Chapter

Endophytic Fungi: Taxonomy, Isolation, and Current Applications

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Abstract

This chapter is a revision of the realm of endophytic fungi, focusing on how to approach the study of their biodiversity in relation to distribution, richness, host plant defense mechanisms, chemistry, and metabolomic profiles. We will revise the current isolation and identification methods available such as culture-dependent techniques and omics approaches including both targeted and functional metagenomics, and their comparison to give a holistic view of the endophytic fungal biome. Additionally, we will discuss their biotechnological potential for the production of bioactive natural products to be applied in plant protection (against nematodes, fungi, and insect control), soil restoration, or disease control practices based on microbial antagonists.

Keywords: fungal endophytes, omics, bioinformatics, natural products, plant protection

1. Introduction

An endophytic microorganism is commonly defined as a bacterium or fungus that spends most of its life cycle colonizing intra- or intercellularly the inner tissues of a given host plant, without causing apparent disease symptoms [1]. These organisms are present in all terrestrial plant species tested up to date and are considered ubiquitous and omnipresent throughout the whole plant kingdom. The diversity of this group of microorganisms can be remarkable; employing culture-dependent techniques, more than 100 endophytic fungal taxa per plant individual have been identified in some plant hosts, while this diversity can increase between 1 and 1.5 orders of magnitude when more resolution approaches related to the use of high-throughput sequencing methods are used in its characterization [2]. Another factor that influences the great diversity observed in endophytic fungal communities is geographical variation. It has been observed that species isolated from the same plant hosts tend to be different between different localities [3, 4]. The age of the plant host also has a certain effect on the composition of endophytic diversity. As the host lives longer, the exposure time to the fungal inoculum increases, accumulating a greater number of endophytic microorganisms in its tissues. It has been shown that the longest lived parts of a given plant species can harbor more endophytes than new tissues [5].

Another related concept with high biotechnological potential is the so-called chemical diversity that this type of fungal communities can harbor, as a consequence of their particular lifestyle and role played in the ecosystems in which they thrive [6]. This chemical diversity makes these species relatively unexplored sources of new natural products for application in medicine, agriculture, biotechnology, or industry [7]. Additionally, this endophytic fungal diversity has allowed in numerous studies the characterization and exploitation of microbial antagonists useful in conservation biological control programs within the framework of Integrated Pest Management approaches [8].

It is well known that some of the physiological and ecological roles played by endophytic fungal communities have to do with increasing the fitness or biological efficiency of the hosts they colonize, due to the production of certain functional metabolites. Like mycorrhizal species, endophytes interact with their hosts by promoting their growth, eliciting their response to certain environmental stresses (humidity, temperature, salinity, etc.), increasing their ability to resist attack by herbivores, or against infection by other pathogenic species of fungal origin [9]. In this sense, the ability of endophytic fungi to influence the interactions of host plants with their environment, as well as to modify their interaction processes with other pathogenic organisms, has been known for some time. In the last 10–20 years, numerous studies have shown that endophytic microorganisms can play a substantial role in the outcome of the different plant-pathogen interactions preceding the onset of the disease [10]. In this sense, various mechanisms have been described by which this type of fungi can prevent and/or limit the development of pathogens. For example, some endophytic species can induce systemic resistance and defense mechanisms against the attack of certain pathogens in their host plants [11], or even produce antibiotics capable of inhibiting the growth of other fungi [12]. Furthermore, endophytic species may eventually compete for space and nutrients with pathogen populations [13]. Finally, another key aspect of the biology of this type of organism is that some plant pathogens can behave as endophytes at some point in their life cycle [14]. This question is of great interest for the study of plant infection phenomena. Among all the fungal taxa that can penetrate and colonize the tissues of a given host, only a small proportion of them, the pathogens themselves, trigger pathogenesis.

The present work deals with different aspects of this large type of microorganism, from certain general aspects such as the definition and characteristics of this lifestyle, some milestones in the history of their research, or key aspects of its biology and distribution, going through a review of the different approaches and methodologies usually used in its sampling, isolation, and characterization, to finally review some of the main practical applications derived from its knowledge and exploitation, including human and animal medicine or industry, with special emphasis on sustainable approaches in agroecosystems (natural products, biological control agents, etc.).

2. Endophytic fungi: definition, main characteristics, infection mode

Fungi are widespread organisms with the ability of growing in multiple niches. Regarding their lifestyle, fungi can be divided in different groups: saprobes (growing on dead or dying material); pathogens (mainly infecting animals or plants); symbiotic (in beneficial interaction with other organisms); epiphytes (living on the surface of plants); or endophytes (inhabiting plants with no sign of harm or disease).

This chapter will provide an overview of key aspects regarding endophytic fungi. The main endophytic organisms are mostly bacteria and fungi, and in some cases, archaea and some protists [15]. Endophytic fungi are considered to be a polyphyletic group, but the genera and species encompassed in it can also be found among the aforementioned, including some that are traditionally considered pathogens or parasites. In addition to the classic definition of endophyte organisms, it is noteworthy to mention that in some cases, endophytic fungi may also be beneficial for the host plant. Thus, endophytic fungi can alter and increase the production of phytohormones, resulting in plant growth promotion and/or defense against pathogens. For instance, lettuce plants inoculated with an endophytic strain of *Penicillium pinophilum* aid in enhancing protection against *Verticillium*, resulting in a higher growth rate and reduced visible symptoms in leaves [16]. In another example, inoculation with two endophytes led wheat to induce systemic response and subsequent resistance to *Rhopalosiphum padi,* increasing the upregulation of defense-related genes and the content of jasmonic acid and other hormones and metabolites [17]. In some cases, during *in vitro* experiments, endophytic fungi do not show direct antagonistic effects against fungal phytopathogens, but their presence *in vivo* may prevent the infection from other fungi due to the occupancy of the same niches inside the plant [18, 19]. Since endophytes do not present any threat to plants, they are presented as a potential method in Integrated Pest Management (IPM) to develop more sustainable agricultural practices [20].

