



REVIEW ARTICLE

Endophytic fungi: Nature's hidden vault of biomolecules

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Abstract

The alarming rise of antibiotic-resistant bacteria has emerged as a major global health concern, intensifying the urgent need for novel bioactive compounds from natural sources. In recent years, scientific interest in endophytic fungi, which reside asymptotically within plant tissues, has grown substantially. These microorganisms have demonstrated the capacity to produce a diverse range of bioactive secondary metabolites, exhibiting anticancer, antimicrobial, antiviral and anti-inflammatory properties. The metabolites exhibit antibacterial activity by disrupting membranes and interfering with essential cellular processes like protein and nucleic acid synthesis. Such compounds hold immense promise for both drug discovery and the development of agricultural biocontrol agents. The role of fungal endophytes in pharmaceutical innovation is becoming increasingly apparent. The unique microenvironment within plant hosts, coupled with the genetic diversity of endophytic fungi, fosters the biosynthesis of structurally novel and biologically potent molecules. However, harnessing this potential is not without challenges. Difficulties in isolating, culturing and characterising endophytic fungi and their metabolites continue to impede progress. Artificial intelligence, omics technologies, and metabolic engineering could be integrated into future research to improve the sustainable production and therapeutic utilisation of endophytic fungal metabolites. This review highlights recent advances in the discovery and characterisation of novel compounds from endophytic fungi, while also addressing ongoing challenges in understanding endophyte biology and optimising metabolite production.

Keywords

bioactivity; biosynthetic gene clusters; co-culture; endophytes; fungal endophytes; plant-endophyte interactions

Introduction

Nearly all plant species harbour diverse microflora, including endophytes and organisms inhabiting the phyllosphere and rhizosphere, collectively forming the plant microbiome. Endophytic fungi are widespread microorganisms that live inside plants that inhabit various ecosystems, including tropical and temperate forests, agricultural fields, grasslands, mangroves, aquatic habitats, and other extreme environments, demonstrating their unique ecological adaptability and host diversity. Recent estimates suggest that the total fungal diversity worldwide may range from 11.7 to 13.2 million (1) and is increasingly recognised as a rich source of structurally and biologically unique natural products.

Certain endophytic fungi can synthesise metabolites identical to those found in their host plants. Capsaicin (2), piperine (3) and azadirachtin (4) are examples of such metabolites. It is postulated that this capacity may have arisen from genetic recombination events during the long evolutionary co-existence of endophytes and their hosts (5, 6). This phenomenon suggests a form of molecular mimicry or shared biosynthetic pathways that can be exploited for biotechnological purposes.

Several studies underscore the value of endophytes as a reservoir of genetic diversity and a prolific source of novel bioactive secondary metabolites. These metabolites have already demonstrated utility in drug discovery, with notable examples including Nodulisporic acids D–F:

Potent insecticidal agents produced by *Nodulisporium* sp., isolated from *Bontia daphnoides* (7); Cyclooctadepsipeptide: A broad-spectrum anthelmintic compound from *Mycelia sterilia*, associated with *Camellia japonica* (8); Vincristine: A well-known anti-leukaemic agent produced by *Nigrospora zimmermanii*, an endophyte of *Catharanthus roseus* (9); antibacterial compound parengyomarin A from *Parengyodontium album*, an endophytic fungus from *Avicennia marina*, shows activity against *Staphylococcus aureus* (10); subplenone A from *Subplenodomus* sp., which resides within *Gentiana stramina*, a Chinese medicinal plant, exhibits potent activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (11); pestalotinone A from *Pestalotiopsis trachicarpicola*, obtained from *Blechnum orientale*, also exhibited activity against *S. aureus* and MRSA (12); Griseofulvin: A potent antifungal compound from *Xylaria* sp. associated with *Garcinia hombroniana* (13); and Sporothriolide: an anti-phytopathogenic metabolite from *Nodulisporium* sp. of *Ginkgo biloba* (14).

Moreover, up to 51% of metabolites derived from endophytic fungi possess unknown chemical structures, underscoring the immense untapped biotechnological and pharmaceutical potential of this microbial group (15). Despite these promising prospects, several challenges persist, particularly regarding the isolation, cultivation, and characterisation of metabolites from endophytic fungi. Addressing these obstacles is essential for advancing the field. This review aims to critically examine current limitations in understanding endophyte biology and to highlight recent breakthroughs in the biosynthesis of novel secondary metabolites mediated by fungal endophytes.

Fungal endophytes

Endophytic microorganisms reside within plant tissues for part or all of their life cycle without harming their host plants (16). It is now well established that most plant species on Earth serve as reservoirs for a diverse array of endophytes; however, fungal endophytes have drawn more attention due to their remarkable metabolic diversity and ability to synthesise distinct bioactive secondary metabolites with potential applications in medicine and agriculture.

Fungal endophytes have garnered increasing interest due to their capacity to synthesise novel bioactive compounds, many of which are previously unknown to science, and hold immense promise across pharmaceutical, agricultural, and industrial sectors (17). Endophytic fungi are broadly classified into two evolutionary and ecological groups (18): a) Clavicipitaceous endophytes: Predominantly associated with grasses and often form systemic, vertically transmitted symbioses; b) Non-Clavicipitaceous endophytes: found in a wide range of vascular and non-vascular plants and are generally more diverse and ecologically flexible.

Based on lifestyle and transmission, endophytes can be grouped into a) Systemic (True) endophytes: are rare, co-evolved, mutualistic, vertically transmitted, strongly contribute to host plant stability and do not trigger host defence responses; b) Non-Systemic (Transient) endophytes: are highly diverse with varied ecological roles and modes of transmission. They can exist as mutualists, commensals, or parasites and often stimulate host defence mechanisms (19). Systemic endophytes are characterised by long-term, mutualistic relationships and coevolution with their host plants. Mutualistic symbiosis represents a beneficial continuum for both partners (20). Within this partnership, endophytic fungi enhance host tolerance to a variety of biotic and abiotic stresses,

promote nutrient uptake, bolster defence mechanisms against pests and pathogens, and regulate secondary metabolite production. In return, host plants provide symbiotic niches and support for fungal growth and help fungi complete their life cycles during colonisation (21). In contrast, non-systemic endophytes form transient associations, often shifting from symbiotic to parasitic in response to environmental changes (22).

