

Arbuscular mycorrhizal colonization of a Cd hyperaccumulator *Viola baoshanensis* at Baoshan Pb/Zn Mine

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Research Article

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Abstract

Despite great potential for arbuscular mycorrhizal (AM) fungi associated with heavy metals (HMs) hyperaccumulators in the phytoremediation of contaminated sites, rather limited information is available in literature about the community structure of AM fungi (AMF) in the rhizosphere of hyperaccumulators in nature. A field survey was conducted to investigate the colonization status and community structure of AMF of *Viola baoshanensis*, a Cd hyperaccumulator, growing at Baoshan Pb/Zn Mine. Shoot/root ratios of 1.78 for Cd, and 2.57 for Zn in *V. baoshanensis* indicate that these two metals were preferentially transported from roots to shoots, whereas Pb was mainly stored in roots. The roots of *V. baoshanensis* were extensively colonized by AMF with M% of 69.1% , and A% of 46.9% , whereas mycorrhizal colonization was not affected by concentrations of Cd, Zn and Pb in the soil. 15 AMF species (5 *Glomus*, 3 *Rhizophagus*, 2 *Claroideoglomus*, 2 *Septoglomus*, and each one of *Ambispora*, *Funneliformis*, and *Sclerocystis*) were identified by a morphological method. The dominant AMF genus was *Glomus*, showing high tolerance to excess Cd, Zn and Pb, while *Glomus ambisporum* and *Claroideoglomus etunicatum* were the most abundant species in the rhizosphere of *V. baoshanensis*.

1. Introduction

Many mining sites were characterized by great toxicity of HMs, and poor nutrients and soil structure leading to destroyed vegetation cover (Wong 2003; Orłowska et al. 2005), meanwhile revegetation of these sites is urgent in order to inhibit HMs contamination of the surrounding soil via erosion and runoff (Shetty et al. 1994; Vieira et al. 2018). However, there are many plants called metallophytes endemic to the contaminated soils have developed tolerance mechanisms to HMs (Perrier et al. 2006; Antosiewicz et al. 2008). The use of these plants to remediate the HMs contaminated sites are known as phytoremediation, which is environmentally friendly and cost-effective compared with conventional methods (McGrath & Zhao 2003; Arthur et al. 2005; Rajkumar et al. 2012).

Hyperaccumulators, a particular group of metallophytes, are able to accumulate HMs in shoots at extraordinary high levels and can be used in one of the key processes of phytoremediation called phytoextraction (Yang et al. 2019). An ideal candidate for efficient phytoextraction requires rapid growth and big shoot biomass of the plant (Wei et al. 2014). Nevertheless, hyperaccumulators often have slow growth and low biomass (Khan et al. 2000), optimization of their fitness and growth need to consider the symbiotic microorganisms in soil (Sánchez-Castro et al. 2017; Wang et al. 2021) .

The most important symbiotic partner is AMF, as they can form symbiosis with most of higher plants, providing a direct connection between soil and plant roots (Smith & Read 1997). AMF can efficiently improve water and minerals uptake of plants, therefore plants are widely colonized by AMF in many polluted soils (Shetty et al. 1994; Pawłowska et al. 1996; Del Val et al. 1999; Whitfield et al. 2004). However, until *Berkheya coddii* was found to be colonized by AMF (Turnau & Mesjasz-Przybyłowicz 2003), hyperaccumulators had been thought as non-mycorrhizal (Leyval et al. 1997), since there may be a trade-off between hyperaccumulation and mycorrhization because of both requiring carbon investment

from plants (Audet 2013). Thereafter, several mycorrhizal hyperaccumulators have been documented (Regvar et al. 2003; Vogel-Mikuš et al. 2005; Perrier et al. 2006; Wu et al. 2007; Rashid et al. 2009; Wei et al. 2014). It has been demonstrated that AMF can increase biomass and survival of hyperaccumulators, thus showing a great potential for phytoextraction (Al Agely et al. 2005; Orłowska et al. 2013).

