Isolation, Identification and Characterization of Two Aluminum-Tolerant Fungi from Acidic Red Soil

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Abstract Acidic red soil from a forest in Jiangxi Province was selected to isolate aluminum (Al)-resistant microbes, from which eight fungi were isolated. Two strains (S4 and S7) were found to be extremely tolerant to Al concentrations of up to 550 mmol L⁻¹ and could grow at low pH levels (3.20–3.11). Morphological and 26S rDNA sequence analyses indicated that strain S4 belonged to *Eupenicillium*, while strain S7 was an unclassified *Trichocomaceae*. Further investigation showed that both strains were endowed with the ability to resist Al; strain S4 accumulated such a substantial amount of Al that its growth was limited to a larger extent than strain S7. The lower amounts of Al adsorbed in the mycelium and the much larger amounts of Al retained in the medium, in addition to the color change of the culture solution, implied that these two strains may resist Al by preventing Al from entering the cell and by chelating Al by secreting unique metabolites outside of the cell.

Keywords Aluminum toxicity · Al tolerance · Al tolerant microbe · Fungi

Introduction

As one of the most abundant elements in Earth's crust, Aluminum (Al) is non-essential and usually lacks of biological function in organisms [1, 2]. In acidic and neutral soils, Al remains mainly in a trivalent oxidation state and as stable mineral forms, which have no biological toxicity. However, as soil pH decreases, Al will become increasingly available its active monomeric form (monomeric Al³⁺), in the soil solution and become toxic to organisms [3]. For decades, increasing acid deposition and immoderate application of acidic fertilizers have led to a significant release of active Al in the soil and other environments [4]. As a result, Al has been recognized as one of the major factors that restrict crop production in acidic soils [5]. To solve this problem, some agronomic measures, such as the use of lime and alkali fertilizer, have been applied to modify surface soil [6–8]. However, these approaches cause secondary pollution to the soil [9].

As an important component of the soil environment, soil microorganisms are inevitably affected by Al toxicity [10]. However, some microbes can survive very well in acidic soil and possess the ability to alleviate Al toxicity through enhancing Al tolerance in crops. For example, *Andropogon virginicus* (broomsedge) can grow in seriously Al-toxic soil due to colonization by the arbuscular mycorrhizal fungus *Glomus clarum* [11]. It is undeniable that the isolation and characterization of microbes highly resistant to Al is the premise of bioremediation. Several researchers in this field have identified several highly Al-resistant fungi [12, 13]. In fact, microbial Al endurance is closely correlated with the

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particular environment. As such, there is opportunity to isolate highly Al-resistant microbes from soils worldwide.

In the present study, we reported the isolation and characterization of two *Trichocomaceae* fungi that exhibited extremely high Al resistance from the typical acidic red soil that is widespread in the Jiangxi province of China. The aims of this study were (1) to isolate and identify aluminum-tolerant fungi from acidic red soil, (2) to investigate the morphological, physiological and biochemical characteristics of the fungi under different Al stressful conditions, and (3) to study the Al resistance ability of isolated fungi.

Materials and Methods

Sample Preparation

Acidic red soil was collected from the wild forest of Chinggang Mountain in Jiangxi province, Eastern China. The GPS coordinates of the field are 27°06'N and 115°01'E. The soil solution was prepared by adding 10 g soil to 90 mL sterile deionized water. After shaking for 2 h and allowing to stand for 30 min, the supernatant was carefully collected for tests.

Enrichment of Al Resistant Microorganisms

We added 5 mL of fresh soil solution to 45 mL enrichment medium (Tryptone 10 g L⁻¹, NaCl 5 g L⁻¹, glucose 5 g L⁻¹, beef extract 3 g L⁻¹ and agar 20 g L⁻¹, natural pH) containing 20 mM Al³⁺ (AlCl₃·6H₂O), and the mixture was shaken in culture for 3 days at 28 °C, 220 rpm. Then, 1 mL of the suspension was used for plate streaking on potato-Martin substratum for fungi enrichment and on LB medium for bacterial enrichment. No colonies were found on the LB medium. Eight colonies from the potato-Martin substratum with different morphologies were picked for further separation. Finally, the purified strains were kept in slant culture, preserved at -80 °C and marked as S1–S8.

Colony Morphology of Al Resistant Fungi

A small bit of mycelium was picked from the slant culture, inoculated onto potato-Martin substratum and allowed to recover for 3 days. Then, a small amount of spores were inoculated onto potato-dextrose-agar medium (glucose 20 g L⁻¹, agar 18 g L⁻¹ and potato extract 1000 mL, natural pH) containing different concentrations of Al³⁺ and were cultured in an incubator at 28 °C. Six days later, we observed colony morphology and determined the diameter of the colony and the diameter of the spore-producing area.

