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Review

Endohyphal bacteria; the prokaryotic modulators of host fungal biology

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ABSTRACT

Fungi interact with bacteria in specific environmental niches through microbial cell–cell interactions and carry out various ecological functions collectively. However, there are a number of known associations wherein some bacteria reside within the hyphae of fungi, leveraging their growth, development, distribution and secondary metabolism. These bacteria are referred to as endohyphal bacteria (EHB). The EHB have been found to regulate key components of host reproductive machinery, induce the production of phytohormones, and play a complementary protective role for the host fungus under stress conditions. In a unique endohyphal association of *Burkholderia* with *Rhizopus* sp., it was found that the phytotoxin which is essential for pathogenecity of the fungus, was produced by the endosymbiont rather than the host fungus causing the rice seedling blight. The EHB were also found to influence the ecology and diversity of endophytic fungi colonizing higher plants. In some cases, the EHB help in activation of genes involved in the recognition processes, transcription regulation, and synthesis of primary metabolism proteins. Although, methods have been developed to isolate EHB in axenic culture, this symbiotic association provides enormous opportunities for new discoveries and new insights into fungal biology. In this review article, we present a discussion on EHB, their significance, and diverse functions in nature as unraveled by the latest research to understand this unique microbial association.

1. Introduction

Fungi play diverse ecological functions in the biosphere by virtue of their metabolic proficiency and unique biochemical pathways (Shaw *et al.*, 2015). They grow in various ecological niches as free living organisms as well as indispensable partners in symbiotic associations. Secondary metabolites of fungi are often bioactive, usually of low molecular weight, and are produced as families of related compounds, with production often correlated with a specific stage of morphological differentiation (Keller *et al.*, 2005). Fungi are regarded as incredible chemical factories for being able to produce

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numerous bioactive molecules, including some life saving drugs (Keller *et al.*, 2005; Kumar *et al.*, 2013; Wang *et al.*, 2015).

Fungi interact with bacteria in specific environmental niches and their associations have positive effects on agriculture, environment and medicine (Scherlach *et al.*, 2013). Apart from the most frequently seen microbial cell–cell interactions, there are a number of known endosymbiotic associations in which bacteria reside within fungal hyphae, thus strongly leveraging their secondary metabolism (Bonfante and Anca, 2009; Frey-Klett *et al.*, 2011). This phenomenon was first discovered in mycorrhizal fungi wherein "bacterium-like organalles" (BLOs) were detected inside the fungal mycelia (MacDonald and Chandler, 1981). Recent studies have shown that foliar endophytes frequently harbor highly diverse bacteria, now called endohyphal bacteria (EHB), of unknown importance. These bacteria occur in living hyphae of phylogenetically diverse endophytes isolated from various plant lineages and in multiple biogeographic provinces. Those found in foliar endophytes are phylogenetically distinct from the apparently obligate symbionts of mycorrhizal fungi (e.g., Glomeromycota) (Hoffman and Arnold, 2010). In certain cases, the role of EHB has been elucidated and in some others their diversity and effects on the secondary metabolites of the fungal host have been explored (Fig. 1). One of the most exciting is the endohyphal association of *Burkholderia* with *Rhizopus* sp., wherein the characteristic phytotoxin, rhizoxin was found to be produced by the endosymbiont rather than the phytopathogen causing rice seedling blight (Partida-Martinez and Hertweck, 2005). Thus, *Rhizopus* sp. is able to cause the disease by virtue of the endosymbiont that it hosts. In some cases, EHB were found enhancing the production of plant growth promoting enzymes by the fungus (Hoffman *et al.*, 2013). In case of some arbuscular mycorrhizal fungi, EHB have been found to facilitate phosphate acquisition and transport (Ruiz-Lozano and Bonfante, 1999). Thus, EHB may play an important role in modulating the secondary metabolism of the fungus and loss of the endohyphal partner may result in attenuation of cultures for the production of key metabolites. This remarkable symbiotic association has been poorly studied and the implications of the presence of the endohyphal symbiotic partner on the secondary metabolism of the fungal host have been thus inadequately understood.

