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Diverse Arbuscular Mycorrhizal Fungi (AMF) Communities Colonize Plants Inhabiting a Constructed Wetland for Wastewater Treatment

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Abstract: Constructed wetlands (CWs) are biological wastewater treatment systems that comprise several components where plants and associated organisms play an important role in water depuration. Microbial studies emphasize bacterial dynamics, whereas studies of arbuscular mycorrhizal fungi (AMF) are scarce and the functional role of AMF in aquatic and wetland plants is poorly understood. The aim of this study was to analyze the AMF communities colonizing the roots of *Canna indica*, *Canna flaccida*, and *Watsonia borbonica* inhabiting a CW treating wastewater of a tourism unit. The dynamics of the AMF communities were evaluated by Denaturing Gradient Gel Electrophoresis (DGGE) of 18S rRNA gene amplification products along cold (C) and hot (H) seasons for three consecutive years. DGGE profiles allowed the estimation of AMF species richness (*S*), and Shannon-Wienner (*H*) and Pielou (*J*) indexes, for the different plant species, showing differences between species and along the years. Excised bands from DGGE were analyzed and identified through sequencing for arbuscular mycorrhiza, revealing the presence of AMF strains closely related to *Glomus* sp., *Rhizophagus* sp. and *Acaulospora* sp. genera. Concomitant water quality analyses showed that the system was effective in organic and nutrient removal during the sampling period. Findings from this study suggest that AMF diversity found in the CW is influenced by the water constituents, season, and plant species.

Keywords: mycorrhizal fungi; tourism; domestic wastewater; AMF diversity; phytoremediation; constructed wetland; water quality

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are an important group of soil microorganisms that form symbiotic associations with 80% of vascular plant species [1]. Despite their high prevalence in the roots of terrestrial plants, in the last decades, several studies have also demonstrated that AMF occurrence in wetland habitats is widespread, including salt marshes [2] and mangroves [3,4]. According to the review published by Xu et al. [5], mycorrhizal colonization has been found in 99 families of wetland plants, and their role on the composition and diversity of wetland plant communities in some cases have been demonstrated. The prominent effect of AMF on plant development in terrestrial environments under various stress conditions, including salinity [6,7] and drought [8], and their capacity to promote the biodegradation of organic pollutants [9] or the phytoremediation of inorganic ones [10,11], is well recognized. However, the diversity of AMF in aquatic and wetland plants is still poorly recognized.

Constructed wetlands (CWs) have been successfully used to improve the quality of various types of water, being identified as a sustainable wastewater management option around the world [12,13]. They are considered multifunctional systems that provide ecosystem services. For that, they integrate several components, including the treatment basin, substrate, selected plant species, and a panoply of associated organisms [13,14]. Although there are reports on the microbial dynamics and their association to macrophytes, these studies are mainly focused on bacteria [15–17], underestimating other important microbial organisms, such as fungi. Both bacteria and fungi have important roles in the assimilation, transformation, and recycling of chemical constituents present in various wastewaters. In addition, fungi (mycorrhizae) increase the efficiency of the host for sorption of nutrients from air, water, and soil [13]. To date, only a few studies have analyzed AMF communities on plants inhabiting CWs for wastewater treatment [5,18,19]. This is probably related to the assumption that low levels of oxygen in these environments may limit the survival of AMF in the roots of plants [20]. Nonetheless, Fester et al. [18] showed the ability of AMF to colonize roots of *Phragmites australis* inhabiting a CW, implemented for the phytoremediation of groundwater contaminated with benzene, methyl tert-butyl ether, and ammonia. However, AMF roots' colonization was highly dependent on the presence of solid substrate in the CW, since no colonization was observed in plants growing in free water. Likewise, Xu et al. [19] reported that roots of *P. australis* plants inhabiting two CWs for the treatment of metal-contaminated water harbored different species of AMFs, and these fungi seemed to play an important role in metal removal from contaminated water.

