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Endophytic Fungi from Medicinal Plants– At a Glance

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Abstract: -

Endophytic micro-organisms are hidden companions of plants livingmutually beneficiallife inside the host plant. Though these endophytes are supposedto be associated and evolvedwith land plants, endophytes are recognised in last century. Beneficial effects of endophytes are attaining importance with thepossibility of obtaining novel medicinally important compounds as well as theirrole in increasing crop productivity because they produce a variety of compoundsand interact with other micro-organisms, pathogenic and non-pathogenic. With the development of modern tools and techniques of molecular biology, it has becomepossible to establish correct identity of these micro-organisms and know theinteractions with host and other micro-organisms. In this overview, we present current scenario about endophytes and their use for human welfare.

Keywords: - Endophytes, fungi, medicinal plants, Diversity, microbes, endosymbiosis, metabolites

Introduction:

Endophytic fungi have been known to possess a dynamic potential in improving growth of host plants during favorable and non-favorable conditions. Inaddition to the resilience of medicinal plants in the traditional health care system, they offer a unique opportunity to offer bio-prospective endophytes, which could be used for a diverse array of beneficial applications.

endophytes can play a significant role in enhancing the tolerance of plants. Endophytes systematically colonize different parts of the host, but plants use a variety of defense mechanisms towards microbial infection. However, they have to survive the oxidative environments, and endophytes like *Enterobacter* sp. encode superoxide dismutases, catalases, and hydroperoxide reductases to cope up the oxidative stress duringcolonization.Fungal endophytes are now generally highly rated for their relevance in plant growthpromotion, plant physiology balancing, plant nutrients restoration and phytoremediation.Due to their huge importance, endophytic fungi as well as other microorganisms, plants,animals and their by-products are now commonly targeted for the production of novelmedicinal products and for other industrial applications. In 2015 Neha Chadha and Manjita Mishra stated that 'It is our consensus that plants survive and flourish in stressed ecosystems because of endosymbiotic organisms that have co-evolved and were essential for their adaptation to changing environments.

Himanshi Godara and Wurisika Ramkrishna in 2023 have recognized that plants' distinct and immensely dynamic microbial communities are more than just "passengers," but instead, play an important role in their development, and shielding against abiotic and biotic stresses.

Endophytic fungi living asymptomatically in plant tissues may present in almost all plants (Saikkonen et al. 1998). One species of an endophyte may be associatedwith many plant species, and many species of endophytes may be present in thesame species. Some endophytes remain as latent in the host plant, while others mayinteract with other endophytes, pathogenic or non-pathogenic (Zabalgogeazcoa2008).Endophytes have evolved mechanisms to live within the plant by defendingthemselves against all physical and chemical weapons of the plants, e.g. in plant like *Camptotheca acuminata* produces anticancer compound camptothecin which binds to topoisomerase I to

stop cell divisions. The endophytic fungus *Fusarium solani* modified its topoisomerase biding site by alterations in amino acids to escapefrom harmful effects of camptothecin (Kusari et al. 2011).

