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Review

Diversity of Endophytic Mycobiota through Metagenomic Approach and Bioprospecting the Phytoconstituent

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Abstract: Endophytic fungi are important organisms that live and thrive in the intercellular or intracellular regions of host tissues of plants without causing immediate or adverse effects on the host plant. Endophytes constitute a large diversity of microbial adaptations that have been developed to survive adverse environments. Plant endophytic fungi play an important role to enhance the uptake and transfer of nutrients from the soil to plants simultaneously influencing the physical and chemical properties of soil. The discovery of fungal endophytes in their natural habitat is quite difficult due to few of its non-sporulating and non-culture-dependent endophytes by the traditional methods. The molecular characterization of isolated fungal endophytes can be carried out by isolation of genomic deoxyribonucleic acid (DNA), polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) regions, DNA sequencing and analysis. One of the culture-independent approaches known as "metagenomics" is capable of reading the variety of ambient microbes that do not include cultivation from the environmental sample. Several researches on fungal endophytes in plants are in progress and have been under exploitation. Several secondary metabolites with diverse biological activities have been discovered in these fungi, suggesting that they have the ability to create a vast selection of secondary metabolites. In the hunt for new medications such as bioactive properties or innovative drug-like compounds, their variety and specialized habituation make them an attractive subject of research. The review highlights the biology of fungal endophytes, their discovery, isolation and identification by molecular methods.

Keywords: endophytic, non-sporulating, metagenomics, bioactive, habituation

1. Introduction

Plants, particularly perennials, are colonised by a number of endophytic microorganisms that live within plant tissues for the whole of their lives or for a portion of their life cycles without producing obvious harm or morphological changes to their hosts [1, 2]. It's been more than a century since endophytes were first discovered. Most of the time, they exist as imperfect fungi and have been referred to be either harmless parasites or real symbionts by researchers [3]. This has been speculated that they may have an impact on the distribution, ecology, physiology, and biochemistry of host plants [4]. An extensive variety of microbial adaptations has emerged in endophytic habitats due to the isolation and specialization of these habitats. Recent investigations have shown the widespread presence of these fungi, with an estimated 1 million species of endophytic fungus dwelling in plants [5, 6]. Endophytic fungi make up a large and important part of fungal diversity, and they have been shown to have an effect on the diverse selection and structure of plant communities where they live [7]. Fungi are the most common endophytes detected in petrified stem and leaf

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tissues, which suggests that endophytic-plant interactions started around 400 million years ago [8, 9]. Members of the Ascomycota and the class Deuteromycota make up the majority of endophytic fungi, although there are also some species from the Basidiomycota, Zygomycota and Oomycota [10, 11]. Endophytic fungi can produce a variety of bioactive chemicals that promote host growth and resistance to environmental stress, as well as decompose litter [12, 13]. The plant parts like roots, stems, leaves, twigs, bark, fruit and seeds, as well as meristems of petioles and flowers, are all possible locations for fungal endophytes, while certain fungi may only be found on a single kind of plant [14-18]. Bioactive compounds are thought to be produced in significant part by their surroundings, according to current understanding. As a result, it is beneficial to research fungal isolates from various ecological conditions in order to identify new compounds that trigger the formation of both primary and secondary metabolites [19]. Of the estimated 1.5 million distinct fungus species, researchers only known around 7% of them [20] and even fewer of them have been tested for drug development. In this light, it is reasonable to assume that we have only uncovered a tiny fraction of fungal metabolites that are commercially relevant. The objective of this review is to accustom the researcher to the perspectives of endophytic fungi and the biology of endophytes.