However, there is a lack of clear elucidation about AMF role in hyperaccumulation of HMs, as the effects of AMF on metal uptake are diversified (Alford et al. 2010). Studies have found that AMF reduce (Liu et al. 2005; Jankong et al. 2007), increase (Wu et al. 2009; Orłowska et al. 2013), have mixed effects (Vogel-Mikuš et al. 2006), or no effects (Liu et al. 2009) on HMs uptake by hyperaccumulators. Up to date, only 4% of the over 700 reported hyperaccumulators have been studied in relation to AM symbiosis (Wang et al. 2021), thus resulting in little understanding of the mechanisms involved in the interactions between plants, AMF and HMs (Dietterich et al. 2017). In nature, an equilibrium have been reached between AMF, host plants, and HMs over time (Zarei et al. 2008), therefore investigations of AMF communities at abandoned mining sites can greatly enhance the their utilization in phytoremediation (Sánchez-Castro et al. 2017).

Viola baoshanensis has been identified as a Cd hyperaccumulator, and also a strong accumulator of Zn and Pb by field survey at Baoshan Pb/Zn Mine, and by hydroponic and pot experiments (Liu et al. 2004; Wu et al. 2010). This species could extract Cd and Zn with high efficiency in a field study (Zhuang et al. 2007) and could be well colonized by AMF in Cd and/or Pb contaminated soils in pot experiments (Zhong et al. 2012). However, the AM status of indigenous *V. baoshanensis* at the mining site remains unclear and need further research. The objectives of the present study were (1) to determine the accumulation of Cd, Zn and Pb by *V. baoshanensis* in relation to soil HMs concentrations; (2) to investigate the AM colonization status of *V. baoshanensis* in relation to soil chemo-physical properties ; (3) to examine the AMF community structure in the rhizosphere of *V. baoshanensis*.

2. Material And Methods

2.1 Site description and sampling

Field sampling was conducted at Baoshan Pb/Zn Mine (25°42'N, 112°35'E), Guiyang County, Hunan Province, China, with a long history of mining and being abandoned in 1996 (Liu et al. 2004). Guiyang County has a humid subtropical monsoon climate with an annual average temperature and rainfall of 17.2°C and 1385 mm, respectively. *V. baoshanensis* is a perennial herb and endemic to the mine at an elevation of 400–650 m (Liu et al. 2004). Soil samples and plant material were collected at the flowering stage of *V. baoshanensis* in May, 2018. In total, 35 plants of *V. baoshanensis* were randomly collected at a slope near the mining pit, and soil samples were taken from the rhizosphere of the plants at a depth of 0–20 cm and stored at 4°C for further analysis.

2.2 Chemical analyses

The fresh soil samples were passed through 2-mm sieve and then divided into two parts. One portion was stored at 4°C at its real moisture status for the spore counting and identification, while the other was air-dried for the chemical analyses. The methods described by Zhong et al.(2012) were used to determine the total and DTPA extractable concentrations of Cd, Zn, and Pb of the soil samples, and total phosphorus of soil and plant samples. Soil pH was determined in a suspension (5:1, deionized water:soil), and measured by a pH electrode.

Plant shoots and roots were separated and about 1g fresh fine roots was left for observation of root AM colonization. The concentrations of Cd, Zn, and Pb of shoots and roots were determined by the methods described by Zhong et al.(2012). The characteristics of uptake and transfer of Cd, Zn and Pb by *Viola baoshanensis* were described by translocation factor (TF) and the bioconcentration factor (BF), which are defined by McGrath &Zhao (2003).