In the present study, we analyzed AMF communities from the roots of *Canna flaccida*, *Canna indica*, and *Watsonia borbonica*, the most representative plants inhabiting a horizontal subsurface flow CW, for the treatment of wastewater from a tourism unit in the North of Portugal. In order to assess changes on AMF communities throughout time, plant roots were collected during the hot and cold seasons across three consecutive years.

2. Material and Methods

2.1. Constructed Wetland Design

A horizontal subsurface flow CW (area: 40.5 m^2) was set-up after a septic tank for wastewater treatment coming from a tourism house (Figure 1). Briefly, the system had randomly planted species of *Zantedeschia aethiopica*, *Agapanthus africanus*, *C. flaccida, C. indica,* and *W. borbonica* in a substrate of expanded clay—Leca®M (from Saint-Gobain Weber Portugal, S.A.). The setup conditions and detailed description of the CW, located at a tourism guest house in North of Portugal, are described in Calheiros et al. [21].

Figure 1. Constructed wetland (CW) treating wastewater from a tourism house (Portugal).

2.2. Water Sampling and Physicochemical Analysis

Wastewater samples (n = 20) were collected from the CW during the cold (autumn/winter—C) and hot seasons (spring/summer—H), during three consecutive years (1, 2, and 3). Air temperature and relative humidity were registered with a logger at the sampling time.

Physicochemical parameters for the wastewater samples of the CW were determined based on Standard Methods [22]: Chemical oxygen demand (COD; Closed Reflux, Titrimetric Method), biochemical oxygen demand (BOD_5; 5-day BOD Test), total suspended solids (SS; Total Solids Dried at 103–105 °C Method), ammonia nitrogen (NH_3–N; phenate method), phosphates (PO_4^{3-} flow injection analysis), pH and conductivity.

2.3. Analysis of AMF Communities

For the analysis of AMF communities, eight subsamples of roots of the most representative plants (*C. flaccida*—CF; *C. indica*—CI, and *W. borbonica*—W), in the CW polyculture, were randomly collected at the end of cold (C) and hot season (H) for three consecutive years (1, 2, and 3). Roots of each plant species were pooled to form a composite sample.

2.3.1. DNA Extraction from Plant Roots

Roots were ground with liquid nitrogen using a mortar and pestle previously disinfected with 95% ethanol for 5 min. Total genomic DNA was extracted from 250 mg of ground roots using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturers' protocol. The genomic DNA was stored at −20 °C until use.

2.3.2. PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

The PCR amplification of AMF 18S rRNA fragments was assessed according to Liang et al. [23]. Briefly, 18S rRNA was amplified using the universal primer NS31 (5′-TTGGAGGGCAAGTCTGGTGCC-3) [24], and the AMF fungus-specific primer AM1 (5′-GTTTCCCGTAAGGCGCCGAA-3′) [25], in a 50 µL reaction containing 100 ng of DNA template, 1.5 mM of $MgCl_2$, 1x PCR buffer (Promega, Madison, Wis, USA), 1.25 mM dNTP, 0.4 mM of each primer, 1 µL of bovine serum albumin (BSA), and 2.5 U of Go *Taq* DNA polymerase (Promega, Madison, Wis, USA). PCR was performed in a Bio-Rad MJ Mini PTC-1148 Thermal Cycler, using the following PCR conditions: 5 min at 94 °C, followed with 31 cycles of 1 min at 94 °C, 1 min at 66 °C, 1 min 30 s at 72 °C, and 72 °C for 10 min. Amplified products were visualized on a 1.5 % agarose gel stained with SYBR® Safe (Invitrogen, Carlsbad, CA, USA) for 45 min at 100 V. A nested-PCR was performed in a Bio-Rad MJ Mini™ thermocycler using the primers NS31-GC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAAGT CTGGTGCC-3′) [26] and Glo1 (5′-GCCTGCTTTAAACACTCTA-3′) [27] under the following PCR conditions: 5 min at 95 °C, 35 cycles for 45 s at 94 °C, 45 s at 52 °C, 1 min at 72 °C, and a final extension step at 72 °C for 30 min. PCR products were visualized as described above and stored at −20 °C until DGGE analysis.

A DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used for DGGE analysis. Samples containing approximately 800 ng of nested-PCR amplicons were loaded onto 8% (*w*/*v*) polyacrylamide gels (40% acrylamide/bis-acrylamide 37:5:1) in 1 × TAE buffer (20 mM Tris-acetate pH 7.4, 10 mM sodium acetate, 0.5 mM disodium EDTA) using a denaturing gradient ranging from 35 to 60% (100% denaturant solution contained 7 M urea and 40% (*v*/*v*) formamide). Electrophoresis was performed in 1× TAE buffer at 60 °C, initially at 20 V (15 min) and then at 70 V (960 min). A commercial reference marker (Hyperladder IV, Bioline, London, UK) was also included for internal normalization of the gel. Gels were stained using 5 × GelGreen in 0.1 M NaCl solution (Biotium, Fremont, Canada). DGGE images were acquired using a Safe Imager™ Blue-Light Transilluminator (Invitrogen™) and a micro DOC gel documentation system (Cleaver Scientific Ltd., Warwickshire, UK).

2.3.3. Sequencing of DGGE Bands

After gel image acquisition, several bands were selected for excision based on prevalence and frequency of appearance with a sterile scalpel, and eluted in 20 mL of sterile water overnight at 4 °C. Five microliters of the supernatant were used for re-amplification with the original primer set [28]. The accuracy of the bands and the position in the gel were checked on DGGE gels, together with the original sample. Whenever necessary, bands were processed again as described above. PCR products were purified (PCR and Gel Band Purification Kit, GRISP, Portugal) for sequencing analysis by Macrogen Inc. (Seoul, Republic of Korea). Band sequences were analyzed for similarity with other sequences in BLASTn software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/), to determine the closest sequence identity. The nucleotide sequences were deposited in Genbank data library under accession numbers KJ639002-KJ639010.

2.4. DGGE Data Analysis

DGGE fingerprints were analyzed using Bionumerics® software (version 6.6; Applied Maths, St-Martens-Laten, Belgium, version 6.6) and clustered according to their similarities. DGGE sample profiles were compared using Pearson correlation coefficient and clustered according to UPGMA method (group average method).

Species richness (S) was calculated based on the total number of distinct bands in a lane. The Shannon-Wienner (H) index [29] was determined based of the band intensity imitated as peak heights in the densitometric curve as follows:

$$H = -\sum\left(\frac{\mathrm{ni}}{\mathrm{N}}\right)\log\left(\frac{\mathrm{ni}}{\mathrm{N}}\right) \quad (1)$$

where ni is the height of the peak, and N is the sum of all peak heights of the densitometric curve.

The Pielou's (J) index [30] was calculated as follows:

$$J = \frac{H}{\log S} \quad (2)$$

3. Results and Discussion

The CW investigated in this study was implemented in a seventeenth century guest house in Calheiros—Ponte de Lima—in the North of Portugal [21], being in operation to date. During the present study, in cold seasons, air temperature varied between 10.0 and 19.4 °C and relative humidity between 29 and 83%; while in hot seasons, air temperature varied between 19.7 and 30.0 °C and relative humidity between 35 and 67%.

The wastewater before and after the CW had a pH around 7 (for inlet: 7.34 ± 0.24; and outlet: 7.17 ± 0.32). The conductivity, varied between 142 and 2120 µS/cm for the CW inlet and between 84 and 1060 µS/cm for the CW outlet, and the water was considered non-saline to slightly saline [31]. In Table 1, water quality parameters measured at the inlet and outlet of CW are shown. There was an effective wastewater depuration by the treatment system, despite the inlet variation, attributed to the fluctuations in the number of guests in the house during the different seasons. This is in agreement with other previous reports [21,32].

Values of the BOD/COD ratio varied between 0.3 and 0.8, which is in the range of typical values for untreated domestic wastewater, being in several occasions above 0.5, indicating that the water is considered easily treatable by biological means [33]. In general, higher values of COD and BOD_5 were registered in hot seasons, corresponding to the higher number of overnight accommodations in the tourism house—a trend also mentioned by Calheiros et al. [21].

