing—original draft preparation, Y.H. and L.Z.; writing—review and editing, Y.H., D.T., C.Z. and X.Y.; supervision, D.T. and X.Y.; funding acquisition, X.Y. and C.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Program at Department of Natural Resources of Anhui Province (2021-K-4), the Science and Technology Major Project of Anhui Province (202103a06020012), the Natural Science Foundation of Anhui Province (2008085QD187), and the Program (yj2019-20) at Anhui Agricultural University.

Data Availability Statement: Not applicable.

Acknowledgments: We appreciated the assistance of Yang Xu from the Biotechnology Center of Anhui Agricultural University in the technical support of the SEM in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zeng, G.M.; Wan, J.; Huang, D.L.; Hu, L.; Huang, C.; Cheng, M.; Jiang, D.N. Precipitation, adsorption and rhizosphere effect: The mechanisms for Phosphate-induced Pb immobilization in soils—A review. *J. Hazard. Mater.* **2017**, *339*, 354–367. [CrossRef] [PubMed]
2. Gadd, G.M. Geomycology: Biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycol. Res.* **2007**, *111*, 3–49. [CrossRef] [PubMed]
3. Hou, D.; Ding, Z.; Li, G.; Wu, L.; Hu, P.; Guo, G.; Wang, X. A sustainability assessment framework for agricultural land remediation in China. *Land Degrad. Dev.* **2018**, *29*, 1005–1018. [CrossRef]
4. Shen, Z.; Tian, D.; Zhang, X.; Tang, L.; Su, M.; Zhang, L.; Hou, D. Mechanisms of biochar assisted immobilization of Pb^{2+} by bioapatite in aqueous solution. *Chemosphere* **2018**, *190*, 260–266. [CrossRef]
5. Tian, D.; Cheng, X.; Wang, L.; Hu, J.; Zhou, N.; Xia, J.; Zhang, C. Remediation of lead-contaminated water by red yeast and different types of phosphate. *Front. Bioeng. Biotech.* **2022**, *10*, 775058. [CrossRef]
6. Gadd, G.M. The geomycology of elemental cycling and transformations in the environment. *Microbiol. Spectr.* **2017**, *5*, 1. [CrossRef]
7. Hou, D.Y.; Qi, S.Q.; Zhao, B.; Rigby, M.; O'Connor, D. Incorporating life cycle assessment with health risk assessment to select the 'greenest' cleanup level for Pb contaminated soil. *J. Clean. Prod.* **2017**, *162*, 1157–1168. [CrossRef]
8. Meng, L.; Pan, S.; Zhou, L.; Santasup, C.; Su, M.; Tian, D.; Li, Z. Evaluating the survival of *Aspergillus niger* in a highly polluted red soil with addition of phosphogypsum and bioorganic fertilizer. *Environ. Sci. Pollut. R.* **2022**, *29*, 76446–76455. [CrossRef]
9. Guo, C.M.; Tian, W.T.; Wang, Z.J.; Han, F.Y.; Su, M.; Wu, Y.L.; Hu, S.J. Reduction of Pb availability during surficial leaching in different types of soils with addition of apatite and oxalic acid. *J. Soils Sediment.* **2019**, *19*, 741–749. [CrossRef]
10. Feng, Y.; Zhang, L.; Li, X.; Wang, L.; Yusef, K.K.; Gao, H.; Tian, D. Remediation of lead contamination by *Aspergillus niger* and phosphate rocks under different nitrogen sources. *Agronomy* **2022**, *12*, 1639. [CrossRef]
11. Li, Z.; Wang, F.W.; Bai, T.S.; Tao, J.J.; Guo, J.Y.; Yang, M.Y.; Hu, S.J. Lead immobilization by geological fluorapatite and fungus *Aspergillus niger*. *J. Hazard. Mater.* **2016**, *320*, 386–392. [CrossRef] [PubMed]
12. Kapoor, A.; Viraraghavan, T.; Cullimore, D.R. Removal of heavy metals using the fungus *Aspergillus niger*. *Bioresour. Technol.* **1999**, *70*, 95–104. [CrossRef]
13. Li, X.; Luo, L.; Yang, J.; Li, B.; Yuan, H. Mechanisms for solubilization of various insoluble phosphates and activation of immobilized phosphates in different soils by an efficient and salinity-tolerant *Aspergillus niger* strain An2. *Appl. Biochem. Biotechnol.* **2015**, *175*, 2755–2768. [CrossRef] [PubMed]
14. Gadd, G.M. Interactions of fungi with toxic metals. *New Phytol.* **1993**, *124*, 25–60. [CrossRef]
