# MICROBIAL SYMBIONTS OF AN INVASIVE GRASS DIFFER IN URBAN AND EX-URBAN ENVIRONMENTS

By

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**Abstract**

Buffelgrass (*Cenchrus ciliaris*) is a widespread, invasive plant in the Sonoran Desert. It establishes readily, weathers drought, alters fire regimes, and is costly and labor-intensive to eradicate. Investigating microbial symbionts of buffelgrass may identify factors that promote its establishment and rapid spread, as microbial symbionts of plants often are associated with enhancing host physiology and stress tolerance. The goal of this project was to evaluate the microbiome of buffelgrass under a wide range of environmental conditions in a portion of its invasive range. Specifically, we quantified the abundance, diversity, and composition of fungi associated with buffelgrass in urban areas (alleyways with poor soil and limited plant cover) and ex-urban areas (sites with natural soil and vegetation) in and near Tucson, AZ, USA. We isolated fungal endophytes from healthy roots and shoots and characterized distinctive strains via DNA barcoding following restriction fragment length polymorphism (RFLP) analysis. We found that endophytes of buffelgrass were more abundant in urban areas, but more diverse in ex-urban areas. The composition of endophyte communities also differed between urban and ex-urban sites. Our data are consistent with context-specific symbioses, in which buffelgrass recruits distinctive microbiomes under different environmental conditions. Future research will focus on identifying plant-microbe interactions that influence the fitness, germination, and stress tolerance of buffelgrass as an invasive plant.

***Keywords:*** Cenchrus ciliaris*, buffelgrass, endophytes, invasive species, plant-microbial interactions*

## Introduction

In concert with pressures from climate change, habitat fragmentation, and pollution, invasive species contribute markedly to global biodiversity loss (Soulé, 1991). In semi-arid and arid ecosystems like the Sonoran Desert, invasive species pose an especially urgent ecological threat (Marshall et al., 2012). Under the challenging environmental conditions of such ecosystems, the lasting impacts of disturbance and low resource availability mean native vegetation may be especially vulnerable to invasion by species that are able to exploit limited resources (Marshall et al., 2012). One such species is buffelgrass (*Cenchrus ciliaris*, syn. *Pennisetum ciliare,* Poaceae), an invasive bunchgrass that is becoming widespread in the Sonoran Desert.

Native to Africa, buffelgrass was initially introduced to the Sonoran Desert as cattle forage because of its strong tolerance for abiotically challenging environments and high forage value (Syamaladevi et al., 2015). However, buffelgrass readily establishes and spreads in a variety of environmental conditions, including urban landscapes, roadsides, and native desert soil (Marshall et al., 2012). This species flourishes after disturbances such as fire, displacing and crowding out native, keystone species like the saguaro cactus (*Carnegiea gigantea*) (D'Antonio & Vitousek, 1992; Stevens & Falk, 2009). The accumulation of buffelgrass on the landscape also alters the regional fire regime, such that increasingly frequent and intense fires harm native species of the Sonoran Desert that are not adapted to such severe fire conditions (McDonald & McPherson, 2011). Once established, buffelgrass creates a unique mitigation challenge because management is costly and labor-intensive (Stevens & Falk, 2009).

The physiological factors that contribute to the success of buffelgrass as an invasive species are well-known (Marshall et al., 2012). However, the degree to which these physiological traits reflect the impacts of symbiotic microbes has not been investigated.

Symbiotic microbes such as bacteria and fungi affiliate with plants in diverse environments (Arnold & Lutzoni, 2007). Those that occur within healthy tissues – endophytes – are especially intimate symbionts with their hosts, as they colonize and live within roots and leaves without causing symptoms of disease (Wilson, 1995). Endophytes often confer fitness benefits to their plant host, including drought tolerance, increased growth, and nutrient acquisition (Clay, 2009; Rodriguez et al., 2009). Invasive species often affiliate positively with the soil microbiome of their introduced range by engaging in habitat specific symbioses (Rout & Callaway, 2009). Given this context, what role do microbial interactions play in the ability of buffelgrass to exploit abiotically challenging environments such as those of the Sonoran Desert?

