MEDICINAL PLANT *HELICHRYSUM STOECHAS* AND ENDOPHYTIC FUNGI – HINTS OF ECOLOGY

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Abstract: The genus *Helichrysum* is a valuable source of essential oils with medicinal properties. Eighty endophytic fungi have been isolated from 8 *Helichrysum stoechas* plants belonging to two different biotypes of Catalonia. Based on their morphology, 10 different genera have been identified while other have been assigned to 21 OTUs. The colonization rate was similar in both locations (71.53% and 67.36%) and in the entire plants (60-75%). In plant organs values ranged between 50 and 91.67. The PDA was the nutrient medium which provided the highest number of different colonies considering all three media used (PDA, MEA and LCA). The colonization frequency index showed a tendency of the genera and OTUs to specificity of a single organ (21 OTUs and genera were obtained from a single organ, 8 from two organs and only 1 from all the organs used), and nutrient medium (18 OTUs and genera were isolated in a single nutrient medium, 9 in two nutrient media and only 3 in all media used). The most frequent genera / OTUs were *Alternaria* and OTU1.

Key words: *aromatic plant, microbiome, ecology*

INTRODUCTION

Traditionally the term "endophyte" has been used for all microorganisms (mostly fungi and bacteria) that inhabit plants, but more recently mycologists have begun to use the term "endophytic fungi" to refer to microorganisms that reside asymptomatically, without causing any visible symptoms of disease, in the inter- and intra-cellular tissues of healthy plants (Schulz & Boyle, 2002; Stone et al., 2000; Strobel & Daisy, 2003). The term only refers to fungi at the time of detection, regardless of the future state of the interaction (Schulz & Boyle, 2005).

Endophytic fungi represent an important component of fungal biodiversity, knowing that they are involved in the diversity and structure of the plant community. Some species appear to be ubiquitous, being found in hosts belonging to different families and in various environmental conditions (Sanchez Marquez et al., 2010).

There is a wide variety of symbiotic relationships between endophytes and their host plants, ranging from mutualism to antagonism or slight pathogenesis. The transmission of these endophytes can be horizontal, when the inoculum comes in contact with a plant and infects it, or vertical, when the already infected plant produces seeds that carry the fungus (Schulz & Boyle, 2005). Several fungal species appear to be specific, having preference for organs of a certain host community (Khan et al., 2010; Wang et al., 2010).

The *Helichrysum* genus with more than 600 species is widespread throughout the world. In the Mediterranean area it is represented by approximately 25 native species (Leonardi, 2013). It is a natural source of essential oils with bioactive compounds, having potential

pharmacological applications (Giuliani et al., 2016). The species *H. stoechas* (flor de San Juan, chamomile or sempreviva borda) is an aromatic bush of circum-Mediterranean distribution, being relatively common in Catalonia. It inhabits stony and preferably open lands with good sunshine. It extends from coastal areas to low mountains of Mediterranean influence, generally below 1000 m (Masalles et al., 1988).

The present study evaluates the endophytic fungal community of *Helichrysum stoechas* from eight plants collected in two locations.

MATERIALS AND METHODS

Isolation of fungal strains. Eight plants were collected from two areas of Catalonia: Sant Feliu de Codines and El Garraf (Table 1).

Table 1. Plant collection locations

Location	Latitude	Longitude	Altitude	Plants (no.)
Sant Feliu de Codines	41º41'40.90" N	2º09'41.24" E	500 m	1 to 4
Garraf	41º14'59.03" N	1º53'30.11" E	76 m	5 to 8

The selected plants showed no symptoms of disease, were cut and transported to the laboratory in double zip closure plastic bags. Fragments were cut from the selected areas (stem and leaf) and superficially sterilized by immersing them in sterile water, one minute in 70% ethanol, one minute in bleach (15% sodium hypochlorite), another minute in 70% ethanol and once again in distilled water (Cosoveanu et al., 2016). Control of the surface sterilization was maintained by rolling the sterile fragments in PDA nutrient medium and pipetting water from the last step of the sterilization protocol.