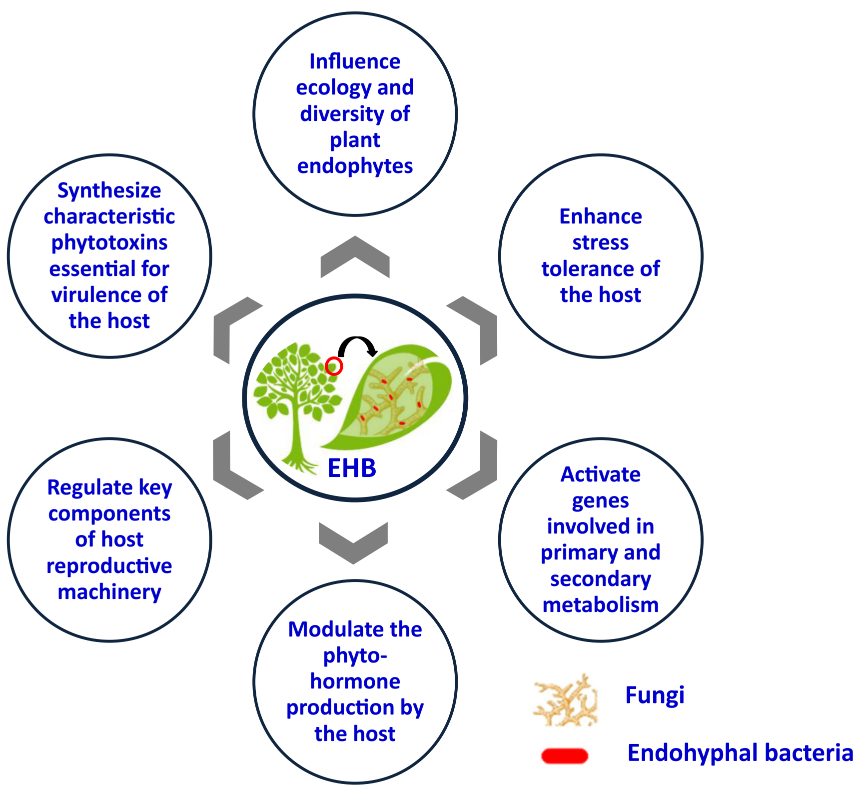

Fig. 1 – Modulation of fungal biology by EHBs: A diagrammatic representation of the ecological functions performed by the endohyphal bacterial. The endophphal bacteria: (i) have the ability to affect the ecology and diversity of plant endophytes (Hoffman and Arnold 2010), (ii) may play a complementary protective role for the host fungus and/or the host plant under biotic stress condition (Pakvaz and Soltani, 2016), (iii) help in activation of genes involved in recognition processes, transcription regulation, and synthesis of primary metabolism proteins (Deveau *et al.*, 2010), (iv) influence the phytohormone production by the host fungus (Hoffman *et al.*, 2013), (v) help regulate key components of host reproductive machinery (Mondo *et al.*, 2017) and (vi) are responsible for production of characteristic phytotoxins by the host e.g. rhizoxin production (Partida-Martinez and Hertweck, 2005).

There are numerous reports on the complexity of a plant's microbiome, both fungal and bacterial, including mutualists and pathogens (Vorholt, 2012; Philippot *et al*., 2013; Hardoim *et al*., 2015). With regard to the plant–microbe interactions, the fungus-endohyphal bacteria symbioses may have a key effect on the host plant's lifestyle and may thus represent the third component of plant–endophyte associations (Fig. 1). The endophyphal bacteria influence the ecology and diversity of plant endophytes (Hoffman and Arnold, 2010), play a complementary protective role for the host fungus and/or the host plant under biotic stress condition (Pakvaz and Soltani, 2016), help in activation of genes involved in recognition processes, transcription regulation, and synthesis of primary metabolism proteins (Deveau *et al*., 2010), influence the phytohormone production by the host fungus (Hoffman *et al*., 2013), help regulate key components of host reproductive machinery (Mondo *et al*., 2017) and are responsible for production of characteristic phytotoxins by the host e.g. rhizoxin production (Partida-Martinez and Hertweck, 2005). The beneficial endohyphal associations are widespread and pervasive but underlying mechanisms are still unexplored due to problems in isolation, cultivation and maintenance of all types of bacterial partners. The formation and consequences of the fungal and bacterial associations are presumably the outcome of chemical communication, wherein a metabolite from one partner evokes a response on other (Griffin, 2006). With the emergence of modern techniques, these "ping-pong" events could be explored by the use of transcriptomics (Mela *et al*., 2011; Gkarmiri *et al*., 2015) and proteomics (Moretti *et al*., 2010). Also, the resolution of events during the formation of the fungal and bacterial association could be better understood through metabolomics.

There are increasing evidences that bacteria–fungi associations are broader than expected. This review highlights the recent reports of these associations thereby illustrating the complex interactions between these partners, their impact on growth and development of host fungi and the host plant.

