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The influence of inoculation and drought on the diversity of fungal communities in the roots of tomato plants

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Abstract

Arbuscular mycorrhizal fungi (AMF) are microorganisms with very important functions in agricultural systems. Since arbuscular mycorrhiza is one of the most common types of mycorrhizae, the diversity of AMF is very varied. Their diversity can be influenced by various biotic and abiotic factors. Of these, drought is one of the most common abiotic stresses in agriculture. In this study, we evaluated the influence of drought and inoculation with three species of AMF (*Rhizoglomus irregulare*, *Funneliformis mosseae* and *F. caledonium)* on the fungal genetic diversity in the roots of tomato plants (*Solanum lycopersicum* L.) using partial 18S rRNA gene in molecular fingerprinting method. Two conserved primer pairs NS1–NS4 and NS31–AM1 in Nested PCR were used. The second primer pair is specific for AM fungi from *Glomeromycota*, but also amplifies DNA from *Ascomycota* and *Basidiomycota* to a very small extent. Drought caused a decrease in fungal alpha diversity in tomato roots of non-inoculated plants. On the other hand, an increase of this diversity due to drought in inoculated plants was observed. Based on principal component analysis, a statistically significant shift in the composition of fungal communities in non-inoculated and inoculated plants due to drought was not detected. At the same time, the most variable fungal communities were in control well-watered and non-inoculated plants, but this variation was not significant.

Introduction

Arbuscular mycorrhizal fungi (AMF) which form a symbiosis with approximately 80 % of terrestrial plant species have an important role in processes such as increasing mobilization and nutrient transfer, plant tolerance to root pathogens, production of plant growth hormones, plant biodiversity, adaptation of the plant to adverse environmental conditions, the absorption capacity of plant roots, and many others (Garg and Chandel 2010). Adverse external conditions for plants whose effect AMF can decrease are drought, salinity, heavy metal toxicity, soil acidification, or heat (Finlay 2008). Of these, drought is one of the world's most abundant abiotic stress (Zhang *et al.* 2018). Many studies have been done to examine the effect of drought on the plants inoculated with AM

fungi. However, the effect of drought on the diversity of AMF as such was much less studied (Bahadur *et al.* 2019) and how AMF respond to this stress in agricultural ecosystems is not fully known (Millar and Bennett 2016). Some studies have not found the impact of drought on AMF communities (Furze *et al.* 2017; Maitra *et al.* 2019; Kozjek *et al.* 2021). Other studies found a shift in their diversity (Deveautour *et al.* 2018; 2020), or others even found an increase in AMF biomass (Karlowsky *et al.* 2018; Mackie *et al.* 2019; Kundel *et al.* 2020). For this reason, our work aimed to investigate the effect of drought as well as the effect of inoculation of tomato plants with three AMF species on the fungal diversity in the roots of such plants. When comparing two sets of samples (inoculated and non-inoculated plants) it could be expected that the drought simulation would have a stronger effect on the genetic diversity of fungi in the roots of non-inoculated than inoculated plants.

Experimental

Pot experiment and AMF inoculation

In the experiment, tomato plants (*Solanum lycopersicum* L.) of variety Karla were used. The soil substrate was mixed from topsoil and sand in proportion 3 : 1. Inoculation was done during seed sowing with a commercially available inoculum INOQ Advantage (INOQ GmbH, Germany) containing a mixture of 3 species of AM fungi: *Rhizoglomus irregulare*, *Funneliformis mosseae* and *Funneliformis caledonium* with a concentration of 550 × 10^4 mycorrhizal units.mL^{-1}. The concentrated inoculum was diluted at 1 : 50 in autoclaved, finely homogenized soil and applied to a seed hole at a dose of 0.5 mL of inoculum per seed. The control group of plants was without inoculation. The plants were cultivated under greenhouse conditions in pots with a diameter of 22 cm, 4 pots per variant with 3 plants per pot. For the first 8 weeks (until the flowering stage), a standard irrigation was applied to keep the substrate humidity at a level of 25 % of volumetric water content. Then the control and inoculated plants were divided into 2 groups, the first one being irrigated with the standard dose of water (3 times a week at a dose of 500 – 500 – 1000 mL of water per container), the second group with a half dose of water (which corresponded to 12 % of volumetric water content). The cultivation of plants under this regime took place for 4 weeks.