Based on the degree of host dependency and transmission patterns, endophytes are further classified as obligate and facultative endophytes. obligate endophytes, meaning they are entirely dependent on the host plant's metabolism and are transmitted predominantly through vertical inheritance or via biological vectors. The clavicipitaceous endophytes like *Epichloë* spp., associated with grasses, represent the classic example of obligate mutualists. Their symbiotic association with grasses enhances the host's fitness by improving resistance to herbivores, environmental stress, and pathogen attack through the production of protective alkaloids (23). In contrast, facultative endophytes can survive independently of the host for part of their life cycle. These are often found associated with plants growing in proximity to the primary host (24). These endophytes transmit horizontally via spores and under favourable conditions, they colonise. *Fusarium*, *Alternaria*, *Colletotrichum*, *Phomopsis*, *Aspergillus*, and *Talaromyces*, which belong to non-clavicipitaceous endophytes, generally exhibit facultative endophytic lifestyles (18, 25).

The differences in host specificity, transmission patterns, and ecological adaptation indicate that endophyte interactions often overlap and do not always fit into rigid categories.

Roles of fungal endophytes in the sustainability of plants

Fungal endophytes establish beneficial symbiotic relationships with their hosts, providing various functional benefits, such as enhanced plant growth and development and the production of bioactive secondary metabolites. Furthermore, their roles in phosphate solubilisation, siderophore production, nitrogen fixation or mobilisation, and micronutrient uptake highlight their importance. In addition, endophytes contribute to the production of growth-enhancing hormones, disease resistance, drought and salinity resilience, heavy metal detoxification, nutrient cycling, and soil fertility enhancement (26). Some examples of fungal endophytes and their reported functional roles are incorporated in Table 1.

Endophytic fungi support host plant health and productivity through a combination of direct and indirect functional mechanisms. Direct mechanisms include the synthesis of phytohormones, biological nitrogen fixation, phosphate solubilisation, siderophore production and antimicrobial metabolite production (33). Phytohormones like Indole Acetic Acid (IAA) promote root development and nutrient uptake, and phosphate-solubilising endophytes stimulate the availability of phosphorus by the secretion of organic acids and phosphatases. Similarly, siderophore-producing endophytes facilitate iron uptake by chelating ferric ions in the rhizosphere, making them more available to plants. Indirect mechanisms encompass the enhancement of plant resistance to abiotic and biotic stresses through metabolic modulation, as well as contributions to biocontrol, bioremediation, and phytoremediation (34, 35). Some processes associated with endophyte-mediated stress tolerance include activation of antioxidant defence systems, regulation of stress-responsive genes, osmolyte accumulation and modulation of phytohormone signalling pathways. Even though these multiple functions have

Table 1. Functional roles of fungal endophytes and their representative examples

Functional role	Endophyte	Host plant	Reported effect	Reference
Plant growth promotion	<i>Serendipita indica</i>	<i>Vaccinium corymbosum</i> L. (Blueberry)	Effect on the plant growth, root development and rooting of blueberry	(27)
Phosphate Solubilisation	<i>Curvularia geniculata</i>	<i>Parthenium hysterophorus</i> L.	Improves plant growth through phosphate solubilisation	(28)
Siderophore Production	<i>Candida membranifaciens</i>	<i>Euphorbia milii</i> L.	Enhanced the growth attributes of maize and mitigated the adverse effects of salinity stress.	(29)
Phytohormone production	<i>Fusarium proliferatum</i>	<i>Rhazya stricta</i> Decne	Endophyte can improve crop productivity in dry areas	(30)
Drought and salinity tolerance	<i>Nigrospora</i> spp.	<i>Oryza sativa</i> L.	Isolates exhibited tolerance to high salinity and drought stress	(31)
Heavy metal detoxification	<i>Cladosporium cladosporioides</i>	<i>Gentiana yunnanensis</i> Franch	Arsenic bioremediation	(32)

been reported across different plant taxa, some plant groups exhibit more specialised and obligate interactions with endophytic fungi. Among these, the fungal symbioses of orchids are among the most thoroughly studied examples.

Orchids represent one of the largest, most diverse and widely distributed families of flowering plants, making significant contributions to habitat biodiversity. Endophytic fungi play a crucial role in orchid seed germination and growth, as orchid seeds lack an endosperm. A key aspect of their growth and development is the establishment of mycorrhizal symbioses with compatible endophytic fungi. These intimate associations help to initiate germination by supplying essential nutrients sustained throughout the plant's life, influencing root development, nutrient uptake, and resilience to environmental stress (36).

Plant-endophyte interactions

Plant-endophyte interactions represent a complex and dynamic symbiosis that ranges from mutualism to latent pathogenicity, depending on the host species, endophyte identity, and prevailing environmental conditions (37). Following colonisation, endophytes alter the host physiology by modulating phytohormone signalling, nutrient metabolism, antioxidant defence pathways and stress-responsive pathways. This helps to establish a balanced association with both partners. For example, endophyte-mediated regulation of antioxidant enzymes and phytohormone homeostasis has been reported to increase plant tolerance to drought and salinity (38). These interactions often begin at the root-soil interface, where endophytes gain entry through natural openings or wounds or by active penetration (39, 40). Once inside, they colonise intercellular or intracellular spaces, typically without eliciting any visible disease symptoms. The nature of this association may evolve, with some endophytes maintaining a lifelong mutualistic presence, while others may shift toward a parasitic or saprobic phase under stress or senescence conditions. The plants provide a secure niche for endophyte colonisation, supplying essential nutrients and a protected environment conducive to microbial growth and persistence. This reciprocal exchange underpins the evolutionary success of many endophytes and the ecological fitness of their host plants.