Identification of Highly Al Resistant Fungi

26S rDNA identification was carried out as follows: purified S1–S8 strains were inoculated onto potato-Martin substratum containing 50–600 mmol L⁻¹ Al³⁺ for more stringent selection. Among the eight strains, S4 and S7 showed the strongest Al resistance ability. These two strains were then subjected to further purification and preserved for subsequent tests. After genome DNA extraction, the specific primers NL-1 (5'-GCATATCAA-TAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') were used to amplify the 26S rDNA partial sequence of the S4 and S7 strains, respectively. The PCR procedure: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 57 °C for 30 s and 72 °C for 1 min, with final extension at 72 °C for 10 min. PCR products were collected, purified and cloned into the pMD18-T plasmid vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5 α competent cells. Ten colonies from each strain were randomly selected, and the 26S rDNA inserts were confirmed using colony-PCR with two vector primers M13F (5'-CGCCA GGGTT TTCCC AGTCA CGAC-3') and M13R (5'-GAGCG GATAA CAATT TCACA CAGG-3'). A positive colony from each strain was sequenced at Sangon Biotech (Shanghai) Co., Ltd., China. The sequence was then submitted to GenBank to blast against the 26S rDNA sequence database. A phylogenetic tree was constructed using the neighbor-joining (NJ) method.

Hematoxylin Staining

Strains S4 and S7 were inoculated onto potato-Martin substratum containing 50 mmol L⁻¹ Al³⁺ and cultured at 28 °C for 6 days and then hematoxylin dye (0.2 % hematoxylin, 0.01 % KIO₃ and 0.1 mmol L⁻¹ NaOH) was carefully added into the culture dish until the medium surface was just submerged. After 5 min, the strains were observed and photographed.

Evaluation of Acid-and Al-Tolerance in the S4 and S7 Strains

The S4 and S7 strains were inoculated onto potato-Martin substratum (containing 20 mmol L⁻¹ Al³⁺) for 5 days and then the spores were collected with sterile water and made into a suspension with a final concentration of 10⁸ spores mL⁻¹. Five microliters of 10⁸ spores mL⁻¹ of the S4 and S7 strain suspensions were point-planted onto the centers of plates of potato-Martin substratum containing 0 mmol L⁻¹ Al³⁺ (pH 6.10), 50 mmol L⁻¹ Al³⁺ (pH 3.20), 100 mmol L⁻¹ Al³⁺ (pH 3.18), 150 mmol L⁻¹ Al³⁺ (pH 3.16) and 200 mmol L⁻¹ Al³⁺ (pH 3.11). The above

pH values were measured after Al^{3+} addition. Then, five microliters of the above suspension were point-plated onto the centers of plates of potato-Martin substratum (without Al^{3+} addition) at different pH values (were adjusted to 3.20, 3.18, 3.16, and 3.11 to keep consistent with aforementioned pH values). We determined the diameters of the colonies at 1, 2, 3, 4, 5, 6 and 7 d.

Determination of the Dry Weight of Mycelium and Al Content

We again inoculated 1 mL of 10^8 spores mL^{-1} of the strain S4 and S7 suspensions onto 150 mL of potato-Martin liquid medium containing 0, 50, 100, 150 or 200 mmol L^{-1} Al^{3+} . After shaking the culture for 5 days at 30 °C and 180 rpm, we filtered and collected the mycelium. Then, we washed the mycelium with distilled water at least 3 times and subsequently dried the mycelium to a constant weight at 105 °C. The weight of the dry mycelium was then determined.

The Al content of the mycelium was determined using ICP-MS (ELAN9000, PerkinElmer Inc., USA), and the samples for testing were prepared according to Shi's method (2008). Li (6) was used as an internal standard element, and the instrument parameters were set as 1.35 kW power, 1.18 L min^{-1} carrier gas flow rate and 7 mm sampling depth.

Statistical Analysis of Data

The data were presented as the Mean $\pm$ SE ($n = 3$). All data were analyzed using SPSS Statistics 19 for windows (IBM Corporation, United States). Significance analysis was performed using one-way analysis of variance (ANOVA) followed by a least significant difference test (LSD, $p \leq 0.05$).

Results

Physicochemical Properties of Soil Samples from the Forest

We determined some of the physicochemical properties of the tested soil and found that it was typical acidic soil (pH 4.46) with a relatively high content of soluble Al^{3+} (shown in Table 1), suggesting excessive acidity.