2. An overview of endophytes

There have been numerous reviews and various books written on the term “endophyte”, however, it is best defined as “all organisms inhabiting plant organs that may colonise interior plant tissues at some point in their lives without inflicting visible damage to the host” [21]. Endophytic fungi were connected with plants for more than 400 million years [7] and they’ve been extensively investigated in a range of topological and meteorological environments. Microbial habitats in plant tissues are multi-layered, geographically and ecologically diversified environments, and as a result, they sustain a diverse and rich endophytic microbiota that forms specialised relationships with distinct plant species. An approximate 1.5 million fungi on Earth is particularly based on a ratio of land plants to fungal species of 1:6 [22]. Additionally, [21] put forward that there were over a million species of endophytic mycobiota that are yet to be identified and characterised. The endophytic mycobiota are present in diverse plant species and a few of them contains endophytic microbes including rice and wheat; tomato; cowpea; maize; strawberry; chickpea; mustard; sugarcane; chilli; citrus; soybean; cotton; pearl millet; and sunflower [23, 24]. A lot of experts are interested in the world of endophytes because of their important functions in encouraging plant development and in boosting plant endurance in severe environments [25]. Endophytes may effectively promote plant development by using a variety of modes of action and boosting the plant tolerance to severe environments [26]. For the production of various bioactive metabolites, the potential for endophytic fungal secretion is enormous. Cancer and heart disease may be reduced by certain of these plant secondary metabolites, such as polyphenol and anthocyanin. Species diversity estimates from endophytic fungus from tropical and temperate forests are supported by recent research.

3. Endophytic fungi and soil nutrients

Endophytic fungus on plants may enhance soil and organic matter absorption of macronutrients like phosphate, nitrogen, potassium, and magnesium as well as micronutrients like zinc, iron and copper [27]. Endophytes have also been demonstrated to enhance the elemental composition of roots and shoots of endophyte-inoculated plants, with nitrogen and phosphorus being the most notable increases in the elemental composition [28-32] indicating that phosphorus and zinc concentrations in rice roots and shoots of endophytic fungus *Piriformospora indica*-inoculated rice seeds were substantially greater than those of control plants the effectiveness of sulphur absorption in plant roots was shown to be directly related to the availability of phosphate, and an increase in the sulphur transfer was seen only when the soil phosphate concentration was low [33, 34]. Endophytes’ capacity to transport unrestricted amounts of soil nutrients to their plant hosts is poorly understood [35]. Seasonal changes may have a direct effect on colonisation and species composition, and they can also have an indirect impact through modifying soil microenvironments [36].

4. Endophytic fungi isolation

Traditional strategies for identifying endophytic fungi inside plant tissues include direct observation and cultivation-dependent procedures. Deckert et al. [37] made a direct observation of endophytic fungi under the light and electron microscope by employing various staining techniques (e.g. thionin/phenol) to differentiate the fungal mycobiota from the plants [38]. Due to the absence of spore-producing structures and sexual or asexual spores, the majority of endophytic fungi inside plant tissue have just a hyphal structure and cannot be assigned to any taxonomic group based on morphology. Cultivation-dependent procedures, rather than direct observation methods, have been frequently employed for endophyte diversity studies [13] (Figure 1). When working with endophytic fungi using cultivation-dependent approaches, the isolation procedure is a key and essential phase in the research process [39]. Plant samples, such as leaves, stems, and roots, are usually taken from the field and stored in a cool place. The samples have been grown in-vivo, where the endophytes are encouraged to leave the host and grow on agar. In order to eliminate microbial epiphytes from the plant's surface, the living plant tissues are treated through a successive process of surface sterilisation. Only internal fungi are isolated from plant samples that have been incubated on nutrient plates for a period of time. The hyphal tips of fungus may be observed emerging from the leaf pieces after a week of incubation at 24 °C. After being moved on fresh, nutrient-rich potato dextrose plates, the cultivated microbial fungal colonies are purified. The morphology, shape, and colour of endophytes may be used to identify them at a preliminary level [40]. The microstructural identification of endophytic fungi by mycologists is significantly important [41]. While the culture-dependent isolation technique is an effective method for rapidly cultivating a large endophytic fungal mass from the tissue of a plant where they are directly affected by a variety of factors, including surface sterilisation techniques, incubation conditions, and whether the isolates sporulate. The imprinting of surface-sterilised plant tissues onto nutrient medium, as well as the culturing of an aliquot of water from the previous rinse and placing it onto nutrient media, should not yield any fungal growth in practice. When performing the isolation method, other media may be utilised, such as normal potato dextrose agar (PDA) and malt extract agar (MEA). A variety of media, including PDA, potato carrot agar, and water agar, were used to inoculate the fungal strains in Petri plates in order to induce sporulation. Mold characteristics were created in water mounts, and then the slides were mounted in lactophenol and sealed with nail varnish or parafilm. The non-spore-producing cultures were classified as sterile mycelia, and they were further subdivided into distinct morph species based on their morphological traits [42, 43]. Numerous fungi have uncultivable traits and cannot be cultivated [44]. Some fungus grows very slowly [45], whereas others need particular conditions [46]. Culture-based identification of endophytic fungi from natural environments has been limited due to the non-culturable and non-sporulating nature of most of the endophytic fungi. The method of isolation must be modified so that it is appropriate for the particular plant species, tissues, and fungus being studied in order for it to be successful. The size of the plant tissue may have an effect on the observed diversity and community composition of the fungi. Molecular approaches have the ability to overcome the various technical biases that are present in conventional endophytic investigations [39].