Both vertical and horizontal transmission pathways shape endophyte interactions. In vertically transmitted systems, endophytes are inherited through seeds or vegetative propagules, often forming long-standing, co-evolved relationships with the host plant. These systemic associations are typically stable and persist across multiple generations without inducing disease symptoms (41). In contrast, horizontally acquired endophytes are recruited from the surrounding environment, primarily from the rhizosphere, phyllosphere or endosphere, and usually form transient, non-

systemic associations that may vary depending on ecological conditions. Phyllosphere-associated endophytes colonise aerial tissues via hydathodes, stomata, lenticels or surface injuries. Through root hairs, natural openings, or the lateral root emergence site, the rhizosphere-associated endophytes enter the plant tissues. Following entry, these microorganisms settle in intercellular or intracellular spaces without causing visible disease symptoms (42).

Recent advances in molecular biology, particularly the development of high-throughput sequencing (HTS) technologies, have revolutionised our understanding of endophytic communities. Techniques such as amplicon sequencing, metagenomics, transcriptomics, and comparative genomics have revealed an unprecedented level of taxonomic and functional diversity among endophytes, with many species being unculturable or undetectable by conventional microbiological methods (43). These technologies have not only enabled the identification of novel endophytic taxa but have also provided insights into their functional roles, gene expression profiles and complex interactions with host plants.

In the context of sustainable agriculture, endophytes are increasingly recognised as valuable biological resources. Their ability to act as natural bio-fertilisers, biopesticides, and stress mitigators makes them promising candidates for the development of climate-resilient cropping systems and low-input agricultural practices (44). The practical potential of endophytes is further highlighted by the commercial application of beneficial microorganisms in agriculture as bioinoculants and bio stimulants. One such example is the root endophytic fungus *Piriformospora indica* (also known as *Serendipita indica*), which shows great promise for crop productivity, nutrient uptake, and tolerance to abiotic stresses under greenhouse and field conditions (45). Such applications demonstrate the importance of endophytes in sustainable crop management and agricultural biotechnology.

Endophytes-bioactive metabolites

Endophytes were first isolated as early as 1904; however, for much of the twentieth century, they remained largely overlooked by the scientific community, until interest surged in 1993 following the landmark discovery of *Taxomyces andreanae*, an endophytic fungus isolated from the Pacific yew (*Taxus brevifolia*). This organism was reported to produce Taxol, a highly potent anticancer compound, within its culture broth (46). This finding generated considerable excitement and catalysed global interest in the metabolic potential of endophytic fungi. However, more recent studies have cast doubt on the original claims. Genomic investigations have failed to identify Taxol biosynthetic genes in the genome of *T. andreanae*, questioning its actual role in Taxol production (47). Furthermore, taxonomic revisions have since reclassified *Taxomyces* within a

basidiomycete genus, further complicating its identity. Subsequent studies have pointed out several significant issues of reproducibility in Taxol production using endophytic fungi. Repeated subculturing of many reported strains under laboratory conditions leads to a reduction or complete absence of Taxol biosynthesis, implying that metabolite production may involve complex microbial interactions present in the native environment, epigenetic regulation or host-associated factors. In addition, recent comparative genomic studies have been unable to detect the complete Taxol biosynthetic pathway in many of the claimed Taxol-producing endophytes (48, 49). Despite these controversies, the initial discovery played a pivotal role in bringing endophytes to the forefront of natural product research, spurring extensive exploration into their diverse biosynthetic capabilities.

Endophytic fungi are prolific producers of a diverse array of bioactive metabolites, encompassing terpenoids, indole alkaloids, polyketides, and non-ribosomal peptides. Non-ribosomal peptides are assembled by multinodular non-ribosomal peptide synthetases (NRPSs), which utilise both proteinogenic and non-proteinogenic amino acids. Polyketides, in contrast, are synthesised from acetyl-CoA and malonyl-CoA units through the catalytic action of polyketide synthases (PKSs). Terpenoids, which are constructed from isoprene subunits, are formed via the mevalonate pathway and catalysed by terpene cyclase enzymes. Alkaloids, nitrogen-containing secondary metabolites, are typically derived from aromatic amino acids and dimethylallyl pyrophosphate, and their biosynthesis often involves both the shikimic acid and mevalonate pathways (50). The major classes of endophyte-derived secondary metabolites, their biosynthetic precursors, pathways and key enzymes are summarised in Table 2.

Through mechanisms that target bacterial metabolic pathways and key cellular structures, endophytic fungal metabolites exhibit antibacterial activity. Certain metabolites interfere with cell membrane permeability, protein synthesis, quorum sensing, nucleic acid replication, and other cellular processes (51). Despite the remarkable antibacterial properties of endophyte-derived secondary metabolites, the precise mechanisms of action of these metabolites remain poorly understood. So advanced approaches like genome mining, structural biology and omics technologies are essential for the development of antibacterial drugs from endophytic fungi.

Importantly, the structural diversity and biological activities of endophyte-derived secondary metabolites have attracted considerable interest in pharmaceutical applications (52). A comprehensive review of the literature reveals several key classes of bioactive compounds produced by fungal endophytes, including antioxidants, industrially valuable enzymes, antifungal agents, antimycobacterial compounds, anticancer agents and antibiotics.

Endophytes as *in vitro* secondary metabolite production platforms

The rising global demand for plant-derived bioactive compounds is accompanied by several notable limitations that hinder the efficiency and sustainability of traditional production methods. These include: 1) Inherently low yields, often subject to environmental fluctuations and seasonal variability, 2) Restricted availability of plant material, which may lead to overharvesting and consequent ecological degradation, and 3) Structural complexity of many phytochemicals, making their total or semi-synthetic production highly challenging and economically prohibitive. In contrast, cultivating endophytes presents a more sustainable and cost-effective alternative. Endophytic fungi can be grown *in vitro* with minimal nutrient requirements and do not necessitate the use of expensive plant hormones, unlike plant cell cultures. Moreover, low-cost substrates such as molasses or whey liquid, often considered agro-industrial waste, can be utilised in large-scale fermentation processes, further reducing production costs (53).

