Arbuscular Mycorrhizal Fungi Community Structure in Rhizosphere Soil and Colonization in Root of BADH Transgenic Maize BZ-136 and Receptor Parental Maize

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Abstract

Betaine aldehyde dehydrogenase (*BADH*) transgenic maize has a capability to grow under drought and salt stress, yet problems remain about the risk of cropping *BADH* on symbiotic microbes. A pot experiment was carried out to assess the impact of *BADH* transgenic maize BZ-136 on arbuscular mycorrhizal fungi (AMF) colonization in plant and community structure in rhizosphere soil compared with that of parental maize Zheng58 in neutral and saline-alkaline soil. Microscope observation found that BZ-136 only had a significant effect on AMF colonization at elongation stage. High-throughput sequencing analysis revealed that the AMF taxonomic composition kept consistency at the genus level between transgenic BZ-136 and non-transgenic parental Zheng58. NMDS analysis verified the slight difference in community structure between BZ-136 and Zheng58 presented a agrotype-dependent pattern. AMF community indices showed that BZ-136 had a higher richness at flowering stage in saline-alkaline soil and had a higher diversity at mature stage in neutral soil. Heatmap analysis also illuminated AMF community structure of transgenic maize at species level was similar to that of non-transgenic maize. In summary, cropping transgenic *BADH* maize has minor or transient effects on AMF colonization and soil AMF community structure, while agrotype have a stronger effect on AMF community structure.

1 Introduction

Salinity is a very notorious environmental factor that not only decreases the crop production, but also changes the soil properties and upsets the ecological balance (Guo et al., 2012, Prochazkova et al., 2013). It has been estimated that the salinized areas increase at a rate of 10% annually and more than 50% of the arable land would be salinized by the year 2050 (Jamil et al., 2011). Transgenic plants have been gradually adopted throughout the world due to their persistently agronomic and economic benefits in agriculture. Glycine betaine (GB) is considered as one of the most important osmolytes resistant to abiotic stress (Hayashi and Murata 1998; McNeil et al., 1999), and betaine aldehyde dehydrogenase (*BADH*) is the key enzyme regulating GB synthesis. *BADH* transgenic plants have the capability to tolerate a wide range of abiotic stresses, especially salt and drought (Zhang et al., 2011). However, *BADH* gene or GB can also convey salt tolerance to pathogenic fungi (Pearce and Strange, 1977) or be taken as nutrients by microbes (Kappes and Bremer, 1998; Boncompagni et al., 1999), which may increase the potential of persistence and invasiveness, and unpredictable non-target effects.

Arbuscular mycorrhizal fungi (AMF) are a group of non-target microorganisms that can form mutualistic symbioses with the roots of most plant species, provide nutrients (mainly P and N) and water (Wang and Qiu, 2006; Smith and Read, 2008), and enhance the disease resistance and stress tolerance of plants (Hajiboland et al., 2010; Steinkellner et al., 2012). AMF are more sensitive to changes in the physiology of the host plant than other soil microorganisms (Johnson and Pfleger, 1992; Giovannetti and Avio, 2002), thus they should be taken as a potential key non-target microorganism to be monitored in assessment of the environmental influence of transgenic plants. Since some studies have revealed that transgenic crops may have positive or negative effects on the structure and function of the AMF community (Glandorf et al., 1997; Turrini et al., 2005), the European Union and non-government organizations suggest that non-target effect on AMF should be evaluated before the release of transgenic plants (Anderson et al., 2005; Knox et al., 2008).

As maize is one of the most heavily mycorrhizal-dependent plant species, the interactions between transgenic maize and AMF should be preferentially investigated in natural and abiotic stress circumstance. In this study, we conducted experiments in neutral and alkaline soils to evaluate the environmental risks of transgenic *BADH* maize BZ-136, mainly exploring the effect of cropping BZ-136 on AMF colonization and rhizosphere AMF community structure at four growth stages via comparison with parental maize Zheng58.

2 Materials And Methods

2.1 Plant and soil materials

Two maize lines including the receptor parental maize (Zheng58) and the transgenic *BADH* maize (BZ-136) were provided by agricultural college, Northeast Agricultural University, China. Maize BZ-136 was developed by inserting betaine aldehyde dehydrogenase (*BADH*) gene into genome of inbred maize line of zheng58 under the control of ubiquitin promotor (Di et al, 2015). The plants of transgenic maize BZ-136 express higher amount of betaine aldehyde dehydrogenase and grow better than the wild type plants under NaCl stress. Both transgenic and parental maize seeds were planted in neutral and saline-alkaline soil. Neutral soil was taken from horticulture experimental station of Northeast Agricultural University, China, and saline-alkaline soil was taken from Lindian, Heilongjiang province, China. The chemical properties of two soils were listed in Table 1.

Table 1
Chemical property of soil before planting maize

	Natural soil	Saline-alkali soil
pH	7.38 ± 0.14b*	8.08 ± 0.09a
organic C (%)	4.43 ± 0.07a	4.39 ± 0.06a
total N ($g \cdot kg^{-1}$)	0.78 ± 0.04a	0.60 ± 0.05b
available N ($mg \cdot kg^{-1}$)	102.37 ± 2.36a	80.92 ± 1.88b
available P ($mg \cdot kg^{-1}$)	26.25 ± 1.65a	9.64 ± 1.07b
available K ($mg \cdot kg^{-1}$)	187.39 ± 4.10a	118.73 ± 3.82b
electrical conductivity (Ec) ($ds \cdot m^{-1}$)	0.106 ± 0.005b	0.653 ± 0.006a
*All values were given as mean ± SD. The standard error is based on the average of three biological replicates.		

