CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

Unidad Irapuato
Unidad de Genómica Avanzada

Functional role of endophytic fungi within the coralloid roots of cycads.

Thesis presented by

Fernando López Restrepo

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in

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Thesis Director: Dr. Angélica Cibrián Jaramillo

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Papel funcional de hongos endófitos dentro de raíces coraloides de cícadas.

Tesis que presenta

Fernando López Restrepo

Para obtener el grado de

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en

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Dedicatorias

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Abstract

Cycads are an endangered gymnosperm order that has existed since the Permian period. These plants are a model for symbiosis in eukaryotes, as they constitute the only order that fixes nitrogen through a symbiotic relationship with cyanobacteria within a specialized organ, the coralloid root. These root structures contain a great diversity of microorganisms, among which are many species of fungi. Root-noduling systems in angiosperms show that fungi help increase the rates of nitrogen fixation of cyanobacteria, but in cycads, the functional role that fungi have within the coralloid root is currently unknown. In this research we characterized the fungi inside the coralloid root using multidisciplinary approaches. Using autofluorescence microscopy we confirmed the presence of endogenous fungi within the cells of the coralloid root. In order to determine the effect that fungi have within the coralloid root, we collected samples from cycads from research greenhouse collections and from their natural habitat. Using meta-transcriptomics we detected a marked mycorrhiza-like response to symbiosis within the coralloid root. However, all fungal activity detected in our study was not exclusive to the coralloid root, but equal in both types of sampled roots. As we found no evidence of specific fungi being favored by the cycad nor their activity being specific to the coralloid root, our results seem to indicate that root colonization by fungi is not the result of a specific inter-organism relationship, but rather an opportunistic process that does not distinguish between root types.

Resumen

Las cícadas son un orden de gimnospermas amenazadas que ha existido desde el periodo Pérmico. Estas plantas son consideradas un modelo de simbiosis en eucariotas debido a que constituyen el único orden de plantas que fija nitrógeno por medio de una relación simbiótica con cianobacterias dentro de un órgano especializado, la raíz coraloide. Estas estructuras contienen una gran diversidad de microorganismos, entre los cuales se encuentran diversas especies de hongos. Los sistemas de nodulación en angiospermas muestran que los hongos ayudan a incrementar las tasas de fijación de nitrógeno de cianobacterias, pero en cícadas el papel funcional de los hongos dentro de la raíz coraloide es desconocido. En este estudio caracterizamos los hongos dentro de la raíz coraloide utilizando un enfoque multidisciplinario. Utilizando microscopía de autofluorescencia confirmamos la presencia de hongos endógenos dentro de las células de la raíz coraloide. Para determinar el papel de los hongos en la raíz coraloide, se colectaron muestras de cícadas en invernaderos de investigación y en su hábitat natural. A través de un análisis meta-transcriptómico detectamos en la cícada una respuesta a la simbiosis en la raíz coraloide muy similar a la observada en micorrizas. No obstante, la actividad fúngica detectada en nuestro estudio no fue exclusiva a la raíz coraloide, sino igual en los dos tipos de raíces estudiadas. Dado que no encontramos evidencia de que hongos específicos fueran favorecidos por la cícada, así como de actividad fúngica específica a la raíz coraloide, nuestros resultados parecen indicar que la colonización fúngica de la raíz no es resultado de una relación específica entre los organismos, sino un proceso oportunista que no distingue entre tipos de raíces.

1. Introduction.

1.1. Cycads.

Cycads (order *Cycadales*) are the only plants from class *Cycadopsida* still living in the present day and the most ancient extant seed-bearing plants. Cycads are of scientific interest due to the age of their lineage, with some fossils dating back to the Permian (between 298.9 and 251.902 million years ago; Mamay, 1969; Norstog and Nicholls, 1997; Cai *et al*., 2018). These plants show minimal changes to their external morphology despite having survived several major environmental changes during their existence. Cycads are adapted to environments with a thin soil layer, where they occupy ecological niches with very little competition from other species (Lobakova *et al*., 2002a; Lobakova *et al*., 2002b). Cycads are considered to be endangered, and thus their study and conservation is considered a priority (González-Astorga *et al*., 2003; González-Astorga *et al*., 2009; Mora *et al*., 2013; *International Union for Conservation of Nature*, 2019).

1.2. Coralloid roots.

Cycads are the only gymnosperms to fix nitrogen through symbiosis (Kneip *et al.*, 2007). Nitrogen fixation is achieved in cycads in a specialized structure known as the apogeotropic or coralloid root (Thajuddin *et al*., 2010), which hosts endophytic organisms and allows the formation of symbiotic relationships with bacteria, with cyanobacteria being the most prevalent symbionts (Gutierrez-Garcia *et al*., 2018; Suarez-Moo *et al*., 2019; Zheng *et al*., 2018). Coralloid roots develop a mid-cortex cyanobacterial zone (previously named algal zone), which hosts diverse bacteria that form a symbiotic relationship with the plant.

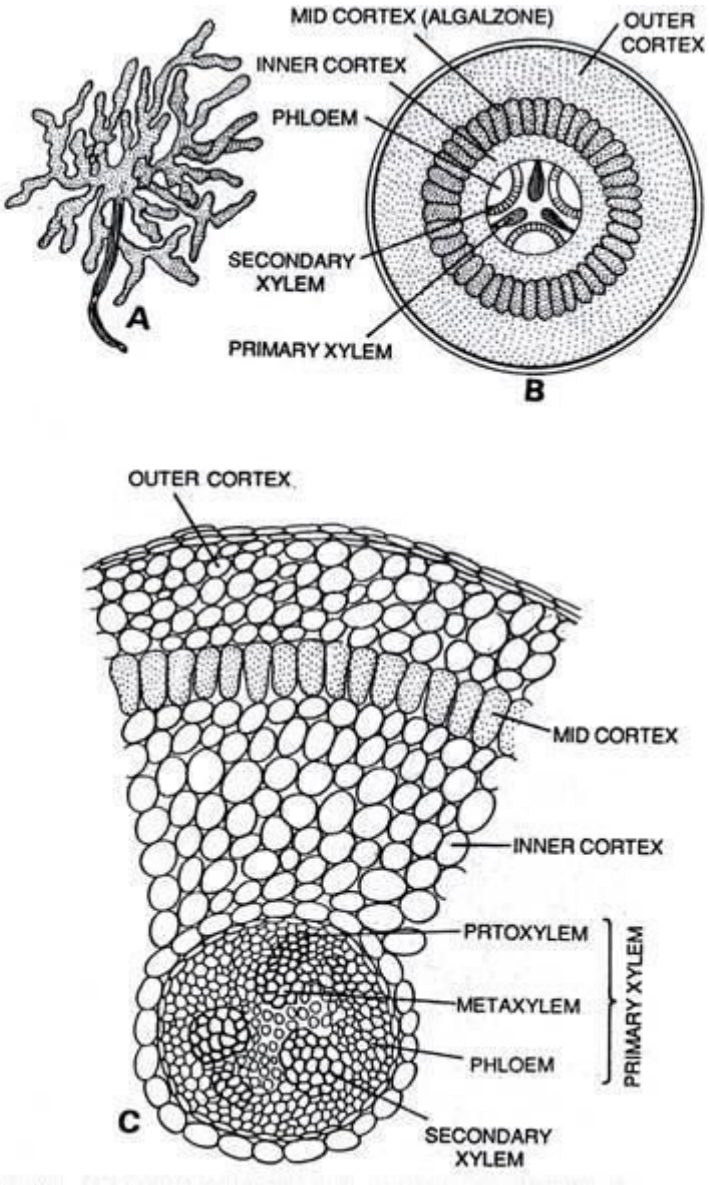

Figure 1. Anatomy of a cross section of *Cycas* coralloid root.

Coralloid roots are structures exclusive to the order *Cycadales* and have been reported in every cycad species studied so far (Lindblad, 2008). Development of the coralloid root begins with the differentiation of the secondary root into a pre-coralloid root, which matures into a coralloid root after the entry of microorganisms present in the rhizosphere, the layer of soil in direct contact with the plant roots (Ahern and Staff, 1994; Lynch and de Leij, 2012). The rhizosphere is home to bacteria and saprotroph fungi capable of degrading the pre-coralloid roots cell wall, creating channels through which cyanobacteria enter the root (Lobakova *et al*., 2002b). Once cyanobacteria have entered the pre-coralloid root, their presence seemingly stimulates the irreversible maturation of the root into the coralloid-root through indeterminate means (Ahern and Staff, 1994). Afterwards, the coralloid root divides dichotomically into large root masses, looking similar to coral. At the same time,

coralloid roots grow towards the soil surface, showing negative geotropism. The development of the coralloid root ends with the senescence of the structure and the formation of new coralloid roots from the base of dead coralloid roots (Norstog and Nicholls, 1997). While the factors that promote the initial development of the pre-coralloid root are unknown, it has been reported that the initial formation of the pre-coralloid roots depends solely on the plant, independently of the presence or activity of microorganisms that may constitute potential endosymbionts (Norstog and Nicholls, 1997).

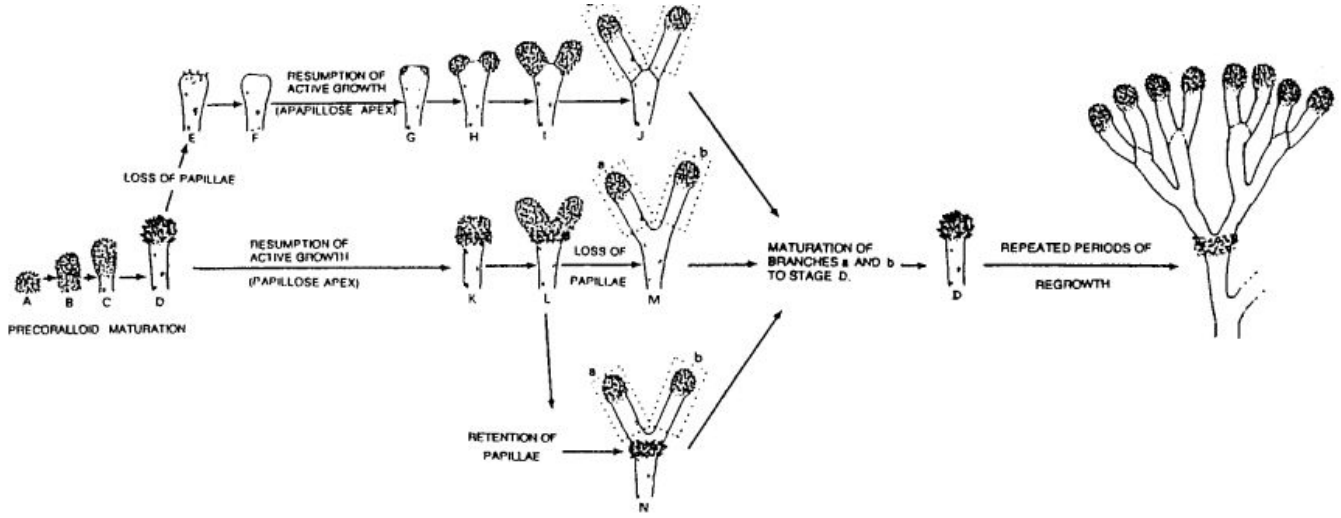

Figure 2. Pre-coralloid root maturation (Ahern and Staff, 1994).

1.3. Diversity and role of endogenous bacteria

The coralloid root has been reported to host a wide diversity of endophytic organisms that vary from environment to environment and even from individual plant to individual plant, indicating that the relationship between the cycad and its endosymbionts is based in functionality rather than specificity of the microorganisms (Suarez-Moo *et al*., 2019). Most studies regarding the microbiota of cycads have centered on bacteria and, most commonly, on cyanobacteria, prokaryotes capable of oxygenic photosynthesis. The morphology of these microorganisms can vary from unicellular to filamentous. Cyanobacteria belonging to genus *Nostoc* (reported as being present within the coralloid roots) showing the latter morphology. Filamentous cyanobacteria present three types of cells: nitrogen fixing heterocysts, motile filaments called hormogonia, and akinetes, spore like cells (Rai, 2018; Sukenik *et al*., 2019).

As mentioned before, cyanobacteria inhabiting the interior of the coralloid root are located inside a distinct cell layer present in mature coralloid roots, known as the cyanobacterial zone (historically named the algal zone before reclassification of

cyanobacteria). This layer is composed of enlarged cells that connect both cortical layers, being generated through dedifferentiation during the first stages of the coralloid root development (as shown in figure 1; Lindblad *et al*., 1985; Ahern y Staff, 1994); within these cells cyanobacteria take nitrogen from the environment and incorporate it into nitrates or ammonia ions (Norstog and Nicholls, 1997; Wittmann *et al*., 1965; Kanesaki *et al*., 2018). This process occurs through the activity of nitrogenase, an enzyme produced by the *nif* genes. This enzyme is composed of two subunits: Dinitrogenase and Dinitrogenase Reductase. Each subunit is formed by a metal ion associated protein, the specific metal ion varying between species (Mulligan and Haselkorn, 1989; Igarashi and Seefeldt, 2003). Nitrogen fixation by these cyanobacteria occurs only in isolation from oxygen, such as that procured by the coralloid root, or conditions similar to it. Nitrogen fixation activity is interrupted by an increase in environmental oxygen levels (over 1%), but seems to increase in the presence of light (Lindblad *et al*., 1991; Lindblad, 2009).

Evidence of co-evolution between cycads and their endophytic cyanobacteria has been reported, as seen by the phylogenetic closeness of cyanobacteria known to be cycad symbionts (Papaefthimiou *et al*., 2008). Among these endophytic microorganisms, the predominance of nitrogen fixing bacteria has been confirmed; a biosynthetic gene cluster (BGC) was also found to be unique to the clade of cycad-associated bacteria (Gutierrez-Garcia *et al*., 2018). Symbiotic cyanobacteria, have also been shown to have a less marked regulation of nitrogenase synthesis and form heterocysts, which gives them an increased capacity to fix nitrogen constantly, rather than based on external cues (Bustos Díaz *et al*., 2019). While this symbiosis is entirely facultative, it has been proposed that the ability to form these relationships has allowed the evolutionary conservation of cycads, making them an ideal system for the study of the evolution of nitrogen fixation as a result of symbiosis (Norstog and Nicholls 1997; Lobakova *et al*., 2002a).

As mentioned before, the coralloid root also houses a wide array of endophytic microorganisms other than cyanobacteria, including other bacteria and fungi (Norstog and Nicholls, 1997). The diversity of microorganisms living within the coralloid root varies greatly between plants in a single population, between coralloid roots from the same plant and even between developmental stages from a single coralloid root. This is taken as an indicator that the relationship between the cycad and its microbiota is based on the functional role of the microorganisms living in the rhizosphere rather than on a specific interspecies relationship (Costa *et al*., 2001; Thajuddin *et al*., 2010). In the following section I will review the possible relationship between cycad coralloid roots and fungi, which is much less understood.

1.4. The cycad-fungi relationship.

Preliminary observations in our own laboratory, as well as the findings in previous reports, have shown the presence of fungi either within or around the plant cells that form the cyanobacterial zone. The presence of fungi from the genera *Fusarium*, *Penicillium* and *Gibberella*, has been noted as being predominant in the coralloid roots of the cycad *Cycas bifida* (Zheng *et al*., 2018), yet no statistically meaningful differences were detected between the fungal taxa present in the coralloid and the non-symbiotic secondary root. Mycorrhizal fungi have also been reported in both the primary and secondary roots (Muthukumar and Udaiyan, 2002; Fisher and Vovides, 2004); however, none of the previous studies go into detail regarding the effect of mycorrhizal fungi on the host plant during this symbiosis. It is also to be noted that the impact that these fungi have on the microhabitat created within the coralloid root has, to our knowledge, never been studied before.

The role of symbiotic fungi has been widely studied in different plant-fungi relationships which, while lacking the of formation of specialized plant structures, are very similar to the coralloid root. Endophytic fungi usually have a role related to host defense (previously reported on *Theobroma cacao* endophytic fungi*;* Arnold *et al*., 2003), and an increase in the expression of plant genes related to the defense and stress response has been observed after contact with endophytic fungi (observed in *Medicago truncatula* mycorrhizae; Liu *et al.*, 2007).