The most recent way to identify endophytic fungi is to use the polymerase chain reaction (PCR) and then do deoxyribonucleic acid (DNA) sequencing. PCR can be done on endophytes grown in a culture that has primers that can amplify DNA that code for ribosomal ribonucleic acid (rRNA). Amplification of fungal endophytes can be done best with the help of internal transcribed spacer (ITS) regions for ID. People who study the fungal tree of life, called Assembling the Fungal Tree of Life (AFTOL), have a huge dataset of fungal DNA sequences on Genbank and the Fungal Tree of Life project. Another sequencing method that isn't used very often in the identification of fungal species is the 18S ribonucleic acid (RNA) sequencing (Figure 1). This method isn't used very often, either. As many of the endophytic mycobiota found in plants are likely to be new, it is important to have primers for as many taxonomic groups as possible. Thus, the molecular sequencing techniques are very useful for figuring out the fungal diversity in natural ecosystems down to the lowest level of classification. The enumerated studies researchers discovered that endophytes improve the fitness of their host plants via the synthesis of bioactive secondary metabolites where these compounds are concerned in the protection of the host against herbivores and infectious microorganisms. Further development in this field is the discovery that endophytes are capable of biosynthesizing medicinally significant "phytochemicals", which were previously considered to be generated solely by their host plants.

