Archives of Microbiology (2021) 203:1211–1219
https://doi.org/10.1007/s00203-020-02119-z

ORIGINAL PAPER

Assessment of fungal diversity in soil rhizosphere associated with *Rhazya stricta* and some desert plants using metagenomics

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Received: 31 March 2020 / Revised: 19 May 2020 / Accepted: 8 November 2020 / Published online: 24 November 2020

Abstract

This study aimed to compare the fungal rhizosphere communities of *Rhazya stricta*, *Enneapogon desvauxii*, *Citrullus colocynthis*, *Senna italica*, and *Zygophyllum simplex,* and the gut mycobiota of *Poekilocerus bufonius* (Orthoptera, Pyrgomorphidae, "Usherhopper"). A total of 164,485 fungal reads were observed from the five plant rhizospheres and Usherhopper gut. The highest reads were in *S. italica* rhizosphere (29,883 reads). Species richness in the *P. bufonius* gut was the highest among the six samples. Ascomycota was dominant in all samples, with the highest reads in *E. desvauxii* (26,734 reads) rhizosphere. Sordariomycetes and Dothideomycetes were the dominant classes detected with the highest abundance in *C. colocynthis* and *E. desvauxii* rhizospheres. *Aspergillus* and *Ceratobasidium* were the most abundant genera in the *R. stricta* rhizosphere, *Fusarium* and *Penicillium* in the *E. desvauxii* rhizosphere and *P. bufonius* gut, *Ceratobasidium* and *Myrothecium* in the *C. colocynthis* rhizosphere, *Aspergillus* and *Fusarium* in the *S. italica* rhizosphere, and *Cochliobolus* in the *Z. simplex* rhizosphere. *Aspergillus terreus* was the most abundant species in the *R. stricta* and *S. italica* rhizospheres, *Fusarium* sp. in *E. desvauxii* rhizosphere, *Ceratobasidium* sp. in *C. colocynthis* rhizosphere, *Cochliobolus* sp. in *Z. simplex* rhizosphere, and *Penicillium* sp. in *P. bufonius* gut. The phylogenetic results revealed the unclassified species were related closely to Ascomycota and the species in *E. desvauxii*, *S. italica* and *Z. simplex* rhizospheres were closely related, where the species in the *P. bufonius* gut, were closely related to the species in the *R. stricta,* and *C. colocynthis* rhizospheres.

Keywords Fungi · Metagenomics · Rhizosphere · *Rhazya stricta* · Gut · Usherhopper

Introduction

The climate in Saudi Arabia is hot and dry throughout most of the country. Due to its arid climate, the flora of Saudi Arabia has been neglected like other Gulf countries in the Arab peninsula. Folk medicine, which is widely practiced, maintains an important part of Saudi Arabia's health legacy (Al-Essa et al. 1998). Public interest in traditional medicine, especially

Communicated by Erko Stackebrandt.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00203-020-02119-z) contains supplementary material, which is available to authorized users.

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in towns, has decreased with the integration of western modern medicine in Saudi Arabia.

Rhazya stricta Decne (Gentianales, *Apocynaceae*) is a widely distributed dogbane plant that is a dominant species in the rangeland of Saudi Arabia due to its allelopathic effects (Assaeed 1996; Allred 1968). To characterize the fungal diversity among the flowering plants, *Aristolochia bracteolate* (Piperales, Aristolochieae), *Haplophyllum tuberculatum* (Sapindales, Rutaceae), *Nigella sativa* (Ranunculales, Ranunculaceae), *Rhazya stricta*, *Teucrium muscatense* (Lamiales, Lamiaceae), *Trigonella foenum-graecum* (Fabales, Fabaceae), and *Zataria multiflora* (Lamiales, Lamiaceae) have been monitored (Elshafie et al. 2003). Twenty-four fungal species were isolated from the different parts of plants and there were no differences in significance among the herbal plant species mycoflora. The common fungal species isolated were *Aspergillus niger* and *Penicillium* sp. (Eurotiales, Trichocomaceae), followed by *Rhizopus* spp., and *Aspergillus flavus* (Elshafie et al. 2003).

The study of soil fungal flora associated with *R. stricta* showed that there were 14 species belonging to five genera, representing three orders: *Penicillium* (Eurotiales, Trichocomaceae), *Aspergillus* (Eurotiales, Trichocomaceae), *Mucor* and *Rhizopus* (Mucorales, Mucoraceae), and *Fusarium* (Hypocreales, Nectriaceae). *Aspergilli* were most frequently *A. terreus,* the most dominant among *Aspergillus* spp. These results suggest that soil fungal flora of the *R. stricta* plant is complex and diverse (Baeshen et al. 2014). In another study, fungal communities at Mecca old road showed that three phyla, *Ascomycota*, *Basidiomycota* and *Chytridiomycota* were recorded (Moussa et al. 2017). Metagenomic techniques have been utilized to study the fungal diversity of soil and rapidly characterize the biodiversity of many soils all over the world (Schmit and Mueller 2007; DeSalle et al. 2008; Hirsch et al. 2010; Blackwell 2011). Few metagenomic studies have been used to characterize microflora of soils in developing countries, or in Saudi Arabia (Yasir et al. 2015).