Previous studies have also shown that the formation of symbiotic relationships between plants and fungi results in an increase of gene expression related to ion transport, mainly phosphorus and nitrogen, including ammonia and nitrates; this increase in gene expression has been observed both in the plant and in symbiotic fungi (Brechenmacher *et al.*, 2004; Willman *et al.*, 2007; Lucic *et al.*, 2008; Cappellazzo *et al*., 2010; Javaid, 2010). The presence of arbuscular mycorrhizae within the secondary roots of cycads *Zamia pumila* and *Dioon edule* has already been observed; given the similarity to other studied systems, it has been proposed that the formation of this symbiosis results in an increase in phosphorus uptake (Fisher and Vovides, 2004).

1.5. Relationships between endophytic organisms.

Interactions between organisms in this kind of relationships are multidirectional, meaning each component of the symbiosis is likely to have an effect on each and every other component of the relationship. Fungal growth, for example, has been shown to dramatically alter the dispersion and organization of microbial communities, as well as the establishment of different microenvironmental conditions

that has a direct effect on such communities, by affecting nutrient availability and transport (Lobakova *et al*., 2002a; Javaid, 2010; Zhang *et al.*, 2018). Other possible effects of fungal growth on micro and macro communities includes the halting of pathogen growth or infection (Garbelotto *et al.*, 2018.). Given that microbial communities are composed of elements with varying metabolic pathways that allow a certain independence from external environmental conditions, it is possible that the cycad´s micro symbiont communities are indirectly involved with the nitrogen fixation process that takes place within the coralloid root, be it by preserving the coralloid roots microbiota or by allowing the establishment of conditions that favor or even stimulate nitrogen fixation.

Interactions between different organisms have also been observed to affect the activity rates of nitrogen fixing bacteria. According to previous reports, the interaction between cyanobacteria and other bacterial species has been shown to increase the nitrogen fixation rates of cyanobacteria (Zvyagintsev *et al*., 2010). Saprophytic bacteria can also affect nitrogen fixation, as shown by reports that establish the growth of these bacteria on the coralloid root can allow the establishment of microaerophilic conditions (low oxygen concentration), which, as mentioned before, are appropriate for anaerobic nitrogen fixation (Lobakova *et al*., 2002a); thus, it is clear that microorganisms interacting with cyanobacteria have a very important role in symbiotic nitrogen fixation.

Mycorrhizae, a biological symbiosis with many similarities to the coralloid root, have been widely studied in legumes and other plant taxa in order to determine the nature of the relationship between the plant and its symbionts. In mycorrhizae, fungi colonize the plants roots forming nodules which also contain nitrogen fixing bacteria. In this symbiotic relationship fungi associated with the plant host ease solubilization and uptake of nutrients present in the soil, mainly phosphorus, nitrogen and potassium. Phosphorus is then used by the plant as an energy source during photosynthesis, leading to an increase in the rates of the plants photosynthetic activity. The higher amounts (compared to non-symbiotically associated plants) of products obtained from photosynthesis are spread along the entire plant, and reach the symbiotic nodules where the fungi and nitrogen fixing bacteria are localized. This results in the plant providing more nutrients that bacterial symbionts feed on, thus increasing their metabolic activity and rates of nitrogen fixation (Javaid, 2010).

Given the similarities between mycorrhizae and the coralloid root, there is the possibility that the fungi living within the coralloid roots of cycads have a role similar to that of mycorrhizae forming fungi. Since this role has not been explored to date, it is the aim of this study to determine how fungi alter their metabolism and physiology within the coralloid root.

D. edule as a system of study

D. edule, known colloquially as *chamal*, is an arborescent cycad endemic to Mexico, inhabiting tropical deciduous forests (figure 3; González-Astorga *et al*., 2003). This species populations, as the populations of many other species of cycads, are considered close to being endangered due to the loss of their habitat due to agriculture, exploitation for illegal commerce and extermination due to its toxicity to cattle (the consumption of young *Dioon* leaves and cones results in diarrhea, hind limb paralysis and death; Yáñez-Espinosa *et al*., 2009).

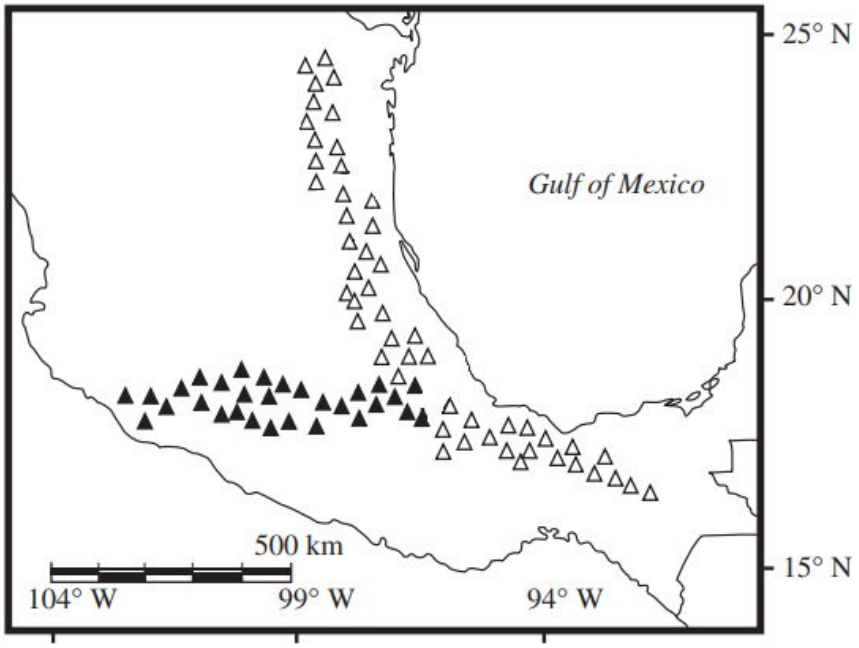

Figure 3. Geographic distribution of *D. edule*. White triangles show its distribution at the "Sierra Madre Oriental"; black triangles show its distribution at the "Eje Volcánico Transversal" (modifier from González-Astorga *et al*., 2003).

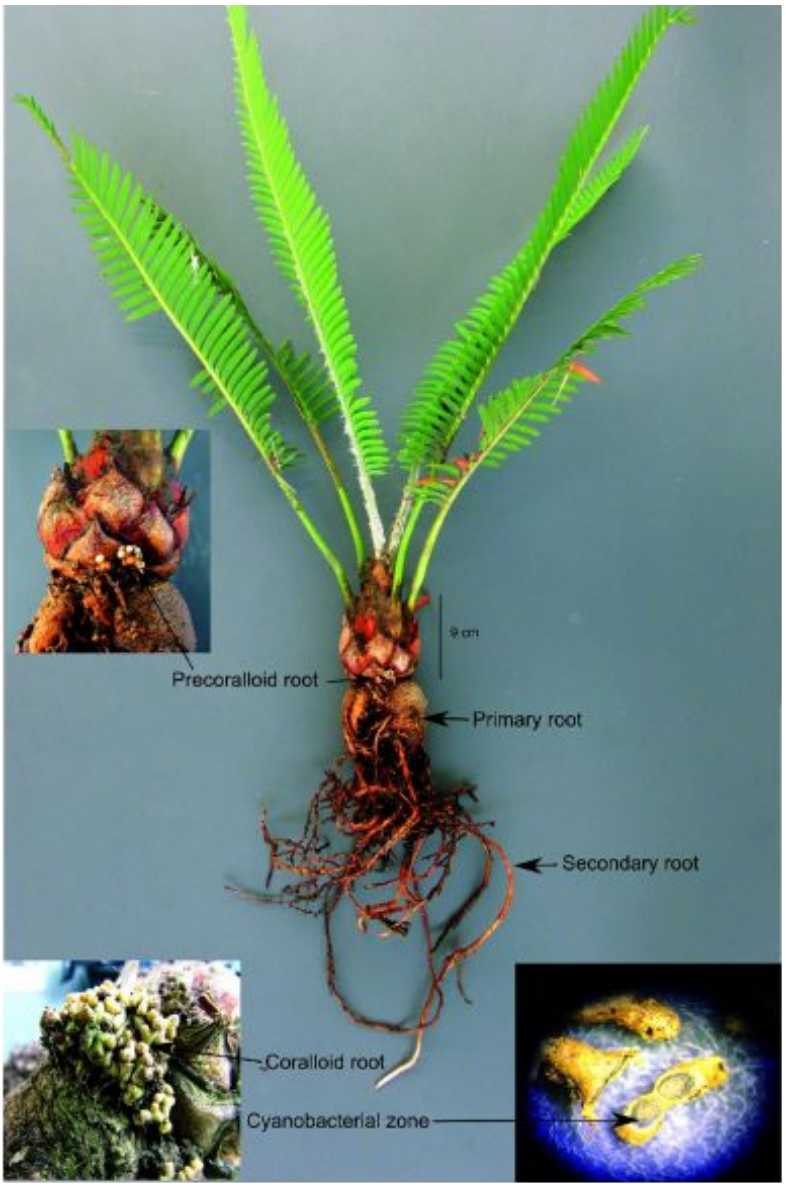

Figure 4. The anatomy of *D. edule* (Suarez-Moo *et al*., 2019).

2. Hypothesis.

Fungi living within coralloid roots have a nitrogen-fixation related role.

3. Objectives.

Main objective: To characterize the functional role of fungi inhabiting the coralloid root with respect to nitrogen-fixation.

Specific objectives:

1. Documentation of the presence of fungi in the coralloid roots of the cycad.

2. The generation of a fungal microbiological collection from cycad coralloid roots.

3. Identification of differentially expressed cycad and fungal genes in order to infer the possible role of fungi in the coralloid root.

4. Material and methods.

4.1. Experimental design and sample collection.

We collected mature coralloid roots, as well as non-symbiotic secondary roots to be used as control, from different *D. edule* specimens kept in three different conditions:

1. *D. edule* individuals kept in pots inside greenhouses at the Ecology Institute (INECOL) at Xalapa, Veracruz.
2. Pot-grown plants kept in our own laboratory. These plants were not isolated from external environmental conditions.
3. Natural cycad populations from different locations in Queretaro and San Luis Potosi (figure 5).

Samples were separated in two experimental groups based on the analysis to be performed: coralloid and secondary roots obtained from greenhouse conditions and pot-grown plants were reserved for the first two objectives of this study (observation of fungal structures through a microscope and generation of a microbiological stock); meanwhile, samples taken from natural *D. edule* populations were used for differential expression analysis.

Figure 5. Location of our three sampling sites

D. edule specimens were identified through a series of diagnostic

characteristics (Vovides, 1983):

- Grayish to brownish bark with a rough texture, covered in bases of persistent petioles.
- Pale green coloured leaves, measured between 0.8 and 1.3 m long and between 15 and 23 cm wide at the middle portion, showing intermediate stiffness.Juvenile leaves are pale green-yellowish in color, and present trichomes localized on the leaf underside.
- 75 to 110 pairs of subopposite leaflets with a linear-lanceolate shape, measured between 6 and 10 cm long and between 5 and 9 cm wide, coriaceous or rigid. Entire margins, sometimes pungent in juvenile plants. Leaflets also present a pungent apex and 9 to 12 midribs. Petioles are 17 to 22 cm long; cataphylls are between 7 and 12 cm long and between 3 and 3.5 cm wide at the base.
- Microstrobili are cob shaped, with a conic to almost cylindrical shape, erect, greenish or light brown in color once they reach maturity. Microstrobili are 20 to 35 cm long and 6 to 8.5 cm in diameter.

Mature coralloid roots were identified visually based on external anatomy and the presence of the cyanobacterial zone (visually, a dark green ring) detectable at plain sight from a cross section of the coralloid root. After collection, all samples to be used for meta-transcriptome analysis were immediately stored in 50 ml Falcon tubes and frozen in liquid nitrogen. Samples taken for stock generation were stored in 50 ml Falcon tubes and resealable bags without a freezing step. Samples taken for microscopy were processed right after removal from the cycad.

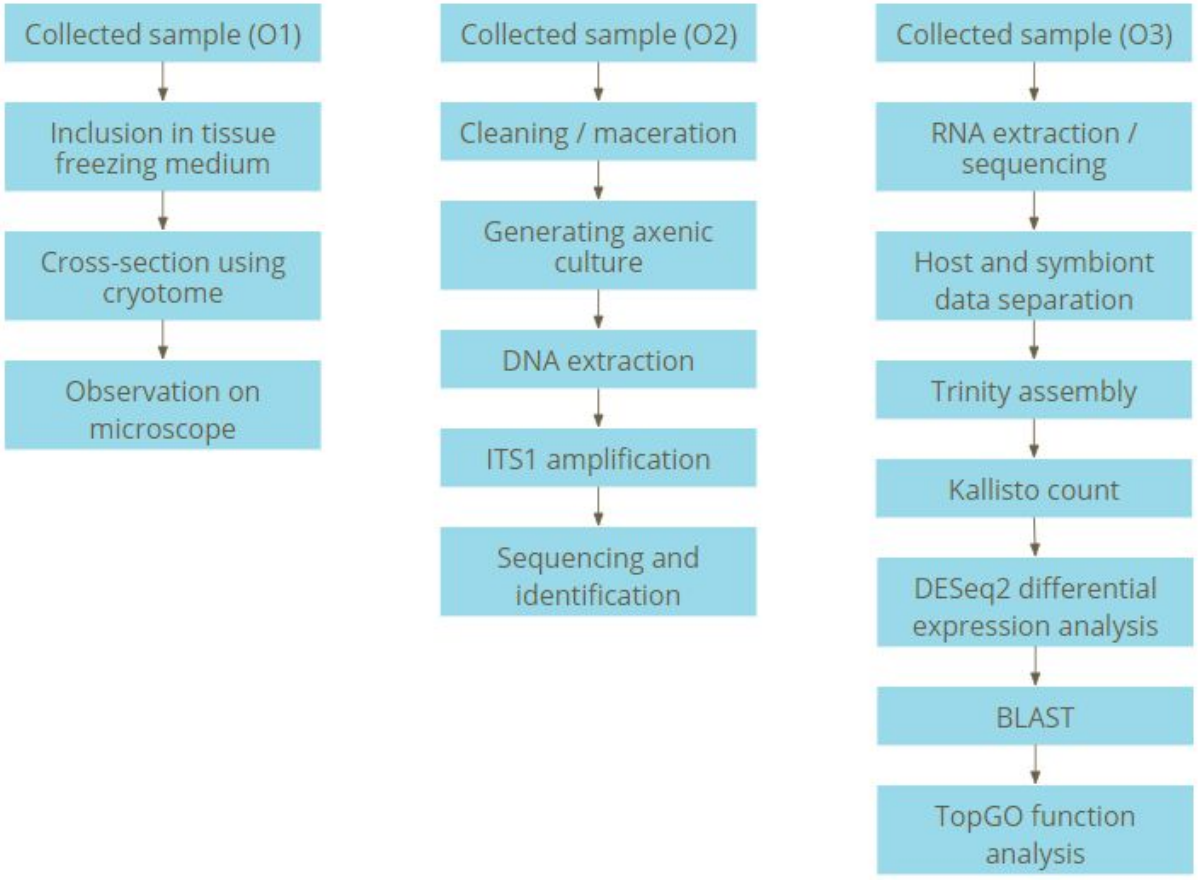

Figure 6. Procedural flowchart for all three objectives.

4.2. Microscopy.

To study the anatomy, localization and positioning of fungal endophytes we took fresh samples to be fixed in tissue freezing solution in order to conserve the root structure. We cut 60 µm cross sections of both the coralloid and secondary root using a cryotome at -19° C. Cross sections were fixed to a poly-L-lysine treated slides. We observed fungal anatomy by staining the samples with lactophenol-cotton blue, which stains the chitin present in the fungal cell wall while preserving fungal structures (Leck, 1999; Golabgir *et al*., 2015). Tissue was then washed using glycerol, and the samples were kept in glycerol during and after observation and storing of the tissue. Slides were sealed using varnish to fix the coverslip to the slide and stored at 4° C to avoid degradation of the tissue. Slides were observed under a digital microscope Keyence VHX-5000 in order to determine the position and distribution of fungal structures within the coralloid root, and under a fluorescence microscope Olympus BX50 in order to observe and identify specific fungal structures based on their morphology.

