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Cultivable fungi associated with tumors caused by *Hypogeococcus pungens* in *Pilosocereus royenii*: a first report

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Endophytes of columnar cacti have been studied very little and less in association with tumors or galls produced by an insect infestation. This research is the first report of endophytes in *Pilosocereus royenii* infested with *Hypogeococcus pungens*. This parasitic insect is an invasive species in Puerto Rico, usually infesting columnar cacti. Sampling was done with two specimens infested in the Fish and Wildlife Refuge (F & W) Cabo Rojo. A total of 20 fungal morphotypes were isolated, four of which correspond to yeasts. Only four of the morphotypes of mycelial fungi were identifiable though morphological characteristics, which correspond to two species of *Penicillium*, and one species of *Cladosporium* and *Curvularia*, each. Future considerations include morphological and molecular identification of unidentified morphotypes.

Introduction

Columnar cacti are considered keystone species in the ecosystem because a large number of other organisms depend on them for subsistence. For example, insects may function as pollinators, as well asbirds and bats, and the cacti provided food and shelter for these species (4, 5). Many of these relationships have become so specific that some organisms could not exist without the other's presence. For example, *Melocactus intortus* is pollinated by hummingbirds with a much higher success rate than by ants or bees (4).

Puerto Rico has 14 species of native and endemic cacti, with seven species affected by the mealybug, *Hypogeococcus pungens*. The affected species includethe columnar cacti *Pilosocereus royenii*, *Harrisia portoricensis* and *Stenocereus fimbriatus*; the semi-epiphytes *Hylocereus trigonus*, *Leptocereus quadricostatus* and *L. grantianus*, and the globular *Melocactus intortus* (3). *Leptocereus grantianus* is endemic to Culebra island and *L. quadricostatus* to the Big Island, Puerto Rico, and together are considered endangered (6,10), but only *L. grantianus* is protected by the federal government. *Harrisia protoricensis* is endemic to Puerto Rico and the islands of Mona, Monito, Desecheo, and Caja de Muertos and it is also protected by the federal government. Currently, only one population of *Ha. portoricensisis* found on the Big Island, located in the forested areas surrounding the Cabo Rojo lighthouse, attempt to reintroduce the species to the Big Island.

Hypogeococcus pungens is a pest of columnar cacti that has been used successfully as biocontrol in Australia and South Africa to control and eliminate invasive Harrisia cacti in these areas. (7, *Hypogeococcus pungens* was described from material collected in Argentina in 1981. Prior to this, this species was wrongly classified as *Hp. festerianus* and separation as a separate species was justified due to small morphological differences and a different host range as *Hp. festerianus* usually affects succulents (12).

Outside a description of the symptoms associated with *Hp. pungens* infestation in the seven species of cacti affected (3) and its potential threat to dry and arid ecosystems of Central and North America (12), no information on their biology and ecology has been published. So details on their interaction with the host cacti and cacti endophytes are unknown.

Cacti fungal endophytes have not been well studied, with the oldest study published more than a decade ago. To our knowledge, only four articles have been published (1, 2,8, 9). Suryanarayanan et al (9) conducted a sampling in Arizona of 21 species of cacti, including the saguaro, *Echinocereus engelmannii* and *E. fasciculatis*, both considered columnar cacti and *Mammillaria viridiflora*, a globular cactus. They reported that species richness of fungal endophytes consisted of two to seven species. In most cases the community of fungal endophytes was dominated by one or two species. Bezerra et al (1) reported the community of endophytes of *Opuntia ficus-indica*, consisting of 44 species of fungi, being *Cladosporium cladosporioides* and *Cl. spharospermum* the most common species. Bezerra et al (2) describes the endophytic community of *Cereus jamacaru* comprising 59 species, but with more than 30% of cultured mycelia lacking reproductive structures. Finally, Silva-Hughes et al (8) reported that the fungi isolated from plant tissue of *O. humifusa* represented about 17 different species identified by molecular methods.