The fragments were dried on sterile filter paper. In the case of the root and stem they were cut to obtain 1-2 cm sub-fragments, which were subsequently cut lengthwise. For leaves, the central part was cut discarding the apical and basal part. Once the final fragments were obtained, four of each organ were placed in a Petri dish. Three culture media were used: MEA (Malt Extract Agar) rich in carbon, proteins and essential nutrients for fungi and sugar; PDA (Potato Dextrose Agar) synthetic and rich in sugar; and LCA (Lignocellulose Agar) modified, replacing KCl with NaCl, which was used due to its low glucose content which suppresses the proliferation of fast growing species (Osono et al., 2009). The fragments were placed on the Petri plate so the internal part is in direct contact with the medium to favour the growth of the fungal mycelium. Streptomycin sulphate has been added to the media to prevent bacterial growth.

When fungal growth was detected in the plates, the endophytes were replicated to a new Petri dish with PDA culture medium to obtain pure isolates. All steps were performed under laminar flow chamber.

Microscopic identification. Preparations were made to observe their morphological characteristics (mycelium, spores, conidiophores and sexual structures). The fungal biomass was placed on a slide with a drop of lactophenol mixed with cotton-blue (10% vol). A technique used to promote sporulation was to inoculate a fragment of PDA medium with fungal mycelium on a fragment of sterile carrot and / or spinach placed on a Petri dish with agarized water medium (1.2% m/v).

Colonization rate and colonization frequency. The colonization rate (CR) indicates the percentage of fragments that have been colonized from the total number of sampled

fragments. This analysis is applied to plant, different organs and isolation media. CR = Total no. of stem fragments in a sample (plant) yielding at least 1 isolate / Total no. of stem fragments in that sample.

The frequency of colonization (CF) offers a perspective of the abundance of each genus/OTU in each plant, organ, medium and in the set of plants. CF% = [Total no. of fragments in a sample (plant/organ/medium) colonized by a species / Total no. of fragments plated] x 100.

RESULTS AND DISCUSSIONS

Microscopic characteristics did not allow identification of all fungi; therefore, the unknown strains were assigned to morphological groups called OTUs.

In all plants (P1 to P8) the colonization rate ranged between 63-73% (Figure 1). The colonization rate values showed important differences in the colonization of the fragments when all three nutrient media were compared, observing the highest value in PDA (with a maximum value of CR% = 100 and a minimum of CR% = 83.33), followed by MEA (maximum value CR% = 91.67 and minimum CR% = 66.67) and the LCA with the lowest values (maximum value CR% = 50 and minimum CR% = 16.67). Regarding the values obtained when using different organs, the differences are smaller, which suggests a similar colonization in leaves (maximum CR% = 91.67 and minimum CR% = 58.33), stem (maximum CR% = 75 and minimum CR% = 50) and roots (maximum CR% = 83.33 and minimum CR% = 58.33). Both locations provided similar values (minimum CR% = 63.89 and maximum CR% = 72.22).

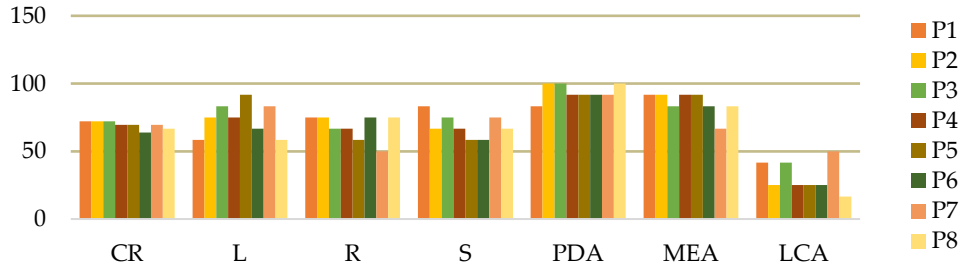

Figure 1. Colonization rate per plant (CR), organ (L - Leaf, R - Root, S - Stem) and medium (PDA, MEA, LCA); P1 – plant no. 1

