SUMMARY

of the doctoral thesis

RESEARCH ON THE DIVERSITY AND BIOACTIVITY OF ENDOPHYTE FUNGI IN SPONTANEOUS FLORA IDENTIFIED ON POLLUTED SOILS WITH HYDROCARBONS

Ph.D-student: **GURĂU M. Lorena-Roxana**

Scientific coordinator: ***Professor Dr.*** **ILIE Leonard**

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From the earliest times, evolutionary biology has highlighted the close connection between fungi, plants, and ultimately, the entire biosphere. Global problems, such as environmental degradation and biodiversity loss, undermine ecosystem balance and directly affect the quality of life. Endophytes, hidden microorganisms within plants, represent an untapped resource with enormous potential in various fields, from agriculture to medicine.

The doctoral thesis entitled "***RESEARCH ON THE DIVERSITY AND BIOACTIVITY OF ENDOPHYTIC FUNGI IN SPONTANEOUS FLORA IDENTIFIED ON SOILS POLLUTED WITH HYDROCARBONS***" represents an in-depth analysis of the importance of the endophytic community and their biomechanisms involved in the degradation of certain contaminants, under laboratory conditions.

The doctoral thesis comprises two parts, totalling four chapters. The first part includes a literature review, while the second part presents the original research. The literature review comprises the first two chapters, entitled "***The current state of knowledge regarding endophytic fungal communities***" and "***Involving endophyte communities in the process of hydrocarbon degradation***".

The first chapter presents a variety of aspects related to endophytic microorganisms and their principles. Based on previous studies, information is provided regarding the importance, transmission, colonization, ecology, distribution, interaction with the host plant, and applicability of endophytic fungi.

Chapter II presents data on the current situation of hydrocarbon-contaminated soils in Romania, as well as the effects of hydrocarbon pollution on spontaneous flora and soil. In addition, the chapter is complemented by concepts related to the fungal biomechanisms involved in the degradation of contaminants.

The second part of the thesis, detailed in two chapters, outlines the purpose and objectives of the study, the natural setting and climatic conditions, the materials and methods used, and the results obtained regarding the diversity of endophyte communities in spontaneous flora and the evaluation of their bioactivity under the influence of the investigated factors.

The purpose of this doctoral thesis was to conduct research on the evaluation of the diversity and bioactivity of an endophyte fungal community isolated from the roots of plants identified in the spontaneous flora present on hydrocarbon-polluted soils. To ensure the best possible approach to achieving this and its objectives, the research was conducted exclusively in the laboratory.

Laboratory examinations were much more complex and aimed at isolating endophytic fungi and identifying them using morphological and molecular methods, conducting analyses on the ecology and diversity of the endophyte fungal community, and evaluating their ability to degrade pollutant compounds on a synthetic nutrient medium. Field research was limited to selecting locations with the potential for hydrocarbon pollution and collecting plant and soil samples.

Chapter III, entitled "***Research on the diversity of endophytic communities in spontaneous flora***", initially presents data on the natural setting, the primary location being the city of Băicoi, Prahova county, and secondary sampling points that targeted areas in the vicinity of oil extraction wells and a high salinity zone.

The methodology for sampling soil and plant specimens (*Cichorium intybus, Xanthium strumarium, Salicornia europaea*, and *Suaeda maritima*) is presented, including their description, as well as the laboratory procedures for isolating endophytic fungi from root fragments on an amended nutrient medium (PDA with diesel/PDA with motor oil), followed by their purification and preservation. Also presented in this chapter is the identification of isolates at the macroscopic, microscopic, and molecular levels. According to the results of DNA sequence similarity using the ITS region, 12 genera and 23 species were identified in *Cichorium intybus*, 5 genera and 8 species in *Xanthium strumarium*, 5 genera and 9 species in *Suaeda maritima*, and 8 genera and 17 species in *Salicornia europaea*.

In order to analyse the diversity of endophyte communities, the colonization frequency (CF%) was calculated statistically as the total number of fragments in a sample (plant/root zone) colonized by a strain divided by the total number of fragments in all plates, as well as the Margalef, Shannon, and Simpson diversity indices. Using RStudio, a programming language for statistical computing and graphics, the data was interpreted regarding the analysis of ecological elements of endophytic fungal communities and their association. This chapter also includes the results and discussion sections regarding the frequency and colonization of fungal genera in each plant species, root part, and nutrient medium. In the case of *Cichorium intybus*, considering the two nutrient media (PDA + diesel and PDA + motor oil), a total of 10

genera were observed, of which five genera were common to both media (*Alternaria, Colletotrichum, Diaporthe, Fusarium, Mucor*), and five genera were unique (*Aspergillus, Beauveria, Clonostachys, Paraphoma, Penicillium*). The genus *Fusarium* showed the highest frequency, followed by *Alternaria* and *Diaporthe*, in both media. Considering only one medium (PDA + motor oil), the highest frequency value was recorded for the genus *Aspergillus* (FC% = 19.45), followed by *Beauveria* and *Clonostachys* with much lower values (FC% = 5.5 and 2.8, respectively).