Table 1 Partial physicochemical properties of acidic red soil from the forest

Sample	pH	OM ^a /g kg^{-1}	N/mg kg^{-1}	P/mg kg^{-1}	K/mg kg^{-1}	Al^{3+} /mg kg^{-1}
Soil	4.46	17.8	127.5	4.05	48.5	11.5

^a OM organic matter

Isolation of Al-Resistant Fungi and the Determination of Colony Parameters

We successfully isolated eight strains from the forest acidic soil that grew well under 20 mmol L^{-1} Al^{3+} stress. We further investigated their growth conditions under different Al^{3+} concentrations (0, 10, 15, 20, 25 and 30 mmol L^{-1}). All fungi produced obvious growth under different Al^{3+} conditions, but their growth abilities varied. Among the eight strains, S4 and S7 exhibited the best growth. Although S1, S3, S5 and S6 could grow under different Al^{3+} stresses, they failed to produce spores, suggesting an inhibitory effect of Al toxicity on their propagation (Table 2). As the Al^{3+} concentration increased, both the diameter of the colony and the diameter of the spore-producing area decreased. In particular, under the 30 mmol L^{-1} Al^{3+} condition, only S2, S4, S7 and S8 could produce spores, among which S4 and S7 had relative higher spore-producing capacities (Table 2). Therefore, the S4 and S7 strains could be considered highly resistant to Al. Further investigations revealed that strains S4 and S7 could even survive in up to 550 mmol L^{-1} Al, which might be recognized as the highest Al-tolerant fungi to be reported (Table 3).

Morphological and Molecular Identification of Strains S4 and S7

We point-plated the S4 and S7 spores onto potato-dextrose-agar medium and cultured at 28 °C for 5 days. The diameters of the colonies reached approximately 6–7 cm for each strain. Both S4 and S7 colonies grew in a circular manner with a villiform surface that was white at first and gradually became yellow. S4 mycelium was nonseptate. Its conidiophore was verticillate and had fastigiated branching. The conidium was spherical, single or catenate. The S7 mycelium was also nonseptate, but it had podocytes. Its conidiophore differed in length and grew basally. The acrosomal vesicle was semi-spherical and there were catenate conidia on top (Fig. 1).

PCR amplification of 26S rDNA revealed that there was a single 528 bp amplicon in the S4 strain and a single 616 bp amplicon in the S7 strain. The two PCR products were then collected for sequencing. The sequences of the S4 and S7 strains represented the 26S rDNA D1/D2 domain fragments (DDBJ Accession No. AB712029–AB712030). The RDP classifier (<http://rdp.cme.msu.edu/classifier>) demonstrated that both S4 and S7 belonged to *Trichocomaceae*. Specifically, the S4 strain was an

Table 2 The diameter of colony and the diameter of spore-producing area under different concentrations of Al^{3+}

No.	0 mmol L^{-1}		10 mmol L^{-1}		15 mmol L^{-1}		20 mmol L^{-1}		25 mmol L^{-1}		30 mmol L^{-1}	
	DC	DS	DC	DS	DC	DS	DC	DS	DC	DS	DC	DS
S1	55.0	31	48.3	18.2	41.1	0	42.3	0	38.5	0	38.5	0
S2	33.4	29.2	27.0	26.8	24.7	24.0	24.2	23.0	23.5	22.5	24.2	22.2
S3	55.2	22.7	49.3	0	44.5	0	44.0	0	44.0	0	41.4	0
S4	72.5	70.3	56.2	53.8	45.0	42.3	43.0	40.2	41.1	39.2	40.3	39.0
S5	46.1	6.4	44.0	0	42.2	0	41.5	0	38.5	0	35.5	0
S6	52.0	12.0	40.2	0	39.8	0	39.0	0	37.5	0	36.5	0
S7	88.0	87.0	88.0	87.0	86.5	86.0	84.3	53.2	75.0	44.0	73.2	42.2
S8	28.5	24.5	20.0	17.4	19.0	16.5	18.5	16.2	15.0	12.3	14.0	10.2

DC diameter of colony (mm), DS diameter of spore-producing area (mm)

Table 3 Growth of strains S4 and S7 in the presence of inorganic monomeric Al

Strains	Inorganic monomeric Al (mM)								
	0	50	100	200	300	400	500	550	600
S1	+++++	+++	++	++	+	–	–	–	–
S2	+++++	+++	++	+	–	–	–	–	–
S3	+++++	+++++	+++++	++	+	–	–	–	–
S4	+++++	+++++	+++++	+++++	+++++	+++++	+++	++	–
S5	+++++	++	+	–	–	–	–	–	–
S6	+++++	+++	+	+	+	–	–	–	–
S7	+++++	+++++	+++++	+++++	+++++	+++	++	+	–
S8	+++++	+++	++	–	–	–	–	–	–

Medium used: GM, pH3.5. Shaking at 28 °C and for 7 d. Growth +++++, very good; +++++, good; +++, fair; +, little; –, no growth

Fig. 1 Spore morphology of strains S4 and S7

Eupenicillium while the S7 strain was an unclassified genus. Moreover, The GenBank blast results also confirmed this prediction. Six strains that had over 97 % sequence similarity to S4 and S7 were picked to construct a neighbor-joining phylogenetic tree (Fig. 2).