DNA extraction and molecular analysis

Root samples were obtained by carefully removing all three tomato plants from the soil from each pot, gently removing the soil from the roots, and placing the entire three roots in a sterile container. Therefore, one sample was composed of roots from three plants cultivated in one pot. In this way, root samples from individual treatments (each treatment consisted of 4 replicates, which formed separate samples for subsequent analyses) were collected in the following order: 4 pots = 4 separate root samples from non-inoculated well-watered control plants, 4 samples from non-inoculated drought-affected plants, 4 samples from inoculated well-watered control plants, and 4 samples from inoculated drought-affected plants. After root collection, the samples were transferred to the laboratory, washed 3 times with sterile water, and thoroughly dried.

DNA was extracted from 100 mg of dry roots using the modified CTAB protocol. One hundred mL of each root sample was homogenized to a powder with a mortar and pestle using liquid nitrogen. Seven hundred µL of 2x CTAB solution (2 M NaCl, 0.2 M Tris, 50 mM EDTA, 2 % CTAB) and 3 µL of 2-mercaptoethanol were added to the pulverized root samples, mixed and thoroughly vortexed, and incubated in a water bath for 30 min at 60 °C. After incubation, the mixture was cooled to room temperature and centrifugated for 5 min at 14,000 × g. The supernatant was transferred to a new tube to which 5 µL of RNase A was added and incubated for 20 min at 37 °C. An equal volume of chloroform/isoamyl alcohol (24 : 1) was added. The mixture was vortexed for 5 s and centrifugated for 1 min at 14,000 × g to separate the phases. The upper aqueous phase was transferred to a new tube. This step with chloroform/isoamyl alcohol was repeated until the upper phase was clear. DNA was precipitated by adding 0.7 volume of chilled isopropanol, incubated for 15 min at -20 °C and centrifugated for 10 min at 14,000 × g. Supernatant was discarded without upsetting the

pellet, which was subsequently washed with 500 µL of ice-cold 70 % ethanol. The ethanol was removed, and the samples were dried at room temperature until residual ethanol was completely removed. The DNA was dissolved in 50 µL of pre-heated TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The quantity and purity of DNA were measured spectrophotometrically with NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), samples were diluted to the same final concentration (25 ng.µL^{-1}), and stored at -20 °C.

Molecular analysis of partial fungal 18S rRNA gene using two conserved primer pairs NS1–NS4 (Lee *et al.* 2008) and NS31–AM1 (Mummey *et al.* 2005) in terminal restriction fragment length polymorphism (T-RFLP) was performed according to our previous study (Ondreičková *et al.* 2019). The second primer pair is specific for AM fungi (*Glomeromycota*); however, it should be noted that also amplify DNA from *Ascomycota* and *Basidiomycota* to a very small extent (Clapp *et al.* 1995, 1999; Ondreičková *et al.* 2019).

Data analysis and statistical evaluation

Outputs from T-RFLP analysis in the form of electrophoregrams were analysed by the Peak Scanner 2 (Applied Biosystems, Thermo Fisher Scientific Inc., Wilmington, USA), and operational taxonomic units (OTUs) in range 60 – 550 bp were used for the evaluation. Only peaks above the threshold of 50 fluorescence units were considered.