4.3. Stock generation.

We cleaned the exterior of 18 coralloid roots from different cycad species using commercial bleach (diluted 1:10), oxygenated water and sterile water. The coralloid roots were macerated in sterile water and the extract diluted 1:10 and 1:100, then grown in Petri dishes with Potato Dextrose Agar (PDA), Sabouraud and Czapek culture medium. Water from the last washing step was used as a negative control to confirm that fungal growth did not originate on the exterior of the coralloid root. All petri dishes were kept in the dark at 24° C to allow the growth of fungi.

We transferred fungi exhibiting different morphologies within the same Petri dish into new Petri dishes until axenic cultures were achieved (pictures in Annex 1). When bacterial growth was observed, fungi were transferred to a new Petri dish with medium with streptomycin as antibiotic. Once enough biomass had grown, the sample was transferred to a new Petri dish as a backup, which was stored at 4° C. Fungal biomass was macerated with liquid nitrogen and DNA was extracted from each sample; fungal biomass was stored at 4° C in glycerol after DNA extraction. We amplified the ITS1 intergenic region, which was sequenced and the fungi were identified by performing BLAST against six different databases (BLAST, BOLD, CBS Database, Q-Bank, ISHAM and Unite). Best hits were selected based on E-value and bit score. We only considered identification as successful if the results of four or more databases coincided (in some cases, identification was not achieved through every database; in this case, we considered identification successful if most databases coincided); otherwise, the sample was discarded.

4.4. Transcriptomic analysis.

We extracted total RNA from previously collected samples through a protocol perfected in our laboratory to obtain quality RNA from difficult plant tissue (modified from Yockteng *et al*., 2013; Annex 2), by using Plant RNA Reagent (by Thermo Fisher) as a purification reagent, mixing all samples by inverting rather than using a vortex, and adding a Lithium Chloride cleaning process (the details of which are contained in Annex 1). Successful RNA extraction was tested by electrophoresis on 1% agarose gel, and RNA integrity was tested using NanoDrop 2000 and Bioanalyzer (we only used samples with an RNA integrity number over 8 and an RNA ratio higher than 2.1.

We generated cDNA fragments from extracted RNA and prepared libraries for sequencing by Illumina, performing a paired-end 100 bp run on platform HiSeq4000. Once sequenced, we discarded low quality reads based on Phred score, which represents the possibility that bases are incorrect. Bases with a Phred score under 5

were discarded to reduce sequencing-error induced noise and confirmed read quality through the use of FastQC. Samples for each type of root were treated as biological replicates.

In order to explore whether gene expression differs between the coralloid (symbiotic) and secondary (control) roots, as well as which genes show differential expression between these structures, we generated alignment indexes for the host cycad transcriptome. These indexes were created from a reference *D. edule* transcriptome, available online as part of the 1000 plants online project (Matasci *et al*., 2014). Afterward, we performed a differential expression analysis by using the R package DESeq2, using a p-value < 0.01 and a log2 fold change > 0, indicating any fold change greater than 1. Sample groups (coralloid root vs secondary root) were compared to each other, as DESeq2 allows for differential expression analysis with different sample group sizes. We used BLAST to compare all of our reads to the Uniprot online database (https://www.uniprot.org/) and selected the best hits based on E-value and bit score. For this study we only used reads that were identifiable through BLAST and showed differential expression. Afterward, we used the R package TopGO for a GO term analysis, in order to identify which functions were enriched in our differentially expressed genes. The R script used to perform this analysis can be found in GitHub as “Differential expression + GO.txt”, or in the following link:

https://gist.github.com/flopezr-code/71dd16812edaba973c7e0f66cb2a192c

Fungi transcriptome analysis, on the other hand, required the removal of all reads that mapped to a cycad reference transcriptome, followed by the generation of an alignment of all available metatranscriptomes (performed using Trinity). The index used for this analysis was created from the resulting alignment. Available meta-transcriptomes were mapped by using Kallisto as well, which also counted the reads in each transcriptome. We performed BLAST on our reads in the same manner as with the cycad reads, followed by differential expression and GO term analysis in the same manner as with the cycad transcriptome.

5. Results.

5.1. Microscopy shows presence of fungi in the coralloid root.

Visual analysis under a fluorescence microscope revealed the presence of fungal hyphae and spores in coralloid root cross sections (figures 8 and 10), with higher concentrations around the cyanobacterial zone and inside the vascular bundle. We also observed what we identified as a fungal vesicle, a structure that stores lipids and can be used by arbuscular mycorrhizae as a propagule (figures 8-10; Sullia, 1991; Müller *et al*., 2017). Endophytic fungi presence was also observed in the secondary root, where the parenchyma of the root showed the highest concentrations of fungal structures. However, fungal structures were found in higher concentrations and much closer to one another in the coralloid root compared to the secondary root (figures 8 and 10). Both roots presented golden cells which are toxin containing idioblasts (figures 7-10; Vovides, 1993; Brenner *et al*., 2003).

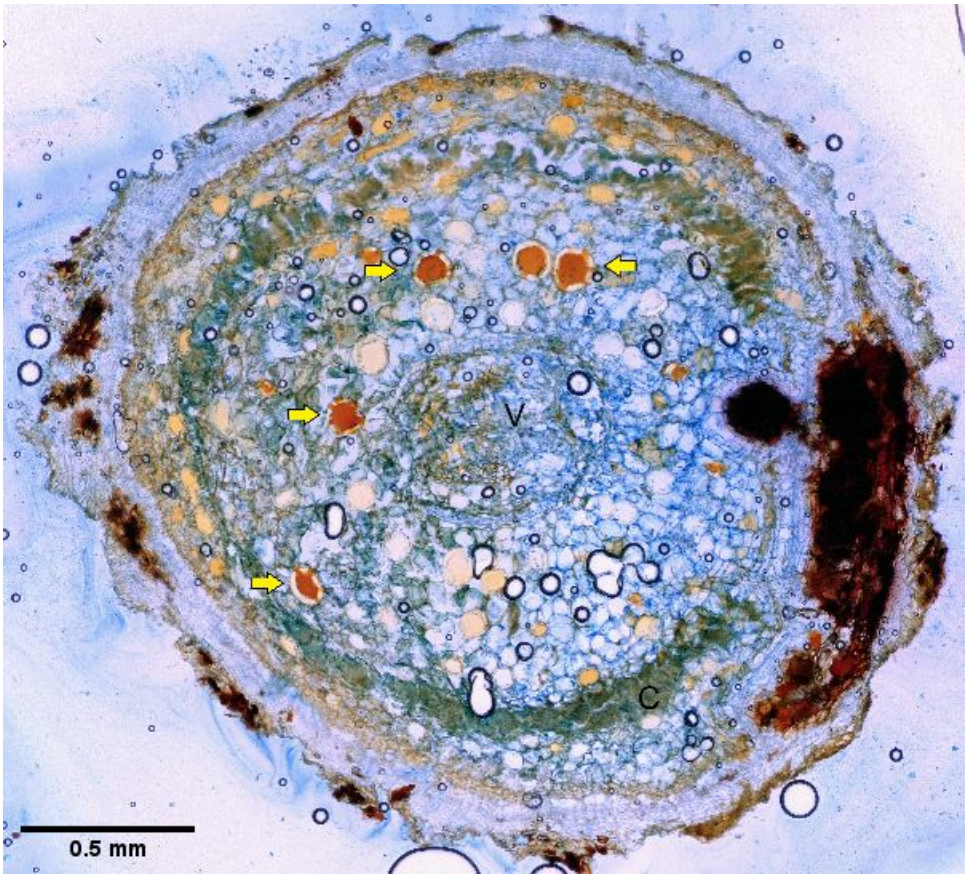

Figure 7. Cross section of *Dioon* coralloid root observed under a microscope after lactophenol-cotton blue staining. Lettering shows the position of the cyanobacterial zone (C) and the vascular bundle (V). Yellow arrows indicate the position of golden cells.

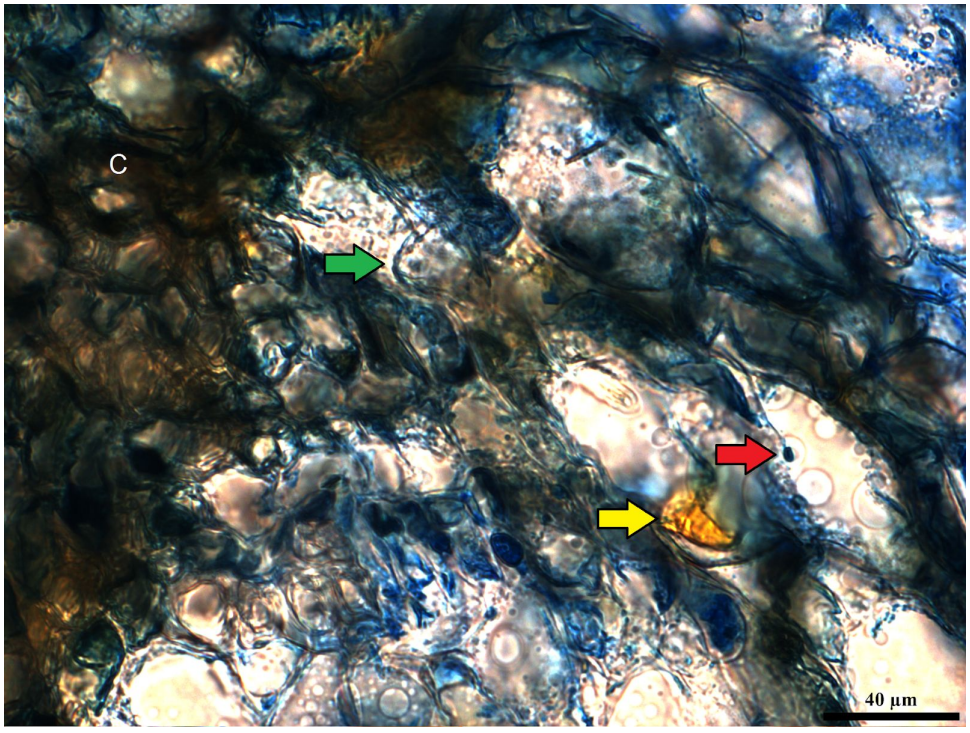

Figure 8. Cross section 40x micrograph showing the presence of fungal hyphae (green arrows) and sporangia (red arrows) inside the coralloid root of *Dioon*, as well as a golden cell (yellow arrow). Lettering shows the position of the cyanobacterial zone (C).

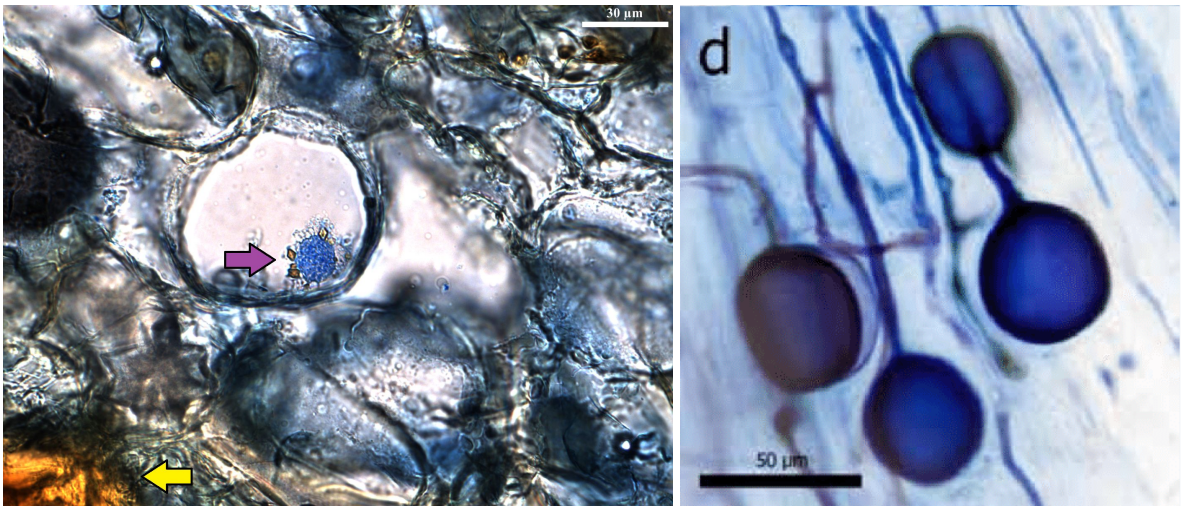

Figure 9. 40x micrograph (left) showing the presence of a structure identified as a vesicle (purple arrow) within the coralloid root of *Dioon,* as well as a golden cell (yellow arrow). Micrograph (right) used as an anatomical reference for identification, showing fungal vesicles within the roots of mycorrhiza forming herbaceous plant *Dianthus superbus* (Stokłosa *et al*., 2016).

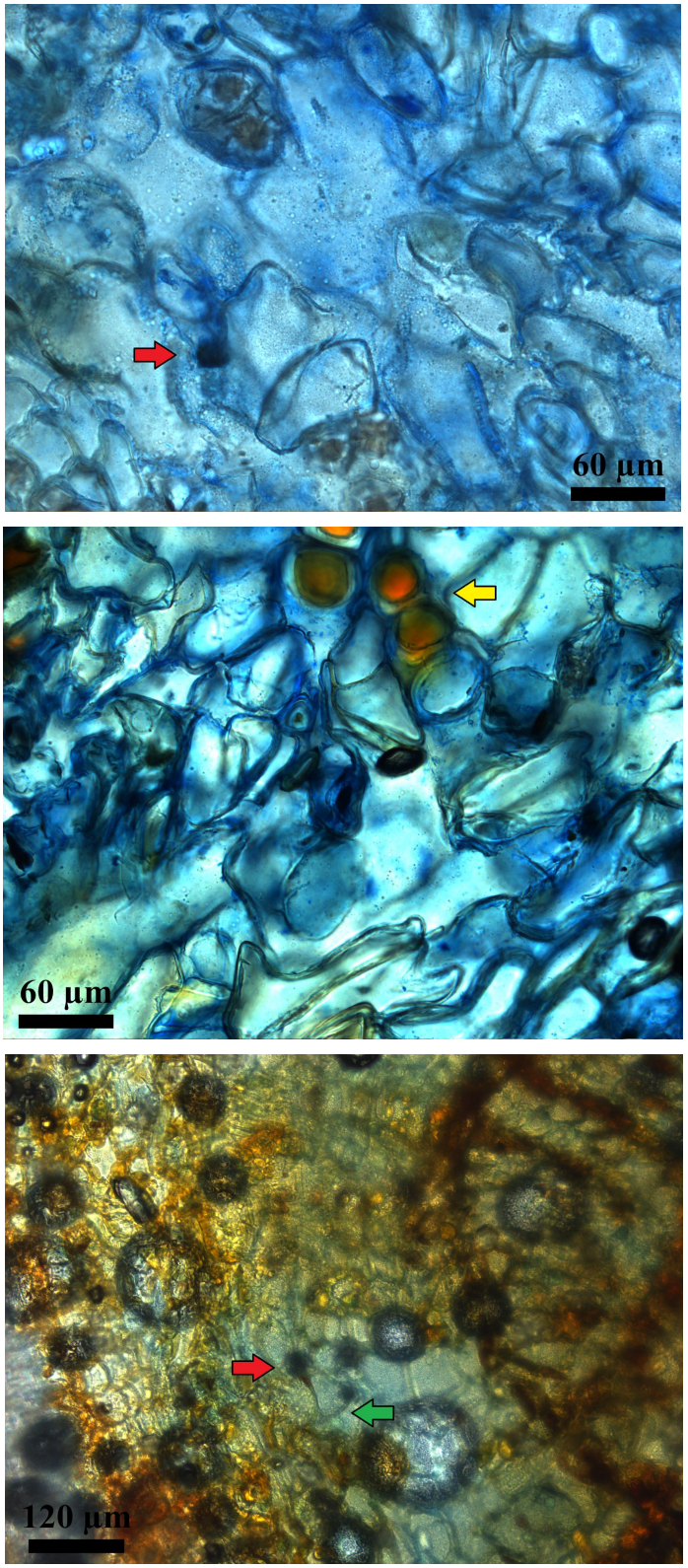

Figure 10. Three 100x micrographs showing the presence of fungal hyphae (green arrows), sporangia (red arrows) and golden cells (yellow arrows) within *Dioon*'s secondary root.