Acid tolerance and Al Tolerance of the S4 and S7 Strains

As shown in Fig. 3, the S4 and S7 strains both had favorable tolerance to acid and Al. However, Al had an inhibitory effect on them; that is, the higher the

concentration of Al^{3+} , the lower the growth of the fungi. Furthermore, the longer the incubation time, the lower the propagation rate of the fungi. S4 and S7 seemed to have little difference in growth when they suffered under the same Al^{3+} condition and culture time, except that the S7 strain grew faster than the S4 strain under 150 mmol L^{-1} Al^{3+} during 7 days of cultivation. Both strains seemed to be adaptive to acidic conditions. They could grow well in pH 3.20–3.11 solution, which is considered to be a strongly acidic environment. These results indicated that, in acidic soil, it was the Al itself and not the acidity that retarded the growth of these microbes.

Fig. 2 Acquisition of 26S rDNA sequences of S4 and S7 strains and the phylogenetic analysis with other strains (Phylogenetic tree was evaluated by bootstrap analysis of the neighbor-joining method with 1000 resamplings using MEGA 5.0)

Fig. 3 The growth condition of strains S4 and S7 under different concentrations of Al and different pH levels

Al Absorption in Strains S4 and S7

As shown in Fig. 4 (left), the content of Al^{3+} significantly increased in both the S4 and S7 strains due to the

availability of Al in the growth medium. In strain S4, the Al content varied from 0.026 mg kg^{-1} in the control to 8.31 mg kg^{-1} in the 200 mmol L^{-1} Al-treated solution, while in strain S7 these values were, respectively,

Fig. 4 Al absorption of strains *S4* and *S7* (left) and hematoxylin staining of the colonies (right). *a, b* indicated strain *S4* without/with hematoxylin and *c, d* indicated strain *S7* without/with hematoxylin

0.029 mg kg⁻¹ and 7.39 mg kg⁻¹, implying that strain *S4* might have a stronger absorbent ability toward Al³⁺ than strain *S7*. This finding could be confirmed by the hematoxylin staining results shown in Fig. 4 (right). After the two strains were cultured on potato-Martin substratum (50 mmol L⁻¹ Al³⁺) for 6 days, the colony of strain *S4* became gray, while the colony of strain *S7* was yellow. Obviously, the morphologies of the colonies of the two strains differed greatly following the addition of hematoxylin. The centers of the plates of the two strains were darker than the rest, but the difference was that *S4* was fuchsia, while *S7* was orange. As the dye mordant of hematoxylin, Al can form an amaranthine complex with hematoxylin, and the greater amount of Al, the darker the complex. The color changes in the colonies revealed that both *S4* and *S7* could absorb Al³⁺; however, strain *S4* seemed to absorb much more Al³⁺, implying that strain *S4* might have suffered much more Al stress, leading to growth inhibition by Al toxicity. This was consistent with previous findings in arbuscular mycorrhizal fungi [14].

We continued to investigate the dry weight of the *S4* and *S7* strains after 5 days of suspension culture at different Al³⁺ concentrations. As shown in Fig. 5, Al stress significantly reduced the dry weights of both strains; however, strain *S4* experienced a greater reduction than strain *S7*. Under 200 mmol L⁻¹ Al³⁺, strain *S4* fell by nearly 85 %, while strain *S7* was down by only 27 % compared with the controls. This difference might be attributed to excessive Al³⁺ accumulation in strain *S4*, such that its growth rate was lower than strain *S7*, which could be confirmed by the sizes of the diameters of the two colonies (shown in Table 2).

Fig. 5 The dry weight of strains *S4* and *S7* after 5 days suspension culture at different concentrations of Al³⁺

Discussion

Due to strong rain leach, acidic red soils contain low pH, high level of exchangeable aluminum and low content of organic matter. The exchangeable aluminum can be very toxic to plants and microorganisms. Our previous study reported that fungi could survive at a higher concentration of Al³⁺ than bacteria [15]. Kanazawa et al. [16] also reported that fungi accounted for most highly Al-resistant microbes. In the present study, eight Al-resistant fungi were successfully isolated from the acidic forest soil, but no bacteria were isolated, which is consistent with the results of Kawai et al. [13], suggesting that fungi were more resistant to acidity and Al toxicity [17].

