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HOST PREFERENCES OF ARBUSCULAR MYCORRHIZAE ALONG THE SOIL NUTRIENT AND HYDROLOGICAL GRADIENT

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This study compared the composition of arbuscular mycorrhizal fungal (AMF) communities colonizing the roots of two common native plants along a slope-related moisture gradient on a small hillside in a natural east Texas (USA) forest. PCR amplification of a region of the small subunit ribosomal 18S RNA gene with Glomeromycota-specific primers was used to detect AMF in the roots of *Chasmanthium sessiliforum* (Poir.) Yates and *Callicarpa americana* L. The PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) to obtain community profiles.

Comparison of profiles revealed significant differences between the AMF communities associated with the two hosts. Additionally, communities of AMF colonizing *C. sessiliforum* differed according to their location along the hill slope gradient.

Key words: Arbuscular mycorrhizal fungi, denaturing gradient gel electrophoresis, DGGE, AMF diversity, 18S rRNA, multivariate analysis.

Arbuscular mycorrhizal fungi (AMF) (Phylum Glomeromycota) [1] form mutualistic symbiotic associations with the roots of roughly 80% of all terrestrial plant species [2] acting as an extension of the plant root system to support plant development under nutrient-limiting and stress conditions. AMF alleviate water stress via the direct uptake and transfer of water and nutrients through the fungal hyphae to the host plants resulting in a significant increase in plant height, stem diameter, and fresh weight of the plant, which in turn provides carbohydrates to the fungus. Plant growth is greatly affected by environmental stresses such as drought, nutrient limitation and salt loading; nutrient exchange by AMF may be critically important when soil fertility and water availability are low. A number of studies reported benefits of AMF associations to plants under environmental stress [3–6]. Several studies show that the presence of AMF alters plant communities by affecting the relative abundance of plant species and plant-species diversity [7–9]. The plants and soil in turn also influence the species composition of the AMF community. Eom et al. [10] suggest that AMF do not show host specificity and are randomly distributed in natural ecosystems. However, others suggest some preference may exist between host plant and AMF since the effect of symbiosis on individual plants can differ by AMF species and small scale variation in soil factors such as pH, nutrient concentration, moisture, temperature or organic matters may influence AMF communities [11–15]. In this study we use PCR with Glomeromycota-specific primers and denaturing gradient gel electrophoresis (DGGE) to investigate the effect of local environmental variation (a hydrological and soil nutrient gradient along a hillslope) as well as the effect of plant host on natural AMF populations colonizing two widespread, native eastern Texas USA plants. Previously we sequenced the PCR products from root samples from these two plants and confirmed glomalean origin of the amplicons [16].

Materials and methods

Study Area

Far-east Texas has a humid, subtropical climate with 1,250 mm mean annual precipitation, hot humid summers, mild winters, occasional frost, and negligible snowfall [17]. Natural upland vegetation consists of pine and

broad-leaved deciduous forest. The study site, in Stephen F. Austin Experimental Forest, Nacogdoches county, Texas, USA is on a south-facing hill slope adjacent to the floodplain of the Angelina river in a natural mixed pine broad-leaved deciduous forest. The upper part of the hill is subjected to intermittent drought and high temperatures in summer while the bottom may occasionally flood in spring and fall. Significant populations of *Callicarpa americana* L. (Verbenaceae) and *Chasmanthium sessiliforum* (poir.) Yates, (Poaceae) occur in the understory on the upper, middle, and lower portions of the hill slope. These plants were chosen as AMF hosts for the study as they are common native forest species.

Field samples

Random points were selected from the upper, middle and lower thirds of an approximately 100 m transect running from the top to the bottom of the hill slope. The three individuals each of *C. americana* and *C. sessiliflorum* closest to each point were carefully excavated, transported to the laboratory and stored at 4 °C. In addition, soil pH, moisture, and levels of phosphate, potassium, calcium, magnesium, sulfur, manganese, zinc and copper were determined at the Stephen F. Austin State University Soil Testing Laboratory for soil samples collected adjacent to the selected plants.