5.2. Stock generation indicates presence of several ecological niches.

After discarding Petri dishes that showed fungal growth in the negative control we obtained a total 50 different morphologies from a total of 101 axenic cultures. Sequencing the ITS genomic region, we identified fungi belonging to 9 genera. These fungi have been reported to have different roles in their interactions with plant hosts. Results are shown in Figure 11.

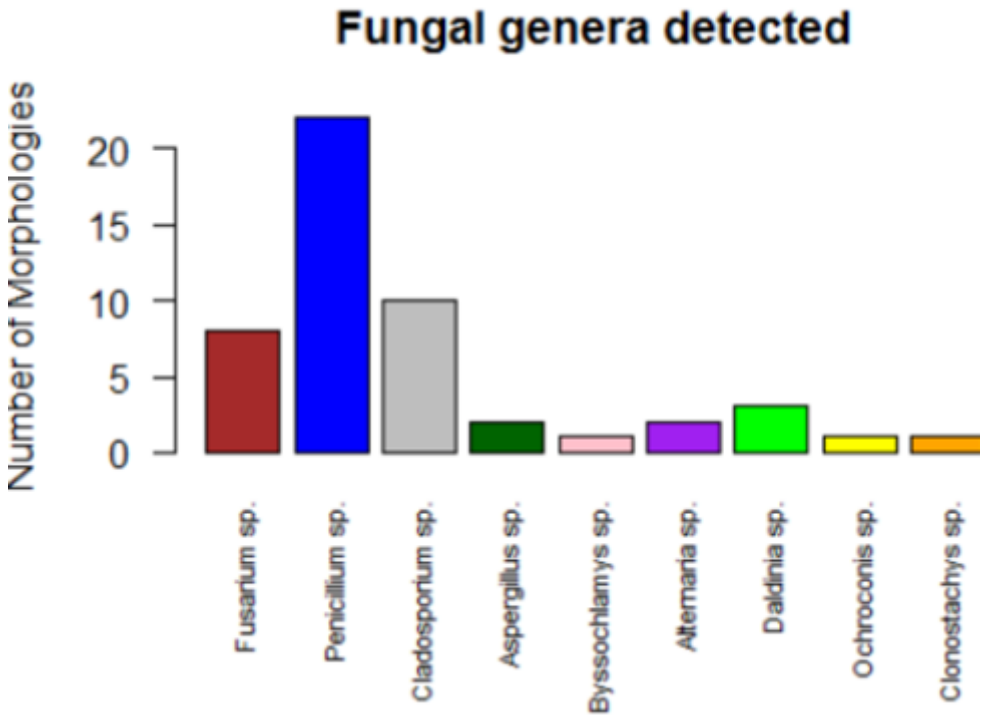

Figure 11. Fungal taxa identified from the coralloid roots of different cycad species by morphology and ITS sequencing. Y axis shows the number of different morphologies found within the coralloid root for each genus.

The generation of a microbiological fungal stock allowed us to detect the presence of different genera which have been identified in the literature as saprophytes, pathogens or both. Fungi belonging to genera *Cladosporium*, *Aspergillus*, *Byssochlamys spectabilis*, *Daldinia sp.* and *Clonostachys sp*. have been identified as saprophytes (Johannesson *et al*., 2001; Ravnskov *et al*., 2006; Hamayun *et al*., 2010; Marfenina *et al*., 2013; Mioso and Toledo Marante, 2015). On the other hand, pathogenic fungi include *Fusarium* (specially the well studied pathogen species *F. oxysporum*; Nelson *et al*., 1994) and *Ochroconis sp*. (Giraldo *et al*., 2014). A third group of fungi detected in our microbiological stock includes fungi that have been reported as both saprophytes and pathogens or parasites, such as *Alternaria* (Thomma, 2003) and *Penicillium* (Dupont *et al*., 2016).

The most interesting of all these fungi are species from the genus *Penicillium* since they have been reported previously as mycorrhizae forming fungi (Fan *et al*., 2008). Also interesting is the presence of species belonging to genera *Cladosporium*, *Aspergillus* and *Clonostachy*s since these are fungi that benefit the host plant through their interaction with mycorrhizae, as well as solubilizing phosphorus present in the rhizosphere (Singh and Kapoor, 1998; Cabello *et al*., 2005; Medina *et al*., 2007), promoting plant growth (Ravnskov *et al*., 2006; Hamayun *et al*., 2009), and overall improving the plants performance (Caravaca *et al*., 2004).

Presence of *Fusarium oxysporum*, a common plant pathogen that causes great agricultural losses worldwide, was frequent in this and previous studies that also analyzed the coralloid root of the cycads (Zheng *et al*., 2018). This was accompanied by the presence of fungi from genera *Cladosporium* and *Alternaria*, also pathogenic fungi. The pathogenic effects of these fungi have been observed to be negatively affected by the activity of mycorrhizal fungi (Fritz *et al*., 2006; Abohatem *et al*., 2011; Nair *et al*., 2014; Wang *et al*., 2018). The presence of *Byssochlamys spectabilis* within the coralloid root was also interesting, as this fungus has been employed as a biocontrol agent that halts the growth of pathogenic fungi (Bosso *et al*., 2016).

5.3. Transcriptomic analysis of cycad genes reveals mycorrhiza-like symbiosis

5.3.1. Cycad GO term clustering

Transcriptomic analysis of *D. edule* coralloid and secondary roots revealed that 794 genes out of 42,000 considered in this study were differentially expressed (totalling 1.8 %). 552 (1.3 %) of these genes were upregulated and 242 (0.5 %) were downregulated. Clustering of GO terms through gene expression was performed using the REVIGO website, which reduces redundancy to explore the most representative terms and processes (Supek *et al*., 2011). As seen in Figure 4, the most represented biological processes among highly expressed genes in the coralloid root include those involved with the development of the structure, such as hydrogen sulfide biosynthesis, GABA transport, cell wall organization, bundle sheath cell fate specification and wax biosynthesis. Also overrepresented are processes that likely result from the symbiosis between the plant and nitrogen-fixing bacteria, such as iron assimilation by chelation and transport and processes involved with nitrogen, such as GABA transport and response to zinc ion. Selenium compound metabolism was also represented by our analysis. While selenium uptake and metabolism in plants has been reported previously, its role in the coralloid root is unknown at the moment.

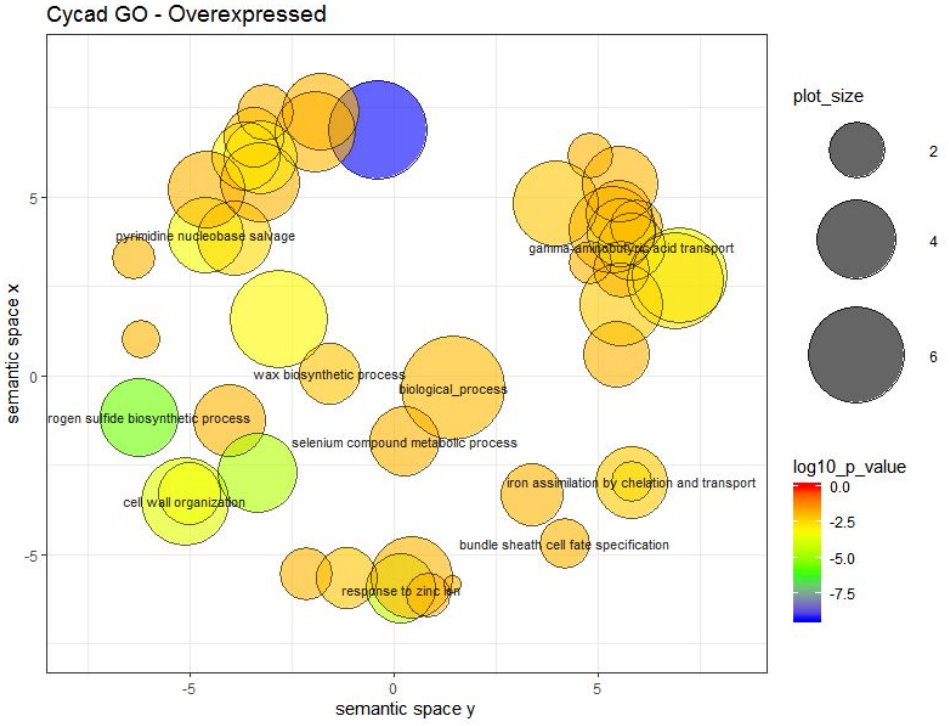

Figure 12. Bubble chart showing the overlap between the GO terms enriched by genes with greater expression in the coralloid roots of the cycad after transcriptome analysis of *D. edule*. Bubble size indicates frequency of the term in the Gene Ontology Annotation database; bigger bubbles indicate more general terms. Bubble color indicates p-value of each GO term according to the color scale (bottom right).

Representation of less expressed cycad genes in the coralloid root, on the other hand, shows two key processes: plant defense (defense response, jasmonic acid metabolism) and root integrity (pectin biosynthesis, cell wall organization), the relevance of both of which was explored above. Perhaps related to these two processes are leaf senescence and viral process. The representation of leaf senescence could be explained by the co-opting of related genes to serve a function in root development or integrity, which is severely altered during the formation of the coralloid root and may lead to down-regulation of these related genes. Viral process, on the other hand, indicates a reduction in the response of the cycad to the presence of a virus. Virus presence in the cycad has been reported previously (Baker and Adkins, 2007; Alvarez-Quinto *et al*., 2020), and preliminary work on our laboratory indicates viral presence on the coralloid root; however, further investigation is required to understand the activity of virus in this structure and their potential interaction with other elements of the cycad microbiome. With processes such as negative regulation of protein binding and protein K63-linked ubiquitination it is harder to reach a conclusion as to their relevance due to the vast array of processes they may be associated with; however, K63-linked ubiquitination has been observed to be related to DNA repair in yeast (Spence *et al*., 1995).

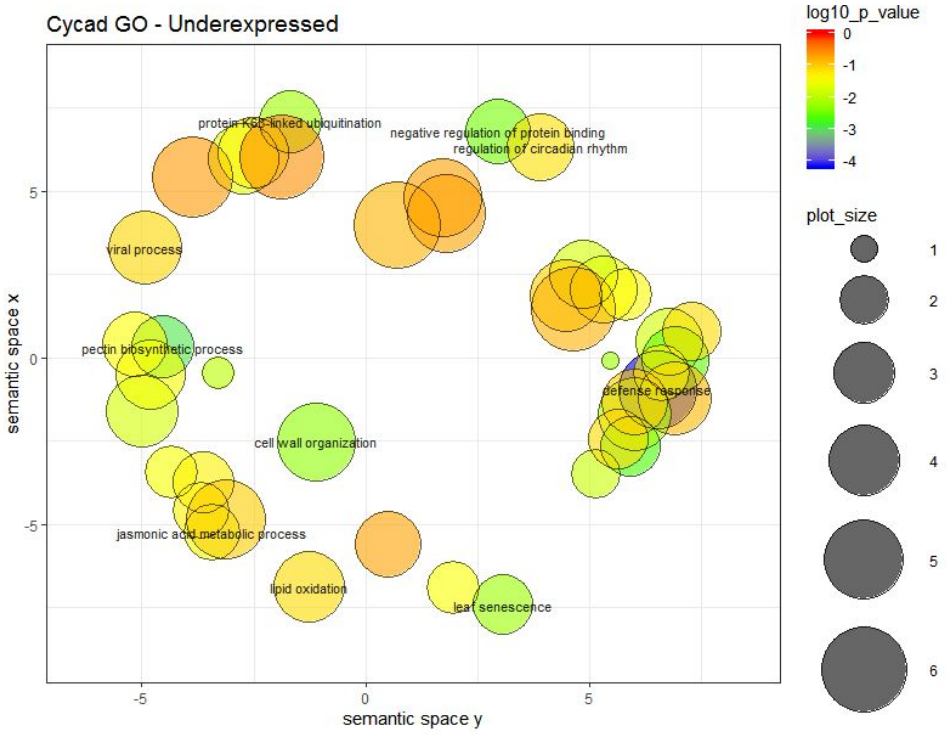

Figure 13. Bubble chart showing the overlap between the GO terms enriched by genes with lower expression in the coralloid roots of the cycad after transcriptome analysis of *D. edule*. Bubble size indicates frequency of the term in the Gene Ontology Annotation database; bigger bubbles indicate more general terms. Bubble color indicates p-value of each GO term according to the color scale (top right).

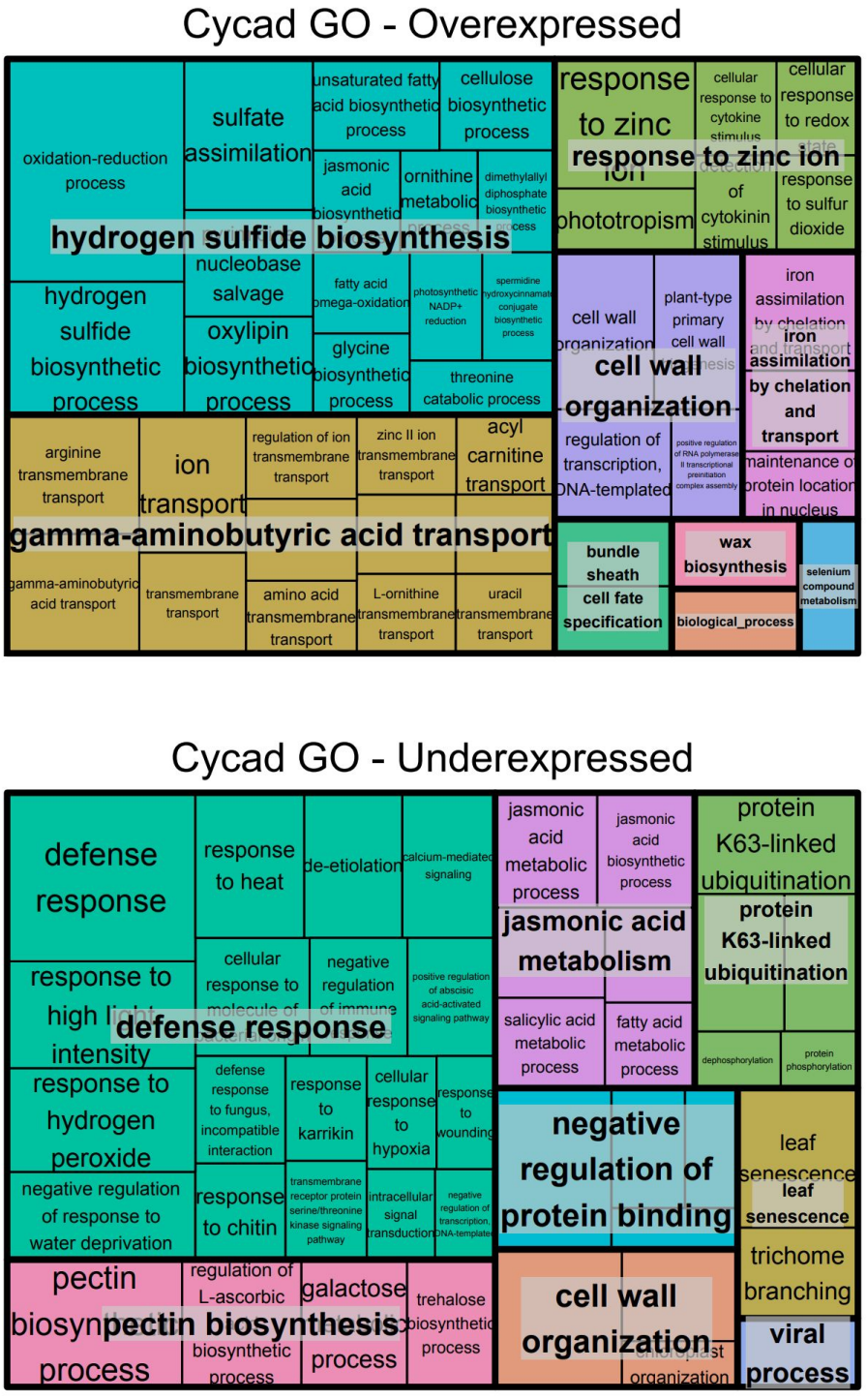

Figure 4. Biological processes associated with the top enriched GO terms associated with genes overexpressed (above) and underexpressed (below) in the coralloid root of *D. edule*. Large, uniformly colored rectangles represent superclusters of related terms, which are represented by smaller rectangles of the same color. Rectangle size is given by the p-value of each GO term.