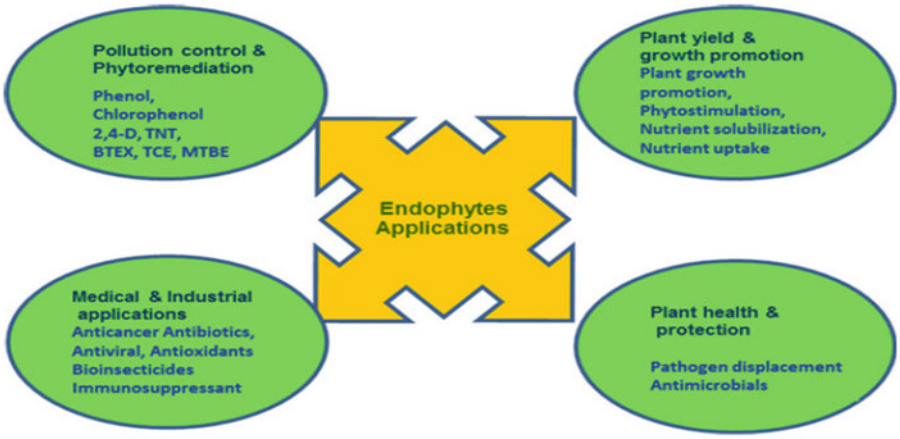


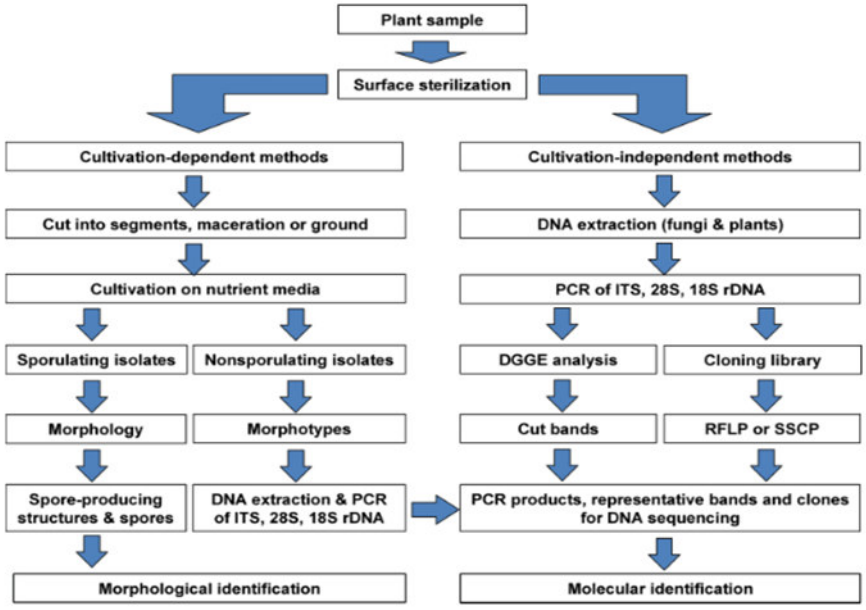
Fig. 1.1 Applications of endophytes in various fields. Examples in each category are symbolic representatives. Pollutant like 2,4-dichlorophenoxyacetic acid (2,4-D) is used as weedicide; petroleum-based products such as benzene–toluene–ethylbenzene–xylene (BTEX), methyl tertiary-butyl ether (MTBE); explosives such as trinitrotoluene used in mining, road and dam making (TNT); trichloroethylene (TCE) is a common solvent.

Methods: -

Traditional techniques used in endophyte studies

Traditionally, endophytic fungi inside plant tissues can be recognised by two basic techniques, i.e. direct observation and cultivation-dependent methods. In the direct observation method, endophytic fungal structures within living plant tissues are directly examined under a light and electron microscope, which can show all endophytic mycobiota within the plant tissue, particularly biotrophic fungi that cannot be cultured on standard growth media (Deckert et al. 2001; Lucero et al. 2011). However, most endophytic fungi within plant tissue have only a hyphal structure, and therefore cannot be identified to any taxonomic category according to morphology due to lack of spore-producing structures and sexual or asexual spores. In addition, endophytic isolates cannot be obtained as microbial resources for further use with the direct observation method. Therefore, this is not commonly used in endophyte diversity studies (Deckert et al. 2001)

advances in methodologies to study endophytic fungi, several barriers come up at all stages of commonly used protocols, requiring well-established strategies to avoid misinterpretation. Here, we discuss some methods mostly used to study endophytic fungi, emphasizing the main limitations derived from the different approaches, with alternatives to circumvent and/or minimize the existing barriers.