2. EHB associated with Mucoromycota

Mycorrhiza Helper Bacteria (MHB): a breakthrough in the endobacteria research

MHB is a common name given to bacteria which stimulate the formation of mycorrhizal symbiosis (Garbaye, 1994). Mycorrhiza helper bacteria were initially evidenced by indirect experiments in which it was found that the effect of fumigation to inhibit the mycorrhizal infection is correlated to different microorganisms colonizing the soil. Similar results indicated that there exists strong interaction between the soil microflora and the mycorrhizal infection (Fitter and Garbaye, 1994). The mechanism of action and functions of MHB included an increase in the germination of fungal spores and/or the survival, a stimulation of the pre-symbiotic growth of the mycelium, an increase of the receptivity to fungal signals of the short root, a stimulation of the root mycelium recognition and an alteration in the physicochemical properties that would aid in the mycorrhizal formation. It was also revealed that the interactions can't only be understood in terms of competition, as synergism also plays a key role in the ecology and distribution of the mycorrhizal symbiosis. However, it was not understood whether these results were the consequence of individual microorganisms or complex interactions. Later on, it was well demonstrated with ectomycorrhiza that the helper response of the microflora of the soil was due to the individual bacterial strains (Oliveira and Garbaye, 1989).

The true helper bacteria are adapted to living in association with the fungus as they are more abundant in the close proximity of the fungus. Several groups have isolated bacterial strains from the surface-sterilized ectomycorrhiza of which 80% were found to possess a positive effect on mycorrhiza establishment while 20% were neutral. Following this, several investigators suggested that such associations are more widespread than expected.

Thus, MHB are considered to be an important aid towards controlled mycorrhization techniques in forestry practice, by extending their use in poor nursery soils, saving fungal inoculum, improving the mycorrhizal quality or suppressing the use of soil disinfectants (Garbaye, 1994).

Bacteria-like organisms: Emergence of the third component of plant fungal interactions

In 1970, Mosse in her attempt to study the changes in fine structure during spore development in honey colored *Endogone* spores, found three unusual organelles *viz*., pigment granules, large crystals and self duplicating bacteria-like organisms. This third unusual structure, not much abundant as the pigment granules, was present in the germ tubes as well as in the vegetative hyphae. It was found that these organisms reside freely in the host cytoplasm without any outer membrane and are seen to be dividing independently with no visible symptoms on the host. When spores transit from the dormant to the resting state, such generally latent organisms become much more active, forming extensive colonies in the host. Consequently, they are seen to be expanding into enlarged entities and appear much more like bacteria (Mosse, 1970).

Wilson and Hanton (1979) described BLOs as Gram negative pleomorphic rods ranging from 0.5 to 1 μm $\times$ 1.0–3.9 μm which are believed to be enclosed in vacuoles or host membranes. Many other authors also described BLOs as rod shaped and enclosed in vacuoles, or coccal and devoid of host membranes unless degeneration of BLOs had occurred in fungal vacuoles.

A comparison between BLOs associated with *Glomus caledonius*, *Acaulospora laevis*, *Glomus mosseae*, *Gigaspora margarita*, *Gigaspora heterogama* and an unidentified white reticulate vesicular-arbuscular mycorrhizal spores was carried out (Macdonald *et al*., 1982). They were found to be transient prokaryotic cytobionts which could be acquired or lost by the host frequently. At the time of emergence of BLOs, the phylogeny of arbuscular mycorrhizal fungi was confined and the classification was carried out exclusively by morphological studies, so it led to unanswered questions about the repeated infections of the endobacteria and their existence as free living organisms or as obligate symbionts.

Characteristic phytotoxin-producing endo-fungal bacteria of a plant pathogen

Another example of well studied endohyphal associations is the one between *Rhizopus microsporus* and its bacterial endosymbiont. The fungus which is the causative agent of rice seedling blight was found to produce a phytotoxin known as rhizoxin. Application of the pure compound induces an abnormal swelling of rice seedling roots, the typical symptoms of the plant disease at nano-molar concentrations. It has also attracted considerable interest as a potential antitumor agent due to its antimitotic activity in various tumor cell lines (Tsuruo *et al.*, 1986). In an interesting breakthrough, it was shown that rhizoxin is not biosynthesized by the fungus itself, but by the endosymbiotic bacteria of the genus *Burkholderia*. The unexpected findings unveiled a remarkably complex symbiotic–pathogenic relationship that extends the fungus–plant interaction to a third key-player (Partida-Martinez and Hertweck, 2005). Similarly, the endosymbiont, *Burkholderia endofungorum* was found to produce rhizonins which are cyclopeptides and were wrongly regarded as mycotoxins (Lackner *et al.*, 2009). Later, it was reported that in the absence of the endosymbionts, the host, *R. microsporus* is not capable of vegetative reproduction. Formation of sporangia and spores is restored only upon reintroduction of the endobacteria (Partida-Martinez *et al.*, 2007). This showed a complex interaction which broadens the scope of plant–fungal associations leading to its third component.