DNA extraction and amplification

Root samples were screened for the presence of AMF by light microscopy [15]. Genomic DNA was recovered from 0.3 g of the ground root material using the UltraClean™ Soil DNA Kit (Mobio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol. PCR experiments were performed using a Mastercycler (Eppendorf, Inc., Westbury, NY) thermocycler. Previous studies [18, 19] reported low resolving power for AM1/NS31GC amplification product. To improve the quality of DGGE we attached a GC clamp to the AM1 primer and, in addition, designed a new primer to amplify a single melting domain region (~ 280 bp) as calculated with the Melt 94 program [20]. The resulting amplification product was used for DGGE analysis. The AMF generic forward primer sequence with a GC clamp was 5′-CGC CCG CCC CGC CCC GTG CCC CGC GCC GCC CGC CCC GCC CCC AAC TAT CCC TAT TA-3′. The standard PCR reaction contained 2 μl of 100 ng/μl of primers (Operon Biotechnologies, Inc., Alabama), 4 μl (3 to 10 ng/μl DNA concentration) of DNA extract, 4 μl of MgCl at 25 mM (Promega, USA), 5 μl of Buffer 10X (Promega, USA), 1 μl of 10 mM dNTP's (Promega, USA) and 0.5 μl of Taq Polymerase at 5.0 units/μl (Promega, USA). The total volume of PCR reaction was brought to 50 μl with de-ionized water. The PCR program included an initial denaturation cycle of 95 °C for 3 min. followed by 35 cycles of denaturation at 94 °C for 40 sec, annealing at 58 °C for 40 sec, and an extension at 72 °C for 2 min. The final extension step was increased to 7 min at 72 °C.

Analysis of PCR products by DGGE

Formamide (Sigma Inc.) and urea (EMD Chemicals, Inc., Gibbstown, NJ) were used to prepare a 40 to 60% denaturing gradient in a 40% acrylamide/bis (Bio-Rad) gel. The PCR products were loaded onto the gel on the basis of equal amounts of DNA per PCR sample. The DGGE marker used in the current project was constructed from bacterial and AMF DNA to cover the full range of the gel. The gels were run for 4 h at a constant 250 V in 50X TAE buffer in a C.B.S. Model DGGE 2401 tank (C.B.S. Scientific Company, Inc., California, 92014). Staining was performed with 100 μl/L Sybr Green (Invitrogen Corp., California 90028) and photographed with a BioRad Imager System equipped with a Gel Doc XR camera and Quantity-One software (BioRad Inc., Hercules, CA).

Data analysis

Gel images were aligned and band intensities standardized between samples in GIMP (GNU Image Manipulation Program) and images were digitized and converted to presence/absence data with a custom Python script. The script used the Python Imaging Library (PIL) to convert the image to gray scale and extract the pixel values along the wells of the gel. Local maxima points were marked as 1 and the rest of the data were marked as 0. Statistical analysis was carried out in R [21] using the VEGAN [22] and ADE4 [23] packages. Distances between the sample profiles were calculated using Bray-Curtis indices [24]. Hierarchical clustering was generated using Ward's linking algorithm and the clades were tested for statistical significance with PVCLUST [25] using 10,000 iterations. To assess the significance of covariance of community and location with clade topology we used the Treeclimber program [26] with 100,000 iterations. Principal coordinates analysis (PCOA) of DGGE profiles was used to

provide statistical evidence and a concise assessment of the differences in AMF community structures between the host species and locations. The significance of fitted environmental vectors into principal coordinates analysis ordination was assessed with 10,000 random permutations.

