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ASSESSMENT OF THE ENDOPHYTIC FUNGAL COMMUNITY OF THE SPECIES *CICHORIUM INTYBUS* AND *XANTHIUM STRUMARIUM*

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Abstract. Hydrocarbon polluted agricultural areas contain spontaneous plants possibly involved in the soil bioremediation process. This study highlights the diversity of the endophyte microfungal community of *Cichorium intybus* and *Xanthium strumarium*, the dominant plant species in a hydrocarbon- contaminated area in the Prahova region, in September 2020. Plants release organic compounds through roots, which increase the density, diversity and activity of specific microorganisms that in turn degrade hydrocarbons. For the isolation of endophyte fungi, fragments of roots were used, the plant organ being sectioned in the basal, median and apical area. The nutrient medium (PDA) was fined with diesel and motor oil to 1 mg/l. There were obtained 92 endophyte fungal strains, which following the observation procedures of morphological similarity, namely elements of shape, texture and color of the colonies, as well as mycelium and spores under the microscope, 39 different morphologically different strains resulted. To partially characterize the ecological aspects of the endophyte fungal communities in the two plants, from two locations contaminated with hydrocarbons, considering three root areas, Margalef diversity indices, Shannon, Simpson's dominance and frequency of colonization were used. The diversity expressed by the Shannon index indicated a superior net value for the endophyte fungal communities of *C. intybus* (Shannon_H= 3.0) compared to *X. strumarium* (Shannon_H= 2.0). The same host plant, *C. intybus*, was also noted for the richness of the species, the value of the Margalef index being 6.6, compared to *X. strumarium*, where the index had a value of 2.9. The dominance of endophyte fungal communities showed similar values in both plants. These preliminary results point to a higher diversity of endophyte fungal communities in the host species *Cichorium intybus*, colonized by the highest number of different organizational taxonomic units (OTUs) compared to the other host plant *Xanthium strumarium* (i.e 28 versus 11).

Keywords: *endophytic fungi diversity, bioremediation, spontaneous flora*

INTRODUCTION

In recent years, the degree of soil pollution with hydrocarbons has increased significantly, and this caused the occurrence of negative phenomena in terms of plant growth and development. The soil polluted with hydrocarbons forms on its surface a waterproof, compact film that prevents water circulation, gas exchange, reduction processes are installed, and the roots are suffocated. Furthermore, they have a negative biological impact due to their carcinogenic and mutagenic effects (Marin et al., 2018). Thus, pollution has a major impact in all biochemical and chemical processes of plants, their resistance to pathogens and pests being diminished (Gavrilescu, 2007).

Endophytic fungi are microorganisms capable of inhabiting a plant tissue (inter- or intracellular, localized or systemic) and describe a transitory status (see review on definitions Cosoveanu & Cabrera, 2019). Fungal endophytes help plants to tolerate unfavorable environmental conditions such as drought (Swarthout et al., 2009), high temperatures and salts (Malinowski & Belesky, 2000), unsuitable soil conditions (Malinowski et al., 2004) and also promote plant growth.

In Romania, like in other countries where petroleum is extracted, the areas nearby refineries are contaminated with polyhydroxyalkanoates (PAHs). Thus, these areas are not used for agriculture. An option to remediate these soils is phytoremediation. Phytoremediation is one way to treat these soils. Using plants and their microbial colonizers, phytoremediation can effectively clean up contaminated soils at a little cost (Pilon-Smits, 2005). The microbial extracellurar enzymes released by endophytic fungi from plants could digest some of the toxic substances that are present in the soil, and therefore improve the phytoremediation process. Endophytic bacteria have a great potential for improving phytoremediation effectiveness, according to recent studies (Lumactud et al., 2016; Marchut Mikolajczyk et al., 2018) as well as endophytic fungi (Dhayanithy et al., 2019).

Cichorium intybus is belonging to the family *Asteraceae* and has been introduced within agriculture for its ability to produce high quality forage for sheep or dairy production (Iqbal et al., 2021). It is known that all parts of this plant species synthesize important secondary metabolites. Also chicory is considered a biomonitor of heavy metals such as Pb, Zn, Cu, and Cd (Sahan et al., 2017; Aksoy, 2008; Wilson et al., 2004; Dalar & Konczak, 2014; Mares et al., 2005; Abbas et al., 2015; El-Sayed et al., 2015; Mulabagal et al., 2009).