Here we evaluate the composition of the microbial community of buffelgrass in a variety of environmental conditions in a portion of its invasive range. Specifically, we quantified the abundance, diversity, and composition of fungi associated with buffelgrass in urban areas (alleyways with poor soil and limited plant cover) and ex-urban areas (sites with natural soil and vegetation) in and near Tucson, AZ, USA. Given the nature of microbial communities previously evaluated in the Sonoran Desert (Massimo et al., 2015), we expected that endophytes of buffelgrass would be more abundant, more diverse, and distinctive in ex-urban plants found in native desert locations with low disturbance vs. urban plants found in degraded soils and high disturbance.

**Methods**

In June and July 2018, we collected buffelgrass from five locations in and around the Tucson, AZ metropolitan area (*Figure 1*). These locations were categorized as either urban or ex-urban based on the history of development and the level of soil degradation near the collection site.

Urban sites feature degraded, nutrient-poor soil with limited plant cover, while ex-urban sites featured natural soil and native desert vegetation. A total of 42 buffelgrass plants (18 urban and 24 ex-urban) were sampled from these five locations.

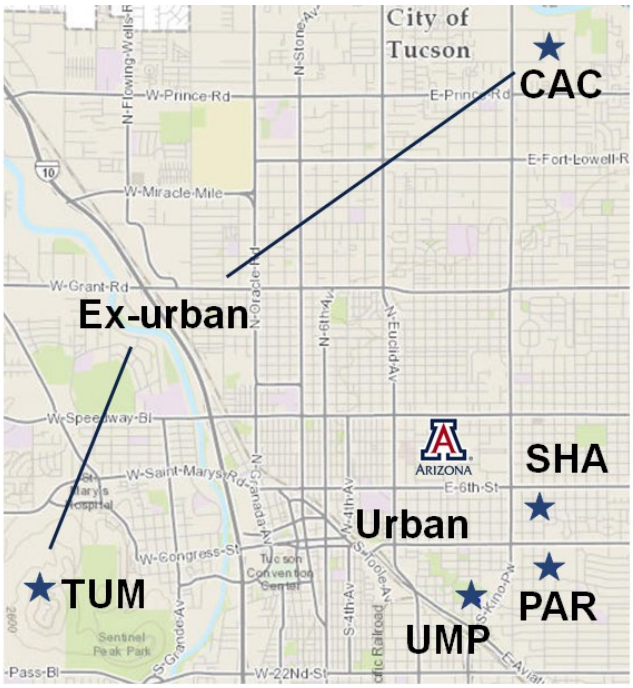


**Figure 1.** *A map of the city of Tucson, AZ, featuring the urban collection sites of Sam Hughes Alleyway (SHA), University of Arizona (UArizona) Motorpool (UMP), and the UArizona Park and Ride Lot #9008 (PAR) as well as the ex-urban collection sites of Tumamoc Hill (TUM) and the UArizona Campus Agricultural Center (CAC).*

The urban locations were a municipal alleyway in the Sam Hughes neighborhood located off N. Plumer Ave. (32.226215 N, -110.940079 W), the roadside of the University of Arizona (UArizona) Motorpool on E. 16th St. (32.214695 N, -110.948684 W), and an alleyway adjacent to the UArizona Park and Ride Lot #9008 on S. Plumer Ave. (32.218762 N, -110.939204 W). The ex-urban locations were the east and south slopes of Tumamoc Hill, courtesy of the UArizona Desert Laboratory (32.217692 N, -111.002377 W and 32.207500 N, -111.008611 W,

respectively), and a natural arroyo located in the UArizona Campus Agricultural Center (CAC) near the Rillito River (32.280369 N, -110.937547 W).

Each urban location contained two sub-areas separated by at least 10 m. Each sub-area had one collection site where three buffelgrass plants were obtained, for a total of six plants obtained per urban location. Each ex-urban location had two sub-areas; the Tumamoc Hill sub-areas were approximately 1000 m apart while the CAC sub-areas were about 150 m apart from each other. Each ex-urban sub-area consisted of two collection sites where three buffelgrass plants were collected for a total of 12 buffelgrass plants obtained per ex-urban location.

*Sample Processing*

Following collection, plants were placed in cold storage (4°C) for a maximum of three days before processing. After removal from cold storage, shoots (leaves and stems) and roots were thoroughly washed in running tap water to remove residual soil. Next, the roots and shoots of each plant were cut into 1 cm long segments.