Table 1. Mean ± SD inlet and outlet composition of the wastewater in the CW system during the cold (C) and hot (H) seasons across three consecutive years (1, 2, and 3). (n = 4 for each season).

Season/ Year	TSS (mg/L)		BOD_5 (mg/L)		COD (mg/L)		PO_4^{3-} (mg/L)		NH_4^+ (mg/L)	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
C1	46 ± 23	9 ± 4	37 ± 28	3 ± 1	73 ± 74	7 ± 5	5.4 ± 6.0	1.5 ± 0.5	7.5 ± 1.9	3.8 ± 1.5
H2	297 ± 237	15 ± 9	386 ± 182	15 ± 4	1214 ± 685	41 ± 15	32.0 ± 6.0	16.1 ± 2.7	19.3 ± 13.3	15.1 ± 12.5
C2	105 ± 87	16 ± 15	86 ± 48	13 ± 6	261 ± 105	34 ± 18	13.1 ± 5.3	5.1 ± 0.7	12.5 ± 17.6	7.0 ± 9.4
H3	148 ± 35	13 ± 6	265 ± 212	13 ± 5	885 ± 733	26 ± 2	27.3 ± 6.2	8.9 ± 2.2	67.6 ± 39.9	26.0 ± 28.3
C3	31 ± 7	4 ± 1	123 ± 94	15 ± 11	258 ± 154	36 ± 21	25.5 ± 7.6	4.7 ± 2.1	66.1 ± 26.2	25.1 ± 12.1

Vegetation influences, to different extents, organic and nutrient removal in treatment wetlands; however, the activity of their associated microbial communities, such as bacteria and fungi, also play important roles in the transformation, assimilation, and recycling of chemical constituents present in wastewater [13]. In wetland systems, AMF's colonization may be influenced by several environmental factors, namely flooding [34], levels of nutrients [35], toxic metals [13], pH and conductivity [2]. In the present work, despite the unfavorable environmental conditions for the establishment of AMF communities, namely continuous water flow and high nutrient levels, DGGE profiles exhibited bands in all root samples (Figure 2), indicating that AMF successfully colonized the roots of the plants in the CW. Several other studies have shown the presence of AMF inside the roots of wetland plants [5,18,19,36]. This occurrence seems to be related to the well-developed aerenchyma of wetland plants [35], which facilitate the diffusion of O_2 from leaves to the rhizosphere, producing an oxidant layer around the roots [37]. Nonetheless, despite AMF's ubiquity in wetland systems, the influence of abiotic and biotic factors on AMF viability in CW is still unclear [5].

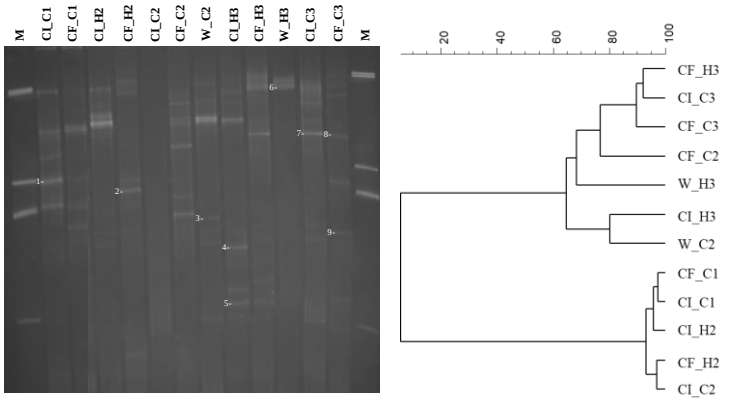

Figure 2. Cluster analysis generated by the 18S rRNA DGGE patterns of arbuscular mycorrhizal fungi (AMF) communities colonizing roots of *C. indica* (CI), *C. flaccida* (CF), and *W. borbonica* (W) inhabiting a constructed wetland during cold (C) and hot (H) seasons across three consecutive years (1, 2, and 3). The dendrogram was created using the UPGMA method (group average method) and based on the Pearson correlation coefficient. A commercial reference marker (M) was included for internal normalization of the gel. Excised bands are marked in gel with numbers (1–9).