The aim of this research was to determine the efficiency of different cultivation mediato describe the community of mycelial endophytes in *Pilosocereus royenii* that may be associated with tumors caused by *Hypogeococcus pungens*. It is expected to observe an association between endophytes (with pathogenic potential) as the anatomy of tumors caused by *Hp. pungens* is not characteristic of galls caused by insect infestations (3). This is not only the first report of *P. royenii* endophytes and the first study of endophytes that may be associated with *Hp. pungens* infestation, but also the first attempt to describe the endophytic community of a cactus in Puerto Rico.

Methods

Preparation of culture media: Culture media was prepared from commercial Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) as described by the manufacturer (Difco, Becton, Dickinson and Co.) and also mixed with extracts of fresh cactus (PDAC and MEAC) and cactus extract agar. Sabouraud Dextrose Agar (SDA) and Corn Meal Agar (CMA) were prepared as described by the manufacturer (Oxoid Micobiology, Thermo Scientific) and without cactus extract. Cactus extract was obtained from apical tissue of a branch from a healthy specimen of *Pilosocereus royenii* at the F & W Refuge at Cabo Rojo. The branch was processed no more than three days after its collection, while preserved in a cooler. The branch was surface sterilized with 70% ethanol three times and the thorns were removed. Thorn-free tissue was then homogenized in a surface sterilized blenderwithout adding water. The homogenate was stored in a refrigerator and later use to enrich the commercial media. Ten grams of undiluted cactus extract, was added per 300mL of water added to the commercial media. Two additional media were prepared using only agar with cacti extracts as its only source of nutrients, differing only in the amount of cacti extract added: 5g/300ml (CA5) or 10g/300ml (CA10). All culture media were autoclaved for 15 minutes at 121°C and later distributed uniformly in sterile Petri dishes (100mm x 15 mm).

Inoculation: Three tumors from *P. royenii* infested by *Hp. pungens* were removed from the same branch of two different plants, classified as heavily infested, as described in Carrera-Martínez et al (unpublished). The exterior of tumors and branches were surface sterilized spraying 70% ethanol three times before making the cuts. The cuts were made using a sterile scalpel to the interior of the tumors and then inoculated as a single streak on the culture media three times. For the first *P. royenii*, inoculations from three tumors were made on CMA, SDA, MEA and MEAC, for a total of 12 inoculated plates (four plates, one of each media, per tumor). Cultures from four tumors from the second cacti were made on PDA, PDAC and MEAC, for a total of 12 inoculated plates (three plates, one of each media, per tumor). Growth was observed on the first and second weeks after inoculation. Afterwards each colony was purified on MEAC, PDA, PDAC, CA5 and CA10 to stimulate sporulation. Colonies that appear to be

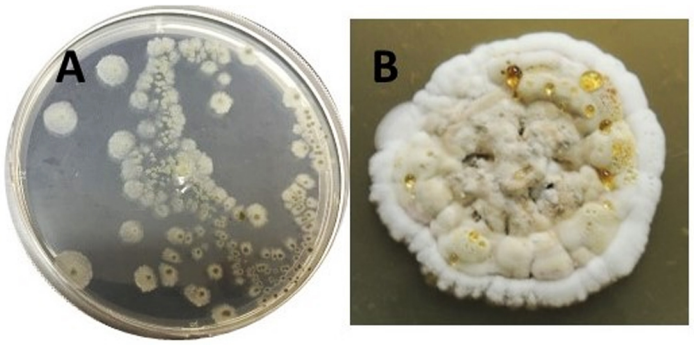

Figure 1. (A) *Penicillium* sp1 isolates in SDA, and (B) detail of exudates in a mature culture of *Penicillium* sp2 growing in PDAC. Both species differ from each other by their coloration, size and the absence of exudates in *Penicillium* sp1