Given these advantages, plant-associated endophytes are increasingly recognised as promising and viable sources for the sustainable production of high-value phytochemicals. Their ease of cultivation, scalability, and metabolic potential make them attractive candidates for industrial bioprocessing, offering a practical solution to the limitations of conventional plant extraction methods. Therefore, endophyte-based production methods offer significant promise for economically and environmentally viable biotechnological applications.

Isolation of endophytes

The initial step in bioprospecting endophytes for metabolite synthesis is isolating the endophyte. This process begins with the selection of appropriate plant explants, followed by surface sterilisation to eliminate external contaminants and epiphytic organisms. Typically, surface sterilisation involves thorough rinsing of plant material with sterile water, chemical sterilisation using agents such as ethanol, sodium hypochlorite, or mercuric chloride, and inoculation of sterile explants onto suitable culture media under aseptic conditions. Various protocols for surface sterilisation are available, depending on the type of plant parts (e.g., leaves, stems, roots, or flowers), and generally differ based on three key parameters: the type of surface sterilant used, its concentration, and the duration of exposure (54). To confirm the effectiveness of surface sterilisation, contamination-check procedures are generally used, including imprinting the sterilised explants onto culture media or plating the final rinsed water. The absence of microbial growth in these controls is typically regarded as evidence of a successful surface sterilisation (55). Even though mercuric chloride is an effective sterilising agent, due to its toxicity, environmental persistence and disposal issues, it has prompted the usage of safer

Table 2. Major classes of secondary metabolites produced by endophytic fungi and their biosynthetic pathways

Metabolite class	Biosynthetic precursors	Biosynthetic pathway	Key enzymes	Representative metabolites
Terpenoids	IPP, DMAPP	Mevalonate pathway	Terpene synthases/cyclases	Taxol, gibberellins
Alkaloids	Aromatic amino acids, DMAPP	Shikimate and mevalonate pathways	Prenyl transferases, methyl transferases	Ergot alkaloids, indole alkaloids
Polyketides	Acetyl-CoA, Malonyl-CoA	Polyketide pathway	Polyketide synthases (PKSs)	Chaetoglobosins, curvularides
Non-ribosomal peptides	Proteinogenic and non-proteinogenic amino acids	NRPS-mediated pathway	Non-ribosomal peptide synthetases (NRPSs)	Beauvericin, enniatins

alternatives like sodium hypochlorite and ethanol in the endophyte isolation procedure.

After sterilisation, endophytes can be isolated using various methods, including leaf cutting, blender shaft, mortar and pestle, or enzymatic digestion. The enzymatic digestion of *Oxalis acetosella* L. plant tissue yielded the highest number of species compared with other isolation methods (56).

Molecular methods that utilise DNA sequencing analysis of ribosomal RNA genes (ITS/18S rRNA gene sequence for fungal identification) or the higher-resolution "Multilocus sequence typing (MLST)" methodology enable accurate identification at the species or strain level (57). Sometimes, ITS-based identification may lead to ambiguous species identification or to the absence of species in the reference database, because it has limited discriminatory power for closely related species. MLST facilitates the efficient taxonomic identification and phylogenetic inference in such cases by simultaneously analysing multiple genetic loci (58). The knowledge of plant-microbiome interactions has expanded with the introduction of omics tools (metatranscriptomics, metabolomics, and metagenomics) in endophyte research. By directly sequencing endophyte DNA from plant samples, the culture-independent method known as "metagenomics" removes the burden of isolating and sustaining microbial cultures (59). Moreover, the multi-omics approach will provide a broad perspective of endophyte biology, incorporating taxonomic, functional and metabolic information. Metagenomics reveals community composition and genetic potential, metatranscriptomics reveals which genes are expressed, and metabolomics reveals metabolites produced and host-microbe interactions. These strategies can be used together to better understand the diversity, functions and biosynthetic potential of the endophytes.

Screening of fungal endophytes

Endophytic fungi are prolific producers of structurally diverse bioactive secondary metabolites, including quinones, steroids, terpenoids, tetralones, xanthenes, flavonoids, phenolic acids, and benzopyranones (60). Many endophytes produce growth-inhibitory compounds, including antibiotics, antibacterials and antifungals, which have considerable commercial value. These strains can be initially screened using co-culture inhibition assays, wherein test strains are observed for growth suppression on the same agar plate (61). Advanced molecular approaches enhance the discovery and selection of promising endophytic fungi. Genome mining and the use of molecular markers facilitate precise identification and evaluation of biosynthetic potential (62). For instance, *Dactylonectria alcacerensis*, isolated from the medicinal plant *Corydalis tomentella*, was shown to possess 45 secondary metabolite biosynthetic gene clusters (63). However, genome mining alone cannot confirm metabolite production, as many biosynthetic gene clusters remain transcriptionally inactive or are weakly expressed under standard laboratory conditions. So, the anticipated biosynthetic potential of the endophyte may not result in metabolite synthesis. Epigenetic modification, co-cultivation, and culture-condition optimisation are some strategies to activate these cryptic biosynthetic pathways (64, 65).

Sensitive analytical tools have significantly advanced the field of metabolite profiling. Techniques such as liquid chromatography-high-resolution mass spectrometry (LC-HRMS) and matrix-assisted laser desorption/ionisation-high-resolution mass spectrometry

(MALDI-HRMS) enable the detection of even trace-level compounds. Liquid chromatography-high-resolution mass spectrometry is ideal for analysing complex biological extracts, offering high sensitivity, comprehensive metabolite coverage and robust chromatographic separation. Matrix-assisted laser desorption/ionisation-high-resolution mass spectrometry, on the other hand, allows rapid analysis, minimal sample preparation, and high-throughput screening (66). These methods support rapid and accurate identification of novel bioactive molecules, potentially leading to the development of new therapeutics. Such scientific progress not only broadens our understanding of fungal biodiversity but also accelerates the discovery of innovative natural products and drug leads, highlighting the immense biotechnological potential of fungal endophytes.

