**PI – Prof. Segula Masaphy**

**Research Program**

**I. Scientific Background**

The nutritional capacity of fungi determines their fitness and adjustment to different habitats. Their important role in degradation processes and organic matter recycling is well documented. Members of this kingdom are especially important in the degradation of complex biological carbon (C) macromolecules such as lignin due to their unique enzymatic production and activities. The ability of different fungal phyla to degrade complex C biomolecules determines their distribution in diverse habitats, where they express various trophic states—saprotrophic, pathotrophic, parasitic, endophytic or mycorrhizal, as presented by Nguyen et al. (2016) (41). While many fungi have restricted lifestyles, others may shift their trophic state (30) and act as facultative mycorrhizal (51).

For their nitrogen (N) source, fungi use either biomolecules from the decomposition process or soluble mineral N as ammonia or nitrate. Although as early as 1916, Duggar & Davis (1916) (15) suggested nitrogen fixation by fungi, only in recent years have there been increasing reports of fungi showing N fixation (3, 12, 17, 18, 24-27, 31, 32, 34, 52). It is largely accepted that only prokaryotic organisms can fix N (52). Yet, nitrogen fixation by eukaryotes has been proven to exist through bacterial associations (30). Therefore, fungal N fixation must be associated with symbiotic N-fixing bacteria (NFBs; diazotrophs). Evidence of fungal-bacterial symbiosis is well established in the literature (6, 14, 58). Evidence of fungal-N-fixing associated bacteria has been provided mainly in mycorrhizal fungi (3, 12, 17, 30-32, 43), suggesting cooperation between the fungi and associated NFBs that might also facilitate N nutrient supply to the growing plant in natural systems (24, 48, 68). This cooperation is very important in nature as it ensures the availability of N sources in environments having low soluble N content.

In addition to mycorrhizal fungi, several saprotrophic fungi have been shown to use atmospheric N2 obtained from their associated NFBs, and there are reports on fungal growth on nitrogen-free medium (56). For saprotrophic fungi, association with NFBs mainly serves fungal growth and protein accumulation due to low levels of fixed N in the degraded plant and wood tissue. NFBs were reported to be important contributors of N to fungal communities in nature, including in forests (23). Moreover, mushroom-producing fungi that require high concentrations of N to produce the protein-rich biomass (up to 60% of dry matter) (63) benefit from N-supplementation by NFBs in the environment as demonstrated in reports related to mushrooms such as *Pleurotus* (26). A more unique phenomenon of N fixation in the fruiting body tissue itself has recently been reported (13, 34).

Fungal-associated NFBs may be found as endobacteria within the mycelium (56). Endobacteria in general are important in the life of fungi and their fitness (19). They are transferred from one growing site to the other by the growing mycelium and by spores. Efforts to cure fungal mycelium from endobacteria to study their role in fungal life included the use of several successive generations of spores transferred and mycelium in bacterial-free medium (37) or the use of antibiotics such as ciprofloxacin (47).

The presence of associated NFBs in the fungal-bacterial association was shown to be accompanied by nitrogenase genes evidence, nitrogen-15 (15N) isotope accumulation in an (15N)-enriched environment, and nitrogen-fixing activity as determined by acetylene assay. For example, using serial assays, Koch et al. (2021) (31) showed that the fruiting body of *Guyanagaster necrorhizus* can use nitrogen fixation due to a range of associated bacteria. Although evidence exists of the assimilation of atmospheric N2 by mushroom-producing fungi in their mycelium as well as in the fruiting bodies, the role of fungal-associated NFBs in the flexibility and adaptation of the fungal species/strain to different habitats, where the fungi may exhibit as free living or in a plant-associated growth mode and grow in low-nutrition environments, is still far from being understood. An effort to correlate between mushroom-associated bacteria of *Tuber indicum*, a similar species that grew in different geographical regions, with nitrogen-fixing bioactivity was conducted by Chen et al. (2019) (12), but it focused on mycorrhizal mushrooms only, without trophic state flexibility.

In order to address the question of the role of fungal nitrogen fixation in the flexibility of mushroom-producing fungi to be adapted to different ecological niches (i.e., in plant-associated or free-living saprotrophs), we suggest using the Morchellaspecies. Morels (*Morchella* spp., Pezizales, Ascomycota) are highly appreciated edible mushrooms (61). The *Morchella* genus includes over 50 species based on molecular identification (56). Species in this genus have high variability with respect to phenotypic appearance and trophic state. Morchella species have been reported as being mycorrhizal, including facultative mycorrhization (9, 10, 13, 40), saprotrophic (21, 32, 46) and even endophytic (4). Morels are collected mainly from their natural growth habitats. This mushroom was subjected to artificial cultivation systems with several successful systems, as reviewed by Liu et al. (2018) (35), including in our lab (39). However, knowledge of the factors required to establish mushroom colonies, fruitification and mushroom health, which depend mainly on the exploitation of environmental nutritional sources, is still lacking.