2.2 Experimental design

The pot experiment was conducted at experimental station of Northeast Agricultural University (longitude 126°73', latitude 45°75'), China. All plantlets were well watered to three-leaf stage, and then supplied with 100 mL 0.5 Hoagland nutrient solution in salt-treated group and control group every 3 days (0.3 mol NaCl was added to the nutrient solution in the salt-treated group on the first day). On the seventh day, plants of each maize line treated with NaCl were transplanted in the saline-alkaline soils and non-treated plants were

transplanted in the neutral soils. Each pot (20 cm diameter, 20 cm deep) containing ca. 5 kg of soil was placed at 75 cm spacing into a 3.0 m×3.4 m plot (a total of four plots) in a greenhouse, watered every 3 days, fertilized five times (on the 7th day; the 27th day; the 47th day; the 67th day and the 87th day), applying a similar fertilizer regime (pure nitrogen 150 kg hm^{-2}, P_2O_5 content 70 kg hm^{-2}, K_2O content 80 kg hm^{-2}) to ensure a basic fertility for plant growth in pot experiment.

2.3 Soil and root samples treatment

Maize and soil samples in each pot were collected at seedling (the 17th day), elongation (the 52th day), flowering (the 85th day) and mature (the 118th day) stage, respectively. First, the maize plants were removed from the pot, and then rhizosphere soils were shaken off and collected in a plastic bag. Each soil sample was a composite of soils from three different pots. The soil samples were sieved through 2 mm meshes and stored at -80℃ for DNA extraction. The one hundred fresh root tips (1cm) were washed repeatedly with distilled water and bleached in chlorine for 1–2 min, then the tips were acidified with 1% HCl and stained with 0.05% Trypan Blue according to the method of Phillips and Hayman (1970). AMF colonization was quantified under a microscope and the percentage of AMF colonization including arbuscules, vesicles and hyphae for each maize was calculated as following equation (Giovannetti and Mosse1980): % of colonized roots $= \frac{the amount of roots with AMF}{the amount of total roots} \times 100.$

2.4 DNA extraction and high-throughput sequencing analysis

Total genomic DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, USA). Next generation sequencing library preparation and Illumina MiSeq sequencing were performed at Majorbio Bio-Pharm Technology (Shanghai, China). The 18S rRNA gene was amplified using fungal primers AMV4-5NF (5'-AAGCTCGTAGTTGAATTTCG-3')/AMDGR (5'-CCCAACTATCCCTATTAATCAT-3'). PCR products were pooled and purified using the DNA Extraction Kit. QuantiFluor-ST Fluorometer was used to quantify the purified amplicons (Promega, Madison, WI, USA), and then a composite sequencing library was constructed by combining equimolar ratios of amplicons from all samples. The reads that were shorter than 200 bp were removed, then all the remaining sequences were considered in the subsequent analyses. DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument (Majorbio Bio-Pharm Technology, Shanghai, China). Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.8.0, http://bio.cug.edu.cn/qiime/). Alpha diversity was calculated by Silva (Release128 http://www.arb-silva.de) at 97% sequence identity. Beta diversity analysis was calculated using weighted and unweighted UniFrac distances among groups, and non-metric multidimensional scaling (NMDS) was performed in R software to compare AMF community structure across all samples. Unweighted pair group method with arithmetic mean (UPGMA) clustering tree was constructed to compare the hierarchical relationships among groups. The Mann-Whitney U-test was performed to compare diversity indices between two groups. Heat maps were performed using Mothur and R software (http://www.mothur.org/wiki/MainPage).

2.5 Statistical analysis

Statistical analyses were implemented using one-way analysis of variance (ANOVA). Biochemical characteristics data were analyzed with SPSS 18.0. Statistical significance was calculated by Student's t-test

and a probability value $p < 0.05$ was considered significant. ANOSIM differences were calculated using the vegan package in R software. Data were expressed as mean and standard deviation.

3 Results

3.1 AMF colonization

In this study, some mycorrhizal parameters, including arbuscular, hyphal, vesicle and total AMF colonization in the roots, were measured and compared between *BADH* transgenic maize BZ-136 and non-transgenic maize Zheng58 (Fig. 1 and Fig. 2), and the arbuscular, vesicular, and hyphae structures of arbuscular mycorrhizal fungi in maize roots were observed under microscopy at 40 times (Fig. 3). The arbuscular colonization ranged from 3%-17% for BZ-136 and 3%-14% for Zheng58 in neutral soil, while 9%-19% and 13%-19% for BZ-136 and Zheng58 respectively in saline-alkaline soil. The colonization of hyphae and vesicles were relatively higher than that of arbuscules for BZ-136 (40–70%) and Zheng58 (27%-70%) in neutral soil as well as in saline-alkaline soil (54%-71% for BZ-136 and 46%-73% for Zheng58). It was found that colonization of hyphae, vesicles, and total AMF in *BADH* transgenic maize at elongation stage was significantly lower than that in non-transgenic parental maize in both neutral and saline-alkaline soil ($p < 0.05$). However, ANOVA showed that the maize lines had no significant impact on total AMF colonization in both neutral ($F = 0.054$, $p = 0.820$) and saline-alkaline soil ($F = 0.063$, $p = 0.806$) throughout the growth cycle (Table 3). However, growth stage in neutral soil ($F = 33.932$, $p < 0.001$) and agrotype ($F = 8.330$, $p = 0.006$) seemed to greatly affect AMF colonization as compared between *BADH* transgenic maize and non-transgenic maize.