Production and extraction of secondary metabolites

Two principal cultivation systems underpin the commercial exploitation of fungal endophytes: Submerged fermentation (SmF) and Solid-state fermentation (SSF). Submerged fermentation enables rapid production of fungal biomass and metabolites under tightly controlled environmental conditions. This offers cost-effectiveness, efficient space utilisation and reduced contamination risk. Submerged fermentation enables precise control over temperature, pH, aeration and media composition, thereby enhancing metabolite yields (67). The endophytic fungi *Ceratobasidium ramicola* IBRLCM127, isolated from the rhizome of *Curcuma mangga* Valetton & Zijp, demonstrated notable anti-MRSA activity when cultivated via SmF. The optimised conditions led to an 11.72% increase in antimicrobial output (68). Solid-state fermentation offers more technical and economic merits over SmF, as it involves simpler equipment and operations. Solid-state fermentation usually consumes less energy than SmF and requires a lower moisture content, which inhibits bacterial growth. Solid-state fermentation promotes the differential expression of secondary metabolites, potentially enhancing yield diversity (69). Various investigations have shown that using SSF rather than SmF can produce better aroma compounds, which are crucial for commercialising food, feed, and cosmetic products (70). A total of 23 flavour compounds were identified from *Talaromyces funiculosus* by solid-state fermentation. One of the key flavour metabolites, 4-methyl-5,6-dihdropyran-2-one, exhibited a strong sweet aroma with floral flavour (71). SSF is a valuable technique for biodegradation and bioremediation of hazardous substances (72). Evaluation of the SSF technique for pesticide waste disposal significantly reduced pesticide leachability and bioavailability (73). In addition, SSF has been extensively used in the production of enzymes like cellulase, pectinase, lipases, and xylanase, as well as the conversion of agricultural waste into value-added products (74, 75). It is expected that the synthesis of secondary metabolites using SSF will become the technology of the future and will continue to develop into a safer, natural bioprocess (76). Table 3 summarises the comparison of SmF and SSF for endophytic fungal metabolite production. Despite significant improvements in endophyte-based metabolite synthesis through SmF and SSF, several biological and technological challenges remain. Overcoming these barriers is critical to maximise metabolite productivity and ensure the successful commercialisation of endophyte-based products.

Bottlenecks in endophyte biology

Researchers working in the field of endophytic fungal biology encounter numerous challenges that hinder both scientific advancement and broader practical applications. A major obstacle lies in the fact that many fungal endophytes are unculturable,

Table 3. Comparison of SmF and SSF

Parameter	SmF	SSF
Culture medium	liquid nutrient medium	Cultivated on solid substrates with little or no free water
Process Control	Allows precise control of temperature, pH, aeration and nutrient availability	More difficult to control environmental parameters
Biomass production	Supports rapid fungal growth and biomass accumulation	Biomass production is often lower, but may favour metabolite synthesis
Contamination	Controlled conditions help reduce contamination risks.	Low moisture content restricts bacterial growth.
Equipment and operation	Requires specialised bioreactors and continuous monitoring.	Uses simpler equipment and operational procedures.
Energy requirement	Higher due to agitation, aeration, and mixing requirements.	Lower energy consumption makes the process economically attractive.
Product recovery	Downstream processing and metabolite extraction are relatively straightforward.	Extraction may be more challenging depending on the substrate used.
Industrial applications	Widely employed for the production of pharmaceuticals, enzymes, and bioactive compounds.	Commonly used for flavour and fragrance production, bioremediation, and other value-added bioproducts.
Major limitations	High operational costs and the generation of large volumes of liquid waste.	Scale-up may be difficult due to challenges in heat transfer, aeration, and process monitoring.

species that cannot be grown on artificial media. As a result, their biological characteristics, capacity for producing secondary metabolites, and ecological roles remain largely unexplored.

In the standard bioprospecting approach, a wide array of distinct endophytes is typically isolated from a single host plant and screened for desirable bioactive secondary metabolites. However, using classical methods, it is common for only a limited number, or in some cases, none of these endophytes to exhibit their full biosynthetic potential. Those deemed "incompetent" without further investigation are often discarded, leading to the loss of valuable natural products that might have been expressed under more host-associated conditions.

The primary bottlenecks in harnessing endophytes for secondary metabolite production is the marked decline in metabolite output upon successive *in vitro* subculturing. For instance, 3 endophytic fungi, *Trichoderma atroviride* LY357, *Aspergillus* sp. LY341 and *Aspergillus* sp. LY355 was isolated from *Camptotheca acuminata*, capable of producing Camptothecin (CPT). The fermentation broths of these fungi produced CPT at concentrations of 7.93, 42.92, and 197.82 µg/L, respectively. However, strains LY341 and LY355 lost their ability to produce CPT after repeated subculturing, while strain LY357 exhibited a drastic decline in CPT production by the second generation (77). This finding underscores the inherent instability of secondary metabolite production in endophytic fungi over time.

Although it is still unclear how this decline occurs, previous studies indicate that the decreased metabolite could be related to epigenetic modifications, silencing of gene clusters involved in biosynthesis, changes in gene expression profiles, etc (53). Fig. 1 provides an overview of the key challenges involved in employing endophytic microorganisms as micro-factories for the sustainable production of secondary metabolites.

Approaches for the enhanced production of secondary metabolites

The *in vitro* production of secondary metabolites by endophytic fungi is often low, posing a significant barrier to their commercial and therapeutic applications. Several innovative approaches have emerged to address this challenge, each designed to enhance

secondary metabolite production in endophytic fungi (Fig. 2). Endophytic fungi possess specialised genomic regions, known as biosynthetic gene clusters (BGCs), that encode the regulatory elements and enzymes necessary for producing a variety of secondary metabolites. The PKS clusters, terpene synthase clusters, non-ribosomal peptide synthetase (NRPS) clusters and hybrid PKS-NRPS clusters are the main types of BCGs. These gene clusters are involved in the biosynthesis of several biologically active compounds (65). However, under typical *in vitro* conditions, a significant fraction of BGCs in endophytic fungi are "cryptic" or silent, meaning they do not release metabolites without specific activation.