Rhazya stricta, *Enneapogon desvauxii*, *Citrullus colocynthis*, *Senna italica*, and *Zygophyllum simplex* were chosen because they are native plants that Bedouin use in folk medicine to treat many diseases. This study aimed to compare rhizosphere fungal communities of the five selected plants and the gut mycobiota of *Poekilocerus bufonius*, which is a type of grasshopper, known as Usherhopper that feeds on milkweed plants.

Methods

Area of study

The chosen study site was a *Rhazya stricta* community and public site (Hadda, Mecca-Jeddah road) at **N**21° 45′ 04.03", **E**39° 53′ 88.92". Field samples were taken from the rhizosphere of five plants (1A, *Rhazya stricta*; 2A, *Enneapogon desvauxii*; 3A, *Citrullus colocynthis*; 4A, *Senna italica;* soil5, *Zygophyllum simplex*) in the morning in June 2014 at 40 ºC. Soil cores were taken and pooled. Samples were stored in Falcon tubes (50 ml) at − 20 ºC until analysis. In addition, the gut of *P. bufonius* (Usherhopper DNA) was collected and stored.

DNA Extraction, library construction, and PCR

Next-generation sequencing followed Nilsson et al. (2008). Genomic DNA was extracted using MoBio Power kit from 5 g soil. A quality test was done, once the DNA samples were obtained, and then the whole qualified DNA was used to construct libraries. PCR products were converted into blunt ends. To add adapters, an 'A' nucleotide was added to each 3′ end. The fusion primer with dual adapters and index was used for PCR, and fragments that were too short were removed. Only the qualified library was used for sequencing. Illumina HiSeq/MiSeq encoded amplicons were used for monitoring the rhizosphere fungal diversity in the five plants and usherhopper gut. The primer pair ITS1 and ITS4 were used to amplify the qualified DNA (Moussa et al., 2017).

Bioinformatics analysis

Bioinformatics analysis for sequencing data was carried out. Filtration of the raw data was done to obtain clean reads, and tags were clustered at 97% sequence identity to an operational taxonomic unit (OTU), which assigned its taxonomy.

Processing of data and statistical analysis

Data statistics

The raw data were processed by an in-house procedure to obtain more reliable and accurate results (Douglas et al. 2014). For pooling of libraries, clean reads were assigned to samples. The results of data processing are listed in Table S1.

Paired-end reads

Both two paired-end reads overlapping regions were merged using **FLASH** v1.2.11 (Table S2) (Magoc and Salzberg 2011).

Analysis of community patterns

The sequences of the Internal Transcript Spacer [ITS1 and 4] were screened using the gold database (v20110519) for chimeras and UNITE (v20140703) separately for 18S rDNA

sequences. The de novo chimera detection was done, and all tags were mapped to each representative OTU sequence. Sequences representing OTUs were taxonomically classified using 0.8 confidence values as a cutoff. Databases used for species annotation: 18S rDNA and ITS for the fungal community were used Silva V119 (Quast et al. 2013) and UNITE Version 6 (Abarenkov et al. 2010). OTUs that were not assigned to certain species were removed. A Venn diagram was drawn to the illustrated overlap of OTUs for each group. Different colors represent different samples or groups.

Results

OTU cluster and abundance

OTU statistics

Filtered tags were clustered into OTU at 97% similarity (Table 1). The results revealed that the *P. bufonius* gut sample was the richest and most diversity (162 OTUs), while the least diversity was obtained in the *S. italica* rhizosphere sample (66 OTUs), where the degree of sample diversity represents primarily the number per sample. In addition to the OTU count, the *C. colocynthis* rhizosphere sample had the highest tag sequence (29,883 bp), while the least tag sequences number was obtained in the *P. bufonius* gut sample (18,816 bp) (Table 1).

Venn chart of OTUs

A Venn diagram was used to visualize the number of common and unique OTUs in multiple samples. The microbiome core from different environments could be estimated from the OTU representative species. The overlapping area and/or intersections represents the set of OTUs shared with the counterpart. There were 15 shared OTUs among all five samples (Fig. 1). The rhizospheres of *S. italica, C. colocynthis*, *E. desvauxii*, *R. stricta*, and the gut of *P. bufonius* were composed of 10, 19, 26, 42, and 117 unique OTUs, respectively.