Tissue segments were placed in clean Petri dishes (60 mm) for surface sterilization. All tissue segments were surface sterilized following the standard lab protocol of 10 seconds in 95% EtOH, two minutes in 0.5% NaOCl, and two minutes in 70% EtOH (Arnold & Lutzoni, 2007). This surface sterilization technique allows for the isolation of endophytes by removing microbes from the exterior of the tissue segments while keeping microbes inside the plant tissue intact (see Shaffer et al., 2017). Surface sterilized tissues were kept in a laminar flow hood to air dry in sterile conditions prior to plating.

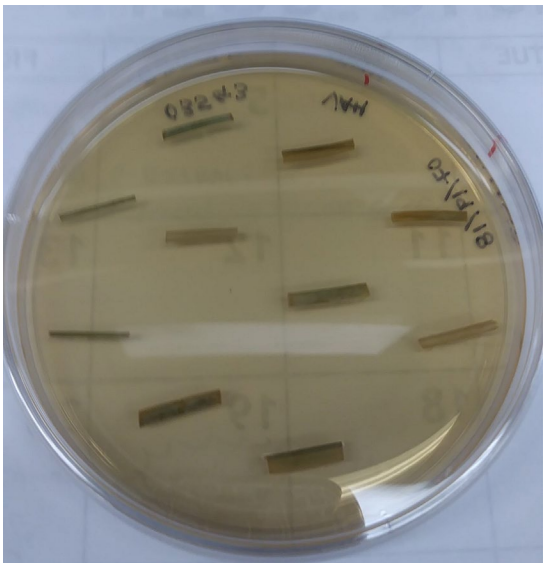

**Figure 2.** *Sterilized green shoot tissue plated on 100 mm 2% MEA plates.*

We sorted and plated the surface-dried tissue segments on 2% malt extract agar (MEA, 20.0 g malt extract, 20.0 g agar, 1.0 L Milli-Q water) in 100 mm Petri dishes containing (*Figure 2*). We generated seven plates per plant: three plates of green shoots, one plate of brown shoots, and three plates of roots. We included brown shoots to capture endophytes that may have been present in recently living plant tissue. Every plate had 10 tissue segments for a total tissue segment total count of 2,940. We sealed the plates with Parafilm and stored them in a stable location at room temperature (~23 °C) until microbial growth was observed. We checked for microbial growth on a daily basis over the course of two months.

*Microbial Isolation*

Once microbial growth appeared, individual isolates of bacteria and fungi were transferred to 60 mm MEA plates under sterile conditions in a biosafety cabinet via sterile, autoclaved toothpicks. Isolates were stored at room temperature (~23 °C) until microbial growth was observed. Once isolates were given ample time to grow, they were sorted into morphotypes on the basis of

physical characteristics like mycelium color, texture, shape, growth pattern, and general appearance (Ko Ko et al., 2011).

*DNA Extraction and Barcode Sequencing*

We chose candidate isolates from each morphotype to characterize by barcode sequencing. DNA extraction followed Shaffer et al. (2017). We used the polymerase chain reaction (PCR) following Shaffer et al. (2017) to amplify the barcode loci for fungi (nuclear internal transcribed spacer and 5.8S region) using the ITS1F and LR3 primers. Positive PCR products were confirmed through visualization by staining and gel electrophoresis on a 1% agarose gel. Due to the subjective nature of morphotyping, restriction fragment length polymorphism (RFLP) analyses were completed prior to genetic sequencing (Ko Ko et al., 2011). RFLP analyses followed Oono et al. (2015) as modified by Leo (2019).

Successful PCR products were purified (see Shaffer et al., 2017) and sent to the UArizona Genetics Core, where they were sequenced bidirectionally on the Sanger sequencing platform (Heather & Chain, 2016). We edited the sequences manually in Sequencher v5.1 to ensure quality. We compared edited sequences to records in GenBank via the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to tentatively identify strains. Sequence data were assembled into operational taxonomic units (OTU) on the basis of 95%, 99%, and 100% sequence similarity (Vu et al., 2019) in the Tree Based Alignment Selector (TBAS) toolkit (Carbone et al., 2016). Taxonomy and OTU designations then were assigned to the entire collections of isolates based on RFLP band patterns.

**Results and Discussion**

Overall, 638 bacterial isolates and 586 fungal isolates were obtained from buffelgrass roots and leaves over the course of this study. Based on isolation data, each buffelgrass individual harbored at least 15 endophytes on average, indicating endophyte colonization of buffelgrass tissue is common. The fungal isolation frequency (19.9%) was higher than has been observed previously for leaves of many plants native to the region (see Massimo et al., 2015), in part due to the relatively high isolation frequency in roots of buffelgrass. Overall our data indicate that buffelgrass readily associates with endophytes in its invasive range.