Elicitation by chemical modulators

Biosynthetic gene clusters expression has been observed *in vitro* when elicitors are added to microbial growth media. Elicitors, which are substances that induce the production of secondary metabolites, can be categorised as either biotic (proteins, polysaccharides, cell-wall fragments, glycoproteins, etc.) or abiotic (UV rays, heavy metals, methyl jasmonate, salicylic acid, etc.) (53). One plant alkaloid being considered a potential therapy for Alzheimer's disease is huperzine A (HupA). The production of HupA from endophyte *Colletotrichum gloeosporioides* ES026 can be enhanced by ethanol and methanol by 51.89 % (78).

Epigenetic modifiers: Target strains are cultivated with one or more chemical epigenetic modifier drugs, such as DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors, to achieve chemical epigenetic modification. It has been discovered that adding these substances to growing cultures at micromolar or even nanomolar concentrations can either activate or suppress the related enzymes, thereby reengineering secondary metabolite biosynthesis pathways in fungi (79). The epigenetic modification was applied to *Nigrospora sphaerica*, which is responsible for the production of phomalactone from *Adiantum philippense* L. DNA methyltransferase inhibitors such as 5-azacytidine and hydralazine hydrochloride, as well as histone deacetylase inhibitors such as sodium butyrate, suberohydroxamic acid (SAHA) and valproic acid, were employed to investigate epigenetic modification in the endophytic fungus *N. sphaerica*. Histone deacetylase inhibitors and DNA methylation were both effective in causing the endophytic fungus to produce cryptic

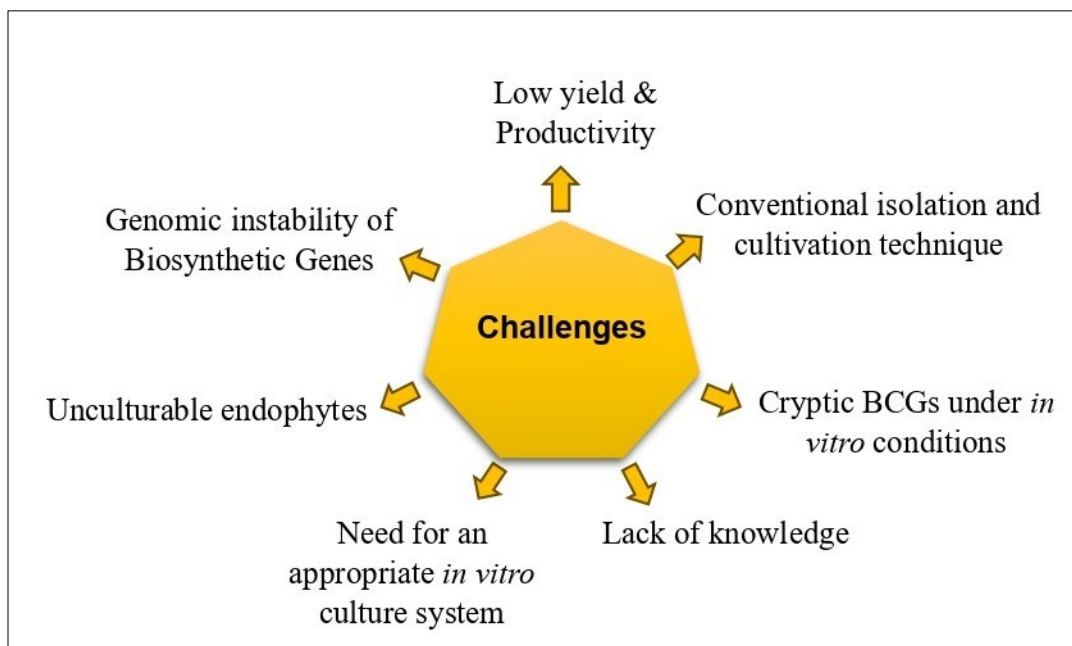


Fig. 1. Challenges faced during secondary metabolite production.

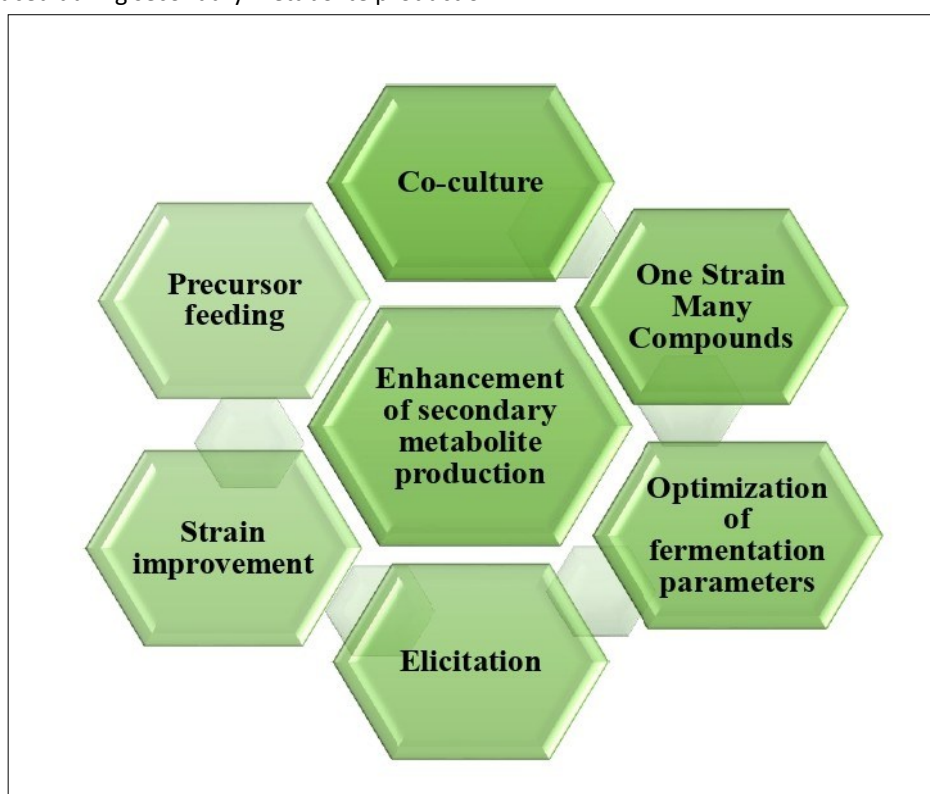


Fig. 2. Approaches for the enhanced production of the secondary metabolites.

metabolites (80).