2.3 Root AM colonization

Fine roots of *V. baoshanensis* were cut into 1 cm segments cleared with 10% KOH and stained with 0.01% trypan blue (Koske & Gemma 1989). 50 root segments per plant were examined under a compound microscope at 200× magnification to determine the mycorrhizal colonization by the magnified intersection method (McGonigle et al. 1990). Mycorrhizal parameters such as mycorrhizal frequency (F%), relative mycorrhizal root length (M%), relative arbuscular richness (A%), relative vesicular richness(V%) were used to assess the mycorrhizal status of *V. baoshanensis*.

2.4 Spore extraction and identification

Spores were isolated from soils by wet sieving and decanting followed by sucrose density gradient centrifugation (Tommerup & Kidby 1979). The extracted spores were mounted on the slides in polyvinyl alcohol-lactic acid glycerol (PVLG) and Melzer's reagent for observation under a light microscope at 400-fold magnification. Spore identification was based mainly on morphological characteristics such as spore colour, wall structures, and subtending hyphae (Aguilera et al. 2014). Classification and taxonomic identification of AMF is according to identification reports (Redecker et al. 2013; Bills et al. 2015), species descriptions from INVAM homepage (<http://www.invam.caf.wvu.edu>), and other institutional collection of original species. Relative abundance of each species in one sample was determined by the percentage of the number of spores of the species in relation to the total number of spores in the sample (Del Val et al. 1999).

2.5 Statistical analysis

Relative abundance of AMF species was tested for normality and analyzed by one-way analysis of variance (ANOVA), followed by the Duncan test at the level of 5% significance. Pearson's correlation coefficient (r) was used when calculating correlations between mycorrhizal colonisation parameters, HMs concentrations of plants, and soil physico-chemical properties.

3. Results And Discussion

3.1 Soil chemical properties

The soil from the mining site was slightly alkaline (pH = 7.60) and heavily polluted by Cd, Zn and Pb with maximal concentrations of 990 mgkg⁻¹ Cd, 16548 mgkg⁻¹ Zn, and 20111mgkg⁻¹ Pb. A significant variation in soil metal concentrations between 35 samples was observed as shown by large S.E.s (Table 1), indicating the heterogeneous nature of the mining soil. Total and DTPA-extractable concentrations of Cd, Zn and Pb were shown in Table1, while the total soil phosphorus was 857 ± 57 mgkg⁻¹.

Table 1
Cd, Zn and Pb concentrations of the soils from the rhizosphere of *V. baoshanensis* at Baoshan Pb/Zn Mine (Mean ± S.E., n = 35)

Total Metal (mgkg ⁻¹)			DTPA-extractable metal (mgkg ⁻¹)		
Cd	Zn	Pb	Cd	Zn	Pb
147 ± 30	7290 ± 720	7239 ± 694	36 ± 7	421 ± 42	506 ± 57

DTPA-extractable fractions ranged from 6.1 to 33.6% for Cd, from 0.9 to 9.7% for Zn, and from 4.5 to 13.1% for Pb. Significant correlations between total and DTPA-extractable concentrations were found for Cd ($r = 0.990$, $p < 0.01$), Zn ($r = 0.767$, $p < 0.01$), and Pb ($r = 0.871$, $p < 0.01$), respectively. DTPA-extractable Cd, Zn, and Pb were 25%, 6% and 7% of the total soil Cd, Zn and Pb, respectively. As the soil was slightly alkaline, it is reasonable that the bioavailability of these metals was low, which is in accordance with findings at the same site (Wu et al. 2010).

3.2 Cd, Zn, Pb and P accumulation in *V. baoshanensis*

As shown in Table 2, the mean shoot Cd concentration was > 100 mgkg⁻¹, and the TF value was > 1, thus confirming the hyperaccumulation of Cd by *V. baoshanensis*. Meanwhile, the BF value for Cd is 3.06, also showing high capacity of *V. baoshanensis* for the accumulation of Cd. Significant correlations were found between shoot Cd concentration and total Cd ($r = 0.484$, $p < 0.01$), DTPA-extractable Cd ($r = 0.499$, $p < 0.01$) concentration in soil, and between root Cd concentration and total Cd ($r = 0.753$, $p < 0.01$), and DTPA-extractable Cd ($r = 0.747$, $p < 0.01$) concentration in soil, which is consistent with the results of Liu et al. (2004).