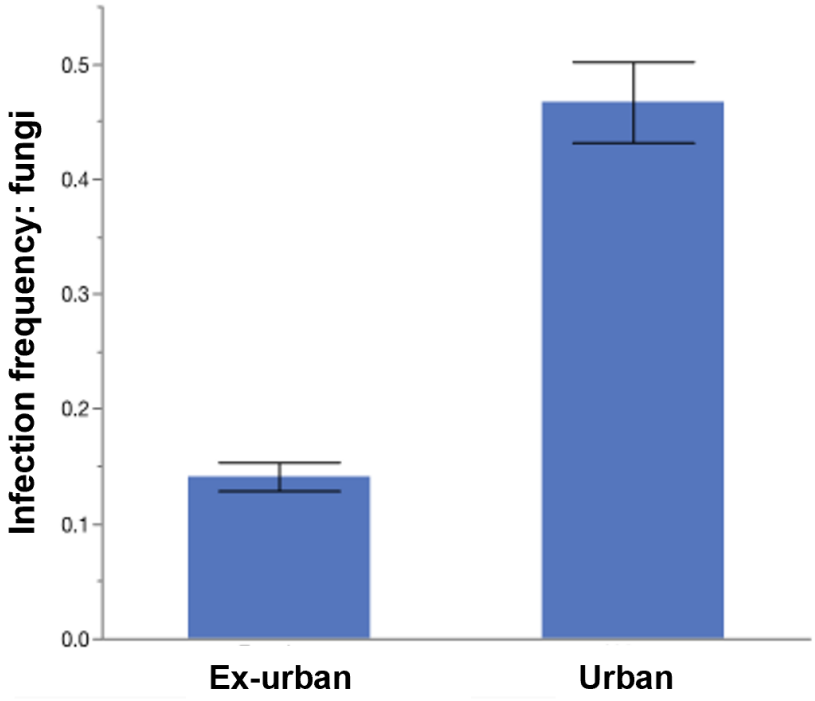


**Figure 3.** *Mean isolation frequency for fungal endophytes, calculated as the percent of tissue segments that yielded an isolate in culture.*

We found that isolation frequency varied among the types of sites surveyed here: endophytes were isolated significantly more frequently from ubran plants than from ex-urban plants ($t = 4.85$, $p < 0.0001$, data logit-transformed prior to analysis) (*Figure 3*). This difference was nearly threefold higher in the urban areas (*Figure 3*). It is possible that plants had been

established longer in the urban vs. ex-urban areas, or that their roadside positions generally afforded them more water and more extensive growth, thus encouraging additional endophyte colonization.

Although bacteria and fungi were observed, we focused on fungi because of their higher abundance (above) and greater degree of morphological diversity. A total of 57 morphotypes were observed on the basis of physical characteristics, including mycelium color, shape, texture, growth pattern, and overall appearance. RFLP analysis following PCR was able to eliminate 18 morphotypes as redundant. Thus, 39 fungal samples were chosen for DNA sequencing and identification. These results highlight that morphotyping can be misleading with regard to estimating fungal biodiversity, as morphology in culture can be quite flexible.

Of those 39 fungal samples, 35 produced high-quality sequencing results (89.7%), which were analyzed with TBAS and BLAST to establish OTUs and tentatively assign taxonomy (*Table 1*). The endophytes were generally filamentous Ascomycota with species of *Fusarium* especially common in roots and highly pigmented fungi (e.g., *Cladosporium*) more common in shoots. Many of the taxa are common in this region (Massimo et al., 2015; Leo 2019), suggesting that buffelgrass has co-opted local symbionts in this area rather than arrived and established with a non-native symbiont community

**Table 1.** *OTU identity based on TBAS and BLAST results. Includes tissue type and collection location. B = brown, G = green. Different taxonomic IDs between TBAS and BLAST reflect anamorph/teleomorph names and/or different taxa in each reference set. Tissue type and urban/ex-urban data are compiled based on the occurrence of each OTU.*

| **100% OTU** | **B or G Shoots/Roots** | **Urban/Ex-urban** | **Closest Match (TBAS)** | **Closest Match (BLAST)** |
|---|---|---|---|---|
| OTU00 | Roots | Both | *Magnaporthe grisea* | ***Gaeumannomyces hyphopodioides*** |
| OTU01 | B shoots | Ex-urban | *Preussia minima* | ***Preussia sp.*** |
| OTU02 | Both shoots | Ex-urban | *Aspergillus terreus* | ***Aspergillus sp.*** |