2.1 Transmission of endophytes

There are essentially two modes of transmission of fungal endophytes: vertical and horizontal. Vertical transmission refers to the transmission from the original plant to its descendance through the seed, while horizontal implies transmission either through soil, air or vectors, or through the pollen of another plant hosting this fungus [21]. In general, vertical transmission is less common, with most of the studied examples in the genus *Epichlöe* and its narrow relationship with tall fescue grasses [22, 23]. Nevertheless, this phenomenon is not exclusive to this genus, and it can be found in other endophytic fungi such as *Alternaria* and *Cladosporium,* among others [24, 25]. This transmission can be modulated by some factors that can be determinant for the process. Thus, plant genotype seems to play a major role, promoting the proliferation of certain taxa and/or communities on seeds and seedlings augmenting selective pressure for some species in favor of others [24]. Moreover, vertical transmission of endophytes seems to be important to reduce the presence of phytopathogenic fungi in seeds and plants. However, there are other secondary factors that can also modulate vertical transmission and settlement of fungal communities, such as host defense mechanisms [26], temperature [27] or water availability [28]. Regarding horizontal transmission, it is the most common way of endophyte dissemination into other plants. From an ecological point of view, horizontal transmission is very important to establish symbiotic relationships with other plants. While vertical transmission is only disseminating endophytic fungi through a genetic line, horizontal transmission can help their dispersion to wider areas or whole ecosystems, inside the same or different species [29]. Horizontal transmission might contribute to increased plant fitness and stress resistance in whole local populations such as forests or grasslands [30]. Apart from the natural ways of dispersion of fungi (soil, air, etc.), their dissemination is sometimes mediated by external vectors (i.e., insects), whose digestive tract can contain a diverse mycobiome that sometimes includes endophytic species that can pass to new plants during herbivory [31]. Some researchers hypothesize that the pollen microbiome may play a role in maintaining stable microbiomes within plants of the same species. Exploring its potential impact on the presence of plants across various ecosystems offers promising opportunities for future investigation in microbial ecology and how it can shape plant communities [32]. In any case, both strategies of transmission, vertical and horizontal, can frequently be found at the same time in the same fungal species, increasing its possibilities to disseminate through entire populations in an ecosystem [33].

2.2 Discovery and importance of endophytic fungi: *epichloe* and *Serendipita indica* as cases of study

Endophytes are very important for the development and fitness of the host plants. Thus, endophytic fungi may confer adaptability, protection, and enhanced nutrients intake to the plants they inhabit [34]. Probably, one of the first and most studied cases of fungal endophytes is the case of the genus *Epichlöe* (formerly *Neotyphodium).* One of the first hints of the beneficial relationship between endophyte and its host was documented in 1977 by Bacon et al. [35]. This studio was connecting the presence of *Epichlöe typhina* in tall fescue grasses with the diseases in cattle after consuming plants infected by this fungus. Subsequent studies determined that the toxicity was associated to the presence of multiple alkaloids inside of the plants, among other secondary metabolites, that had been produced by some fungi [36–38]. Later, some works determined that grasses infected with these fungi were predominant in pasture and outcompeted non-infected plants [39]. Additionally, some studies have connected the higher resistance of some plants to pests and diseases to their infection with *Epichlöe* and its secondary metabolites*.* For instance, one of the main compounds derived from this interaction, N-formoline, is toxic to insects. For example, decreased *in vitro* survival rate of *Stenotus rubrovittatus* in the presence of this compound [40], or the decreased survival of *Trigonotylus caelestialium* when this compound is present in plants, even at low doses [41]. A wide range of metabolites produced by these fungi can protect the plant against insect pests [42]. Apart from insects, *Epichlöe* has also been reported to reduce significantly the presence of other pathogens. For example, strong nematicidal effects have been observed against *Pratylenchus scribneri,* inducing a significant decrease in nematode population when the fungus was inhabiting the plant [43]. Similarly, other authors noted a decrease in *Meloidogyne javanica* infection in plants colonized by this fungus [44]. Furthermore, several authors have reported toxicity in some metabolites derived from *Epichlöe* against fungal phytopathogens such as *Phytophthora infestans* [45–47]. It has also been observed that some fungi that colonize plants are able to influence the bacterial microbiome of the soil where they develop [48, 49], being a potentially useful tool to reduce pathogenic microorganisms from the soil.