Table 3
Results of the statistical analysis (ANOVA) on mycorrhizal parameters

	Hyphae	Vesicle	Arbuscular	AMF colonization
Maize (neutral)	1.02 (0.328)	0.154 (0.700)	1.088 (0.312)	0.054(0.820)
Maize (saline-alkaline)	0.271 (0.610)	0.079 (0.782)	1.014 (0.329)	0.063(0.806)
Stage (neutral)	46.921 (<0.001)	61.004 (<0.001)	4.913 (0.013)	33.932 (<0.001)
Stage (saline-alkaline)	2.778 (0.075)	1.935 (0.165)	1.634 (0.221)	0.266 (0.849)
Soil	8.865 (0.005)	19.663 (<0.001)	40.480 (<0.001)	8.330 (0.006)

The numbers indicate the F-value of the ANOVA (*p*-value). Response variables were hyphae, arbuscular, vesicle and arbuscular mycorrhizal fungi (AMF) colonization.

3.2 Richness and diversity of AMF

According to high-throughput sequencing analysis of 18S rRNA gene, a total of 454722 high quality sequences from 16 samples with an average of 28420 sequences per sample were obtained. The raw sequences were deposited to the NCBI Sequence Read Archive with a group accession No. SRP145063. For alpha and beta diversity analyses, OTU tables were rarefied at 19636 reads. A total of 371 OTUs ranging from 8 to 51 were identified based on the conventional criterion of 97% similarity (equal to species level). Based on Shannon and Chao1 index showed in Table 2, the range of total number of OTUs estimated by Chao1 estimator was 12–25

(Zheng58, neutral soil), 9–32 (BZ-136, neutral), 8–48 (Zheng58, saline-alkaline soil) and 20–53 (BZ-136, saline-alkaline soil). Shannon index of BZ-136 was 1.07–2.63 in neutral soil and 1.44–2.75 in saline-alkaline soil, and Zheng 58 was 1.02–1.77 in neutral soil and 1.77–2.97 in saline-alkaline soil, respectively. The diversity and richness of AMF community of BZ-136 were only different from those of parental Zheng58 in saline-alkali soil at flowering stage and in neutral soil at mature stage.

Table 2
Different arbuscular mycorrhizal fungi diversity indices in different samples

Group	Sequences	OTUs	Chao1	Shannon	Group	Sequences	OTUs	Chao1	Shannon
NZS	37533	11	12	1.77	AZS	21781	35	35	2.70
NBS	19832	12	13	1.77	ABS	21838	41	41	2.75
NZE	25301	17	17	1.02	AZE	31231	48	48	2.97
NBE	38378	12	12	1.07	ABE	33264	51	53	2.73
NZF	22945	12	12	1.67	AZF	26900	8	8	1.77
NBF	37085	9	9	1.70	ABF	21242	25	28	2.13
NZM	22254	17	25	1.67	AZM	22716	23	23	1.98
NBM	38305	30	32	2.63	ABM	34117	20	20	1.44

N, means neutral soil; A, means saline-alkaline soil; Z, means Zheng58 maize line; B, means BZ-136 maize line; S, means seedling stage; E, means elongation stage; F, means flowering stage; M, means mature stage.

3.3 Relative abundance of AMF taxonomic composition

The phylogenetic classification based on 18S rRNA sequences at genus level for all samples was showed in Fig. 4. A total of four primary genus including *Glomus f Glomeraceae*, *unclassified c Glomeromycetes*, *Diversispora*, *Acaulospora* and other extreme minority were found in tested samples. BZ-136 had similar AMF taxonomic composition with Zheng58 at each stage at genus level (Fig. 4a). *Glomus* was the most dominant genera on average in both maize lines, and its relative abundance of BZ-136 was 8.23%, 24.36% and 10.35% higher than Zheng58 in alkaline-saline soil at seedling, flowering and mature stage and 12.63% lower than Zheng58 in neutral soil at seedling stage, respectively. In addition, the relative abundance of *Diversispora* of BZ-136 was decreased in saline-alkaline soil. Figure 4b showed the relative abundance of AMF in the rhizosphere soils of two maize lines at specie level. *Unclassified g Glomus f Glomeraceae, Glomus mosseae VTX00067 and Glomus group B Glomus lamellosum VTX00193* were the most dominant species on average. BZ-136 showed some differences from Zheng58 in the relative abundance of a few species at different stage.

3.4 AMF community composition

The relative distributions and abundances of AMF were analyzed on the base of the top 50 OTUs and the results were showed in Fig. 5a. In each soil type, the obvious difference of relative abundance between the two maize lines appeared at seedling stage. In saline-alkaline soil, compared with Zheng58, the relative abundances of ten OTUs (OTU8, OTU10, OTU22, OTU23, OTU25, OTU27, OTU31, OTU42, OTU50 and OTU58) were decreased in BZ-136. In neutral soil, BZ-136 had higher

abundance of five OTUs (OTU44, OTU64, OTU67, OTU69 and OTU78) and lower abundance of four OTUs (OTU19, OTU49, OTU77 and OTU81). Among all these OTUs, only OTU69 belonged to *Diversispora*, the other OTUs all belonged to *g Glomus f Glomeraceae*. Clustering analysis manifested that AMF communities closely to one another in the same type of soil (Fig. 5b), indicating that *BADH* transgenic maize BZ-136 had insignificant effect on soil AMF community composition. As seen in Fig. 5c, the NMDS ordination of the AMF community composition in different soil samples showed an acceptable stress level of 0.14, indicating a good representation of AMF taxonomic composition. Based on the analysis of relative abundance of OTUs, it was found that soil type contributed more on AMF community composition than maize line, which was confirmed by ANOSIM (Neutral soil vs. Saline-alkaline soil; R = 0.7271, p=0.001). There was no significant difference between two maize lines in AMF community composition (p 0.05) both in neutral soil (R = -0.0313) and saline-alkaline soil (R = 0.0729).