The Venn diagrams were constructed using Venny 2.1 online program (Oliveros 2007-2015). Statistically significant differences among samples were tested using t-test at the 95 % confidence interval for the means using the software Statgraphics x64 (Statpoint Technologies, Inc., Warrenton, USA). Alpha diversity indices were calculated from standardized profiles of individual samples using the number and height of peaks in each profile as representations of the number and relative abundance of phylotypes. Fungal communities in different samples were compared from T-RFLP profiles using the height of fluorescence in individual OTUs. These data were subsequently used for the Principal component analysis (PCA) using the scores of the first two principal components. Analysis of Similarities (ANOSIM) was used to determine if significant effects occurred among samples using 2 factors – inoculation and drought (Two-way ANOSIM), and only 1 factor – all principal component values (One-way ANOSIM) with Euclidean distance measure with 9999 permutations. Alpha diversity indices, PCA, and ANOSIM were evaluated by using the PAST (PAleontological STatistics) software version 3.19 (Hammer and Harper 2006).

Results

Fungal alpha diversity

Most fungal species were detected in the roots of non-inoculated control tomato plants and the least in non-inoculated drought-affected plants (56 *vs* 27, respectively; Fig. 1A).

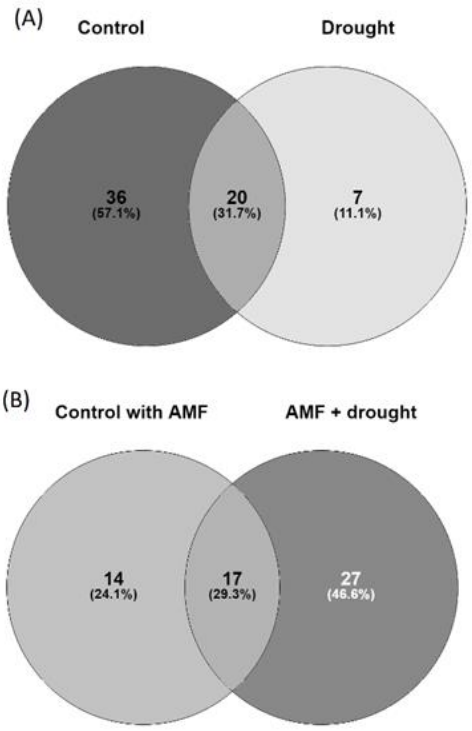

Fig. 1. Venn diagrams constructed from T-RFLP data representing the number of shared and unique OTUs between fungal communities from the roots of non-inoculated control and non-inoculated drought-affected tomato plants **(A)** and inoculated control and inoculated drought-affected tomato plants **(B)**.

In non-inoculated plants, the fungal species richness in the roots of drought-affected plants was reduced by 51.8 % compared to the control samples watered properly. The opposite effect was observed in inoculated plants. The increase of 41.9 % of fungal species was in the roots of drought-affected tomato plants compared to controls. Roots of control plants contained 31 fungal species, while 44 were in drought-affected plants (Fig. 1B). From this number of fungal species in the roots of non-inoculated and inoculated plants, in both cases approximately the same percentage of common species was detected between controls and drought-affected plants (31.7 % *vs* 29.3 %, respectively; Fig. 1).

Fungal alpha-diversity between control and drought-affected non-inoculated and inoculated plants was evaluated using 10 different diversity indices (Fig. 2). Statistically significant differences between control and drought-affected plants were detected in two indices, Margalef and Fisher alpha, in non-inoculated tomato plants (t-test, $\alpha = 0.05$). Control plants, as well as drought-affected plants without and with inoculation, showed exactly opposite values. When the index value was higher in the non-inoculated controls, it was lower in the inoculated controls and *vice versa*. The same trend was observed in drought-affected plants without and with inoculation.