15. Tian, D.; Wang, W.; Su, M.; Zheng, J.; Wu, Y.; Wang, S.; Hu, S. Remediation of lead-contaminated water by geological fluorapatite and fungus *Penicillium oxalicum*. *Environ. Sci. Pollut. Res.* **2018**, *25*, 21118–21126. [CrossRef]
16. Tian, D.; Lai, Z.; Zou, X.; Guo, C.; Tang, L.; Su, M.; Hu, S. A contrast of lead immobilization via bioapatite under elevated CO_2 between acidic and alkaline soils. *Soil Use Manag.* **2018**, *34*, 542–544. [CrossRef]
17. Tian, D.; Li, Z.; O'Connor, D.; Shen, Z.; Hou, D. The need to prioritize sustainable phosphate-based fertilizers. *Soil Use Manag.* **2020**, *36*, 351–354. [CrossRef]
18. Mendes, G.D.O.; Murta, H.M.; Valadares, R.V.; Silveira, W.B.; da Silva, I.R.; Costa, M.D. Oxalic acid is more efficient than sulfuric acid for rock phosphate solubilization. *Miner. Eng.* **2020**, *155*, 106458. [CrossRef]
19. Tian, D.; Jiang, Z.; Jiang, L.; Su, M.; Feng, Z.; Zhang, L.; Hu, S. A new insight into lead (II) tolerance of environmental fungi based on a study of *Aspergillus niger* and *Penicillium oxalicum*. *Environ. Microbiol.* **2019**, *21*, 471–479. [CrossRef]
20. Xiong, Y.L.; Kirkes, L.; Westfall, T.; Roselle, R. Experimental determination of solubilities of lead oxalate (PbC_2O_4(cr)) in a NaCl medium to high ionic strengths, and the importance of lead oxalate in low temperature environments. *Chem. Geol.* **2013**, *342*, 128–137. [CrossRef]
21. Palmieri, F.; Estoppey, A.; House, G.L.; Lohberger, A.; Bindschedler, S.; Chain, P.S.G.; Junier, P. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. *Adv. Appl. Microbiol.* **2019**, *106*, 49–77. [CrossRef] [PubMed]
22. Tian, D.; Wang, L.; Hu, J.; Zhang, L.; Zhou, N.; Xia, J.; Gao, H. A study of P release from Fe-P and Ca-P via the organic acids secreted by *Aspergillus niger*. *J. Microbiol.* **2021**, *59*, 819–826. [CrossRef] [PubMed]

23. Mäkelä, M.R.; Hildén, K.; Lundell, T.K. Oxalate decarboxylase: Biotechnological update and prevalence of the enzyme in filamentous fungi. *Appl. Microbiol. Biot.* **2010**, *87*, 801–814. [CrossRef] [PubMed]
24. Mandal, S.K.; Banerjee, P.C. Submerged production of oxalic acid from glucose by immobilized *Aspergillus niger*. *Process. Biochem.* **2005**, *40*, 1605–1610. [CrossRef]
25. Gadd, G.M.; Bahri-Esfahani, J.; Li, Q.; Rhee, Y.J.; Wei, Z.; Fomina, M.; Liang, X. Oxalate production by fungi: Significance in geomycology, biodeterioration and bioremediation. *Fungal. Biol. Rev.* **2014**, *28*, 36–55. [CrossRef]
26. Li, Z.; Bai, T.; Dai, L.; Wang, F.; Tao, J.; Meng, S.; Hu, S. A study of organic acid production in contrasts between two phosphate solubilizing fungi: *Penicillium oxalicum* and *Aspergillus niger*. *Sci. Rep.* **2016**, *6*, 25313. [CrossRef]
27. Behera, B.C. Citric acid from *Aspergillus niger*: A comprehensive overview. *Crit. Rev. Microbiol.* **2020**, *46*, 727–749. [CrossRef]
28. Dutton, M.V.; Evans, C.S. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* **1996**, *42*, 881–895. [CrossRef]
29. Mattevi, A.; Obmolova, G.; Schulze, E.; Kalk, K.H.; Westphal, A.H.; de Kok, A.; Hol, W.G. Atomic structure of the cubic core of the pyruvate dehydrogenase multienzyme complex. *Science* **1992**, *255*, 1544–1550. [CrossRef]
30. Amary, M.F.; Bacsi, K.; Maggiani, F.; Damato, S.; Halai, D.; Berisha, F.; Flanagan, A.M. IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours. *J. Pathol.* **2011**, *224*, 334–343. [CrossRef]
31. Wang, L.; Guan, H.; Hu, J.; Feng, Y.; Li, X.; Yusef, K.K.; Tian, D. *Aspergillus niger* enhances organic and inorganic phosphorus release from wheat straw by secretion of degrading enzymes and oxalic acid. *J. Agric. Food. Chem.* **2022**, *70*, 10738–10746. [CrossRef] [PubMed]