Results and Discussion

We analyzed a total of 18 samples of DNA extracts (three from the roots of each of the two host plant species from the top, middle and the bottom of the hill). The mean number of DGGE bands was significantly fewer for *C. americana* than for *C. sessiliforum* at both the bottom ($p = 0.032$) and the top ($p = 0.0065$) but not in the middle ($p = 0.6597$) of the hill (Fig. 1). The calculated Bray-Curtis similarity coefficients ranged from 0.72 to 1.0 (between two profiles from *C. sessiliforum* from middle of the hill) with an average of 0.92. Fig. 2 displays a hierarchical cluster obtained using the calculated similarity coefficients and Wards linking method. Two strong groups were identified (Fig. 2), A and B, with a high p-value as assessed by pvclust. The first group included 6 of the 9 samples from *C. americana* and the second group included all samples from *C. sessiliforum* along with the remaining *C. americana* samples. Treeclimber analysis of the cluster showed that the groups were significantly different between the hosts ($p = 0.006$) while no significant covariance in groups was observed between locations ($p = 0.34$).

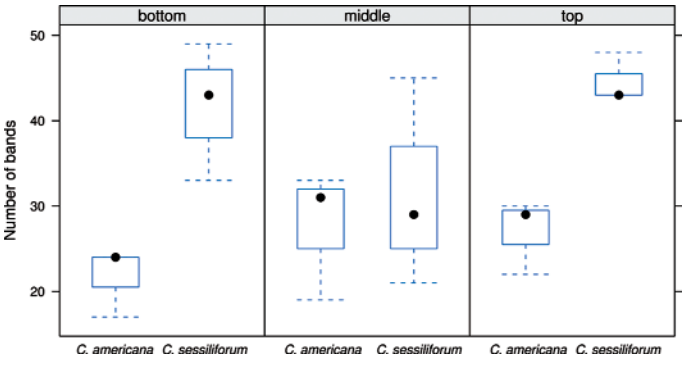

Fig. 1. **Total number of bands calculated from DGGE profiles for *C. americana* and *C. sessiliforum* from the bottom, middle and top of an east Texas USA hill slope.**
Solid dots within each box indicate median of the data; boxes show the 25th and 75th percentiles of the data; whiskers indicate range. If the whiskers for two boxes do not overlap, the medians are significantly different ($p < 0.05$), thus allowing for visual estimates of statistical significance

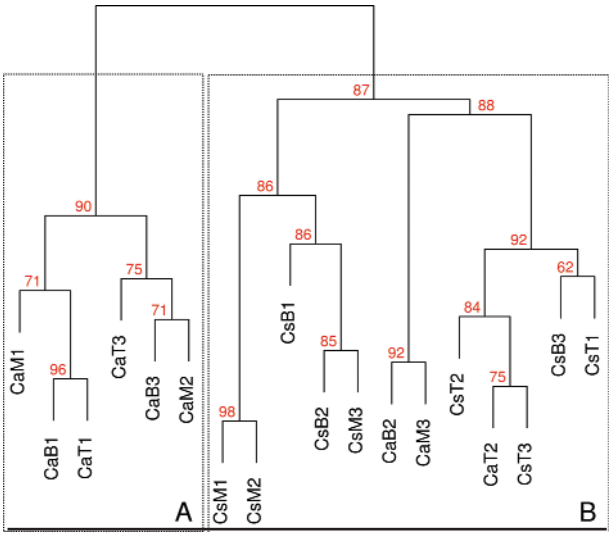

Fig. 2. **Hierarchical cluster showing similarities between DGGE profiles. Numbers above the internal branches correspond to p-values.**
Abbreviations at branches denote *C. americana* — Ca; *C. sessiliforum* — Cs; middle — M; top — T, bottom — B; and sample numbers. Clade A contains 6 out of 9 samples from *C. americana* and clade B has all the samples from *C. sessiliforum*. Clades do not clearly separate by location

The results of PCOA are plotted in Fig. 3, which displays similar samples close together and dissimilar samples far apart. They showed a clear separation between samples from *C. americana* and *C. sessiliforum* on the first axis. The second axis separated samples from *C. sessiliforum* by their respective locations. However, profiles from the second host, *C. americana*, were not clearly separated.