As rhizoxin posesses potential antimycotic activity against a number of eukaryotes including animals, vertebrates and fungi by targeting their β-tubulin gene, the host *R. microsporus* must be resistant to the toxin produced by its endosymbiont (Tsuruo *et al.*, 1986, Partida-Martinez and Hertweck 2005). Interestingly, several fungal groups are resistant to this toxin (Iwasaki *et al.*, 1984; Takahashi *et al.*, 1987). The study to unfold the resistance of rhizoxin was successfully carried out by the analysis of 49 β-tubulin sequences obtained from 36 different fungal species belonging to Ascomycota, Basidiomycota and Zygomycota. The selected rhizoxin resistant fungal species were consequently subjected to analysis and alignment of β-tubulin sequences which inferred that besides the presence of amino acids Ile-100 and Val-100, Ser-100 and Ala-100 also conferred resistance against the toxin. Further studies confirmed the residue 100 as the hotspot playing a major role in the rhizoxin resistance. The evolution of rhizoxin resistance is an important key to maintain a stable association between the host and its partner (Schmitt *et al.*, 2008). Also, type 3 secretion system of the endosymbiont was found to be a crucial component for the foundation of the symbiotic relationship (Lackner et al., 2011).

The whole structure of lipopolysaccharides (LPS) from endobacteria *B. rhizoxinica* indicated that the O-antigen was an important constituent for a stable association (Leone *et al.*, 2010). Bacterial cell surface polysaccharides are classified generally into two major groups; those that are bound to the cell wall as a capsule, exopolysaccharides (EPS) and others attached to the lipid A, lipopolysaccharides (Weiner *et al.*, 1995). EPS are secreted to the surrounding environment and accumulated on the cell surface whereas LPS are anchored on the cell membrane and help in membrane stabilization (Lepek & D'Antuono, 2005). The EPS was not found to play a significant role in the foundation of partnership between *R. microsporus* and *B. rhizoxinica* (Uzum *et al.*, 2015). The exploration of the biosynthesis of rhizoxin by the endobacterial partner, *B. rhizoxinica* led to the finding that the enzymes required to produce 2,3-oxirane ring of the precursor in the rhizoxin biosynthesis pathway was contributed by the host fungus (Scherlach et al., 2012). Thus, both the partners play an important role in the biosynthesis of this phytotoxin.

A novel endohyphal bacterium, **Mycoavidus cysteinexigens** *gen. nov. associated with* **Mortierella elongata**

Sato and co workers in their study to screen and analyze the strains of soil fungi producing nitrous oxide, found the presence of an intra-mycelial bacterium (Sato et al., 2009). They further explored the presence of endobacteria in ten fungal strains identified as *Mortierella elongate*. 16S rRNA gene sequences generated from the DNA of the fungal strains containing the endobacteria showed that all the sequences were closely related to that of *Candidatus* Glomeribacter gigasporarum belonging to Burkholderiaceae. A negligible amount of bacterial endotoxin was quantified in the endobacterium-negative fungal strains as compared to positive fungal strains. No remarkable correlation was found between the presence of endobacteria and N_2O production (Sato et al., 2010). Since the previous attempts were unsuccessful to isolate the endobacterium by the use of conventional media, they employed whole genomic sequencing analysis to evaluate their metabolic requirements. The crucial genes involved in the cysteine biosynthesis, as well as the glycolytic pathway genes, were missing in these bacteria. Thus, by using cysteine supplemented media they successfully isolated the endobacterium. The characterization of their 16S rRNA gene sequences revealed that the endobacteria forms a distinct clade in the family Burkholderiaceae which is named as *Mycoavidus cysteinexigens* gen. nov. (Ohshima et al., 2016).

In continuation to this, the comparative genome analysis of earlier known endobacteria, *Burkholderia rhizoxinica* HKI 454T and *Mycoavidus cysteinexigens* B1-EBT, isolated in Japan, and *M. cysteinexigens* Mc-AG77, isolated in the USA was performed (Tsuruo *et al.*, 1986; Partida-Martinez *et al.*, 2007; Sato et al., 2010). Interestingly, the genomes of both the endosymbionts B1-EBT and Mc-AG77 lacked the genes for chinolytic enzymes (chitinase, chitosanase) which may facilitate the entry of bacteria into the cells of the fungus. However, there are many proposed toxin-antitoxin systems along with an insecticidal toxin complex and a PIN domain required for the controlled growth of the endobacterium while colonization (Sharmin et al., 2018).