Morel mushrooms are found in a range of habitats, from healthy forests to various disrupted soils and harsh environments with little plant material. In some cases, the same species was reported to appear in diverse habitats with different nutritional qualities, as we reported for *M. rufobrunnea*, which grew in healthy groves as well as in nutritionally poor soil (41, 46). The wide range of Morchella ecotypes is mainly related to the availability of C-source types exhibiting carbohydrate-active enzyme (CAZyme)-proteins adjustment (11). As for Nsources, onlythe use of already fixed N has been reported (i.e., biological N molecules and soluble mineral sources (28, 29, 36, 64, 66)). More recently, we reported on the presence of high NFBs population in natural morel soil habitats (46), whereas Yu et al. (2022) (65) reported the same in cultivated outdoor morel soil, suggesting their role in N supplementation to the fungal colonies.

Helper bacteria were reported for the Morchella species with cooperative relationships of exo-bacteria, facilitating both partners’ growth and nutrient supply (35, 50). In an earlier study, Buscot (1992) (8) showed a shift from the saprotrophic mode of *Morchella esculenta* to the mycorrhizal mode, with *Picea abies* stimulated by helper bacteria added to the medium under controlled culture conditions. Morchella mycelium was reported to harbor endobacteria (9, 38), but its role, if any, in nitrogen fixation has not been reported in the literature. Recently, in an as yet unpublished work, we obtained successful growth and sclerotia formation of one examined isolate of Morchella sp. grown on agar N-free medium even in the third generation (see preliminary data). Moreover, we succeeded to show the presence of bacterial nitrogenase gene sequence related to *Bradyrhizobium* sp. and bacteria from the Burkholderiales order. This could lead to further study on this phenomenon within diverse morel populations, as proposed here.

**II. Research Objectives and Expected Significance**

**Study Aim**

N fixation is an important process, providing available N in soil habitats. Since fungi play a dominant role in the ecosystem, N fixation in fungi may also contribute to N enrichment of the soil or associated plants. For the fungus itself, N fixation may facilitate adaptation to harsh environments and play a role in its adaptation plasticity to nutritionally different habitats. This is important for all fungi, especially the Morchella species, where a great deal remains to be understood about its life and colony establishment in nature.

The primary aim of the proposed research is to shed light on the role of mycelial-harbored NFBs on the nitrogen fixation of Morchella isolates and determine the extent of the nitrogen fixation ability of different Morchella species and their ecotypes (i.e., those growing in diverse natural habitats).

The objectives of this proposal are :

1. To determine the diversity of the trophic state of different collected morel populations from various sites throughout Israel (i.e., different species from different habitats) by determining their isotopic signature (% 15N and % 13C).

2. To compare the ability of isolates’ mycelial cultures of different ecotypes to grow on N-free medium in culture and their N accumulation (i.e., N fixation).

3. To determine the specificity between the defined NFBs and Morchella isolate’s habitat origin bydetermining and identifying culturable and non-culturable NFBs. The latter will be based on the presence of nitrogenase genes sequences in the fungal culture biomass.

4. To study the effect of curing the mycelium from endobacteria on fungal growth and protein accumulation.

This will enhance our understanding of the connection between the N-fixation ability of Morchella ecotypes and their adaptation to their habitats.

**III. Detailed Description of the Proposed Research**

**Working hypothesis**

Mushroom-producing fungi require high nitrogen concentrations to build up their protein-rich fruiting bodies. This requirement is not fulfilled in harsh and nutritionally poor soil or from degrading wood material. Hence, NFBs are expected to play a role in contributing N to the fungal substrate, as was already reported in earlier studies (23). Morchella species are found in a wide range of habitats – from nutritionally poor soils, post-fire soils, healthy forests, orchard soils and fertilized soils – as free-living or plant-associated. NFBs were found in high abundance in natural (46) and cultivated (65) morel-growing sites. They play a role in enriching the soil with fixed N sources exploited by the fungal mycelium.