Table 1 The operational taxonomic unit (OTU) and tag numbers of the rhizosphere of five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and gut of *P. bufonius*

Sample name	Tag number	OTU number
R. stricta	29,560	114
E. desvauxii	29,686	105
C. colocynthis	29,883	84
S. italica	27,558	66
Z. simplex	28,982	92
P. bufonius	18,816	162
Total	164,485	326

Principal component analysis (PCA)

The fungal metagenomic differences among the five samples were reflected in a Principal Component Analysis (PCA) plot. The PCA plot compares OTU distribution among the different rhizosphere soil samples. The 1st ordination axis (35.39% of the total variance) separated the fungal community in the rhizosphere soil of *R. stricta* from those of the other samples, whereas the 2nd ordination axis (29.55% of the total variance) distinguished the communities of fungi in *C. colocynthis* rhizosphere soil and *Z. simplex* from the other samples (Fig. 2).

Sampling depth and species richness

The horizontal asymptote was reached in only the curves representing the rhizosphere samples of *C. colocynthis* and *S. italica*, suggesting sufficient sampling depth from these samples. The curves of *R. stricta* and *E. desvauxii* rhizosphere samples appeared to have reached the plateau (Figs. 3 and S1–S4).

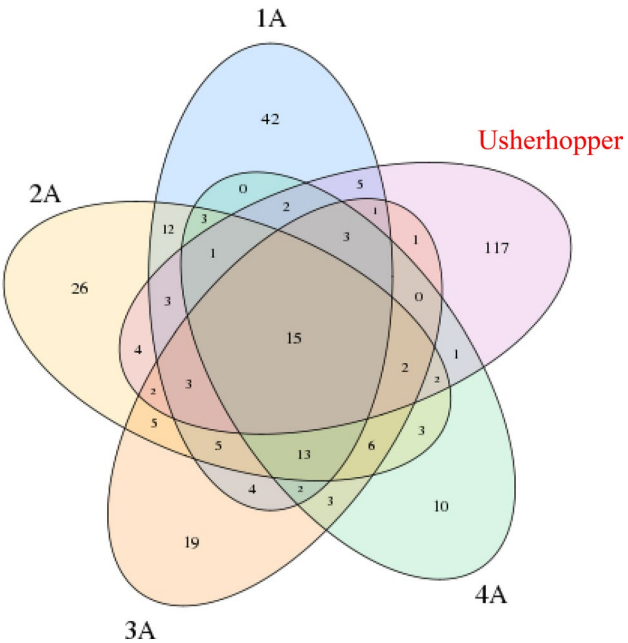

Fig. 1 The unique and shared operational taxonomic unit (OTUs) across different rhizosphere samples of the four plants rhizosphere (**1a**, *R. stricta,* **2a,** *E. desvauxii,* **3a**, *C. colocynthis,* **4a**, *S. italica*) and the gut of usherhopper (*P. bufonius*)

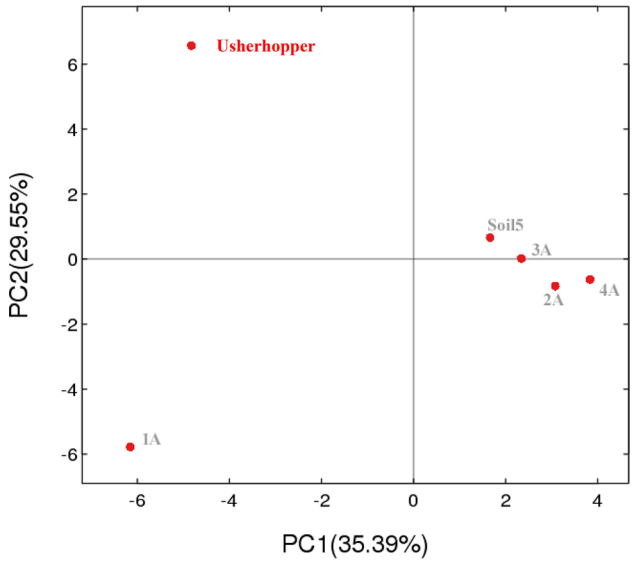

Fig. 2 The principal component analysis (PCA) based on OTU abundance of different rhizosphere samples of the five plants rhizosphere (**1a**, *R. stricta,* **2a,** *E. desvauxii,* **3a**, *C. colocynthis,* **4a**, *S. italica and Z. simplex* Soil5 and the gut of usherhopper (*P. bufonius*)