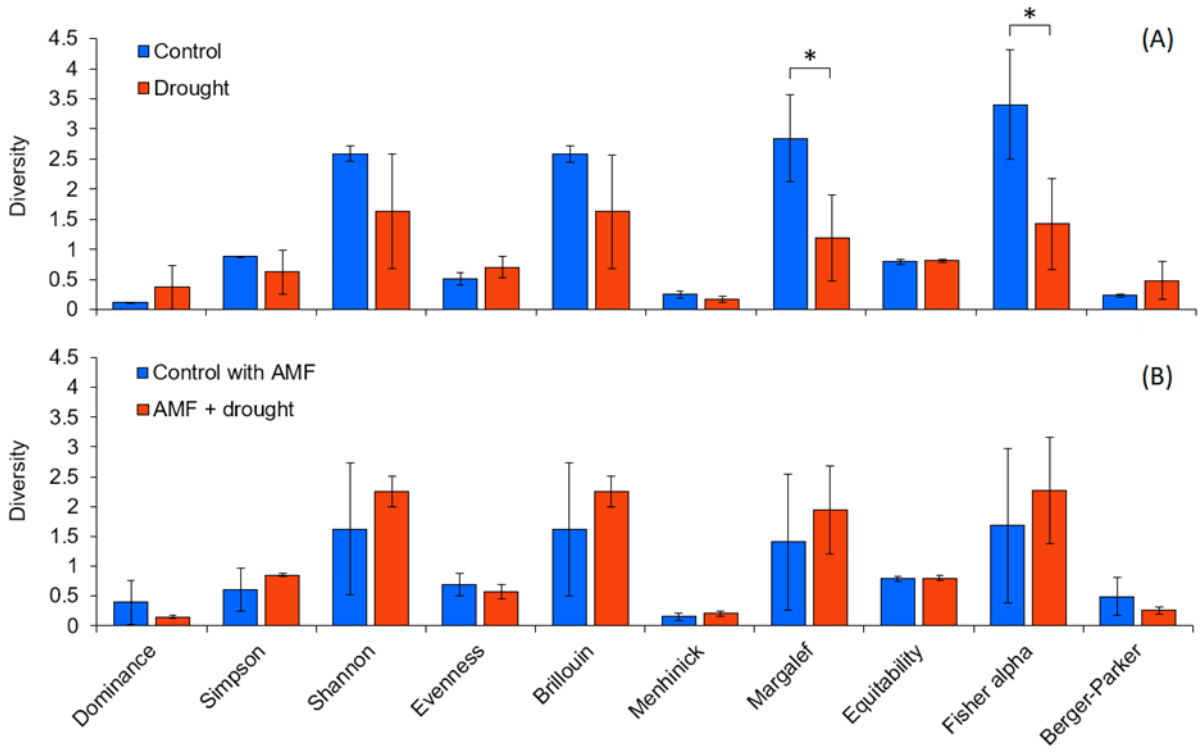

Fig. 2. The values of genetic diversity indices between fungal communities in the roots of non-inoculated control and non-inoculated drought-affected tomato plants (**A**) and inoculated control and inoculated drought-affected tomato plants (**B**). Displayed values represent the average values calculated from four replicates; bar represents the standard deviation; and * represents statistically significant difference (t-test, $\alpha = 0.05$).

Impact of drought and inoculation on the fungal genetic diversity

Based on the principal component analysis (PCA), fungal community from the roots of one non-inoculated control sample represented the most different community from other non-inoculated samples (Fig. 3A). In this case, one control sample is in the far right of the PCA graph, with the horizontal axis expressing up to 84.16 % variability. Non-inoculated drought-affected samples showed very similar fungal communities based on the PCA graph. On the other hand, fungal communities from the roots of inoculated drought-affected tomato plants are more different (Fig. 3B) than those from non-inoculated drought-affected tomato plants. In particular, one such sample was separated from the others by PC1 and this axis

represented only 48.92 % variability. At the same time, PC1 mutual separated 2 and 2 inoculated control samples from each other on the opposite sides of this graph, which means that these samples represent two types of fungal communities. Based on two-way ANOSIM analysis, inoculation with AMF and drought did not statistically significantly affect the fungal communities in the roots of tomato plants (P = 0.3456, P = 0.4827, respectively).