An endosymbiotic association of a fungus and a cyanobacterium: **Geosiphon pyriformis & Nostoc punctiforme**

A very rare association has been reported between *Geosiphon pyriformis* and *Nostoc punctiforme*, which is the only known unique association between a fungus and a cyanobacterium (Knapp, 1933; Kluge *et al.*, 2002). *G. pyriformis* is a monotypic species belonging to this genus. This species is characterized by the formation of arbuscular mycorrhiza and it forms unicellular, multinucleated bladders containing cyanobacteria. *N. punctiforme* cells are not enclosed by fungal cell walls but they live freely in the cytoplasm of the host (Schüssler, 2002).

Nostoc cells fix carbon dioxide into organic materials and supply sugars. Additionally, under certain conditions they fix N_2 which serves as a source of nitrogen compounds to the host. *Nostoc* is also benefitted by the host as it can supply mineral nutrients like phosphate and carbon dioxide. The stable association between both the partners is because of the supply of sugars by cyanobacteria, and essential products required for the photosynthesis produced by the host, *Geosiphon* (Schüßler, 2012). However, the mechanism behind the exchange of metabolites between the two partners is poorly explored.

An endobacterium of the arbuscular mycorrhizal fungus, Gigaspora margarita: Candidatus Glomeribacter gigasporarum

Bianciotto *et al.* (1996) reported the presence of prokaryotic rod shaped cells in the spores of *Gigaspora margarita* isolate BEG 34. It was also confirmed by electron microscopy and by using the bacterial counting kit (Molecular probes). Bacterial counting kit helps to accurately enumerate bacteria in a sample by the use of flow cytometery. The sample is diluted, stained with high affinity nucleic acid stains, mixed with a fixed number of microspheres, applied to a flow cytometer and the density of bacteria in the sample is determined from the ratio of bacterial signals to microsphere signals in the cytogram. The 16S RNA genes were amplified using universal prokaryotic primers from the spore DNA of *G. margarita*. The sequence analysis indicated that BLOs associated with it were closely related to the genus *Burkholderia*, a member of the *β-Proteobacteria*.

In order to explore whether this association was a common feature of the family Gigasporaceae, Bianciotto and coworkers (2003) studied 11 isolates belonging to *G. margarita, G. rosea, G. gigantean, G. decipiens, Scutellospora persica* and *S. castanea*. It was reported that all of them were associated with intracellular bacteria except *G. rosea* and their morphology was similar except for BLOs of *G. gigantean,* which were smaller and round in shape.

The properties of these uncultured microorganisms were recorded under *Candidatus* and it was concluded that the endobacteria of *G. margarita*, *S. persica* and *S. castanea* belong to a single taxon with similar morphology. The cytoplasmic stability of the endosymbiont in their host fungus and their transmission during fungal reproduction demonstrated that a vertical transmission occurs through the sporulation of the arbuscular mycorrhizal fungus as active bacterial proliferation took place in the fungal mycelium. These associated bacteria were found to be obligate endocellular partners of their host fungi. The EHB of *G. margarita* enhances the fungal sporulation, raises the fungal bioenergetic capacity by increasing ATP production, and elicits mechanisms to detoxify reactive oxygen species, thus playing an important role in host metabolism, reproduction and stress tolerance (Salvioli *et al.*, 2016).

Mollicutes *related endobacteria (Mre)*

In the study to identify the BLOs and to explore their distribution among the members of the Glomeromycota, a diverse inventory of 28 cultured AMF belonging to four continents was assessed. It was reported that the BLOs live within fungal cytoplasm and that divergent but monophyletic bacterial lineages coexist in single spores. They are identified as a novel bacterial taxon clustering with the *Mollicutes* and suggested as sister to a clade encompassing the *Mycoplasmatales* and *Entomoplasmatales* within *Mollicutes* (Naumann et al., 2010).

To find out an answer to whether *Mollicutes* related bacteria are associated with wild AM fungi, three species of liverwort were taken in a study. The 16S rDNA sequences of endobacteria associated with the wild liverworts clustered with the sequences of *Mollicutes* depicting the presence of novel phylotypes. Further, by using specific fungal and bacterial probes in FISH, it was seen that *Mollicutes* related endobacteria (Mre) reside within the cytoplasm of the AMF hyphae, isolated from the liverworts (Desiro et al., 2013).

Since AMF harbor two groups of endobacteria, *Candidatus* Glomeribacter gigasporarum (CaGg) and *Mollicutes* related endobacteria (Mre), therefore a study was carried out to analyze their distribution pattern and coexistence among their AMF hosts. It was found that an individual AMF can harbor both the types of endobacteria *viz.* CaGg and Mre, and that the Mre are more abundant, variable and prone to recombination as compared to CaGg (Desiro *et al.*, 2014).