32. Tian, D.; Xia, J.; Zhou, N.; Xu, M.; Li, X.; Zhang, L.; Gao, H. The utilization of phosphogypsum as a sustainable phosphate-based fertilizer by *Aspergillus niger*. *Agronomy* **2022**, *12*, 646. [CrossRef]
33. Geng, Y.; Pan, S.; Zhang, L.; Qiu, J.; He, K.; Gao, H.; Tian, D. Phosphorus biogeochemistry regulated by carbonates in soil. *Environ. Res.* **2022**, *214*, 113894. [CrossRef] [PubMed]
34. Ma, Q.Y.; Traina, S.J.; Logan, T.J.; Ryan, J.A. In-situ lead immobilization by apatite. *Environ. Sci. Technol.* **1993**, *27*, 1803–1810. [CrossRef]
35. Ruby, M.V.; Davis, A.; Nicholson, A. In-situ formation of lead phosphates in soils as a method to immobilize lead. *Environ. Sci. Technol.* **1994**, *28*, 646–654. [CrossRef]
36. Liang, X.J.; Kierans, M.; Ceci, A.; Hillier, S.; Gadd, G.M. Phosphatase-mediated bioprecipitation of lead by soil fungi. *Environ. Microbiol.* **2016**, *18*, 219–231. [CrossRef]
37. Purchase, D.; Scholes, L.N.L.; Revitt, D.M.; Shutes, R.B.E. Effects of temperature on metal tolerance and the accumulation of Zn and Pb by metal-tolerant fungi isolated from urban runoff treatment wetlands. *J. Appl. Microbiol.* **2009**, *106*, 1163–1174. [CrossRef]
38. Fomina, M.; Hillier, S.; Charnock, J.M.; Melville, K.; Alexander, I.J.; Gadd, G.M. Role of oxalic acid overexcretion in transformations of toxic metal minerals by *Beauveria caledonica*. *Appl. Environ. Microb.* **2005**, *71*, 371. [CrossRef]
39. Su, M.; Meng, L.; Zhao, L.; Tang, Y.; Qiu, J.; Tian, D.; Li, Z. Phosphorus deficiency in soils with red color: Insights from the interactions between minerals and microorganisms. *Geoderma* **2021**, *404*, 115311. [CrossRef]
40. Tian, D.; Su, M.; Zou, X.; Zhang, L.; Tang, L.; Geng, Y.; Li, Z. Influences of phosphate addition on fungal weathering of carbonate in the red soil from karst region. *Sci. Total Environ.* **2021**, *755*, 142570. [CrossRef]
41. Zhang, L.; Yang, X.; Li, S.; Tang, L.; Chen, T.; Gu, T.; Chen, G.; Gadd, G.M.; Li, Z. A contrast of Pb(II), Cd(II), and Cu(II) toxicities to *Aspergillus niger* through biochemical, morphological, and genetic investigations. *J. Hazard. Mater.* **2023**, *446*, 130691. [CrossRef] [PubMed]
42. Kobayashi, K.; Hattori, T.; Honda, Y.; Kirimura, K. Oxalic acid production by citric acid-producing *Aspergillus niger* overexpressing the oxaloacetate hydrolase gene oahA. *J. Ind. Microbiol. Biot.* **2014**, *41*, 749–756. [CrossRef] [PubMed]
43. Kim, J.W.; Barrington, S.; Sheppard, J.; Lee, B. Nutrient optimization for the production of citric acid by *Aspergillus niger* NRRL 567 grown on peat moss enriched with glucose. *Process. Biochem.* **2006**, *41*, 1253–1260. [CrossRef]
44. Knox, K. Le Châtelier's Principle. *J. Chem. Educ.* **1985**, *62*, 863. [CrossRef]
45. Gautam, A.K.; Bhadauria, R. Diversity of fungi and mycotoxins associated with stored triphala churn and its ingredients. *J. Biol. Sci.* **2011**, *11*, 226–235. [CrossRef]
46. Susca, A.; Proctor, R.H.; Morelli, M.; Haidukowski, M.; Gallo, A.; Logrieco, A.F.; Moretti, A. Variation in fumonisin and ochratoxin production associated with differences in biosynthetic gene content in *Aspergillus niger* and *A. welwitschiae* isolates from multiple crop and geographic origins. *Front. Microbiol.* **2016**, *7*, 1412. [CrossRef]
47. Han, X.; Jiang, H.; Li, F. Dynamic ochratoxin A production by strains of *Aspergillus niger* intended used in food industry of china. *Toxins* **2019**, *11*, 122. [CrossRef]
48. Fiema, M.; Wlodarczyk, A.; Wojkowska-Mach, J.; Garlicki, J.; Gregorczyk-Maga, I. Atypical presentation of *Aspergillus niger* infection in the oral cavity as a prediction of invasive pulmonary *Aspergillosis* in a patient with COVID-19: Case report and literature review. *Microorganisms* **2022**, *10*, 1630. [CrossRef]