In contrast, no significant correlations were found in Zn concentrations between plants and soils. Although the TF of Zn was 2.57 indicating very efficient transport of Zn from roots to shoots, the shoot Zn concentration was much lower than the threshold, thus no hyperaccumulation of Zn was observed in *V. baoshanensis*, which was confirmed by the low BF value (average 0.15) as shown in Table 2.

Table 2

Cd, Zn and Pb concentrations (mg kg^{-1} , DW), BF and TF of *V. baoshanensis* collected at Baoshan Pb/Zn Mine (Mean $\pm$ S.E., n = 35)

Metal	Shoot	Root	BF	TF
Cd	250 $\pm$ 19	165 $\pm$ 17	3.06 $\pm$ 0.43	1.78 $\pm$ 0.13
Zn	719 $\pm$ 32	705 $\pm$ 134	0.15 $\pm$ 0.02	2.57 $\pm$ 0.46
Pb	159 $\pm$ 12	546 $\pm$ 44	0.03 $\pm$ 0.004	0.32 $\pm$ 0.02

In contrast, the TF of Pb for *V. baoshanensis* was 0.32, indicating that most of the Pb accumulated was immobilized in roots. Shoot Pb concentration significantly correlated with total Pb ($r=0.398$, $p<0.05$), DTPA-extractable Pb ($r=0.361$, $p<0.05$) concentration in soil, while root Pb concentration significantly correlated with total Pb ($r=0.621$, $p<0.01$), DTPA-extractable Pb ($r=0.673$, $p<0.01$) concentration in soil. The BF value for Pb was much lower than those for Cd and Zn. In addition, shoot and root P concentrations of *V. baoshanensis* were 1882 ± 93 and $4948 \pm 357 \text{ mg kg}^{-1}$, respectively. Significant correlations were found between soil total P concentration and P concentrations in shoots or roots ($r = 0.411$, $p < 0.05$; $r = 0.454$, $p < 0.01$), respectively.

Accumulation and exclusion are two basic strategies taken by metallophytes growing on sites with high concentrations of HMs, the accumulation characteristics of which are commonly described by BF and TF values (McGrath & Zhao 2003). TF values over 1 in metal accumulator species are normal, whereas those in metal excluder species are often less than 1 (Baker 1981). In this survey, the shoot Cd concentration of *V. baoshanensis* exceeded the threshold, and the BF and TF for Cd were both greater than 1, thus confirming that *V. baoshanensis* is a Cd hyperaccumulator (Liu et al. 2004; Wu et al. 2010). The TF for Cd (1.78) of *V. baoshanensis* is comparable to that of a Cd, Zn hyperaccumulator, *Sedum alfredii*, naturally growing at a mining site with a TF of 1.87 (Yang et al. 2019). In addition, *Thlaspi praecox*, another Cd, Zn hyperaccumulator sampled from the vicinity of a lead mine and smelter in Slovenia, had a TF of 1.4 for Cd (Vogel-Mikuš et al. 2005). As hyperaccumulators are ideal candidates for phytoextraction, hyperaccumulation of Cd is of particular importance since so far only 10 species has been identified across the world (Wang et al. 2021).

As shoot concentration of Zn was lower than 10000 mg kg^{-1} , *V. baoshanensis* was not a Zn hyperaccumulator. However, the TF of *V. baoshanensis* for Zn was 2.57, suggesting there was efficient transfer of Zn from roots to shoots, while the TFs for Zn of *S. alfredii* and *T. praecox* were 1.24 and 3.2, respectively (Vogel-Mikuš et al. 2005; Yang et al. 2019). As for Pb, the TF is 0.32, indicating that most of Pb uptake were stored in roots. TFs of 0.3 were common for Pb in *Thlaspi* species (Vogel-Mikuš et al. 2005), meanwhile the TF of *S. alfredii* for Pb was 0.28 (Yang et al. 2019).