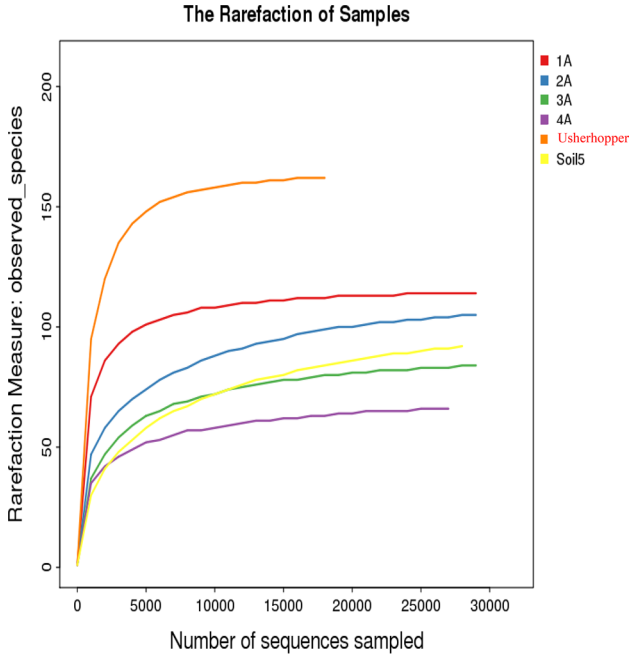

Fig. 3 Species accumulation curves of multiple rarefactions based on observed species value across the rhizosphere of the five plants (**1a**, *R. stricta,* **2a**, *E. desvauxii,* **3a**, *C. colocynthis,* **4a**, *S. italica* and **Soil5**, *Z. simplex*), and the gut of usherhopper (*P. bufonius*)

Species composition and abundance

The total number of fungal reads was 164,485 from all six metagenomes. The number and ranking of fungal reads are shown in Table 2. The highest number was in the *S. italic*a rhizosphere (29,883), and the lowest was in the gut of *P. bufonius* (18,816 reads).

Species annotation

At phylum level, the rhizospheres of the five plants and the gut of *P. bufonius* were composed of only two fungal phyla (Ascomycota and Basidiomycota), with Ascomycota as the dominant phylum (Fig. 4). At the class level, the two classes *Sordariomycetes* and *Dothideomycetes*, which belong to Ascomycota, were dominant over all the classes of Ascomycota and Basidiomycota (Fig. 5). At the order level, the most abundant order was Eurotiales (Eurotiomycetes), followed by Hypocreales (Sordariomycetes) and Pleosporales (Dothideomycetes) (Table S4 and Fig. 6). At the family level, the dominant families detected were Trichocomaceae and Didmellaceae (Ascomycota), also family Ceratobasidiaceae, (Basidiomycota) (Table S5 and Fig. 7).

The most abundant genera and species were *Aspergillus* (*Aspergillus terreus*), and *Ceratobasidium* (*Ceratobasidium* sp.) in the *R. stricta* rhizosphere, while *Fusarium* (*Fusarium* sp.) and *Penicillium* (*Penicillium* sp.) in the rhizosphere of *E. desvauxii* and *P. bufonius* gut. *Ceratobasidium* (*Ceratobasidium* sp.), and *Myrothecium* (*Myrothecium roridum*) in the *C. colocynthis* rhizosphere, *Aspergillus* (*Aspergillus terreus*) and *Fusarium* (*Fusarium* sp.) in the *S. italica* rhizosphere, and *Cochliobolus* (*Cochliobolus* sp.) in the *Z. simplex* rhizosphere (Tables S6 & S7, and Figs. 8 & 9).

Phylogenetic analysis

From Figures S5A-F, the unclassified species were closely related to *Ascomycota* and the species in the rhizosphere of *E. desvauxii*, *S. italica*, and *Z. simplex* were closely related to each other, while that of *P. bufonius* gut (Usherhopper) and the rhizospheres of *R. stricta* and *C. colocynthis* were closely related to each other. From the phylogenetic tree at the genus level (Fig. 10), the genera *Cladosporium, Arthrobotrys, Phoma, Kabatiella, Aspergillus, Myrothecium*, and *Candida* were classified under Ascomycota, while the genus *Jaminaea* was classified under Basidiomycota.

Data availability

All sequence data were submitted to NCBI and have accession numbers: SRR6447717, SRR6447718, SRR6447719, SRR6447720, SRR6447721, and SRR6447722, with direct link http://www.ncbi.nlm.nih.gov/sra?term=SRP128163.

Table 2 The number of reads in the fungal phyla of the five plants rhizosphere and gut of *P. bufonius*

Taxon	*R. stricta*	*E. desvauxii*	*C. colocynthis*	*S. italica*	*Z. simplex*	*P. bufonius*	Total
Ascomycota	15,272	26,734	24,339	16,580	24,787	15,447	123,159
Basidiomycota	257	18	372	7	5	2083	2742
Unclassified	14,031	2934	5172	10,971	4190	1286	38,584
Total	29,560	29,686	29,883	27,558	28,982	18,816	