| | | | | |
|---|---|---|---|---|
| OTU03 | Both shoots | Urban G shoots, Ex-urban both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU04 | Roots | Urban | *Curvularia geniculata* | ***Bipolaris sp.*** |
| OTU05 | Roots | Urban | *Chaetomium elatum* | ***Acrophialophora fusispora*** |
| OTU06 | Roots | Both | *Nectria haematococca* | ***Fusarium sp.*** |
| OTU07 | Roots | Urban | *Acremonium persicinum* | ***Unknown*** |
| OTU08 | Roots | Both | *Cladosporium cladosporioides* | ***Acremonium persicinum*** |
| OTU09 | G shoots | Ex-urban | *Cladosporium cladosporioides* | ***Cladosporium sp.*** |
| OTU10 | Roots | Ex-urban | *Fusarium graminearum* | ***Fusarium sp.*** |
| OTU11 | B shoots | Ex-urban | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU12 | Both shoots | Urban G shoots, Ex-urban both | *Ascochyta rabiei* | ***Phoma sp.*** |
| OTU13 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU13 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU13 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU14 | Roots | Both | *Aspergillus fumigatus* | ***Aspergillus fumigatiaffinis*** |
| OTU15 | Roots | Ex-urban | *Fusarium polyphialidicum, Fusarium graminearum* | ***Fusarium sp.*** |
| OTU16 | Roots | Urban | No close match | **Unknown** |
| OTU17 | B shoots | Ex-urban | *Nectria haematococca* | ***Fusarium sp.*** |
| OTU18 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU19 | Roots | Ex-urban | *Melanopsamma pomiformis* | ***Fusarium sp.*** |
| OTU20 | Roots | Ex-urban | *Phaeodothis winteri* | ***Periconia sp.*** |
| OTU21 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU22 | Roots | Ex-urban | *Aspergillus terreus* | ***Aspergillus terreus*** |
| OTU23 | Roots | Urban | *Eutypa lata* | ***Diatrypella pulvinata*** |
| OTU24 | Roots | Both | *Eutypa lata* | ***Daldinia vernicosa*** |
| OTU24 | Roots | Both | *Eutypa lata* | ***Diatrypella pulvinata*** |
| OTU25 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU26 | Both shoots | Urban B shoots, Ex-urban G shoots | Eurotiomycetes sp. | ***Aspergillus sp.*** |
| OTU27 | Roots | Urban | No close match | ***Fusarium sp.*** |
| OTU28 | B shoots | Ex-urban | *Ascochyta rabiei* | ***Phoma sp.*** |
| OTU29 | B shoots | Ex-urban | *Chaetomium elatum* | **Unknown** |
| OTU30 | Both shoots | Both | *Curvularia geniculata* | ***Bipolaris sp.*** |
| OTU31 | Roots | Urban | No close match | ***Cladosporium cladosporioides*** |

| | | | | |
|---|---|---|---|---|
| OTU32 | Roots | Both | *Fusarium polyphialidicum* | ***Fusarium sp.*** |
| OTU33 | Roots | Both | *Aspergillus terreus* | ***Aspergillus terreus*** |
| OTU34 | B shoots | Both | No close match | **Unknown** |
| OTU35 | Roots | Both | No close match | **Unknown** |

*Microbial Community Composition*

As fungi were more common in roots than in shoots, we focused on root endophytes in evaluating variation in endophyte diversity between urban and ex-urban areas. We measured diversity as Fisher's alpha (see Massimo et al., 2015) (*Figure 4*). Endophyte diversity was significantly higher in ex-urban plants compared to those in urban settings ($t = -3.23$, $P = 0.0458$).