3.3 Root colonization by AMF

High levels of colonization for *V. baoshanensis* collected from the Baoshan Pb/Zn mine at flowering stage was observed with mean F% of 94.5%, and mean M% of 69.1% ranging from 51.3 to 83.8%. The roots were extensively colonized by intraradical hyphae forming well-developed arbuscules, but few vesicles were observed. The average A% was 46.9%, while the average V% was only 1.7% (Fig. 1).

There were positive correlations between F% and M% ($r = 0.628$, $P < 0.01$), between F% and A% ($r = 0.401$, $P < 0.05$), and between M% and A% ($r = 0.538$, $P < 0.01$). However, root colonization was not correlated with Cd, Zn and Pb concentrations in the soils, indicating that no inhibitory effect of HMs on mycorrhizal colonization of plant roots was evident.

Plant stage of development is one of the factors influencing AM colonization and flowering is a development reference point for the determination of AM status in calamine plants (Pawlowska et al. 1996), as the enhanced elemental demand during flowering favors mycorrhizal colonization, thus offering adequate essential elements (Pongrac et al. 2007). Therefore, the plants of *V. baoshanensis* in this survey were collected at the flowering stage.

It has been suggested that selective pressure would decrease colonization unless the symbiosis supplied some benefits to the host (Whitfield et al. 2004). In contrast to several hyperaccumulators belonging to the genus *Thlaspi* poorly colonized by AMF (Regvar et al. 2003; Vogel-Mikuš et al. 2005), *V. baoshanensis* was well colonized by AMF. Colonization rate of 30% was considered as the threshold of a functional symbiosis (Zarei et al. 2010), the M% of *V. baoshanensis* was well above this level, indicating that AMF provide some benefits to their hosts in toxic soils (Whitfield et al. 2004; Rashid et al. 2009). Nevertheless, no significant correlation between the mycorrhizal status of *V. baoshanensis* and concentrations of metals in the soils was observed, which is consistent with the findings of Pierrier et al. (2006). In addition, *Viola tricolor* L. grown on a calamine spoil mound (rich in Cd, Zn and Pb) was also found to be well colonized by AMF with percentage of root colonized of above 60% (Pawlowska et al. 1996), suggesting that the colonization status of a plant species may have a family basis.

As arbuscules are the major site of nutrient exchange (Smith & Read 1997), the arbuscular richness is considered as the most useful parameter for measuring mycorrhizal status (Orłowska et al. 2005). A% of *V. baoshanensis* is considerably higher than those of Cd hyperaccumulators *Desmostachya bipinnata* and *Dichanthium annulatum*, which were 7% and 6%, respectively (Rashid et al. 2009), further indicating the existence of an effective symbiosis between *V. baoshanensis* and indigenous AMF.

3.4 AMF spore abundance and diversity

A total of 15 AMF species from 7 genera and 3 families of Glomeromycota were isolated and identified from the rhizosphere soil of *V. baoshanensis* (Table 3). Most of the species found belong to the family Glomeraceae (12 species), and most of spores identified belonged to this family (Table 3). In terms of species richness, the most abundant genus was *Glomus* (5 species), and followed by the genus *Rhizophagus* (3 species), while the relative abundance of *Glomus* was 44.0%, followed by *Claroideglomus* (18.5%) (Fig. 2).