4 Discussion

Transgenic *BADH* maize BZ-136 was developed for its high GB dehydrogenase activity and extra accumulation of GB against salinity (Di et al., 2015). However, as a regulatory element in stress tolerance mechanism, the *BADH* gene may influence the expression of other genes in adversity, which may consequently affect non-target soil microflora (Mallory and Zapiola, 2008; Warwick et al., 2009; Wolt, 2009). In the previous study, we found that *BADH* transgenic maize BZ-136 has marginal effects on bacterial community diversity (Bai et al., 2019). AMF are the most important soil microbes that should be monitored in the risk assessment of transgenic plant due to their sensitivity to environmental changes such as soil type and climate factor. Previous studies have shown that GB content in plants increased with salinity stress and AMF inoculation (Hashem et al., 2006). In this study, the impact of cropping transgenic maize on AMF was detected in neutral and saline-alkaline soil for the first time. In essence, both individual colonization and community structure of AMF are fundamental for many of the biochemical processes in soil. The similar rates of hyphal, vesicle, arbuscular and AMF colonization in the maize BZ-136 and Zheng58 indicated that *BADH* or GB had no or minor effects on AMF colonization in roots of transgenic maize. However, some reports have suggested that transgenic plants have significant effects on AMF colonization, symbiotic mycelial growth, and the normal development of appressorium (Turrini et al., 2005). In the rhizosphere, with the constitutively expression and accumulation of GB, the intraradical or extraradical development of arbuscules and hyphae of AMF were insignificantly affected, indicating that transgenic *BADH* didn't decrease or increase the affinity of maize with AMF. High-throughput sequencing technology provided us with a comprehensive insight into the AMF community in rhizosphere soils of maize. According to the obtained data, no evidence was found that *BADH* maize affected the AMF community structure, but Shannon index and Chao1 index showed that BZ-136 had a greater community richness than Zheng58 at flowering stage in saline-alkaline soil and greater community diversity at mature stage in neutral soil. Our results were consistent with that of Liang et al (2015), who found that there were no significant differences in the AMF community structure of transgenic high-methionine soybean ZD91 and its parental isoline ZD in the same year.

AMF symbiotic development are subjected to the changes in environmental factors, including soil type, fertility level, temperature and agronomic practices. Previous study showed that higher percentage of AM colonization occurred at lower soil salinity level (Tian et al., 2004; Asghari et al., 2008). In this study, to eliminate the effect

ed in neutral and saline-alkaline soil, where obvious difference in

AMF colonization and community structure was found in BZ-136 as well as Zheng58. Therefore, it was denoted that transgenic maize would not affect AMF colonization in maize and community structure in soil, but the soil type might slightly impact the colonization or community structure. In this study, *Glomus* was the most dominant genus among AMF community, which was in agreement with earlier studies by Wu et al. (2013). In fact, the dominance of *Glomus* in AMF community of rhizosphere soil reflects the higher tolerance of plants to abiotic stress in agricultural systems (Hassan et al., 2011; Shi et al., 2012; Wetzel et al., 2014). Factors such as annual variation, weather, agricultural management, soil type and plant growth stage are more influential on AMF community structure than the genetic modification (Hannula et al., 2014). For example, the diversity and richness of BZ-136 in saline-alkaline soil was higher than that in neutral soil in most stages. In addition, we found the AMF species *Glomus-mosseae*-VTX00067 had a higher abundance in neutral soil at seedling, elongation and flowering stage, but a lower abundance in saline-alkaline soil. Therefore, environmental factors must be taken into account when evaluating the effects of transgenic crops on rhizosphere microorganisms.

Early study on seasonal variation in AMF colonization with perennial grasses was reported by Giovannetti (1985). Current reports have also shown that AMF colonization in root and AMF community in soil often present a seasonal dynamic (Tian et al., 2011). Generally, AMF experience three phases to colonize plant root, including a lag phase where percentage infection increases slowly, then a linear increasing phase and finally a constant phase (Varela-Cervero et al., 2016). For plant species with short-lived roots, AMF colonization increased rapidly with the growth of roots and then decreased when roots senesced. In this study, significant differences in AMF colonization between Zheng58 and BZ-136 were observed at elongation stage in both kinds of agrotype, but no adverse effects were found throughout the growth cycle based on ANOVA. At elongation stage, leaves widened, roots prolongated and internodes differentiated. Accompanied with the vigorous growth of maize, AMF colonization also increased, indicating that seasonality or growth stage had an impact on AMF colonization. Meanwhile, NMDS and cluster analysis showed that not only maize line but also growth stage had a greater effect on composition of AMF community than soil type. Previous studies have reported that non-Bt corn grown on soils previously planted with Bt maize for five seasons had minor impact on the AMF community (Zeng et al., 2014). Luo et al (2017) found that the colonization rates of AMF in rice fluctuated with growth stages, reaching their peak at jointing stage and then decreasing at flowering and ripening stages. In contrast, some studies found that the AMF community composition either in colonized roots or soil was not affected by seasonality (Santos-González 2007, Öpik et al., 2008, Davison et al., 2012). The AMF community is also significantly influenced by growth stage, where distinct seasonal trends in the AMF community were reported for maize roots (Hijri et al., 2006) (Hijri et al., 2006). These contradictory results may be caused by other important determinants such as soil physiochemical properties, environmental conditions or plant species, (Gavito et al., 2003), which needs further investigation.