The root endophyte *Serendipita indica* (formerly *Piriformospora indica)* has been extensively proven to confer tolerance to biotic and abiotic stresses to its host plants. *Serendipita indica* can affect the pattern of production of phytohormones and plant secondary metabolites [50] which trigger and increase the plant response to biotic and abiotic stresses. For example, the colonization by this fungus improves *Arabidopsis thaliana* growth under salt stress, through the modulation of the expression levels of the main Na + and K+ ion channels [51]. Other study describes and characterizes two ENA ATPases proteins in *S. indica* involved in the molecular mechanism of tolerance against high salinity [52]. On the other hand, the inoculation of *Pinus taeda* plants with this fungi enhanced its tolerance to drought, changing the metabolome and proteome of the plant and increasing the protein content of the leaves and of several metabolites such as suberine and 2-oxocarboxylic acid among other flavonoids and organic acids that protect the plant from drought stress [53]. Besides this, *S. indica* colonization has been reported to increase nutrient uptake. Thus, in tobacco, it has been demonstrated that *S. indica* can enhance nitrogen uptake by inducing the expression of ammonium and nitrate transporter genes, promoting plant growth [54]. Similarly, the fungal colonization increases phosphorus intake, due to the transport to the host plant mediated by high affinity phosphate transporters [55], magnesium [56], zinc [57], or iron [58]. Conversely, *S. indica* reduces the accumulation of boron [59] and arsenic [60, 61] among other heavy metals that can be harmful to both plants and humans. In addition to the ability of inducing tolerance in host plants against biotic and abiotic stress, *S. indica* has also been proven as a potential tool for the biocontrol of insect pests. Thus, its presence in onion plants significantly reduced (>50%) the damage caused by *Spodoptera exigua,* promoting the defensive response through the upregulation of genes and routes related to defense and oxidative stress [62]. In the same way, *S. indica* enhanced herbivory resistance to *Cnaphalocrocis medinalis* through higher production of jasmonic acid and mitigation of oxidative stresses [63]. Furthermore, *S. indica* has been successfully applied in the biocontrol of other plant pests and pathogens such as root-knot nematodes or fungi. For example, root colonization by this fungus promotes cucumber tolerance to *Meloidogyne javanica* by modulating photosynthesis and salicylic hormone levels [64]. Several examples can also be found for biocontrol of fungi, for example, against some common pathogens such as *Verticillium dahliae* or *Fusarium graminearum* [65, 66].

In addition to the aforementioned examples, many other can be found in literature about endophytic fungi that are also known to improve the fitness and tolerance of the host plants to biotic and abiotic stresses: osmotic pressure due to salinity [67]; to heavy metals [68–70]; to drought [71–74]; to temperatures [74, 75]; to nutrient deficiency [76, 77] and to pests and pathogens [16, 17, 78].

2.3 Diversity of endophytic fungi

Based on the ratio of endophytes found in vascular plants, approximately up to 1 million fungal species are estimated to exist [79] but other authors set this number in up to 12 million [80]. Moreover, with the development of NGS methods (discussed later in this review), particularly shotgun metagenomics, numerous new species are being described as endophytes every year, making predictions far from accurate at this moment. Notwithstanding, the study of diversity of endophytic fungi can be challenging, especially due to the difficulty to determine if some species are real endophytes or latent pathogens. Additionally, classifying endophytes can be complex, requiring a multifaceted approach to their biology [79]. Latent pathogens are fungi that may become pathogenic at certain stages of a plant's development but remain harmless for most of the time. Often, additional pathogenicity assays are required to determine whether an isolated fungus is endophytic or pathogenic [81, 82].

Some of the most extended fungal pathogenic species along most ecosystems have an equivalent endophytic strain that is only not harming the plants they inhabit, but their presence is favorable to their host. Several cases of this phenomenon can be found in literature. For instance, *Fusarium* species infect up to 120 horticultural plants [83], causing wilt disease and head blight disease, among others. It is a soil-borne fungus spread in multiple agrosystems worldwide and its infection and mycotoxins cause important crop and economic losses [84–86]. There have been described several strains of *Fusarium spp.* with beneficial effects on its host: *Fusarium sp.* isolates from *Withania somnifera* were reported to display growth promotion effects on tomatoes in addition to disease suppression ability against other pathogenic *Fusarium* strains [87]. *Fusarium* is also one of the main pathogens in bananas, but when the effects of the application of non-pathogenic *F. oxysporum* strains were studied, banana crop yields were enhanced along with a reduction in the populations of phytopathogenic nematodes in soil and their damage on the plants [88]. Another example of fungi considered a pathogen displaying endophytic behavior is *Verticillium.* Species of this genus also cause important economic losses in agriculture every year [89]. The strain Vt305 of *V. isaacii* demonstrated to be effective in controlling wilt disease caused by *V. longisporum*, reducing severe disease symptoms in both greenhouse and field conditions [90]. Differences in endophytic/pathogenic behavior of *V. dahliae* have been studied, finding that this species can be found as an endophyte or a pathogen depending on its host, suggesting a possible process of speciation based on endophytic specialization from pathogenic lines [91]. Additionally, several other examples of traditional pathogens with endophytic behaviors have been documented for several fungi genus such as *Alternaria* [92]*, Colletotrichum* [76, 93], and *Acremonium* [94, 95], among many others.

Most endophytic fungi are considered generalists, capable of inhabiting a wide range of host plants, whereas there are relatively fewer fungi that are specialized to specific host plant species. Certain species and genera of fungi frequently appear and commonly form an important part of the host plant's microbiome. This includes fungal genera such as *Fusarium*, *Trichoderma*, and *Aspergillus*, among others reported in the literature. In broad terms, abundance and diversity of fungi can be studied in several scales. Globally, many environmental factors shape endophytic communities in different hosts and ecosystems [96]. Essentially, the highest diversity and abundance of fungal endophytes can be found in tropical areas (being considered as hyper diverse group), while in colder areas, its diversity and abundance decrease [97]. Furthermore, there is a higher tendency to find greater host-endophyte specificity in Arctic and boreal zones than in tropical and temperate zones [98]. This suggests that generalist endophytic fungi are more likely to be more abundant in warm and temperate zones, while the relative abundance of specialist endophytic fungi tends to increase in colder ones. Furthermore, it is noteworthy that islands and endemic plants may serve as very potential reservoirs of yet undiscovered endophytic fungi with prospective applications [99–101].