In this study, DGGE profiles exhibited variation in the number and intensity of bands among samples, suggesting differences in the composition of AMF's communities that colonize the root systems of the three plants. According to the cluster analysis (Figure 2), communities clustered in two distinct major groups with low similarity between them. The first group mainly comprised samples of the third year, which were clustered irrespective of plant type or of sampling season (C/H), while the second group enclosed samples from first and second years (similarity > 90%).

Species richness (*S*) also varied between samples; the highest value ($S = 13$) was obtained for the roots of *C. indica* plants collected in the cold season of first year (CI_C1), while the lowest value ($S = 2$) was found in roots of *C. indica* collected in the cold season of second year (CI_C2) and in roots of *W. borbonica* collected in the hot season of third year W_H3 samples (Table 2). In general, AMF

diversity was higher in plants collected in the cold season of first year (C1) than in the other sampling campaigns, with an overall decrease of Shannon-Wienner (*H*) and Pielou (*J*) indexes, as well as species richness (*S*), throughout the time.

Table 2. Diversity indexes of AMF communities colonizing roots of *C. indica* (CI), *C. flaccida* (CF), and *W. borbonica* (W) inhabiting a constructed wetland (CW) during cold (C) and hot (H) seasons across three consecutive years (1, 2, and 3). *H*—Shannon-Wienner index; *J*—Pielou index; *S*—Species richness.

Season/Year	Diversity Indexes						Species Richness		
	H			*J*			*S*		
	CI	CF	W	CI	CF	W	CI	CF	W
C1	0.83	0.78	*	0.74	0.75	*	13	11	*
H2	0.94	0.30	*	0.87	0.36	*	12	7	*
C2	n.d.	0.63	0.53	n.d.	0.58	0.59	2	12	8
H3	0.72	0.57	*	0.76	0.57	*	9	10	*
C3	0.11	0.60	n.d.	0.13	0.63	n.d.	7	9	3

n.d.: not determined (bands with very low intensity); * absence of plant species in the CW.

The observed changes in the composition and diversity of AMF's communities can be related to fluctuations in the wastewater organic load in the CW throughout different seasons/years. The higher diversity of AMF found in the roots of plants collected during the cold season of the first year (C1) can be associated to low conductivity and nutritional levels registered in the wastewater during that season, when compared to the other seasons and years (Figure 3). According to Carvalho et al. [2], high levels of salinity negatively affect AMF establishment inside roots. Moreover, Wang et al. [38] also reported that the intensity of AMF colonization in two mangrove species was highly dependent on the concentration of nutrients present in a municipal effluent, since high levels of N, P, and organic matter decreased AMF colonization.

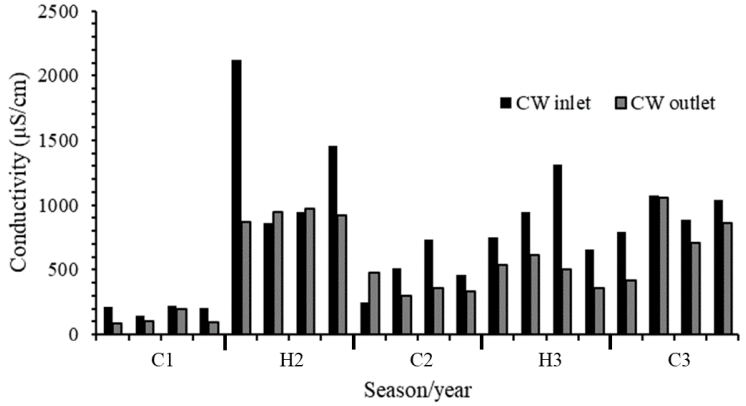

Figure 3. Constructed wetland inlet and outlet water conductivity (µS/cm) for different seasons (cold—C and hot—H) and years (1, 2, and 3).