5 Conclusion

Cropping transgenic BZ-136 had no obvious effect on AMF colonization in neutral or saline-alkaline soil throughout the growth cycle, except significantly reduced the colonization rate of AMF at elongation stage. In terms of AMF community richness and diversity rhizosphere soil, transgenic BZ-136 showed similar pattern with non-transgenic maize Zheng58. The AMF community structure of transgenic BZ-136 was stable during

the potting period in neutral soil, but not in saline-alkaline soil. Our results demonstrated that transgenic *BADH* maize BZ-136 had minor or transient impact on AMF colonization and AMF community structure.

Declarations

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

Rui Li and Xing Zeng conceptualized the idea of the review; performed literature search, data analysis, and data curation; and prepared the original draft of the manuscript.

Xin Bai helped in the drafting of the manuscript.

Zhenhua Wang helped in the drafting of the manuscript.

Juanjuan Qu supervised the team and reviewed the manuscript.

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Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

Not applicable

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Figures

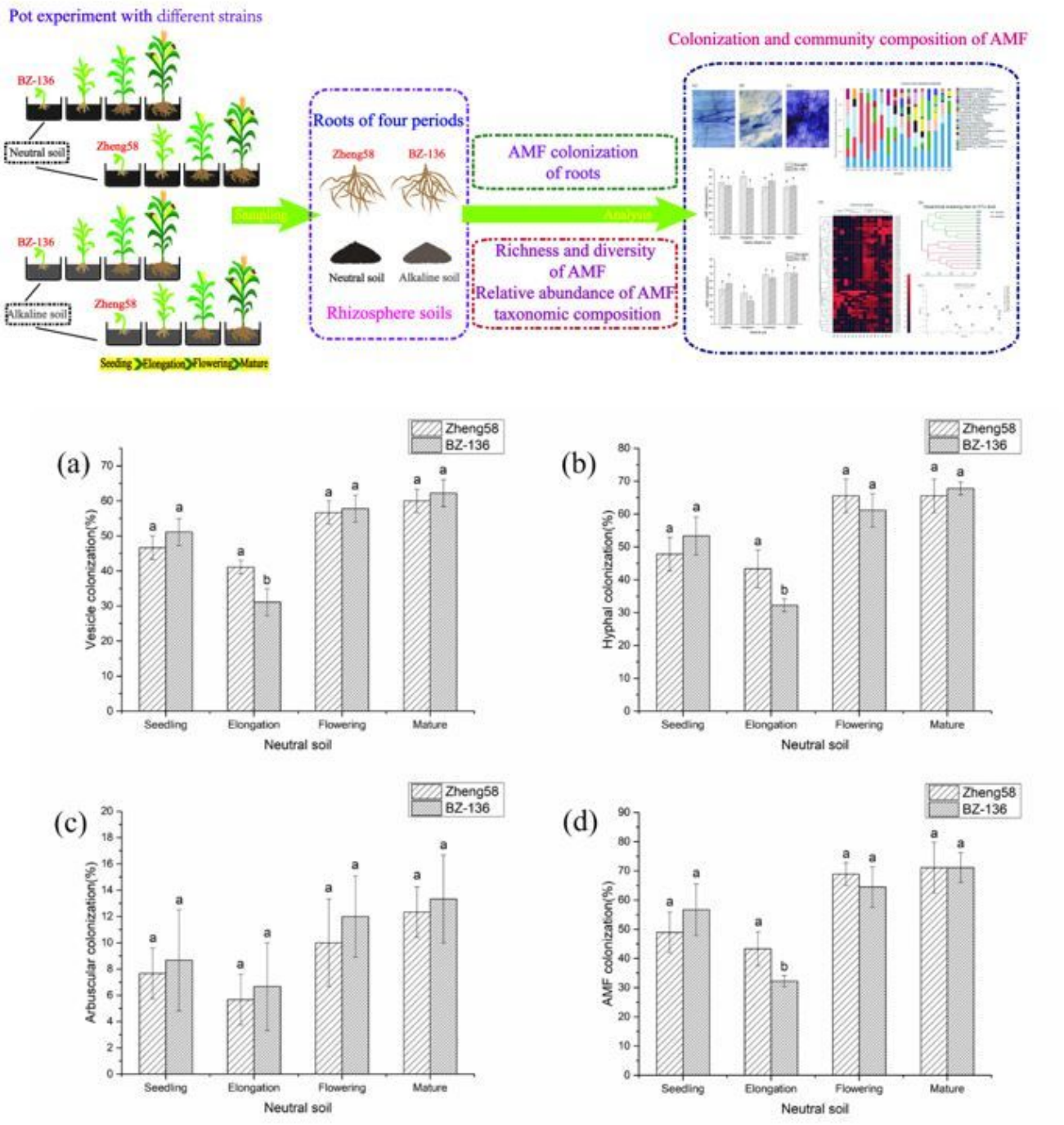

Figure 1

Colonization of different genotypes of maize roots by indigenous arbuscular mycorrhizal fungi in neutral soil. Vertical bars indicate the standard error of the means. Same letter above each bar represents the value that is unsignificantly different ($p > 0.05$) according to the LSD test ($n= 3$) at the same stage. The standard error is based on the average of three biological replicates.