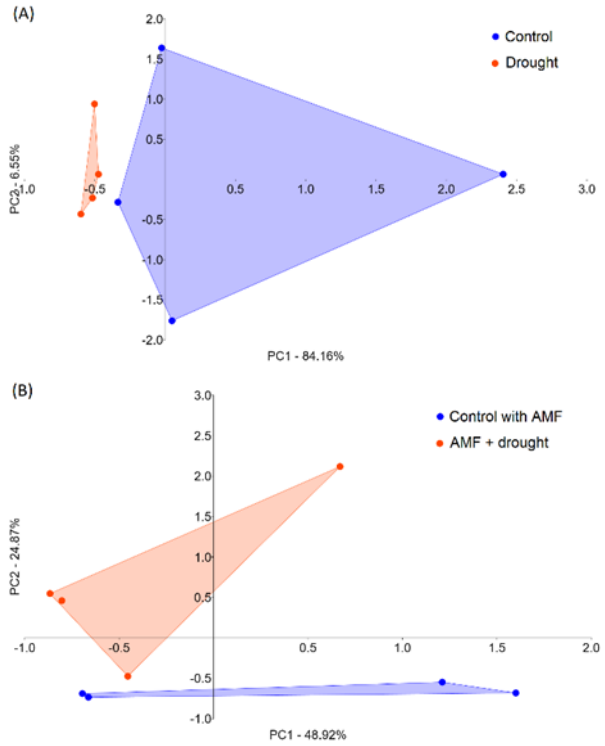

Fig. 3. The principal component analysis constructed from T-RFLP fluorescent data of fungal communities from the roots of non-inoculated (**A**) and inoculated tomato plants (**B**). PCA graphs explained a total of 90.71 % and 73.79 % (respectively) of the variability in the data.

Matrix plots generated from data of all principal components (PCs) provide a comprehensive view of these values and were different for non-inoculated and inoculated plants (Fig. 4). The value of PC1 from non-inoculated control sample 1 (abbreviated as C1 in Fig. 4A) was in the red spectrum. This PC1 value was crucial for the separation of this control sample from other samples and therefore was in the right part of the PCA graph (Fig. 3A). As mentioned above, the PCA result from the inoculated plants showed that the control plants contained two types of fungal communities in their roots. Their values of the principal components also correspond to this (Fig. 4B). These fungal communities of control samples 1 and 4 (abbreviated as CA1 and CA4 in Fig. 4B) formed the first community type in the lower left part of the PCA graph, and control samples 2 and 3 (abbreviated as CA2 and CA3 in Fig. 4B) formed the second community type located in the right part of the graph in Fig. 3b. Different values for PC1 and PC2 which characterize inoculated drought-affected sample 3 (abbreviated as AD3 in Fig. 4B) determine its separation from other drought-affected samples in the upper right part of the PCA graph in Fig. 3B. However, when comparing all the values of the principal components, not only PC1 and PC2, which are shown in the PCA graphs in Fig. 3, statistical differences between controls and drought-affected samples were not detected in non-inoculated and inoculated plants (ANOSIM, α = 0.05; Fig. 4).