Earlier studies were focused on the isolation of Mre from Glomeromycota but recently an attempt was made to explore the association of Mre as endosymbionts associated with Mucoromycotina. Out of 29 *Endogone* spp., 13 were found to be associated with Mre and the cluster analysis of these with the earlier reported Mre from Glomeromycota formed a separate clade (Desiro et al., 2015). All these findings provide new opportunities to explore the ecological role of endosymbionts and their underlying mechanisms. Molecular evolution patterns of Mre residing within AMF revealed that the diversity of Mre are divergent within the individual AMF but there is not much differentiation between Mre associated with AMF from different continents. Mre may be parasites of AMF Glomeromycota and this stable association is the outcome of a combination of both the transmissions, vertical and horizontal. However, this inference left an unanswered question that whether this association shifts repeatedly between antagonism and mutualism (Toomer et al., 2015).

Candidatus Moeniiplasma glomeromycotorum was isolated as a novel endobacterium associated with AMF, Glomeromycotina. Based on 16S rRNA gene sequencing, this endobacterium resembles with *Candidatus moeniiplasma* but phylogenies of 19 genes showed similarities with the members of the family Mycoplasmataceae (Naito et al., 2017).

In a recent study, 12 out of 394 Mortierellomycotina strains were found to be associated with Mre representing new bacterial phylotypes. This study concluded that the association can occur within fungi across Mucoromycota. It was also confirmed that Mre could be antagonistic to their hosts and adapt to a non-lethal lifestyle (Desiro *et al.*, 2018).

3. EHB associated with Basidomycota

***Intracellular bacterium inside the mycelium of the ectomycorrhizal fungus, Laccaria bicolor* S238N**

In the earlier reports, EHB were known to be associated with Mucoromycota and Glomeromycota. The first report of association of EHB with an ectomycorrhizal fungus is that of *Laccaria bicolor* belonging to Basidomycota. *L. bicolor* S238N was known for its commercial use to inoculate seedlings of Douglas fir in French nurseries in order to obtain high-performance planting material for reforestation. However, recurrent bacterial proliferations were observed in cultures grown in fermentors. So this prompted the exploration of the presence of intracellular bacteria residing within the hyphae of *L. bicolor*. The investigation illustrated the presence of a bacterium, within the hyphae of *L. bicolor* S238N, which was identified as *Paenibacillus* sp. by FISH analysis with a specific 16S rRNA-directed oligonucleotide probe, and was clearly found localized inside the hyphae (Bertaux *et al.*, 2003). The endosymbionts inhabited both live and dead cells of their host. However, the role this bacterium in the ectomycorrhizal symbiosis is not clear.

Novel intracellular nitrogen fixing association between a fungus and a bacterium

A pathogenic fungal strain *Ustilago maydis* was found to have the capability to grow on nitrogen free media, inferring its ability to fix atmospheric nitrogen. This surprising finding led to the assumption of the presence of a nitrogen-fixing endosymbiotic bacterium within this fungus. Consequently, the presence of an intracellular bacterium was confirmed by the amplification of bacterial 16S rRNA and nifH genes. The EHB was identified as *Bacillus pumilus* which is a Gram positive free-living N_2 fixing bacterium. The ability of the fungus *U. maydis,* harboring the bacterium, to fix nitrogen was confirmed by nitrogenase activity and ^{15}N incorporation into the cells (Ruiz-Herrera *et al.*, 2015).

***The endosymbiont of* Piriformospora indica*; *Rhizobium radiobacter**

In an attempt to illustrate the stable association of bacteria with fungal species belonging to the order *Sebacinales*, it was reported that *Piriformospora indica* DSM 11827 was found to be symbiotically associated with *Rhizobium radiobacter* strain F4 (RrF4) (Sharma *et al.*, 2008). It was suggested that this association is not only complex but also the affinity of these symbionts towards each other includes key traits in bacterial-*Sebacinales* interactions. The bacteria–fungi associations belonging to *Sebacinales* were diverse with three distinct genera (*Paenibacillus*, *Acinetobacter* and *Rhodococcus*) associated with *S. vermifera* whereas *R. radiobacter* was only found to be associated with *P. indica* (Guo *et al.*, 2017). Furthermore, the isolation of *R. radiobacter* from *P. indica* and subsequent axenic cultivation was successful, and the attempts to obtain bacteria-free clones failed repeatedly. Many strategies were employed so as to break the association, like high antibiotic concentrations, *in vitro* single spore cultivation and exposure to antibiotics in the regenerating medium. However, none of these were successful whereas surprisingly, in the presence of antibiotics, the fungus grew slowly presumably due to the inhibitory effects on the bacterial growth, but the number of bacteria was similar as found in untreated *P. indica*. Therefore, the bacteria may be protected inside the fungus or conversely absence of the bacterium may be reducing the fitness of the host fungus (Glaeser *et al.*, 2016). Further, the factors responsible for influencing the load of endobacteria under different growth conditions of the host fungus were analyzed and it was observed that the endobacterium, *R. radiobacter* improves the fitness of *P. indica* and thus contributes to the success of the tripartite *Sebacinalean* symbiosis.