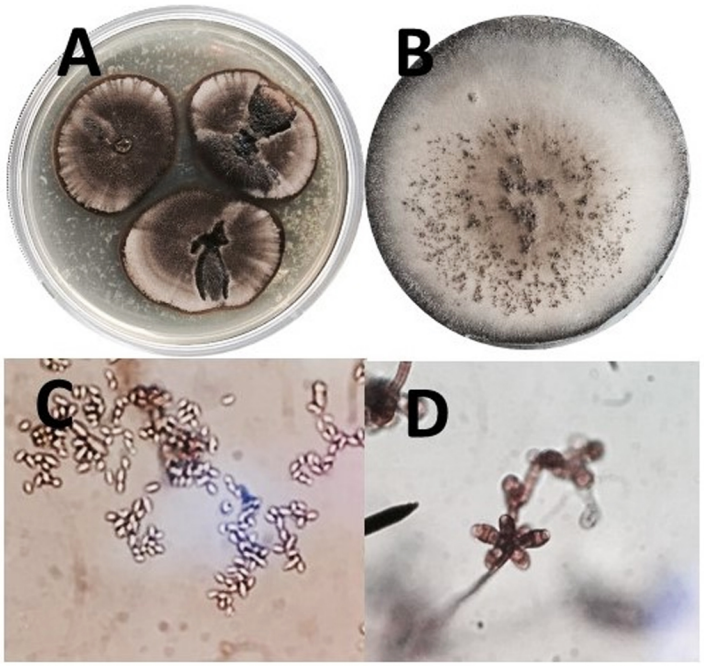

Figure 2. (A, C) *Cladosporiumsp*. (A) colonies and (C) conidia; (B, D) *Culvulariasp*. (B) colony, and (D) multiseptate-conidia growing from mature conidiophore.
Both growing in PDAC

bacteria and/or yeasts were purified on PDA with lactic acid to determine its identity. To avoid additional metabolic stress on the fungi recovered, each colony was transferred to a new Petri dish containing the same media where the colony was initially recovered.

Results

In total, 20 morphotypes were isolated from *P. royenii* tumors, from which four were yeasts and the remaining 16 werefilamentous fungi (Figure 1-3, Table 1). From the first sampling, 14 morphotypes were isolated, four of which correspond to yeasts (Figure 3) and the rest, were filamentous fungi (Table 1). About 9 morphotypes were isolated from the second sample, all representing filamentous fungi (Table 1). Of these, only three fungal morphotypes were shared between samples. A dark green filamentous fungus was observed as a contaminant in some dishes with the media CA5 and CA10. Isolated morphotypes on these mediawere considered as contaminants and discarded.

Only four morphotypes between the two samples were identified to the genus level: wo species of *Penicillum*, one *Curvularia* sp. and one *Cladosporiumsp*. *Penicillium* sp1 and *Cladosporium* sp. belong to the first sample, while the *Curvularia* sp. and *Penicillium* sp2 belong to the second sampling (Figures 1 and 2, Table 1). *Penicillium* sp2, *Culvularia* sp. grow on PDAC, while *Cladosporium* sp. grown MEAC. *Penicillium* sp1 grow on PDA, PDAC, MEA, and MEAC. The other morphotypes did not developed reproductive structures. Overall, growth on CMA, SDA, CA5 and CA10 of all inoculates was poor and no reproductive structures were observed.

Discussion

This study represent the first report of possible endophytic fungi associated to *Pilosocereus royenii*. About 20 isolated morphotypes, from which only two species of *Penicillium*, one species of *Curvularia* and another of *Cladosporium* were identified. Fourmorphotypes were yeasts, with remaining 12 fungal morphotypes. If each morphotype represent a different species, comparable with results reported in *O. humifusa*, with 17 species (8). Still, is less than those reported for *O. ficus-indica*, with 44 species (1) and *Cereus jamacaru*, with 59 species (2), but greater than all those reported for 21 cacti species in Suryanarayanan et al (9), ranging from two to seven isolated fungal species per cacti species.