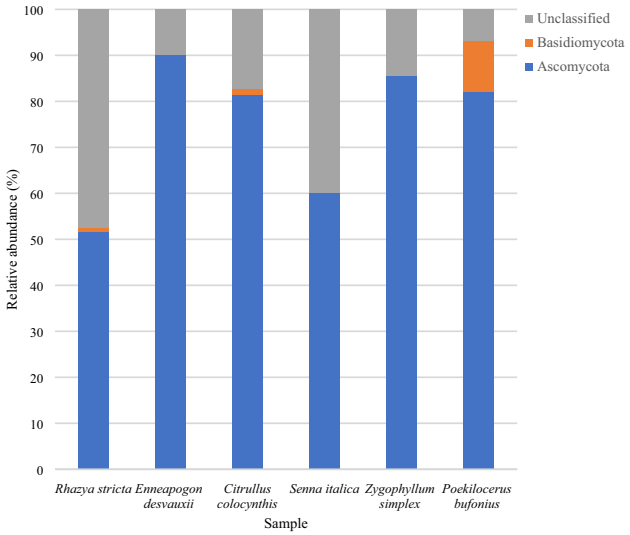

Fig. 4 The taxonomic composition distribution at Phylum-level in the rhizosphere of the five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and the gut of *P. bufonius*

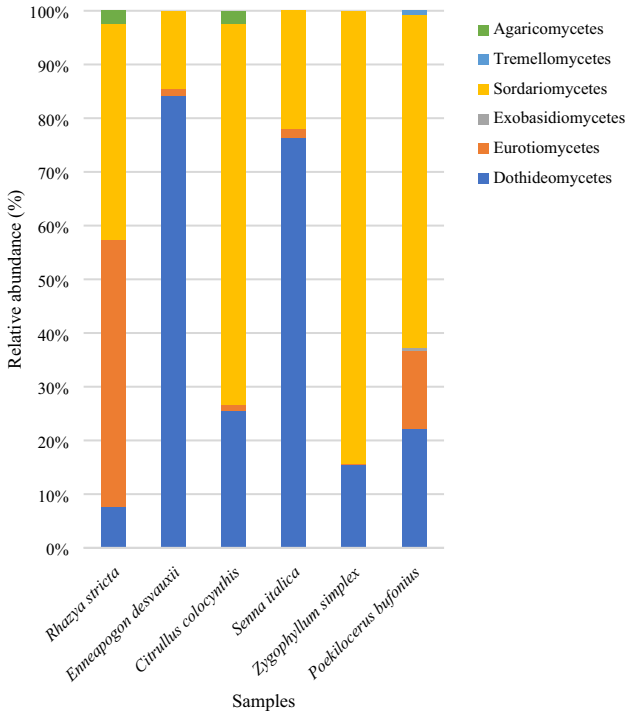

Fig. 5 The taxonomic composition distribution at Class-level in the rhizosphere of the five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and the gut of *P. bufonius*

Discussion

The rapid evaluation of the genetic structure of complex communities in diverse environments was carried out using DNA fingerprinting (Muyzer and Smalla 1998). This permits the correlation of environmental disturbances and the extent of changes (Massol-Deya et al. 1997; Smit et al. 1997; Engelen et al. 1998; Schäfer et al. 2001). Fungal diversity, especially the mechanisms leading to their large-scale ecological and geographic ranges, have not been previously understood. Fungi associated with roots play a crucial role in the nutrition and health of plants (Verbruggen et al. 2012).

Microbiomes of the rhizosphere differ among plant species and in bulk soil. Pea plants had a stronger effect than wheat and oat on the rhizosphere, resulting in a great difference in the rhizosphere community. The eukaryotes relative abundance in the rhizospheres of pea and oat was higher than five-fold the abundance in bulk soil or the wheat rhizosphere (Turner et al. 2013). Our findings were in agreement, as the fungal community of *R. stricta* had 42 unique OTUs, *E. dessvauxii* had 26, *C. colocynthis* had 19 and *S. italica* had 10 unique OTUs.

Over the last decade, rapid development in microbiome research has shown that the microbial communities make significant contributions to immunity, reproduction, digestion, and other functions in insect hosts (Warnecke et al. 2007; Werren et al. 2008; Lehman et al. 2009; Fraune and Bosch 2010; Lee and Mazmanian 2010; Sharon et al. 2010). Our findings showed that the gut of *P. bufonius* was rich in saprophytic fungi like *Penicillium* spp., which may supply the insect with enzymes that aid in digestion, and antimicrobial peptides that boost insect immunity.

The root-associated fungal diversity was studied using 454 pyrosequencing and revealed 164 non-singleton OTUs (Yu et al. 2013). In this connection, our findings concluded that there are 15 non-singleton OTUs assigned to all five plants.

The richness of plant species diversity increases soil microbe diversity, particularly fungal communities (Hollister et al. 2010; Kernaghan 2005; Peay et al. 2013; Wu et al.