When reducing the scale of the study to the host plant, differences can also be observed. Thus, in the different parts of the plants, there are differences in the communities of fungal endophytes. Generally, several authors have observed higher abundance and diversity of fungi species in roots than in aerial parts [102–104]. This is likely attributable to soil being a primary entry point for endophytic fungi into plants, as well as the presence of various fungi that can function both as saprobes in the soil and as endophytes [105]. As a matter of fact, there frequently exists partial overlapping between soil and endophytic fungi communities [106]. However, sometimes, diversity and abundance patterns in host plants are occasionally affected by external factors, that is, infection by pathogens [107].

2.4 Insights in the interaction between endophyte and its host

The interaction between endophytes and plants is not merely the result of a random arrival of the fungus near the plant. Rather, in many cases, it is an interaction driven by bidirectional communication between fungi and plants, often resulting from mutual signaling mechanisms. Given the ability of plants to detect microbe-associated molecular patterns (MAMPs), this interaction can sometimes be promoted by the plant itself for its benefit. For instance, the production of root exudates rich in chemoattractants eases the entrance of endophytes to the plants [108]. In some other cases, to colonize a plant, endophytic fungi alter host metabolism, by decreasing the defensive response or shaping the host metabolism [109–112]. For instance, colonization by some endophytes can modulate gene expression in plants, finding downregulation of some biotic stress and primary metabolism genes [113]. Besides, the production of certain small secreted proteins or SSPs by endophytic fungi seems important to reduce the defensive mechanism of the plants and facilitate host colonization, [114–116]. In fact, in some cases, SSP expression may be found to be higher in endophytic fungi than in pathogenic strains. Additionally, hormone production patterns can also be altered by the entrance of determined endophytic fungi in plants [117]. Plant hormones seem to have a key role in this interaction, particularly abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA). Hu and Bidochka [118] reported ABA as an hormone capable of modulating different plant defensive responses during colonization either endophytes or pathogens. When an endophytic fungus colonized the plant, ABA-related genes were downregulated, while when a pathogenic one did, they were upregulated. Furthermore, in their study, the external application of ABA was used to hypothesize its potential as a useful tool for promoting positive endophyte colonization, demonstrating that ABA treatment decreased endophytic colonization while increasing pathogen colonization when applied to the plants. Similar effects have been demonstrated for SA, where its exogenous application suppressed *Epichlöe* in leaves of ryegrass [109]. In this context, it has been demonstrated that *Epichlöe* is able to suppress the SA pathway and simultaneously increase auxin production. This highlights its ability to manipulate plant hormone pathways and suggests that the ability to shape and suppress plant defense responses is essential for successful endophytic colonization [119]. Additionally, it underscores the importance of defense suppression in the evolution of endophytic fungi and plants, as SA application also reduced the presence of characteristic *Epichlöe* alkaloids in leaves, leading to lower pest resistance.

In addition to alterations in primary plant metabolism during the interaction, Horizontal Gene Transfer (HGT) between fungi and plants is a phenomenon yet to be elucidated. In the past, some authors proposed HGT as a hypothesis to explain why some endophytic fungi were able to produce compounds of their hosts. Several authors have reported the importance of HGT in the evolution of certain organisms. For instance, non-pathogenic strains of *Fusarium* may become pathogenic due to the transfer of chromosomes from pathogenic strains when cultured together [120], and conversely, loss of accessory chromosomes may lead pathogenic fungi to become endophytes [121]. HGT has been observed between bacteria-bacteria [122], fungus-fungus [123], and bacteria-fungus [124], but it is a very rare phenomenon between fungi and plants.

Additionally, in the last years, it has come to attention how mycoviruses could be modifying the genome and behavior of endophytic fungi. The mycovirus (PtCV1) from an endophytic strain of the fungus *Pestalotiopsis theae* conferred high resistance to the host plants against the virulent pathogen *P. theae* strains and reduced the virulence of its fungal host [125]. Similarly, plants colonized by a hypovirulent *Sclerotinia sclerotiorum* strain, with mycovirus (SlaGemV-1), showed a resistant phenotype to virulent *S. sclerotiorum* infection [126]. Therefore, plant immunity induced by endophytic colonization of mycovirus-induced hypovirulent fungal pathogen provides an alternative approach to biological control of plant diseases caused by fungi.

The role of HGT in inter-kingdom interactions remains an area of significant interest and debate, especially due to the absence of knowledge about how some fungi are able to produce compounds of the plants they inhabit. The case of taxol is likely one of the best-known instances of endophytic fungi producing a plant molecule. In 1993, Stierle et al. described the ability of *Taxomyces andreanae*, a fungus isolated from *Taxus brevifolia*, of producing taxol, an important drug used in cancer therapy [127]. There are reports of other taxol-producing fungi, and some authors have hypothesized that HGT could explain this phenomenon [128]. Nevertheless, current evidence does not fully support this hypothesis, as the fungal and plant taxol pathways appear to be a case of convergent evolution [129]. However, there are other cases of HGT between endophytic fungi and plants, such as a ß-1,6-glucanase (an enzyme that degrades fungal cell walls) [130] or the Fusarium Head Blight resistance gene [131]. Additionally, apart from taxol, other plant compounds have been observed to be produced by endophytic fungi [132, 133]. These observations are consistent with the hypothesis and keep it viable for further studies.