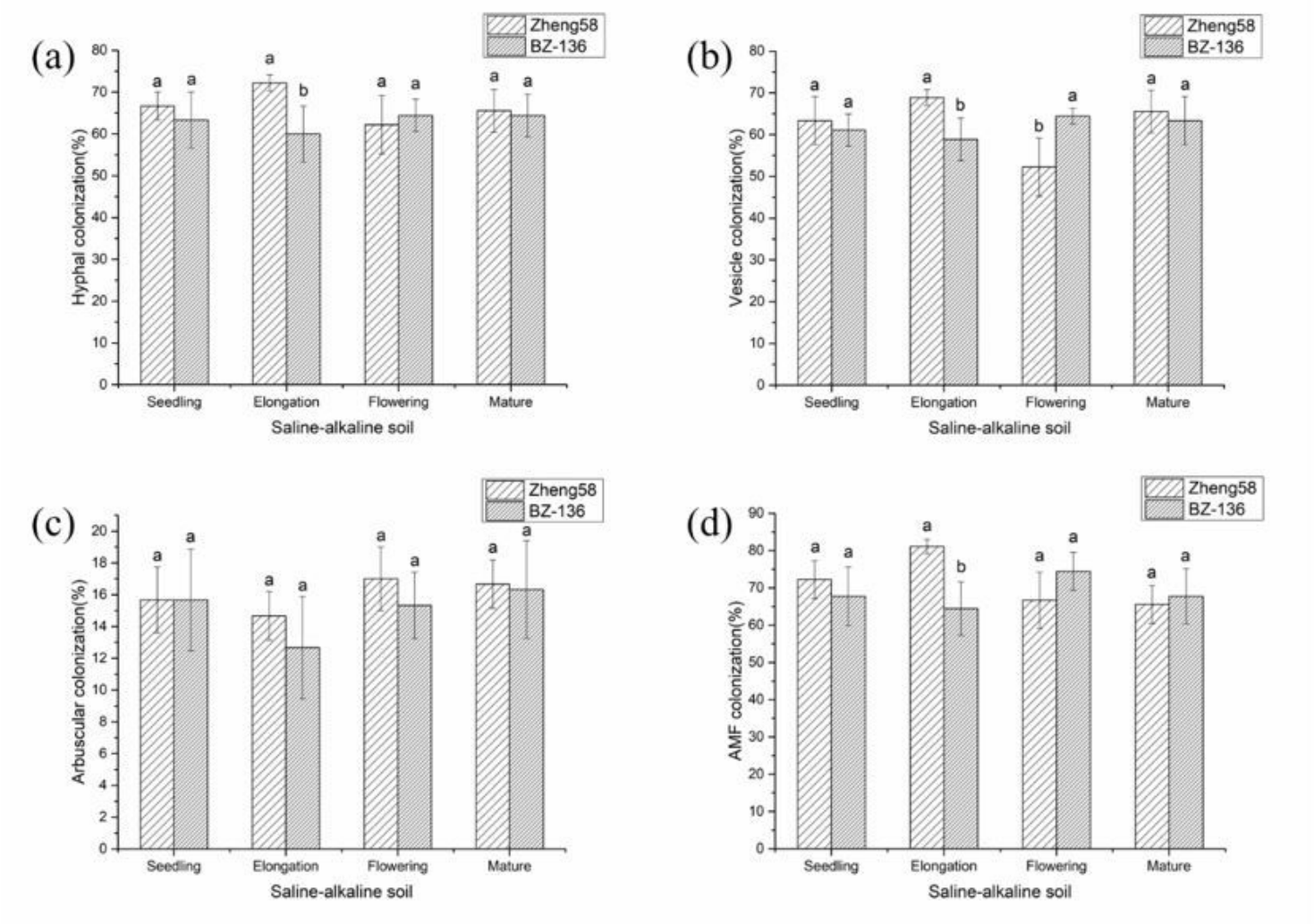

Figure 2

Colonization of different genotypes of maize roots by indigenous arbuscular mycorrhizal fungi in saline-alkaline soil. Vertical bars indicate the standard error of the means. Same letter above each bar represents the value that is unsignificantly different ($p > 0.05$) according to the LSD test ($n= 3$) at the same stage. The standard error is based on the average of three biological replicates.