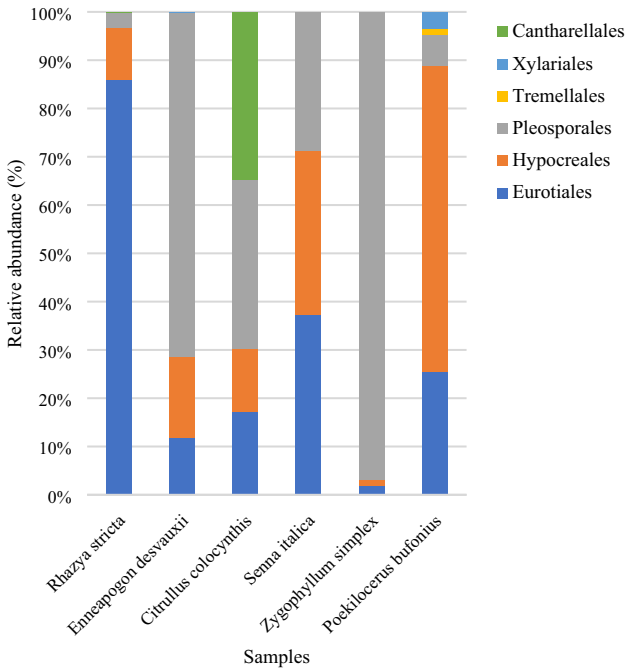

Fig. 6 The taxonomic composition distribution at Order-level in the rhizosphere of the five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and the gut of *P. bufonius*

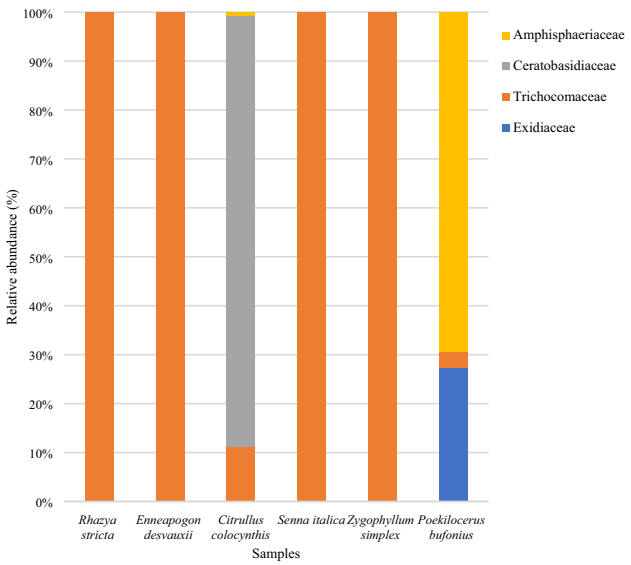

Fig. 7 The taxonomic composition distribution at Family-level in the rhizosphere of the five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and the gut *P. bufonius*

2013; Zak et al. 2003). Our study found that areas rich in *R. stricta* showed the highest number of fungi in its rhizosphere (42 OTUs). Fungal diversity is directly proportional to plant diversity. However, precipitation levels may have a

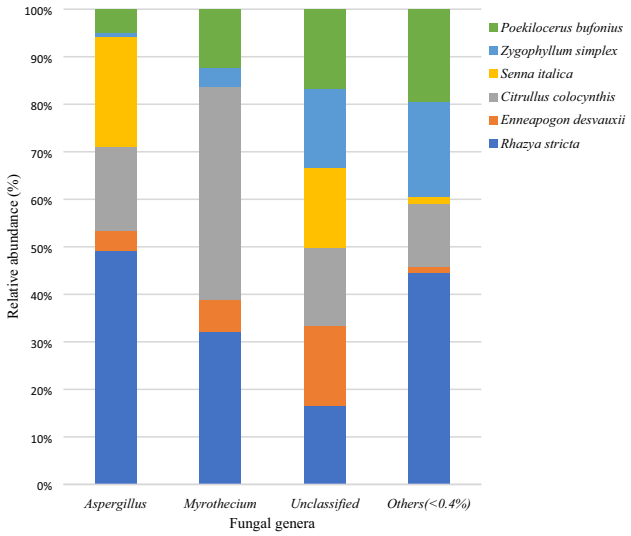

Fig. 8 The taxonomic composition distribution at Genus-level in the rhizosphere of the five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and the gut of *P. bufonius*

greater effect (McGuire et al., 2012). Despite essential fluctuations due to different conditions in the host (Hongoh et al. 2005, 2006; Moran et al. 2008; Andert et al. 2010; Huang et al. 2013), a specific set of microbes is often associated