We hypothesized that Morchella mycelium could harbor endobacterial NFBs that could facilitate Morchella colony establishment and fruit bodies development in N-poor soil. On the other hand, plant-associated morels harboring NFBs could help the plant with its N requirements. However, different species of morel mushrooms produce a range of secondary metabolites, including antibacterial activity (20) that might influence the type of harbored endobacteria. The type and concentration of metabolites can vary according to environmental conditions and from one growing site to the other (20). We hypothesize that growing different Morchella species and different isolates under the same controlled cultural conditions will enable us to determine the harbored NFBs in each isolate and to correlate between the specific endobacterial NFBs and the origin of the isolate species versus habitat.

Our preliminary data confirm the growth and sclerotia formation of the mycelium on N-free agar medium and the presence of the nitrogenase gene related to *Bradyrhizobium* sp. and bacteria from Burkholderiales in one isolate of *M. donalli.* We will examine additional Morchella isolates for their related NFBs and ability to fix nitrogen in order to correlate between the Morchella isolate origin and its N-fixing abilities. We have been studying the ecology and cultivation of morel mushrooms in our laboratory. We monitored the growth of the Morchella species in different habitats throughout Israel and procured many isolates, some from the same species, growing in different habitats or geographical zones, suggesting their ecological flexibility, which could confirm our hypothesis.

**Research design**

The research will focus on two directions. One is the comparison of harbored NFBs of different Morchella isolates obtained from various species of morel populations from diverse geographical and habitats (different species and different habitats). The isolates will be obtained from morel ascocarpes collected throughout Israel, with a determined trophic mode according to isotope signature (22). The second approach will focus on an in-depth study of N fixation by selected isolates to confirm the role of harbored NFBs in the culture growth in N-free medium. The selected isolates will be from different species showing different NFBs identity and quantity under the same growth conditions.

1. Morel isolate origin versus harbored NFBs identity.

a. Morel mushrooms will be collected from different geographic zones and habitats, and their isotopic signature will be analyzed to determine their suspected trophic mode.

b. A comparison will be made of biomass and nitrogen accumulation of the different isolates grown on N-supplemented and N-free medium.

c. The NFBs present in the mycelial cultures will be determined by (a) screening the nitrogenase gene presence in the mycelial cultures in N-supplemented and N-free medium and (b) determining the related bacteria.

2.Contribution of the NFBs to fungal growth by examining up to five isolates showing different NFBs species genes.

a. Observing the endobacteria using confocal microscopy.

b. Curing the fungal mycelium from the endobacteria using antibiotics and determining its effect on fungal biomass and nitrogen accumulation on N-free medium.

c. Determining the N fixation capacity of the cured and uncured fungal cultures using 15N enrichment assay.

d. Determining the culturable NFBs bacterial by isolation on N-free medium and identification using 16s r-RNA sequences.

**IV. Methods**

**1. Mushroom and habitat characterizations**

Morel mushrooms from different populations (ecotypes) will be collected from diverse geographical zones, habitats and areas in Israel: healthy natural forest mushrooms (including plant-associated mushrooms); post-fire mushrooms; garden plant-associated mushrooms; bare soil-associated mushrooms; and orchard mushrooms. We will use freeze-dried cultures from our collection and additional fresh morels will be collected by citizen scientists. For the proposed study, at least five isolates of each species collected from different sites will be used according to their availability.

**2. Isotopic signature of the collected mushrooms**

Isotopic signature will be determined in the fruiting bodies of the collected morel mushrooms. The freeze-dried tissue will be powdered and sent to an external service laboratory for isotopic signature (% 15N and % 13C) determination (**Carbon and Nitrogen Stable Isotopes Facility, INRA** Centre de Nancy**, France).** Relative abundances of carbon and nitrogen isotopes were shown to be a useful tool in discriminating between saprophytic and mycorrhizal fungi (20) as well as determining the use of recent atmospheric fixed nitrogen (49). The % 15N and % 13C method was previously used to determine the trophic state of saprotrophic morel mushrooms (21). The stable isotopes 14N and 15N occur naturally at a ratio of 0.3663 atom% 15N in atmospheric N2; differences exist between the atmosphere and soil and plants caused by preferential use of the lighter 14N isotope in biological processes. Hence, the 15N:14N ratio is an acceptable indicator of atmospheric N2 fixation (57). For example, nodules of legume plants, known for their nitrogen-fixing associated bacteria, are commonly highly enriched in 15N (60).

**3. Fungal isolates biomass accumulation on N-supplemented or N-free medium**

Different isolated cultures will be screened for their growth on N-free medium and N-supplemented agar medium. Isolates from our Morchella culture collection along with newly collected mushrooms will be grown on a membrane-covered mineral agar medium according to Ruiz‐Herrera et al. (2015) (56) with or without supplementation of a nitrogen source for three successive generations. The growing biomass will be subjected to: (a) dry biomass determination; (b) total nitrogen and total protein accumulation (c); and nitrogenase gene determination in wet biomass (d).