4. EHB associated with Ascomycota

Diverse bacteria within hyphae of endophytic fungi

Hoffman and Arnold (2010), for the first time, reported the presence of phylogenetically diverse endobacteria within the hyphae of endophytic fungi. The occurrence was explored in the Ascomycetous endophytic fungi which included numerous phyto-pathogens, saprotrophs and pathogens. They have provided strong evidence indicating that this association is widespread. Also, the occurrence of endobacteria was examined using Live/Dead straining (a mixture of SYTO9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide) and confirmed by FISH analysis. Fluorescent *in situ* hybridization (FISH) is a molecular technique employing fluorescent probes that bind to specific position of the chromosome with a high degree of sequence complementarity; for endobacteria, a specific probe (a universal 16S rRNA gene oligonucleotide probe) is usually used to confirm the presence within the fungus.

Further, Arendt and co-workers (2016) explored the effects of endobacteria on the host fungus, *Pestalotiopsis neglecta,* an endophyte of *Cupressaceae sempervirens*. The growth rate of the fungus was found to be independent of the presence of endobacteria characterized as *Luteibacter*. They were able to isolate the bacteria in axenic culture successfully. The endophyte with bacteria produced indole acetic acid (IAA) in higher quantities as compared to the fungus without the bacteria. Later, they successfully reintroduced the endohyphal bacteria (EHB) into the host fungus. Thus, the third component of the plant–fungal association opens up many doors yet to be explored.

Similarly, Pakvaz and Soltani (2016) explored the occurrence, diversity and bioactive potential of diverse endosymbiotic bacteria associated with fungal endophytes of *Cupressaceae sempervirens*. Almost, 31% of the fungal endophytes isolated from *C. sempervirens* harbored endohyphal bacteria.

It was illustrated that a single fungal culture harbors a single bacterial species. Further, *Bacillus subtilis* was isolated as an endophyte as well as endohyphal bacteria by both the groups. So it was not clear how a single genotype of bacteria can together adapt both the life styles. The endohyphal bacteria associated with fungal endophytes of cypress were found to have potential bioactivity. Further investigations may lead to the discovery of novel bioactive molecules associated with this third level of association.

5. How bacteria invade the fungal cells?

Despite the growing number of reports of fungal endobacterial symbionts, there is a manifest lack of knowledge about the direction and active mechanisms that allow their integration with a fungus or entry into the fungal hyphae, where the fungus is left intact to serve as a host for the endobacterial symbiont (Lackner *et al.*, 2009; Frey-Klett *et al.*, 2011). The key striking feature of prokaryotes is the transport of proteins from the cytoplasm into the environment or other compartments of the cell through protein secretion and it is important for growth and an array of processes. A number of bacterial secretion systems are known depending on whether the protein substrates need to cross single or more phospholipid membranes based on the association. Many reports in the recent times have depicted the manner in which microorganisms enter into the host cell, suppressing its defenses, usually due to the bacterial secretions (Table 1).

Gram negative bacteria depend on firm secretion systems to transport protein or protein substrates into the cytoplasmic membrane of eukaryote or a prokaryote target cell directly from the bacterial cell. It is a complex task for Gram negative bacteria to secrete extracellular proteins since these must pass through two phospholipid membranes. Two separate steps are followed by some proteins secreted by Gram negative bacteria to traverse these membranes, in which they enter into the periplasm via the Sec- or Tat-systems and then pass through the outer membrane by another transport system (Green and Mecsas, 2016). Also, many proteins are secreted via Sec- or Tat-independent secretion systems. The firm protein secretion system employed by Gram negative bacteria includes Type I (T1SS) through Type VI (T6SS) secretion systems, including distinct set of proteins to be transported. Due to basic differences in cell wall structure of Gram negative and Gram positive bacteria, it is not surprising that they differ in their mechanism of secreting extracellular proteins. Similar to Gram negative bacteria, Gram positive bacteria also involve both Sec- and Tat-protein secretion systems. Additionally, few Gram positive bacteria employ a distinct secretion system (T7SS) to transport some proteins across the membrane and possibly through the cell wall (Green and Mecsas, 2016).