Collection and sampling of plant material

The first step to study endophytic microorganisms is the collection of plant material. However, there is no universal consensus on the adequate number of individuals and/ or samples per individual that should be considered. It is strongly recommended a representative sampling according to the objectives proposed by the study. Considering the geographic factor, Yao et al.

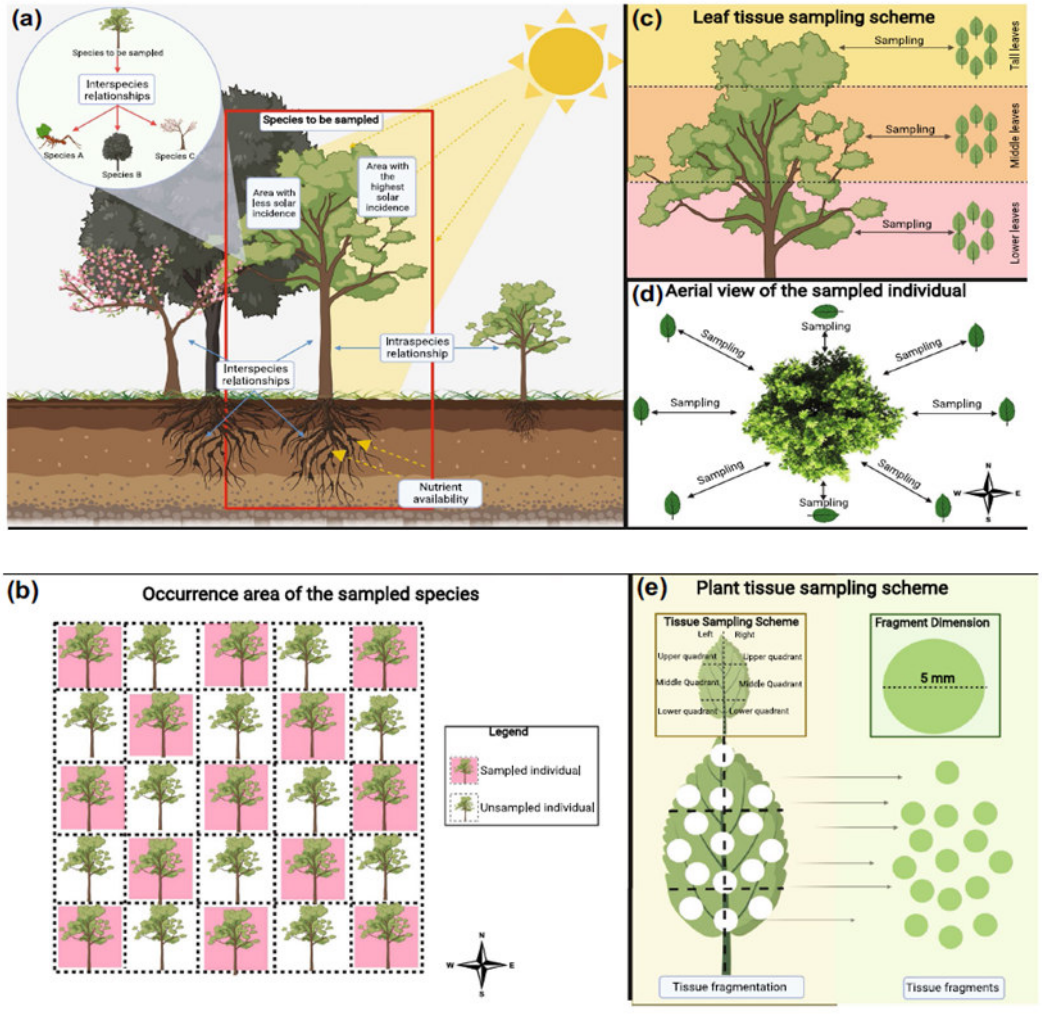
(2019) accounted eight individuals per plant species sampled and recommended a minimum distance of at least 50 m between each plant species in studies on diversity of endophytic fungi of different tropical mangroves. Wang et al. (2019) aimed to describe the three-years-old mycobiota of *Zanthoxylum bungeanum* and for these ten specimens were sampled excluding the spatial criteria. Fan et al. (2020) studied the endophytic mycobiome of the cultivated *Huperzia serrata* and three specimens were randomly chosen. Therefore, the number of plant individual ssampled depends on the objective of the research, but the quantity and quality of sampling plus biotic and abiotic factors that can interfere with the endophytic mycobiota must be considered (Fig. 2).Interestinglymost fungal genera and/or species are found in specific host plant species probably because the phytochemistry and micro/macronutrient contents present in the leaf tissue that acts by selecting the fungal species for colonization (Arnold et al. 2003).