Co-culturing with other microorganisms

Co-culturing is a potent new technique for reawakening BGCs. Fungal–fungal, fungal–bacterial, and fungal–host co-cultures are the three usual methods. This technique aims to produce artificial microbial communities that mimic natural ecosystems. Microbe interactions can influence metabolic pathways, which in turn can lead to the production of natural products. Thus, developing natural products can be substantially aided by obtaining a thorough grasp of these interactions in microbial co-culture communities (81). In a recent study on mangrove endophytic fungi from *Rhizophora mangle*, an established co-culture of the *Phomopsis asparagi* strain DHS-48, along with another *Phomopsis* genus fungus, DHS-11, was

highly effective in both increasing yields of the target metabolites and inducing the synthesis of new metabolites (82). Table 4 lists examples of co-culture systems and their outcome.

One strain many compounds

The continuous use of traditional cultivation and isolation techniques has considerably slowed the rate of discoveries of novel bioactive secondary metabolites. A simple yet efficient method called One Strain Many Compounds (OSMAC) allows the expression of multiple metabolites from a single microbial strain by adjusting various cultivation parameters (such as aeration, incubation duration, pH, addition of enzyme inhibitors, and culture media composition) (87). This approach produced metabolites, including polyketides, azaphilones and an isochromanone from the

Table 4. Examples of co-culture systems and their outcome

Fungus	Co-cultured Strain	Product	Outcome	Reference
<i>Chaetomium</i> sp	<i>Bacillus subtilis</i>	3- and 4-hydroxybenzoic acid methyl esters	8.3-fold yield increase	(83)
<i>Fusarium tricinctum</i>	<i>B. subtilis</i> 168	Lateropyrone, Fusaristatin A, Citreoisocoumarin	78-fold yield increase	(84)
<i>Aspergillus oryzae</i>	<i>Epicoecum dendrobii</i>	Epilactones A and B, Epioxochromane and Aoergostane	Production of the spore inhibitor fumigermin, antifungal agent kojic acid	(85)
<i>Paraconiothyrium variable</i>	<i>Fusarium oxysporum</i>	13-Oxo-9,11-octadecadienoic acid	Reduced the amount of mycotoxin that <i>F. oxysporum</i> produces	(86)

endophytic fungus *Dothideomycete* sp. CR17. By simply altering the sources of potato and malt extract in the culture media, CR17 was able to produce these novel metabolites, demonstrating the effectiveness of the OSMAC strategy in activating diverse metabolic pathways (88). The mangrove-derived fungus *Phomopsis* sp. QYM-13 treated with 3 % NaBr or 3 % KI in potato liquid medium yielded twelve new cytochalasins, phomopchalasins D–O, including one brominated and two iodinated cytochalasins, along with six recognised counterparts (89).

Strain improvement

Strain improvement refers to the science and technology of modifying and enhancing microbial strains to increase their metabolic capabilities for biotechnological applications (90). Numerous techniques, including genetic engineering, mutagenesis, genome editing, and genome shuffling, can be employed to achieve this goal. The synthesis and yield enhancement of the anticancer medication vinblastine by *Alternaria alternata* using gamma irradiation mutagenesis has been reported for the first time (91).

Using CRISPR-Cas9 technology and genome mining, BGCs that produce secondary metabolites can be found and altered. The CRISPR-Cas, an adaptive immune system of prokaryotes, was initially identified in 1987. Because of its exceptional effectiveness, adaptability, and user-friendliness, CRISPR-Cas9 can be used for gene editing in fungi (92). A recent study utilised this technology, mediated by double-guide RNAs, to inactivate the stress signal-transducing gene of the mitogen-activated protein kinase in an endophytic fungus strain, *Phomopsis liquidambaris*. As a result, several flavonoid metabolites, including kaempferol, quercetin, and naringenin, were biosynthesised, protecting the mutant cell against possible stressors (93).

Precursor feeding

Precursor feeding is one method for producing secondary plant metabolites in plant cell culture. It can also be utilised in microbes that mimic the plant's biosynthesis pathway (94). Studies have been conducted on the efficacy of precursor feeding. In *Talaromyces neorugulosus* R-209 cultures, L-phenylalanine increased the synthesis of phenolic compounds that benefit health. Optimising the feeding phase for 6 days and the L-phenylalanine dose to 0.16 g/L significantly increased the yields of significant phenolic compounds, such as apigenin, genistein, CSA and biochanin A, by 7.20-fold, 15.59-fold, 10.30-fold and 25.93-fold, respectively (95).

Optimisation of fermentation parameters

Several variables regulate the production of fungal secondary metabolites. Some known variables are culture media composition, nutrition ratio, pH, aeration, temperature, and cultivation duration.

Shao *et al* used stationary liquid fermentation to optimise the production of N-methylsalsalvamide (SA) by *Fusarium* sp. R1. A combination of approaches, including One-Factor-at-a-Time and Response Surface Methodology, was applied to determine the key conditions and medium compositions under which the SA yield from these cultures might be nearly tenfold increased, from 54.05 ± 3.45 mg/L to 536.77 ± 2.67 mg/L. Standard conditions included 22.5 g/L sucrose, 16.5 g/L tryptone, 0.024 g/L yeast extract, and specific fermentation parameters, including 13 days at 24 °C with a pH of 6.5 (96). *Neopestalotiopsis vitis*, cultured in a medium containing 3 % malt extract and 5 mM ammonium sulfate at pH 7.0, grew rapidly with the sustainable production of paclitaxel and other taxane products, such as 10-deacetyl baccatin III and cephalomannine (97). So, it is critical to optimise parameters such as culture media, pH, and temperature to maximise fungal secondary metabolite production. Optimised conditions yield higher yields, making the process highly efficient and viable for large-scale industrial and pharmaceutical applications.