Soil moisture on the top (7.87%) and the bottom (9.76%) of the hill was higher than in the middle (6.9%). The soil pH decreased from 5.35 at the top to 4.9 at the bottom, potassium from 74 to 29 ppm, calcium from 127 to 115 ppm, manganese from 127 to 47 ppm and phosphate from 2 to 1 ppm (Table). In contrast amounts of sulfur, zinc and copper increased from the top to the bottom of the hill. Both host species factor ($p = 0.0026$) and the total number of bands present on the gel ($p = 0.008$) were statistically significantly correlated with the ordination configuration. When statistical analysis was repeated for AMF of each host separately, none of the environmental vectors were statistically significant with the ordination of AMF associated with *C. americana* whereas for *C. sessiliforum*, soil moisture ($p = 0.066$) and the location ($p = 0.026$) were significant.

Our results have implications for understanding the basis of the relationship between

AMF communities and local environmental variation. We anticipated that if the environmental conditions and soil chemistry are the main factors that shape the AMF community composition then the profiles from different hosts within same environment would be more similar to each other then profiles from different locations. The pattern that emerged was different from that anticipated. If host species are classified as 1 and 2, their AMF profiles cluster into groups that correspond to these categories. Thus host species appeared to be of more importance in determining the pattern of clustering among samples than environment, as shown by both the cluster analysis (Fig. 2) and principal coordinates analysis (Fig. 3). Investigations covering a larger spatial scale and more environmental heterogeneity, however, may reveal a greater importance for environment.

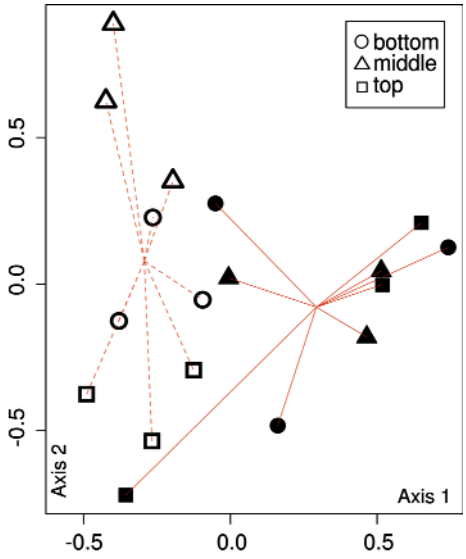

Fig. 3. **Principal coordinates analysis (PCOA) showing relationships of AMF populations among sites.**
The PCOA is based on similarities (Bray-Curtis coefficient) as calculated with absence/presence data obtained from digitized gel images of denaturing gradient gel electrophoresis. Similar sites appear close together and dissimilar sites are far apart. Symbols are used to identify sites from the bottom, middle and the top of the hill, as well as the host species *C. americana* (solid symbols) and *C. sessiliflorum* (open symbols). Black and dotted lines connect samples to their weighted group centroids of *C. americana* and *C. sessiliflorum*

It is, however, evident from our results that different combinations of AMF species occur on the same host plant in the natural environment across a relatively small spatial scale (~100 m in the case of the current study) and it is likely that some of these communities of AMF can buffer the environmental stress levels on plants better than the others. Although relationships between these differences and the environment were weak—especially for *C. americana*, it remains unclear if the observed differences between the profiles are random or a result of the physiology of individual plants. Small-scale differences in AMF may play a role in the relative success of individuals within a population and may affect competitive interactions.