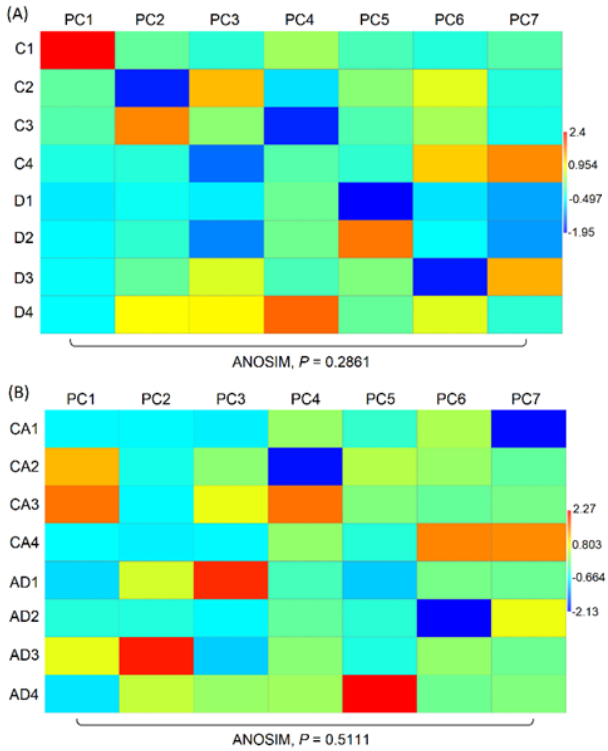

Fig. 4. Matrix plots generated from data of all principal components from PCA from non-inoculated (**A**) and inoculated tomato plants (**B**). C1-C4 represent non-inoculated control samples; D1-D4 represent non-inoculated drought-affected samples; CA1-CA4 represent inoculated control samples; and AD1-AD4 represent inoculated drought-affected samples. P represents the values obtained from all PCs using Analysis of similarities (ANOSIM) with Euclidean similarity index.

Discussion

The results of this study represent an ecological view of the fungal communities in the roots of tomato plants under the influence of drought and inoculation with three species of arbuscular mycorrhizal fungi. A positive effect of inoculation with *Rhizoglomus irregulare*, *Funneliformis mosseae*, and *F. caledonium* of tomato plants in drought condition on the overall communities of mainly AM fungi in the roots of these plants was observed. This effect was manifested as an increase in species diversity as well as in the values of diversity indices (Fig. 1 and 2). However, this alpha-diversity increase in inoculated plants was not statistically significant. On the other hand, root fungal alpha-diversity was decreased in drought-affected non-inoculated tomato plants. Andreo-Jimenez *et al*. (2019) in their study observed the opposite trend in the rice root fungal microbiota community in comparison with our non-inoculated samples. Their Shannon diversity index was higher under drought for all 15 rice cultivars tested and was strongly statistically significant. Also, they detected that these differences between treatments were rice cultivar dependent. However, in contrast to our results especially from *Glomeromycota* and to a limited extend from *Ascomycota* and *Basidiomycota* (Clapp *et al.* 1995, 1999; Ondreičková *et al.* 2019), the statistical significance which was detected by the above mentioned Andreo-Jimenez *et al*. (2019) was noticed from the entire diversity of the fungal community including *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Zygomycota*, *Glomeromycota*, and unclassified fungi. Though, based on diversity richness and Shannon index at OTU level between control and drought samples, they detected no significant effects on AMF community structure in *Glomeromycota*. Additionally, Alguacil *et al.* (2021) observed that drought did not significantly affect AMF community richness and Shannon-Wiener diversity index at OTU level in the rhizosphere samples from *Helianthemum syriacum* and *Gypsophila struthium*. In the study of Emery *et al.* (2022), drought as a single factor was also not significant for AMF OTU richness from soil associated with two cultivars of switchgrass. On the other hand, they detected that drought treatment had a significant direct effect on AMF root colonization in switchgrass, and reduced root colonization by 6 %. An experiment similar to our was performed by He *et al.* (2019) inoculating the trifoliate orange with *F. mosseae*. They observed that the fungal richness in the roots of control non-inoculated trifoliate orange plants was higher than in the roots of drought-affected non-inoculated plants. However, in the plants inoculated with *F. mosseae*, the fungal richness in the roots of control and drought-affected plants was the same. In addition, they detected statistically higher OTUs and more variable diversity in the roots than in the rhizosphere soil. In their experiment, environmental factors such as inoculation with *F. mosseae* and drought had a much less impact on fungal diversity in the rhizosphere than in the roots.

Conclusion

Inoculation of tomato (*Solanum lycopersicum* L.) plants with arbuscular mycorrhizal fungi increased, but not significantly, fungal richness and alpha-diversity in the roots of drought-affected plants. Drought in non-inoculated plants did not cause a shift in the composition of the fungal community. In inoculated plants, the shift caused by drought was observed only in one sample. It was also observed that control well-watered, non-inoculated plants showed higher variability in fungal community composition than in drought-affected plants. Although fungal richness and the increase in alpha-diversity due to inoculation with AMF were not significant, the use of AMF as an inoculant appears to be an appropriate way to prevent the effects of drought on root fungal diversity.

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Conflict of interest

The authors declare that they have no conflict of interest.

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