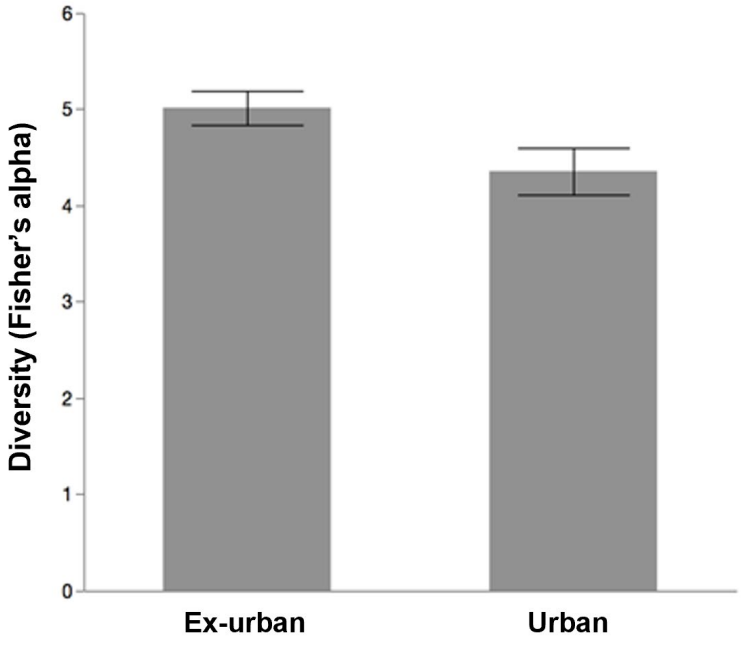


**Figure 4.** *Mean diversity of fungal endophytes in root tissue, as measured by Fisher's alpha.*

We used non-metric multidimensional scaling (NMDS) to visualize communities of fungi as a function of tissue type and location type. The NMDS analysis was done at the 95% OTU level based on abundance data (*Figure 5*) and presence-absence data (*Figure 6*). In each case, proximity between points indicates similarity in community. For both abundance and presence-absence data, there are distinctive endophyte communities based on both tissue type (roots vs. shoots) and collection location (ex-urban vs. urban).

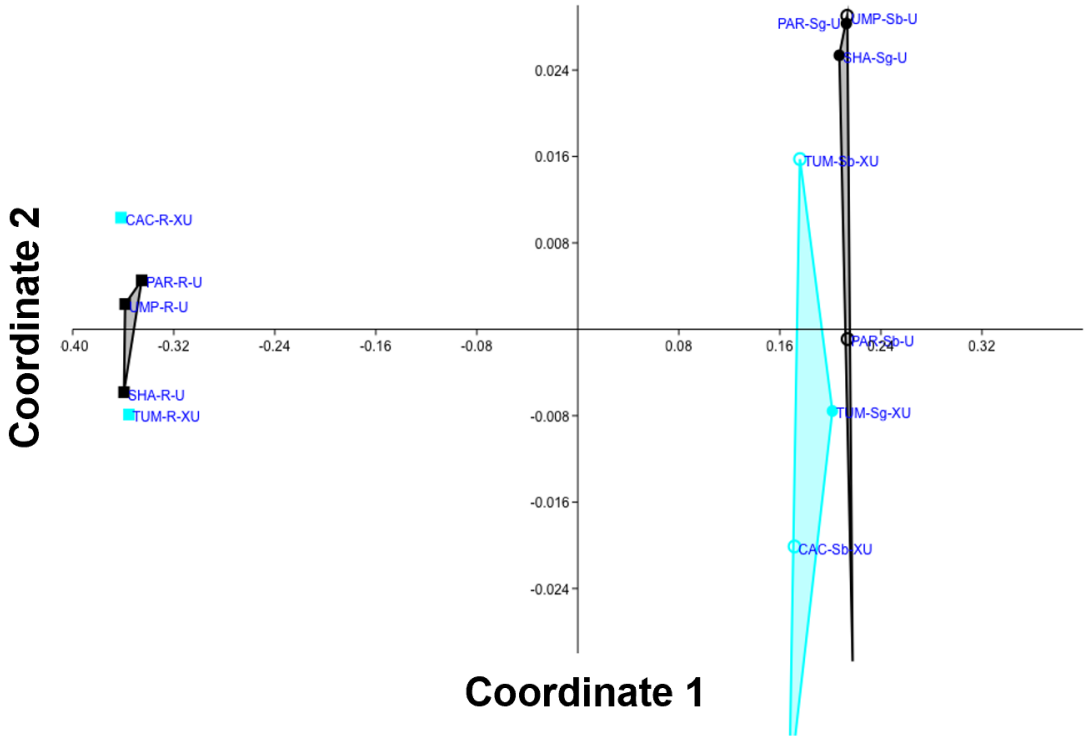


**Figure 5.** *Non-metric multidimensional scaling (NMDS) of fungal endophyte communities based on abundance data at the 95% OTU level. The grouping of points to the left is the fungal community in root tissue while the right grouping is shoot tissue. The cyan points are ex-urban sampling locations, and the black points are urban locations.*