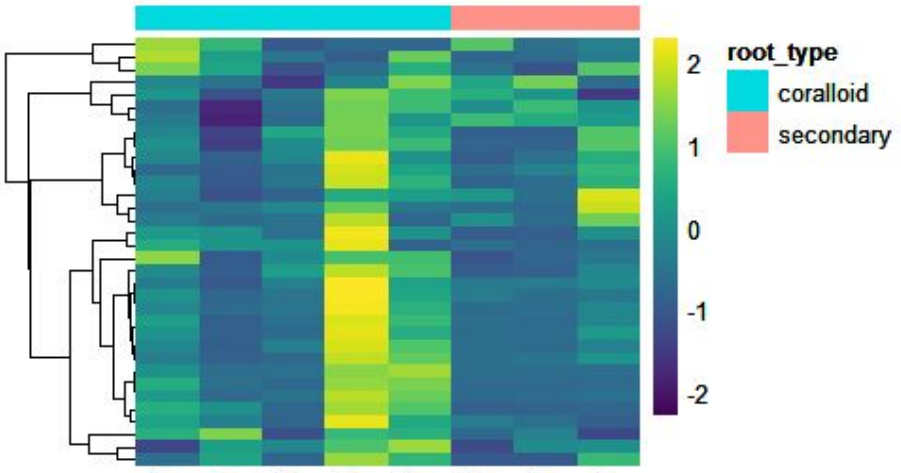

Figure 15. Expression profiles of *D. edule* coralloid and secondary roots. Color bar (top) indicates the type of root (blue for coralloid, pink for secondary) for each sample. Clustering (left) indicates correlation-based distance between genes. Gradient color barcode (right) indicates log2 Fold Change value.

5.3.2. Coralloid root formation

Among the GO terms enriched in the cycads coralloid root (Figure 16) we found were phototropism (GO:0009638). This is potentially related with negative geotropism, that is, the growth of coralloid roots towards the soil surface rather than away from it, therefore growing towards a source of light (Norstog and Nicholls, 1997). Possibly related to the coralloid root formation as well, we saw enrichment of cell wall organization (term GO:0071555), plant type primary cell wall biogenesis (GO:0009833), regulation of endodermal cell differentiation (GO:1903224), bundle sheath cell fate specification (GO:0090610), wax biosynthesis (GO:0010025) and cellulose biosynthetic process (GO:0030244). All of these processes are directly related to the growth and formation of the coralloid root through the expression of cellulose synthases from the CESA family (in some cases showing up to 41 times higher expression) and detection of cytokinin stimulus, along with other genes (Bischoff *et al*., 2009).

Biosynthesis of compounds reported to have a direct effect on root development, such as spermidine-hydroxycinnamate conjugates and lignin, as well as upregulation of cytokinins detection (hormones related to plant development), may also be involved in the formation of the coralloid root (Kakimoto, 2001; Couée *et al*., 2003; Vanholme *et al*., 2013). These results seem to indicate an upregulation of cycad processes involved in the coralloid root formation.

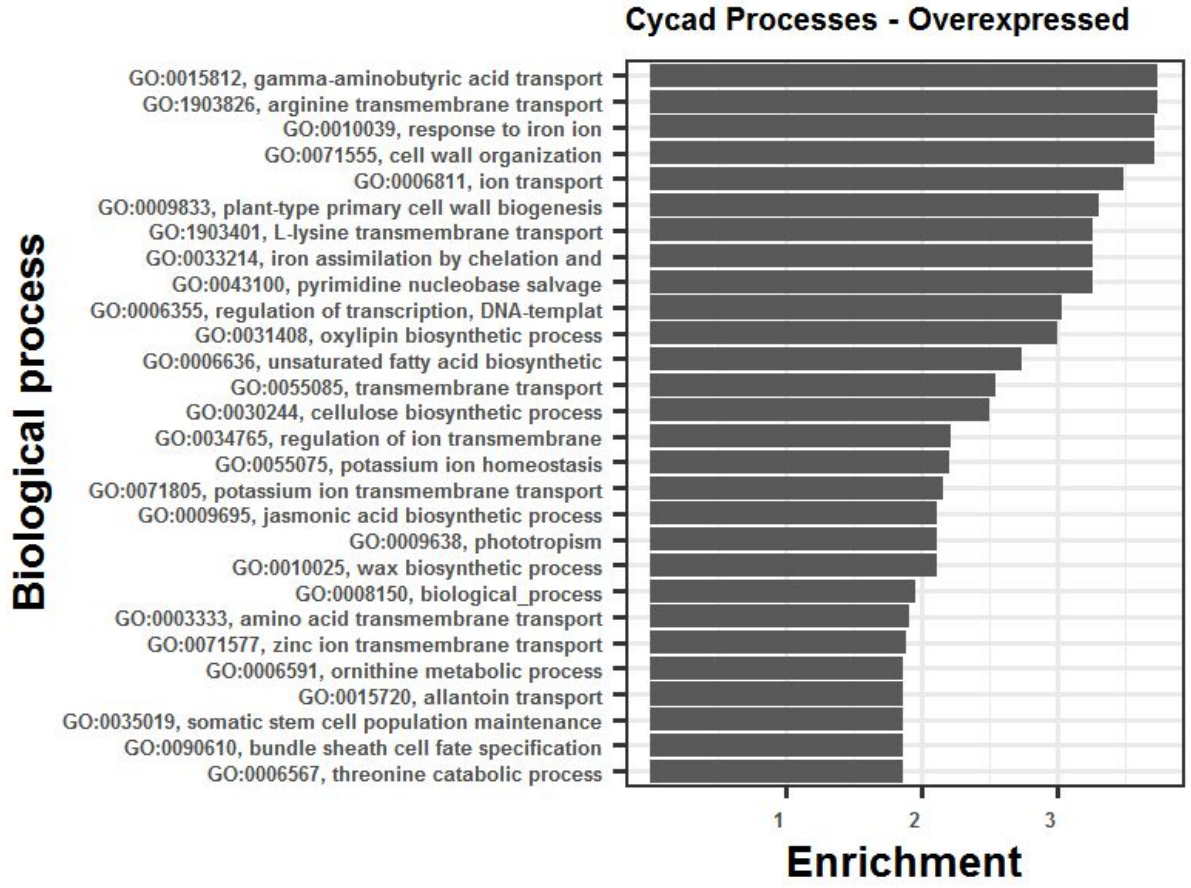

Figure 16. Enrichment of the top GO terms corresponding to different biological processes expressed in greater amounts in *D. edule*'s coralloid roots when compared to secondary roots. Enrichment is given by Fisher F value (scale denotes negative exponential).

We also found a series of processes that were repressed on the coralloid roots of *D. edule* when compared to the expression in secondary root (Figure 17). Among these processes, we found enrichment of cell wall biogenesis (term GO:0042546), response to wounding (GO:0009611) and pectin biosynthetic process (GO:0045489), all of which might be related to the formation of the coralloid root. Pectin is a component of the plant cell wall, usually conforming around a third of its structure, as well as regulating intercellular adhesion in certain areas (Willats *et al*., 2001). This could be related to the opening of papillae on the cell wall during the formation of the coralloid root, thus allowing the entry of microorganisms. However, further research into the composition of the cell wall of coralloid roots may provide more information. Enrichment of xyloglucan metabolic process (term GO:0010411) is related to a compound involved in growth of the root tip (Peña *et al*., 2012), triggering the halting of such process in the coralloid root. Enrichment of term regulation of circadian rhythm (GO:0042752) was also observed, possibly a result of a relaxation of the environmental cues that regulate the circadian rhythms of the coralloid root.

5.3.3. Symbiosis in the coralloid root

Another GO term that was enriched in the coralloid root of *D. edule* was oxylipin biosynthetic process (term GO:0031408). Oxylipins are compounds produced by the plant during mycorrhizal formation, and they have been reported to be involved in wound-related functions, plant defense and symbiosis regulation (León *et al*., 2001; López-Ráez *et al*., 2010; León Morcillo *et al*., 2012). In addition, we observed enrichment of ion transport (term GO:0006811), regulation of ion transmembrane transport (GO:0034765), response to zinc ion (GO:0010043), and zinc ion transmembrane transport (GO:0071577). The enrichment of these processes is likely a result of symbiosis-induced increase in soil nutrient solubilization in the plants rhizosphere, resulting in an increased available ion transport. This may also be related to nitrogen fixation of the coralloid root, as high zinc concentration has been shown to increase efficiency of nitrogen fixation (Gupta *et al*., 2002). Glycine biosynthetic process (term GO:0006545) was also enriched; glycine containing proteins have been reported to be implicated in symbiotic nodule development in arbuscular mycorrhizae forming plants, such as *Medicago spp.* (Kevei *et al*,., 2002).

We also found enrichment of potassium ion transmembrane transport (GO:0071805) and potassium ion homeostasis (GO:0055075). Potassium is a very important nutrient present in the soil and its uptake is increased by the formation of arbuscular mycorrhizae (El-Mesbahi *et al*., 2012). Similarly, we found enrichment of terms response to iron ion (GO:0010039) and siderophore-dependent iron import into cell (GO:0033214) which points towards a phenomenon similar to one that has been observed in mycorrhizae where fungi and bacteria solubilize iron through the production of siderophores. Soluble iron can then be transported to the plant. Siderophores are iron-chelating ligands produced by microorganisms in order to solubilize and transport iron, and are produced under iron-limiting conditions (Renshaw *et al*., 2002; Winkelmann, 2007); genes such as YSL1, and YSL2 (showing up to 71 times more expression), as well as plant ferritins (7 times as much expression in coralloid roots in our study) have been reported as being involved in iron uptake, storage and transport (Briat *et al*., 2010; Montgomery *et al*., 2015). Enrichment of molybdate ion export from vacuole (GO:0090414) was also found; interestingly, molybdenum is often used by nitrogenase as a cofactor (Burgess, 1996).

Enrichment of term sulfate assimilation (GO:0000103) is also relevant, as it relates to the high sulfate concentration previously reported in the cyanobacterial zone of the coralloid roots (Caiola, 2001). Enrichment of gamma-aminobutyric acid transport (term GO:0015812) indicates the upregulation of GAT1 mediated transport of gamma-aminobutyric acid (GABA), a compound with a wide variety of functions

including plant defense, plant development and nitrogen storage. Upregulation of such processes could be possibly related to the increased uptake of nitrogen from either cyanobacterial fixation or fungal symbiosis (Shelp *et al*., 1999). This is possibly related to the enrichment of allantoin transport (term GO:0015720), as allantoin is a form of nitrogen and storage translocation in plants (Streeter, 1979). Enrichment of photosynthetic NADP+ reduction (term GO:0009780) could possibly function as an indication of higher rates of photosynthesis in the coralloid root resulting from the symbiosis in this structure, and enrichment of cellular response to redox state (GO:0071461) could indicate a necessity of the plant to maintain redox state control, due to the generation of oxygen and nitrogen reactive species during symbiosis (Matamoros and Becana, 2019). Interestingly, we also found enrichment of amino acid transmembrane transport (GO:0003333); amino acids are known to be a source of nitrogen from soil (Chen *et al*., 2018a).

On the other hand, we saw enrichment of unsaturated fatty acid biosynthetic process (GO:0006636) and fatty acid omega-oxidation (GO:0010430), which are related with the biosynthesis of compounds produced by the roots in response to drought; previous reports have shown that, during symbiotic relationships between plants and fungi, the plants physiological reaction can be very similar to drought response (Zhang *et al*., 1987; Sebastiana *et al*., 2019). GO term enrichment also points towards the biosynthesis of hydrogen sulfide (GO:0070814), a compound related to stress resistance, as well as plant development (Li *et al*., 2016a). Interestingly, genes necessary for establishing mycorrhizal symbiosis, such as RAM1, have been shown to be related to the production of fatty acids, which feed the arbuscular mycorrhizae forming fungi (Luginbuehl *et al*., 2017). This is further supported by enrichment of acyl carnitine transport (term GO:0006844), involved in the metabolism of fatty acids (Bourdin *et al*., 2007).

5.3.4. Lowered defense response of *D. edule*

We also found a series of processes that were underexpressed in the coralloid roots of *D. edule*. Among these processes, we found an enrichment of GO terms involved in the plants defense response. Defense response (term GO:0006952) and protein autophosphorylation (GO:0046777) are processes that participate in processes that have been reported to be involved in gene response to wound formation in plants (León *et al*., 2001). Processes related to jasmonic acid were also enriched, such as jasmonic acid metabolic process (GO:0009694) and jasmonic acid biosynthetic process (GO:0009695); jasmonic acid is a compound involved in plant growth and development, and it is reported that it could be related to mycorrhizae regulation; jasmonic acid also affects defense against herbivores and pathogens since biosynthesis of jasmonic acid inhibits the growth of pathogenic fungi

(Wasternack and Hause, 2013; Nair et al., 2014). Most tellingly, enrichment of cellular response to molecule of bacterial origin (GO:0071219), response to chitin (GO:0010200) and defense response to fungus (GO:0009817) indicates a lowered response to the microorganisms that inhabit the coralloid root, possibly as a necessity for the plant to reduce defense responses that may negatively impact beneficial bacteria or fungi within the coralloid root. Interestingly, one of the genes that was most upregulated in the coralloid root is GNK2 (241 times more expressed in the coralloid root), which has been reported as being involved specifically in antifungal activity by inhibiting the growth of phytopathogenic fungi such as *F. oxysporum*, *Trichoderma reesei*, and *Candida albicans* (Sawano *et al*., 2007; Miyakawa *et al*., 2014).

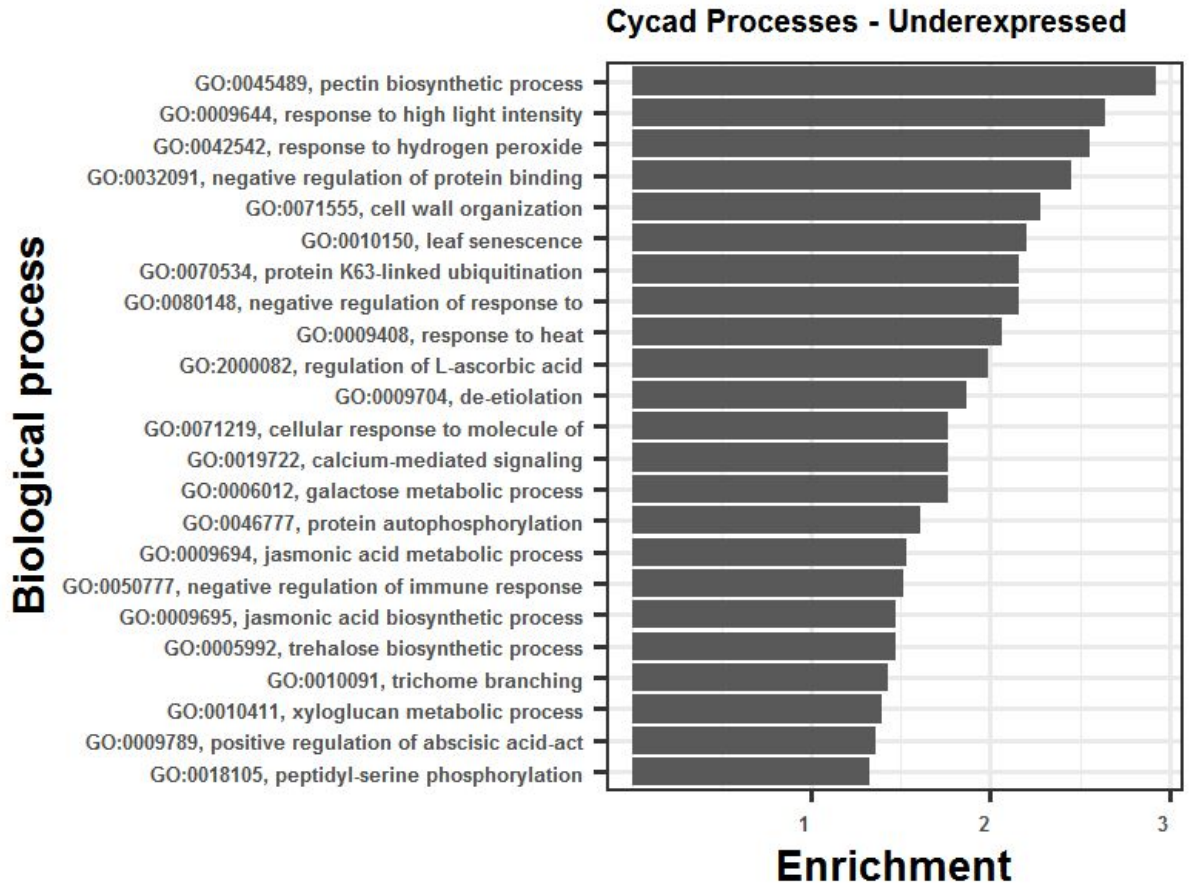

Figure 17. Enrichment of the top GO terms corresponding to underexpressed biological processes in *D. edule*'s coralloid roots when compared to secondary roots. Enrichment is given by Fisher F value (scale denotes negative exponential).