AMF communities may also be influenced by plant species. Overall, species richness and diversity indexes were higher for *C. indica* than for *C. flaccida* and *W. borbonica*, the latter having a low prevalence in CW over time, which was reflected in the composition and low diversity of AMF's communities in these plants. Indeed, according to Su et al. [39], the season and host plant are main factors influencing spore density, species richness, and diversity of AMF, since plants can regulate carbon allocation to roots, produce secondary metabolites, and change soil environmental conditions during different growth seasons. The morphology of the root system may also justify differences in AMF communities among plants [40]. In the case of *Canna* spp., they have a variable root length (18–20 cm) consisting of a large root area and biomass [41], unlike *Watsonia* spp. that are characterized by having basal-rooting corms

with associated dormancy, where corm tunics consist of the thickened fibrous remains of cataphylls and lower leaf bases [41,42]. However, for the latter plants, very little information is available concerning radicular system development and association with wetland cosmos.

The analysis of some DGGE bands (marked in the gel presented in Figure 2) revealed the presence of AMF strains closely related to *Glomus* sp., *Rhizophagus* sp., and *Acaulospora* sp. genera (Table 3).

Table 3. Phylogenetic affiliation of AMF strains colonizing roots of *C. indica* (CI), *C. flaccida* (CF), and *W. borbonica* (W) inhabiting a constructed wetland during cold (C) and hot (H) seasons across three consecutive years (1, 2, and 3). Data based on bands excised from DGGE gel.

Plant Species	Season/Year	Band Number	Phylogenetic Affiliation	Accession No.	Closest Relative (Accession No.)	Similarity (%)
C. indica	C1	1	*Acaulospora* sp.	KJ639002	Uncultured *Acaulospora* (JN559796)	99
	C3	7	*Glomus* sp.	KJ639009	Uncultured *Glomus* (JN788351)	97
	H3	4	*Glomus* sp.	KJ639003	Uncultured *Glomus* (HF913474)	100
	H3	5	*Glomus* sp.	KJ639006	Uncultured *Glomus* (AY641821)	96
C. flaccida	H2	2	*Rhizophagus* sp.	KJ639008	Uncultured *Rhizophagus* (KF134509)	100
	C3	8	*Glomus* sp.	KJ639004	Uncultured *Glomus* (HG004524)	99
	C3	9	*Glomus* sp.	KJ639010	Uncultured *Glomus* (EU340290)	100
W. borbonica	C2	3	*Acaulospora* sp.	KJ639007	Uncultured *Acaulospora* (JN559797)	95
	H3	6	*Rhizophagus* sp.	KJ639005	Uncultured *Rhizophagus* (HF913502)	99

These findings are in agreement with the results obtained by Xu et al. [19], who also reported AMF species affiliated with Glomeraceae and Acaulosporaceae families in a CW for metal-contaminated wastewater bioremediation.

The presence and absence of AMF associated with plant species across the years, as well as their diversity and richness, may be associated, to a certain extent, with the variability of the water constituents, its flow (due to tourism overnight fluctuation), and season (hot and cold).

To our knowledge, this is the first time that AMF were studied in a real scale CW treating with this typology of water and consisting of a polyculture planting scheme. This new knowledge will help to develop a strategy to better select the plant species that are associated with mycorrhizae fungi. It reinforces the advantages of the fungi-plant associations for water depuration processes, and possible adaptations to climate change effects.

4. Conclusions

Reports related to AMF in CWs are scarce, and the present work contributed to increasing the knowledge on AMF diversity in such wastewater treatment systems. Common species of AMF, such as strains closely related to *Glomus* sp., *Rhizophagus* sp., and *Acaulospora* sp. genera, were able to establish and survive in such inhospitable conditions. Deeper investigations on AMF prevalence and functions in CWs are still needed.

Findings from this study corroborate that plants and associated biota influence water depuration in the CW, and emphasize that the water constituents, the season, and plant species have an effect on AMF diversity, which was the issue specially addressed in the present study. This work will contribute to increasing the knowledge on AMF diversity in CWs, and to infer about their role as plant allies in phytoremediation processes for domestic wastewater treatment.

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