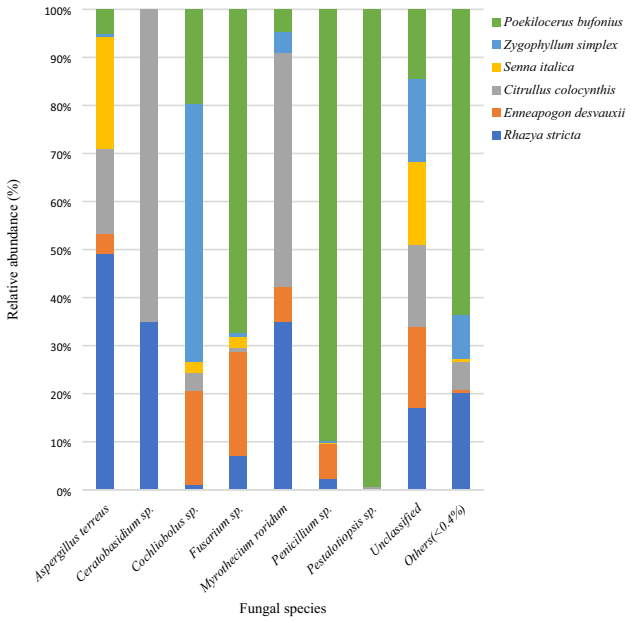

Fig. 9 The taxonomic composition distribution at Species-level in the rhizosphere of the five plants (*R. stricta, E. desvauxii, C. colocynthis, S. italica* and *Z. simplex*), and the gut of *P. bufonius*

with a specific host (Turnbaugh et al. 2007; Hamady and Knight 2009; Huse et al. 2012). The set of microbes can be

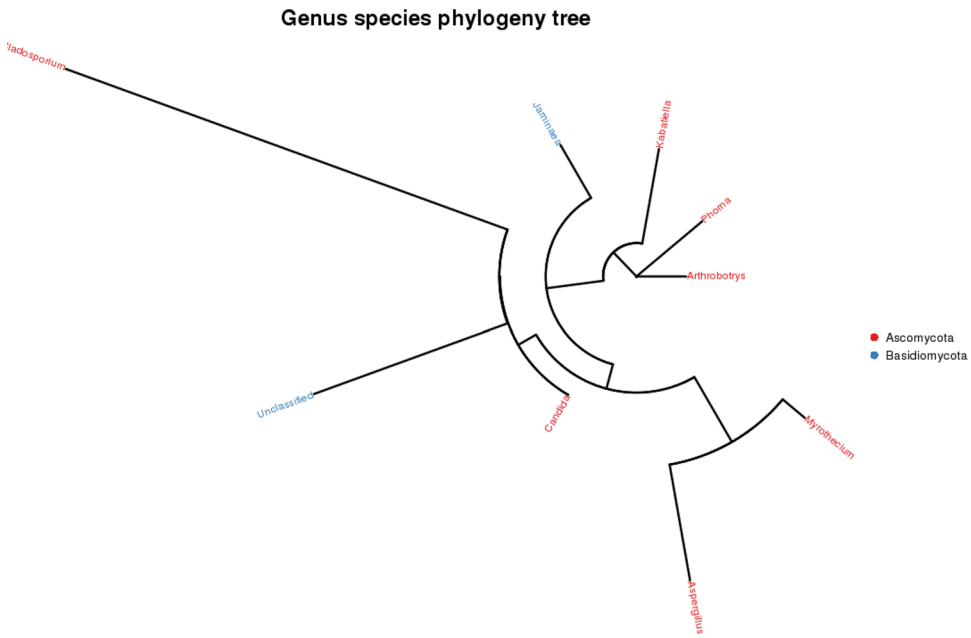

Fig. 10 Genus level phylogenetic tree (The same Phylum is shown as the same color)

considered as a host family-, genus-, or species-specific that may play important co-evolutionary roles (Hongoh 2010; Hongoh et al. 2005; Andert et al. 2010; Brucker and Bordenstein 2012). Our findings concluded that *Aspergillus terreus* and *Ceratobasidium* spp. were abundant in the *R. stricta* rhizosphere while *Fusarium* spp. and *Penicillium* spp. were abundant in the *E. desvauxi* rhizosphere and *P. bufonius* gut.

Baldrian et al. (2012) concluded that fungal sequences closely related to Basidiomycota represented 53.5% of the OTUs in forest soil, and sequences close to Ascomycota represented 41.1%. Geml et al. (2014) reported that Ascomycota was the dominant genus, representing 33.18% of the OTUs, followed by Basidiomycota with 22.73% of the OTUs, Glomeromycota with 5.29%, Mucoromycotina, *incertae sedis* with 1.94%, and Chytridiomycota with 0.53% of the OTUs, while 36.33% of the fungal OTUs were unidentified. In spruce plots, 28% of the soil fungal OTUs belonged to Basidiomycota and in spruce plots, Basidiomycota represented 65% of OTUs (Buée et al. 2009). The heterogeneity of the spatial distribution of populations is determined by the soil organic matter composition and host tree. Ascomycota was the most dominant of fungal community across Italy and France with OTU percentages ranging from 36.7% to 93% for samples from different ecosystems (Orgiazzi et al. 2013). As a result of our work, we noticed that OTUs were 51.7%, 90.1%, 81.4%, 60.2 and 85.5% belonging to Ascomycota in the rhizosphere of *R. stricta*, *E. desvauxi*, *C. colocynthis*, *S. italica* and *Z. simplex*, respectively, while the unidentified fungal OTUs were 47.5%, 9.9%, 17.3%, 39.8% and 14.4% in the rhizosphere of *R. stricta*, *E. desvauxi*, *C. colocynthis*, *S. italica* and *Z. simplex*, respectively.