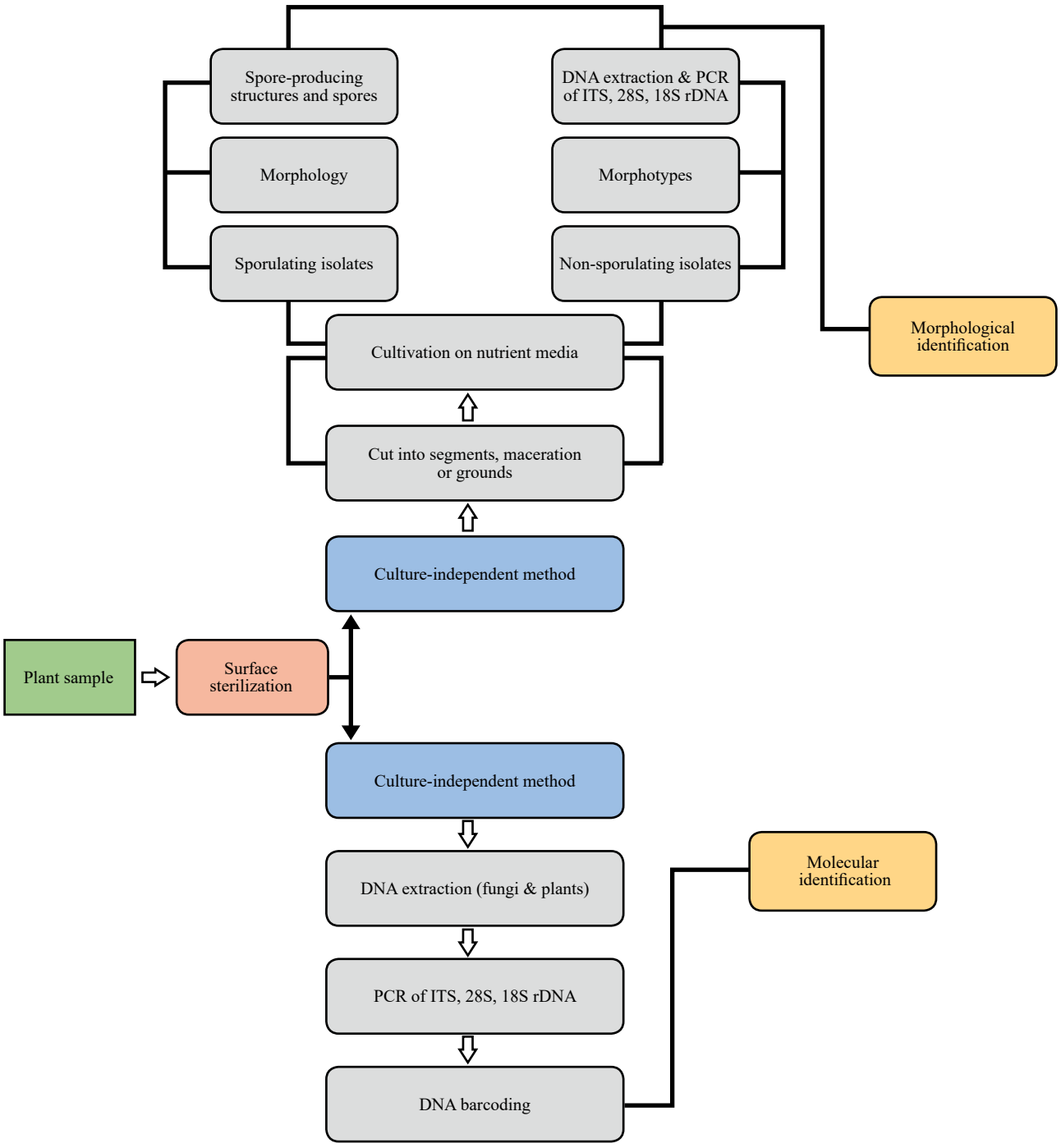

Figure 1. The schematic representation of studying the endophytic fungal community

5. The prospect of metagenomic research

Metagenomics is a method for analyzing genomes from ambient microbes that do not include cultivation [47]. This approach is capable of reading the variety of microorganisms in environmental samples up to 99% of the total numbers of microbes present [48]. The concept of exploring and analyzing the whole microbiome in its entirety has transformed

the knowledge about the ecosystem around us [49]. Recently, genomic investigations of environmental samples have emerged as an important tool for better understanding the evolutionary history, structure and functional variety and ecological diversity of the organisms [50].

After more than three decades of using the Sanger technique, researchers developed the second-generation sequencing technology. Roche/454, Ion torrent and Illumina are among the technological platforms that make up the second-generation sequencing approach [51]. The sequencing techniques usually target the full ITS region, which is typically spaced between 450 and 700 base pair (bp). Either ITS1 or ITS2 has been identified as a potential target [52-54]. Fungal diversity in an environment may be studied in great detail using high-throughput sequencing, which analyses hundreds of genomes in the sample [55]. All the portions of the ITS region may be amplified by using different primers. The primers that are most often used were developed in the early 1990s when only a tiny portion of the molecular diversity in ribosomal deoxyribonucleic acid (rDNA) throughout the fungal kingdom was known [56, 57]. Generally, the reading of genus and species identified can be recognized at a minimum of 95% for the genus and 97% for the species, conversely reading of strain levels can be distinguished at a minimum of 99% can normally be distinguished [58].

6. Metagenomic methodologies

The approach is separated into two parts: Molecular and Bioinformatic methods [59]. Environmental community genomes (metagenomes) are the focus of metagenomic research in recent days. This study is a departure from the typical genome research that focuses on an individual (single genome). Metagenomics analysis relies heavily on method selection [60].

7. Metagenomic DNA extraction

Environmental samples are used directly to extract DNA metagenomes. DNA metagenome exploring begins with this approach. Depending on the study sample, some researchers use a variety of approaches [61]. The simplest technique for obtaining metagenomic DNA is to utilize a commercial kit, which just necessitates chemicals given by the manufacturer. Some of the kits such as Qiagen DNeasy power max and power soil kits for soil samples and plant samples. Standard procedures need a greater amount of time to complete than commercial kits. As a result, researchers favor kits since they are more effective in terms of time management [60, 62]. The size of the fragment generally employed for metagenomic analysis ranges from 600 bp to 25 kilobase pair (kbp). The concentration and purity of DNA are accomplished by using the ultraviolet (UV) absorbance technique. DNA absorbs UV light at a wavelength of 260 nm, whereas proteins absorb it at a wavelength of 280 nm which is used to determine the purity of nucleic acids [63]. Nucleic acids may be separated, identified and purified using gel electrophoresis. The factor of molecular weight, the concentration of the gel, and the electrical voltage utilized all have an impact on the speed of the transfer process [64, 65]. The molecular identification of fungi via the use of DNA barcoding has become an essential and important part of fungal ecology, bringing new insights into the diversity and ecology of a number of different fungal species [55]. The International Nucleotide Sequence Database contains more than one hundred thousand fungal ITS sequences. These sequences were acquired using the conventional Sanger sequencing method. This database has been approved as the official major barcoding marker for fungi [66]. The metagenomic analysis employed with the bioinformatic tool is used to identify the diversity of samples as well as forecast the metabolic pathways of microbes present in the samples.