The only genus colonizing all plants is *Alternaria*, followed by OTU1 (6 plants) and *Diaporthe* (5 plants). If colonization frequency data is segregated into plants we observe that *Alternaria alternate* is the major colonizer in four out of eight plants, namely P1, P3, P5 and P6 (Figure 2). Followed by OTU 18, OTU 5 and OTU 1 as major colonizers in one plant, namely P4, P7 and P8, respectively. In plant no. 2 both OTU 15 and *Ulocladium* registered same colonization rate, same as in plant no. 3 with *Alternaria* and OTU 2. Some morphotypes were not found both two locations - in Sant Feliu de Codines OTUs 7, 8, 9, 13, 14, 16 and 17 were not found, while Garraf lacked in *Botritys*, *Fusarium*, *Penicillium*, *Trichoderma* and OTUs 6, 10, 11, 12, 15, 18, 21.

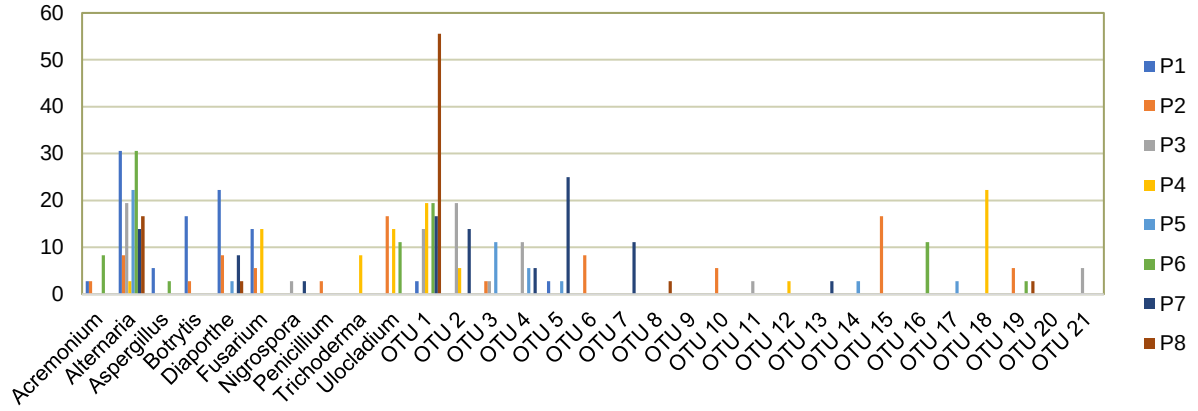

Figure 2. Colonization frequency (CF) of each genus/OTU per plant; P1 – plant no.1

Regarding the colonization frequency index, when analysing the data of each plant, values showed a tendency of the genera and OTUs to specificity for organ and nutrient medium. Considering the data set, we observe that the specificity is maintained by 18 OTUs and genera isolated in a single nutrient medium, 9 in two nutrient media and only 3 in all media used. This suggests the necessity to broad both culture media (Cosoveanu et al., 2018) and plant organs to obtain a higher richness of endophytic fungal taxa.