The most abundant phylum at all four sites in the western coastal region of Saudi Arabia was Ascomycota followed by Basidiomycota (Moussa et al. 2017). Our results are consistent with this, in that Ascomycota was the most abundant phylum in all rhizosphere of the five plants studied.

Our previous work in the traditional isolation of fungi from the soil rhizosphere of *R. stricta* showed that there were 14 fungal species belonging to five genera: *Aspergillus, Fusarium, Mucor*, *Penicillium,* and *Rhizopus,* with *Aspergillus* spp. most frequent. (Baeshen et al. 2014). This study confirmed the previous findings using next-generation sequencing (NGS), where *Aspergillus* spp. were the most abundant in *R. stricta* rhizosphere.

In the western coastal region of Saudi Arabia, Sordariomycetes was observed predominantly at Asfan road, Thuwal and Khulais, while at Mecca old road, Pezizomycetes was dominant, but absent at Asfan road. At Mecca old road, Agaricomycetes was present only; while Tremellomycetes, Microbotryomycetes and Malasseizomycetes were found at Asfan road only (Moussa et al. 2017). Due to the dominance of Ascomycota, the dominant classes were Dothideomycetes in the rhizosphere of both *E. desvauxi* and *S. italica*, Eurotiomycetes in the rhizosphere of *R. stricta*, and Sordariomycetes in the rhizosphere of both *C. colocynthis* and *Z. simplex*.

Nine fungal species of genera *Cladosporium*, *Alternaria*, *Exserohilum*, *Pyrenochaeta*, *Phoma*, *Neosartorya*, *Aspergillus*, *Penicillium*, and *Fusarium* have been isolated from the plants roots at Ulleungdo Island (South Korea), which belonged to classes Eurotiomycetes, Dothideomycetes, and Sordariomycetes (Kim et al. 2013). Fourteen fungal species (*Eurotium*, *Aspergillus*, *Penicillium*, *Cladosporium*,

Talaromyces, *Exserohilum*, *Fusarium*, *Acremonium*, *Gibberella*, *Microsphaeropsis*, *Pestalotiopsis*, *Mucor*, *Zygorhynchus* and *Umbelopsis* have been isolated from the plants roots at Dokdo Island (S. Korea/Japan); eleven of which belong to four classes (Dothideomycetes, Ascomycetes, Sordariomycetes, and Eurotiomycetes) and three species belonging to *Mucoromycotina* (You et al. 2011a; b, 2013). In our study, we found four fungal genera (*Aspergillus*, *Ceratobasidium*, *Fusarium* and *Myrothecium*) in the *R. stricta* rhizosphere, two genera (*Fusarium* and *Penicillium*) in the rhizosphere of *E. desvauxii*, three genera (*Aspergillus*, *Ceratobasidium* and *Myrothecium*) in the *C. colocynthis* rhizosphere, two genera (*Aspergillus* and *Fusarium*) in the *S. italica* rhizosphere, and three genera (*Cochliobolus*, *Fusarium* and *Myrothecium*) in the *Z. simplex* rhizosphere.

Ranjard et al. (2001) investigated 18 genera belonging to Basidiomycota, 78 genera belonging to Ascomycota, 3 genera belonging to Zygomycota, one genus belonging to Chytridiomycota, 3 genera belonging to Plasmodiophoromycota and one genus belonging to Oomycota. In our study 226 genera belonging to Ascomycota and 17 genera belonging to Basidimycota were investigated.

Conclusion

The most dominant phyla in all samples were *Ascomycota* and *Basidiomycota*. The fungal species found in the rhizosphere of the plants under study included saprophytic and pathogenic fungi only (*Aspergillus, Fusarium, Penicillium, Ceratobasidium Myrothecium, Cochliobolus, Phoma, and Pestalotiopsis*). Glomeromycota were absent. The *P. bufonius* gut mycoflora was composed of *Fusarium* and *Penicillium, Candida* that aid in the digestion of cellulose and hemicellulose feeds.

Acknowledgment This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. (HiCi 1-363-1434H). The authors, therefore, acknowledge with thanks DSR for technical and financial support.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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