8. Endophytes - repository of bioactive compound

Endophytes are microorganisms that have piqued researchers' attention in recent decades, mostly because of the discoveries of key secondary compounds that have been isolated from them. The fungal endophytic has emerged as a major source of naturally occurring bioactive metabolites with a wide range of applications in the pharmaceutical sector during the past few years [67, 68]. Due to the mutual relationship between fungal endophytes and their host plant, a variety of secondary metabolites are produced [69]. Microorganisms are significant producers of bioactive natural

compounds with immense promise for pharmaceutical research, industrial usage, and agricultural uses. The bioactive chemicals found in medicinal plants are either synthesized by the plant or by endophytes that live within the plant. They have recently been recognised as an exceptional source of secondary metabolites and bioactive chemicals including the natural antimicrobials and nutrient uptake process [70]. Antimicrobial and anticancer drugs might be generated from plant-derived bioactive chemicals synthesized by endophytic fungi [71, 72]. The fungal endophytes are a good source of antibacterial, anti-viral, anti-fungal and anti-cancer properties [73] and when in association with higher plants are a rich source of novel anti-oxidants [74]. In addition to being an excellent source of biological metabolites, fungi-derived natural products are also useful as antibiotics, antifungal agents and immune suppressants. Fungi-derived natural products also have potential applications in treating parasitic infections and cancer prevention [71, 75-78] (Figure 2).