In the case of *Xanthium strumarium*, considering both nutrient media (PDA + diesel and PDA + motor oil), five fungal genera were identified, of which three genera were common *(Alternaria, Macrophomina, Fusarium*), followed by two unique genera (*Aspergillus, Talaromyces*). Similarly, to *Cichorium intybus*, the genus *Fusarium* had the highest colonization frequency (FC%= 36.1), followed by *Alternaria* and *Macrophomina* (FC% = 11 and 8.3, respectively), in both media. In *Salicornia europaea*, for the two nutrient media (PDA + diesel and PDA + motor oil), nine fungal genera were identified, of which six genera were isolated on both media (*Aspergillus, Fusarium, Monosporascus, Phaeosphaeriaceae* sp., *Pleospora*), followed by three unique genera (*Dimorphosporicola, Laburnicola, Tremateia*).

The results of the colonization frequency also indicate that the genera *Fusarium* and *Pleospora* showed high values (FC% = 22.2) compared to the other four common genera. Of the three unique genera, *Tremateia* had the lowest frequency compared to the other two (FC% *Laburnicola* = 5.5 and FC% *Dimorphosporicola* = 8.3). Following the determination of genera in the plant species *Suaeda maritima*, a total of five fungal genera were observed isolated on both nutrient media (PDA + diesel and PDA + motor oil), of which four genera were common (*Alternaria, Laburnicola, Monosporascus, Phaeosphaeriaceae* sp.), and a single unique genus (*Clonostachys*), obtained only from PDA amended with diesel.

Regarding the calculation of fungal genera diversity indices in plant species, *Cichorium intybus* recorded the highest number of genera detected, in other words, the greatest fungal species richness (Species Richness index = 14), compared to the other three plants: *Salicornia europaea* (Species Richness index = 9); *Xanthium strumarium* (Species Richness index = 5) and *Suaeda maritima* (Species Richness index = 5). The Shannon diversity index showed similar values of abundance and evenness of species distribution for *Salicornia europaea* (Shannon-Weaver Index = 1.87) and *Cichorium intybus* (Shannon-Weaver Index = 1.77), a slightly reduced diversity in *Suaeda maritima* (Shannon-Weaver Index = 1.42), and very low diversity in *Xanthium strumarium* (Shannon-Weaver Index = 0.98). Simpson's diversity index, which measures species richness and evenness, showed similar values for three of the four plant species: *Salicornia europaea* (Simpson Index = 0.82), *Cichorium intybus* (Simpson Index = 0.77), and *Suaeda maritima* (Simpson Index = 0.74), while *Xanthium*

strumarium had the lowest value (Simpson Index = 0.49), indicating a dominant fungal community.

Chapter IV presents two *in vitro* tests aimed at evaluating the bioactivity of endophytic fungi through the behaviour of fungi on a contaminated medium and assessing their biodegradation capacity of hydrocarbons.

Initially, the growth rate of the fungi was monitored by measuring the diameter of the mycelium formed at 24, 48, 72 hours, and up to 10 days. Based on these measurements, the fungi were classified into two categories: fast-growing fungi and slow-growing fungi.

Subsequently, evaluations and observations were conducted to assess the potential of these strains obtained from the spontaneous species *Cichorium intybus*, *Xanthium strumarium*, *Salicornia europaea*, and *Suaeda maritima* to grow on media with concentrations of up to 20% hydrocarbons (motor oil, diesel, and petroleum) and to examine the pollutant degradation capacity of those that showed the highest tolerance index on the contaminated medium, using a technique based on the redox decolourization indicator 2,6-Dichlorophenol-indophenol. The growth potential of endophytic fungi on hydrocarbon-amended media was verified in the first experiment, which was conducted in two stages, using two different sets of amendment concentrations (0.1%, 0.5%, and 1% (set 1) and 5%, 10%, and 20% (set 2). There were also control variants, to which no contaminants were added (noted CTRL – control).

The entire experiment was conducted in a sterile environment, in a laminar flow hood, and in 90 mm diameter Petri dishes. The stock solutions with amendments were stored at 5°C for 1-2 months. The testing was performed in isolated groups of fungi, a maximum of 10 fungi, to avoid possible contamination. The quantity of PDA for each working volume was sterilized beforehand without the contaminant (motor oil/diesel) to avoid hydrocarbon evaporation. The final volume for each Petri dish was 5 ml, consisting of PDA+ SS nutrient medium. For the first set of tests, at a concentration of 0.1%, the resulting volume was 5 µl SS + 4995 µl PDA, for a concentration of 0.5%, the resulting volume was 25 µl SS + 4975 µl PDA, and for a concentration of 1%, the resulting volume was 50 µl SS + 4950 µl PDA in a final volume of 5 ml. For the second set of tests, the resulting volumes were 250 µl SS + 4750 µl PDA for a concentration of 5%, 500 µl SS + 4500 µl PDA for a concentration of 10%, and 1000 µl SS + 4000 µl PDA for a concentration of 20%. For the inoculation of each strain onto the nine Petri dishes corresponding to the treatments, control, and each concentration/test set, 72 inocula with a diameter of 4 mm were cut from the most recent purifications. For each of the eight inocula/each plate, two diameters of growth were measured. The optimal time for measurements was chosen so that none of the eight mycelial growths would intersect with another. Consequently, for each fungal isolate, 144 measurements were taken, which were recorded in tables, along with the date on which they were taken and the time between measurements (fungal growth), forming an electronic database.