Table 3

Relative abundance (mean, n = 35) of AM fungal species associated with *Viola baoshanensis* at Baoshan Pb/Zn Mine

AM Fungal species	Relative abundance (per cent)
Family Glomeraceae	
<i>Funneliformis mosseae</i>	3.58 a,b,c
<i>Glomus ambisporum</i>	14.20 h
<i>Glomus brohultii</i>	6.44 b,c,d,e,f,g
<i>Glomus formosanum</i>	7.59 c,d,e,f,g
<i>Glomus</i> sp	9.80 f,g
<i>Glomus hoi</i>	6.00 a,b,c,d,e,f,g
<i>Rhizophagus aggregatus</i>	4.89 a,b,c,d,e
<i>Rhizophagus fasciculatus</i>	3.83 a,b,c,d
<i>Rhizophagus invernium</i>	1.82 a
<i>Sclerocystis rubiformis</i>	7.92 d,e,f,g
<i>Septoglomus constrictum</i>	8.86 e,f,g
<i>Septoglomus deserticola</i>	2.39 a,b
Family Claroideoglomeraceae	
<i>Claroideglomus claroideum</i>	6.75 c,d,e,f,g
<i>Claroideglomus etunicatum</i>	11.79 g,h
Family Ambisporaceae	
<i>Ambispora leptoticha</i>	4.17 a,b,c,d
Values followed by the same letters do not differ statistically by the Duncan test at the level of 5% significance.	

There was a dominance of Glomeraceae species in HMs polluted areas such as minging tailings, which is a common observation worldwide (e.g. Zarei et al., 2010; Sun et al. 2016; Sánchez-Castro et al. 2017), showing that fungi in this family are more disturbance-tolerant than those in family Acaulosporaceae and Gigasporaceae (van der Heyde et al. 2017). In HMs polluted sites, soil pH has been found as an important factor in determining the AMF community structure (Sun et al. 2016; Vieira et al. 2018; Yang et al. 2019). *Acaulospora* was widely observed in acid soils and *Gigaspora* are tolerant for acid pH (Clark 1997; van der Heyde et al. 2017), while species of *Glomus* are common in neutral and alkaline soils (da Silva et al. 2005). In consistent with this observation, the most abundant genus from the rhizosphere of *V. baoshanensis* was *Glomus*, as the pH of the soil is slightly alkaline at Baoshan Pb/Zn mine. In addition,

previous studies show that species from genus *Claroideglomus* are often tolerant to HMs (Cornejo et al. 2013; Park et al. 2016) and the results of this survey showed that *Claroideglomus* was the second abundant genus.

The number of identified spores was 146 per 100 g soil on average. *Glomus ambisporum* and *Claroideglomus etunicatum* were the representative species, followed by *Glomus* sp and *Septoglomus constrictum* (Table 3). Most of the AMF species identified in this survey have already been found in soils with high concentrations of HMs. Spores of *G. ambisporum* were found at a mining site contaminated with Zn, Cd, and Pb in southeast Kansas (USA), where *S. constrictum* was abundantly found (Shetty et al. 1994). *C. etunicatum* was identified from five of six sites in a copper mining area in northeastern Brazil (da Silva et al. 2005), meanwhile *C. etunicatum* was the most dominant species in the post-mining area (Korea), where *S. constrictum* and *C. claroideum* were also abundantly observed (Park et al. 2016). *F. mosseae* is normally found in soil contaminated with Zn and Pb (Turnau et al. 2001; Zarei et al. 2008), although the relative abundance of this species was only 3.58% in this study. The species identified in this survey have practical implications since metal-tolerant AMF should be isolated and inoculated in plants for phytoremediation (Vieira et al. 2018).

The proper integration of AMF and hyperaccumulators has been considered as the solution to pollution of HMs and finding appropriate AMF is very important (Vogel-Mikuš et al. 2006; Miransari M. 2011). The use of indigenous AMF species from long-term polluted sites has been widely observed as more suitable than laboratory strains (Sánchez-Castro et al. 2017). Up to now, information about interactions between native AMF and hyperaccumulators has been rather limited in published literature (Wang et al. 2021). Further work, therefore, needs to focus on isolating AMF from the rhizosphere of native hyperaccumulators in the light of community structure to explore their roles in phytoremediation under laboratory and field conditions.