After collecting the plant material, the samples should be placed in a sterile plastic container or bag, transferred to a cool box at a controlled temperature, and preferably processed within 24 h after sampling (Lundberg et al. 2012; Sharma et al. 2016; Li et al. 2016a, b; Ibrahim et al. 2017,2021; Pietro-Souza et al. 2017; Yao et al. 2017, 2019; Szűcs et al. 2018; Hamzah et al. 2018; Gong et al. 2019; Dhayanithy et al. 2019; Thi et al. 2019; Jayatilake and Munasinghe2020; Du et al. 2020; Fan et al. 2020; Chowdappa et al. 2020) under aseptic conditions.

Surface disinfection of plant material

Several methodological obstacles appear when studying endophytes, includingthe complete removal of microorganisms that make up theepiphytic microbiota. Effective methods of surface disinfectionto remove the epiphytic microbiota are mandatoryfirst steps for studying endophytic microorganisms, whichincludes chemical or physical procedures.The chemical sterilization of the sample surface is themost used method for removing microorganisms from the rhizoplane and many protocols are

available (Table 1). Inmost cases, they consist of three basic steps: (1) submersionof the tissue in ethanol; (2) immersion of the tissue inthe main sterilizing agent, and (3) successive washing withdistilled water previously autoclaved. Themain sterilizingagents used in rhizoplane disinfection are sodium hypochlorite(NaOCl) (Yao et al.
2019) and mercury chloride (HgCl2)(Du et al. 2020). Other sterilizing agents can also be usedsuch as hydrogen peroxide (H2O2), paraquat, or 1% peroxyaceticacid (CH3COOOH) in 30% ethanol (Sieber, 2002).According to some authors (Pietro-Souza et al. 2017; Ibrahimet al. 2021),the samples should be washed in runningtap water for some times to remove debris, dust, other particles,and main epiphytes (e.g. epiphytic bacteria, yeasts andfilamentous fungi) prior to the superficial disinfection of thematerial to enhance the process.



Culture medium for growth of endophytic fungi: -

There are many cultures medium protocols available to recoverendophytic fungi, with PDA asthe most frequently used. Other growth media such as Malt Extract Agar (Liet al. 2016a, b), Agar containing Murashige and Skoog (MS)vitamins and sucrose (Lundberg et al. 2012), Hagem MinimalMedium (Khan et al. 2016), Czapek medium (Chand et al.2020), Tryptone Soybean Agar, Tryptone Bovine Extract Agar,and Luria–Bertani medium (Gong et al. 2019), which is alsoknown as LB broth, Lysogeny broth or

Luria broth, can beefficiently used to recover endophytic fungi from plant samples.Although several culture media are used for recoveryand cultivation of endophytic fungi, they must have a slightlyacidic pH range (5.8–6.0 pH) (Lundberg et al. 2012; Li et al.2016a, b; An et al. 2020). Depending on the purpose of thestudy and the characteristics of the habitat (i.e. host plant species),the medium commonly used can be nutritionallysupplementedand/or replaced by specific medium to better achievethe objectives of the study (Pietro-Souza et al. 2017; Hamzahet al. 2018; Hamzah et al. al. 2018).