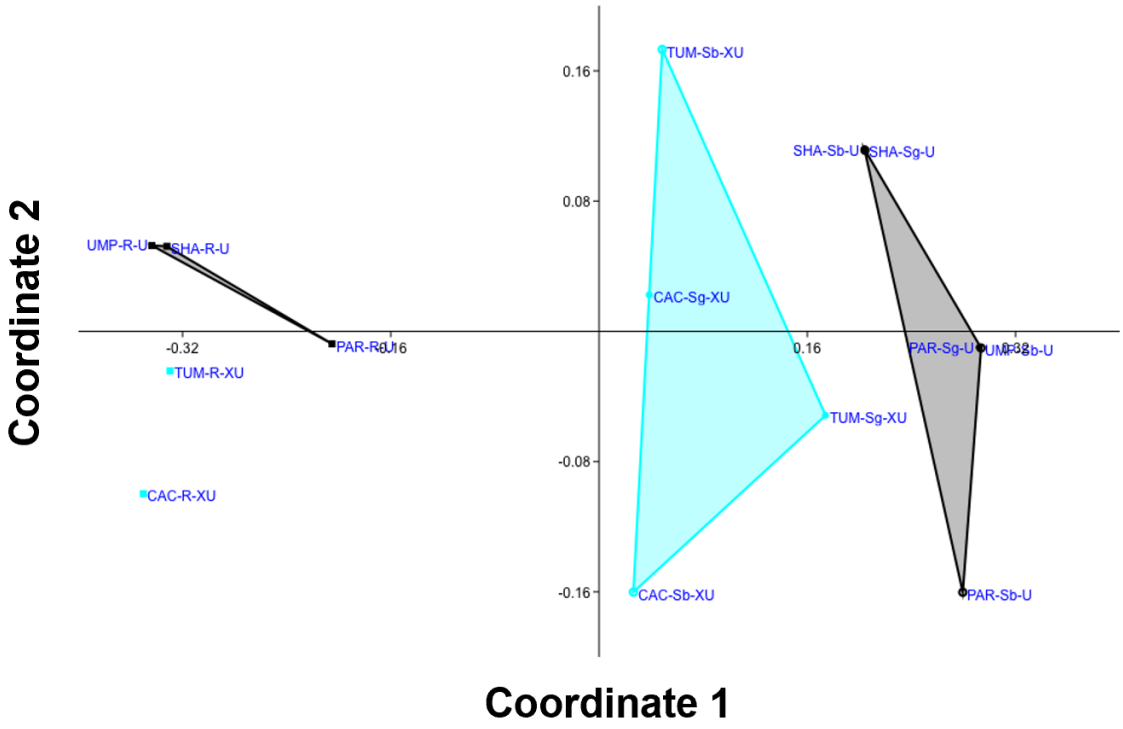


**Figure 6.** *NMDS of fungal endophyte communities based on presence-absence data at the 95% OTU level. The grouping of points to the left is the fungal community in root tissue while the right grouping is shoot tissue. The cyan points are ex-urban sampling locations, and the black points are urban locations.*

At the 100% OTU level, the composition of endophyte communities was visualized for each tissue type and location. Several OTUs were unique to each community, but there are a few ubiquitous OTUs. For example, OTU 12 is found in both green and brown shoots, while OTU 34 is unique to green shoots (*Figure 7, Table 1*). Overall, communities differed most strikingly between urban vs. ex-urban shoots, whereas communities often had similar members (albeit at different relative abundances) for roots in urban vs. ex-urban plants.