4. Conclusions

Field survey confirmed that *V. baoshanensis* was a Cd hyperaccumulator but not a Zn hyperaccumulator, although efficient Zn transfer from roots to shoots was observed, while most of Pb uptake was stored in roots. This study showed that *V. baoshanensis* growing at Baoshan Pb/Zn mine with high concentrations of HMs were extensively colonized by AMF and forming well-developed arbuscules, suggesting that the host plant may derive some benefits from the symbiosis, and those AMF were HMs tolerant. *Glomus* was the dominant genus in the rhizosphere of *V. baoshanensis*, while *G. ambisporum* and *S. etunicatum* were the most abundant species.

Declarations

Authors' Contributions: Zhong Weiliang is the only author of this manuscript and no other author contributes to the manuscript.

Data availability statements

The data that support the findings of this study are not openly available due to sensitivity of the data and are available from the corresponding author upon reasonable request.

Statement of competing interests

The author declares no competing interests.

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Figures

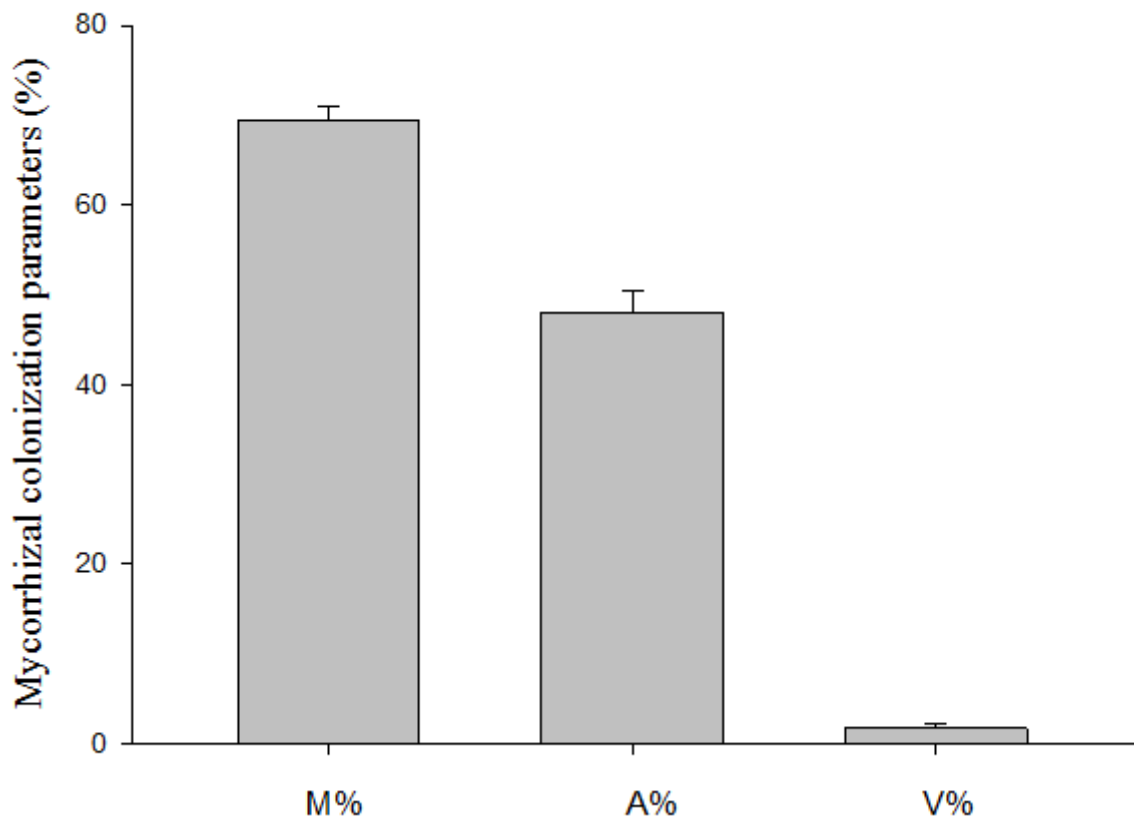


Figure 1

Mycorrhizal status of *V. baoshanensis* collected at Baoshan Pb/Zn Mine. Bars represents standard errors (n=35).