Cultivation conditions, isolation and culture purification

According to the objectives of the study, the cultivation conditionsof endophytic fungi in artificial culture medium are diverse. The temperature commonly used for incubation of Petri dishes usually ranges from 25 oC (Li et al. 2016a, b;Du et al. 2020) to 28 oC (Yao et al. 2017), with period of incubation between 3 and 20 days (Li et al. 2016a, b; Yaoet al. 2017; Du et al. 2020), which can be extended for upto 6 weeks if necessary (Yao et al. 2017). In the case ofcultures that require a longer period of incubation or arekept under environmental conditions outside greenhousesor BOD incubators (Bio-Oxygen Demand), it is necessaryto seal the plates with Parafilm to minimize risks of contaminationand/or drying of the culture medium (Szűcs et al.2018; Ababutain2021). Cultivation can also be carried outwith or without incidence of light (Duan et al. 2019; Agbessenouet al. 2020). In this sense, the endogenous circadianclocks present in both prokaryotes and eukaryotes providethe machinery by which they keep in synchrony with theexternal environment. In fungi, the clock has been shownto control daily rhythms (Dunlap and Loros2017) in spore development and liberation, supporting their survival.Plates seeded with fragmented plant tissue should bemonitored daily. Fragmentation of plant tissue can beachieved with the aid of a scalpel, eyelet pliers or scissors,then printed on a culture medium and incubated. Althoughfragments with 5 mm × 5 mm are commonly used, otherdimensions have also been employed (Table 1). As fungalgrowth frequently occurs from the edges of the inoculatedplant tissue, the isolation is recommended by collecting thehyphae from the edges of the fungal colonies, followed byseeding in fresh culture medium with or without antibiotics(Li et al. 2016a, b; Ibrahim et al. 2017; Yao et al. 2017;Ababutain2021). This step should be repeated severaltimes until a monoculture of a endophytic fungus strain isreached with a uniform colony (Ibrahim et al. 2017). Then,the monosporic and/or hypha tip purification must be performed (Supaphon et al. 2013; Duan et al. 2019) for laterdeposit in a mycological culture collection and/or molecularidentification of the isolates.

Morphological or morphotype grouping and storage of endophytic fungi colonies: -

After the purification process, the fungal isolates are grouped based on their macro- and micromorphological characteristics (Dhayanithy et al. 2019; Jayatilake and Munasinghe2020). For this, macroscopic vegetative characteristics that includes color, texture, topography, and diffuse pigmentation of structures, and color and topography of the colony's dorsum, are considered, and also analyses of their microscopic reproductive structures, using microculture and/or sporulation methods (Dos Banhos et al. 2014). The macroscopic classification allows grouping the isolates into morphotypes, while the microscopic classification allows the morphological classification of the isolates. However, the arrangement in morphotypes and/or morphological features does not reflect the real phylogeny of the organisms, even more considering non-sporulating fungal endophyte species (Li et al. 2016a, b; Yao et al. 2017; Du et al. 2020) that requires robust tools such as molecular methods to achieve the taxonomic classification of the isolates. To avoid losses of isolated colonies caused by contamination, re-isolation on PDA prepared inclined cryotubes and subsequent storage in a cold chamber at 4o C and freezer – 80o C is recommended (Li et al. 2016b; Wang et al. 2019). For long-term preservation of cultures, mycelia and spores should be transferred to 20% glycerol in ultrapure distilled water and stored at − 80° C (Wang et al.

2019). Another alternative for long-term preservation of monocultures is to transfer small pieces of pre-inoculated culture medium to sterile microtube containing 30% glycerol (v/v) and sterile rice medium, followed by incubation at 25 oC. After a fungal growth is observed, the microtubes should be transferred to a cold room (4 °C), and storage in a freezer at − 20 oC, respectively (Ibrahim et al. 2017).