The average values of the measurements were necessary for the final evaluation of the isolates' behaviour on a polluted medium at different concentrations and for the calculation of the stress tolerance index on a hydrocarbon medium. It was considered that the higher the index values were above 1, the higher the stress tolerance was.

The analysis of endophytic fungi bioactivity and the interpretation of statistical data were performed using the RStudio program. To observe and analyse trends and preferences among strains within groups and between treatments at the same amendment concentration, summary graphs (Clustering Key) and local regression (loess regression) were created. Therefore, based on all the calculations and measurements, we can conclude that the response to treatments (diesel and motor oil) varies significantly among strains, indicating genetic or physiological differences in the ability to cope with petroleum pollutants.

All fungal genera analysed exhibited greater sensitivity to diesel fuel than to motor oil. Diesel fuel has a strong inhibitory effect on fungal colony growth for most genera, with significant decreases in diameter at high concentrations. Motor oil tends to have a slightly stimulatory effect at low concentrations for many genera, followed by moderate inhibition at high concentrations. Genera such as *Aspergillus*, *Diaporthe*, and *Talaromyces* tend to exhibit good tolerance to both diesel fuel and motor oil, making them potential candidates for bioremediation studies. Genera such as *Alternaria*, *Fusarium*, and *Mucor* are extremely sensitive to diesel fuel but tolerate motor oil relatively well.

The second part of ***Chapter IV*** focused on examining the hydrocarbon degradation capacity of fungal isolates that exhibited a high tolerance index in a medium amended with motor oil/diesel and petroleum, utilizing a technique based on the redox indicator 2,6-Dichlorophenol-indophenol. By adding this reagent to the culture medium, we were able to observe the fungi's ability to utilize hydrocarbons as a carbon and energy source through the decolourization reaction (from blue to colourless). Initially, this experiment required the results of soil sample analyses corresponding to the four plant species studied from the three locations. All four soil samples were analysed at the Laboratory of Physico-Chemical Analyses for Soil Sciences, Agrochemistry, and Environmental Protection within the National Research and Development Institute for Pedology, Agrochemistry, and Environmental Protection (ICPA Bucharest) with the aim of determining the total petroleum hydrocarbon (TPH). Analytical results showed a total hydrocarbon content (TPH) of 5400 mg/kg in the soil sample collected from a site with older oil spills, where *Xanthium strumarium* plant samples were collected, compared to soil samples collected from saline sites with a THC of 500 mg/kg, where *Salicornia europaea* and *Suaeda maritima* plant samples were collected. For this experiment, the previous amendments were used with the addition of 10% petroleum. Therefore, in addition to the degradation reaction, we could also analyze mycelial growth on a new contaminant. Five strains were selected

that showed mycelial growth for seven days. The selection criterion was based on the uniformity of the mycelium in the plate and the growth category (all strains being fast-growing). Three inocula were immersed in each plate at relatively equal distances. Thus, 54 inocula were cut from each fungus, which were necessary for all test variants and the control variant (engine oil/diesel/petroleum amendment). Observations focused on measuring the radius of the discoloration zone at seven days, measuring the mycelial diameter for the tolerance index, following the oxidation reaction, for each isolate/Petri dish, and visually examining the degree of discoloration. Furthermore, photographs were taken to monitor the formation of the discoloration halo at 7, 10, and 15 days. Statistical data visualization was performed using box plots (employing the "ggplot2" library in RStudio), with the distribution representing the minimum value, lower quartile, median, upper quartile, and maximum value.

The halo analysis considered the average radii of the discoloration zones for each isolate/each amendment (engine oil, diesel, and petroleum) and each PDA concentration (0.1%, 1%, and 100%). Based on visual and percentage observations, we can conclude that CEH19 exhibited the highest biodegradation capacity for 10% petroleum, with the most representative values. For engine oil, CEH34 demonstrated the highest degradation capacity, while for diesel, CEH43 showed the most significant percentage of discoloration. All three isolates belong to the genus Fusarium, making it representative for this study. Therefore, it is considered that the evaluated endophytic fungi have significant potential for hydrocarbon biodegradation, especially under optimal absorption conditions, with higher PDA concentrations stimulating degradation activity and indicating that nutrients play a major role in biodegradation processes.

This doctoral thesis comprises 159 pages, including 6 tables and 54 figures. The results obtained from the conducted research have been utilized in 3 published articles, as follows: one article indexed in ISI with an Impact Factor of 0.161 (2024) and two articles (BDI database indexed). The thesis bibliography includes 199 sources (books, articles in specialized journals, scientific papers, web sources).

The results obtained in this study contribute to the completion and improvement of existing knowledge.