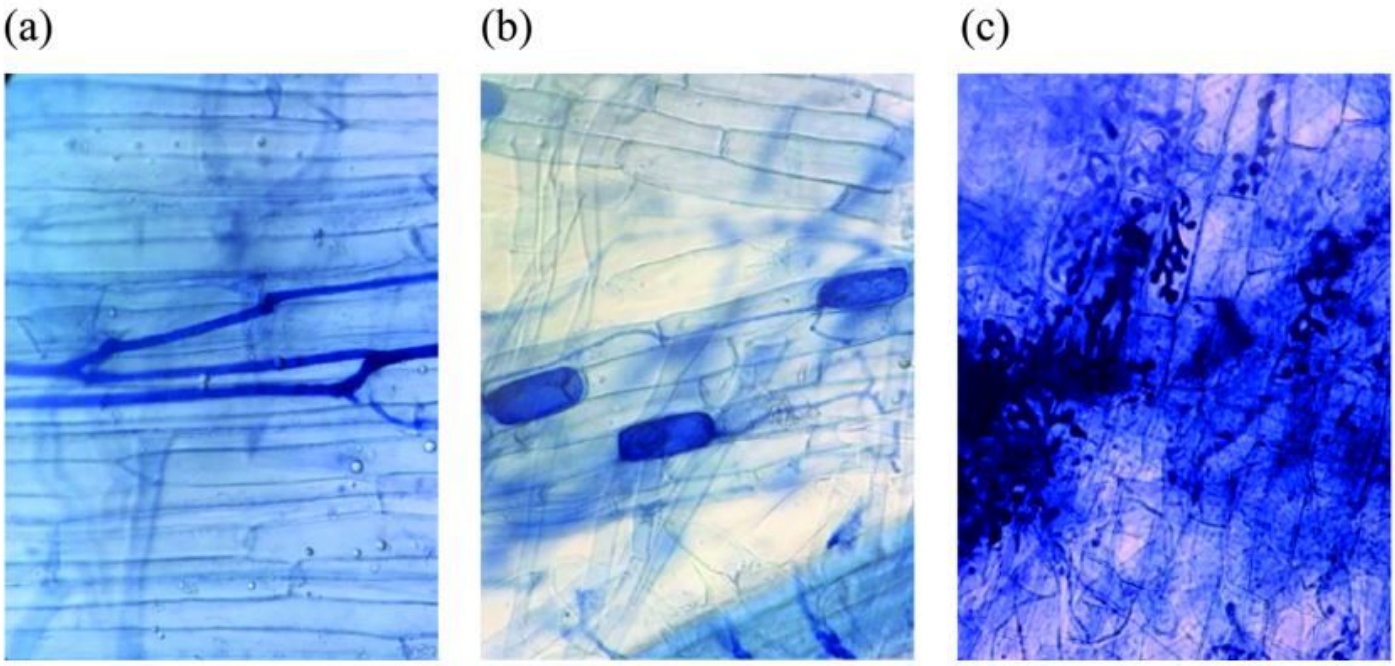

Figure 3

Pictures of AMF mycelia (a), vesicles (b) and arbuscular (c) colonization on the roots of maize