3. Isolation and identification of endophytic fungi

In the last five decades, the term endophyte, or more specifically endophytic fungi and many of the variants associated with the concept (endophytic colonization, endosphere, endophytism, etc.) have become more familiar and present in scientific and popular literature, to refer in a precise way to these fungal microbial communities (also for bacterial ones) that, as another type of symbiosis defined as the "coexistence of different organisms," names the lifestyle of those fungi that live asymptomatically in the internal tissues of higher plants. Regarding the different isolation techniques of this type of endophytic microbial diversity, the different methods commonly employed have generally pivoted until now on aspects related to the way of sampling, treating, and surface sterilizing the plant materials of origin, or on the composition and selectivity of synthetic nutritional media used in the management (isolation, sporulation, maintenance, etc.) of these microorganisms. On the other hand, as for other groups of fungi, the identification of different taxonomic entities has long been a laborious task that requires deep multidisciplinary knowledge (anatomy, cytology, histology, nomenclature, etc.), which usually limited speed and resolution of reliable taxonomic determinations. The arrival and generalization toward the end of the 90s of molecular characterization techniques greatly improved the speed of taxonomic determination work, while the phylogenetic affinities existing between the groups of organisms studied were drawn more precisely. More recently, it has been shown that the sequence diversity existing in small DNA fragments of standardized genomic regions allows diagnostic identification in numerous groups of microorganisms (including both eukaryotes and prokaryotes) [134], which today we know it as DNA Barcoding. This approach, based on a standardization of identification systems using these genetic barcodes, has led to greater confidence in the diagnosis and identity of species, facilitating numerous practical applications, including the study of fungal endophyte populations. Finally, the generalization of the use of high-throughput sequencing techniques, based largely on the identification of organisms from the mentioned barcode sequences, has also allowed, in the case of endophytic fungi, mass characterization, and identification of microbial diversity in a much more efficient way than that obtained through classic culture-dependent microbiological techniques.

3.1 Isolating methods of endophytic fungi. Classical taxonomic characterization and molecular systematics approaches

3.1.1 Isolation and culture

As a rule, it has been widely accepted up to date that some basic microbiological procedures are essential to yield the greatest amount of microbial diversity possible when studying endophytic communities in each plant species. Most of these methods are routine protocols in a microbiology context, not especially difficult for well-trained personnel [106]. The first critical step in the isolation procedures has to do with the processing of fresh plant material in good condition, where there must be an acceptable balance between the degree of desiccation and aeration of the samples, to avoid both the death of the tissue and its colonization by contaminant microorganisms. Following these handling protocols, removing epiphytic, contaminating fungal species usually present in plant tissues must be carried out by surface sterilization methods to ensure the isolation of true endophytic diversity. The most employed methods for surface sterilization of plant tissues are based on the sequential immersion of materials in EtOH and NaOCl solutions. Other chemical agents less employed for removing epiphytic microorganisms are mercury chloride ($HgCl_2$), hydrogen peroxide (H_2O_2), paraquat, or 1% peroxyacetic acid (CH_3COOOH) in 30% ethanol [2]. Some authors have proposed alternative protocols for surface sterilization that include alternative germicidal agents such as the sequential use of calcium hypochlorite, sodium hydrogen carbonate, and finally sodium azide [135]. Together with the dip in the mentioned germicidal agents, the processing can be completed with washing materials with sterile distilled water either before or after treatment with sterilizing solutions. In the case of very delicate plant tissues or for those that do not withstand the action of ethanol or bleach well, cleaning with serial washings only with sterile deionized water or the continuous action of running tap water is usually indicated [106]. In these latter cases, it is usually necessary to use a synthetic isolation medium supplemented with some type of antibiotic. Another reported alternative method for removing surface contaminants consists of intense washing and subsequent sonication of the tissues before plating and incubation [136]. The immersion times and concentrations of sterilizing solutions of ethanol and bleach will be a combination of each plant studied (monocots, dicots, lichens, mosses, ferns, etc.) and the type and nature of the tissue or organ processed (leaves, stems, twigs, trunk, roots, leaf litter, etc.). In the literature, there are numerous examples of surface sterilization protocols for endophytic fungi (dipping times, concentrations, etc.) as a guide [106].

Following the surface sterilization processes, the design and choice of incubation, culture, and isolation protocols for endophytic microorganisms are key when it comes to characterizing the greatest amount of fungal microbial diversity possible. In this sense, the size of the plant sample under study is crucial, since a clear and direct relationship is established between it and the recovery/identification rate of species. In fact, it is commonly assumed that the smaller the sample size, the greater the isolated cultivable diversity, while on the contrary, the larger the size of the material analyzed, the possibility of overlooking rare or very slow-growing species will be greater [106].

As a rule, fragments of variable size of host tissues are usually placed on the surface of certain synthetic nutritive agar medium in a way that ensures a distance between fragments that allows the emergence and growth of individual fungal colonies. To avoid overlap and cross-contamination in colonies due to the existence of fast-growing species, the use of microwell plates is highly recommended, in combination with a maximum fragmentation of plant material. These processes of cutting and separating tissues into the smallest possible fragments are often laborious and time-consuming in the laboratory, so alternative material atomization methods can be used. One of the most frequently used is the maceration of the tissues, the filtration of particles, and their subsequent dilution and plating. This is a technique originally developed for high-throughput bacterial cultivation of slow-growing bacteria that has been adapted by several authors [137–139], to improve the recovery rates of culturable fungi from certain complex substrata.