Mean values of measured soil characteristics in different locations of an east Texas USA hill slope

	(%) soil moisture	pH	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)
Bottom	4.70	4.86	1	29	115	47
Middle	4.57	5.34	2	56	127	127
Top	6.28	5.35	2	74	127	127

In spite the local variations in environment and the variability of AMF communities from the three locations, the AMF ordination was not significantly correlated to the gradient of soil chemistry or nutrient variables when both species were analyzed together. However, when the AMF data were analyzed separately for each plant, AMF associated with *C. americana* showed no correlation with any of the environmental variables but *C. sessiliforum* AMF species profiles were correlated with both location and soil moisture. This suggests that the *C. americana* AMF communities maybe more uniform across a heterogeneous environment than the AMF communities from *C. sessiliforum*, which showed location and moisture dependence; different plant hosts in an ecosystem do not interact in identical ways with their AMF. The relationships between mycorrhizal symbionts, the environment, and host may differ from one host plant species to another; these complex interactions may be one of the levels at which emergent properties of an ecosystem, such as plant species composition is determined.

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СПЕЦИФІЧНІСТЬ, ПОВ'ЯЗАНА З ХАЗЯЇНОМ РОЗГАЛУЖЕНОЇ МІКОРИЗИ, ЩОДО ЖИВИЛЬНОГО І ГІДРОЛОГІЧНОГО ГРАДІЄНТА ҐРУНТУ

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У даній роботі порівнювали склад грибкових угруповань розгалуженої мікоризи (AMF), що колонізують коріння двох споріднених нативних рослин, які ростуть на невеликому природному узгір'ї в лісі східного Техасу (США), залежно від нелінійного градієнта вологості. Для виявлення AMF у корінні *Chasmanthium sessiliforum* (Poir.) Yates і *Callicarpa americana* використовували ПЦР-ампліфікацію ділянки гена малої субодиниці рибосомної 18S РНК з Glomeromycota-специфічними праймерами. Продукти ПЦР досліджували за допомогою денатуруючого градієнтного гель-електрофорезу з метою отримання електрофоретичних профілів угруповань. При порівнянні профілів було виявлено достовірні відмінності між угрупованнями AMF, пов'язані з належністю до двох хазяїв. Крім того, угруповання AMF, що колонізували *C. sessiliforum*, відрізнялися за положенням їх піку на нелінійному градієнті вологості.

Ключові слова: гриби розгалуженої мікоризи, *Arbuscular mycorrhizal*, денатуруючий градієнтний гель-електрофорез, DGGE, різновиди AMF, 18S рРНК, багатовимірний аналіз.

СПЕЦИФИЧНОСТЬ, СВЯЗАННАЯ С ХОЗЯИНОМ ВЕТВЯЩЕЙСЯ МИКОРИЗЫ, ОТНОСИТЕЛЬНО ПИТАТЕЛЬНОГО И ГИДРОЛОГИЧЕСКОГО ГРАДИЕНТА ПОЧВЫ

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В данной работе сравнивали состав грибковых сообществ ветвящейся микоризы (AMF), колонизирующих корни двух родственных нативных растений, произрастающих на небольшом естественном косогоре в лесу восточного Техаса (США), в зависимости от нелинейного градиента влажности. Для обнаружения AMF в корнях *Chasmanthium sessiliforum* (Poir.) Yates и *Callicarpa americana* использовали ПЦР-амплификацию участка гена малой субъединицы рибосомной 18S РНК с Glomeromycota-специфическими праймерами. Продукты ПЦР исследовали с помощью денатурирующего градиентного гель-электрофореза с целью получения электрофоретических профилей сообществ. При сравнении профилей были выявлены достоверные различия между сообществами AMF, связанные с принадлежностью к двум хозяевам. Кроме того, сообщества AMF, колонизирующие *C. sessiliforum*, отличались по положению их пика на нелинейном градиенте влажности.

Ключевые слова: грибы ветвящейся микоризы *Arbuscular mycorrhizal*, денатурирующий градиентный гель-электрофорез, DGGE, разновидности AMF, 18S рРНК, многомерный анализ.