Artificial intelligence: Role in fungal endophyte research

Artificial intelligence (AI), including machine learning and deep learning techniques, plays a crucial role in evaluating large datasets. The use of these technologies in fungal endophyte research is revolutionising the optimisation and discovery of secondary metabolites. Traditional screening methods for bioactive compounds are mostly time-consuming. AI-based methods offer data-driven insights for identifying novel biosynthetic pathways, optimising culture conditions, and predicting high-yielding strains. Fig. 3 displays some of the applications of AI in fungal endophyte research.

Plant microbiome distribution patterns in the context of environmental niches could ideally be predicted by machine learning or deep learning frameworks, which could also facilitate the prediction of natural products derived from endophytes. This would replace extensive, worldwide, molecular surveys of plant microbiomes, which are difficult for all but a few clades (98). The effectiveness of culture conditions on the amount of cellulase produced by *Taxus cuspidata* endophytic fungi was examined using a single-factor analysis of response surface methodology (RSM) and an artificial neural network-genetic algorithm (ANN-GA) to obtain factors for enhancing the enzyme activity process of production (99).

AI-based technologies are primarily used as predictive and analytical tools rather than as genetic manipulation tools, unlike genome editing technologies like CRISPR–Cas9. CRISPR–Cas9 can be used to precisely edit the genome and study the function of biosynthetic genes for targeted strain improvement and pathway engineering. The use of this approach might be restricted by low transformation efficiency, off-target effects, and the lack of genome

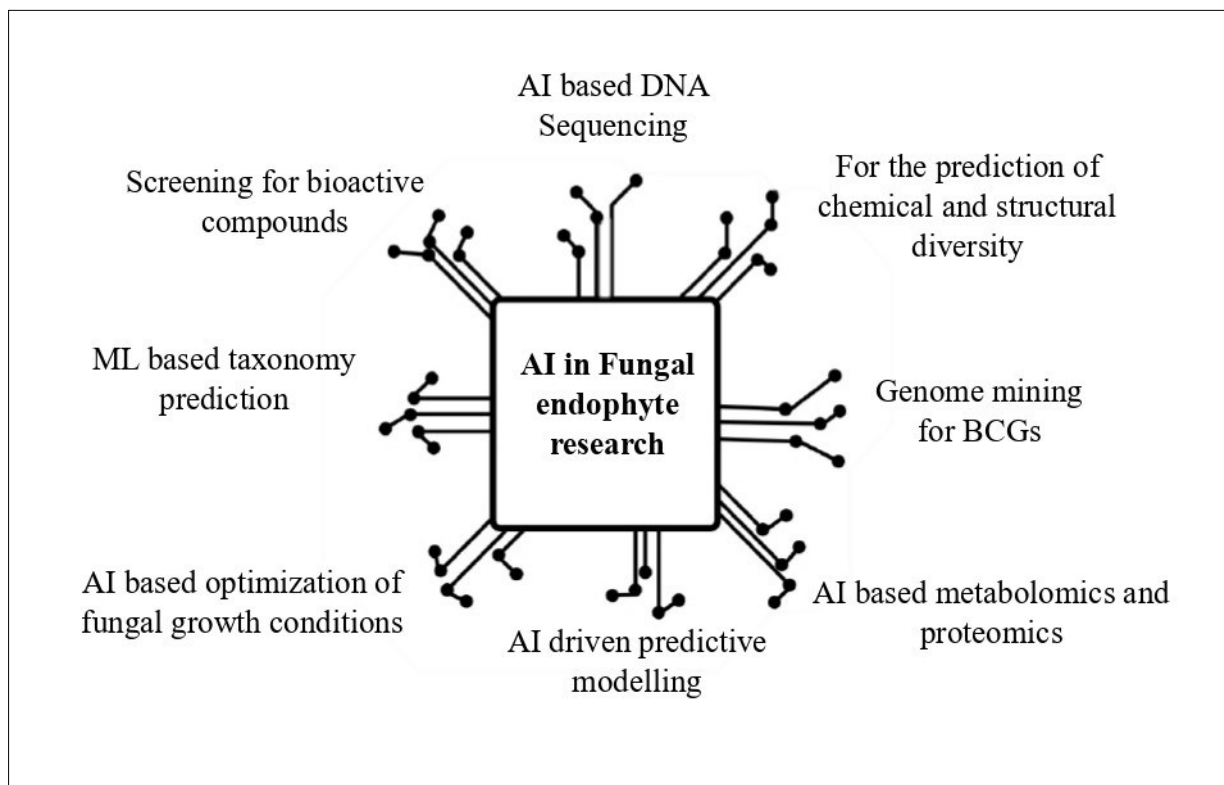


Fig. 3. Artificial intelligence in fungal endophyte research.

information for many endophytic fungi. AI-driven methods, on the other hand, can be used to analyse large amounts of data, forecast biosynthetic potential, and optimise culture conditions, but their usefulness depends on the quality and availability of the datasets. A combination of AI-based prediction and CRISPR-based validation could then accelerate the discovery and production of valuable secondary metabolites from endophytic fungi.

Conclusion

Endophytic fungi are considered promising producers of novel secondary metabolites; however, various challenges in endophyte biology limit their full potential. The challenges include the inability to isolate non-culturable strains, the difficulties of sustaining steady metabolite production and the absence of a thorough understanding of their biochemical processes, etc. Emerging developments in molecular biology, genetic engineering and omics technologies are increasingly overcoming these limitations, offering new ways to improve metabolite production. Methods such as co-culturing, epigenetic modulation and metabolomic profiling are now revealing new possibilities to tap the metabolic richness of endophytes. Broader mysteries may be solved by incorporating computer learning techniques into the pipeline for predicting and validating endophyte metabolites. With continued technological and methodological advancements, endophyte research holds a considerable promise for sustainable sources of novel bioactive metabolites and natural product-based innovations.

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Authors' contributions

AS prepared the original draft of the manuscript. SRS conceived and conceptualised the study, provided the necessary resources, supervised the research, and critically reviewed and revised the manuscript. Both authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: The authors have no conflicts of interest to declare.

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