5.3.5. Cycads environmental stress response

Response to sulfur dioxide (GO:0010477) and response to karrikins (GO:0080167) were also overexpressed in the coralloid root. Sulfur dioxide is a

pollutant produced by volcanic activity and decomposition, but mostly as a residue of human activities such as coal burning and ore smelting (Rakwal *et al*., 2003). Karrikins, on the other hand, are compounds generated by the burning of plants; detection of this compound through gene KAI2 allows plants to detect wildfires (Guo *et al*., 2013; Waters *et al*., 2014). This could be an indication of the negative effect that human activities have on natural populations. However, genes belonging to this term and related to different processes were also overexpressed on the coralloid root. Proteins PSBS, GPT2 and HY5 have a role in the plant response to light stimulus through photoprotection by energy dissipation, acclimation to increased light radiance and photomorphogenesis (Li *et al*., 2000; Athanasiou *et al*., 2009; Srivastava *et al*., 2015), very possibly related to the above mentioned growth of the coralloid root, as the coralloid root approaching the surface would require an adaptive response to the presence of photic stress. Protein FLS2, interestingly, is involved in defense against bacteria through the detection of flagellin, a component of the bacterial flagellum, possibly as part of the selection of bacteria that enter the coralloid root (Zipfel *et al*, 2004).

On the other hand, we observed underexpression of genes related to the biosynthesis of compounds involved in the plants response to environmental stress, including hydrogen peroxide (GO:0042542, response to hydrogen peroxide), salicylic acid (GO:0009696, salicylic acid metabolic process; GO:0009627, systemic acquired resistance), trehalose (GO:0005992, trehalose biosynthetic process) abscisic acid (GO:0009789, positive regulation of abscisic acid-activated signaling pathway; GO:0009738, abscisic acid-activated signaling pathway; GO:0009737, response to abscisic acid) and ascorbic acid (GO:2000082, regulation of L-ascorbic acid biosynthetic process). Hydrogen peroxide is a compound that acts as a signal generated by the plant when faced with high light intensity-induced stress, drought, cold, wounds and pathogen infection (Neill *et al*., 2002). Hydrogen peroxide, however, has also been shown to be involved with the regulation of the interaction between plants and fungi during mycorrhizal formation (Puppo *et al*., 2013). Salicylic acid, on the other hand, is involved in drought stress by relieving oxidative damage, while trehalose participates in plant development (Fernandez *et al*., 2010; Li *et al*., 2016b; Phan *et al*., 2019). Abscisic acid regulates response to abiotic stress, (Sah *et al*., 2016), and ascorbic acid is an antioxidant that responds to oxidative stress, with its biosynthesis being reduced during drought-like conditions (Seminario *et al*., 2017). Similarly, enrichment of response to heat (term GO:0009408), cellular response to oxidative stress (GO:0034599) and response to water deprivation (GO:0009414) indicates a lower response of the coralloid root to changes in external conditions. The reduced expression of genes related to these processes shows that the coralloid root presents a lower response to environmental as well as biotic stress, perhaps as a way to maintain the internal microenvironment of the coralloid root and

keep it from suffering plant responses that may alter or damage the bacterial communities present in this structure.

The above mentioned results indicate that the coralloid roots gene expression allows the survival of certain microorganisms within the structure. Enrichment of cellular response to hypoxia (term GO:0071456) is potentially supportive of this, as cellular hypoxia in the roots tends to favor mycorrhizal formation (Kumari *et al*., 2019). A lowered response to hypoxia could then favor relations with other organisms, such as those present in the coralloid root. While reduced defenses from the plant and an increase in conditions that allow symbiosis might point towards favoring mycorrhizal symbiosis, we also report a decreased expression of genes related to transmembrane receptor protein serine/threonine kinase signaling pathway (term GO:0007178) and galactose metabolic process (GO:0006012), which would potentially result in a decrease of mycorrhizal symbiosis with the host cycad. Serine/threonine kinases have been reported to regulate mycorrhizal symbiosis, while galactose containing polymers help interaction with microorganisms (Koroney *et al*., 2016; Roth *et al*., 2018).

5.4. Transcriptomic analysis of fungal genes reveals non-specific symbiosis

Differential expression analysis of our samples showed no statistically meaningful differences between the expression of fungal genes in the coralloid and the secondary root (Figure 18). As our previous results show the presence of mycorrhiza like structures in both the coralloid and secondary roots, and given that the presence of mycorrhizal fungi was previously reported in both the primary and secondary roots (Muthukumar and Udaiyan, 2002; Fisher and Vovides, 2004), it is likely that, while fungi do show mycorrhiza and pathogen like functions, these are not exclusive to the coralloid root. Seemingly paradoxically, we found contradictory enrichment of GO terms for genes with both high and low expression in the coralloid and secondary roots of *D. edule*. This is likely a result of different sequences being identified as similar genes belonging to different fungal species, which show differential expression to each other due to their different ecological niches and interaction with the plant.

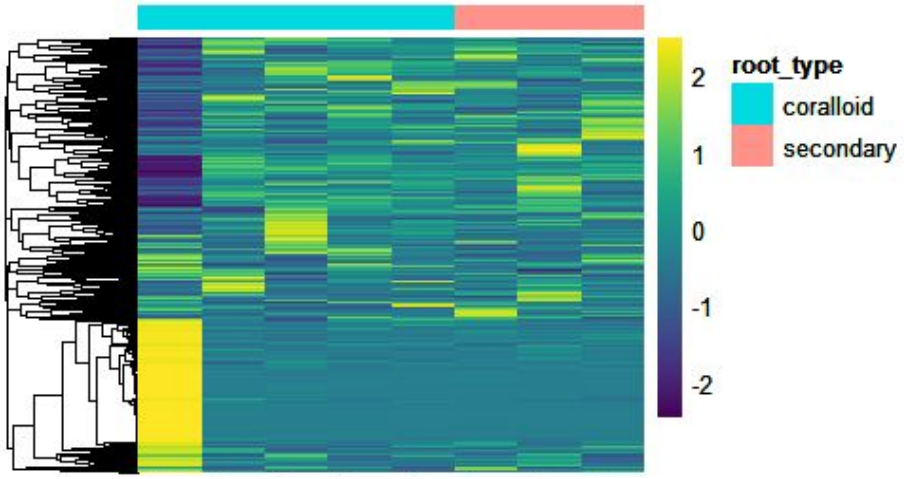

Figure 18. No differential expression was found in fungi present in *D. edule*'s roots. Color bar (top) indicates the type of root (blue for coralloid, pink for secondary) for each sample. Clustering (left) indicates correlation-based distance between genes. Gradient color barcode (right) indicates log2 Fold Change value.

5.4.1. Fungal GO term clustering

REVIGO clustering of GO terms containing the most and least expressed fungal genes in the coralloid root (Figure 21) revealed representation of key processes that show the same contradictions presented above, including fungal reproduction (kinetochore assembly, cell cycle, intracellular cell transport, cell division), stress

resistance (positive regulation of transcription from RNA polymerase II promoter, rRNA processing), pathogenesis and virulence (macroautophagy, protein transport, piecemeal autophagy of nucleus), with intracellular cell transport containing genes that have been reported to have functions related to fungal growth and symbiosis-related stress response.

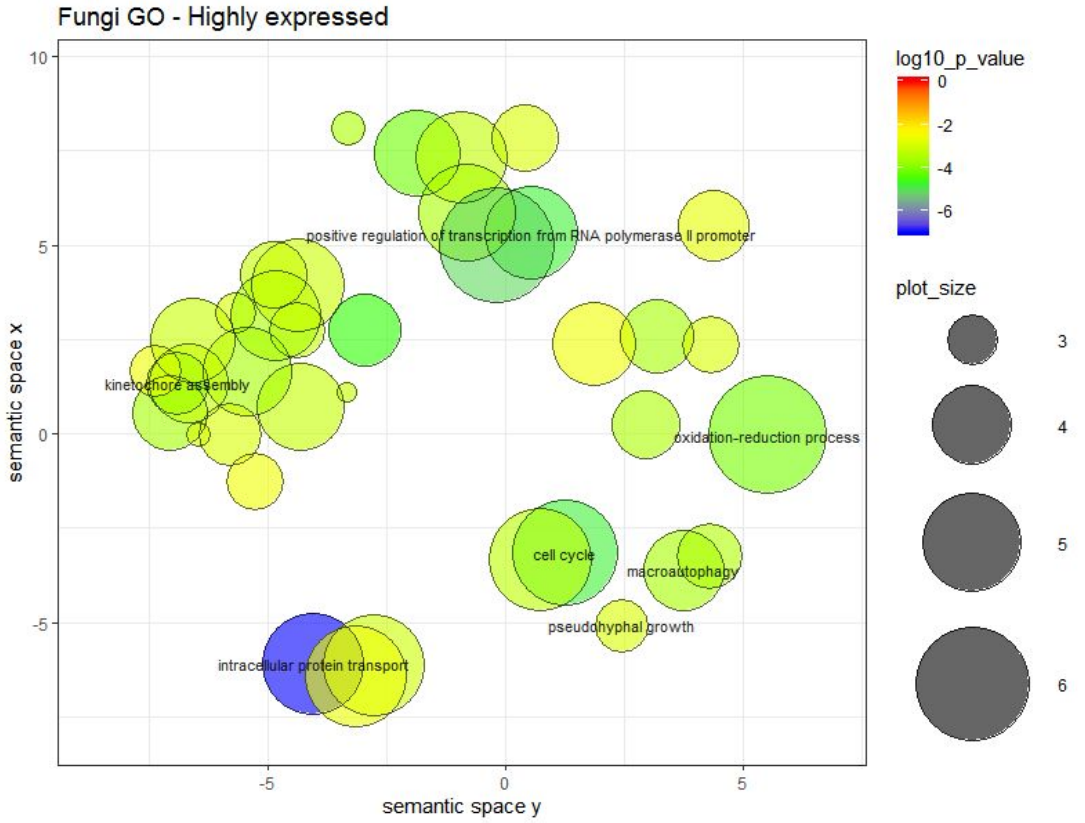

Figure 19. Bubble chart showing the overlap between the GO terms enriched by genes with high expression in endophytic fungi within the roots of *D. edule*. Bubble size indicates frequency of the term in the Gene Ontology Annotation database; bigger bubbles indicate more general terms. Bubble color indicates the p-value of each GO term according to the color scale (top right).

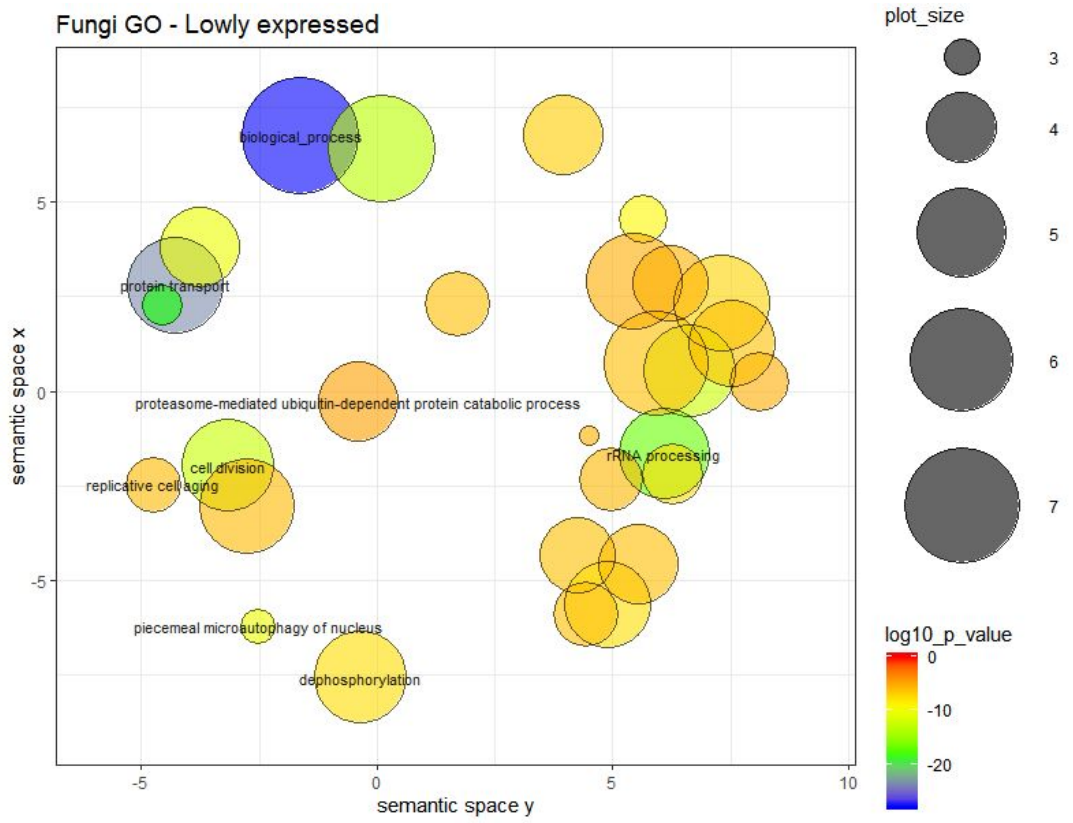

Figure 20. Bubble chart showing the overlap between the GO terms enriched by genes with low expression in endophytic fungi within the roots of *D. edule*.Bubble size indicates frequency of the term in the Gene Ontology Annotation database; bigger bubbles indicate more general terms. Bubble color indicates the p-value of each GO term according to the color scale (bottom right).