Xanthium strumarium, is also belonging to the family *Asteraceae*, and is one of the emerging invasive plant species. However, chinese pharmacopeia has documented 60 different compounds from the fruit of this plant species that are used for treating various diseases, including rhinitis, nasal sinusitis, headache, gastric ulcer, urticarial, rheumatism, bacterial and fungal infections, and arthritis (Kamboj & Saluja, 2010). Many studies reveal that heavy metal concentration and *X. strumarium*'s phytoremediation of it are significantly influenced by the soil's textural characteristics and plant biomass (Ullah et al., 2021).

Here we present preliminary data on the diversity of the endophytic microfungal community from the roots of two spontaneous dominant plants present on hydrocarbons contaminated soil, *Cichorium intybus* and *Xanthium strumarium*.

MATERIALS AND METHODS

Field sampling. Two individual plants of *Cichorium intybus* were collected from a distance of about 3 meters from the oil extraction points, in Baicoi, Prahova county, Romania (N 45°02’543"; E 25°49’859"). The second location chosen was in the immediate vicinity of Tufeni Forest where numerous mounds with drilled substrate were observed (N 45°02’938"; E 25°48’971"). Two individual plants of *Xanthium strumarium* were collected directly next to the mounds with freshly drilled substrate. Samples were collected in zip-lock bags and stored at low temperature (4°C) until processed (within 48 hours).

Isolation of fungal strains. For endophytic fungal isolation, only roots were analyzed. The roots were divided in base, medium and apex. Root fragments (1 cm long) were surface sterilized by immersion for 1 minute each in: sterile deionized water, 15% sodium hypochlorite, 70% ethanol and sterile deionized water and dried on sterile filter paper. In order to obtain fungal isolates with potential for growth on the medium contaminated with hydrocarbons, two nutrient media were used: potato dextrose agar (PDA) + motor oil (MO) and PDA + diesel (D). To obtain the emulsion, MO and D were initially mixed with Tween 80 solution in a final concentration of 0.05%. The final concentration of each amendment in the nutrient medium was 1 mg/ml. Stock solutions (MO + Tween 80 and D + Tween 80) were stored at 5 °C. To avoid bacterial growth nutrient media were amended with a mix of antibiotics: chloramphenicol + ampicillin + tetracycline at 0.2 mg/l each. Three fragments per root zone from each individual plant were plated in 90 mm Petri dish on each medium with two replicates. Plates with the plant fragments were incubated at 25 °C in the dark for seven days and observed daily for fungal growth. When fungal outgrowth from the plant tissues occurred, observations on emerged fungi were made. Only the fungi with different morphological characteristics were subcultured. Eventually, when an endophyte was acquired in pure culture it was preserved in mineral oil, at 5 °C and in glycerol 20% in deionized H_2O, at 24 °C. To analyze the fungal diversity, each replicate of the distinct stem fragments was noted. A preliminary classification was made to avoid the selection of identical strains arising from the same plant individual, separating isolates into morphotypes. Observations targeted characteristics related to the colony and medium as: colony shape, texture and color; exudates, medium color and growth rate. For the microscopic observations, each strain was inoculated onto a PDA Petri plate and a sterile cover slide was attached at two centimeters. Once the growth of the fungus partially covered the cover slide, the slide was removed, inverted on a slide with cotton blue (for the slightly colored colonies) and observed under microscope. Isolates were categorized into OTUs (organizational taxonomic units) until further taxonomic identification (Cosoveanu et al., 2018).

Data analysis. Colonization frequency (CF%) was calculated as the total number of fragments in a sample (plant/root zone) colonized by an OTU divided by the total number of fragments plated. For the diversity of endophytic fungi, the Margalef index, the Shannon index and Simpson's index of diversity were used. Margalef index measures species richness while Shannon index combines both richness and evenness. The Margalef index was calculated using formula $d=(S-1)/\ln N$, where S is the number of OTUs and N is the number of individuals in the sample (Margalef, 1958). The Shannon diversity index was calculated according to the formula:

$$H' = -\sum_{i=1}^{s} pi \ln pi$$

where *s* is the total number of species and *pi* is the relative proportion of each OTU (*Shannon, 1948*). Simpson's index of diversity was calculated according to the formula $D=1-\Sigma[ni(ni-1)/N(N-1)]$, where *ni* is the number of individuals belonging to *i* species and *N* is the total number of individuals (Simpson, 1949). For the diversity indices, PAST software version 3.15 (copyright Hammer & Harper, Natural History Museum, University of Oslo, Norway) was used. Venn diagrams were performed using the web-based tool InteractiVenn (Heberle et al., 2015).