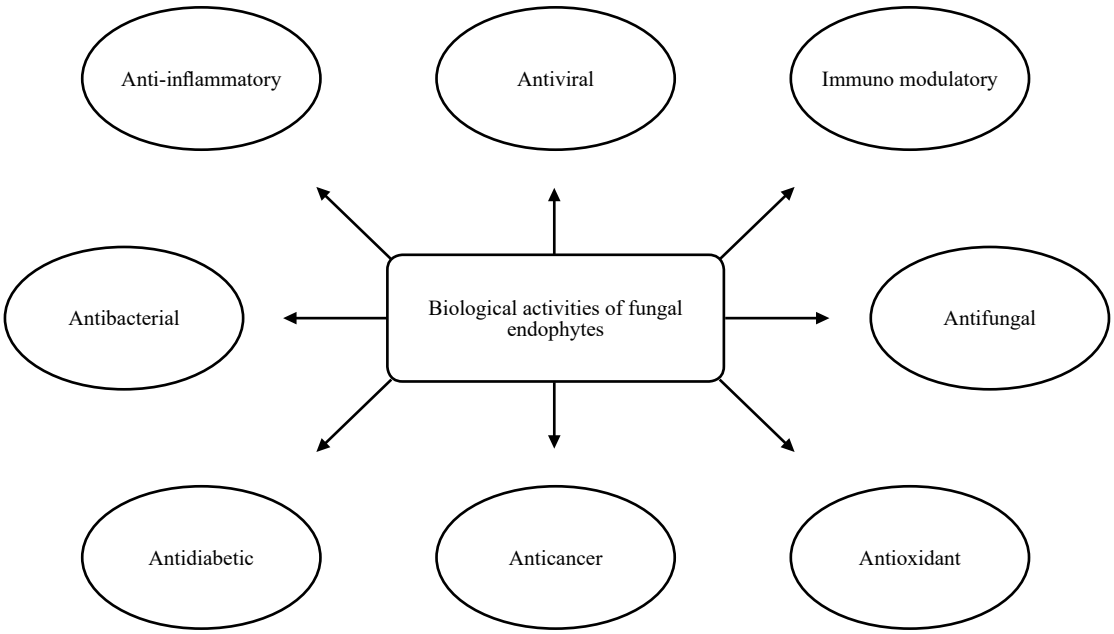

Figure 2. Biological activities of fungal endophytes

9. Overview of secondary metabolites

Endophytic fungal and host secondary metabolites are diverse and may be classified into a variety of categories, the majority of which are as follows: alkaloid, terpene, lignans, flavonoids, saponins, phenols, phenolic acids, chlorinated metabolites, peptides, and steroids. Alkaloids include indole derivatives, pyrimidine and pyrrolizidine, quinoline and isoquinoline, amines, and amides [79]. Additionally, alkaloids contain bioactive features such as fungicidal, antibiotic, and anticancer activity. As a result, they have been the focus of various pharmacological research and development efforts due to their complicated chemical properties and biological activities [80, 81]. The second major group of plant secondary metabolites is grouped to be terpenoids which are obtained from endophytic mycobiota. Terpenoids and diterpenoids are efficiently extracted from endophytic cultures without breaking down the compounds [82]. Another important class of plant natural products are the polyphenolic flavonoids which have a wide range of antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic effects. The antimicrobial properties of saponin play a protecting role in the symbiotic association with host plants while the other applications are related to their anticancer, anti-nutritive, and anti-cholesterol properties [83]. Bioactive compounds such as phenols and phenolic acids may be found in a wide variety of plant and microbial sources [84]. Endophytic fungal peptides are another kind of secondary metabolites that may be used as a defensive agent against pathogens. The anti-carcinogenic and antifungal peptide leucinostatin was discovered from the fungal endophyte *Acremonium* sp., which was recovered from *Taxus baccata* (European yew plant). Leucinostatin has been shown to inhibit the growth of cancer cells [85]. The microbial communities are rich in

naturally occurring steroids that are predominantly present in plants and animals [86]. The steroids obtained from fungal endophytes such as ergosterol manifests the additional pharmaceutical properties and natural roles in their producers [87]. Initially, secondary metabolites extracted and separated from endophytes were employed industrially [88]. According to [89], certain natural compounds, such as mycotoxins, are dangerous to humans, while others, such as antibiotics, are helpful. The secondary endophytic fungal metabolites generated are critically important for a variety of metabolic interactions, including signalling, control, and defence of the plant-fungal symbiosis.

10. Future prospects and challenges

Recently, interest in prospecting physiologically significant fungal endophytes has developed owing to their biochemical variety, which may be used to create new natural products or bio-transformed products with therapeutic uses. Although endophytic fungi are found in vast environmental conditions, gathering knowledge on their ecology, evolution, and interactions with the plant hosts and other microbiota may also be complicated. Plant research may greatly benefit the better knowledge of the selection and isolation of diverse endophytes, their pathways into host plants, growth promotion and inhibition, and resistance to disease. There are several endophytic fungi that may produce bioactive metabolites that are similar to those of their host plants or novel metabolite [90]. The research on endophytic metabolites obtained from fungi has fascinating importance that has shown the cost-effective yield of bioactive compounds from it in a cost-effective manner. As a result, it is necessary to develop technologies for commercializing the production of secondary metabolites that are cost-effective. Endophytic fungus interacts with the nearby bacterial communities and interacts with the plant host which results in the production of promising secondary metabolites under natural conditions [91]. It is possible that the natural circumstances, such as co-cultivating more than two endophytic fungal strains together or adding up the plant extract in the growth medium, might trigger the production of undiscovered compounds [92]. Additionally, it is essential to determine the mode of action of novel bioactive metabolites that are derived from fungal endophytes, in addition to their preclinical and clinical development. This is something that can only be done by developing a specific methodology that combines ecology, biology, biochemistry, biotechnology, and bioinformatics into a single cohesive whole.

11. Concluding remarks

Extensive research has shown that endophytic fungi contain an abundance of new secondary metabolites with broad potential uses in the pharmaceutical industry. In recent years, a considerable increase in the number of studies showed a major increase in interest in endophytes derived from plant sources. It makes it mandatory to understand the relationship of fungal endophytes and the communication with the host plant as well as with other endophytic microbiota in the environment. Extensive research on the relationship between the host plants and environmental variables on the ability of fungal endophytes to create bioactive secondary metabolites may have commercial implications in the near future. Endophytic microorganisms are being collected, catalogued, and exploited all over the globe, which may provide chances in the fields of agriculture, industry, and medicine. The unique bioactive substances released by endophytes, when considered as a whole, might make significant contributions to the resolution of current and future difficulties in agriculture, the environment, and medicine.

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Conflict of interest

There is no conflict of interest for this study.

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