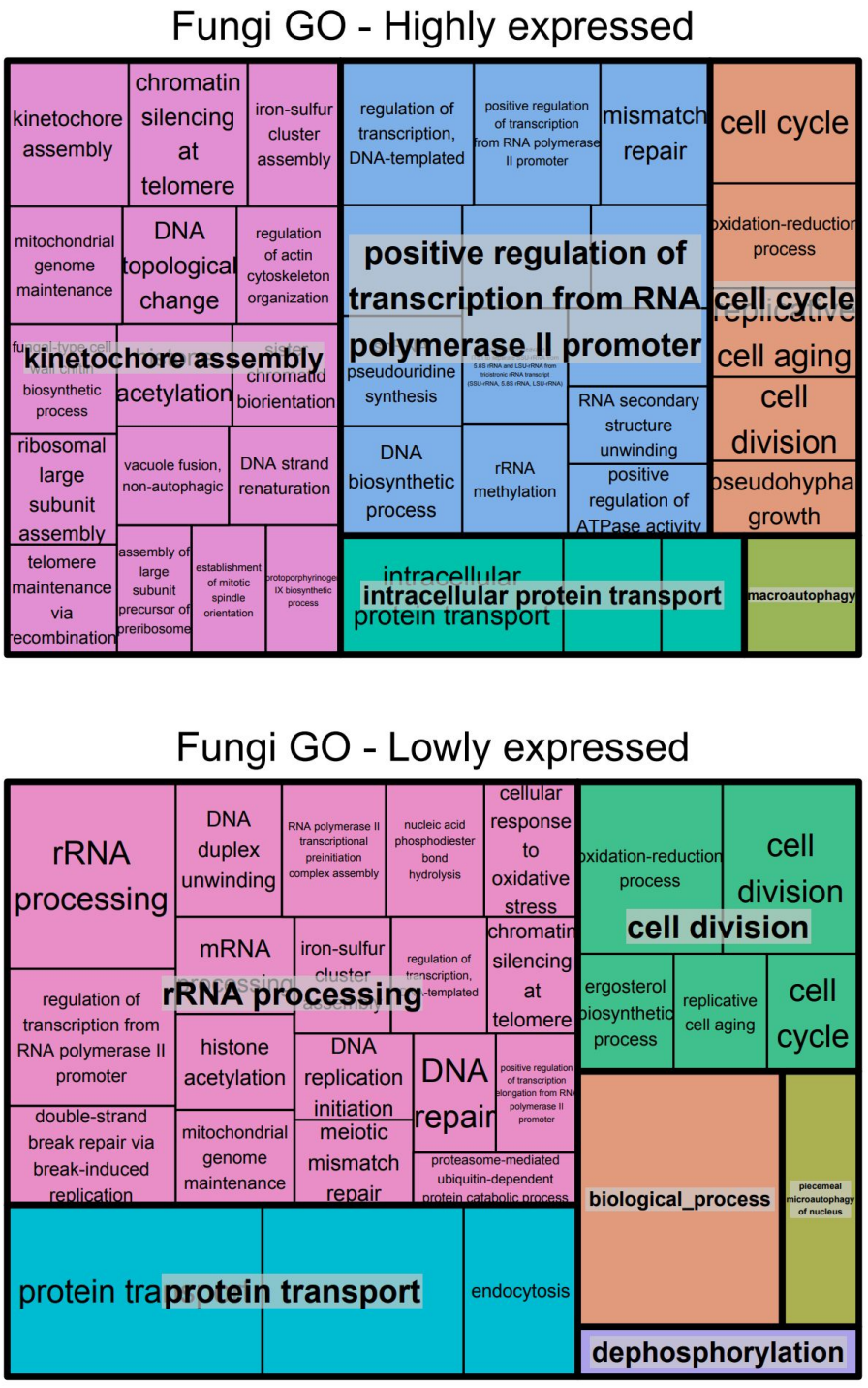

Figure 21. Biological processes associated with the top enriched GO terms associated with highly (above) and lowly (below) expressed genes in fungi present in *D. edule*'s roots. Large, uniformly colored rectangles represent superclusters of related terms, which are represented by smaller rectangles of the same color. Rectangle size is given by the p-value of each GO term.

5.4.2. Fungal growth and reproduction inside the coralloid root

Among the most expressed fungal genes in the cycad's roots (Figure 22), many processes were directly related to growth through cell division and hyphal morphogenesis, as observed through the enrichment of processes such as fungal-type cell wall chitin biosynthetic process (GO:0034221), pseudohyphal growth (GO:0007124), which contain proteins such as BCH1 and GFA1, which participate in the transport of chitin synthase III and the chitin biosynthesis pathway, respectively (Lagorce *et al*., 2002; Sanchatjate and Schekman, 2006). This is supported by enrichment of processes such as mismatch repair (GO:0006298), DNA strand renaturation (GO:0000733), establishment of mitotic spindle orientation (GO:0000132), mitotic spindle orientation checkpoint (GO:0031578) and mitotic spindle assembly checkpoint (GO:0007094), as well as ceramide biosynthetic process (Hastings, 1992, Li *et al*., 2006; Lu and Johnston, 2013).

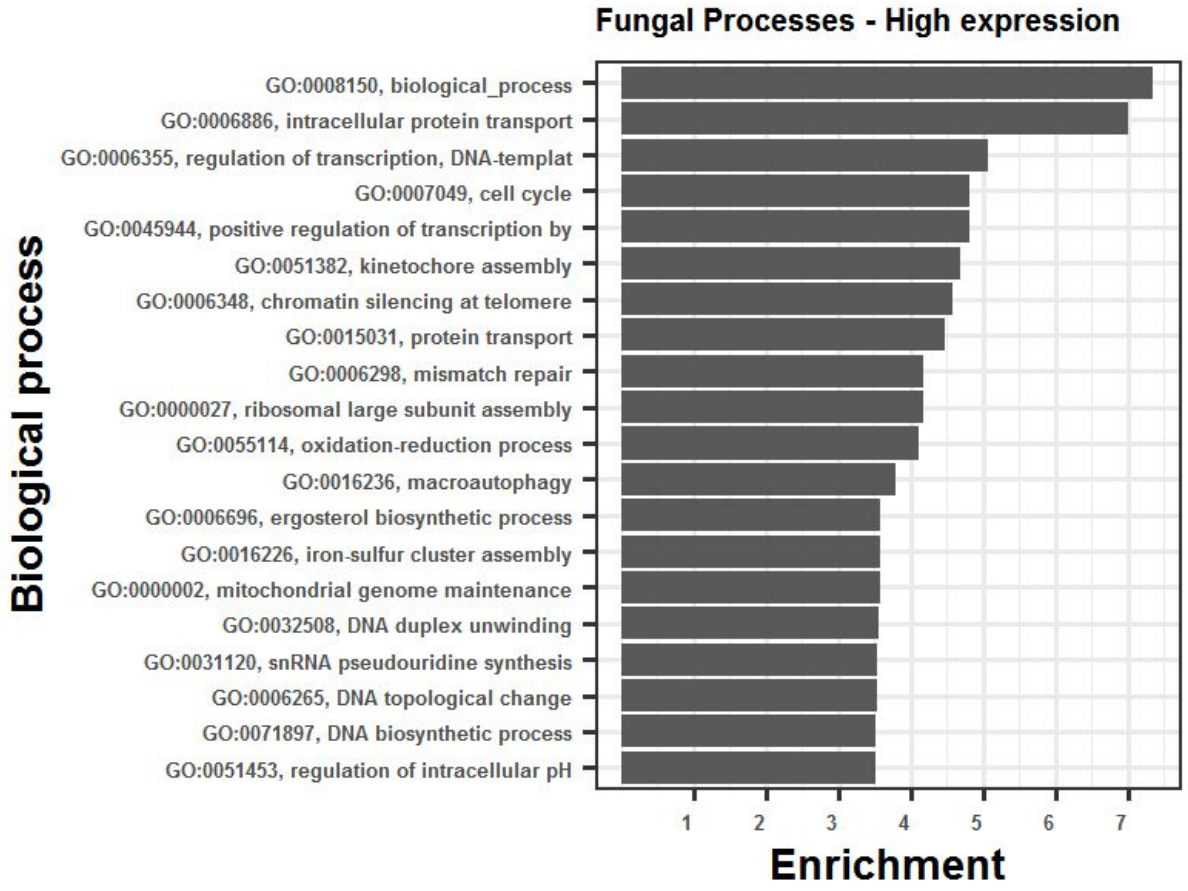

Figure 22. Enrichment of the top GO terms corresponding to different biological processes expressed in great amounts in endophytic fungi within *D. edule*'s coralloid roots. Enrichment is given by Fisher F value (scale denotes negative exponential).

We also found evidence of contradictory expression (explained above) of processes related to the growth of fungi such as fungal-type cell wall organization (GO:0031505) and ergosterol biosynthetic process (GO:0006696); ergosterol is a lipid that helps conform the fungi membrane (Rodrigues, 2018). GO term mRNA splicing (GO:0000398) also varied a lot in expression in the roots of *D. edule*. Along with similarly expressed intracellular protein transport (GO:0006886), these GO terms include upregulated helicases DED1 and DBP5, as well as ubiquitin-ligases and proteins required for correct actin polymerization and efficient mRNA export from the nucleus to the cytoplasm (Abe and Iida, 2003; Gjetting *et al*., 2007; Hilliker *et al*., 2011; Kaminska *et al*., 2011). The relevance of these processes to the fungi contained in the cycads roots is currently unknown, but there is evidence that ubiquitin-ligase affects the integrity of the actin cytoskeleton, potentially being involved in the development of structures different fungal structures (Kaminska *et al*., 2011; Takeshita *et al*., 2014).

Other processes that showed varying expression in the roots include kinetochore assembly (GO:0051382), regulation of actin cytoskeleton organization (GO:0032956), reciprocal meiotic recombination (GO:0007131), mitotic sister chromatid segregation (GO:0000070), sister chromatid biorientation (GO:0031134), covalent chromatin modification (GO:0016569), cell cycle (GO:0007049) and cell division (GO:0051301), which are involved in cell fusion, regulation of the cell cycle, sporulation, as well as terms involved in protein transport such as protein transport (GO:0015031) and intracellular protein transport (GO:0006886); this is done mainly through the expression of proteins GPA1, SPN1 and MLP1 (An *et al*., 2004; Osmani *et al*., 2006; Li *et al*., 2007). Protein GPA1 (expressed in secondary and coralloid roots), is involved with pheromone reception and the mating pathway (Fujimura, 1989; Li *et al*., 2007; Seike, 2019). We found this very interesting given the evidence of internuclear recombination and sexual reproduction in arbuscular mycorrhizae forming fungi, previously considered to be “ancient asexuals”, clonal organisms with no way of purging deleterious mutations (Corradi and Brachmann, 2017; Chen *et al*., 2018c). Evidence of genes in the cycads roots being related to fungal reproduction is in agreement with evidence gathered through microscope observations in this same study regarding the noticeable presence of sporangia in the coralloid and secondary roots.

We also found highly variable expression in processes such as protein transport (GO:0015031), intracellular protein transport (GO:0006886), vesicle-mediated transport (GO:0016192), ER to Golgi vesicle-mediated transport (GO:0006888), regulation of transcription (GO:0006355) and positive regulation of transcription by RNA polymerase II (GO:0045944) as well. Proteins representing these terms included SEC13, SEC24, GLE2, YOP1, NUP85, and MLP1; these proteins are involved in vesicle traffic, and show functions that include forming the

coat complex protein II (COPII), which deforms the endoplasmic reticulum membrane in order to form vesicles and select cargo to be transported to the Golgi apparatus, as well as forming the nuclear pore complex and associating with it (Murphy *et al*,., 1996; Brands and Ho, 2002; Lutzmann *et al*., 2002; Osmani *et al*., 2006 Mancías and Goldberg, 2008; Nickel and Rabouille, 2008; Schwartz, 2016). Genes with functions related to vesicle fusion with the endoplasmic reticulum (the main protein-synthesizing organelle) and Golgi apparatus, as well as vesicle transport along actin filaments, have a role in stress response during symbiosis, and it is of note that vacuoles and vesicles are also involved with iron metabolism, mentioned above as being realized by endophytic fungi (Travers *et al*., 2000; Winkelmann, 2007; Krishnan and Askew, 2014). Genes GLE2 and RSP5 in particular (GO:0006364; rRNA processing), both expressed in varying amounts in our study, are involved in growth during stress conditions (Kaminska *et al*., 2011). Cell cycle related processes (GO:0007049; mentioned above) were also variably expressed in the cycads roots. This GO term contains proteins HAC1 and NPL4, which participate in environmental stress response, a common cause of incorrect protein folding (Travers *et al*., 2000; Bosis *et al*., 2010; Krishnan and Askew, 2014).

5.4.3. Mycorrhiza-like activity in fungi

Among the genes most expressed in the coralloid root we found some that seem to be related to mycorrhizal formation. Enrichment of regulation of intracellular pH (term GO:0051453) was found to be of interest as intracellular pH has been identified as a marker of fungi-root interaction during mycorrhizal symbiosis (Jolicoeur *et al*., 1998). Protein PMA1 (present in the term above mentioned) has been reported as a hydrogen pump in parasitic fungi. This protein has been reported to have a positive effect in growth mediation, particularly in stressful environmental conditions (Bowman *et al*., 1997; Struck *et al*., 1998). Growth of fungi within the host has been observed in previous studies that report fungal structures growing in between the hosts cells (Voisey *et al*., 2016); these observations have been confirmed through microscopy in the present study.

5.4.4. Fungal pathogens in the coralloid root

As was the case in our analysis on cycad differential expression, we found a series of processes that showed very little expression on the coralloid roots of *D. edule* (Figure 23). One of the key biological processes found in our study was autophagy, a process that has a big role in host infection, root colonization and pathogenicity (Pollack *et al*., 2009, Estrada-Navarrete *et al*., 2016; Zhu *et al*., 2018). In this regard, we saw enrichment of autophagy of peroxisome (term GO:0030242),

autophagosome assembly (GO:0000045), piecemeal microautophagy of the nucleus (GO:0034727), late nucleophagy (GO:0044805) and autophagy of mitochondrion (GO:0000422), acting mainly through proteins from the ATG and VPS family (Zhu *et al*., 2018). Several genes belonging to the VPS family (late endosome to vacuole transport via multivesicular body sorting pathway), as well as genes related to regulation of response to osmotic stress, have been correlated with fungal development, conidiogenesis and conidiation, infection of the host and pathogenesis (Chen *et al*., 2014; Li *et al*., 2019). The reduced expression of these proteins seems to imply a reduction in autophagy from endophytic fungi, perhaps due to a reduction of pathogenic activity from fungi within the coralloid root.

On the other hand, we found contradictory expression of genes directly related to pathogenesis. Proteins related to pathogenicity include SEC14 (Golgi to vacuole transport; vesicle-mediated transport; protein transport) and CYT1 (phosphorylation; proton membrane transport); these genes have been observed to be directly related to pathogenicity and virulence in fungi (Chayakulkeeree *et al*., 2011; Sun *et al*., 2016).

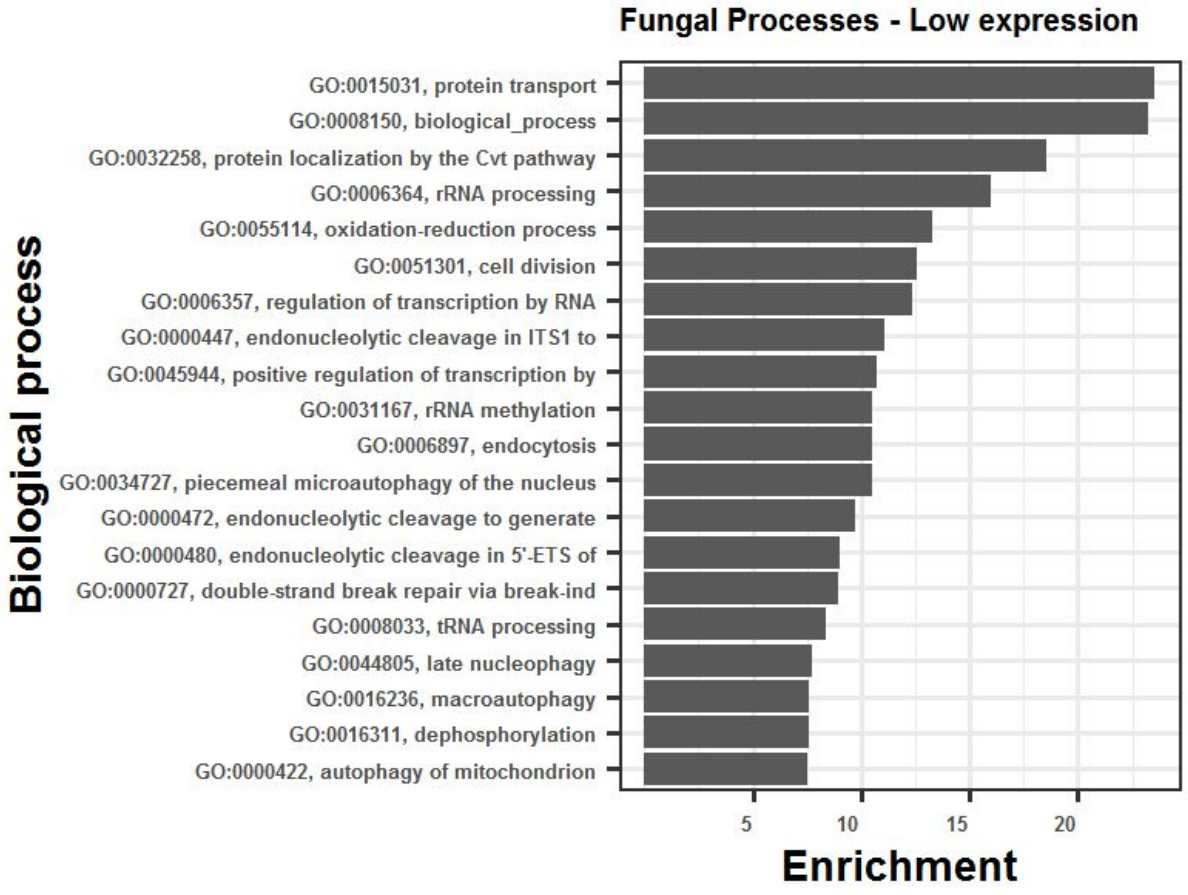

Figure 23. Enrichment of the top GO terms corresponding to biological processes with low expression in endophytic fungi within *D. edule*'s coralloid roots. Enrichment is given by Fisher F value (scale denotes negative exponential).