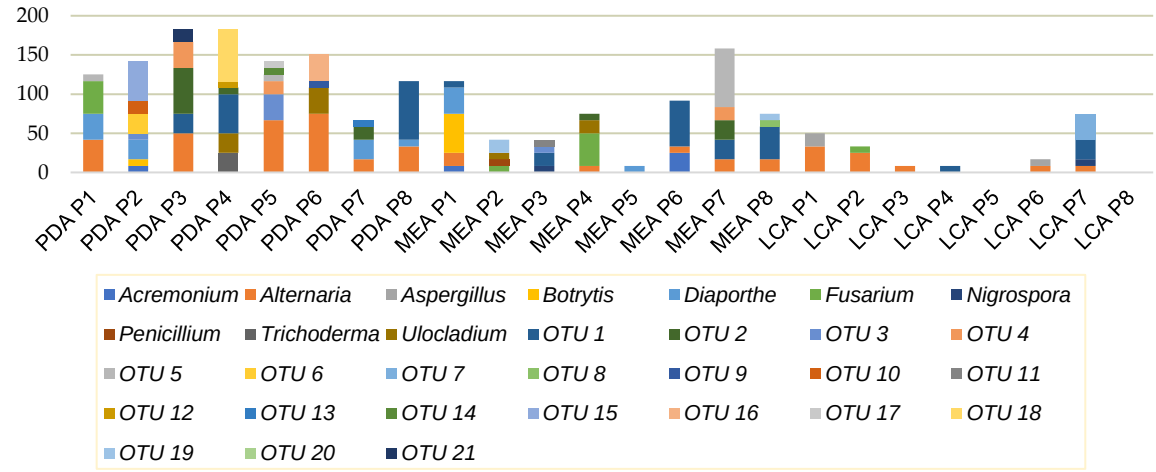

Figure 3. Colonization frequency of each genus/OTU per medium (PDA, MEA, LCA); P1 - plant no.1

As for the plant organs, 21 OTUs and genera were obtained from a single organ, 8 from two organs and only 1 from all the organs used (Figure 4). Myrchiang et al. (2014) investigated the endophytic fungi associated with *Artemisia nilagirica* and comparing the colonization of three organs (i.e., root, stem and leaf), the authors obtained the highest diversity in roots (i.e., 14 species), less in stem (i.e., 10 species) and the smallest number in the leaves (i.e., 6 species). Similarly, in *Artemisia thuscula*, Cosoveanu et al. (2012) isolated 29 distinct morphotypes: 20 from roots, 7 from stem and 2 from leaves. In addition, Myrchiang et al., 2014 observed that from all fungal endophytic species, only *Phoma eupyrena* was found to be a common occurrence in all plants sample, the other species having a certain preference for one or maximum two organs. Interestingly, in *Artemisia* as host, a tendency on host specificity of most endophytic fungal species was observed (Cosoveanu et

al., 2018). In the present study were isolated 31 morphotypes, with the stem the most colonized organ (i.e., 17 species), followed by the leaves (i.e., 14 species) and the roots (i.e., 9 species).

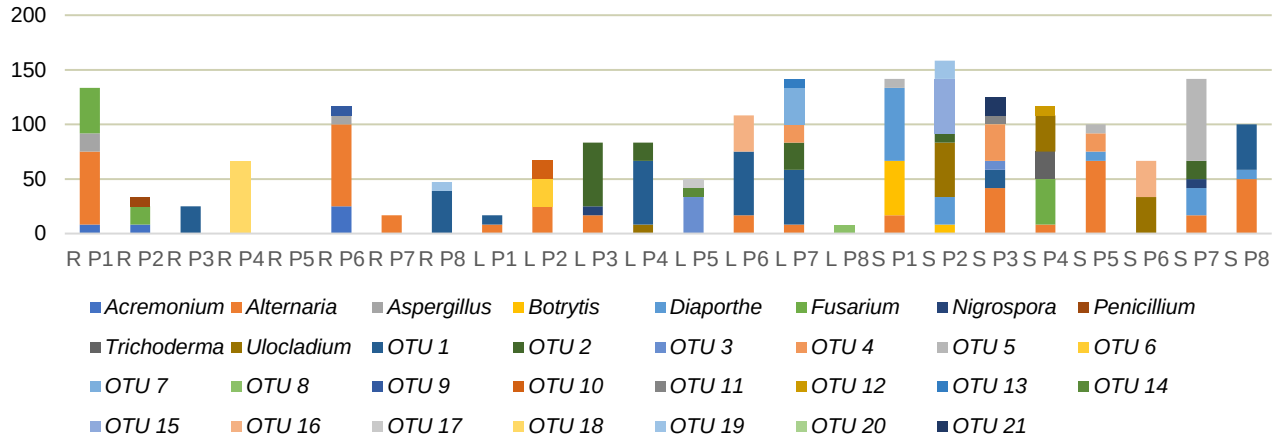

Figure 4. Colonization frequency of each genus/OUT per organ (RP1 - root plant no.1; LP1 - leaves plant no. 1; SP1 - stem plant no. 1)

CONCLUSION

The endophytic fungi isolated from *Helichrysum stoechas* showed tendency for plant organs specificity and preference for single nutrient medium. Molecular identification is required to reveal which fungal species are propitious to narrow environment.

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