3a. Biomass accumulation: The biomass grown on N-free and N-supplemented medium will be collected from the agar medium surface two weeks after growth and freeze-dried. The total accumulated biomass will be determined and compared to other isolates.

3b. Nitrogen accumulation in the mycelial culture: The freeze-dried biomass will be subjected to total N and % protein in the biomass determination using the Kjeldahl method (2) and an elemental analyzer.

3c. Screening for *NifH* gene (nitrogenase)sequences: Total DNA will be extracted from the growing cultures using the ZymoBIOMICSTM DNA Miniprep Kit (Zymo Research, Irvine, CA). Universal primer sets IGK3/DVV (1) will be used on the gDNA extracted from the whole culture. In our preliminary work, we had obtained positive nitrogenase sequences in the mycelial culture using universal primer sets IGK3/DVV (1), but we will also try the primer pair PolF (5’- TGC GAY CCS AAR GCB GAC TC -3’) and PolR (5’- ATS GCC ATC ATY TCR CCG GA -3’) previously used for *NifH* sequences of a natural mixed population of NFBs in forest soil (23) to cover other potential *NifH* sequences. The extracted gDNA will be subjected to the relevant PCR conditions. The *NifH* gene will be partially amplified and sequenced on the Illumina MiSeq platform at the Genome Research Core (University of Illinois). The related NFBs will be identified using bioinformatics tools.

3d. Determination of nitrogenase activity: Up to five fungal isolates that showed different NFBs species according to *NifH* sequences determination will be studied further to determine the role played by the harbored NFBs in fungal growth on N-free medium. To compare N-fixing activities by the different fungal isolates, we will use the 15N enriched assay of Ruiz-Herrera et al. (2015) (56), with some modifications. Basically, fungal mycelium grown on N-free medium will be transferred to the same medium in tubes. Sterile air will be injected into the tube followed by 15N2. The tubes will be incubated for 72 h at 22oC. After incubation, the biomass will be collected, freeze-dried and sent to the **Stable Isotopes Facility, INRA** Centre de Nancy**, France,** for biomass 15N concentration determination. As a control, mycelial culture without 15N enrichment will be incubated under the same conditions and biomass 15N will be determined.

**4. Isolation and identification of endobacteria and culturable fungal NFBs**

The g-DNA extract used for *NifH* sequence determination in the fungal culture biomass will be used for molecular determination of all endobacteria using 515F and 926R (46) and primer sets 338F and 806R (12, 44). The first primer set had been used previously in our earlier work to screen for bacterial populations in natural soils in which *M. rufobrunnea* was grown, and several different genera of NFBs were identified colonizing the soil. The second primer pair based on the work of Mori et al. (2014) (44) was designed to amplify prokaryotic 16S rRNA with a low likelihood of amplifying eukaryotic DNA.

For culturable NFBs bacteria isolation and identification, wet fungal mycelium obtained from N-free medium will be homogenized by a beater homogenizer. The bacteria-containing suspension will be separated from the mycelial suspension by centrifugation. For the isolation of NFBs, the bacterial suspension will be inoculated on top of N-free medium and N-supplemented medium. The isolated bacterial colonies will be identified using 515F and 926R (46) and primer sets 338F and 806R (12, 44).

**5. Endobacterial curing by antibiotics**

In the literature, endobacterial curing was achieved by transferring the conidia for several generations in culture (37) or by using antibiotics (19, 47). In the present work, we will use antibiotics to obtain endobacterial-free mycelium. The fungal cultures will be grown on top of agar medium supplemented with a range of antibiotics including spectinomycin and ciprofloxacin, which were suggested for curing the endofungal bacterium *Rhizobium radiobacter* in the tripartite *Sebacinalean* symbiosis (19). To ensure curing, the mycelium will be subjected to confocal microscopy after staining with SYTO 9. The cures mycelium will then be transferred to N-free medium without antibiotics to follow its re-growth.

**6. Localization of the endobacteria**

Microscopically observed cells in fresh preparations will be stained with SYTO 9 and fluorescence will be observed in a fluorescence microscope (Leica, Wetzlar, Germany) with GFB filter for fluorescence, with excitation BP 470/40 and emission BP 525/50 (56) or by DAPI staining of the mycelial culture and observation by a Hermes image analyzer.