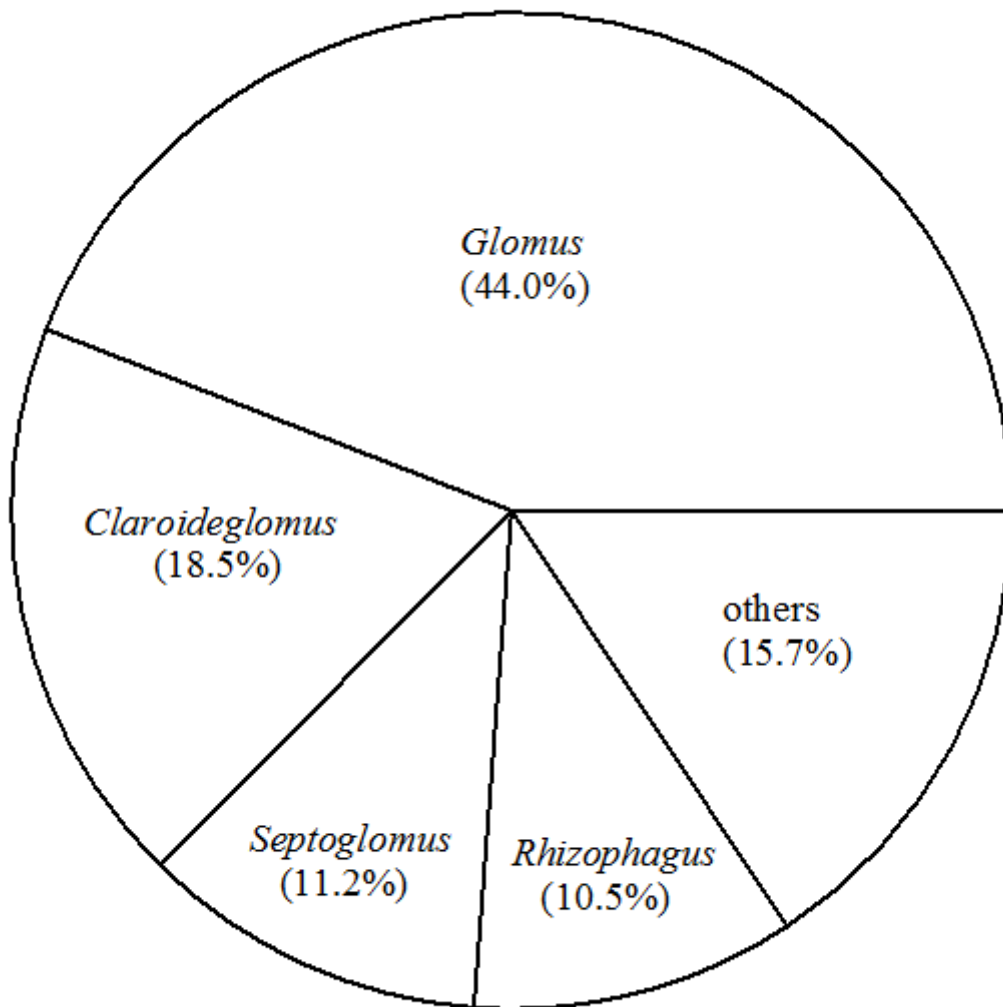


Figure 2

Proportion of genera (% of the total spores) of Glomeromycota in the rhizosphere of *V. baoshanensis* at Baoshan Pb/Zn Mine