Concerning culture media employed to isolate, maintain, and characterize endophytic fungi, synthetic routinary microbiology media are considered to be suitable for a primary recovery and isolation of fungal endophytes, also for subsequent subculturing and purify isolated strains. In this sense, PDA is the most used and widespread medium for these purposes [2], although another commonly used media is Malt Extract Agar (1–2%), sometimes in combination with yeast extract [106]. Other less common synthetic culture media used for the isolation of this type of microorganisms would be Agar containing Murashige and Skoog (MS) vitamins and sucrose [140], Hagem Minimal Medium [141], Czapek medium [142], Tryptone Soybean Agar, Tryptone Bovine Extract Agar, and Luria-Bertani (also known as LB broth) medium [143]. In addition to its composition of basic nutrients and depending on the type of endophytic diversity to be studied, the use of certain supplements in isolation media is also a common practice. Thus, the employment of microbial growth inhibitors such as fungistatics or antibiotics, is usually common practice in the primary isolation of strains. The former are usually used to delay the emergence of fast growing fungal species that can often hide and prevent the isolation of slower-growing taxa, and substances such as Dichloran, Pimaricin, Benomyl, certain antibiotics such as Cyclosporin A or Amphotericin are usually used. Other compounds include surfactants (i.e., benzyltrimethylammonium hydroxide, sodium dodecyl sulfate) and organic acids (tannic acid, lactic acid) to be employed as differentially selective agents in culture media [106]. Regarding the control of bacterial growth, it is usually common practice to supplement the isolation media with one or several broad-spectrum antibiotics such as sodium benzylpenicillin, chloramphenicol, streptomycin, gentamicin, or tetracycline [2].

After purification and obtaining in pure culture of the different isolates, these are usually classified in a first stage based on their micro- and macromorphological diagnostic characteristics in culture, as the basis of a classic taxonomic approach. Thus, endophytic fungi are usually identified at the species level using firstly morphological characterization combined with subsequent molecular methods. Vegetative morphological characters in plate culture usually include pigmentation (including the colony reverse), odor, topology (texture, margin, type, and growth rate), or the cardinal temperatures of the colonies in different axenic media [2], together with the description (when they are available) of the different reproductive (sexual and asexual) or resistance (sclerotia, chlamydospores, etc.) structures [144]. However, for many of the endophytic species routinely isolated, the aforementioned reproductive structures could not be available and the different isolates usually present as sterile mycelia, which can be subjected to certain techniques to promote their sporulation, from the use of specific culture media (including plant components or hydrophobic elements, etc.) [145, 146] to the exposure to certain lengths of UV light [147].

3.1.2 Taxonomic identification of fungal endophytes

Even when an acceptable number of diagnostic macro- and micromorphological characters are available for classical taxonomic identification, this can present different problems, since classical taxonomic work not performed by specialists in fungal systematics can be effective in establishing identifications at the genus or family, but often presents problems in classifying lower taxonomic levels (specific or infraspecific), especially in complex and/or highly diverse groups, where morphological characters can be misleading or insufficiently diagnostic due to the existence of phenomena such as hybridization, cryptic speciation, or evolutionary convergence [148]. As a consequence, it is necessary to have more precise methods for the identification of fungal isolates based on molecular techniques [149].

Although, in the past, molecular techniques have been applied to connect anamorph and teleomorph phases in endophytic species such as Random Amplification of Polymorphic DNA (RAPD) [150], currently, the selective amplification of the ribosomal ITS region and other useful genomic areas and the subsequent analysis and comparison of these sequences, has become the most widespread method of taxonomic identification of isolates, due to its universality, reproducibility, and ease of automating processes. Basically, the routine work of molecular characterization of endophytic isolates involves a series of generic processes and sequential steps, ranging from the extraction of the genomic DNA of the microorganisms to be identified (normally from pure cultures or to a lesser extent from complex environmental samples), the subsequent selective amplification using universal or specific primers by PCR (conventional, RT-PCR, etc.) of certain genomic regions (conserved or not), the sequencing of the PCR products, their subsequent processing and comparison with public databases (preferably curated and taxonomically supervised), and to finally determine and infer both the taxonomic identity and the phylogenetic relationships existing between the characterized species [2]. Although not without limitations (especially present in certain groups of filamentous Ascomycetes and to a lesser extent Basidiomycetes) [151, 152], the ribosomal ITS region that includes its three most commonly employed components, the LSU (26-28S) and SSU (18S) fragments and the ITS region (including the internal spacers ITS1 and 2 and the 5.8S region), is currently considered the most preferred molecular DNA marker to identify fungi, being accepted as a primary barcode region for mass and routinary identification. These DNA barcodes (massively used in other groups of living organisms) are short (400–600 bp), universal, and standardized DNA sequences that are easily amplifiable (unlike other more conserved genomic regions), with a common presence in different groups of the Kingdom Fungi and related organisms, but highly divergent at the species level [153]. For the identification of certain groups and genera that are especially complex or that contain numerous cryptic species (e.g., *Fusarium*, *Cladosporium*, *Penicillium*, *Rhizoctonia*, *Verticillium*), in addition to the use of these DNA barcodes based on the ribosomal ITS region, the sequencing of fragments from other protein-coding genomic regions (i.e., tef-1, RPB1, RPB2, CaM, tub1/BenA) is necessary to, through a multiloci phylogenetic analysis, clarify the molecular systematics of highly complex groups [154].