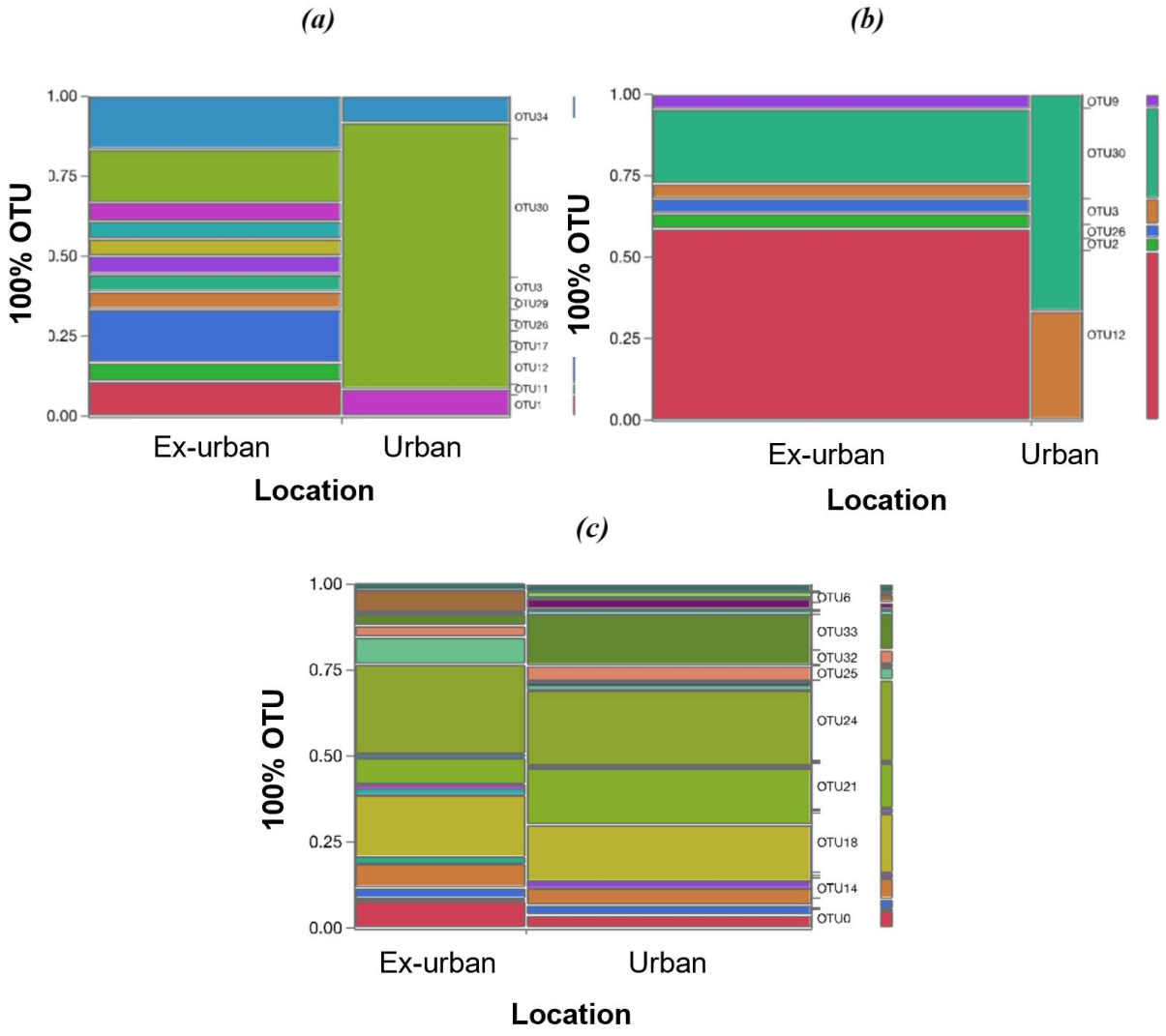


**Figure 7.** *Distribution of endophytic fungi at the 100% OTU level, where* ***(a)*** *is brown shoots,* ***(b)*** *is green shoots, and* ***(c)*** *is roots.*

## Conclusions

Four main takeaways can be discussed from these results. First, endophytes are ubiquitous in the roots and shoots of buffelgrass in a representative area of the Sonoran Desert that encompassed both urban and relatively undisturbed, ex-urban areas. Second, endophytes were more abundant in terms of isolation frequency in urban areas. Third, fungal endophytes in root tissue were more diverse in plants collected at ex-urban sites than urban sites. Lastly, fungal endophyte

community composition differs as a function of both tissue type and urban/ex-urban area. Many endophytes we isolated from buffelgrass can also be found in other plant hosts previously confirmed to reside in the Sonoran Desert (see Massimo et al., 2015), supporting the habitat specific symbiosis where invasive plants integrate with native soil microbiomes (*Table 1*). Overall, our data are consistent with the broad idea of context-specific symbioses, in which buffelgrass recruits distinctive microbiomes under different environmental conditions. In future work we hope to examine how these fungi may interact with buffelgrass in terms of physiological enhancements or other potential benefits, and to examine whether they may play a role in depressing germination of seeds of native plants.

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