Moebius *et al.* (2014) reported the genomics- and proteomics-driven discovery of a new bacterial fusion process that includes the secretion of chitinolytic enzymes. Furthermore, they presented the first electron microscopic snapshots of the actual infection process. In light of the fact that chitosan is the dominant component of the Zygomycete cell wall (BartnickiGarcia and Nickerson, 1962), it is surprising that only the chitinolytic enzyme plays a significant role in the active intrusion of bacteria into the fungal cells. To establish an intimate association, the physical contact between the endobacterial partner and the host fungus should take place at the right time and at the right place, in addition to some other factors (Bright and Bulgheresi, 2010). Subsequently, they identified two molecular mechanisms; a type two secretion system (T2SS) and secretion of chitinase that were employed in the interaction and infection. They suggested that the secretion of extracellular enzyme chitinase and the translocation of the associated proteins was done by T2SS which in turn softens the cell wall of fungi helping the bacteria to invade into the host fungus. This finding may act as the model for several other symbionts and provide the stage for exploration of mechanisms involved in this interaction.

6. Shift from antagonism to mutualism between bacterial–fungal associations

The recent exploration of the mutual association of bacteria with fungi questioned their earlier known antagonist relationship. So as to seek an answer, Lastovetsky and coworkers (2016) studied the unusual association between *Rhizopus microsporus* and its bacterial endosymbiont *Burkholderia*. This symbiosis is gaining much attention and acting as a model to understand the underlying molecular mechanisms as both the partners can be easily manipulated *in vitro* (Partida-Martinez and Hertweck, 2005). The rice seedling blight disease causing agent *Rhizopus* harboring *Burkholderia* becomes resistant to the toxin as a consequence of mutations in its tubulin sequence (Schmitt *et al.*, 2008). Therefore, it is inferred that this symbiosis shifts from antagonism to mutualism (Leon *et al.*, 2010) and the transition is assisted by novel mechanisms including the changes in steps of lipid metabolism by the host. The host and its endobacteria are in a mutualistic relationship, when the lipid metabolism and phosphatidic acid

Table 1 – Overview of major secretion systems in bacteria.

Secretion System	Components	Steps Involved	References
Sec independent			
Type 1	Tripartite System; ABC, Adapter protein and Outer membrane pore	Single step mechanism	Omori *et al.*, 2003
Type 3	Needle complex or injectisome	Single step mechanism	Cornelis *et al.*, 2000
Type 4	homologous to conjugation machinery of bacteria	Two step mechanism	Fronzes *et al.*, 2009
Sec dependent			
Type 2	Multiprotein machinery including general secretory proteins (GSPs)	Two step mechanism	Filloux *et al.*, 2004
Type 5	Two-partner secretion systems including autotransporter proteins	Two step mechanism	Henderson *et al.*, 2004
Type 6	IM proteins and tail complex consisting a tail sheath, an inner tube and a baseplate	Multi step mechanism	Costa *et al.*, 2015 Gallique *et al.*, 2017

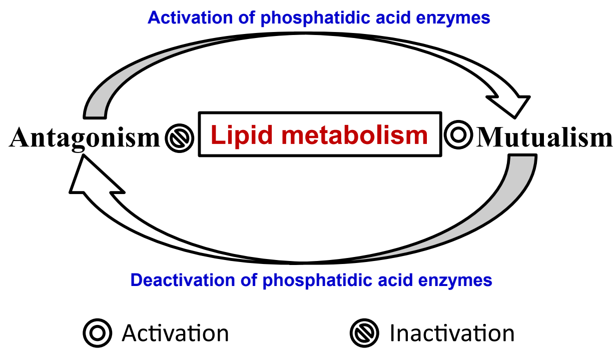

Fig. 2 – Leveraging the symbiotic relationship: The host and its endobacteria are in a mutualistic relationship when the lipid metabolism (LM) and phosphatidic acid (PA) producing enzymes are activated. In contrast, they act as antagonists when the PA producing enzymes are inactivated (Lastovetsky *et al.*, 2016).

producing enzymes are activated. In contrast, they act as antagonists when the phosphatidic acid producing enzymes are inhibited (Lastovetsky *et al.*, 2016) (Fig. 2).

7. Conclusion

Recent studies on "fungal-bacterial" associations, wherein the bacteria reside within the fungal hyphae, unfolded a novel chapter in microbial ecology. These associations are more common and important than previously thought. Moreover, many of these associations are central to agriculture, forestry, and bioremediation. Whereas few of these unique symbioses have been studied to a significant level, many still remain unexplored due to the microscopic scale of the associating partners, the complexity of their communities and the intricate nature of the relations that connect them. Hence, it is imperative to develop approaches allowing for new discovery and characterization of novel links between fungi and their endohyphal bacterial partners.

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Conflict of interest

The authors declare that they have no conflict of interest.

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