3.2 New methods of analysis of endophytic mycobiota: amplicon and shotgun metagenomics. Present application of bioinformatics and potential in the future. Transcriptomic analysis of the plant in the presence/absence of certain endophytes

Among the new techniques for analysis and description of endophytic microbial communities, next-generation sequencing (NGS) methods currently play a crucial role, providing experimental evidence on their diversity, population structure, abundance, metabolic functionality, or ecological role played in the ecosystems they inhabit [155]. Using this type of approach and employing DNA from complex environmental samples (soil, plant tissues, water, etc.), complete microgenomes can be sequenced simultaneously and relatively fast [156]. In purely taxonomic metagenomics (metabarcoding), sequences produced from amplicons of genomic regions of universal DNA barcodes are assigned to Operational Taxonomic Units (OTUs) according to a nucleotide similarity threshold by comparison with curated taxonomic databases [157]. Along with these types of approaches, Shotgun metagenomics allows sequencing the entire genomes of the whole microbial population from a total genomic DNA sample, allowing the identification and annotation of all microbial genes, providing additional information on the potential functionality of each microbiome studied, including the different metabolic pathways present in the microbiome of a given environmental sample [158].

These metagenomic approaches have revolutionized the analysis of endophytic communities without the use of laboratory microbiological routines, providing a more complete view of the microbial composition of plants [159]. In the specific case of Shotgun metagenomics, the simultaneous sequencing of complete genomes has allowed us to begin to access knowledge of the so-called deep biodiversity of microorganisms, made up of all those taxa (and their associated genes) that are usually discarded in metagenomic analyses by not having homologies in the available sequence databases. All this taxonomic and functional diversity probably represents most of the existing microbial diversity [160]. The first studies on endophyte metagenomics date back to 2013, although the generalization and progressive accessibility of NGS methods have rapidly stimulated the taxonomic, functional, and ecological characterization of endophytic (fungal and bacterial) metagenomes. In the last 5–10 years, analyses of microbial communities associated with all types of environments have multiplied, including the inner compartments of different organs and plant tissues [156]. Furthermore, the progressive preference in this type of study for the use of shotgun metagenomics is allowing us to highlight and exploit the potential of the metabolism of this type of microorganisms, in aspects such as knowledge of their secondary metabolism, their biochemical diversity or evolutionary significance [161].

Regarding the development and application of bioinformatics solutions and their potential for the analysis of metagenomic data for the study of endophytic communities, the need to process and manage large datasets has led to the development of computational tools and pipelines capable to integrate into single platforms (QUIIME, Galaxy, etc.), tasks such as the processing of raw sequences, the analysis and taxonomic assignment of marker genes, the evaluation and statistical analysis of diversity, or the prediction of ecological roles and aptitudes of the different OTUs. In the case of Shotgun metagenomics, data analysis is usually more complex than studies based on amplicons, since there are usually numerous difficulties in the assembly and annotation of metagenomes obtained *de novo*, where only a small percentage of the coding genes can be accurately identified. To overcome these obstacles, there are currently different bioinformatics tools such as assemblers (SOAP, Ray Meta, Meta Velvet, Meta SPAdes, etc.), programs for grouping sequence fragments (Kraken, MaxBin2, Meta Bat, etc.), or tools for classification and annotation of metagenomes (PRODIGAL, MetaGenMark, SEED, KEGG, COG, etc.) [162].

In addition to culture-independent genomic approaches, other disciplines such as Transcriptomics or Metabolomics are currently being used to obtain a more complete view of the functioning and ecological role of endophytic microbial communities. This type of methodologies allows us to analyze the levels of gene expression both in an entire community and only for certain specific species of microorganisms, or to characterize the biochemical profiles of a given community, revealing its metabolic state in a certain time frame [163]. In this way, transcriptomics can inform us of alterations in gene expression related to the establishment of fungal-plant mutualism relationships [164], pathogenesis phenomena [165], elicitation of the defensive response in plants [166, 167], or adaptability to different abiotic stresses [168, 169]. Interestingly, all these phenomena can be better understood and explained today with comparative gene expression analyses, either at the level of the plant transcriptome in the presence/absence of certain microorganisms or in the case of the fungal transcriptome in interaction or outside of the plant host studied [170].

4. Uses of endophytic fungi

Endophytic fungi possess numerous biosynthetic gene clusters that possess genes encoding various enzymes, transcription factors, etc., in their genome, responsible for the production of secondary metabolites. The discovery of the anticancer product taxol was a landmark in endophyte research and established potential of the metabolism of plant-associated endophytes. Plant-associated endophytes are recognized reservoirs of plant-derived natural products and novel metabolites of pharmacological and agricultural significance. Endophytic fungi-mediated biosynthesis of well-known metabolites includes taxol from *Taxomyces andreanae*, azadirachtin A and B from *Eupenicillium parvum*, vincristine from *Fusarium oxysporum*, quinine from *Phomopsis sp*. [171, 172], and ginsenosides [173]. Furthermore, synthetic biology engineering of endophytes for yield enhancement has been proposed as a prospective area of research to improve the yield of valuable compounds [174].

Antimicrobial compounds: Compounds with activity against microorganisms have been isolated from both endophytic fungi and actinomycetes. Specifically, the presence of bioactive metabolites with unique skeletons in endophytic fungi could contribute to the prevention of antimicrobial resistance. The major classes of metabolites reported include anthraquinone, sesquiterpenoid, chromone, xanthone, phenols, quinones, quinolone, piperazine, coumarins, and cyclic peptides [175–177]. However, only a limited number of these compounds have been subjected to comprehensive studies regarding their mechanisms of action against bacterial cells, their effects on antibiotic-resistant bacteria, and the identification of biosynthetic gene clusters responsible for synthesizing these secondary metabolites [178].