The fungi identified, *Penicillium* spp., *Cladosporium* sp. and *Curvularia* sp. have been previously reported from cacti (1, 2,8, 9). *Cladosporium cladosporioides* and *Cl. sphaerospermum* were the most common species isolated from *O. ficus-indica*, with a frequency of 20% and 16%, respectively (1). These species have also been isolated from *Ce. jamacaru*, with a frequency of 18.5% and 0.71%, respectively (2), while *Cl. cladosporioides* and *Cl. bruhnei* were isolated from *O. humifusa* (8). Suryanaray-

Table 1. Fungal genera and morphotypes by sample

	Morphotype or Genera	Quantityisolated
Sample 1	Not Reproducing Mycelia	8
	Penicillum sp1	1
	Cladosporium	1
	Yeast	4
	Total	14
Sample 2	Not Reproducing Mycelia	7
	Penicillum sp2	1
	Curvularia	1
	Yeast	0
	Total	9

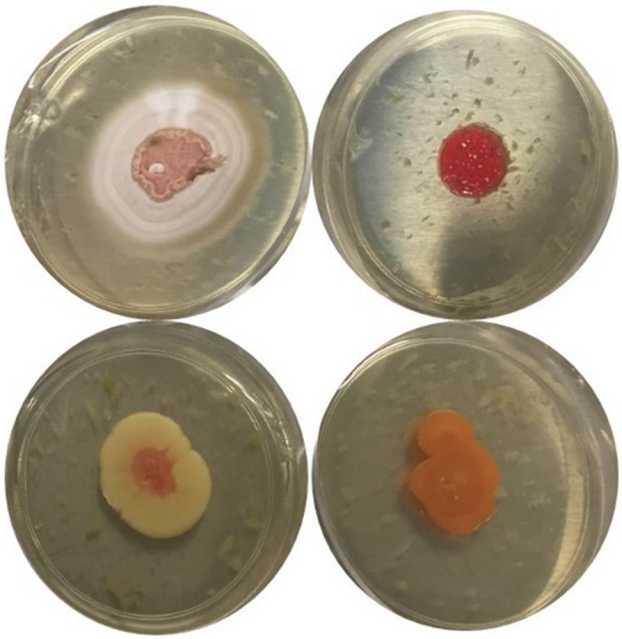

Figure 3. Yeast morphotypes isolated from the first survey, all growing in PDAC media

anan et al (9) reported *Cladosporium* sp. in tissues from *Cylindropuntia* sp., *Cy. californica*, *Cy. versicolor* and *Opuntia* sp. Two species of *Penicillium* have been isolated from *O. ficus-indica* (1), while nine species were isolated from *Ce. jamacaru* (2). Similarly, *Curvularia* has been isolated from *Ce. jamacaru* with two representatives: *Cu. brachyspora* and *Cu. senegalensis*, both isolated with a frequency of 0.17% and, therefore, considered to be rare (2).

Twelve fungal morphotypes (68%) were not identified, as they lack reproductive structures. These morphotypes differ from each other morphologically in color, shape, texture, margin and growing time in the same media. Bezerra et al (2) reported that about 30% of their isolates lack reproductive structures. They explain that these fungi may not obtain the necessary nutrients to sporulate outside the plant tissue. This may explain our results, even after the addition of cacti extract. In addition, many of these studies inoculated a piece of the plant to isolate the endophytes while in our study, inoculation was performed using a scalpel instead of tissue, which may have influenced our results. However, a higher number of isolates were observed in PDAC than on any other media. The efficiency of the PDAC may be due to enrichment of the PDA, with nutrients present in the cacti extract, essential for the fungi to grow and reproduce. These nutrients may include trace elements only present in the soil of that particular location where the cacti and their fungal endophytes were growing. Future studies should use this cultivation media to increase the frequency of isolates.

Several authors report that cacti endophytic communities dominated by a single species tend to have lower species richness in general (8, 9). In contrast, in ecosystems where there is a high diversity and richness, a dominant species is usually not observed (1, 2). Because it does not appear to be a definite dominance in our study, it is possible that it is a system with high diversity and richness of fungi, although no conclusions can be drawn because of a very low sample size (only two individuals).

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