Fig. 6 The growth conditions of strains S4 and S7 in liquid medium containing different Al^{3+} at different incubation time

As one of the most important components of soil ecosystems, soil microbes are involved in the degradation and purification of soil pollutants [18–20], and play crucial roles in the process of nutrient cycling and energy flow in the soil ecosystem. Furthermore, they enhance the stress tolerance of plants [11, 21]. For these reasons, many researchers have made efforts to identify highly Al-resistant microbes that confer Al detoxification effects in acidic soil because this soil type accounts for approximately 40 % of global arable land. Konishi et al. (1994) identified a bacterial strain (*Flavobacterium* sp.) from indigenous tea soil that could tolerate high Al concentrations of up to 2000 ppm (approximately 73 mmol L^{-1}) at pH 3.5; they noticed that the strain could decrease the Al content by increasing the pH value of the medium [22]. An extremely highly Al-tolerant fungus, *Penicillium janthineleum* F-13, could survive in $100 \text{ mmol L}^{-1} \text{ Al}^{3+}$; however, the mechanism of Al resistance was obscure [23]. In the present study, eight fungi that exhibited different Al-tolerant capacities were isolated, among which two strains (S4 and S7) were considered as extremely highly acid- and Al-tolerant. These two strains were capable of surviving in $550 \text{ mmol L}^{-1} \text{ Al}^{3+}$ medium and might be recognized as the most Al-resistant fungi cultured in an artificial acidic environment to date. Morphological and 26S rDNA sequence analysis indicated that strain S4 belonged to *Eupenicillium*, while strain S7 belonged to an unclassified member of Trichocomaceae. Similarly, a previous report revealed that *Eupenicillium parvum* (isolated from acidic tea soil) exhibited high tolerance (100 mmol L^{-1}) to Al in vitro [24].

Even if fairly resistant to Al toxicity, the growth of microbes would be hindered in highly concentrated Al conditions [25, 26]. We observed that the growth condition, especially the propagation rate of the isolated fungi, was notably decreased as the Al concentration increased. However, both strains seemed to be acidophilic; they could grow well at pH 3.11, suggesting that Al may be the chief culprit (s) for retarding the growth of microbes, whereas acidity probably acts as an accessory factor that increased the amount of active Al in the acidic red soil of the forest.

Although the absolute content of Al^{3+} was low in both strains, strain S4 absorbed much more Al^{3+} than strain S7 (Fig. 4). Excessive absorption, however, prevented the growth of strain S4 to some extent and led to limitations in the dry weight of its mycelium. There are two main mechanisms that can be used to interpret Al resistance in microbes: one is the chelation of Al outside of the cell via the cellular secretion of special compounds and the other is the sequestration of Al inside of the cell via the formation of complexes [27–31]. According to our experimental results, the majority of Al^{3+} was still retained in the medium (data not shown), implying that strains S4 and S7 possibly resisted Al stress by preventing soluble Al from entering the cells. We noticed that there were obvious morphological differences between strain S4 and strain S7 in Chavez's liquid medium containing different concentrations of Al for different incubation times. Under 0 or $50 \text{ mmol L}^{-1} \text{ Al}^{3+}$, strain S4 could form a mycelium pellet, while strain S7 could not form this structure, suggesting that these strains have different characteristics. Interestingly, extending the incubation time

and increasing the concentration of Al led to substantial changes in the color of the medium in both strains (Fig. 6). We hypothesized that there might be new special metabolites that were produced and secreted into the medium and that these metabolites were probably connected with Al tolerance in strains S4 and S7. Future work needs to focus on resolving these matters.

Conclusions

The present work reported the isolation and identification, as well as the characterization, of Al-tolerant microorganisms from acidic red soil of the forest in the Jiangxi province of China. Eight fungi were isolated, among which two strains belonging to *Trichocomaceae* were recognized to be extremely highly Al- and acid-resistant. The results suggest that, in acidic red soil, fungi are the major group of microbes able to cope with Al toxicity. Furthermore, Al toxicity, rather than acidity, is one of the main restriction factors that retards the growth of fungi. Limited accumulation of Al in mycelia suggests that these two strains might invoke Al resistance by blocking Al from entering the cell and chelating Al by secreting unknown compounds. However, their ecological roles in forest are seldom known and need further investigation.

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