RESULTS AND DISCUSSIONS

The distribution of the colonization frequency of the endophytic fungal OTUs was extremely skewed with a few common and many unusual OTUs for both plant species and root divisions. Only three OTUs shared by the two plant species and no OTU detected in all three root divisions imply (Figure 1).

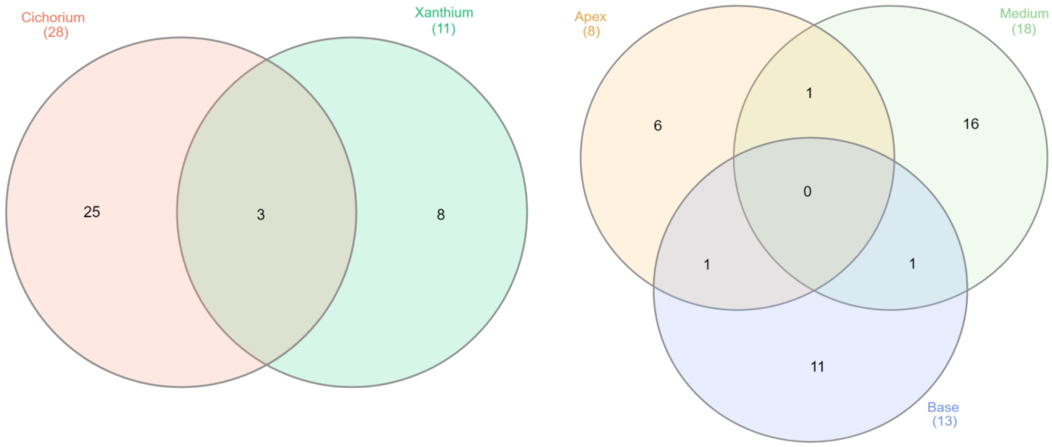

Figure 1. Venn diagram showing common OTUs between *Cichorium intybus* and *Xanthium strumarium* (left) and roots divisions of both plants (right)

Species, plant host and organ specificity were previously documented in multiples studies like screening of *Xanthium strumarium* (Ullah et al., 2021), molecular and phytochemical characterizations of *Cichorium intybus* (Malik et al., 2022), phytoremediation opportunities (Bandiera et al., 2016) and endemic species *Artemisia thuscula* (Cosoveanu et al., 2018).

Following the observation procedures and morphological similarity, respectively elements of shape, texture and color of colonies, as well as mycelium and spores under the microscope, it resulted in 39 morphologically different strains.

Frequency of fungal endophytes in *Cichorium intybus* showed a high number of species as rare — 90% of the OTUs were isolated with a colonization frequency value below 10%, considering all root divisions (Figure 2). Of 28 OTUs from *Cichorium intybus*, six were isolated only from the root apex, other nine from the root medium section and seven from the base zone of the root. Only one OTUs was isolated from all three divisions (OTU9).

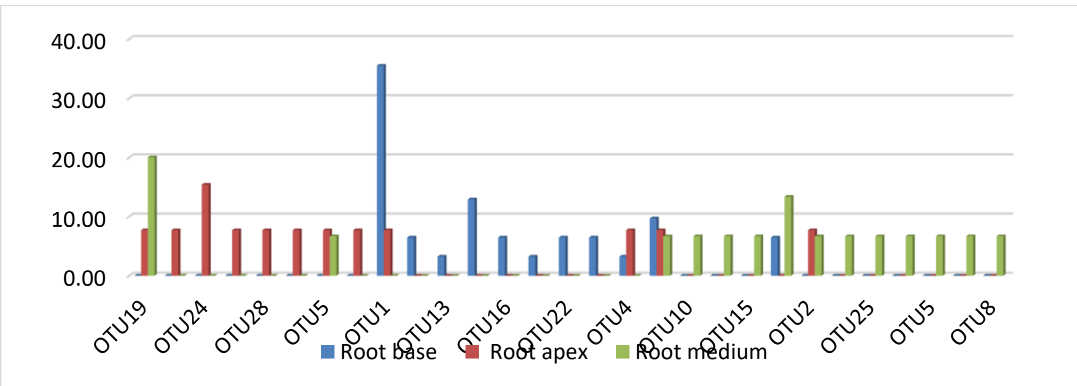

Figure 2. *Cichorium intybus* colonization frequency % of endophytic fungi OTUs considering individually root divisions base, medium and apex

In *Xanthium strumarium* rare species were here also dominant with 58% of them having colonization frequency values below 10%. From a total of 11 OTUs, only two were isolated from all three divisions (OTU19, OTU30) (Figure 3).