Molecular identification of isolatedendophytic fungi

Despite the development of different methods to promote fungal sporulation (Taylor et al. 1999; Sun et al. 2008a), more than 50% of endophytic fungal isolates do not sporulate in cultures (*Mycelia sterilia*) (Wang 2008; Wang and e Guo LD 2007; Sun et al. 2008b, 2011), which makes the conventional classification based on observation of the reproductive structures of fungi impossible. Furthermore, even for those isolates capable of sporulating, a polyphasic approach that includes molecular analysis plus observation of reproductive structures is strongly recommended to achieve a precise fungi identification (Sun and Guo 2012). There are many protocols used for molecular characterization of endophytic fungal isolates (Table 2). Briefly, this approach requires the following steps: (I) genomic DNA (gDNA) extraction; (II) PCR amplification of conserved DNA sequences (rDNA) using specific or universal oligonucleotide primers forward and reverse; (III) sequencing of PCR products; (IV) sequence processing and comparison with related sequences deposited in DNA databases; (V) data interpretation and phylogenetic tree reconstruction (Hamzah et al. 2018; Thi et al. 2019; Jayatilake and Munasinghe2020). In addition, DNA cloning using cloning vectors can also be employed prior to sequencing (Atsatt and Whiteside 2014).

Present endophytic fungi in plants: -

Endophytic fungi	Plant species	Reference
Acremonium sp.	*Taxus chinensis* *Huperzia serrata*	Liu et al. (2009) Glienke-Blanco et al. (2002)
Aspergillus sp.	*Datura stramonium* *Moringa olifera* *Prosopis chilensis*	Mahdi et al. (2014)
Cladosporium sp. *C. herbarum*	*Opuntia ficusindica* *Cinnamomumcamphora* *Lycopersicumesculentum Mill.* *Triticum aestivum*	Bezerra et al. (2012) He et al. (2012) Larran et al. (2001) Larran et al. (2002)
Colletotrichum sp. *C. gloeosporiodes*	*Triticum aestivum* *Citrus plants* *Cinnamomumcamphora* *Pasania edulis* *Ginkgo biloba L.* *Tectonagrandis and Samanea saman* *Huperzia serrata* *Cinnamomumcamphora* *Lycopersicumesculentum Mill.*	Larran et al. (2002) Glienke-Blanco et al. (2002) He et al. (2012) Hata and Sone (2008) Thongsandee et al. (2012) Chareprasert et al. (2006) Wang et al. (2011) He el al. (2012) Larran et al. (2001)
Curvularia sp.	*Datura stramonium* *Moringa olifera*	Mahdi et al. (2014)
Penicillium sp.	*Lycopersicumesculentum Mill.* *Huperzia serrata*	Larran et al. (2001) Wang et al. (2011)
Phyllosticta sp.	*Citrus sp.* *Pasania edulis* *Coffea arabica* *Quercus variabilis* *Centellaasiatica*	Glienke-Blanco et al. (2002) Hata and Sone (2008) Santamaria and Bayman (2005)

	Panax quinquefolium *Ginkgo biloba L.*	Wang et al. (2007) Rakotoniriana et al. (2008) Xing et al. (2010) Thongsandee et al. (2012)
Phomopsis sp.	Pasania edulis Ginkgo biloba L. Tectonagrandis and Samanea saman Taxus chinensis	Hata and Sone (2008) Thongsandee et al. (2012) Chareprasert et al. (2006) Liu et al. (2009)
Stemphyliumglobuliferum	Avicennia	marina Moussa et al. (2016)

Practical application of Endophytic fungi: -

1. Potantial as Growth promoting agent.
2. Use as Biocontrol agent.
3. Protection against abiotic stresses.
4. Application of fungal endophytes via endophyte produced compound,
5. Terpenoides.
6. Alkaloides.
7. Phenols.
8. Enzyme and organic acid.
9. Application of endophytes to pharmacuticals
10. Bioremediation
11. Nanoparticalbiosynthrsizer.

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