Anticancer: There is a high diversity of plant-associated endophytic fungi in nature that are potent producers of anticancer compounds, including terpenoids, flavonoids, alkaloids, phenolic compounds, quinines, steroids, with relevance in chemoprevention and as modulators of miRNA [179]. Some of these anticancer compounds, such as paclitaxel, camptothecin, vinblastine, vincristine, podophyllotoxin, and their derivatives, are currently being used clinically. By increasing the yield of specific compounds with genetic engineering and other biotechnologies, endophytic fungi could be a promising, prolific source of anticancer drugs [180].

Additional medicinal effects: Antiviral fungal endophyte secondary metabolites have potential as inhibitors of various HIV-1 target sites [181]. Some studies describe the presence of extracts and metabolites against diabetes mellitus in fungal endophytes [182], antileishmanial, and antitrypanosomal [183].

Plant protection and biocontrol: Endophytes can directly stimulate plant growth by producing phytohormones or stimulate plant growth by increasing the availability of soil nutrients to plants. Endophytes may also help suppress diseases in plants directly by neutralizing environmental toxic elements, and by inhibiting plant pathogens by antagonistic action, or indirectly by stimulating induced plant systemic resistance. Beneficial fungal endophytes improve plant performance, a condition that seems to be magnified in the presence of stress. There is a clear potential for symbiotically modifying crop plants as a strategy to develop more tolerant varieties to face stress and eventually increase the quality of agricultural products [184]. Fungal endophytes used as bioinoculants (bioformulations of selected microbial strains) confer various benefits to the host, such as elicitation of the immune response, mineralization of essential nutrients, and promotion of plant growth. To design bioformulations, genomics, transcriptomics, metabolomics, proteomics, and microbiome modulation strategies (gene editing and metabolic reconstruction) can be used in synthesizing microbial consortia by evaluating the role of structural and functional elements of communities in a controlled manner [185, 186].

Natural compounds produced by endophytes as secondary metabolites are beneficial to their hosts and can be externally produced for plant protection as an indirect effect [187]. Among the potential applications of fungal endophyte, secondary metabolites are antifungal and insecticidal, among others [188–190].

Enzymatic activity and biotransformation: Endophytic fungi exhibit notable enzymatic activity and biotransformation capabilities. Beyond producing a wide range of secondary metabolites applicable in agriculture and medicine through submerged fermentation in culture media, they can also produce compounds of interest by transforming waste and by-products into compounds of interest such as polyphenols and terpenoids, among others [191]. For instance, an endophytic strain of *Penicillium rubens* can produce genistein, a versatile compound with various applications, from the wastes of *Cajanus cajan* [192]. Similarly, through fermentation of orange-peel residues with *Alternaria arborescens,* transaminase enzymes with economic interest have been produced. It is noteworthy to mention the use of endophytic fungi in saponins production (10.2174/0113892010247700230919053439; [193–195]). Other endophytic enzymes with potential use in the industry have been described in the literature [196–198].

Additionally, their flexible enzymatic activity makes them a valuable asset in bioremediation. Free and inoculated endophytic fungi have been used in the bioremediation of cyanotoxins [199], polycyclic aromatic hydrocarbons [200], endocrine disruptive compounds [201], and lead and cadmium contamination [202, 203], among others.

Notably, a recent study explored the utilization of an endophytic fungus in winemaking as a carrier, reporting enhancements in aroma, polyphenols, and antioxidant capacity [204]. These findings provide significant insights for investigating the application of endophytic fungi and their enzymes in food technology.

5. Conclusions

Endophytic fungi constitute a group of microorganisms associated with practically all terrestrial plant species, whose importance and specific weight in the structure and ecosystems (including agroecosystems) have been being unraveled in the last 50–60 years. Some of the first species described and studied as endophytes have served as study models to further understand plant-microorganism relationships, allowing a more exact understanding of the processes that trigger pathogenic or mutualistic behavior. Currently, we know more precisely aspects such as the existing enormous diversity of this group of fungi, their distribution in the different plant tissues and compartments, what their seasonal patterns are, or how the composition and structure of endophytic populations change and evolve as their host plants do. The routine incorporation of molecular methods in fungal systematics has allowed the implementation of relatively simple and rapid protocols for the taxonomic identification of isolates, standardizing and universalizing approaches such as the determination of species based on the use of DNA barcodes. In a subsequent step, the generalization in the recent years of the use of high-throughput sequencing techniques has allowed, in addition to reveal and identify an amount of microbial diversity that is difficult to characterize employing only culture-dependent methods, to study the functionality of this type of fungal communities. In this sense, the incorporation of this and other omics into the studies of endophytic fungi allows us to discover new bioactive substances and compounds of interest and potential produced by these microorganisms (and their applications in agriculture, drug discovery, bioremediation, etc.), or unravel the complex interactions between microorganisms and the plants that host them, increasing our knowledge about the molecular bases of these relationships. These methods also allow us to elucidate the key role of the microbiome in the ecological adaptability and fitness of its plant hosts (to defend itself against biotic or abiotic stresses), to introduce the paradigm of "holobiont" understood as a biological unit composed of the sum of both the plant and its microbial genomes, or the role of microorganisms in providing ecosystemic services in the ecosystems they inhabit.

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