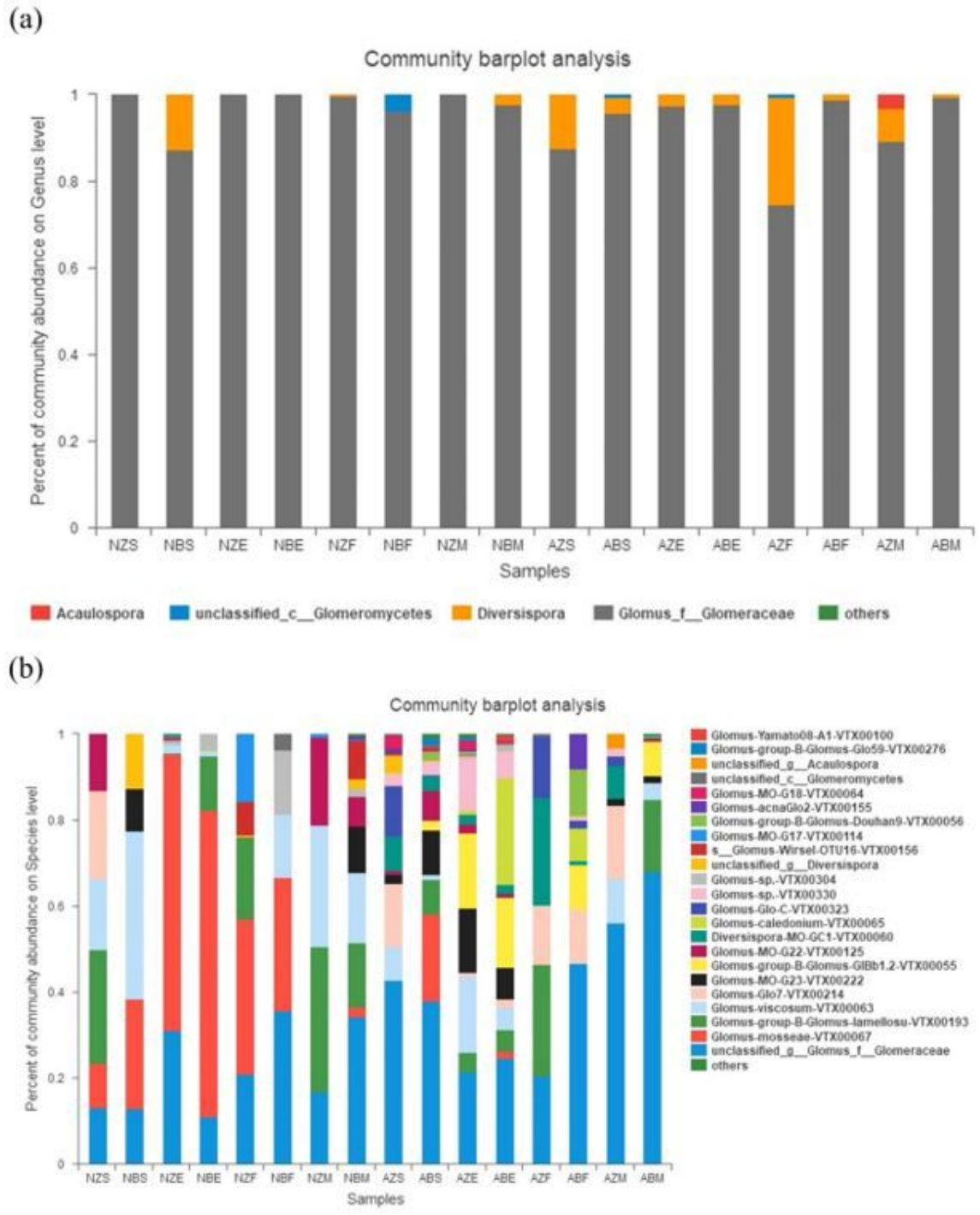

Figure 4

Relative abundance of AMF genus (a) and species (b) present in rhizosphere bacterial communities of transgenic maize BZ-136 and parent Zheng58 following at different growth stage. N, means neutral soil; A, means saline-alkaline soil; Z, means Zheng58 maize line; B, means BZ-136 maize line; S, means seedling stage; E, means elongation stage; F, means flowering stage; M, means mature stage.

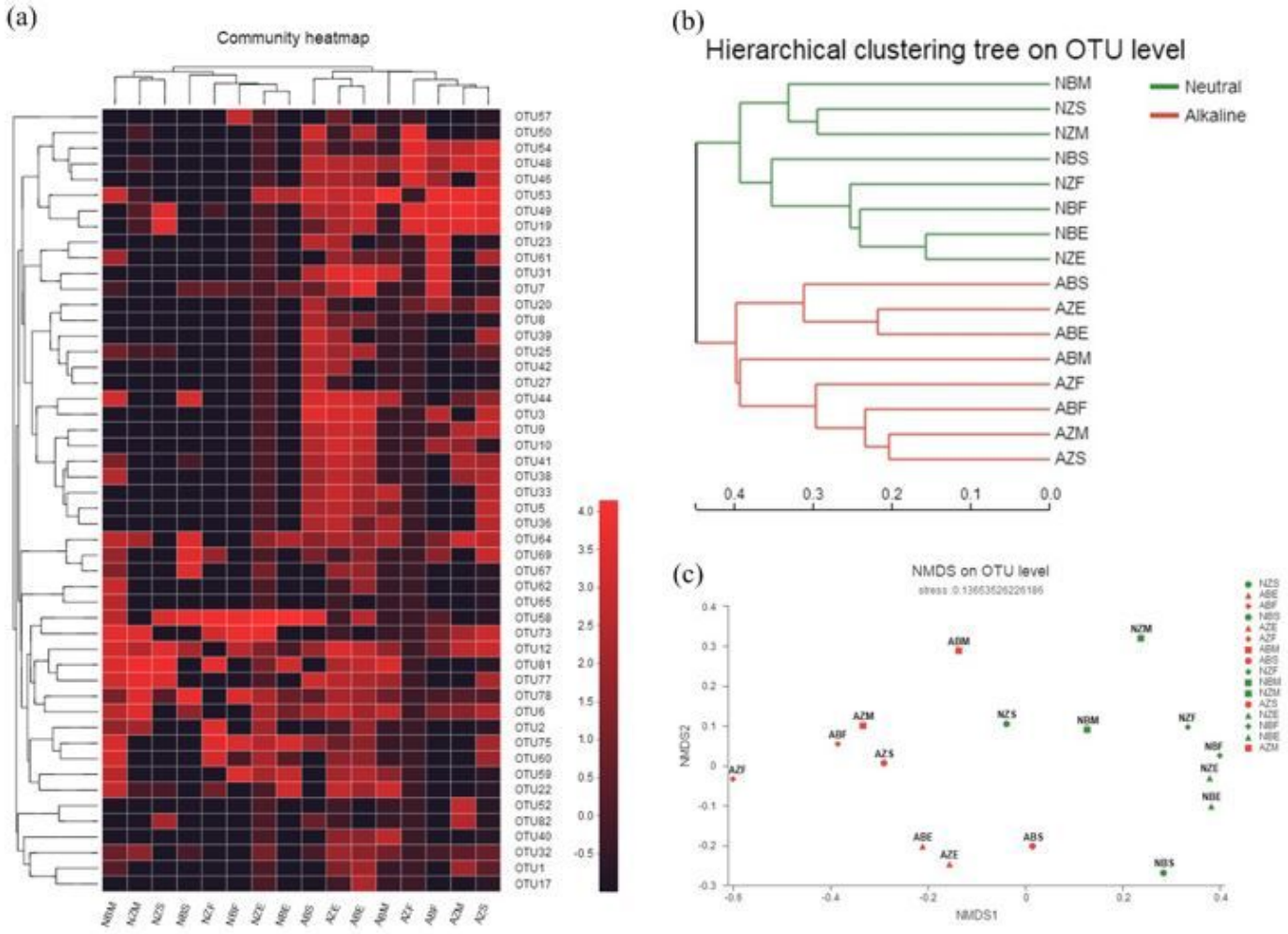

Figure 5

Heat maps (a), clustering analysis (b) and NMDS analysis (c) of the soil rhizosphere AMF composition on OTU level in neutral soil and saline-alkaline soil. N, means neutral soil; A, means saline-alkaline soil; Z, means Zheng58 maize line; B, means BZ-136 maize line; S, means seedling stage; E, means elongation stage; F, means flowering stage; M, means mature stage.