5.4.5. Fungal iron metabolism

We found enrichment of processes with contradictory expression such as iron-sulfur cluster assembly (term GO:0016226), as well as proteins ISU1 and JAC1 related to positive regulation of ATPase activity (term GO:0032781), both involved with fungal iron regulation (Garland *et al*., 1999; Voisine *et al*., 2001; Gupta and Outten, 2020). As observed during the transcriptomic analysis of the cycad, reception of and response to iron ions is increased in the coralloid root; however, as siderophore production seems to be independent of the host plant structure, the observed cycad response is possibly due to siderophore production by bacteria inhabiting the coralloid root. Further research may shed light on the specific structure and class of the siderophores produced by fungi living within the coralloid roots of different cycad species.

6. Discussion

While the relationship between plants and fungi has been widely studied in diverse plant species, the possible role of fungi present in the coralloid root has remained unexplored. In this study we documented for the first time evidence of fungal structures within the coralloid root, performed a cursory analysis of fungal taxa in this root and through a meta-transcriptommic analysis found evidence of a mycorrhiza-like response by the cycad, as well as fungal activity independent of the colonized root.

Similar to our own findings, the growth of fungal structures in the vascular bundle of a variety of plants has been observed previously. As arbuscular mycorrhizae forming fungi have been reported to affect the morphology of the plant's roots after colonization (Miller *et al*., 1997; Chen *et al*., 2018b), fungi inhabiting the coralloid root of the cycad could potentially have an effect on the development and maturation of this structure. Fungal growth was much more noticeable in the vascular bundle of *D. edule'*s coralloid roots compared to the secondary roots. Presence of fungal hyphae in the periphery of the cyanobacterial zone, as well as between the cells of the inner cortex (figure 1), could indicate a close relationship between fungi and cyanobacteria inhabiting the coralloid roots of the cycad.

Previous reports also indicate the presence of fungal structures in the cycad's parenchyma, related to infection by fungal pathogens and the presence of injuries in the tissue caused by their presence (Bernstein and Carroll, 1977; El-Kader *et al*., 2018). However, no fungal created injuries were observed in the present study, neither in the coralloid nor the secondary root. Since the plant cells that host the cyanobacterial ring are located in the parenchyma, we propose a portion of the endophyte fungi present in the coralloid root could have an endogenous origin, being present in the secondary root before differentiation of the pre-coralloid root, rather than entering during its formation. Presence of fungal structures was much more noticeable in the coralloid root, presenting the possibility that microbial communities formed within the coralloid root are creating a microenvironment that favors the growth, mating and propagation of endophytic fungi, thus limiting the growth of fungi in the secondary root. However, as the root cortex allows the entry of bacteria during the formation of the coralloid root, the differences we observed in fungal structure abundance could also be related to the entry of fungi during this process.

The presence of fungi representing different ecological niches within the cycad's coralloid roots supports the idea that fungi enter this structure without being selected by the plant. However, fungi can still greatly impact the plant's rhizosphere and improve its chances of survival. The presence of saprophytic and pathogenic

fungi has been shown to influence, for example, nutrient and nitrogen mineralization (Cline *et al*., 2018).

The impact of fungal symbionts on the rhizosphere can be observed in greater detail through the differential expression of the cycads secondary and coralloid roots. While some of the enriched GO terms present in the coralloid root are related mainly to the development of this structure, other GO terms show similar functions to those observed on other symbiosis models, such as arbuscular mycorrhizae. This is a potential indication that the symbiosis formed in the coralloid root between the cycad, endophytic fungi and cyanobacteria is initiated and maintained in a similar way to the symbiosis observed in other plants, as well as having a similar effect on the host plant.

The diversity of fungi in the coralloid root and their differing activity can be observed in the differential expression of fungal genes, as it resulted in seemingly contradictory expression of genes belonging to different species. We observed gene expression involved with fungal growth and fungal structure development in the roots of the cycad, as well as conidiation and mating pathway; all of this was confirmed by observations made through microscopy in this study. The fact that fungal structures were present in a greater amount in the coralloid root suggests a heightened viability of fungi present in this structure when compared to the secondary root. This could happen due to different conditions within the coralloid root, such as the establishment of a nutrient-rich microenvironment by the cycad's microbiota, in which fungi are favored, whether as commensals, parasites or mutualistic symbionts.

The fact that the cycad shows expression profiles that reveal similar processes to those of mycorrhiza may be a result of the fungi-cyanobacteria-plant interaction that is specific to the coralloid root, and mainly of the cyanobacteria-cycad part of the symbiotic relationship. Meanwhile, fungi within the coralloid and secondary roots seem to belong to a wide diversity of niches that appear to not be selected for by the plant (unlike the case with bacteria), resulting in little difference in diversity (Zheng *et al*., 2018) and functionality of endophytic fungi between different structures (coralloid and secondary roots). This is supported by the presence of mycorrhiza-like structures and mycorrhiza forming fungi being equally located in coralloid, secondary and primary roots of cycads (Muthukumar and Udaiyan, 2002; Fisher and Vovides, 2004).

Apart from a few pathogen-specific genes, plant defense-related genes showed lower expression in the coralloid root despite the evidence for pathogenic fungi present in this structure. This is likely a result of the symbiosis between the cycad and bacteria and the need to preserve these endosymbionts, as the plants

defense response could negatively impact the survival and activity of nitrogen-fixing cyanobacteria that inhabit the coralloid root.

We simultaneously observed expression of both genes previously reported in pathogenic fungi, involved with the virulence of these, and proteins related to stress response present during mycorrhizal symbiosis, suggesting both a possible mutualistic symbiotic relationship and invasion by pathogenic fungi. Therefore, we propose that the cycads roots (both secondary and coralloid roots) present a direct access to microorganisms present in the environment, and so it allows the entry of diverse fungi without a strict selection, as is the case with bacteria inhabiting the coralloid root, resulting in these structures hosting fungi representative of a wide variety of ecological niches which are showing mutualistic, opportunistic or even pathogenic behavior.

7. Conclusions

While evidence of symbiosis between fungi and host cycad was found, there is no evidence of fungi having structure-specific functions in the roots of *D. edule*. Rather, our findings point towards fungi acting in an opportunistic manner rather than having a close relationship with the cycad or being selected through organism-specific signals.

8. Perspectives

Further work following from this study is necessary to study the role of specific fungi in isolation with the plant. Furthermore, primers have been designed from the sequence of fungal genes BCH1 and GFA1, which were upregulated in our study, in order to identify fungal colonization of plants and research more deeply the colonization process of the coralloid root by fungi.

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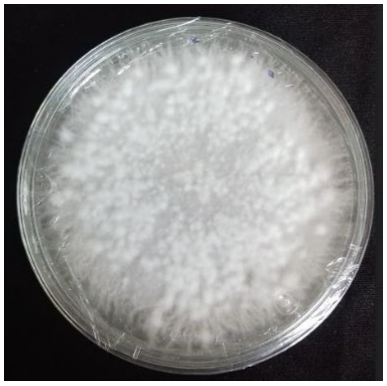
10. Annexes

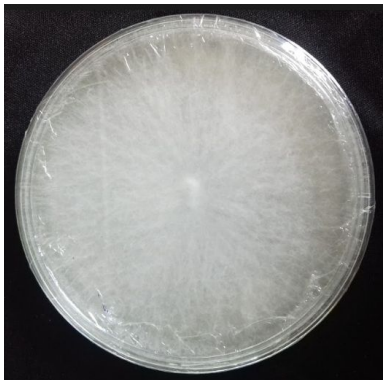
10.1. Annex 1.

Photographs below show the morphology of fungal axenic cultures used for identification.



RC1 D-0 PDAa
Fusarium sp.

RC10 D-1 PDA (SAB)
Penicillium sp.



RC1 D-0 PDAa II
Fusarium sp.

RC5 D-2 PDA
Penicillium sp.

RC1 D-0 SABa II
Fusarium sp.

RC7 D-2 PDA
Penicillium sp.

RC8 D-2 SAB
Penicillium sp.

RC3SAB2
Byssochlamys spectabilis.

RC9 D-2 SAB
Penicillium sp.

RC3PDA10
Penicillium sp.

Cera1RC2CZA3
Aspergillus sp.

RC3PDA3
Penicillium sp.

RC3PDA5
Penicillium sp.

RC3SAB11
Penicillium sp.

RC3PDA7
Cladosporium sp.

RC3SAB3
Penicillium sp.

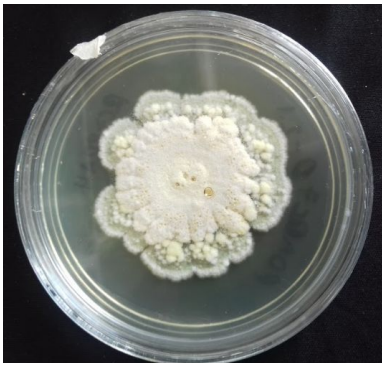
RC3PDA9
Penicillium sp.

RC3SAB4
Cladosporium sp.

RC3SABa2
Cladosporium sp.

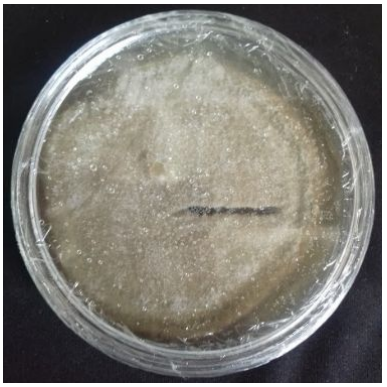
RC6PDA2
Daldinia sp.

RC5SAB2
Cladosporium sp.

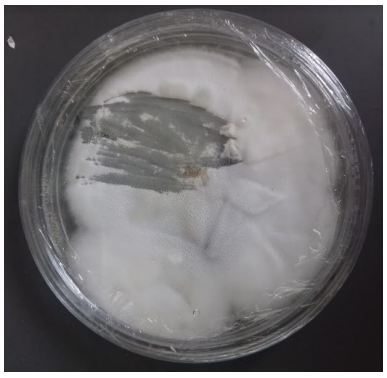


RC7PDA4
Penicillium sp.

RC5PDAa1
Daldinia sp.



RC7PDA6
Alternaria sp.

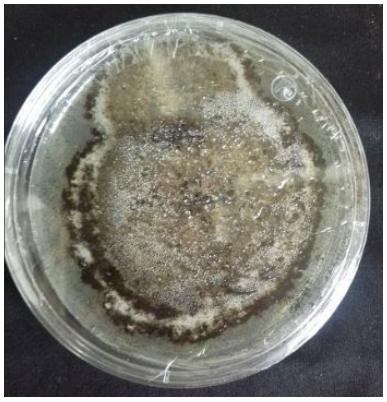


RC7PDAa2
Fusarium oxysporum.

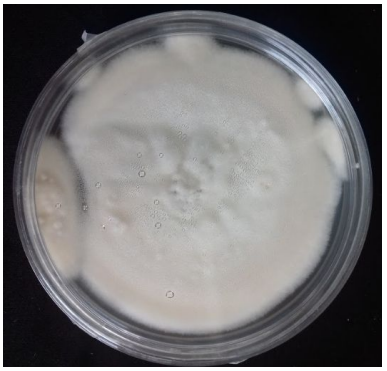
Cmic1RC12SABa1
Ochroconis tshawytschae.

RC9PDA4
Penicillium sp.

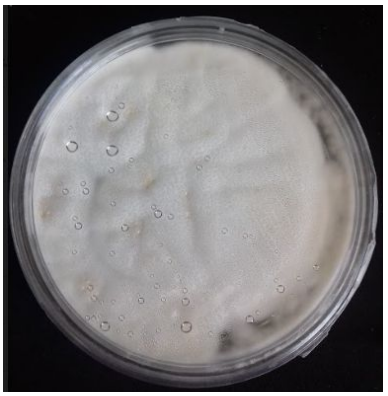
DespRC14SAB4
Penicillium sp.



Cmic1RC10PDA3
Aspergillus niger.



ZfurRC16PDA1
Penicillium sp.



ZfurRC16SAB1
Penicillium sp.

10.2. Annex 2.

Protocol modified from Yockteng et al, 2013.

All reactives and material used was kept refrigerated beforehand and maintained in ice while they were in use.

Samples were washed lightly to remove soil residues and stored in Falcon tubes in liquid nitrogen. Samples were not allowed to unfreeze at any point after collection and until RNA extraction. Tissue was ground under liquid nitrogen in a mortar and pestle that was sterilized. The tissue was ground as finely as possible, and 100 g of the powdered material was placed in a 1.5-mL tube.

1. Add 0.6 mL of cold (4 ° C) Plant RNA Reagent (Life Technologies) to pulverized tissue. Mix by inversion carefully.
2. Place horizontally on rotator and incubate on ice for 5 minutes.
3. Add 15 µl of 2% polyethylene glycol and incubate for an hour at 37° C.
4. Add 100 µl of 5 M NaCl and 400 µl of chloroform–isoamyl alcohol (24 : 1) to the sample. Mix thoroughly by inversion.
5. Centrifuge the sample at 4 ° C for 10 min at 13,000 × g to separate the phases. Transfer the aqueous (top) phase to an RNase-free tube.
6. Add to the sample an equal volume of chloroform–isoamyl alcohol (24 : 1) to the sample. Mix thoroughly by inversion.
7. Centrifuge the sample at 4 ° C for 10 min at 13,000 × g. Transfer the aqueous (top) phase to an RNase-free tube.
8. Add to the aqueous phase an equal volume of a mix of LiCl (4 M) (3/4 v) and isopropyl alcohol (1/4 v). Mix by inversion and let stand at −20 ° C for an hour.
9. Centrifuge the sample at 4 ° C for 20 min at 13,000 × g. Remove supernatant with a pipette, taking care not to lose the pellet.
10. Add 1 mL of 75% ethanol to wash the pellet. Pellet must come loose from the tube during this step.
11. Centrifuge the sample at 4 ° C for 10 min at 13,000 × g. Remove supernatant with a pipette.
12. Repeat steps 10 y 11.
13. Let dry on ice for 15 min at room temperature.
14. Elute pellet in 30 µL of RNase-free water. Pipette the water up and down over the pellet to dissolve the RNA. If the pellet is difficult to dissolve, add more water.

RNA cleaning with Lithium Chloride.

1. Add one equal volume of 4M LiCl to the sample and place on ice for 1 to 2 hours.
2. Centrifuge at 4 ° C for 10 min at 13,000 × g and completely remove supernatant.
3. Resuspend pellet in 100 µL of nuclease-free water.
4. Add 0.1 volumes of 3M NaOAc (10 µL) and 2.5 volumes of 95% EtOH (250 µL).
5. Centrifuge at 4 ° C for 10 min at 13,000 × g and completely remove supernatant.
6. Let dry on ice for 15 min at room temperature.
7. Elute pellet in 20 µL of RNase-free water.