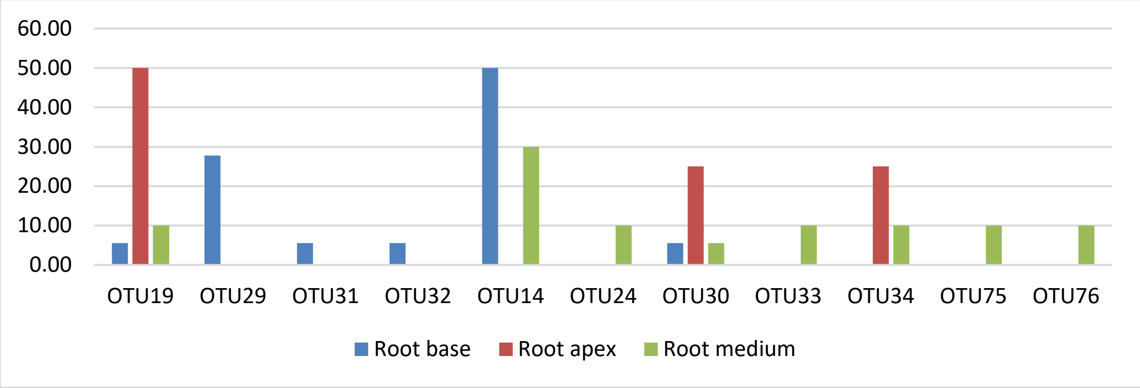

Figure 3. *Xanthium strumarium* colonization frequency % of endophytic fungi OTUs considering individually root divisions base, medium and apex

Fungal endophytic communities are typically composed of highly dynamic organisms wherein the host produces chemicals that alter the fungal "settlement" according to species characteristics and the tissue architecture. This should take precedence over other prevalent elements such as the interplay between rivalry and hostility (Mejia et al., 2008; Rodriguez Estrada et al., 2012; Yan et al., 2015) within the potential settled fungal community or the external factors in terms of ecosystem. The current work offers a part into the divisions of organs since endophytic fungus coexist with their hosts and encourage researchers to investigate the morphology, chemistry, and functions of plants in more detail taking advantage of genomics technologies.

Regarding the similarity of fungal communities in the two plants studied, *C. intybus* and *X. strumarium*, the diversity expressed by the Shannon index indicated a higher net value for the endophyte fungal communities in *C. intybus* (Shannon_H= 3.0) compared with *X. strumarium* (Shannon_H= 2.0) (Jost, 2006). The same host plant, *C. intybus*, was also noted for the richness of the species, the value of the Margalef index being 6.6, compared to *X. strumarium*, where the index had a value of 2.9. The dominance of endophyte fungal communities showed similar values in both plants. These preliminary results point to a higher diversity of endophyte fungal communities in the host species *Cichorium intybus*, colonized by the highest number of different OTUs compared to the other host plant *Xanthium strumarium* (i.e 28 versus 11) (Table 1).

Table 1. Diversity indices of endophytic fungal communities in *C. intybus* and *X. strumarium*

	Taxa_S	**Individuals**	**Simpson_1-D**	**Shannon_H**	**Margalef**
Cichorium intybus	28	60	0,9	3,0	6,6
Xanthium strumarium	11	32	0,8	2,0	2,9

CONCLUSIONS

The research sheds light on the variety and regularity of endophytic communities that are separated from the roots of two naturally occurring plant species, *Xanthium strumarium* and *Cichorium intybus*. According to preliminary findings, both plants have a uniform distribution of rare fungal communities with a composition that is moderately rich. The selectivity of fungal endophytes for particular root divisions is an important point to note. Since the hosts are found on polluted soils that contain hydrocarbons, more research is needed to fully understand the roles played by these uncommon colonies, particularly with regard to their biological activities.

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