**Statistical Analysis**

For the correlation between Morchella isolates and their harbored NFBs, five isolates of each Morchella species from different sites will be studied according to their availability. The effects of the habitat sites on the harbored NFBs communities will be determined by ANOVA at a threshold level of *p* = 0.05 and Fisher’s test. All statistical analyses will be performed by JMP.

**V. Preliminary Results**

1. Functional ecology of soil bacterial population associated with *M. rufobrunnea* fruiting in a natural habitat: we determined the bacterial diversity in *M. rufobrunnea* fruiting body-bearing soil (Fig. 1, from Orlofsky et al. (2021) (46)). The mineral soil population examined had a high proportion of NFBs, suggesting that the NFBs act as helper bacteria by enriching the soil with fixed N and facilitating its accumulation in the fungal mycelium (Fig. 1).
2. Observation of the endo and exo bacteria of different Morchella isolates cultures was demonstrated after staining DAPI (4,6-diamino-2-phenyl indole) for the image analyzer observations (Fig. 2).
3. Sclerotia formation of the third generation (after 30 days) of M. XXX on N-free mineral medium (Fig. 3). This result indicates regular behavior of the fungal colony and accumulation of biomass on N-free medium.
4. Nitrogenase gene presence in the mycelial culture of two different Morchella isolates was determined using IGK3/DVV nitrogenase universal primers set (1). The sequences were related mainly to two groups of bacteria, Burkholderiales and Bradyrhizobiaceae. Both had previously been reported to be endophytic nitrogen fixers in plant and fungi (42, 59). Moreover, inoculation of the saprotrophic Pleurotus with *Pleurotus ostreatus* by *Bradyrhizobium japonicum* was reported to increase yield (67).

**VI. Researcher’s Resources for Conducting the Research**

Prof. Masaphy is studying different aspects of mushroom ecology and biotechnology, with a focus on morel mushrooms and fungal-bacterial interactions. Prof. Masaphy served as Head of the Microbiology Service Laboratory at Migal – Galilee Research Institute, and her work with bacterial cultures and their analysis is part of her expertise. A qualified technician will be hired for work in the laboratory, along with one PhD student and one MSc student. Citizen scientists will locate fresh morel mushrooms throughout Israel.

Prof. Masaphy’s laboratory has the facilities required for the mushroom and mycelial work, including the following equipment: laminar flow hood for clean work, autoclave, incubators and a microscope, as well basic instruments for molecular work (i.e., PCR machine and agarose gel electrophoretic equipment). The following equipment is available at Tel Hai College and Migal – Galilee Research Institute: confocal microscope, fluorescence microscope, image analyzer, quantitative PCR instrument, ELISA reader, scanning electron microscope, gas chromatograph, liquid chromatography-mass spectrometer, and Kjeldahl protein determination systems. Some of the analyses will be made in qualified laboratories: isotopic signature will be analyzed at the INRA facility, and bacterial molecular identification and nitrogenase sequencing will be performed at the University of Illinois’ Genome Research Core and the Tel Hai Lab service lab in Israel.

**VII. Expected Results and Pitfalls**

**Expected results**

We expect to resolve the question as to whether genetic (species) or environmental factors control the ability of fungal mycelium to harbor NFBs and the NFBs defined species. In other words, does a specific fungal-bacterial relationship exist involving specific Morchella and bacterial species that assists in the flexibility of a species to grow in different habitats in nature?

**Pitfalls**

1. Obviously, it is important to determine the nitrogen state in the soil in each habitat, however, the mushrooms appear in unexpected sites and soil characteristics at these points do not represent the nutritional elements used by the fungal mycelium prior to mushroom appearance.

2.The identification of the associated bacteria might encounter technical limitations. In some cases, these may be endobacteria that are not readily culturable. We will try to culture these bacteria under anaerobic conditions or identify them without culturing using the fungal-bacterial g-DNA extract.

3. Efforts to obtain cured mycelium to determine the role of bacteria in N-free medium using antibiotics might end up with no total elimination of the bacteria from the mycelial culture. We will also use the generation transfer (at least three generations of growth on N-free medium supplemented with antibiotics). We expect that in any case the antibiotics might reduce the bacterial number as was reported by Guo et al. (2017) (19).

4. The use of IGK3/DVV (1) universal primer for *NfiH* gene sequencing might be limited in the sequences it takes. Hence, we will use another primer set that was used in soil samples with more diverse NFBs of forest soil according (23).

5. It is possible that unidentified nitrogenase genes sequences will be found in the NGS analysis. This will require additional research to determine their origin.

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